

# ***Perkinsus marinus* susceptibility and defense-related activities in eastern oysters *Crassostrea virginica*: temperature effects**

Fu-Lin E. Chu, Jerome F. La Peyre

Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062, USA

**ABSTRACT:** The relationship of potential defense-related cellular and humoral activities and the susceptibility of eastern oysters *Crassostrea virginica* to the parasite *Perkinsus marinus* were examined at 10, 15, 20 and 25 °C. Oysters were acclimated at experimental temperatures for 20 d and then challenged with *P. marinus*. Total hemocyte counts (TC) and percentage of granulocytes (PG) 20 d after temperature acclimation were higher in oysters at high than at low acclimation temperature. Higher protein (P) and lysozyme (L) concentrations were found in oysters at 10 and 15 °C. No significant differences in hemagglutination (H) titers due to temperature acclimation were observed. Infection prevalence 46 d after challenge by *P. marinus* was 100, 91, 46 and 23 % respectively, for oysters at 25, 20, 15 and 10 °C. Disease intensity increased with temperature. Oysters at higher temperatures had greater PG and TC and hemocyte phagocytic activity. No difference was found in TC and PG between control and challenged oysters within each temperature treatment. Bleeding may to some extent reduce TC and PG in oysters. P did not vary much among temperatures. No reduction of P in oysters was found due to *P. marinus* challenge and infection. L tended to be higher in oysters at lower than at higher treatment temperatures. The oysters at 10 °C had the highest L concentration and lowest *P. marinus* infection. But, it is not known whether the high extracellular L in oysters at 10 °C is attributable to the low *P. marinus* susceptibility in these oysters. There was no significant difference in condition index (CI) between control and challenged oysters and between infected and uninfected oysters. However, CI decreased with increasing temperatures. The H titers were not associated with any measured variables. The greater TC, PG, and phagocytic capability in oysters at higher temperatures did not result in fewer or less intense *P. marinus* infections.

## **INTRODUCTION**

In a host-pathogen relationship, the success or failure of the pathogen in establishing infection in the host depends upon the effectiveness of the internal defense system of the host to eliminate the invading pathogen or the ability of the pathogen to avoid the host defense. It is generally accepted that immunocompetency of the host is governed by genetic factors and mediated by extrinsic environmental regimes. Compensation for excessive environmental stress can compete for energy reserves that might otherwise be available for defense mechanisms. Similarly, pathogen infectivity is genetically controlled and affected by environmental factors.

The protozoan pathogens *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) are the 2 most important disease organisms in eastern oysters *Crassostrea virginica* on the east coast of the United States. *P. marinus* has recently become the primary oysters pathogen in the lower Chesapeake Bay (Burreson 1989, 1990, Barber 1990). It kills eastern oysters mostly during late summer and early fall (Andrews 1988, Burreson 1989). The relationship between temperature and *P. marinus* incidence has been investigated since the 1950s (Mackin 1951, 1956, Andrews & Hewatt 1957, Perkins 1966, Soniat 1985, Craig et al. 1989, Soniat & Gauthier 1989, Crosby & Roberts 1990, Gauthier et al. 1990) in field and laboratory studies. The distribution and abundance of *P. marinus* in the

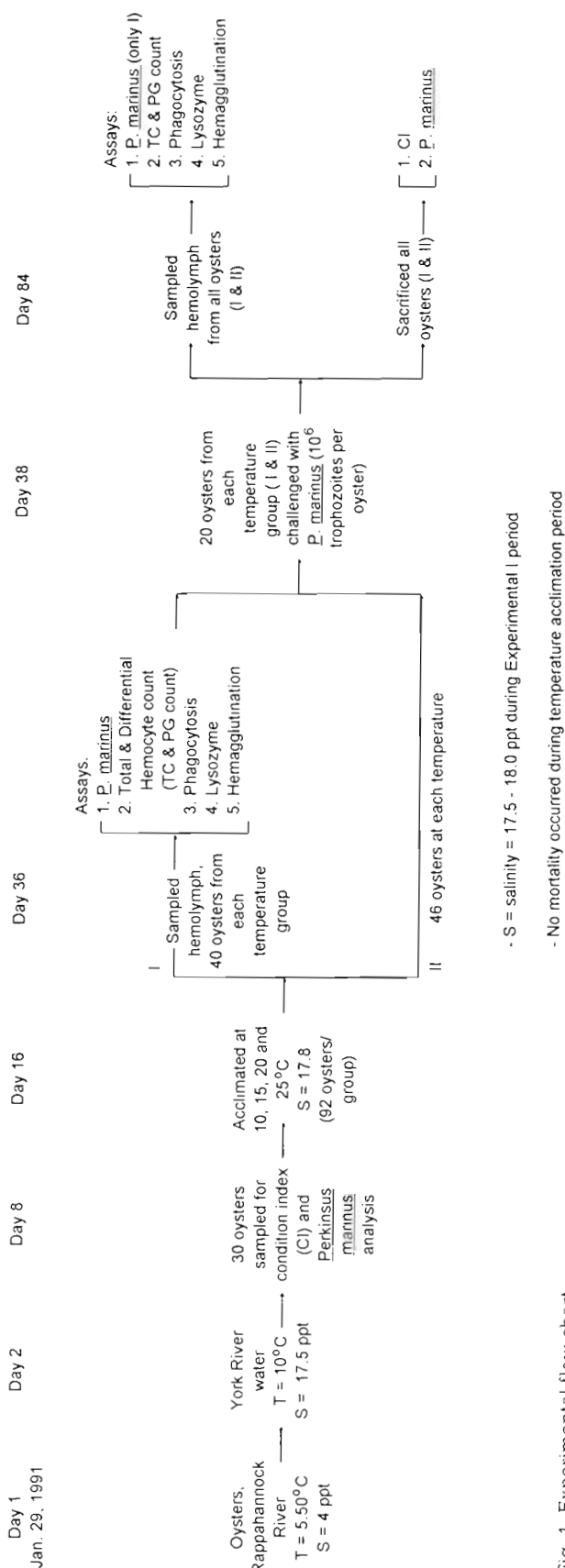


Fig. 1. Experimental flow chart

field are limited by temperature and salinity. Results of laboratory studies support field observation; the prevalence and intensity of *P. marinus* infection in laboratory-maintained oysters were correlated with temperature. Chu & Greene (1989) recently described the temperature and salinity effects on *in vitro* culture of *P. marinus*. They reported that temperature of 28 to 30 °C favored the *in vitro* development of prezoospores to sporulation.

The severe mortality caused by the disease has heightened interest in the study of oyster defense mechanisms and the influence of environmental factors on these mechanisms. The oyster's internal defense involves cellular (hemocytes) and humoral (e.g. lysosomal enzymes and agglutinins/lectins) components (Chu 1988, Feng 1988). Environmental temperatures significantly affect and modify cellular and humoral activities in oysters (Feng & Canzonier 1970, Fisher 1988, Chu & La Peyre 1989). It has been shown that high water temperature inhibits hemocyte spreading and locomotion in *Crassostrea virginica*. Hemolymph lysozyme has been found to vary greatly between seasons, apparently related to water temperature. Serum lysozyme levels in *C. virginica* were reported as being higher in the winter (December) than in the summer (June) (Feng & Canzonier 1970). Similarly, lysozyme concentration was extremely low in the summer (Chu & La Peyre 1989). The above observations suggest that the defense capacity of oysters may be influenced by temperature. The seasonal variability of environmental temperature may contribute to the observed fluctuation in disease susceptibility.

It is not known whether the suppression or change in the defense-related activities of the host is linked to its disease susceptibility. The effects of temperature on defense-related activities of the oyster and subsequent susceptibility to *Perkinsus marinus* have not been defined. The present study investigated the effects of temperature on the interaction between the oyster and its parasite, *P. marinus*, and determined whether there is a linkage between the oyster's defense activities and its susceptibility to *P. marinus*.

## METHODS AND MATERIALS

**Experiment.** The experimental steps involved in this experiment are summarized in Fig. 1. Oysters were collected from Ross Rock, Rappahannock River (Virginia, USA), on January 29, 1991 (ambient temperature = 5.5 °C, salinity = 4 ppt). Oysters were cleaned of fouling organisms and maintained in estuarine water [York River water (YRW), ppt = 17.5] and thereafter in 250 l tanks at 10 °C, and algal paste (a mixture of Tahitian *Isochrysis galbana* and *Thalassiosira pseudo-*

*nana*) was fed to oysters daily ( $0.1 \text{ g oyster}^{-1}$ ). On February 5, 1991, a subsample of 30 oysters was sacrificed for condition index (CI: dry meat weight/dry shell weight  $\times 100$ ; Lucas & Beninger 1985) and *Perkinsus marinus* analysis (Ray 1952, 1966). *P. marinus* diagnosis in all oysters was negative. On February 13, 1991, oysters were distributed to the 4 test temperatures, 10, 15, 20 and  $25^\circ\text{C}$  (80 oysters per temperature, 20 oysters per 38 l aquarium, 4 aquaria per temperature treatment). Twenty days after the transfer to these temperatures, hemolymph was taken from 10 oysters from each aquarium at each temperature treatment to determine cellular and humoral activities, as well as possible *P. marinus* infection, using the hemolymph assay modified from Gauthier & Fisher (1990). For subsequent description, these oysters are referred to as bled oysters to distinguish from their counterpart, nonbled oysters. After hemolymph withdrawal, oysters were labeled and placed back in the aquaria. Each aquarium thus held 10 bled and 10 nonbled oysters. Oysters at each temperature were then divided into 2 groups, control and *P. marinus* challenged. The challenged groups from each temperature treatment were inoculated with a dose of  $10^6$  trophozoites  $\text{oyster}^{-1}$  in 0.1 ml YRW. Control oysters were inoculated with 0.1 ml of YRW. Forty-six days after challenge with *P. marinus*, hemolymph was drawn from both bled ( $n = 20$ ) and nonbled oysters ( $n = 20$ ) at each temperature for cellular and humoral assays. After completing hemolymph sampling, all experimental oysters were sacrificed to determine CI and rectal tissues were removed for tissue assay (Ray 1952, 1966). *P. marinus* analysis was also conducted on individual hemolymph samples of bled oysters.

**Preparation of trophozoite suspension.** Trophozoite suspension was prepared as follows: *Perkinsus marinus* infected oyster tissues were rinsed thoroughly with filtered ( $0.22 \mu\text{m}$ ) YRW and subsequently homogenized in  $0.22 \mu\text{m}$  filtered YRW with a blender (Virtis, Model 23) at high speed for 2 min. The suspension was then passed through  $100 \mu\text{m}$  and  $20 \mu\text{m}$  meshes to remove oyster tissue residues. The number of trophozoites in suspension was counted using a hemocytometer and adjusted to a concentration of  $10^7$  trophozoites  $\text{ml}^{-1}$ .

**Preparation of hemocytes and sera.** Hemocytes and serum were prepared as follows: hemolymph (1.5 ml) from individual oysters was withdrawn from the adductor muscle sinus with a syringe through notches in the shell and the hemolymph was placed in test tubes in an ice bath. Total hemocytes and number of granulocytes and agranulocytes were counted on each sample using a hemocytometer. For humoral activity measurements, serum of each hemolymph sample was separated from hemocytes through centrifugation

( $400 \times g$  at  $4^\circ\text{C}$  for 10 min). Sera were withdrawn and stored in a freezer ( $-20^\circ\text{C}$ ).

**Hemocyte and serum factor activities.** For phagocytosis measurement, hemolymph was obtained only from nonbled oysters. Pooled hemolymph samples ( $5 \text{ oysters sample}^{-1}$ ,  $4 \text{ samples temperature}^{-1}$ ) were assessed for phagocytosis of zymosan (yeast cell extract) at 10, 15, 20 and  $25^\circ\text{C}$ . A 0.02 ml aliquot of each hemolymph sample was added to a glass slide and allowed to adhere at the 4 assayed temperatures for 30 min. Two slides were prepared for each sample. A 0.02 ml aliquot of zymosan ( $1.0 \text{ mg ml}^{-1}$ ) in artificial seawater (ASW), with a salinity similar to the oyster-maintaining water, was added to the hemocyte monolayer. After incubation at assayed temperatures for 1 h, hemocyte monolayers were rinsed with ASW and fixed with Dietrich's fixative. Slides were then stained with Hemal-Stain (Hemal Stain Co., Inc., CT, USA) and attachment and phagocytosis were quantified using a light microscope. Phagocytosis and attachment of zymosan by hemocytes were determined in 5 fields (200 cells) of each slide. Results were expressed as a phagocytic index (no. of hemocytes that phagocytosed at least 1 zymosan particle/total no. of hemocytes) and phagocytic ratio (no. of zymosan particles phagocytized and associated/total no. of hemocytes).

Hemagglutinins were quantified in serum fractions of each oyster by addition of sheep erythrocytes (sheep red blood cells: SRBC) to serially diluted oyster serum in U-well microtiter plates. The titer for a given sample was expressed as the reciprocal of the maximal dilution producing complete hemagglutination (Tripp 1966) and  $\log_2$  transformed. Two percent SRBC suspension was prepared from SRBC stock (Becton Dickinson Microbiology System, Hunt Valley, MD, USA). Hemagglutination activity for all samples was measured using SRBC suspended in ASW of 10 ppt at room temperature. All serum samples were adjusted with distilled water to 10 ppt prior to the assay.

Lysozyme concentration was determined spectrophotometrically according to the method of Shugar (1952) and Chu & La Peyre (1989). Cell-free oyster serum (0.1 ml) was added to 1.4 ml of the bacterial *Micrococcus lysodeikticus* suspension and the decrease in absorbance was recorded at 450 nm on a Shimadzu UV 600 spectrophotometer for 2 min at room temperature ( $21 \pm 1^\circ\text{C}$ ). All measurements were duplicated. Recorded lysozyme activities were converted to lysozyme concentration using a standard curve constructed with different concentrations of egg-white lysozyme.

Serum protein was measured by the method of Lowry et al. (1951) using bovine albumin as a standard. A cell-free hemolymph sample (10  $\mu\text{l}$ ) from individual oysters was used for the serum protein measurement.

**Perkinsus assays.** Two assays, the Ray thioglycollate assay (1952, 1966) and the hemolymph assay (Gauthier & Fisher 1990), were used for *P. marinus* diagnosis. Rectal tissue was removed from each oyster and incubated in thioglycollate medium for 4 to 5 d. Intensity of infection was ranked from 0 (negative) to 5 (heavily infected) based on the number of stained *P. marinus* hyphospores contained in the oyster rectal tissue smear (Ray 1952, 1966). For the hemolymph assay, 0.3 ml hemolymph from each sampled oyster was taken. The procedure modified from Gauthier & Fisher (1990) is as follows: hemolymph samples were centrifuged at  $265 \times g$  at room temperature for 10 min. The pellets (hemocytes plus *P. marinus* trophozoites) were resuspended with 1.0 ml of thioglycollate medium (TM) containing 0.1 ml of  $8.0 \text{ mg ml}^{-1}$  of streptomycin and peni-cillin. Cultures were incubated at room temperature for 4 d, then centrifuged at  $265 \times g$  for 10 min to remove TM. Pellets were resuspended with 1.0 ml 2M NaOH and incubated in a water bath at about  $50^\circ\text{C}$  for 30 min to remove bacteria and hemocytes. Hyphospores were then washed twice with distilled water and stained with 1.0 ml of Lugol's working solution (Ray 1952, 1:10 dilution), transferred into a separate well of a 24-well tissue plate and examined under a Nikon inverted microscope. Intensity of infection was ranked from 0 (negative) to 5 (heavily infected) based on the number of hyphospores contained in the 1.0 ml culture.

**Statistical analysis.** One-way analysis of variance (ANOVA) and a Tukey-Kramer test were used to determine similarities and differences in total hemocyte counts (TC), percentage of granulocytes (PG), protein (P) and lysozyme (L) concentrations, hemagglutinin titers (H) and condition index (CI) among temperature treatments and between control and challenged oysters within each temperature treatment. Differences in

phagocytic ratio (PRA) and CI between temperature treatments and assay temperature were analyzed using a 2-way ANOVA followed by a Tukey-Kramer test. Data were  $\log_{10}$  or arcsin transformed whenever data showed a large variance. Differences were considered statistically significant if  $p < 0.05$ . Linear correlation was calculated among all measured variables.

## RESULTS

Mean ( $\pm 1$  SD) TC, PG, P and L concentrations and H titers in hemolymph of oysters after 20 d acclimation (initial sampling) at 10, 15, 20 and  $25^\circ\text{C}$  are shown in Table 1. Mean TC of oysters in 15, 20 and  $25^\circ\text{C}$  treatments were not significantly different from each other, but were significantly higher than TC in oysters at  $10^\circ\text{C}$ . PG in oysters at 10, 15 and  $20^\circ\text{C}$  was similar but significantly lower than oysters at  $25^\circ\text{C}$ . PG was positively correlated with temperature ( $n = 160$ ,  $r = 0.393$ ,  $p < 0.001$ ). Oysters at 10 and  $15^\circ\text{C}$  had similar P and L concentrations which were higher than the P and L concentrations in oysters at 20 and  $25^\circ\text{C}$ . L concentrations were negatively correlated with temperature ( $n = 160$ ,  $r = -0.598$ ,  $p < 0.001$ ). No significant differences in H titers were observed among groups. *Perkinsus marinus* diagnosis on hemolymph samples of acclimated oysters was negative.

*Perkinsus marinus* prevalence, calculated from results of the Ray tissue assay 46 d after initial exposure, is shown in Fig. 2. Data from bled and nonbled groups were pooled, since there was no difference in prevalence between these 2 groups. Disease prevalence was 100, 91, 46, and 23 % respectively, at 25, 20, 15 and  $10^\circ\text{C}$ . *P. marinus* prevalence and mean infection intensity (Fig. 3A, B) increased with temperature. Only 1 oyster from the  $20^\circ\text{C}$  nonbled control group was found

Table 1. Total (TC) and differential (PG) hemocyte counts ( $10^4$  cells  $\text{ml}^{-1}$ ), protein (P;  $\text{mg ml}^{-1}$ ) and lysozyme (L;  $\mu\text{g ml}^{-1}$ ) concentrations, and hemagglutination titers (H;  $\log_2$ ) in hemolymph of oysters after 20 d acclimation at 10, 15, 20 and  $25^\circ\text{C}$ .  $n = 40$ . Values shown represent mean  $\pm 1$  SD. Lines connect treatments not significantly different (1-way ANOVA, SNK; TC, P and L data were  $\log_{10}$  transformed; PG data were arcsin transformed. L was negatively correlated with temperature ( $n = 160$ ,  $r = -0.598$ ,  $p = 0.001$ . PG was positively correlated with temperature ( $n = 160$ ,  $r = 0.273$ ,  $p = 0.001$ ).

	Temperature ( $^\circ\text{C}$ )				p
	10	15	20	25	
TC	136.3 $\pm$ 188.2	150.6 $\pm$ 94.4	152.6 $\pm$ 65.9	170.8 $\pm$ 104.5	$p < 0.05$
PG	36.2 $\pm$ 19.0	40.0 $\pm$ 14.4	43.3 $\pm$ 16.1	49.2 $\pm$ 17.4	$p < 0.05$
P	8.2 $\pm$ 2.3	6.6 $\pm$ 1.3	5.0 $\pm$ 1.4	5.0 $\pm$ 1.4	$p < 0.05$
L	13.9 $\pm$ 11.4	10.0 $\pm$ 5.8 ( $n = 39$ )	7.3 $\pm$ 3.2 ( $n = 38$ )	6.3 $\pm$ 3.1	$p < 0.05$
H	3.7 $\pm$ 2.4	3.6 $\pm$ 2.4	3.6 $\pm$ 2.1	4.1 $\pm$ 2.4	$p > 0.05$



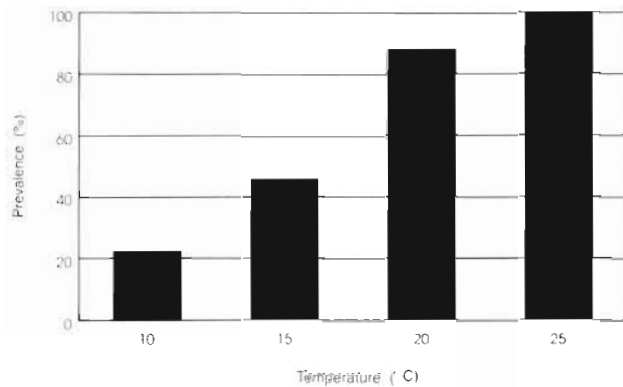


Fig. 2. *Crassostrea virginica*. *Perkinsus marinus* prevalence in oysters at 10, 15, 20 and 25 °C 46 d after challenge with  $10^6$  trophozoites oyster<sup>-1</sup>. Data from bled and nonbled groups combined (n = 44)

to have a light *P. marinus* infection. Infection intensity determined by the hemolymph assay exhibited a linear correlation (n = 73,  $r = 0.764$ ,  $p < 0.001$ ) with the results from tissue assays. Weighted incidence (sum of disease code numbers/number of oysters) calculated from the hemolymph and tissue assay data displayed a pattern similar to disease prevalence and infection intensity (results not shown).

TC in bled and nonbled oysters after 46 d exposure to *Perkinsus marinus* is summarized in Fig. 4. Mean TC was lowest at 10 °C and highest at 25 °C for both bled and nonbled groups. A significant difference was found in TC between control and challenged oysters in the bled group at 15 °C and in the nonbled group at 20 °C. At 25 °C, TC in challenged oysters was significantly higher than in control oysters. Generally, in both control and challenged groups, mean TC appeared to be higher in nonbled oysters than in bled oysters. It is interesting to note that at 10 °C there was a significant decrease in TC between initial and final samples (Table 1, Fig. 4).

A similar trend in PG (Fig. 5) was observed. In both bled and nonbled groups, control and challenged oysters exhibited lower PG at lower temperatures. PG at 10 °C in all groups was significantly lower than at other temperatures. No significant difference was observed between control and challenged oysters of the bled groups; however, PG in control oysters was higher than in challenged oysters in

the nonbled groups. PG of oysters in bled groups sampled at the beginning of the experiment was significantly higher than in oysters sampled at the end of the experiment; but, in nonbled groups, only oysters at 10 °C, PG was lower at the end of the experiment compared to the beginning of the experiment (20 d after exposure at test temperatures, prior to *Perkinsus marinus* challenge).

PRA and phagocytic index (PIN) for hemocytes from each temperature treatment assayed at 10, 15, 20 and 25 °C are shown in Fig. 6A, B. The data shown in this figure were pooled from control and challenged nonbled oysters because no significant differences in mean PRA and PIN were found between control and *Perkinsus marinus* challenged oysters and no effect was observed due to infection status. Subsequent sta-

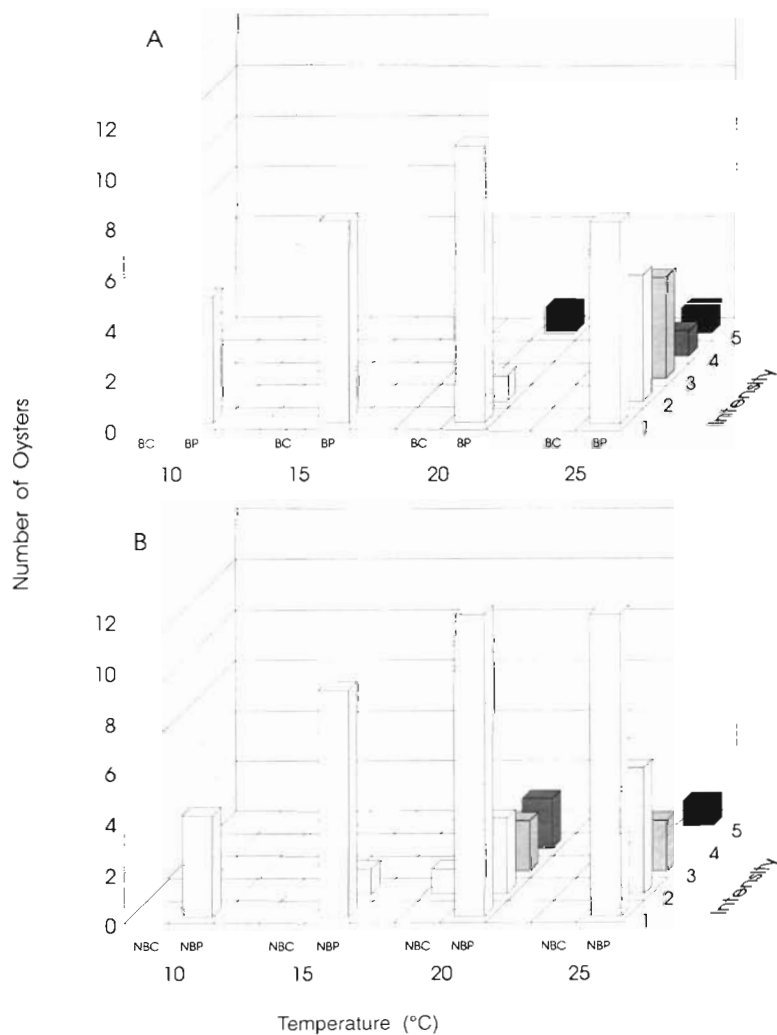


Fig. 3. *Crassostrea virginica*. *Perkinsus marinus* infection intensity in oysters (n = 20) at 10, 15, 20 and 25 °C (1 = light, 2 = light moderate, 3 = moderate, 4 = moderate heavy, 5 = heavy). (A) BC: bled control; BP: bled *P. marinus* challenged. (B) NBC: nonbled control; NBP: nonbled *P. marinus* challenged

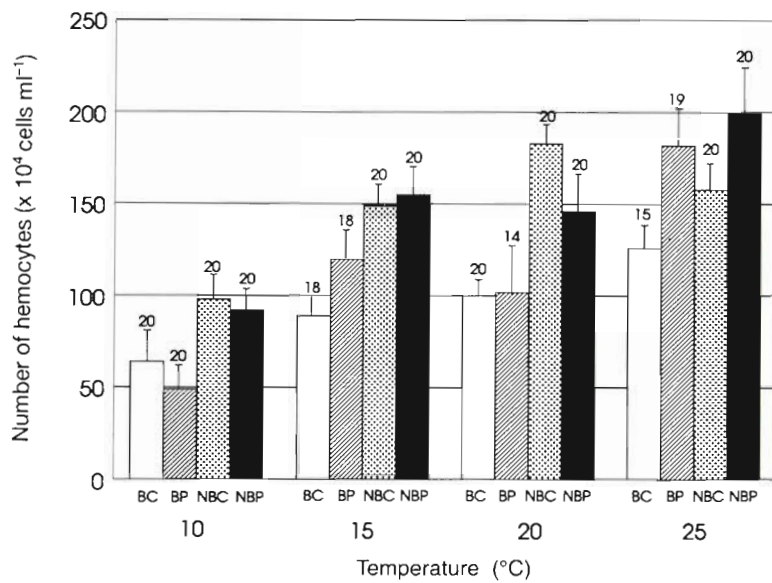


Fig. 4. *Crassostrea virginica*. Mean total hemocyte count (TC) + SE of oysters at 10, 15, 20 and 25 °C 46 d after *Perkinsus marinus* challenge (n = 14 to 20; number of oysters is indicated on the top of each bar). Abbreviations as in Fig. 3

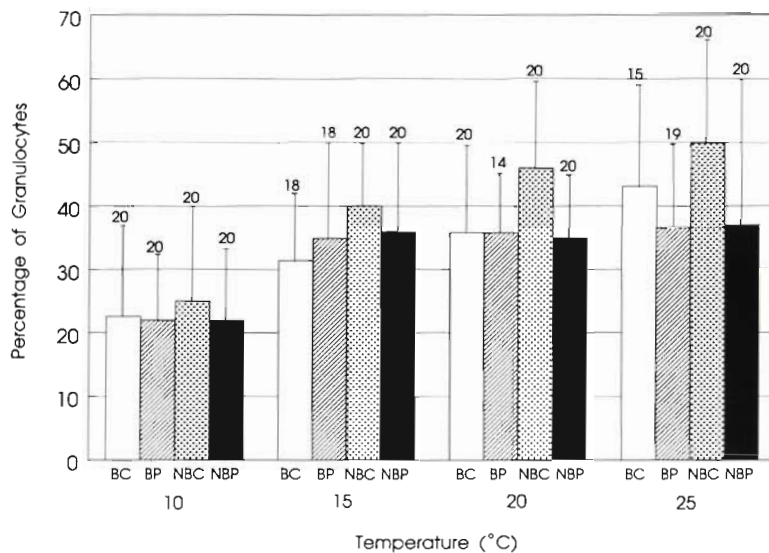


Fig. 5. *Crassostrea virginica*. Mean percentage of granulocytes (PG) + SD in oysters at 10, 15, 20 and 25 °C 46 d after *Perkinsus marinus* challenge (n = 14 to 20; number of oysters is indicated on the top of each bar). Abbreviations as in Fig. 3

tistical analysis was performed on pooled data indicating significant differences ( $p < 0.01$ ) in PRA and PIN due to treatment temperature and assayed temperature. Both PRA and PIN increased with the increase of treatment temperature from 10 to 20 °C, but declined at 25 °C. Significant interaction between assay and treatment temperatures in PIN was detected ( $p < 0.01$ ). The oysters exposed to 15 °C exhibited the highest

PRA at all assay temperatures tested, but the 20 °C treatment group had the highest PIN. Within each treatment group, the highest PRA and PIN occurred at an assay temperature of 20 °C. PRA and PIN were significantly reduced when hemocytes from 20 and 25 °C treatment groups were assayed at 10 °C.

Generally, all oysters exhibited increased hemolymph L concentrations at the end of the experiment compared to the beginning of the experiment (Table 1, Fig. 7). In all temperature treatments, no significant difference in L concentration was observed between control and challenged groups and between infected and uninfected oysters (results not shown). Differences in mean L concentration among temperatures are emphasized when data from challenged and control oysters are pooled (Fig. 8). L concentration in oysters at 10 °C was significantly higher than in oysters at other temperatures. Hemolymph L in oysters sampled at the end of the experiment was negatively correlated with temperature (n = 299,  $r = -0.2682$ ,  $p < 0.001$ ) but positively correlated with CI (n = 295,  $r = 0.2912$ ,  $p < 0.001$ )

Hemolymph P (Fig. 9) did not vary much among temperature treatments. However, when ANOVA analyses were performed on data pooled from control and *Perkinsus marinus* challenged oysters, results indicate that oysters, both bled and nonbled, at 10 °C had significantly higher p than oysters at 15, 20 and 25 °C. P level declined in oysters at 10 and 15 °C over the course of the experiment. The P concentrations of challenged oysters at 25 °C, which were 100 % infected with *P. marinus*, were not significantly different from control (uninfected) oysters. At 10 and 20 °C, P concentration in nonbled *P. marinus* challenged oysters was higher than in control oysters. Similarly at 20 °C, bled challenged oysters, 91 % of which were infected by *P. marinus*, had more P than control oysters.

Results of ANOVA indicated that no difference existed in H titers (Fig. 10) in bled groups among temperatures. However, within nonbled control groups, hemagglutination at 15 °C was significantly higher than other groups. No difference was found between control and challenged groups except in nonbled oysters at 10 and 15 °C and in bled oysters at 25 °C. There was a trend of higher H in nonbled oys-

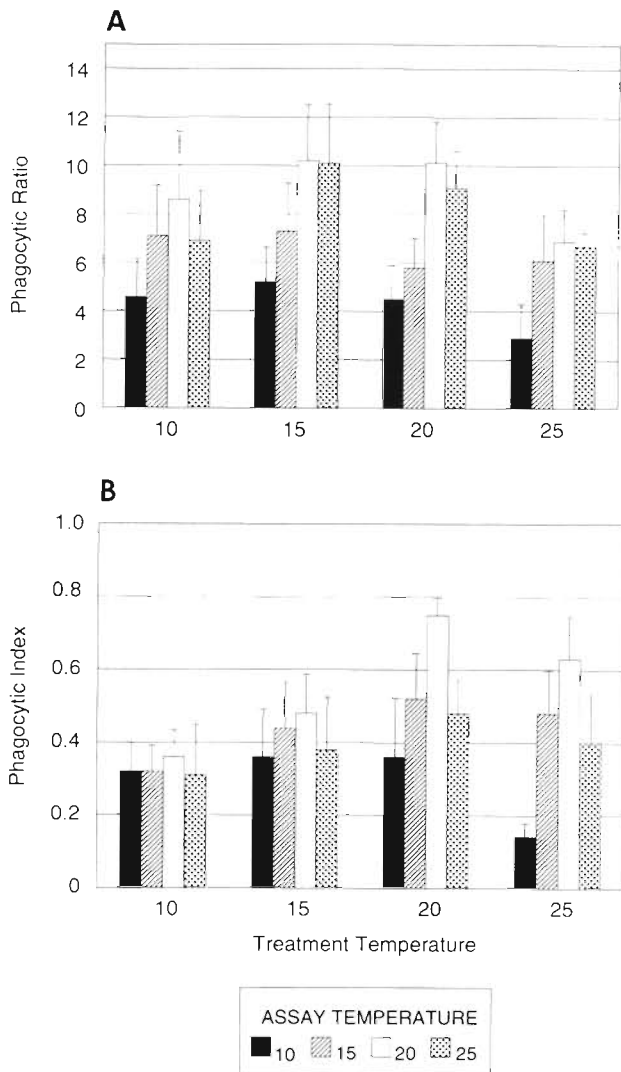


Fig. 6. *Crassostrea virginica*. Phagocytic ratio (PRA) and index (PIN) of hemocytes from nonbled oysters from each temperature treatment assayed at 10, 15, 20 and 25 °C

ters than in bled oysters in both control and challenged groups.

CI of oysters sampled at the end of the experiment is shown in Fig. 11. In both bled and nonbled groups, no difference was found in CI between control and challenged oysters at any temperature. CI was determined to be negatively correlated with temperature (BC: bled control,  $r = 0.5128$ ; BP: bled challenged,  $r = -0.6260$ ; NBC: nonbled control,  $r = 0.4095$ ; NBP: nonbled challenged,  $r = -0.4270$ ). Oyster CI decreased at the end of the experiment compared to the CI measured 8 d after oysters were collected.

Table 2 gives correlation coefficients between cellular and humoral measurements, oyster CI, parasitism and temperature in infected and uninfected oysters at the end of the experiment. Data from bled and nonbled

groups were pooled and only the parameters which displayed significant correlation ( $p \leq 0.05$ ) are shown in the table.

## DISCUSSION

The present study clearly demonstrates that disease prevalence and intensity of the parasite in oysters are positively related to test temperatures. This is consistent with previous findings in field and laboratory studies. It has been suggested that repeated bleeding of oysters may result in increased susceptibility to *Haplosporidium nelsoni* infection (Ford 1986b). Bleeding oysters once did not seem to affect *Perkinsus marinus* susceptibility; the bled and nonbled groups displayed a similar pattern of infection. Although none of the oysters ( $n = 30$ ) collected from the Rappahannock River initially tested positive for *P. marinus*, cryptic infections apparently existed in experimental oysters; 1 control oyster from the nonbled group at 20 °C was found to be lightly infected by the parasite.

Temperature significantly influenced TC and, to some extent, PG in oysters. Higher TC and PG were observed in oysters acclimated at higher temperatures and this continued through the end of the experiment. These results could be explained in terms of a higher heartbeat rate resulting in a larger number of circulating hemocytes (Feng 1965) and/or higher production rate of hemocytes and granulocytes at higher water temperatures. We observed seasonal variation of TC and PG in oysters collected monthly from Deep Water Shoal of James River, Virginia, USA, corresponding to seasonal temperature (Chu et al. unpubl. results). Higher TC and PG occurred in the months of highest water temperature (e.g. June, July and August).

The positive correlation between TC and *Perkinsus marinus* intensity in infected oysters (Table 2) may be an indication of pathological effects in oysters. Ling (1990) also found a significant increase in hemocyte number (hemocytosis) in the hemolymph of oysters infected by *Haplosporidium nelsoni*. The observation of higher TC in oysters of nonbled groups than in oysters of bled groups and the significant decrease of PG in oysters of bled groups at the end of the experiment suggest that bleeding may to some extent reduce TC and PG in oysters. Prolonged low temperature exposure also reduced TC and PG in oysters. At 10 °C, both parameters were lower at the end than at the beginning of the experiment (Table 1, Figs. 4 and 5).

Phagocytosis measured in pooled hemocyte samples at the end of the experiment indicated that phagocytic activity was temperature-dependent (Fig. 6). A temperature increase from 10 to 20 °C heightened phagocytic activity. Increased temperature was reported to

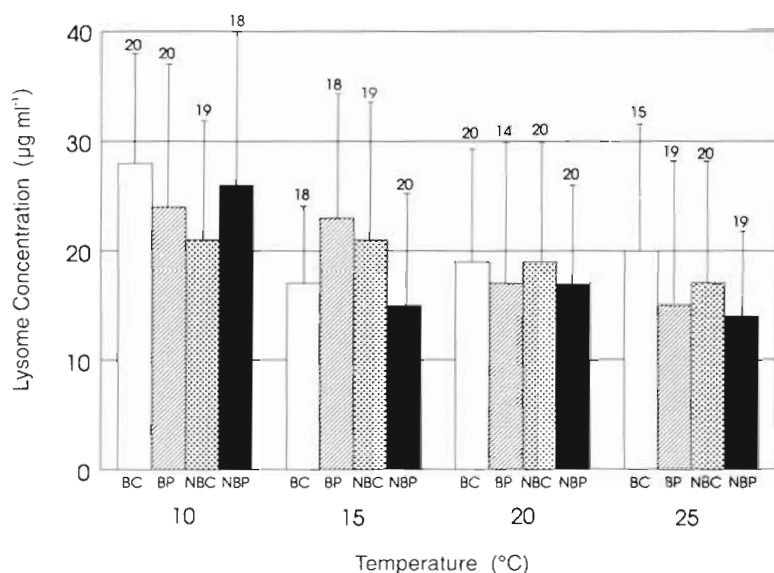


Fig. 7 *Crassostrea virginica*. Mean hemolymph lyszyme concentration (L) + SD of oysters at 10, 15, 20 and 25 °C 46 d after *Perkinsus marinus* challenge (n = 14 to 20; number of oysters is indicated on the top of each bar) Abbreviations as in Fig. 3

enhance hemocyte spreading and locomotion of the eastern oyster (Fisher 1988). Decline of hemocyte activity at temperatures higher than 20 °C was not observed in Fisher's study. Rate of hemocyte locomotion was highest at the highest experimental temperature of 26 °C. However, hemocyte spreading capacity was found to correspond with annual temperature cycle: rapid in late fall, winter and early spring and slow from late spring to early fall (Fisher et al. 1989). In the present study, less effective phagocytic activity at a temperature of 25 °C, which favors parasite development, may be one of the reasons why 100 % of the challenged oysters are infected at 25 °C with the highest disease intensity.

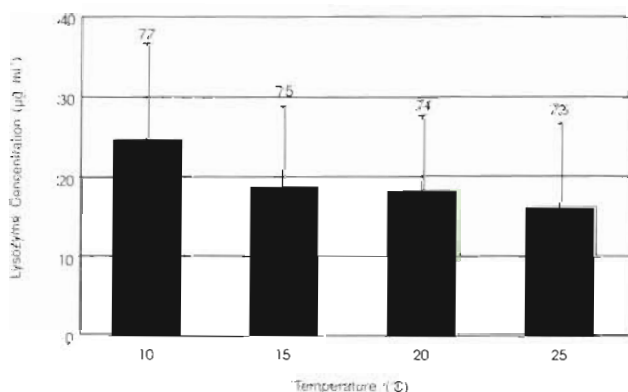


Fig. 8 *Crassostrea virginica*. Mean hemolymph lyszyme + SD in oysters at 10, 15, 20 and 25 °C. Data from each temperature were pooled (n = 80)

It is believed that hemocytes in bivalves are the primary line of defense (Cheng 1981, Feng 1988). Results of this study indicate that the ability of hemocytes to defend oysters against *Perkinsus marinus* may be compromised at higher temperatures. At a higher temperature, oysters had a greater number of circulating hemocytes and phagocytic capability but did not have lower prevalence or less intense *P. marinus* infections. We also observed *in vitro* phagocytosis of *P. marinus* trophozoites by hemocytes from eastern oysters at a temperature of  $15 \pm 1$  °C and at a salinity of about 20 ppt (La Peyre et al. unpubl.). Electron microscopy, however, revealed that only 25 % of the phagocytosed trophozoites were killed. Apparently, oyster hemocytes are able to recognize the parasite *P. marinus* and intracellular killing may have occurred, but the rate of killing may not be sufficient to cope with multiplication of the parasite because of its high metabolic activity.

The observed higher L concentration in oysters acclimated at 10 and 15 °C for 20 d that remained high throughout the whole experiment is a result of temperature effect, not a response to *Perkinsus marinus* challenge and/or infection, since no significant difference in L concentration was observed between control and challenged or between infected and uninfected oysters. A defense role has been suggested for lysosomal enzymes in vertebrates and invertebrates (Ingram 1980, Jolles & Jolles 1984). The significant defense role of lysosomal enzymes in bivalve molluscs has also been documented (e.g. McDade & Tripp 1967a, b, Cheng 1978, Cheng 1979, Huffmann & Tripp 1982, Moore & Gelder 1985), and reviewed and discussed by Cheng (1983a, b, c) and Chu (1988). L concentrations in oysters also decreased with increased salinity (Chu et al. unpubl.). However, it is not known whether extracellular L plays a role in oyster defense through extracellular deterioration of the parasite when its activity is already reduced at low temperature and low salinity (Chu & Greene 1989). It is also unknown whether intracellular L increases in the same manner as extracellular L in oysters at low temperature or at low salinity. Moreover, the lytic activity of L on many species of bacteria has been demonstrated (see reviews by Cheng 1983 and Chu 1988) but its effect on a pathogenic parasite, such as *P. marinus*, has not been tested.

The ability to agglutinate latex beads was greater in *Haplosporidium nelsoni* resistant oysters than in *H. nelsoni* susceptible oysters, and as infection intensified in susceptible oysters, agglutination of latex beads de-



clined (Ling 1990). Decrease of bacterial agglutination was also observed in *H. nelsoni* susceptible oysters (Tamplin & Fisher 1989), but that is not the case with H measured in oysters in this study. H titer was the only parameter unaffected by the temperature change imposed upon the oysters and did not vary with the condition of the oysters. However, bleeding may reduce hemagglutinins in oysters.

The low hemolymph P in oysters at high temperatures may be due to relatively high metabolic activity at high temperature and/or consumption of protein for energy and cell e.g. hemocyte production. It is not certain, at this stage, whether or not the lower P concentration of oysters at 10 and 15 °C at the end of the experiment is attributable to the low feeding rate of these oysters at persistent low temperature.

Oysters infected by *Haplosporidium nelsoni* had low hemolymph and tissue P (Ford 1986a, b, Barber et al. 1988, Ling 1990). In the present study, hemolymph P concentrations in oysters were not affected by *Perkinsus marinus* infections. But, it should be noted that hemolymph P decreased only when oysters were heavily infected by *H. nelsoni* (Ling 1990) and P reduction in tissues occurred only in oysters systematically infected with *H. nelsoni* (Barber et al. 1988). Most (88 %) of the infected oysters in this study were only lightly infected (intensity: 1 to 2) with the parasites, so depletion of P may not have occurred in these oysters.

The negative correlation between CI and infection intensity in infected oysters (Table 2) is analogous to results reported by Paynter & Bureson (1991). The low CI in oysters at 25 °C may be due to higher metabolism. It is not known whether the decrease of CI in oysters at the end of the experiment compared to the CI of the oysters 8 d after collection is due to nutritional stress and/or to the stress of a confined environment.

In summary, prevalence and intensity of *Perkinsus marinus* infection in oysters are positively related to temperature. Temperature also affected the physiology of the oysters. Exposing oysters from an ambient temperature of 5.5 °C to test temperatures of 10, 15, 20 and 25 °C for 20 d induced significant differences in TC, PG and hemolymph P and L concentrations among

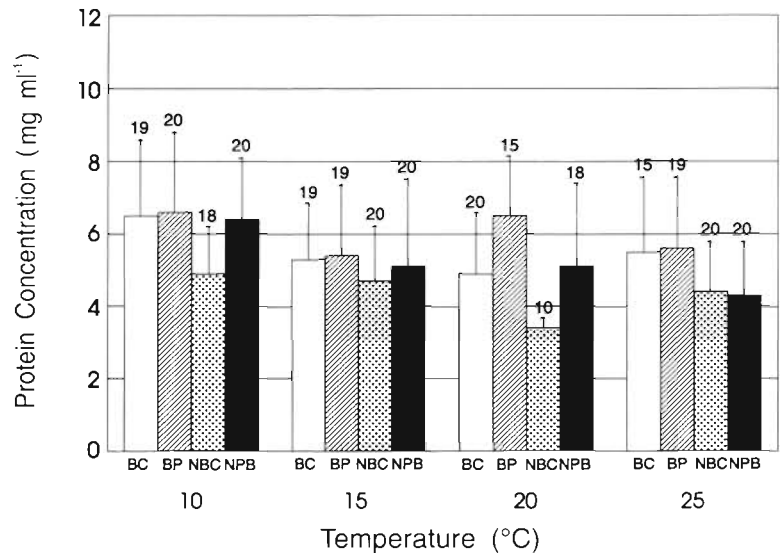


Fig. 9. *Crassostrea virginica*. Mean hemolymph protein concentration (P) + SD in oysters at 10, 15, 20 and 25 °C 46 d after *Perkinsus marinus* challenged (n = 15 to 20; number of oysters is indicated on the top of each bar). Abbreviations as in Fig. 3

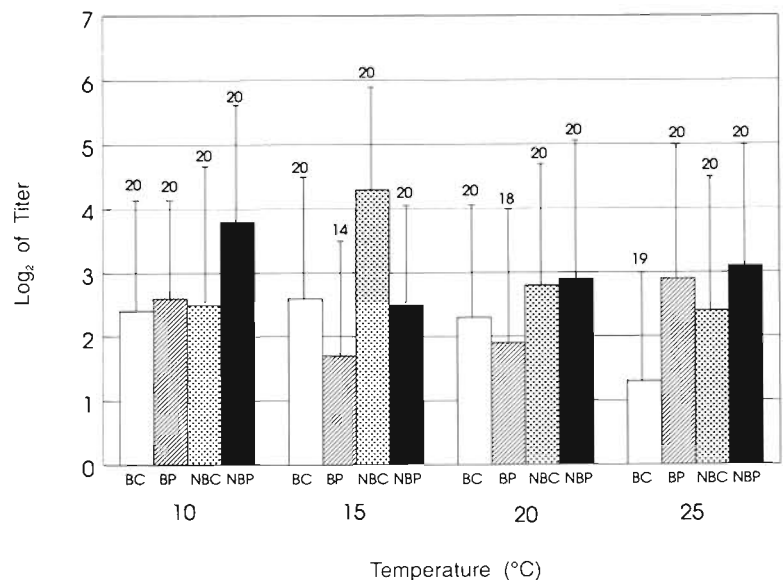


Fig. 10. *Crassostrea virginica*. Mean hemagglutination titer (H) in oysters at 10, 15, 20 and 25 °C 46 d after *Perkinsus marinus* challenged (n = 14 to 20; number of oysters is indicated on the top of each bar). Abbreviations as in Fig. 3

certain treatment groups. These differences persisted throughout the experiment. Oysters with a greater number of circulating hemocytes and phagocytic capability at a higher temperature did not have fewer or less intense *P. marinus* infections. The oysters at 10 °C which had the lowest *P. marinus* prevalence had the highest extracellular L concentration, suggesting the L

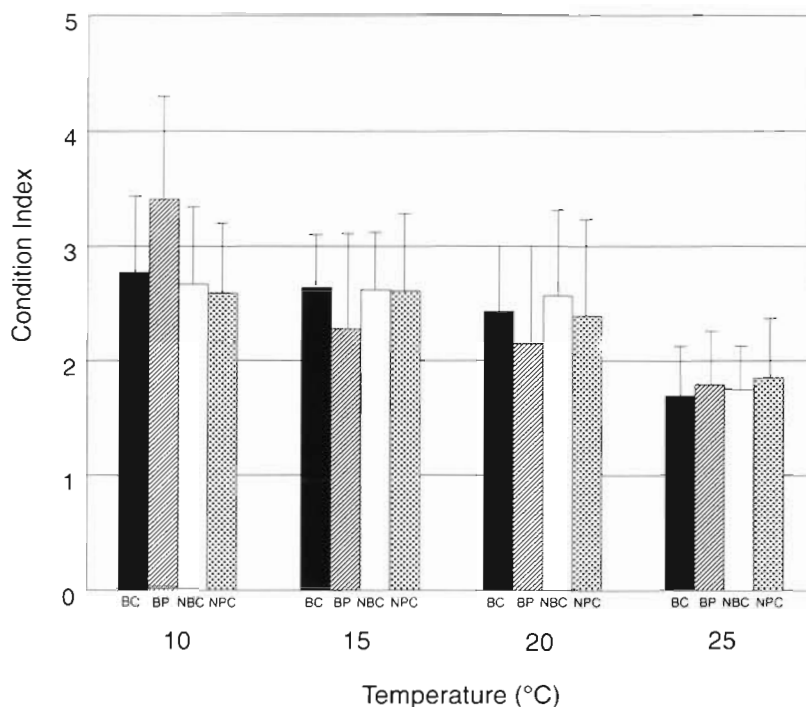


Fig. 11. *Crassostrea virginica*. Mean condition index (CI) ± SD of oysters sampled at the end of the experiment from 10, 15, 20 and 25 °C treatment groups (n = 15 to 20). Abbreviations as in Fig. 3

Table 2. Correlation coefficients between cellular parameters, humoral parameters, oyster condition indices, infection intensity and temperatures of uninfected and infected oysters. TC: total hemocyte counts; PG: % of granulocytes; L: lysozyme; CI: condition indices; PI: infection intensity of *P. marinus*. [Only significant correlations ( $p < 0.05$ ) are shown]

	Uninfected oysters					Infected oysters					
	TC <sup>a</sup>	DC <sup>a</sup>	P <sup>c</sup>	L <sup>b</sup>	CI <sup>d</sup>	TC <sup>d</sup>	DC <sup>d</sup>	P <sup>e</sup>	L <sup>e</sup>	CI <sup>d</sup>	PI <sup>d</sup>
Temperature (°C)	0.34	0.51	-0.27	-0.20	-0.46	0.35	0.28	-0.20	-0.27	-0.57	0.38
TC	-	0.65	-0.19	-	-0.22	-	0.17	-0.29	-	-0.24	0.33
DC	-	-	-0.31	-0.16	-0.24	-	-	-	-	-	-
H <sup>f</sup>	-	-	-	-	-	-	-	-	-	-	-
P	-	-0.31	-	-	0.15	-	-	-	0.18	-	-
L	-	-0.16	-	-	0.30	-	-	-	-	0.26	-0.26
CI	-	-0.24	0.15	0.30	-	-	-	-	0.26	-	-0.26

<sup>a</sup> n = 197; <sup>b</sup> n = 192; <sup>c</sup> n = 183; <sup>d</sup> n = 101; <sup>e</sup> n = 96–99; <sup>f</sup> n = 197 (uninfected oysters) or 96–99 (infected oysters)

may be one of the factors attributing to the low *P. marinus* susceptibility in these oysters. However, at this time, the role of extracellular L in the oyster's defense is completely unknown and remains to be examined.

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