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**Studies on the oyster pathogen *Perkinsus marinus* (Apicomplexa): Interactions with host defenses of *Crassostrea virginica* and *Crassostrea gigas*, and *in vitro* propagation**

La Peyre, Jerome Frederic, Ph.D.

The College of William and Mary, 1993

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

# STUDIES ON THE OYSTER PATHOGEN *PERKINSUS MARINUS* (APICOMPLEXA): INTERACTIONS WITH HOST DEFENSES OF *CRASSOSTREA VIRGINICA* AND *CRASSOSTREA GIGAS*, AND *IN VITRO* PROPAGATION.

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A dissertation presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
of the requirements for the degree of
Doctor of philosophy

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by
Jerome F. La Peyre
1993

## APPROVAL SHEET

This Dissertation is submitted in Partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Jerome F. La Peyre

Approved, August 1993

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DEDICATION

This dissertation is dedicated to my parents

TABLE OF CONTENTS

page

ACKNOWLEDGEMENTS........................................ vi

LIST OF TABLES.......................................... vii

LIST OF FIGURES......................................... ix

ABSTRACT................................................ xii

GENERAL INTRODUCTION.................................... 2

CHAPTER 1. POTENTIAL HOST DEFENSE ACTIVITIES AND PROGRESSION OF *PERKINSUS MARINUS* DISEASE IN EASTERN OYSTERS FROM THE GULF AND EAST COASTS, USA.

Introduction............................................ 23
Materials and methods................................... 24
Results................................................. 29
Discussion.............................................. 35

CHAPTER 2. HEMOCYTIC AND HUMORAL ACTIVITIES OF EASTERN AND PACIFIC OYSTERS FOLLOWING CHALLENGE BY THE PROTOZOAN *PERKINSUS MARINUS*.

Introduction............................................ 46
Materials and methods................................... 48
Results................................................. 53
Discussion.............................................. 60

CHAPTER 3. SIMPLE PROCEDURE FOR THE ISOLATION OF *PERKINSUS MARINUS* MEROZOITES, A PATHOGEN OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.................................. 70

CHAPTER 4. *IN VITRO* PROPAGATION OF THE PROTOZOAN, *PERKINSUS MARINUS*, A PATHOGEN OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.

Introduction............................................ 78
Materials and methods................................... 79
Results................................................. 90
Discussion.............................................. 97

CHAPTER 5. INITIATION OF *IN VITRO* CULTURES OF THE OYSTER PATHOGEN *PERKINSUS MARINUS* (APICOMPLEXA) WITH HYPNOSPORES.


Introduction.......................................... 103
Materials and methods................................. 104
Results and discussion................................ 105


CHAPTER 6. *IN VITRO* INTERACTION OF *PERKINSUS MARINUS* MEROZOITES WITH EASTERN AND PACIFIC OYSTER HEMOCYTES.


Introduction.......................................... 111
Materials and methods................................. 113
Results............................................... 120
Discussion............................................ 129

GENERAL DISCUSSION.................................... 136

LITERATURE CITED...................................... 150

VITA.................................................. 177

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LIST OF TABLES

page

CHAPTER 1:

Table 1........................................... 30

Prevalence, mean intensity and weighted incidence of *P. marinus* infection in control and challenged UJ, LJ and GB oysters prior to challenge and at three and seven weeks post-challenge (N=15).

Table 2........................................... 33

Mean (± SD) density of circulating hemocytes, percentage of granulocytes, percentage of phagocytic hemocytes, number of zymosan particles per phagocytic hemocyte and hemocyte background and net chemiluminescence (CL) in control and challenged UJ, LJ and GB oysters prior to challenge and at three and seven weeks post-challenge. CPM = Counts per minute. N=15.

Table 3........................................... 36

Mean (± SD) plasma hemagglutinin titer and lysozyme concentration in control and challenged UJ, LJ and GB oysters prior to challenge and at three and seven weeks post-challenge (N=15).

CHAPTER 2:

Table 1........................................... 54

*Perkinsus marinus* prevalence and intensity of infection in control and challenged eastern (*C. virginica*) and Pacific (*C. gigas*) oysters from Exp. 1. L-M-H indicates the number of oysters with light, moderate and heavy infections.

Table 2........................................... 55

*Perkinsus marinus* prevalence and intensity of infection in eastern (*C. virginica*) and Pacific (*C. gigas*) oysters from Exp. 2. L-M-H indicates the number of oysters with light, moderate and heavy infections.

Table 3............................................ 59

Density of circulating hemocytes, percentage of granulocytes, percentage of phagocytic hemocytes, number of zymosan particles phagocytosed, net hemocyte chemiluminescence (CL), time of peak CL and plasma hemagglutinin titer, lysozyme and protein concentration in eastern (*C. virginica*) and Pacific (*C. gigas*) oysters from Exp. 2. Values shown are means ± SD. Number of oysters is indicated in parentheses. Asterisks (*) denote significance at $p<0.05$ level.

CHAPTER 4:

Table 1............................................ 81

Composition of the medium JL-ODRP-1. Final pH 7.5; osmolality, 650 mOsm/kg.

## LIST OF FIGURES


page

CHAPTER 2:

Figures 1 and 2.................................... 56

Mean (± SD) density of circulating hemocytes and mean (± SD) percentage of granulocytes in control and challenged, eastern (*C. virginica*) and Pacific (*C. gigas*) oysters from Exp. 1. Number of oysters is indicated on the top of each bar. Asterisks (*) denote significance at $p<0.05$ between challenged and control oysters for either oyster species. Double Asterisks (**) denote significance at $p<0.05$ between eastern and Pacific oysters for either control or challenged oysters

Figures 3-5........................................ 57

Mean (± SD) of plasma hemagglutinin titer, lysozyme concentration and protein concentration, in control and challenged eastern (*C. virginica*) and Pacific oysters (*C. gigas*) from Exp. 1. Number of oysters is indicated on the top of each bar. Asterisks (*) denote significance at $p<0.05$ between challenged and control oysters for either oyster species. Double Asterisks (**) denote significance at $p<0.05$ between eastern and Pacific oysters for either control or challenged oysters.

CHAPTER 3:

Figure 1........................................... 73

Phagosome of a hemocyte filled with *Perkinsus marinus* merozoites (M). Hemocyte nucleus (N). Bar = 10 $\mu$m.

Figure 2........................................... 74

Light micrograph of isolated and partially purified *Perkinsus marinus* merozoites with typical lipid droplets (L). Bar = 10 $\mu$m.

CHAPTER 4:

Figures 1-6........................................ 91

Light micrographs of *Perkinsus*-1 cells. 1. Culture illustrating varying sizes and division stages of cells. 2. Cells showing prominent vacuoles (V) and refractile lipid droplets (L). 3. Mother cells dividing by progressive cleavage of protoplast. 4. Cleavage furrows forming simultaneously throughout a mother cell protoplast. 5. Rupture of mother cell wall due to enlargement of daughter cells. 6. "Budding" in cultured cells. Bar = 10 μm.

Figures 7-9........................................ 94

Electron micrographs of *Perkinsus*-1 cells. 7. Characteristic *Perkinsus*-1 cells with thick cell wall (W), prominent vacuole (V) lined with electron dense material (Va), lipid droplet (L), nucleus (N) with prominent nucleolus (Nu). Bar = 2 μm. 8. Lomosomes (arrow) in a daughter cell. Bar = 1 μm. 9. Higher magnification of Fig 8 illustrating lomosomes. Bar = 0.5 μm.

Figures 10-12........................................ 96

Immunostaining of *Perkinsus*-1 cells and *Perkinsus marinus* in *C. virginica*. Cells in figures 10 and 11 were treated with polyclonal rabbit anti-serum. 10. *Perkinsus*-1 cells. 11. *Perkinsus marinus* in oyster connective tissue (positive control). 12. *Perkinsus*-1 cells treated with normal rabbit serum (negative control). Bar = 30 μm.

CHAPTER 5:

Figure 1........................................ 107

Merozoites (arrow) produced by progressive cleavage of a *Perkinsus marinus* hypnospore. Bar = 15 μm.

Figure 2........................................ 108

*Perkinsus marinus* hypnospore in later stage of division by successive bipartition. Hypnospore discharge pore (DP), discharge tube (DT). Bar = 15 μm.

CHAPTER 6:

Figure 1.......................................... 121

Light micrograph of eastern oyster hemocytes showing a small hyalinocyte (SH), a large hyalinocyte (LH) and a granulocyte (G). Bar = 10 μm.

Figures 2-3....................................... 122

Light micrographs of eastern oyster hemocytes associated with *P. marinus* merozoites (M, Fig 2) and zymosan (Z, Fig 3). Bar = 10 μm.

Figures 4-5....................................... 124

Typical chemiluminescent response curves of eastern oyster hemocytes (Fig 4) and Pacific oyster hemocytes (Fig 5) stimulated with *P. marinus* or zymosan. Arrow indicates time at which either zymosan or *P. marinus* was added.

Figures 6-7....................................... 126

Electron micrographs of *P. marinus* merozoites in eastern oyster granulocytes. 6. Merozoites (P) being ingested, and contained in phagosomes that are possibly fusing (arrow) at 15 min. Pseudopod (PS). Bar = 1 μm. 7. Granulocyte filled with 8 merozoites (P) at 12 hr. Bar = 1 μm.

Figures 8-9....................................... 128

Electron micrographs of *P. marinus* merozoites in eastern oyster granulocytes. 8. Degenerating merozoites (P) at 12 hr. Bar = 1 μm. 9. A merozoite (P) surrounded by electron-dense granules (arrow), suggesting fusion with the phagosome containing the parasite, at 12 hr. Bar = 1 μm.

# ABSTRACT

The disease caused by the protozoan *Perkinsus marinus* has been a major source of mortality in the eastern oyster, *Crassostrea virginica*, along the Atlantic and Gulf coasts of the United States. Variations in susceptibility to *P. marinus* infection among eastern oysters collected from various geographical areas, as well as between eastern and Pacific (*Crassostrea gigas*) oysters have previously been reported. Little is known, however, about the mechanisms leading to these differences in susceptibility. Since oyster host defense may play a role in determining susceptibility to pathogen infection, the present study focused on analyzing cellular and humoral defense activities of the oyster and their interactions with *P. marinus*. An additional objective of this study was to establish procedures for the isolation, purification, and *in vitro* propagation of *P. marinus*.

Progression of *P. marinus* infection and potential host defense activities were determined in eastern oysters collected from the Atlantic and Gulf coasts. Oysters from all sites were found to be highly susceptible to the parasite. Heavy *P. marinus* infection intensity was associated with an increase in the density of circulating hemocytes and hemocyte chemiluminescence, and a decrease in the percentage of granulocytes, plasma hemagglutinin titer and concentration of plasma lysozyme and protein.

Prevalence and intensity of *P. marinus* infection was lower in Pacific oysters than in eastern oysters. The density of circulating hemocytes, percentage of granulocytes and plasma hemagglutinin titer increased in Pacific oysters challenged with *P. marinus* compared to unchallenged Pacific oysters; a similar increase was not observed in challenged eastern oysters with similar infection levels. Pacific oysters may offer a less favorable environment for the development of *P. marinus* compared to eastern oysters, for at least two possible reasons: The elevated cellular and humoral activities may degrade the parasite more effectively and lower plasma protein levels may limit parasite growth.

To investigate the interactions of *P. marinus* with hemocytes from both oyster species *in vitro*, a simple procedure was developed to isolate and enrich *P. marinus* merozoites from infected eastern oysters. The isolated merozoites retained their viability, infectivity and ability to enlarge in fluid thioglycollate medium. Upon exposure to oyster hemocytes, *P. marinus* merozoites became bound to all hemocyte types but in unequal ratios. Electron microscopy of merozoites following incubation with hemocytes of eastern and Pacific oysters for 15 min and 12 hr indicated that the parasites were rapidly phagocytosed, and that a small percentage may be killed by 12 hr. The number of merozoites enlarging in fluid thioglycollate media decreased following

their incubation with hemocytes of both oyster species. These results indicate that limited intracellular killing of *P. marinus* occurred. No increase in chemiluminescence was observed when hemocytes of either eastern or Pacific oyster were exposed to merozoites, indicating that killing was not mediated by oxygen metabolites.

The study of the biology and pathobiology of *P. marinus* has been impaired by the inability to isolate, purify and culture histozoic stage(s) of the parasite. In this study, *Perkinsus marinus* was successfully propagated *in vitro*. The culture medium (JL-ODRP-1) contained most of the known constituents of cell-free oyster hemolymph. Cultures of the parasite were initiated from heart fragments of infected oysters. The cultured protozoan (designated *Perkinsus*-1) was similar in morphology to histozoic stages of *P. marinus* at both the light and transmission electron microscopy levels. *Perkinsus*-1 formed prezoosporangia in fluid thioglycollate medium that stained blue-black in Lugol's solution, reacted positively with polyclonal antibodies raised against prezoosporangia and was infective to susceptible oysters.

A method to initiate numerous cultures within a relatively short time was also developed. Continuous cultures of *P. marinus* were initiated from purified prezoosporangia. Two types of division, progressive cleavage and successive bipartition of the mother cell protoplast were observed when prezoosporangia were placed in the culture medium. Progressive cleavage resulted in the formation of merozoites and successive bipartition resulted in the formation of zoospores. The culture of merozoites provide a markedly simplified system in which to study the biology of *P. marinus* independently of host influences and to produce large quantities of parasites for research material.

# STUDIES ON THE OYSTER PATHOGEN *PERKINSUS MARINUS* (APICOMPLEXA): INTERACTIONS WITH HOST DEFENSES OF *CRASSOSTREA VIRGINICA* AND *CRASSOSTREA GIGAS*, AND *IN VITRO* PROPAGATION.

# GENERAL INTRODUCTION

Much research effort has been devoted to the eastern oyster, *Crassostrea virginica,* because of its commercial importance. The biology of the eastern oyster has been described by Galtsoff (1964). The eastern oyster belongs to the Class Bivalvia of the Phylum Mollusca. The range of the eastern oyster extends from the East Coast of North America as far north as the Gulf of St. Lawrence, Canada, to the Gulf of Mexico and Caribbean Sea as far south as Venezuela (Stanley and Sellers, 1986). In addition to its economic importance, the oyster plays a significant ecological role because of its filter feeding nature (Dame and Zingmark, 1985; Burrell, 1986; Castel et al., 1989). Oysters filter large volumes of water (e.g., up to 24 l/hr), feed on the suspended phytoplankton and deposit organic matter (i.e., feces and pseudofeces) that is important to the benthic organisms associated with oyster reefs (Sornin et al., 1983). It has been proposed that the increase in phytoplankton biomass with the associated increase in eutrophication and low oxygen levels in the Chesapeake Bay, may be in part due to the lowered filtering capacity of the depleted oyster populations (Newell, 1988). Moreover, the oyster shells provide substrate that allow the development of densely populated, diverse communities of benthic organisms (Larsen, 1985).

The Chesapeake Bay was one of the most productive oyster grounds in the world, with an average of seven million bushels harvested per year from 1835 to 1891 (Brooks, 1891 in Haven et al., 1978). As many as 20 million bushels per year may have been produced in the Bay between 1875 and 1885. Virginia was the largest producer of eastern oysters among the Atlantic coastal states for many years. The first sign of decline in the Virginia oyster production began in 1913; in 1932 only 2.4 million bushels were harvested. This decline was followed by relatively stable landings that reached 4.0 million bushels in 1959. An even more drastic decline in oyster production began in 1960 and is still continuing; in 1992, only 45,000 bushels were harvested (Virginia Marine Resource Commission).

Many factors have been implicated in the decline in oyster production in Virginia and the Chesapeake Bay (Hargis and Haven, 1988). These include overfishing, diseases, fresh-water kills, decreased broodstock, reduced setting and predators. Overfishing and diseases are considered the most important factors in the decline of oyster production in the Bay. The oyster epizootics have been caused by two protozoans, the haplosporidian *Haplosporidium nelsoni* and the apicomplexan *Perkinsus marinus* (Haskin and Andrews, 1988; Andrews 1988). Historically disease pressure of *H. nelsoni* (i.e., MSX disease) on oysters has been more intense than *P. marinus* (i.e., Dermo disease) in Chesapeake

Bay. Since 1985, however, there has been a significant increase in oyster mortality caused by *P. marinus*. This has been associated with reduced rainfall and prolonged periods of warm weather. *Perkinsus marinus* now extends into the James River seed area (Andrews, 1988) and the upper Chesapeake Bay. According to Burreson (1989), this parasite is more important than MSX as an oyster pathogen in Chesapeake Bay.

*Perkinsus marinus* was discovered in the Gulf of Mexico in 1948 (Mackin et al., 1950) and named *Dermocystidium marinum* as it was thought to be a fungus. Since Perkins (1976) showed that the motile stage of *P. marinus*, the zoospore, contained an apical complex, the parasite was renamed and included in the Phylum Apicomplexa (Levine, 1978). However, its current taxonomic status is controversial (Vivier, 1982). *Perkinsus marinus* has been detected in oysters along the east and Gulf coasts of North America as far north as Long Island, New York (Ford, personal communication) and as far south as Tabasco, Mexico (Burreson et al., 1993). Ray (1952) discovered the ability of *P. marinus* to enlarge in fluid thioglycollate medium and form prezoosporangia (i.e. also called hypnospores) which stain blue-black in Lugol's solution. This simple and inexpensive diagnostic technique has greatly facilitated the study of the parasite.

Numerous reviews have discussed *P. marinus* morphology, life history, and epizootiology (Andrews, 1988; Perkins 1988; 1991). The life cycle of *P. marinus* consists of reproduction by schizogony in oyster tissue and of zoosporogenesis in sea water. According to Perkins (1991), small, presumably immature, uninucleate merozoites (2-4 $\mu$m) (i.e., previously called aplanospores or trophozoites) enlarge in the oyster to about 10-20 $\mu$m to form uninucleate mature merozoites with an eccentrically situated vacuole. The vacuole of *P. marinus* disappears and the mother cell protoplast divides by schizogony to form a 2-64 cell schizont. Uninucleate immature merozoites (2-4 $\mu$m) are liberated as the schizont wall ruptures. Division of the mother cell protoplast occurs by successive bipartition (alternating karyokinesis followed by cytokinesis) or progressive cleavage (repeated karyokinesis followed by cytokinesis). When infected oyster tissue is placed in fluid thioglycollate medium, presumably any stage can enlarge greatly and form prezoosporangia. Zoosporogenesis occurs when prezoosporangia are liberated into sea water. Successive bipartition of the zoosporangia protoplast can be observed and results in the formation of zoospores with subsequent release into sea water (Perkins and Menzel, 1966). The optimum temperature for sporulation (zoosporogenesis) of *P. marinus* is at 28-30° C with the lowest tested temperature limit for sporulation at 18° C

(Perkins, 1966; Chu and Greene, 1989). Sporulation takes place at salinities of 10 ppt to 35 ppt but is limited at 5-6 ppt (Perkins, 1966; Chu and Green, 1989). The free-swimming biflagellated zoospores are believed to actively penetrate the oyster or to passively be carried into the oyster by hemocytes. Oysters can also become infected by exposure to *P. marinus* stages (i.e., merozoites, schizonts) contained in oyster minced tissue (Ray and Mackin, 1954). Although there is some disagreement, merozoites are considered by some to be the primary agents of disease transmission (Perkins, 1988; Andrews 1988) as suggested by the ease with which infections can be transmitted with infected oyster tissue (Ray and Mackin, 1954; Mackin, 1962). Merozoites are believed to establish infections in the oyster digestive system by either lysing the basement membrane of the stomach, or by being carried into the oyster by hemocytes (Mackin, 1951). Ray (1954) has suggested that *P. marinus* can lyse oyster tissue. Once the parasite gains entrance into the oyster, little is known about factors (i.e., host biochemical composition and internal defenses) that affect the growth, development, and reproduction of *P. marinus*.

The pathological effects of *P. marinus* infection on the normal histology, physiology, and biochemistry of eastern oysters have been described in a number of studies (reviewed by Mackin 1962; Soniat and Koenig, 1982; Wilson et al.,

1988; Chu and La Peyre, 1989, 1993; Paynter and Burreson 1991). Some of these effects include lysis of oyster tissue, cessation of shell growth, glycogen depletion, alteration of hemolymph amino acids composition and decrease in tissue weight. These effects are generally only detected in oysters in oysters with moderate to heavy *P. marinus* infections.

The relationship of *P. marinus* infection with season, temperature, salinity, oyster condition and size have been documented from field studies (Mackin and Boswell, 1953; Andrews and Hewatt, 1957; Mackin, 1962; Quick and Mackin, 1971; Soniat, 1985; Craig et al., 1989; Soniat and Gauthier, 1989; Crosby and Roberts 1990; Gauthier et al., 1990; Paynter and Burreson, 1991). Extreme care must be exercised, however, when interpreting such relationships derived from field data. A factor showing a statistical relationship is not necessarily a major causative factor. Field studies must be substantiated by laboratory experiments in order to understand the complex host-pathogen-environment inter-relationships. The effects of temperature and salinity on host infection by *P. marinus* have been the most thoroughly studied.

Temperature is the most important factor in initiation and termination of *P. marinus* epizootics (Andrews, 1988). Rapid proliferation of the parasite in the host tissue occurs at elevated temperatures (>25° C), with *P. marinus*

being capable of overwhelming the host in several weeks (Andrews, 1988). In the Chesapeake Bay, the number of *P. marinus* cells in oyster tissues does not generally increase below 20° C (Andrews, 1988). In laboratory experiments at 15° C, Hewatt and Andrews (1956) found that oysters challenged with minced tissue from *P. marinus* infected oysters did not develop infections, while Chu and La Peyre (1993) were able to detect infection at that temperature. The parasite can apparently continue to multiply and kill the host at temperatures lower than 20° C, if the initial number of parasites established in the host is high enough (Andrews and Hewatt, 1957). In the colder months, from late fall to early spring, prevalence and intensity of infection in oysters decline in endemic areas of the Chesapeake Bay, and oyster mortalities are generally low. This decrease in prevalence and intensity may be due to an artifact of the failure of *P. marinus* to enlarge in fluid thioglycollate medium, or it may be caused by the active degradation and excretion of *P. marinus* by oyster hemocytes at these cooler temperatures.

Salinity is also an important factor regulating *P. marinus* infection. In the Chesapeake Bay tributaries, *P. marinus* does not usually exist at salinities below 14 ppt (Andrews and Hewatt, 1957). A number of field studies have documented the correlation between prevalence and intensity of infection and salinity (Mackin and Boswell, 1953; Quick

and Mackin, 1971; Soniat, 1985; Craig et al., 1989; Soniat and Gauthier, 1989; Crosby and Roberts 1990; Gauthier et al., 1990; Paynter and Burreson, 1991). The absence of infection in low salinity waters was mainly attributed to the dilution and flushing of infective particles by the influx of fresh water (Mackin, 1956; Andrews and Hewatt 1957; Mackin, 1962). However, several studies have shown that low salinity (around 10 ppt) delays the progression of *P. marinus* infection (Ray, 1954; Scott et al., 1985). The effect of low salinity (i.e., <10 ppt) on *P. marinus* development in eastern oysters under controlled laboratory conditions has only recently been examined (Ragone and Burreson 1993; Chu et al., 1993); *P. marinus* can survive in oysters maintained at very low salinities but disease development appeared compromised.

In spite of abundant information on the epizootiology and morphology of *P. marinus* (Andrews, 1988; Perkins, 1988), many details about its life cycle and infective stages are unknown. Uncertainty about the biology of *P. marinus* can be attributed to the current inability to isolate, purify and culture histozoic stage(s) of the parasite and thus study the parasite independent of the host. In addition, study of the direct interactions of host defenses with *P. marinus* has not been possible because of the lack of purified parasites.

Most of the knowledge of bivalve immunobiology has traditionally been derived from observations on the reaction of the host to a variety of pathogens and from experimental investigations of the fate of inoculated particulate and soluble antigens (Tripp, 1958; Feng, 1962, 1988; Poder, 1980). The internal defense of Mollusca has been reviewed by many authors and includes both cellular and humoral responses (Tripp, 1970; Fletcher and Cooper-Willis 1982; Bayne, 1983; Fries, 1984; Chu, 1988; Feng, 1988).

Cellular responses include inflammation, phagocytosis and encapsulation by hemocytes. Hemocyte structure and function have been reviewed by Cheng (1984) and Fisher (1986). The hemocytes in the hemolymph and interstitial spaces of bivalves are believed to be the primary line of defense. Inflammation is characterized by hemocytosis, either in response to a foreign agent or to damaged tissue or both. Depending on the size of the foreign agent, the hemocytes attempts at phagocytosis either result in internalization by a single hemocyte or encapsulation by more than one hemocyte. Encapsulation by host hemocytes presumably isolates and immobilizes the pathogen which may then be degraded. Phagocytosis can be divided into at least four stages: attraction, binding, internalization and intracellular digestion of the foreign matter. Diapedesis and exocytosis of the undigested particles into an excretory pathway may replace digestion (Fisher, 1986). The

importance of phagocytosis in determining the outcome of a disease has long been recognized (Metchnikoff, 1891; Tripp, 1963; Sindermann, 1979; Cheng, 1975). Molluscan hemocytes readily phagocytose a wide variety of biotic and abiotic particles *in vivo* and *in vitro* (Tripp, 1960; Cheng, 1975; Robohm, 1984; Feng, 1988). Hemocytes also release antimicrobial compounds including lysosomal enzymes and toxic oxygen metabolites (Cheng, 1984; Wishkovsky, 1988; Adema et al., 1991).

Many different lysosomal enzymes, including acid phosphatase, aminopeptidase, beta-glucuronidase and lipase, have been identified in the hemocytes of oysters and other bivalves (Cheng and Rodrick, 1974, 1975; Cheng 1976; Yoshino and Cheng, 1976; Mohandas and Cheng, 1985; Chagot, 1989; Pipe, 1990). These enzymes degrade a wide variety of substrates including proteins, sugars, lipids and nucleic acids and have antimicrobial properties (Dengle, 1977; Holtzman, 1989). Hypersynthesis of lysosomal enzymes upon exposure of hemocytes to bacteria or bacterial products has also been reported (Cheng and Yoshino, 1976; Cheng, 1977). A number of lysosomal enzymes, including aminopeptidase, lysozyme and phospholipase C are present in plasma and are secreted by hemocytes (McDade and Tripp, 1967a; Foley and Cheng, 1977; Mohandas and Cheng, 1985; Cheng, 1992). The potential role of lysosomal enzymes in plasma is the lysis or partial degradation of biotic agents which may, in turn,

enhance their phagocytosis by hemocytes and trigger the inflammatory response (Cheng, 1978, 1983). Among the lysosomal enzymes, lysozymes have received considerable attention, because of their bacteriolytic activities and their "inducible" nature. Lysozymes are enzymes that satisfy the criteria proposed by Salton (1957) and Jolles (1964, 1969): 1) enzymatic activity against *Micrococcus luteus*, and 2) dissolution of isolated bacterial cell walls to liberate reducing groups and N-acetylamine sugars. Some lysozymes may be chitinases and some can catalyse trans-glycolysation (Imoto et al., 1972). In vertebrates, lysozymes are involved in a broad range of defense activities including bacteriolysis, opsonization, immune response potentiation as well as resticted anti-viral and anti-neoplastc activity (Jolles and Jolles, 1984; Lie and Syed, 1986). In addition to their defense role, lysozymes have digestive functions (Dobson et al., 1984). In bivalves, lysozymes are believed to be involved in the mechanisms of host defense and digestion (McHenery and Birbeck, 1982). Lysozyme activity of hemolymph and mantle mucus of the eastern oyster was first described by McDade and Tripp (1967a). Lysozymes or lysozyme-like substances have subsequently been described in many bivalves (Cheng and Rodrick, 1974; Hardy et al., 1976; Conway, 1977; McHenery et al., 1979; Steinert and Pickwell, 1984; McHenery et al., 1986; Maginot et al., 1989; Hawkins et al., 1993).

In addition to lysosomal enzymes, hemocytes of oysters and other molluscs can produce toxic oxygen metabolites (e.g., superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals) much like vertebrate phagocytes (Nakamura et al., 1985; Dikkeboom et al., 1987; Pipe 1992; Anderson et al., 1992; Takahashi et al., 1993). It is well documented that vertebrate phagocytes (e.g., macrophages, polymorphonuclear leucocytes) can undergo a rapid increase in oxygen consumption (i.e. respiratory burst) during phagocytosis which is accompanied by the production of toxic oxygen metabolites (Sbarra and Strauss, 1988; Baggiolini and Wymann, 1990). These toxic oxygen metabolites are part of the armamentarium available to vertebrate phagocytes to combat microbes such as viruses, bacteria, protozoa and yeast (Nathan et al., 1979; Murray et al., 1979; Klebanoff et al., 1983; Murray, 1984; Hughes, 1988). The effects of these oxidants which react strongly with cellular molecules (i.e., proteins, lipids , polysaccharides, nucleic acids), include enzyme inactivation and cell membrane and DNA damage (Borg and Schaich, 1984; Hassan and Schiavone, 1991; Winston, 1991; Frenkel, 1992). In hemocytes, the production of toxic oxygen metabolites (reviewed by Adema et al., 1991; Pipe, 1992, Takahashi et al., 1993), the increase in oxygen consumption (Friedl et al., 1992) and chemiluminescence (Larson et al., 1989; Fisher et al., 1990; Le Gall et al., 1991; Bachere et al., 1991), as well as the presence of

NADPH-oxidase (Chagot, 1989) and peroxidase (Chagot, 1989; Ripley, 1993) suggest that a similar antimicrobial mechanism exists in oysters and other molluscs.

Humoral factors such as agglutinins, hemolysins, opsonins, cidal and lytic factors have been found in the hemolymph of molluscs (Fries, 1984; Chu 1988). Some these may be different stages of a single protein (i.e. agglutinins, hemolysins and opsonins) (Amirante, 1986). Most of these humoral factors have also been found in the hemocytes which presumably can produce and release these factors (Cheng, 1984). It is well known that plasma from oysters and other molluscs contains humoral factors capable of agglutinating foreign particles such as erythrocytes, bacteria and protozoa (Tripp, 1966; McDade and Tripp, 1967b; Pauley, 1974; Fisher and Dinuzzo, 1991). Some of these agglutinins have been isolated and possess lectin-like properties (Hardy et al., 1977; Renwrantz and Stahmer, 1983; Yang and Yoshino, 1990a). Lectins are nonenzymatic proteins or glycoproteins that bind with different degrees of affinity and specificity to cells or glycoconjugates, causing their respective agglutination or precipitation (Goldstein et al., 1980). Knowing that lectins are ubiquitous in nature, combine rapidly, selectively and reversibly with carbohydrate moieties, together with the fact that all cells carry a carbohydrate coat, made lectins prime candidates for recognition molecules (Sharon and Lis,

1972; Gold and Balding, 1975; Barondes, 1981; Sharon and Lis, 1989; Nathan and Lis, 1993). The possible role of lectins in invertebrate immunity has been extensively reviewed (Yeaton, 1981; Ey and Jenkin, 1982; Coombe et al., 1984; Renwrantz, 1986; Olafsen, 1988; Vasta, 1991). The involvement of hemolymph agglutinins in the *in vivo* clearance of particles (e.g., xenogeneic proteins, viruses, bacteria), is suggested by the observed decrease of agglutinin titers following injection of particles and the decrease in clearance rate following a secondary injection (Pauley et al., 1971; McCumber et al., 1979). Agglutinins may immobilize foreign particles and prevent their dispersion in the organism, making them more susceptible to local action by elements of the host defense (Vasta, 1991). In addition, purified agglutinins from plasma have been shown to enhance hemocyte phagocytosis (i.e., opsonization) of foreign particles (Hardy et al., 1977; Renwrantz and Stahmer, 1983; Yang and Yoshino, 1990b). Lectins have also been found on the surface of hemocytes, further suggesting that these molecules are involved in recognition of foreign particles in molluscan defense (Yeaton 1981; van der Knaap et al., 1981; Vasta et al., 1984; Cheng et al., 1984; Olafsen, 1988). In addition to lysosomal enzymes and agglutinins, a number of humoral factors with antimicrobial and cytotoxic properties have been described in molluscs, although little is known about their nature (Cushing et al.,

1971; Anderson , 1981; Wittke and Renwrantz, 1984; Leippe and Renwrantz, 1985; Yoshino and Tuan, 1985; van der Knaap and Meuleman, 1986; Mandal et al., 1991).

One of the requirements for a pathogen to become established in a host is that the pathogen must overcome host defenses (Tripp, 1970; Bayne, 1983; Cheng, 1988; Feng, 1989, van der Knaap and Loker, 1990; Coombes, 1991). The pathogen may passively avoid being recognized, or actively inhibit or interfere with host defense mechanisms (Cheng, 1988). Sometimes, even when the host is able to recognize and kill the pathogen, it may still be unable to avoid rapid proliferation of the pathogen in host tissue. This may possibly result from: 1) infection (entry) by an overwhelming number of pathogens, 2) high rate of multiplication of the pathogen in the host tissues, or 3) depressed host defense due to stress. It is well accepted, that stressful conditions predispose aquatic organisms to infectious disease agents (Wedemeyer, 1970; Meyer, 1970; Sindermann, 1970; Snieszko, 1974; Newell and Barber, 1988). Unnatural conditions such as low dissolved oxygen, crowding, presence of metabolites and chemical contaminants, and high temperature can enhance the adverse effects of infectious agents and as a result be influential in causing outbreaks of disease. Disease arises when infection by a parasite causes pathological effects. The term 'disease' denotes a

negative deviation from the normal physiological state of a living organism (Kinne, 1980). Disease is the result of a complex dynamic interaction between the host, the pathogen, and the environment (Sneiszko, 1974; Kinne, 1980). Factors such as host age, nutritional, and defense status, the pathogen number and virulence and environment parameters such as temperature and salinity all play a role in determining if parasites become established in a host and cause disease. The establishment of a parasite in a host is governed by a complex series of processes during which the parasite must contact the host, gain access in the host tissue, survive, overcome host defenses and propagate in the host (i.e., growth, development and reproduction).

There is little information on the oyster response to *P. marinus* infection. Mackin (1951) examined histologically *P. marinus* infection and reported that the initial response was inflammatory and characterized by a marked hemocytosis at the site of infection. Perkins (1969, 1976) who studied the morphology and ultrastructure of *P. marinus* in oyster tissue reported that layers of hemocytes attempting to encapsulate foci of dividing pathogen cells in the early stages of infection were often seen but disappeared as the number of parasites increased. *P. marinus* can be readily found in oyster hemocytes in heavily infected oysters. It is believed that *P. marinus* can grow and divide in hemocytes

and is liberated as hemocytes rupture, probably facilitating the dissemination of the parasite throughout the host tissues (Mackin, 1951; Perkins, 1976).

Because of the resurgence of *P. marinus* in Chesapeake Bay oysters, there is an increase interest in finding ways to control the disease. Since the use of drugs (for example) to treat the disease in feral oysters is not practical; a more realistic approach is to selectively breed for disease resistance. Differences in resistance may be due to: 1) differences in the ability to satisfy the physiological needs of the parasite, 2) differences in host defenses, 3) differences in the infectivity of the pathogen.

The need to develop strains of oysters that are resistant to disease has long been recognized (Haskin and Ford, 1979). Eastern oysters, for example, have successfully been bred for resistance toward the protozoan *Haplosporidium nelsoni* (MSX) (Haskin and Ford, 1988). There is also the report of natural selection for resistance in eastern oysters exposed to Malpeque Bay disease (Eastern Canada) (Logie et al., 1960). Attempting to increase oyster survival by genetic selection seems reasonable since, in many studies involving invertebrates, disease resistance has been found to be controlled by a single or a small group of genes (Anderson, 1986). Development of resistance to MSX, for example, was quite rapid (Haskin and Ford, 1987).

The first step for selection is to investigate and identify variations of disease resistance between individuals, strains or related species (Chevassus and Dorson, 1990). In the case of *P. marinus* disease, there have been several reports of differences in mortality among oysters collected from various geographical areas (Hewatt and Andrews, 1954; Andrews, 1955; Andrews and McHugh, 1956; Burreson, 1991). Selection using mortality as an end-point over several generations has traditionally been used. These process, however, can take many years. An alternative approach consists of studying the mechanism(s) responsible for disease resistance and then using them as markers for selection criteria (Chevassus and Dorson, 1990; Owen and Axford, 1991). One mechanism that may be involved in disease resistance is host defense (Doenhoff and Davies, 1991). In fact, in the snail-trematode relationship, disease resistance of snails to schistosome parasites has been attributed mainly to the host defense of the snail and not to the unsuitability of the host (i.e., host's inability to provide the physiological needs of the parasite) (Lie et al., 1987).

A long term goal for the future is to produce a stock of oysters resistant to *P. marinus* infection. Unfortunately very little is known about possible differences in susceptibilty to *P. marinus* in individual or groups of

oysters. The first part of this dissertation discusses susceptibiltiy of oysters collected from several geographical locations, including areas enzootic for *P. marinus*, where natural selection might have occurred. Oysters were challenged with *P. marinus* and potential host defense activities were measured in these oysters to determine possible relationships with pathogenesis of *P. marinus* disease. The susceptibility to *P. marinus* and the response of potential host defense activities were also compared between the eastern oyster and the Pacific oyster, *Crassostrea gigas*. Pacific oysters are known to be resistant to a number of diseases, that kill native oysters (Comps, 1988; Grizel et al., 1988), including *P. marinus* (Meyers et al., 1991).

Limitations in these experiments became evident and stemmed in part from the unavailability of isolated histozoic stages of the parasite. Problems encountered with the use of *P. marinus* in minced tissue included: 1) uncertain dosage for challenge, 2) contamination of minced tissue with bacteria and protozoa, and 3) inability to measure the direct interaction between potential host defense activities and *P. marinus*. The need for purified histozoic stages of *P. marinus* to advance our knowledge of the parasite itself and its interaction with its host was clear. The objective of the second part of the dissertation was to develop techniques to isolate and

propagate *P. marinus in vitro*. In the third part of the dissertation, isolated merozoites were used in a preliminary study to investigate hemocyte-parasite interactions *in vitro*, for eastern and Pacific oysters.

## SUMMARY OF OBJECTIVES

1. Determine potential host defense activities and progression of *Perkinsus marinus* disease in eastern oysters from the Gulf and east coasts, USA.

2. Compare hemocytic and humoral activities of eastern and Pacific oysters following challenge by the protozoan *P. marinus*.

3. Isolate and purify *P. marinus* merozoites from oyster tissue.

4. Establish procedures to propagate *P. marinus in vitro*.

5. Investigate the interactions of *P. marinus* merozoites with eastern and pacific oyster hemocytes *in vitro*.

# Chapter 1

# POTENTIAL HOST DEFENSE ACTIVITIES AND PROGRESSION OF *PERKINSUS MARINUS* INFECTION IN EASTERN OYSTERS FROM THE GULF AND EAST COASTS, USA.

# INTRODUCTION

*Perkinsus marinus* was first discovered in the Gulf of Mexico in the late 1940's following research on mortalities in the eastern oyster, *Crassostrea virginica* (Mackin et al., 1950). The parasite was later found along the East Coast of the United States where it has been the cause of serious oyster mortalities (Andrews and Hewatt, 1957; Andrews, 1988). Recently *P. marinus* has expanded to areas traditionally free of the disease and has replaced the protozoan *Haplosporidium nelsoni*, which causes MSX disease, as the most important oyster pathogen in the lower Chesapeake Bay (Burreson and Andrews, 1988).

Several studies have reported differences in susceptibility to *P. marinus* among oysters from various geographical areas (Hewatt and Andrews, 1954; Andrews, 1955; Andrews and McHugh, 1956; Burreson, 1991). According to Andrews and Hewatt (1957), for example, oysters from the Eastern Shore of Virginia were more susceptible to *P. marinus* than Chesapeake Bay oysters while oysters from South Carolina were more resistant. It has been suggested that the continuous exposure of eastern oysters to *P. marinus* in enzootic areas may have resulted in selection for increased survival (Andrews, 1984). There is evidence that selected eastern oysters can develop greater resistance to disease such as in the case of Malpeque Bay disease or MSX (Logie

et. al., 1960; Ford, 1988).

Although there are reports of differences in oyster susceptibility to *P. marinus*, nothing is known about the mechanisms involved. Differences in the pathogenesis of *P. marinus* infection may depend on the cellular and humoral activities of the oyster defense since: 1) the site of infection is often characterized by hemocytosis and attempts by hemocytes to encapsulate clusters of *P. marinus* (Perkins, 1976); 2) the parasites are frequently found in hemocytes (Mackin, 1951; Perkins, 1976; Gauthier and Fisher, 1990); and, 3) hemocytes readily phagocytose and can degrade *P. marinus* (Chapter 6).

Differences in the host defense among oysters from various sites following challenge with *P. marinus* may help explain differences in their susceptibility to the parasite. The objective of this study was to investigate differences in *P. marinus* infection and potential host defense activities among eastern oysters from various geographical areas.

## MATERIALS AND METHODS

### Experimental design

Oysters were collected in June of 1990 from the upper

James River (UJ), Virginia, at Deep Water Shoal (salinity 5 ppt, temperature 21° C) and from the lower James River (LJ) at Nansemond Ridge (salinity 17 ppt, temperature 20° C). In the same month, oysters from Confederate Reef in Galveston Bay, Texas (GB) (salinity 19 ppt, temperature 26° C) were obtained, courtesy of Dr. William Fisher. Galveston Bay (Confederate Reef) and the lower James River, at Nansemond Ridge, are areas enzootic for *P. marinus* (Andrews, 1988; Andrews and Ray, 1988; Soniat and Gauthier, 1989). The upper James River, at Deep Water Shoal, is an area that has been traditionally free of the parasite (Andrews, 1988; Burreson and Andrews, 1988).

Ninety oysters from each site were maintained in 40 l aquaria (12-13 oysters/aquarium), equipped with biofilters and filled with 1 μm filtered York River water (salinity=20 ppt). Oysters were fed daily with 0.1 g of algae paste, composed of a mixture of *Pavlova lutheri*, *Isochrysis galbana* and Tahitian *Isochrysis galbana*. The water in the aquaria was changed weekly. Water temperature during the experiment was maintained at 25°C (± 1 °C). The oysters were acclimated to experimental conditions for a minimum of four weeks prior to the start of the experiment.

The oysters from GB, LJ and UJ were each divided into three groups: the first group of 15 oysters was sampled immediately; the second group of 40 oysters was inoculated with $10^6$ *P. marinus* merozoites/oyster in the shell cavity

(challenged); and, the third group of 35 oysters was inoculated with artificial sea water (control). Fifteen control and 15 challenged oysters from each sites were sampled on the third and seventh week post-challenge. At each sampling time, potential host defense characteristics were measured for individual oysters: these included hemocyte counts, hemocyte phagocytosis and chemiluminescence as well as plasma hemagglutination titer, and lysozyme concentration. The intensity of *P. marinus* infection in oysters was also determined as described below. Oyster mortality was recorded daily.

Measurement of potential host defense activities

About 1 ml of hemolymph was collected with a syringe from the adductor muscle sinus of each oyster through a notch in the shell. The hemolymph samples were immediately placed on ice. The number of circulating hemocytes per unit volume (density) and the percentage of granulocytes in each hemolymph sample was determined with a Bright-Line hemacytometer (Reichert, Buffalo, NY).

Hemocyte phagocytosis of zymosan was measured by adding 20 $\mu$l of hemolymph to microscopic slides in humidified chambers, allowing the hemocytes to adhere for 30 min and then adding 20 $\mu$l of zymosan suspension in artificial sea water (ASW, 20 ppt, Instant Ocean, Aquarium Systems Inc., Mentor, OH). After 45 min incubation, the hemocytes were

rinsed in ASW, fixed with Davidson's AFA (Alcohol/Formaldehyde/Acetic acid) for 30 min and stained with Hemal I and II staining solution (Hemal Stain Company Inc., Danbury, CT). The percentage of hemocytes exhibiting phagocytosis (200 hemocytes counted) as well as the number of zymosan particles phagocytosed per individual hemocytes were determined.

Luminol-enhanced hemocyte chemiluminescence was measured with a Beckman LS 150 scintillation counter operated in the out-of-coincidence mode. Luminol stock solution was prepared according to the method by Scott and Klesius (1981) and diluted 1:500 with ASW (working solution) prior to use in the assay. The chemiluminescence response was initiated by the addition of 500 $\mu$l of 1 mg/ml zymosan suspension to 500 $\mu$l of hemocyte suspension ($10^6$ cell/ml) and 500 $\mu$l of luminol working solution. The background and net hemocyte chemiluminescence were recorded in counts per minute (CPM). The background CPM was the chemiluminescence of hemocytes in luminol prior to the addition of zymosan. The net CPM was calculated by subtracting the background CPM from the peak CPM of hemocytes stimulated with zymosan.

Hemolymph was centrifuged at 400 *g* for 10 min to pellet the hemocytes. The supernate (plasma) was collected and frozen (-20°C). The hemagglutinin titer was determined by the addition of sheep red blood cells (0.5 %, Becton Dickinson Co., Cockeysville, MA) to twofold serially-diluted

oyster plasma in U-well microtiter plates. Results were expressed as the Log2 of the mathematical reciprocal of the maximal dilution showing complete agglutination. Lysozyme activity was measured spectrophotometrically using a modification of the method of Shugar (1952) (Chu and La Peyre, 1989). Each plasma sample (0.1 ml) was added to 1.4 ml of a suspension of *Micrococcus lysodeikticus* (15 mg/ml 0.066 M phosphate buffer of 6.3 pH) and the decrease in absorbance was recorded at 540 nm for 2 min. Hen's egg white lysozyme (Sigma Chemical Co., St Louis, Mo) was used as a standard.

Prevalence and intensity of infection

The level of *P. marinus* infection in oysters was measured according to the method developed by Ray (1952, 1966). Intensity of infection was rated on a scale from 0 to 5, based on the percentage of the rectal tissue occupied by the parasite according to the categories of Ray (1954). Levels of infection were reported as prevalence, mean intensity of infected oysters and weighted incidence. Weighted incidence was calculated according to the formula of Mackin (1962), and was the sum of the intensity rating (0-5) divided by the number of oysters.

Statistical analysis

Data were examined by analysis of variance followed by

SNK's multiple comparison of means when significant differences ($p<0.05$) were found. Data were transformed when necessary to insure homogeneity of variance.

# RESULTS

## Levels of *P. marinus* infection

Oysters from the lower James River (LJ), Virginia and Galveston Bay (GB), Texas, were initially infected with *P. marinus*, whereas the parasite was undetected in oysters from the Upper James River (UJ), Virginia. Prevalences, mean intensities and weighted incidences of infection in UJ, LJ and GB oysters at each sampling time, for both challenged and control groups are reported in Table 1.

While no infections were detected in UJ oysters prior to challenge, prevalence of infection in challenged UJ oysters increased to 67% and 100% at three and seven weeks, respectively. Weighted incidence of infection increased from $1.4 \pm 1.5$ to $2.7 \pm 1.3$ between three and seven weeks. Intensity of infection increased rapidly in some challenged UJ oysters: at three weeks, one oyster was found heavily infected and mean intensity of infected oysters was $2.1 \pm 1.4$. Two out of the forty challenged UJ oysters died between the third and seventh week. No *P. marinus* infection or mortality was detected in the control UJ oysters.

Table 1: Prevalence, mean intensity and weighted incidence of *P. marinus* infection in control and challenged UJ, LJ and GB oysters prior to challenge and at three and seven weeks post-challenge (N=15).

| | Prevalence | Mean intensity | Weighted incidence | Oyster mortality |
|---|---|---|---|---|
| INITIAL | | | | |
| UJ | 0% | - | - | - |
| LJ | 47% | 2.9 ± 1.0 | 1.3 ± 1.6 | - |
| GB | 100% | 3.3 ± 1.3 | 3.3 ± 1.3 | - |
| 3rd WEEK | | | | |
| Control | | | | |
| UJ | 0% | - | - | 0 |
| LJ | 40% | 3.0 ± 2.5 | 1.3 ± 1.9 | 0 |
| GB | 93% | 2.5 ± 1.2 | 2.3 ± 1.4 | 1 |
| Challenged | | | | |
| UJ | 67% | 2.1 ± 1.3 | 1.4 ± 1.5 | 0 |
| LJ | 93% | 2.1 ± 1.4 | 1.9 ± 1.5 | 1 |
| GB | 93% | 2.5 ± 1.5 | 2.3 ± 1.6 | 4 |
| 7th WEEK | | | | |
| Control | | | | |
| UJ | 0% | - | - | 0 |
| LJ | 60% | 1.9 ± 1.4 | 1.1 ± 1.5 | 1 |
| GB | 93% | 2.2 ± 1.1 | 2.1 ± 1.3 | 2 |
| Challenged | | | | |
| UJ | 100% | 2.7 ± 1.3 | 2.7 ± 1.3 | 2 |
| LJ | 93% | 2.6 ± 1.5 | 2.4 ± 1.5 | 1 |
| GB | 93% | 2.5 ± 1.5 | 2.6 ± 1.2 | 1 |

Prevalence of infection in challenged LJ oysters increased from 47% at the start of the experiment to 93% at both three and seven weeks. Weighted incidence of infection in challenged LJ oysters increased from 1.3 ± 1.6 at the beginning to 1.9 ± 1.5 at three weeks, but mean intensity of infection decreased from 2.9 ± 1.0 to 2.1 ± as a result of newly acquired light infections. Only one LJ challenged oyster died during this period. Weighted incidence and mean intensity of infection in challenged oysters were 2.4 ± 1.5 and 2.6 ± 1.5 at week seven, respectively. Prevalence of infection increased slightly in control LJ oysters from 47% at the start of the experiment to 60% at seven weeks and weighted incidence of infection remained at a similar level of 1.3 ± 1.6 at the beginning of the experiment and 1.1 ± 1.5 at week seven. Four challenged and one control LJ oysters died between three and seven weeks.

All GB oysters were initially infected (prevalence 100%) and some oysters (3/15) had heavy infections. Mean intensity of infection in challenged and control GB oysters decreased from 3.3 ± 1.3 at the start of the experiment to 2.5 ± 1.5 and 2.5 ± 1.2, respectively, at week three, as a result of the death of four challenged and two control oysters with advanced infections. The mean intensities of infection in challenged and control GB oysters was 2.2 ± 1.1 and 2.5 ± 1.2, respectively, at week seven. Two challenged and one control GB oysters died between three and seven

weeks.

## Potential host defense activities

Prior to challenge, the number of circulating hemocytes per unit volume was highest in GB oysters and lowest in UJ oysters ($p<0.0001$, Table 2). At week three, hemocyte number in challenged GB oysters was significantly greater than in challenged LJ and UJ oysters ($p<0.0001$) or control GB oysters ($p=0.003$). Although the number of circulating hemocytes tended to be highest in GB oysters and lowest in UJ oysters in both control and challenged oysters at week seven, the differences were not significant. The number of hemocytes in challenged oysters of all groups were significantly greater than in control oysters ($p=0.0359$, 2 factors).

The percentage of granulocytes was higher in UJ oysters than in GB and LJ oysters prior to challenge ($p<0.0001$, Table 2). The percentage of granulocytes remained higher in UJ than in GB oysters in both control and challenged oysters at three and seven weeks. The percentage of granulocytes in UJ oysters tended to decrease during the course of the experiment and was lower in challenged UJ oysters at week seven than at the start of the experiment.

The percentage of phagocytic hemocytes was slightly higher in UJ oysters than in GB oysters prior to challenge ($p=0.0321$) and at week three ($p=0.0268$, Table 2).

Table 2: Mean (± SD) density of circulating hemocytes, percentage of granulocytes, percentage of phagocytic hemocytes, number of zymosan particles per phagocytic hemocyte and hemocyte background and net chemiluminescence (CL) in control and challenged UJ, LJ and GB oysters prior to challenge and at three and seven weeks post-challenge. CPM = Counts per minute. N=15.

| | Hemocyte # ($10^6$/ml) | % Granulocytes | % Phagocytes | # Zymosan/ phagocyte | Background CL ($10^3$ CPM) | Net CL ($10^3$ CPM) |
|---|---|---|---|---|---|---|
| INITIAL | | | | | | |
| UJ | 1.17 ± 0.6 | 58 ± 13 | 44 ± 10 | 6.3 ± 0.8 | 38 ± 9 | 46 ± 24 |
| LJ | 1.69 ± 0.46 | 39 ± 14 | 41 ± 7 | 6.0 ± 1.5 | 47 ± 14 | 94 ± 101 |
| GB | 3.43 ± 1.63 | 26 ± 9 | 33 ± 15 | 5.2 ± 2.0 | 200 ± 97 | 548 ± 194 |
| 3rd WEEK | | | | | | |
| Control | | | | | | |
| UJ | 1.73 ± 0.91 | 52 ± 20 | 55 ± 19 | 4.8 ± 1.4 | 50 ± 24 | 77 ± 133 |
| LJ | 2.16 ± 1.32 | 43 ± 12 | 44 ± 19 | 4.8 ± 1.5 | 66 ± 47 | 118 ± 230 |
| GB | 2.52 ± 0.89 | 37 ± 12 | 36 ± 16 | 4.4 ± 1.4 | 186 ± 90 | 401 ± 261 |
| Challenged | | | | | | |
| UJ | 2.26 ± 0.96 | 48 ± 15 | 49 ± 21 | 4.8 ± 1.7 | 68 ± 38 | 220 ± 248 |
| LJ | 1.88 ± 0.74 | 38 ± 14 | 35 ± 13 | 4.3 ± 1.2 | 55 ± 21 | 127 ± 138 |
| GB | 4.15 ± 1.31 | 32 ± 10 | 42 ± 15 | 4.3 ± 0.8 | 235 ± 141 | 511 ± 228 |
| 7th WEEK | | | | | | |
| Control | | | | | | |
| UJ | 1.41 ± 0.6 | 50 ± 8 | 21 ± 9 | 3.8 ± 0.8 | 43 ± 20 | 41 ± 37 |
| LJ | 1.57 ± 0.61 | 39 ± 13 | 22 ± 11 | 3.4 ± 1.3 | 68 ± 55 | 59 ± 75 |
| GB | 1.91 ± 0.77 | 26 ± 9 | 29 ± 14 | 4.2 ± 1.6 | 169 ± 139 | 350 ± 223 |
| Challenged | | | | | | |
| UJ | 1.74 ± 0.63 | 47 ± 9 | 34 ± 18 | 4.6 ± 1.7 | 66 ± 35 | 109 ± 106 |
| LJ | 2.04 ± 0.86 | 36 ± 8 | 24 ± 18 | 3.7 ± 1.7 | 79 ± 75 | 206 ± 253 |
| GB | 2.51 ± 1.36 | 28 ± 11 | 25 ± 12 | 3.5 ± 1.2 | 198 ± 150 | 449 ± 262 |

The percentage of phagocytic hemocytes in both control and challenged oysters tended to decrease during the course of the experiment. The percentage of phagocytic hemocytes in control UJ oysters at week seven was lower than at the start of the experiment ($p<0.0001$) or at week three ($p<0.0001$). Similarly, the number of zymosan particles per phagocytic hemocyte decreased during the course of the experiment, but no significant differences could be shown between oyster populations (Table 2).

Background and net hemocyte chemiluminescence were higher in GB than in LJ and UJ oysters prior to challenge ($p<0.0001$, Table 2). The same differences in background and net hemocyte chemiluminescence between GB and LJ or UJ oysters were found for control oysters at week three ($p<0.0001$) and week seven ($p<0.0001$), as well as for challenged oysters at week three ($p<0.0001$) and week seven ($p=0.0005$). The hemocyte chemiluminescence measurements of challenged UJ oysters increased with the progression of the disease and was greater than chemiluminescence in control UJ oysters at week seven ($p=0.04$ background; $p=0.0283$ net).

No differences in plasma hemagglutinin titer were found among GB, LJ and UJ oysters prior to challenge. Plasma hemagglutinin titer in challenged UJ oysters, however, was lower than in challenged GB or LJ oysters at week three ($p=0.0011$) and lower than in challenged GB at week seven ($p=0.0378$). The plasma hemagglutinin titer decreased in

challenged UJ oysters (Table 3): plasma hemagglutinin titer was lower in challenged UJ oysters than in control UJ oysters at week three (p=0.0142) and week seven (p=0.0103) The same was true for LJ oysters at week seven (p=0.015).

Seven weeks post-injection, plasma lysozyme concentration in challenged UJ oysters was lower than in control (p=0.001) UJ oysters or challenged LJ oysters (p=0.0438).

## DISCUSSION

Oysters collected from the upper (UJ) and lower (LJ) James River, Virginia, and Galveston Bay (GB), Texas, were highly susceptible to *P. marinus* infection. High intensities of *P. marinus* infection were observed in oysters from each site and apparently caused mortalities.

Mortality was highest in GB oysters probably because these oysters had greater weighted incidence of *P. marinus* infection at the start of the experiment. *Perkinsus marinus* was also initially detected in LJ oysters but at a lower prevalence than in GB oysters. The difference in prevalence between GB and LJ oysters in June, at the time of collection, is probably related to the difference in the seasonal pattern of *P. marinus* pathogenesis between the two sites. Prevalence of infection in GB oysters was generally

Table 3: Mean (± SD) plasma hemagglutinin titer and lysozyme concentration in control and challenged UJ, LJ and GB oysters prior to challenge and at three and seven weeks post-challenge (N=15).

| | Hemagglutinin titer | Lysozyme (μg/ml) |
|---|---|---|
| INITIAL | | |
| UJ | 3.7 ± 1.2 | 19 ± 14 |
| LJ | 4.1 ± 1.7 | 16 ± 12 |
| GB | 3.6 ± 1.9 | 14 ± 5 |
| 3rd WEEK | | |
| Control | | |
| UJ | 3.4 ± 1.6 | 22 ± 11 |
| LJ | 4.4 ± 1.8 | 15 ± 11 |
| GB | 4.1 ± 1.8 | 14 ± 8 |
| Challenged | | |
| UJ | 2.1 ± 1.1 | 15 ± 6 |
| LJ | 4.3 ± 1.4 | 19 ± 15 |
| GB | 3.9 ± 1.6 | 19 ± 11 |
| 7th WEEK | | |
| Control | | |
| UJ | 3.4 ± 1.4 | 22 ± 14 |
| LJ | 4.3 ± 1.2 | 14 ± 7 |
| GB | 3.9 ± 2.2 | 24 ± 14 |
| Challenged | | |
| UJ | 2.2 ± 1.0 | 9 ± 5 |
| LJ | 2.9 ± 1.5 | 12 ± 10 |
| GB | 3.6 ± 1.7 | 16 ± 9 |

above 90 % throughout the year due to winter temperatures that remained high enough to maintain infection of *P. marinus* (Andrews and Ray, 1988). Hence, the Galveston Bay area (i.e., Confederate Reef) provides a constant source of infection for newly recruited spat and juvenile oysters (Andrews and Ray, 1988). In contrast, oysters in the lower Cheseapeake Bay experience a strong seasonal cycle of the disease (Andrews, 1988). *Perkinsus marinus* infections are generally acquired in oysters by July when oysters infected from the previous year develop severe infections, die and release infective stages of the parasite. Oysters from the lower James River (LJ) in June are thus probably comprised of two different age-groups; one group of oysters infected by *P. marinus* in the previous year and another group of uninfected oysters.

*Perkinsus marinus* infection progressed rapidly in UJ oysters. The increase in weighted incidence of infection between three and seven weeks was greater in challenged UJ oysters than in challenged LJ oysters. The upper James River is an area that has been traditionally free of *P. marinus* and the oysters are highly susceptible to infection compared to oysters from other sites (Andrews, 1984). The reasons why these oysters rapidly succumb to the parasite are currently unknown but may be attributed to the following: 1) the absence of possible selection for resistance since UJ oysters have never been challenged with

*P. marinus*; 2) the depression of physiologic functions (including defense) caused by residence in the upper James River, a habitat that has a high sediment load and a salinity that is often below 3 ppt; 3) further suppression of host defense activities caused by transfer of the oysters to the York River and the accompanying increase in water salinity.

The lack of a significant increase in resistance to *P. marinus* over the years in oysters from enzootic areas of the Chesapeake Bay has been attributed to the genetic input of unselected oysters from low salinity refuge areas. Burreson (1991) spawned and raised progeny of UJ and LJ oysters in the York River for two years and found little difference in their susceptibility to *P. marinus*. The faster increase in weighted incidence of *P. marinus* infection in UJ oysters compared to LJ, in this study, may thus be due to environmental rather than genetic factors. Oysters from the upper and lower James River probably form a homogenous population (Rose, 1984; Buroker, 1983; Brown and Paynter, 1991) since larvae originating in the Upper James River (Deep water shoals) are transported to the Lower James River (Nansemond Ridge) in a few tidal cycles (Wood and Hargis, 1971).

There were significant differences in selected potential host defense activities among oysters from the three sites. One factor possibly responsible for these

differences was the difference in the level (i.e., weighted incidence) of *P. marinus* infection among GB, UJ, and LJ oysters.

The density of circulating oyster hemocytes tended to increase with increasing weighted incidence of *P. marinus* infection. At the start of the experiment and at week three, the numbers of circulating hemocytes in GB oysters, which had the highest weighted incidence, were greater than in UJ and LJ oysters. The differences, however, were not significant as the weighted incidence of *P. marinus* infection increased in UJ and LJ oysters. The increase in weighted incidence in challenged UJ and LJ oysters was also accompanied with an increase in the density of circulating hemocytes. Several studies have found similar results in oysters infected with *P. marinus* (La Peyre and Chu, 1988; La Peyre et al., 1989; Chu and La Peyre, 1993; Chu and La Peyre, In Press; Chu et al., In Press). Chu and La Peyre (In Press) found a positive correlation between the intensity of *P. marinus* infection and the density of circulating hemocytes. The elevated counts of circulating hemocytes is not surprising since infection by parasites is often accompanied by increasing densities of hemocytes in oyster tissue and hemolymph (Farley, 1968; Chagot, 1989; Ling, 1990; Ford et al., 1993). Hemocyte density in tissue also increased in oysters infected with *P. marinus* (Mackin, 1951). This increase in the total number of hemocytes, as

indicated by the increase in densities in both hemolymph and tissue of heavily infected oysters, is probably the result of hemopoiesis in response to the parasite or parasite-associated tissue damage.

The increase in circulating hemocyte density in infected oysters may be due to an increase in the percentage of small hyalinocytes. These small hyalinocytes are cells with a low nucleus to cytoplasm ratio and are believed, by some, to be the precursors of other hemocyte types in molluscs (Moore and Eble, 1977; Sminia et al., 1983; Bachere et al., 1988). Increases in the percentage of small hyalinocytes have also been reported for eastern oysters infected with *Haplosporidium nelsoni* (MSX) (Ford et al., 1993), and for edible oysters (*Ostrea edulis*) infected with the protozoan, *Bonamia ostreae* (Chagot, 1989).

*Perkinsus marinus* infection also affected hemocyte chemiluminescence. There were significant increases in hemocyte chemiluminescent responses to zymosan in *P. marinus* challenged UJ and LJ oysters compared to control UJ and LJ oysters. This enhanced chemiluminescence may be due to "activation" of the hemocytes by the parasite or parasite-associated tissue damage. Hemocyte chemiluminescence was consistently greater in GB oysters compared to UJ or LJ oysters which had similar weighted incidence of *P. marinus* infection as GB oysters. The cause of this difference is not yet known but may be due to infection of GB oysters over

a longer time period, and thus increased hemocyte "activation". The difference may also be due to a different composition of the hemocyte population since, for example, the percentage of granulocytes is much lower in GB oysters. Unfortunately, it is unknown which type(s) of hemocytes contribute the most to chemiluminescence. Chagot (1989) has shown that hyalinocytes contain NADPH-oxidase (i.e, the enzyme responsible for the respiratory burst and associated chemiluminescence) and exhibit chemiluminescence. However, granulocytes are the most phagocytic cells and would be expected to have the highest chemiluminescent response. The differences might also be due to environmental differences between habitat, or genetic differences between oyster "stocks".

The elevated production of potentially toxic oxygen metabolites in GB oysters, as reflected by the high hemocyte chemiluminescence, appears to have little effect on *P. marinus*. In fact, Anderson et al. (1992) have speculated that the elevated hemocyte chemiluminescence may actually contribute to the pathological effects of *P. marinus*. Interestingly, La Peyre et al. (1992, Chapter 6) have recently shown that there was no increase in chemiluminescence when hemocytes from either eastern or Pacific oysters were stimulated with isolated *P. marinus* merozoites. It was suggested that the parasite may contain anti-oxidant enzymes such as catalase and superoxide

dismutase, capable of scavenging the toxic oxygen metabolites produced by hemocytes.

Plasma lysozyme concentrations and hemagglutinin titers decreased with increasing weighted incidence of *P. marinus* infection as indicated by the differences between control and challenged UJ oysters. Decreases in plasma lysozyme concentration and agglutinin titer for latex beads have also been observed in eastern oysters heavily infected with MSX (*Haplosporidium nelsoni*) (Feng and Canzonier, 1970; Ling, 1990). The decrease of these factors is probably due to pathological effects of parasite infection. The possible binding of agglutinin to the numerous parasites in heavily infected oysters may also be the cause of the decrease in hemagglutinin titer.

Galveston Bay oysters with similar weighted incidence of *P. marinus* infection as James River oysters exhibited differences in potential host defense activities such as percentage of granulocytes, hemocyte chemiluminescent response and plasma hemagglutinin titer. Although some of these differences may be attributed to difference in the seasonal pattern of pathogenesis, GB oysters being presumably infected over a longer time period, other environmental factors associated with habitat, with past history of the oysters, and with genetic factors may also be responsible.

The percentage of granulocytes, for example, was greater in UJ than in GB oysters with similar intensities of *P. marinus* infection. The high granulocyte percentage of UJ may be in part due to the habitat at which these oysters were collected since the percentage of granulocytes tend to be greater in oysters maintained in low salinity water (Fisher and Newell, 1986; La Peyre et al., 1989; Chu and La Peyre, 1993; Chu et al., In Press).

It is probable that *P. marinus* caused mortalities of oysters from all three sites. It is impossible to assess, however, if any difference in pathogenesis of *P. marinus* among these oysters since GB and LJ oysters were already infected with the parasite at the start of the experiment. Pathogenesis significantly affected certain hemolymph factors; Advanced *P. marinus* infection in oysters resulted in increases in circulating hemocyte density and hemocyte chemiluminescence and, decreases in the percentage of granulocytes, plasma hemagglutinin titer and plasma lysozyme concentration. However, it cannot be excluded that other factors related to the oyster habitat and past environmental history may also be responsible for the differences in potential host defense activities (Fisher, 1988; La Peyre et al., 1989; Chu and La Peyre, 1993; Chu and La Peyre, In press). It is necessary to raise in the same environment progenies of oysters originating from various sites to investigate pathogenesis of *P. marinus* among oyster stocks

and resulting host defense activities.

The use of potential host defense activities as markers of disease resistance in oysters for selection purposes has been proposed (Fisher and Auffret, 1986). Unfortunately, there is a lack of understanding about the role of these factors, in general, and in particular against *P. marinus*. In addition, hemocytes as well as plasma lysozyme and probably agglutinin are involved in other physiological processes such as nutrition and reproduction. Hemocytes have multiple functions. They participate in such processes as wound repair, nutrition and metal detoxification (Feng et al., 1977; Simkiss and Mason, 1985; Sparks and Morado, 1988). Lysozymes of bivalves are also believed to play an important role in food digestion (McHenery and Birbeck, 1982); The function(s) of agglutinins is still unclear. They may serve as transport or storage molecule, or organize macromolecules and multienzyme complexes (Yeaton, 1981, Vasta, 1991). More basic research is needed to better understand the function of hemocytic and humoral activities.

# Chapter 2

## HEMOCYTIC AND HUMORAL ACTIVITIES OF EASTERN AND PACIFIC OYSTERS, FOLLOWING CHALLENGE BY THE PROTOZOAN *PERKINSUS MARINUS*

# INTRODUCTION

Virginia landings of the eastern oyster, *Crassostrea virginica*, in 1992 were at a record low (Virginia Marine Resource Commission). The decline in recent years can be attributed in part to the effects of MSX and Dermo diseases caused by the two protozoan parasites, *Haplosporidium nelsoni* and *Perkinsus marinus* (Hargis and Haven, 1988). *Perkinsus marinus* (Dermo) has become the predominant pathogen since the mid-1980's, replacing *H. nelsoni* (MSX) as the most important pathogen of oysters in the lower Chesapeake Bay (Andrews 1988; Burreson and Andrews, 1988). *Perkinsus marinus* also causes extensive mortalities along the Atlantic and Gulf coasts of the United States (Andrews, 1988).

The introduction of the Pacific oyster, *Crassostrea gigas*, into the Chesapeake Bay has been proposed to revitalize the oyster industry of Virginia (Mann et al., 1991). Pacific oysters have been successfully introduced in many parts of the world (Mann, 1981) because they are resistant to a number of infectious diseases that kill native oysters (Comps, 1988; Grizel et al., 1988). Disease resistance may be due in part to the competence of the host defense system.

Host defenses in oysters include both cellular and humoral mechanisms (Feng, 1988; Chu, 1988). The major role

of hemocytes in molluscan internal defense has long been recognized (Stauber, 1950; Cheng, 1975). Hemocytes readily phagocytose a wide variety of biotic and abiotic particles *in vivo* and *in vitro* (Tripp, 1960; Poder et al., 1982; Feng, 1988). Hemocytes also produce antimicrobial compounds such as lysosomal enzymes and toxic oxygen metabolites (Cheng, 1983; Adema, 1991). Moreover, humoral factors, some of which are released by hemocytes, may also play an important role in host defense (Fries, 1984; Chu, 1988). Lysozyme is a lysosomal enzyme with antimicrobial properties that is secreted by hemocytes and normally found in oyster plasma (McDade and Tripp, 1967; Rodrick and Cheng, 1974; Chu and La Peyre, 1989). Plasma agglutinins, some of which have been demonstrated to be lectins, can act as opsonins (Hardy et al., 1977; Renwrantz and Stahmer, 1983; Yang and Yoshino, 1990). Lectins are believed to play an important role in non-self recognition in invertebrates which do not produce immunoglobulins (Olafsen 1988, Vasta 1991). Changes in potential host defense activities of eastern oysters following infection by *P. marinus* have only recently been investigated (Chu and La Peyre, In Press; Chu et al., In Press).

Meyers et al. (1991) found that Pacific oysters challenged with *P. marinus* developed mostly light infections and that no mortalities could be attributed directly to the parasite. Cellular and humoral activities associated with

possible resistance may thus be identified by comparing these two species. This paper compares the changes in potential host defense activities between eastern and Pacific oysters following challenge with *P. marinus*.

## MATERIALS AND METHODS

### Experimental animals

Eastern oysters (*Crassostrea virginica*) and Pacific oysters (*Crassostrea gigas*) were the progeny of broodstock obtained from Mobjack Bay (VA) and the Coast Oyster Company (Quilcene Bay, WA). The broodstocks were spawned in the spring of 1989 and the spat were grown in flumes as described by Meyers et al. (1991). The flumes were continuously supplied with estuarine water (York River, VA) and the effluents were diverted to a sand retention pond. Oysters used for the experiments were 14 months old and about 40 mm in shell height.

### Experimental design

In the first experiment, 50 oysters from each species were divided into two groups and placed in four separate 250 L tanks filled with filtered (1 $\mu$m) estuarine water. Water temperature was maintained at 25° C, a temperature favorable for the proliferation of *P. marinus* in eastern oysters.

Water was changed every two weeks. Oysters were fed with 0.1 g/oyster/day of algal paste composed of a mixture of *Pavlova lutheri*, *Isochrysis galbana* and Tahitian *Isochrysis galbana*. Oysters from both species were challenged weekly by adding minced oyster tissues infected with *P. marinus* to the water. Control oysters were not exposed to minced tissue. Oysters were sampled three months post-exposure. Potential host defense activities and intensity of infection in 10 individual oysters from each group were determined as described below.

In the second experiment, 175 oysters from each species were maintained in a single flume supplied with flowing estuarine water as described by Meyers et al. (1991). The maintenance of these oysters in a flow-through water system better simulated field conditions and limited stress associated with holding bivalves in closed water systems (Bayne and Thompson, 1970; Ansell and Sivadas, 1973). Minced tissue from infected eastern oysters was added to the flume weekly during the first month of the experiment to supplement infection by *P. marinus* occurring via estuarine water (i.e., enzootic area). Oysters were sampled three months after the start of the experiment.

Measurement of potential host defense activities

In both experiments, the density of circulating hemocytes, percentage of granulocytes, plasma lysozyme and

protein concentration and hemagglutinin titer were measured. In addition, hemocyte phagocytic and chemiluminescent responses to zymosan were measured in the second experiment.

Oysters were notched on the dorsolateral side of the shell closest to the adductor muscle. Hemolymph (1 ml) was withdrawn from the adductor muscle sinus of individual oysters with a syringe equipped with a 27 gauge needle. Hemolymph samples were immediately placed in test tubes in an ice bath.

The density of hemocytes and the percentage of granulocytes in each hemolymph sample were determined with a Bright-Line hemacytometer (Reichert, Buffalo, NY).

Phagocytosis of zymosan particles (Sigma Chemical Company, St. Louis, MO) was measured using hemocyte monolayers. Hemolymph samples (20 $\mu$l) were placed onto microscope slides and the hemocytes were allowed to settle and adhere for 30 min in humidified chambers. Zymosan suspended in 20 $\mu$l artificial sea water (1 mg/ml ASW, 20 ppt, Instant Ocean, Aquarium Systems Inc., Mentor, OH) was added to the hemocyte monolayers. After incubation for 45 min, hemocytes were rinsed in ASW, fixed with Davidson's AFA (Alcohol-Formaldehyde-Acetic acid) for 30 min and stained with Hemal I and II staining solution (Hemal Stain Company Inc., Danbury, CT). The percentage of phagocytic hemocytes in 200 hemocytes and the number of zymosan particles per phagocytic hemocyte were determined.

Hemocyte chemiluminescent (CL) response to zymosan was measured with a Beckman LS 150 scintillation counter operated in the out-of-coincidence mode. Luminol stock solution prepared according to the method of Scott and Klesius (1981) was diluted 1:500 with ASW and was used to amplify chemiluminescence. Chemiluminescent response was initiated by the addition of zymosan suspension (500 $\mu$l, 1 mg/ml ASW) to hemocyte suspension (500 $\mu$l, $10^6$ cell/ml) in diluted luminol (500 $\mu$l). The control received 500 $\mu$l of ASW instead of zymosan suspension. The CL responses are reported as net counts per minute (CPM): Net CPM= peak CPM of stimulated hemocytes - CPM of control hemocytes. The time of peak CPM was also recorded.

Cell-free hemolymph or plasma was obtained by centrifugation at 400 *g* for 10 min. Plasma hemagglutinin titer was determined by the addition of sheep red blood cells (0.5%, Becton Dickinson Co., Cockeysville, MA) to serially diluted oyster plasma in U-well microtiter plates. Results are expressed as the log2 of the mathematical reciprocal of the maximal plasma dilution showing complete agglutination. The remaining plasma was stored at -20° C for measurements of lysozyme and protein concentration.

Lysozyme activity was determined spectrophotometrically according to a modification of the method of Shugar (1952) (Chu and La Peyre, 1989). Briefly, a suspension of *Micrococcus lysodeikteicus* (15 mg/ml) with an absorbance of

0.7 at 450 nm was prepared in 0.066 M phosphate buffer (pH 6.3). Plasma (0.1 ml) was added to 1.4 ml of the bacterial suspension and the decrease in absorbance was recorded at 450 nm for 2 min. Lysozyme activities were converted to concentrations using lysozyme from hen egg white as a standard.

Plasma protein was determined according to the method by Lowry et al. (1951) using bovine serum albumin as a standard.

Prevalence and intensity of *P. marinus* infection

The fluid thioglycollate test described by Ray (1952, 1966) was used for *P. marinus* diagnosis. The intensity of infection was determined in mantle, gill and rectal tissues and rated according to the categories of Ray (1954) by estimating the percentage of tissue occupied by the parasite.

Statistical analysis

Data were analyzed by two-factor and one factor analysis of variance, followed by SNK's multiple comparisons of means when significant differences ($p<0.05$) were found.

## RESULTS

Most (9/10) of the challenged eastern oysters from experiment 1 were infected with *P. marinus* and the majority (7/10) had light infections (Table 1). In contrast, the parasite was detected in only one (1/10) of the challenged Pacific oysters. None of the control oysters from either species were infected. No oyster mortality occurred in experiment 1. Similarly, prevalence of infection in Pacific oysters was lower than in eastern oysters in experiment 2 (Table 2). Infection was detected in all eastern oysters (16/16) and the majority (10/16) of the eastern oysters had heavy infections. In contrast only 60 % (12/20) of the Pacific oysters were infected and all infections were light. Mortality was 76 % in eastern oysters and 25 % in Pacific oysters in experiment 2.

Changes in potential host defense activities varied between species in experiment 1. There were significant increases in the density of circulating hemocytes ($p<0.0001$, Figure 1) and percentage of granulocytes ($p=0.0038$, Figure 2) as well as plasma hemagglutinin titer ($p<0.0001$, Figure 3) in Pacific oysters that were exposed to the minced tissue compared to control Pacific oysters; this was not observed for eastern oysters.
Moreover, hemagglutinin titer was greater in control Pacific oysters than in control eastern oysters ($p<0.0001$), but

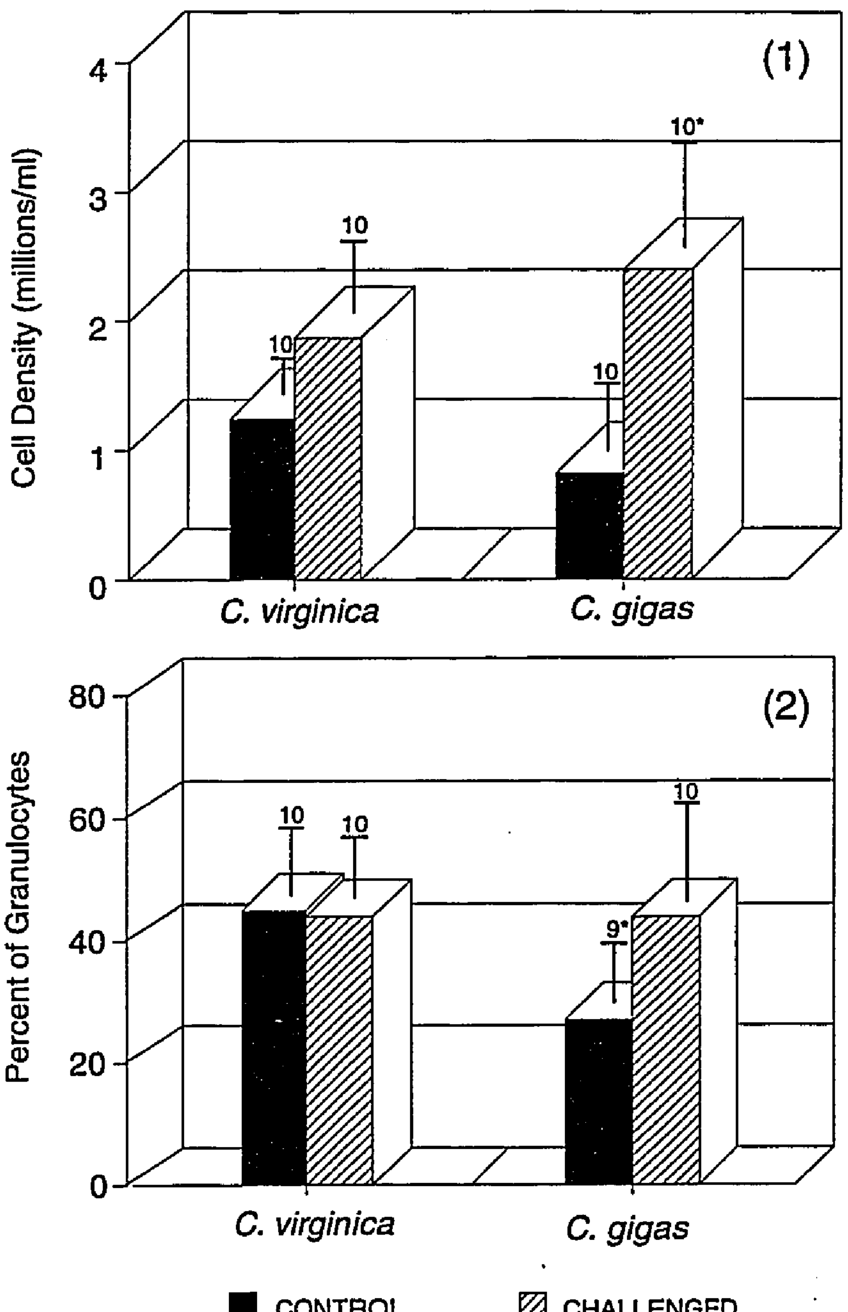
Table 1: *Perkinsus marinus* prevalence and intensity of infection in control and challenged eastern (*C. virginica*) and Pacific *(C. gigas)* oysters from Exp. 1. L-M-H indicates the number of oysters with light, moderate and heavy infections.

| | No. examined | Prevalence | Intensity (L-M-H) |
|---|---|---|---|
| *C. virginica* | | | |
| Control | 10 | 0 % | - |
| Challenged | 10 | 90 % | 7-2-0 |
| *C. gigas* | | | |
| Control | 10 | 0 % | - |
| Challenged | 10 | 10 % | 1-0-0 |

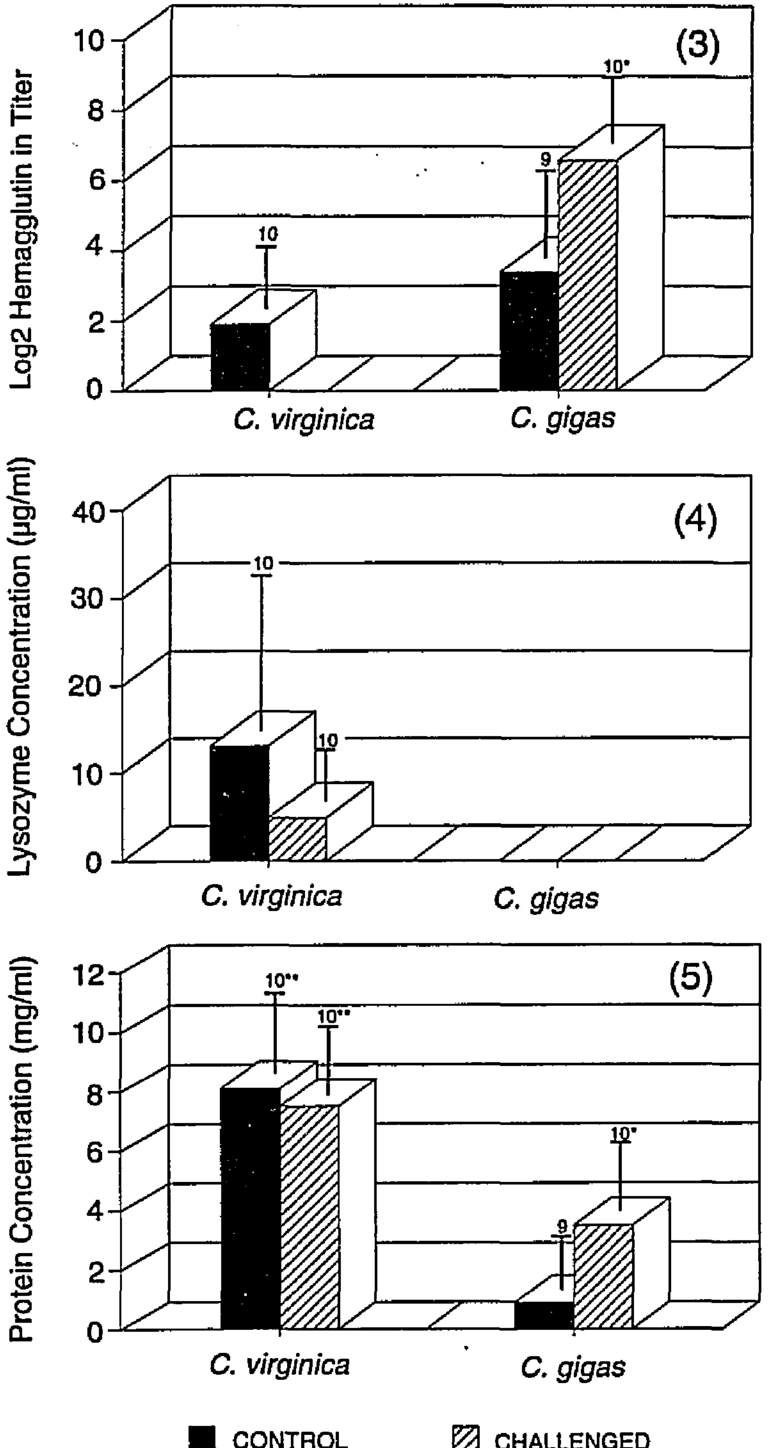
Table 2: *Perkinsus marinus* prevalence and intensity of infection in eastern (*C. virginica*) and Pacific (*C. gigas*) oysters from Exp. 2. L-M-H indicates the number of oysters with light, moderate and heavy infections.

| | No. examined | Prevalence | Intensity (L-M-H) |
|---|---|---|---|
| *C. virginica* | 16 | 100 % | 3-3-10 |
| *C. gigas* | 20 | 60 % | 12-0-0 |

Figures 1 and 2: Mean (± SD) density of circulating hemocytes and mean (± SD) percentage of granulocytes in control and challenged, eastern (*C. virginica*) and Pacific (*C. gigas*) oysters from Exp. 1. Number of oysters is indicated on the top of each bar. Asterisks (*) denote significance at $p<0.05$ between challenged and control oysters for either oyster species. Double Asterisks (**) denote significance at $p<0.05$ between eastern and Pacific oysters for either control or challenged oysters


(1)
Cell Density (millions/ml)
4
3
2
1
0
10
10
10
10*
C. virginica
C. gigas
(2)
Percent of Granulocytes
80
60
40
20
0
10
10
10
9*
C. virginica
C. gigas
CONTROL
CHALLENGED

Figures 3 through 5: Mean (± SD) of plasma hemagglutinin titer, lysozyme concentration and protein concentration, in control and challenged eastern (*C. virginica*) and Pacific oysters (*C. gigas*) from Exp. 1. Number of oysters is indicated on the top of each bar. Asterisks (*) denote significance at $p<0.05$ between challenged and control oysters for either oyster species. Double Asterisks (**) denote significance at $p<0.05$ between eastern and Pacific oysters for either control or challenged oysters



(3)
Log2 Hemagglutin in Titer
10
8
6
4
2
0
10
9
10*
C. virginica
C. gigas
(4)
Lysozyme Concentration (µg/ml)
40
30
20
10
0
10
10
C. virginica
C. gigas
(5)
Protein Concentration (mg/ml)
12
10
8
6
4
2
0
10**
10**
9
10*
C. virginica
C. gigas
CONTROL
CHALLENGED

plasma hemagglutinin titer was undetectable in challenged eastern oysters. Plasma lysozyme concentration varied greatly between individual eastern oysters and was not detected in Pacific oysters (Figure 4). Plasma protein concentration was significantly greater in control eastern oysters than control Pacific oysters ($p = 0.0067$), but increased significantly only in exposed Pacific oysters (Figure 5).

Significant differences in host defense activities were also found between eastern and Pacific oysters in experiment 2 (Table 3). The density of circulating hemocytes was significantly greater ($p=0.0008$) in eastern oysters than in Pacific oysters while the percentage of granulocytes in Pacific oysters was significantly greater ($p<0.0001$) than in eastern oysters. Moreover, the hemocytes of Pacific oysters were significantly more active, both in terms of the percentage of phagocytic hemocytes ($p<0.0001$) and in the number of zymosan particles per phagocytic hemocytes ($p=0.0005$), than hemocytes of eastern oysters. Hemocyte chemiluminescent responses showed great variability, and although chemiluminescence to zymosan in Pacific oysters appeared to be greater than in eastern oysters, the difference was not significant. However, Pacific oyster hemocytes responded significantly faster ($p<0.0001$) than eastern oyster hemocytes, with peak chemiluminescence occurring in about half the time. The plasma hemagglutinin

Table 3: Density of circulating hemocytes, percentage of granulocytes, percentage of phagocytic hemocytes, number of zymosan particles phagocytosed, net hemocyte chemiluminescence (CL), time of peak CL, plasma hemagglutinin titer, lysozyme and protein concentration in eastern (*C. virginica*) and Pacific (*C. gigas*) oysters from Exp. 2. Values shown are means ± SD. Number of oysters is indicated in parentheses. Asterisks (*) denote significance at $p<0.05$ level.

| | *C. virginica* | *C. gigas* |
|---|---|---|
| Hemocyte density ($10^6$/ml)* | 4.97 ± 3.37 (16) | 2.33 ± 1.2 (20) |
| % granulocytes* | 25 ± 9 (16) | 46 ± 14 (20) |
| % phagocytic hemocyte* | 17 ± 6 (10) | 46 ± 25 (10) |
| No. zymosan particles* | 2.2 ± 0.5 (10) | 3.6 ± 1.3 (10) |
| Net CL (Counts.$10^3$/min) | 77 ± 135 (10) | 205 ± 284 (10) |
| Time of peak CL (min)* | 4.3 ± 1.3 (10) | 2.1 ± 0.3 (10) |
| Hemagglutinin titer* | 4.0 ± 2.0 (16) | 6.7 ± 2.2 (20) |
| Lysozyme (ug/ml)* | 10.0 ± 12.4 (16) | 1.6 ± 2.7 (20) |
| Protein (mg/ml)* | 17.0 ± 7.5 (16) | 5.0 ± 2.8 (20) |

titer of Pacific oysters was greater than that in eastern oysters while plasma lysozyme and protein concentration was greater in eastern oysters.

## DISCUSSION

In this study, significant differences in potential host defense activities between Pacific and eastern oysters challenged with *P. marinus* were found. Caution must be exercised when interpreting these results, however, since the cause(s) of these differences are uncertain. In addition to *P. marinus*, minced tissue contains cellular debris and various microorganisms (e.g., bacteria and protozoa). Further, minced tissue added to the water may have also increased the bacterial microbiota. *Perkinsus marinus* in minced tissues was used because it was the only available method to effectively transmit the disease to uninfected oysters at the time these experiments were conducted (Ray, 19854); there was no technique to isolate and purify *P. marinus* from infected oyster tissue.

Whatever the cause(s) of the changes in host defense activities, those changes are clearly different in Pacific and in eastern oysters. Moreover, these changes may be important in limiting the development of *P. marinus* in the Pacific oyster.

The ability of oysters to resist infection may be dependent on their hemocyte numbers and activities at the time of challenge or in response to infection (Fisher and Newell, 1986). It is thus interesting to note that the density of circulating hemocytes in Pacific oysters exposed to *P. marinus* increased significantly compared to the control oysters, whereas no such change occurred in eastern oysters with light *P. marinus* infections. Although the difference in the density of hemocytes in hemolymph of exposed versus control Pacific oysters may not appear significant, it may be important in terms of the total number of hemocytes since hemolymph may account for a large percentage of the oyster's total body volume.

Light infection by *P. marinus* did not significantly change the density of circulating hemocytes in eastern oysters. Similar findings have been observed previously (Chu and La Peyre, In Press; Chu et al., In Press; Chapter 1). The higher hemocyte densities in eastern oysters in experiment 2 compared to experiment 1 are probably due to higher intensities of infection. There are reports of increasing hemocyte densities in tissue and hemolymph with intensification of *P. marinus* infection (Mackin, 1951; Chu and La Peyre, In Press; Chapter 1).

It is presently difficult to asses the significance of hemocyte number in relation to the susceptibility of oysters to *P. marinus*. *In vivo*, *P. marinus* is often found in

hemocytes (Mackin, 1951; Perkins, 1976; Gauthier and Fisher, 1990) and in heavily infected oysters, individual hemocytes can contain as many as 25 parasites (La Peyre and Chu, Submitted). In addition, hemocytes of both eastern and Pacific oysters maintained at 15° C, readily ingest and to some degree degrade *P. marinus in vitro* (La Peyre et al., 1992, Chapter 6). It is currently unknown, however, if and to what extent the capacity of hemocytes to degrade *P. marinus* is affected in oysters maintained at higher temperature. A greater number of hemocytes in the oyster should be beneficial if the hemocytes can effectively kill *P. marinus*. However, hemocytes may accelerate the progression of the infection by spreading the parasite throughout the oyster tissue if the hemocytes ability to kill the parasite is reduced or overwhelmed by multiplication of the parasite.

In addition to hemocyte density, the composition of the hemocyte population may be important since it is well known that hemocytes differ in their level of phagocytosis, chemiluminescence and lysosomal synthesis (Cheng, 1984; Cheng and Downs, 1988; Chagot, 1989; La Peyre et al., 1992, Chapter 6). The percentage of circulating granulocytes increased in challenged Pacific oysters while it did not change significantly in challenged eastern oysters. Granulocytes are the most phagocytic hemocytes and thus they may be important in the outcome of *P. marinus*-oyster

interactions since, according to La Peyre et al. (1992, Chapter 6), granulocytes readily ingest *P. marinus* merozoites *in vitro* and can degrade the parasite to some extent.

The percentage of circulating granulocytes in heavily infected eastern oysters (Experiment 2) was quite low compared to lightly infected eastern oysters (Experiment 1). This difference was also seen in other studies (Chu and La Peyre, In Press; Chu et al., In Press; Chapter 1). The cause of the low percentage of granulocytes may be due to various processes including: 1) hemocyte lysis or bursting following the ingestion and possible intracellular multiplication of *P. marinus* (Mackin, 1951; Perkins 1976); 2) degranulation; 3) migration of granulocytes to the sites of infection; or 4) increase in the number of circulating hyalinocytes. Increase in the percentage of hyalinocytes has been reported in the case of oysters afflicted with MSX or Bonamiasis (Farley, 1968; Chagot, 1989; Ford et al. 1993).

In addition to the number and types of hemocytes, their general level of activity may also be important in the oysters host defense against pathogens such as *P. marinus*. Pacific oysters had greater numbers of phagocytic hemocytes that ingested greater numbers of zymosan particles than eastern oysters. The higher percentage of phagocytic hemocytes in Pacific oysters is probably related to the

greater percentage of granulocytes in Pacific oysters than in eastern oysters. Zymosan particles are avidly ingested by granulocytes. In fact, a strong correlation was found between the percentage of hemocytes ingesting zymosan and the percentage of granulocytes (Chu and La Peyre, In Press; La Peyre et. al, 1992, Chapter 6).

The lower number of zymosan particles ingested by eastern oyster granulocytes compared to Pacific oysters may have been influenced by the presence of *P. marinus* in many of the eastern oyster hemocytes. Alternatively or in combination, the reduced phagocytic activity of eastern oyster hemocytes may be due to pathological effects of infection or to stress related to the high temperature at which the oysters were maintained. Hemocyte phagocytosis of zymosan is reduced in oysters maintained at 25° C compared to 20° C (Chu and La Peyre, In Press). Therefore, hemocyte activities in uninfected Pacific and eastern oysters maintained at elevated temperature should be compared in future studies.

Although hemocyte phagocytosis is an indicator of the level of hemocyte activity, it does not provide information on the capacity of hemocytes to kill *P. marinus*. The degree and mechanisms of killing, the most important step, are still unknown.

Toxic oxygen metabolites are produced by phagocytic cells including oyster hemocytes and are believed to play a

role in the killing of parasites (Klebanoff et al., 1983; Adema, 1991). Although the zymosan-stimulated hemocyte CL of Pacific oysters appears to be greater than that of eastern oysters, the difference was not significant. Pacific oyster hemocytes, however, responded twice as fast as eastern oyster hemocytes in terms of peak CL. The effects of toxic oxygen metabolites on the parasite need to be determined before their role in *P. marinus* infection can be fully elucidated.

Hemagglutinin titers increased in Pacific oyster plasma following challenge with tissue of *P. marinus* infected oysters (experiment 1). Induction of hemagglutinin titer in Pacific oysters exposed to bacteria in water has been reported previously (Hardy et al., 1977; Olafsen et al., 1992). Pacific oysters had higher hemagglutinin titer than eastern oysters in experiment 2. High hemagglutinin titer prior to infection may also be important; Burreson (1991) found that a strain of MSX-resistant oysters succumbed more quickly to *P. marinus* than native oysters from Delaware and Chesapeake Bays. Plasma hemagglutinin titers in these MSX-resistant oysters were lower in the spring, prior to *P. marinus* infection, than in the native oyster stocks (La Peyre, unpublished data). This finding is somewhat puzzling since the plasma agglutinin titers for latex beads (Ling, 1990) and for the bacterium *Vibrio cholerae* CA401 (Chintala and Fisher, 1991) in MSX-resistant oysters are generally

greater than in MSX-susceptible oysters.

The role of hemagglutinin in host defense against *P. marinus* is not yet known. Oyster hemagglutinins are lectins which are molecules believed to serve in non-self recognition (Olafsen, 1988; Vasta, 1991). Plasma hemagglutinin may thus serve as an opsonin by bridging carbohydrates between hemocytes and *P. marinus* or zymosan. The opsonic activity of hemagglutinin of bivalve mollusc plasma has been demonstrated in a number of studies (Hardy et al. 1977; Renwrantz and Stahmer, 1983; Yang and Yoshino, 1989). Phagocytosis of zymosan was greater in Pacific oysters than in eastern oysters and it is possible that hemagglutinin may be partly responsible. Fryer and Bayne (1989), for example, have found that plasma from two strains of the snail, *Biomphalaria glabrata*, resistant to infection by the trematode, *Schistosoma mansoni*, enhanced hemocyte phagocytosis of yeast while plasma from a susceptible strain did not. Plasma hemagglutinin may also bind the parasite to specific receptors on the surface of hemocytes which may trigger phagocytosis and degradation of *P. marinus*. The direct effect of plasma hemagglutinin in the hemocyte-*P. marinus* interaction needs to be investigated to determine the significance of the lectin.

Plasma lysozyme concentration has been reported to vary greatly in individual eastern oysters (Chu and La Peyre, 1989). In this study, plasma lysozyme concentrations

appeared lower in eastern oysters with light infections compared to uninfected oysters, although the difference was not significant. Results of decreasing plasma lysozyme with increasing intensity of *P. marinus* infection or in oysters infected with the protozoan *Haplosporidium nelsoni* have also been reported (Feng and Canzonier, 1970; Chu and La Peyre, In Press; Chapter 1). It is not known why lysozyme was very low or undetectable in Pacific oyster plasma but it may indicate differences in lysozyme gene expression between the two oyster species. It would be interesting to determine the level of lysozyme in Pacific oyster hemocytes. Lysozyme is a lysosomal enzyme that has been shown to be secreted by hemocytes and is normally found in plasma of various molluscs (Cheng, 1983).

Plasma protein concentrations were significantly greater in eastern oysters than in Pacific oysters. Although plasma protein concentrations are variable and influenced by a number of factors, concentrations reported in the literature for Pacific oysters are generally much lower than for eastern oysters (Fisher and Newell, 1986; Ford, 1986; Chagot, 1989; Chu and La Peyre, 1989).

The significance of the low plasma protein level on the development of *P. marinus* is not known, but it may retard the growth of the parasite. In addition, low plasma protein may indicate further differences in the biochemical composition of eastern and Pacific oysters and the

availability of nutrients from the host to *P. marinus*.

It is interesting to note that plasma protein increased in Pacific oysters exposed to *P. marinus* and may be due to the increase in hemagglutinin titer. According to Acton (1970), plasma hemagglutinin can represent a large part (97%) of oyster plasma protein. This finding, however, is controversial. Moreover, correlations between protein concentration and hemagglutinin titer are often found (Ling 1990; La Peyre and Chu, unpublished data).

The factors governing the success of *P. marinus* development in eastern oysters are still unknown. *Perkinsus marinus* does not avoid being recognized since eastern oyster hemocytes readily phagocytose the parasite (La Peyre et al., 1992, Chapter 6). Moreover, there is little evidence suggesting that the parasite can inhibit the host defenses in eastern oysters. There is little change in cellular and humoral activities of eastern oysters with light *P. marinus* infections, whereas the decrease of these activities in heavily infected oysters is probably due to pathological effects. More studies on the direct interaction of *P. marinus* and host defenses are needed. Temperature is the major determining factor in the development of *P. marinus* disease in eastern oyster (Andrews 1988). *Perkinsus marinus* appears to rapidly overwhelm the host defenses of eastern oysters at high temperatures (>25° C). It is likely that temperature greatly influences the multiplication rate of *P.*

*marinus*. In fact, the multiplication rate of *P. marinus* propagated *in vitro* at 15° C is quintupled at 28° C (La Peyre, unpublished data). Additionaly, the oyster host defenses may be depressed by high temperature stress which further favors the development of *P. marinus*.

Results indicate that Pacific oysters may offer a less "suitable" environment for the development of *P. marinus* compared to eastern oysters, for at least two possible reasons: 1) the elevated cellular and humoral activities may degrade the parasite more effectively, and 2) lower plasma protein levels may limit parasite growth. The direct effects of cellular and humoral activities as well as protein concentration on parasite viability and growth will need to be determined. A technique has recently been established to propagate *P. marinus in vitro* that will enable the study of the parasite outside of the host (La Peyre et al., 1993).

# Chapter 3

# SIMPLE PROCEDURE FOR THE ISOLATION OF *PERKINSUS MARINUS* MEROZOITES, A PATHOGEN OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.

The protozoan *Perkinsus marinus* (Apicomplexa) causes heavy mortalities of the eastern oyster, *Crassostrea virginica*. Studies of the pathogenesis of this parasite and the host defense responses have been hampered by the absence of a standard protocol to isolate and enrich the tissue-associated parasitic stages.

The main histozoic stage of *P. marinus*, the merozoite (uninucleate coccoid cells, 2-4 $\mu$m), multiplies within oyster tissues following enlargement and division by schizogony (Perkins, 1991). It has recently been demonstrated that *P. marinus* merozoites can be propagated *in vitro* (La Peyre et al., 1993; La Peyre and Faisal, Submitted). It is unknown, however, if the culture procedure and conditions influence the virulence of *P. marinus*. In order to make this evaluation, it is necessary to isolate merozoites of known high pathogenicity as the reference in comparisons with cultured merozoites. In the present study, we report on a relatively simple procedure that allows the isolation of *P. marinus* merozoites directly from heavily infected oysters in a relatively pure form.

Hemolymph from individual oysters (*Crassostrea virginica*) was withdrawn from the adductor muscle sinus and examined for the presence of *P. marinus* merozoites with phase contrast microscopy. Oysters that had at least 25% infected hemocytes with 5 or more merozoites per hemocyte,

were selected (Figure 1). The visceral mass of each individual oyster was excised, minced finely into 0.1 $mm^3$ fragments, and suspended in sterile filtered (0.2 $\mu m$) York River water (FYW) using 25 ml/g of oyster tissue wet weight. The infected oyster tissue was then homogenized with a blender (Virtis 200, Virtis Company, Gardiner, NY) for 45 seconds. The homogenate was then filtered through a series of Nytex screens (53, 35, 15 $\mu m$ mesh size) (Tetko Inc., Briarcliff Manor, NY) into a 500 ml beaker. In order to separate the larger oyster cells and tissue fragments from the merozoites, the filtrate was allowed to stand overnight at 4°C. The supernate was gently aspirated with pasteur pipets to avoid disturbing the sediment. The supernate was centrifuged at 50 *g* for 10 min in order to pellet any remaining oyster cells and tissue fragments. The supernatant was then collected and centrifuged at 800 *g* for 20 minutes to sediment the merozoites. The pellets were collected, resuspended in FYW and washed six times (800 *g* for 20 minutes) to reduce the number of bacteria, and possibly mitochondria or nuclei. The last step was repeated until the number of bacteria, mitochondria or nuclei in the merozoite suspension was $<$ 1 bacterium or organelle/10 merozoites (Figure 2). Contamination (i.e. bacteria, mitochondria or nuclei) of merozoites was determined by staining DNA with 4'6-diamidino-2-phenylindole (DAPI) followed by examination with an epifluorescent microscope

Figure 1. Phagosome of a hemocyte filled with *Perkinsus marinus* merozoites (M). Hemocyte nucleus (N). Bar = 10 $\mu$m.

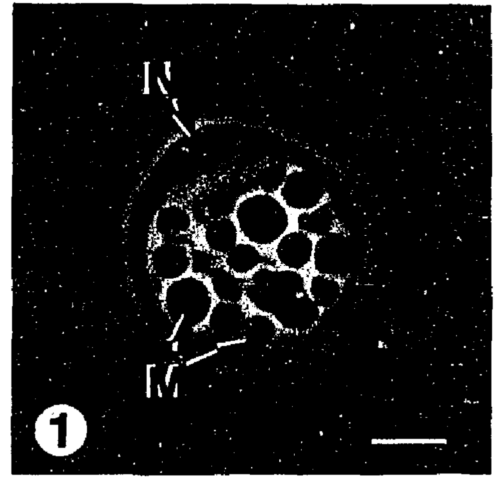
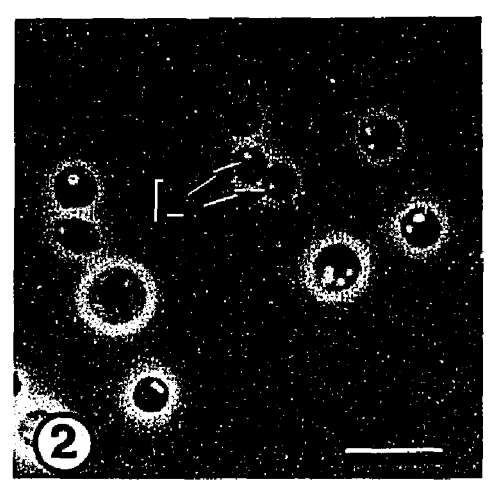

N
M
1

Figure 2. Light micrograph of isolated and partially purified *Perkinsus marinus* merozoites with typical lipid droplets (L). Bar = 10 μm.


2

according (Porter and Feig, 1980).

The viability of merozoites was determined by the uptake of neutral red at 10 mg/l for 12 hr. Some merozoites were boiled for 30 min to verify the validity of the vital stain for *P. marinus.* The ability of the isolated merozoites to enlarge in fluid thioglycollate medium was examined using the method described by S.M. Ray (1952). Moreover, the infectivity of isolated merozoites was determined by injecting $10^6$ cells (suspended in 100 $\mu$l FYW) into the mantle cavity of 25 notched uninfected oysters. Control oysters received 100 $\mu$l FYW. The presence of *P. marinus* in both oyster groups was determined in the rectal tissue of individual oysters using the Ray test (1952, 1966) at day 42 post-injection.

In this study, eight separate trials were performed on individual oysters. The average number of merozoites obtained after purification was $3.1 \pm 2.8 \times 10^8$ cells/gm wet weight of oyster tissue (range of $1.2\text{-}8.6 \times 10^8$ cells). The majority of merozoites were about 3-6 $\mu$m in diameter and contained the characteristic refractile inclusions, presumably lipid droplets, of *P. marinus* (Figure 2). The enriched merozoites were free from oyster cells or tissue fragments, although some tissue debris (5 $\mu$m) was noticed. The viability of merozoites was consistently over 95% (98 $\pm$ 1.4) and heat-killed merozoites did not take up the stain.

The merozoites retained their capacity to enlarge in fluid thioglycollate media (FTM). The isolated merozoites retained their infectivity since the prevalence of *P. marinus* in oysters challenged with isolated merozoites ($10^6$) was 96 % after 6 weeks at 25°C. No *P. marinus* could be detected in the control oysters.

In conclusion, isolation and partial purification of *P. marinus* merozoites were achieved using the described procedure. It is important that the viability and infectivity of the isolated merozoites were not lost. Despite the time involved in this isolation procedure, the simplicity of the technique and the satisfactory enrichment make this protocol useful for obtaining merozoites directly from infected oyster tissue.

# Chapter 4

## *IN VITRO* PROPAGATION OF THE PROTOZOAN *PERKINSUS MARINUS*, A PATHOGEN OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*.

# INTRODUCTION

The pathogenic protozoan *Perkinsus marinus* is the cause of serious and extensive mortalities in eastern oyster populations (*Crassostrea virginica*) along the Gulf and Atlantic coasts of the United States (Andrews, 1988). In spite of abundant information on the epizootiology and morphology of *P. marinus* (Andrews, 1988; Perkins, 1988), details about its life cycle and infective stages remain to be elucidated. It is believed that *P. marinus* is an apicomplexan, although there is some disagreement on its current taxonomic position (Perkins, 1976b; Levine, 1978; Vivier, 1982).

Uncertainty about some aspects of the biology of *P. marinus* can be attributed to the current inability to isolate, purify and culture histozoic stage(s) of the parasite. Culture of *P. marinus* has been limited to studies of sporulation of zoosporangia in sea water (Perkins and Menzel, 1966). Zoosporangia are obtained by incubating oyster tissue in fluid thioglycollate medium which causes enlargement of *P. marinus* (Ray, 1952). Attempts have been made to culture *P. marinus* from minced oyster tissue and hemolymph, in a variety of media, but these attempts failed (Prokop, 1950; Ray, 1954; Mackin, 1962; Perkins, 1966). Inability to control microbial contamination and the absence of an appropriate growth medium for *P. marinus* were the main

obstacles to achieving culture success.

During our attempts to develop an *in vitro* cell culture system for the eastern oyster, *C. virginica*, a culture medium (designated as JL-ODRP-1) was formulated to contain most of the known constituents of the cell-free hemolymph of oysters. Using this medium, supplemented with antibiotics proven to be effective against the contaminating bacteria, we were able to propagate a protozoan (designated *Perkinsus*-1) resembling *P. marinus*. This organism adapted very well to our culture conditions and was successfully subcultured 7 times within a six-month period. The purposes of this communication are 1) to report the culture conditions under which the protozoan *Perkinsus*-1 has been propagated, 2) to give morphological and biological evidence that the protozoan *Perkinsus*-1 and *P. marinus* are identical and 3) to describe the infectivity of the protozoan *Perkinsus*-1 to *P. marinus*-free oysters.

## MATERIALS AND METHODS

### Oysters

Eastern oysters (8-12 cm) (*Crassostrea virginica*) were collected from the Rappahannock River (VA) at Ross Rock (37° 53′ N, 76° 45′ W) in December of 1990. The oysters were maintained in flumes that were continuously supplied with

estuarine water (salinity 20 ppt) from the York River at Gloucester Point (VA) (37° 13′ 30′′ N, 76° 29′ 30′′ W), an area known to be enzootic for *Perkinsus marinus* (Andrews and Hewatt, 1957).

Initiation and maintenance of the culture

Hearts of Ross Rock oysters were removed aseptically and rinsed in a concentrated antibiotic solution that consisted of chloramphenicol (50 mg/L), gentamicin (500 mg/L), kanamycin (1 g/L), penicillin (1,000,000 U/L), polymyxin B (500 mg/L), streptomycin (1 g/L) and rifampicin (50 mg/L) dissolved in sterile filtered (0.2 μm) York River water (YRW). All antibiotics were purchased from Sigma Chemical Company (St. Louis, MO). The hearts were placed in separate petri dishes and finely minced with razor blades. The heart fragments were washed five times with YRW, resuspended in the culture medium JL-ODRP-1 and placed in 25 $cm^2$ culture flasks (Corning Glass Works, Corning, NY) (1 heart/flask). The cultures were incubated at 21° C in the presence of a 5% $CO_2$ atmosphere.

The culture growth medium (JL-ODRP-1) consisted of inorganic salts, trace elements, amino acids, carbohydrates, vitamins, nucleic acid derivatives, lipids, proteins, and other components (Table 1). The ingredients were dissolved in culture grade water obtained from a Milli-Q UF water purification system (Millipore Co., Bedford, MA).

Table 1. Composition of medium JL-ODRP-1. Final pH 7.5; osmolality, 650 mOsm/kg.

| Ingredients | mg/L | Source[a] |
|---|---|---|
| Major inorganic salts and buffers | | |
| Calcium Chloride Anhydrous | 199.8 | S |
| Magnesium Sulfate Anhydrous | 3,371.3 | S |
| Magnesium Chloride Anhydrous | 2,031.3 | S |
| Potassium Chloride | 574.6 | S |
| Sodium Chloride | 15,973.3 | S |
| Sodium Bicarbonate | 2,000.0 | S |
| HEPES | 5,957.5 | S |
| Trace elements | | |
| Boric acid | 0.4 | S |
| Sodium Bromide | 0.686 | S |
| Sodium Fluoride | 0.2799 | S |
| Strontium Chloride Hexahydra | 17.77 | S |
| Cupric Sulfate.7$H_2O$ | 0.00249 | S |
| Ferrous Sulfate.5$H_2O$ | 0.834 | S |
| Zinc Sulfate.7$H_2O$ | 0.1438 | S |
| Manganese Sulfate.$H_2O$ | 0.0000338 | G |
| Sodium Metasilicate.9$H_2O$ | 0.0284 | G |
| Molybdic Acid.4$H_2O$ (Ammonium) | 0.0000248 | G |

Table 1. cont.

| Ingredients | mg/L | Source[a] |
|---|---|---|
| Ammonium Metavanadate | 0.000117 | G |
| Nickel Chloride.6$H_2O$ | 0.0000238 | G |
| Stannous Chloride.9$H_2O$ | 0.0000226 | G |
| Amino Acids | | |
| L-Arginine.HCl | 31.6 | G |
| L-Cystine | 6.0 | G |
| L-Glutamine | 25.0 | G |
| L-Histidine.HCl.$H_2O$ | 10.5 | G |
| L-Leucine | 13.1 | G |
| L-Isoleucine | 13.13 | G |
| L-Lysine.HCl | 18.13 | G |
| L-Methionine | 3.78 | G |
| L-Phenylalanine | 8.25 | G |
| L-Threonine | 11.9 | G |
| L-Tryptophan | 2.55 | G |
| L-Tyrosine | 9.0 | G |
| L-Valine | 11.7 | G |
| L-Alanine | 54.45 | G |
| L-Asparagine.$H_2O$ | 7.5 | G |
| L-Asparctic Acid | 6.65 | G |
| L-Glutamic Acid | 7.35 | G |

Table 1. cont.

| Ingredients | mg/L | Source[a] |
|---|---|---|
| L-Glycine | 28.75 | G |
| L-Proline | 5.75 | G |
| L-Serine | 30.25 | G |
| Taurine | 75.0 | G |
| Carbohydrates | | |
| Glucose | 500.0 | S |
| Galactose | 100.0 | S |
| Trehalose | 100.0 | S |
| Nucleic Acid Precursors | | |
| Adenosine 5'-Monophosphate | 1.0 | S |
| Cytidine 5'-Monophosphate | 1.0 | S |
| Uridine 5'-Triphosphate | 1.0 | S |
| Vitamins | | |
| D-Ca Pantothenate | 0.2 | G |
| Choline Chloride | 0.2 | G |
| Folic Acid | 0.2 | G |
| i-Inositol | 0.2 | G |
| Niacinamide | 0.4 | G |
| Pyridoxal.HCl | 0.2 | G |

Table 1. cont.

| Ingredients | mg/L | Source[a] |
|---|---|---|
| Riboflavin | 0.2 | G |
| Thiamine.HCl | 0.02 | G |
| D-α-Tocopherol Acetate | 2.0 | G |
| | | |
| Miscellaneous | | |
| Coenzyme A | 1.0 | S |
| Cholesterol | 4.5 | G |
| Cod Liver oil | 10.0 | G |
| Pluronic F-68 | 1000.0 | G |
| Ammonia | 10.0 | S |
| Urea | 1.0 | S |
| Tween 80 | 2.5 | G |
| Phenol Red | 1.0 | S |
| Bovine Serum Albumin | 12000.0 | J |
| Chloramphenicol | 5.0 | S |
| Yeastolate | 400.0 | S |

[a]Source of ingredients: G = Gibco laboratories, Grand Island, NY; J = JRH Biosciences, Woodland, CA; S = Sigma Chemical Co., St Louis, MO.

Osmolality of the medium, which was measured with an Osmette Precision Osmometer (Precision System, Framingham, MA), was adjusted to 650 mOsm/kg. The pH of the medium was adjusted to 7.5. Microscopical examination of culture flasks showed that most heart fragments continued to beat and that they were surrounded by adherent oyster cells, presumably hemocytes that had migrated out of the heart fragments. Some of the small heart fragments disaggregated after a few days in culture. As a consequence many solitary cells and small groups of cells were suspended in the medium. In some culture flasks, small, rounded protozoa-like organisms were observed within the cytoplasm of hemocytes, or suspended freely in the culture medium.

Following two weeks of incubation, the protozoal cells appeared to have enlarged and the number of protozoal cells free in the medium increased, as many were apparently released from bursting hemocytes. Cells ($5 \times 10^6$) in suspension in the culture flasks were harvested, rinsed twice in culture medium, suspended in 5 ml of culture medium and transferred into a new flask. The subculture was rapidly dominated by several forms of unicellular protozoa that resembled the developmental stage(s) of *P. marinus*, the most abundant of which appeared identical with mature meronts, the typical vacuolated cells of *P. marinus* found in oyster tissue (Mackin et al., 1950; Perkins, 1976a). The oyster heart cells did not divide in the culture medium and

were diluted out during subsequent subculturing. The terminology used to describe the stages of *P. marinus* is based on Perkins (1991).

The cells were cloned in their 4th subculture using the dilution cloning method of Freshney (1983). Subculturing of the protozoan (designated *Perkinsus*-1) was repeated at 4 wk intervals at a seeding density of $10^6$ protozoal cells per 25 $cm^2$ tissue culture flask. In an attempt to determine the growth of *Perkinsus*-1 in culture, the number of cells at the time of seeding and after 4 wk was measured for each subculture with a hemacytometer.

Characterization of the isolate: Microscopy

The progress of the culture was observed with an Olympus (CK-2) inverted light microscope with phase contrast optics at a magnification of 200 X. To examine the ultrastructural details of *Perkinsus*-1, cultured cells were harvested, rinsed twice in sterile YRW and fixed for 2 h with 3% glutaraldehyde in sterile YRW buffered with 0.025 M sodium cacodylate (pH 7.4, BYRW) at room temperature. Fixed cells were washed three times with BYRW, post-fixed for 3 h in 1% osmium tetroxide in cacodylate buffer (0.025 M) and rinsed in BYRW. The cells were then enrobed with 2% agar in 0.1 M sodium cacodylate, stained with 1% uranyl acetate for 1 h at room temperature, dehydrated through a graded ethanol series and infiltrated and embedded in Spurr's resin (Spurr,

1969). Ultrathin sections were prepared with a Reichert-Jung Ultracut E ultramicrotome (Leica Inc., Deerfield, IL). Sections were double stained with ethanolic uranyl acetate and Reynold's lead citrate, and were examined with a Zeiss CEM 902 transmission electron microscope (Carl Zeiss, Inc., Thornwood, NY). The ultrastructure of more than 500 cells was observed.

Characterization: Ray test

To determine whether *Perkinsus*-1 cells would form zoosporangia that stained blue-black with Lugol's solution after incubation in fluid thioglycollate medium (FTM) (1952), $10^5$ cells suspended in 20 $\mu$l of sterile YRW were added to 2 ml of FTM in 24-well culture plates and incubated for 5 d at room temperature. The diameter of 150 cells from triplicate wells (50 cells per well) was measured with an ocular micrometer on day one and five of incubation. Cells were then harvested, washed in sterile YRW and stained with diluted Lugol's solution (10%) in sterile YRW. The diameter data was transformed using log10 because of the proportionality of standard deviation to the mean, and analyzed with t-test at a level of significance of $p < 0.05$.

Characterization: Immunoassay

The primary antibody used in immunoassays was polyclonal, rabbit anti-*Perkinsus marinus* raised against

zoosporangia (also called hypnospores). The immune serum was prepared and provided by C.F. Dungan (Dungan and Roberson, 1993).

*Perkinsus*-1 cultured cells were harvested, rinsed three times in sterile YRW and added to a glass slide (20 $\mu$l, $2x10^4$ cells) in a moist chamber. The cells were allowed to settle and adhere to the slide for 1 h, fixed for 30 min with Davidson's AFA (30%[v/v]95%EtOH, 20%[v/v]formalin, 20%[v/v]acetic acid and 20%[v/v]glycerin in distilled water) and rinsed in distilled water. Oysters infected with *P. marinus* were preserved with Davidson's AFA and processed for paraffin histology by standard techniques. Sections (5.0 $\mu$m) through the digestive gland, including gill and mantle were affixed to glass slides, dewaxed in xylene, hydrated in a graded ethanol series and utilized for the positive control. Biocell goat anti-rabbit IgG gold probe and the Biocell Light Microscopy Silver Enhancement Kit (Goldmark Biologicals, Phillipsburg, NJ) were used to detect and visualize specific binding of the primary antibody. Briefly, slides of both paraffin sections and cultured cells were washed in tap water, blocked for 30 min with 10.0% v/v normal goat serum in phosphate-buffered saline containing 1.0% v/v bovine serum albumin (PBSA) and incubated for 30 min in a 1:1600 dilution of primary antibody in PBSA. Negative controls were both paraffin sections and cultured cells incubated with normal rabbit serum. All slides were

rinsed thoroughly in PBSA and incubated for 1 h in a 1:100 dilution of affinity purified goat anti-rabbit IgG coated onto 5 nm colloidal gold particles in PBSA containing 0.1% gelatin. After thorough washing in PBSA and distilled water, the bound colloidal gold particles were visualized with silver enhancement reagents (provided in the kit) that produced a brown/black color. Slides were washed in distilled water, counterstained in fast green, dehydrated in ethanol and cleared in xylene.

Characterization: Infectivity

Oysters (6-10 cm) were obtained from Mook Sea Farms, Damariscotta, ME, an area where *P. marinus* infection has never been reported. The absence of *P. marinus* cells in these oysters was confirmed by the Ray test on a sample of 25 oysters. *Perkinsus*-1 cells in the fifth subculture were injected into the mantle cavity of 15 notched oysters ($10^6$ *Perkinsus*-1 cells suspended in 100 $\mu$l YRW/oyster). Fifteen control oysters received 100 $\mu$l of YRW. The oysters were maintained in separate, aerated, 80 L tanks at 25° C for 8 wk. The water in the tanks was changed weekly with treated YRW. To eliminate the possible introduction of *P. marinus* via water, 500 L of YRW were treated with 100 ml of CHLOROX[R] overnight. YRW was then dechlorinated with 100 ml of 1 N sodium thiosulfate (Mallinckrodt Inc., Paris, KY) and used to replace the water in the oyster tanks. The oysters were

not fed to avoid introduction of *P. marinus* via algae.

At day 56 postinjection, the presence of *P. marinus* in both oyster groups was determined in the mantle, gill and rectal tissue of each individual oyster using the Ray test (Ray, 1952; Ray, 1966). The intensity of infection in each oyster was rated according to the categories of Ray (1954) by estimating the percentage of tissue occupied by the parasite.

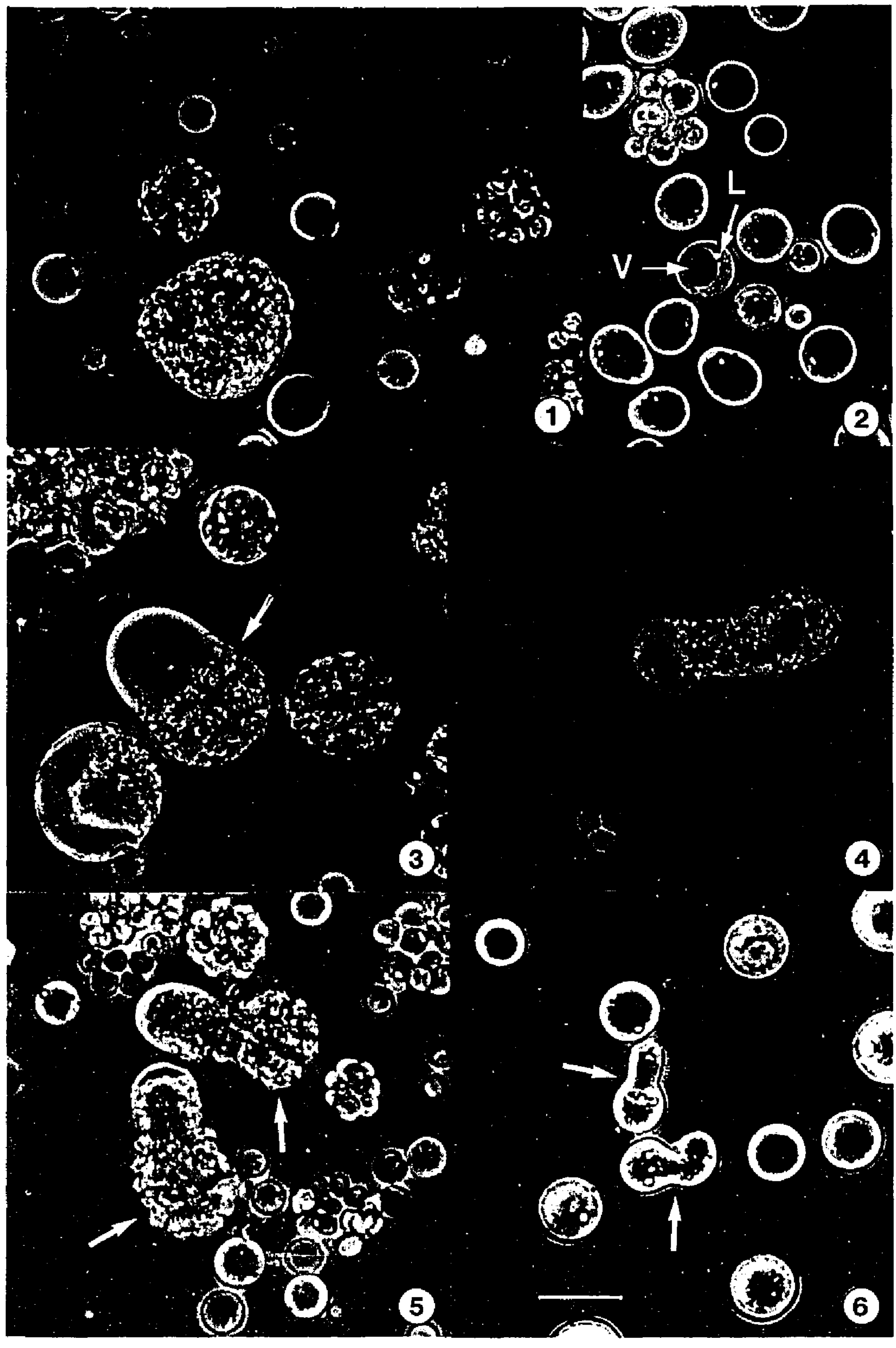
Attempts to reisolate and culture *Perkinsus*-1 from the hearts of experimentally infected oysters were performed as previously described.

## RESULTS

### Morphology of cultured forms

Cultured protozoal cells were heterogenous in size, contour, and contents. The smallest cells measured about 4 μm in diameter whereas the largest cells were about 45 μm in diameter (Figure 1). Most of the small cells occurred in clumps. Although some cells were oval, pear-shaped, or bi-lobed, the contour of most cells was spherical. The chronological examination of the cultures revealed several morphological changes. First, the small cells enlarged 8-12 times their original size generally within the first two weeks of culture. Second, along with the enlargement, a

Figures 1-6. Light micrographs of *Perkinsus*-1 cells. 1. Culture illustrating varying sizes and division stages of cells. 2. Cells showing prominent vacuoles (V) and refractile lipid droplets (L). 3. Mother cells dividing by progressive cleavage of protoplast. 4. Cleavage furrows forming simultaneously throughout a mother cell protoplast. 5. Rupture of mother cell wall due to enlargement of daughter cells. 6. "Budding" in cultured cells. Bar = 10 $\mu$m.



1
2
V
L
3
4
5
6

vacuole became visible within the first week (Figure 2). The percentage of the cytoplasm occupied by the vacuole increased with the enlargement of the cell to as much as 70%. In a few cells (1%), an inclusion body was seen to float freely within the vacuole. Third, one to several minute refractile bodies, presumably lipid droplets, also became visible in the cytoplasm (Figure 2).

During the third and fourth weeks of incubation, the protoplasts of almost all of the large-sized cells (>30 $\mu$m) cleaved (cytokinesis) presumably following nuclear divisions (karyokinesis). Cleavage was generally progressive in that it started at one end of the cell and advanced to encompass the rest of the mother cell (Figure 3). However in a few instances, cleavage furrows developed simultaneously throughout the cytoplasm (Figure 4). The cell walls of most mother cells ruptured early during the cleavage process due to the enlargement of first-formed daughter cells (Figure 5). The numbers and the sizes of daughter cells produced by mother cells varied greatly. Most mother cells produced large clusters of daughter cells that were generally made up of 20-40 cells. However, the maximum number of daughter cells was about 60. Most of the daughter cells measured in the range of 4-8 $\mu$m in diameter. Cell counts for growth measurements were difficult because of the clumping of daughter cells. The number of cells increased by a factor of 14 ± 5 (mean ± sd, n=6) after 4 wk of incubation at 21°C.

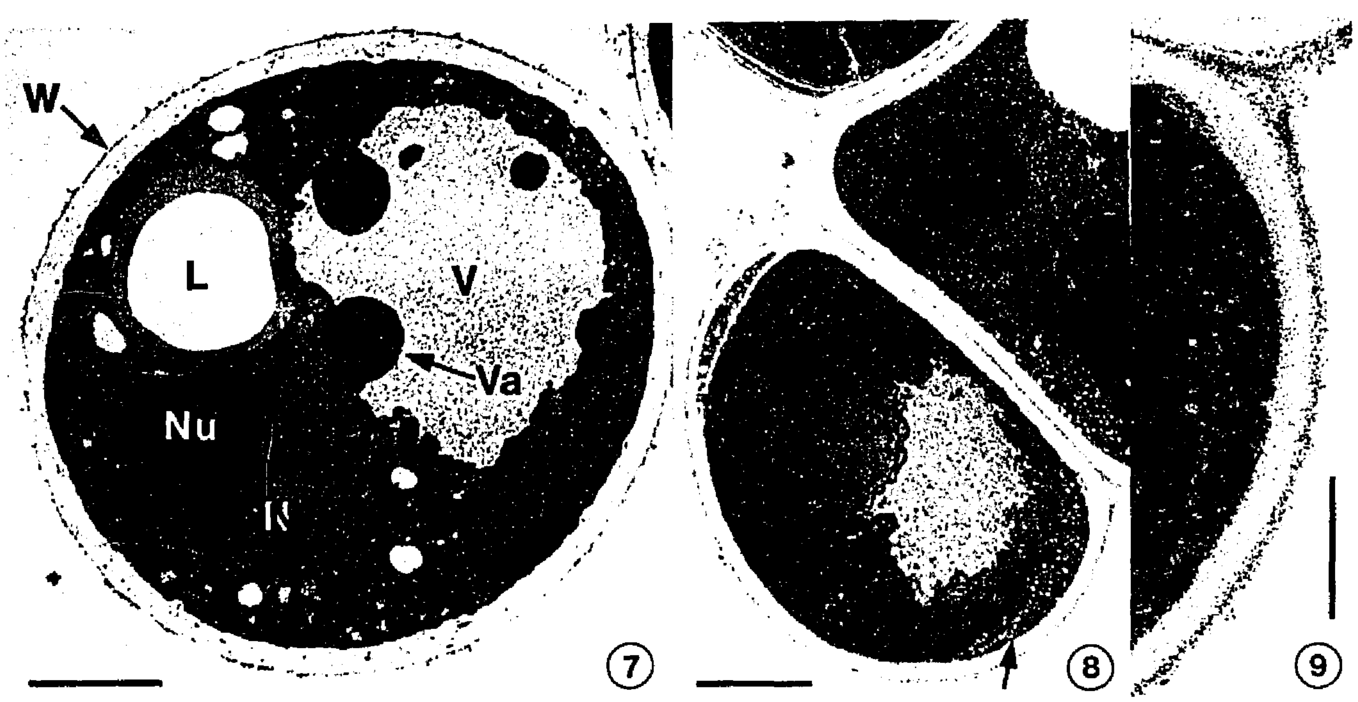
A constriction appeared in about 5% of the cells, dividing each cell into two cells of similar or unequal sizes in a process resembling budding (Figure 6).

Ultrastructurally the cultured cells had granular cell walls that increased in thickness with increasing cell size (Figure 7). Lomosomes were observed between the cell wall and plasmalemma in newly formed cells (Figures 8, 9). Prominent, presumptive, lipid droplets and vacuoles were present in the majority of the cells (Figure 7). The percentage of the cell volume occupied by the vacuole increased with increasing cell size and confirmed observations by light microscopy. Electron dense materials were generally found lining the inside of the vacuole and on occasion suspended in the vacuole (Figure 7). The electron dense material was dispersed throughout the vacuole in the larger cells. Organelles such as tubulovesicular mitochondria and endoplasmic reticulum were commonly found. The nucleus of each cultured cell contained a prominent nucleolus (Figure 7). The nucleolus was composed of a torus-shaped aggregate of ribosomes surrounding a chromatic mass.

Ray Test

Cells incubated in FTM for 5 d enlarged greatly to form mostly rounded cells with thick walls. The average diameter of cells after 5 d was 31 ± 9 $\mu$m (range, 11-61) and was

Figures 7-9. Electron micrographs of *Perkinsus*-1 cells. 7. Characteristic *Perkinsus*-1 cells with thick cell wall (W), prominent vacuole (V) lined with electron dense material (Va), lipid droplet (L), nucleus (N) with prominent nucleolus (Nu). Bar = 2 μm. 8. Lomosomes (arrow) in a daughter cell. Bar = 1 μm. 9. Higher magnification of Fig 8 illustrating lomosomes. Bar = 0.5 μm.


W
L
V
Va
Nu
N
7
8
9

significantly greater ($P<0.001$) than after 1 d (13 ± 5 μm, range, 4-37). The cells stained blue-black with Lugol's solution.

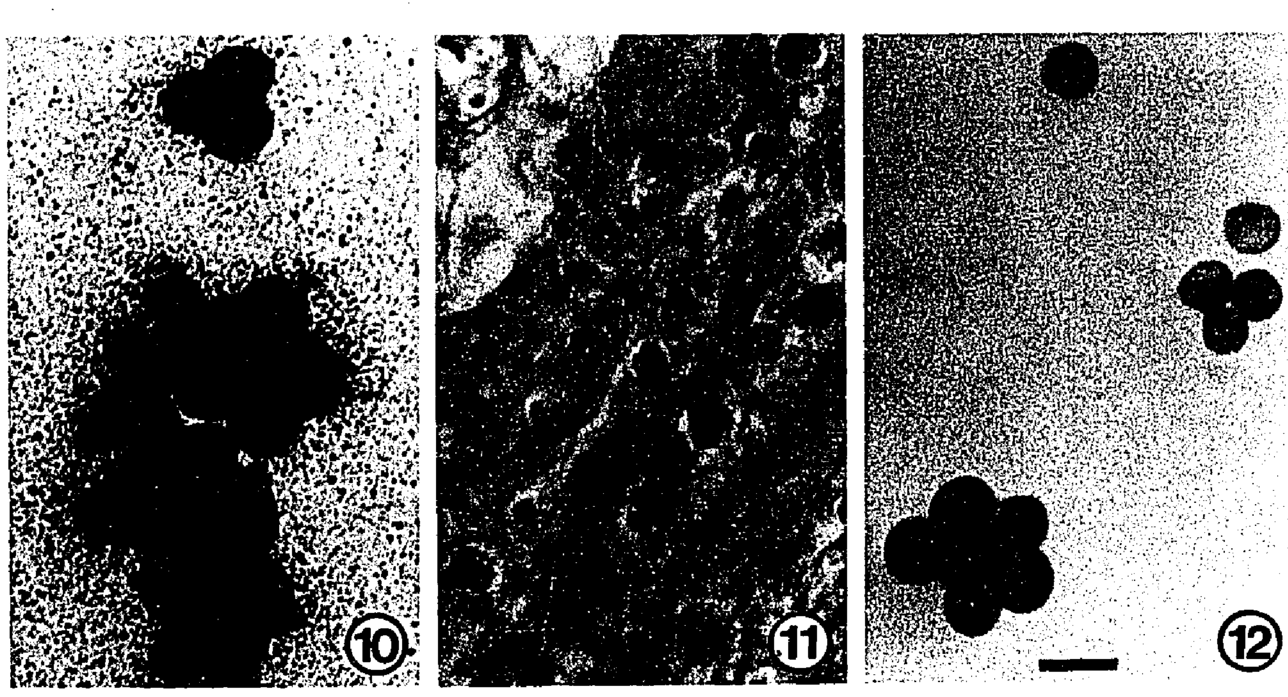
Immunoassay

Cultured cells rinsed in sterile YRW readily adhered to glass slides, as is characteristic of *P. marinus*. The cultured cells and *P. marinus* in infected oyster tissue (positive control) both reacted strongly positive in immunoassays as shown by formation of an intense black color (Figures 10, 11). The surface of the each cultured cell was covered with black spots indicating heavy binding of the colloidal gold label. Colloidal gold label did not bind to either cultured cells (Figure 12) or infected oyster tissue incubated in normal rabbit serum as negative controls.

Infectivity

The prevalence of *P. marinus* in oysters challenged with the cultured cells was 100% as determined eight weeks post-infection. The number of parasites in tissue preparations (1 $cm^2$) of all infected oysters was in the 10-100 range which is categorized as a light infection by Ray (1954). Most of the parasites were found in clusters. No *P. marinus* cells were detected in the control oysters.

Figures 10-12. Immunostaining of *Perkinsus*-1 cells and *Perkinsus marinus* in *C. virginica*. Cells in figures 10 and 11 were treated with polyclonal rabbit anti-serum. 10. *Perkinsus*-1 cells. 11. *Perkinsus marinus* in oyster connective tissue (positive control). 12. *Perkinsus*-1 cells treated with normal rabbit serum (negative control). Bar = 30 μm.



10
11
12

Reisolation

*Perkinsus*-1 was observed in cultures of heart fragments initiated from five of the 15 infected oysters. However, the number of *Perkinsus*-1 cells in the cultures was very low. After two weeks of incubation *Perkinsus*-1 was harvested from the culture with the highest number of parasites and was subcultured successfully thereafter.

# DISCUSSION

Data presented in this paper provide strong evidence that the protozoal isolate *Perkinsus*-1 shares several identical characteristics with *P. marinus*. In particular, the morphological similarities are striking. The cultured cells include cells with a prominent vacuole and one to several refractile lipid droplets, both of which are characteristic of *P. marinus* meronts in oyster tissue (Mackin et al., 1950; Perkins, 1991). Clusters of cells seen in cultures are also frequently found in the tissue of oysters infected with *P. marinus* (Ray, 1954). Moreover, cultured cells divided by schizogony-like processes that are similar to division processes observed for *P. marinus* at the light and electron microscopic level in oyster tissue sections (Mackin et al., 1950; Perkins, 1988). All of the ultrastructural details and cellular organelles described

for *Perkinsus*-1, such as granular cell walls, lomosomes, vacuoles containing electron-dense volutin-like material, presumptive lipid droplets, nuclei with prominent nucleoli and nucleoli with torus-shaped aggregates of ribosomes, were identical with those described for *P. marinus* by Perkins (1969).

There were, however, some differences between *Perkinsus*-1 in culture and *P. marinus* in oyster tissue. The size of the largest cells in culture is 45 µm in diameter, more than double that generally reported for the histozoic stages of *P. marinus* (Mackin et al., 1950; Perkins, 1969), although Ray and Chandler (1955) stated that the diameter of *P. marinus* occasionally reaches 30 µm in overwintering oysters. In general, this difference in size between *Perkinsus*-1 and *P. marinus* is not surprising since the size of protozoa can change in response to nutrient levels (Puytorac et al., 1987). Indeed, we observed a progressive decrease in the size of the largest dividing cells in culture when the medium was diluted to 60, 40, 20, and 10 % with YRW (data not shown). The size difference also may be attributed to the absence of host defense and tissue mediators secreted by the host in response to tissue damage or the parasite. It is interesting to note that the size of *Perkinsus* spp. cell can vary between host species. For example, Goggin (1969) found that *Perkinsus olseni*, in naturally or experimentally infected greenlip abalones

(*Haliotis laevigata*), was significantly larger than in experimentally infected blood cockles (*Anadara trapezia*).

The division process of cultured cells was mostly by progressive cleavage of mother cell protoplasts (i.e., repeated karyokinesis followed by cytokinesis). Although progressive cleavage has been proposed as a type of division for *P. marinus* in oyster tissue (Perkins, 1988), the most frequent type of division described is by successive bipartition of the protoplast (i.e., karyokinesis followed by cytokinesis then repeated) (Perkins, 1976a, 1993). Successive bipartition was not observed in the cultured cells. It is interesting to note that when cells were transferred from the culture media to YRW, a rapid (within a week) division by successive bipartition was observed (unpublished data). The division of the protoplast of *P. marinus* in both oyster tissue and sea water follows closely the division of the nuclei. In contrast, the division of the protoplast of *Perkinsus*-1 cells lags behind the division of the nuclei in the culture medium. The factors influencing the rates of both karyokinesis and cytokinesis may be dependent on the level of nutrients available.

The most intriguing type of division encountered in the cultured cells is a process during which small cells "bud" from larger cells. Although Ray (1954) and Mackin (1962) observed cells, presumably *P. marinus*, that enlarged in

oyster serum and produced tube-like processes from which small cells may detach, little else has been described about the "budding" of *P. marinus* cells. Further studies are required to characterize the division process of "budding" before its significance can be fully appreciated.

One group of organisms commonly found in oysters that may be confused with *P. marinus* because of its similar morphology is the thraustochytrids (Perkins, 1974)). However, one characteristic of these organisms, which is not found in *P. marinus*, is the presence of a sagenogen, an organelle that is involved in the formation of ectoplasmic nets (Perkins, 1974; Porter, 1990). No sagenogen was found in *Perkinsus*-1 cells. In addition, the cultured cells here lacked scales in their cell walls that are characteristic of thraustochytrids, but not *P. marinus*. The cultured cells enlarged in FTM and stained blue-black with Lugol's solution, a property not described for the thraustochytrids.

The positive reaction of *Perkinsus*-1 cultured cells with the anti-*Perkinsus* antibody is further evidence that the cells are *P. marinus*. Although the specificity of this antibody is not completely known, it reacts with *P. marinus* from eastern oysters (*C. virginica*), *Perkinsus* spp. in Baltic macomas (*Macoma balthica*) and softshells (*Mya arenaria*) from the Chesapeake Bay, but does not react with *Perkinsus karlssoni* from bay scallops (*Argopecten irradians*)

from New Brunswick, Canada or with two *Dermocystidium* species from salmonid fishes in Oregon, USA (Dungan and Roberson, 1993).

Based on the above characteristics and the finding that cultured cells infect susceptible oysters, it is evident that *Perkinsus*-1 is the first isolate of *P. marinus* to be successfully propagated in vitro. The problem of microbial contamination was lessened considerably by using decontaminated heart tissue for the establishment of the primary culture of *P. marinus*. The defined medium JL-ODRP-1, formulated to resemble oyster hemolymph composition, supported the growth of *P. marinus*. The culture has the potential to generate large quantities of pure *P. marinus* for research purposes. This will allow further investigation of several unanswered questions about the biology, pathobiology and immunology of *P. marinus*. Furthermore, the culture will aid in screening drugs for their potential use in treating oysters infected with this important pathogen.

# Chapter 5

## INITIATION OF *IN VITRO* CULTURES OF THE OYSTER PATHOGEN *PERKINSUS MARINUS* (APICOMPLEXA) WITH HYPNOSPORES

INTRODUCTION

The resurgence of mortalities of the eastern oyster, *Crassostrea virginica*, in the Chesapeake Bay due to infection with the protozoan, *Perkinsus marinus*, has stimulated attempts to propagate and purify this protozoan *in vitro*. Recently, conditions have been standardized under which a pure continuous culture of *P. marinus*, designated *Perkinsus*-1, was obtained from the hearts of infected oysters (La Peyre et al., 1993).

The heart was chosen initially because of the relative absence of bacterial contaminants in this organ. Using the heart as a source of *P. marinus*, however, has a major disadvantage; the yield of protozoan cells is low and, consequently, a relatively long period (four to eight weeks) is needed to establish a culture and to obtain large number of cells. Therefore, there is a need for a source that will yield more protozoan cells than the heart tissue. The visceral mass is large, easy to excise, and contains a large number of *P. marinus* cells in heavily infected oysters. On the other hand, our previous attempts to culture *P. marinus* directly from the visceral mass have been unsuccessful due to bacterial and protozoan contamination.

Ray (1952) demonstrated that incubating oyster tissue infected with *P. marinus* in fluid thioglycollate medium (FTM) for several days causes a significant enlargement of

the protozoan. The enlarged stage is called a hypnospore or prezoosporangium. Hypnospores do not develop further in FTM (Ray, 1954); however upon their transfer to sea water, the hypnospores divide by successive bipartition to produce numerous biflagellated zoospores (planonts) (Perkins and Menzel, 1966). Through filtration, hypnospores can be isolated and washed thoroughly, a process that eliminates most of the contaminating microorganisms. In this note we report on the initiation of continuous culture of *P. marinus* from hypnospores produced in FTM.

## MATERIALS AND METHODS

The visceral mass of individual heavily infected oyster was excised, minced finely (<1 $mm^3$) with a razor blade and added to 50 ml of fluid thioglycollate media (FTM, Sigma Chemical Co., St. Louis, MO) containing penicillin (50 U/ml) and streptomycin (500 $\mu$g/ml) (Sigma Chemical Co.). After 5 d of incubation in the dark at room temperature, the FTM was filtered through a series of screens with mesh sizes of 100, 75 and 38 $\mu$m (Newark Wire Cloth Company, Newark, NJ). The hypnospores collected on the 38 $\mu$m filter were rinsed thoroughly with sterile filtered York River water (YRW, salinity 20 ppt) and washed ten times with YRW (100 g, 5 min, room temperature) to reduce contaminating bacteria.

Hypnospores were then seeded into tissue culture flasks ($10^5$/25 $cm^2$ flask, Corning Glass Works, Corning, NY) containing 5 ml of culture medium to which chloramphenicol (5 $\mu$g/ml) was added. The growth medium used, designated JL-ODRP-1, contained most of the known constituents of the cell-free hemolymph of bivalve molluscs (La Peyre et al. 1993). The cultures were incubated in a humid atmosphere at 21° C in the presence of 5% $CO_2$ tension and were inspected daily with an inverted microscope (Olympus CK-2, phase contrast) at 200 X. *P. marinus* cells produced following the division of hypnospores were subcultured according to the method by La Peyre et al. (1993).

## RESULTS AND DISCUSSION

The majority of *P. marinus* hypnospores started division 12-17 days after initiation of the cultures. By the end of the fourth week, about 70% of the hypnospores had divided, and the culture flasks were filled with merozoites, making subculturing necessary.

Two types of division were observed in hypnospores. The first type of division was by a schizogony-like process and was observed in about 30% of the hypnospores. The protoplast of the hypnospore divided by progressive cleavage that was usually initiated at one pole of the cell and

advanced to encompass the rest of the cell. In a few cases, cleavage furrows developed simultaneously throughout the cytoplasm. As a result of the schizogony-like division, a large number of daughter cells, sometimes exceeding 300, could be produced from an individual hypnospore (Figure 1). This type of division (progressive cleavage) was similar to that reported recently by La Peyre et al. (1993), although the schizonts in the previous study produced significantly fewer daughter cells (40-60 merozoites) than the larger hypnospore of this study. Progressive cleavage has been proposed to occur in oyster tissue (Perkins, 1988), but has not been previously observed in hypnospores.

The second type of division, observed in about 40% of the hypnospores, was successive bipartition of the protoplast and resulted in the formation of flagellated cells (zoospores) (Figure 2). Prior to division, the protoplast of each hypnospore contracted, a clear cytoplasmic area under the cell wall appeared, and a discharge tube was then formed (Figure 2). Following the successive bipartition of the protoplast, zoospores were produced and could be seen moving inside the hypnospore. A similar division pattern has been described by Perkins and Menzel (1966) upon placing hypnospores, produced in FTM, into sea water.

Interestingly, both division patterns occured in the same culture flask. This might be attributed to the

Figure 1. Merozoites (arrow) produced by progressive cleavage of a *Perkinsus marinus* hypnospore. Bar = 15 μm.

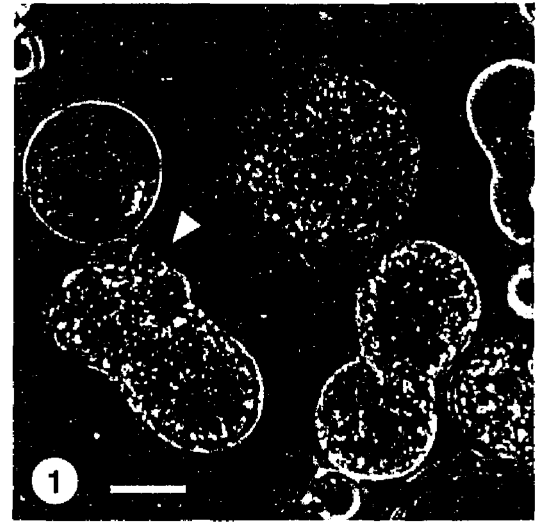
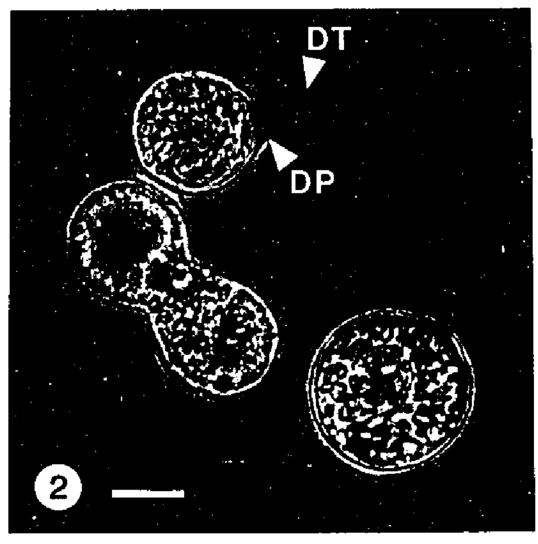

1

Figure 2. *Perkinsus marinus* hypnospore in later stage of division by successive bipartition. Hypnospore discharge pore (DP), discharge tube (DT). Bar = 15 μm.


DT
DP
2

difference in the stage of the development reached by the hypnospores in FTM before transfer into the culture medium. Elucidation of the mechanism(s) leading to each type of division will require further investigation. *P. marinus* cells produced following the division of hypnospores were subcultured and exhibited the same morphology and division type (i.e. progressive cleavage) as the cells described by La Peyre et al. (1993).

Because large numbers of cultured *P. marinus* cells could be obtained in a relatively short time from an individual oyster by preincubation in FTM, we recommend this two-step culture procedure for the routine culturing of *P. marinus*. In addition, the availability of the described developmental stages, and division patterns of *P. marinus* in the same container may facilitate comparative studies on the development and chemotherapeutic treatment of this lethal oyster pathogen.

Chapter 6

# *IN VITRO* INTERACTION OF *PERKINSUS MARINUS* MEROZOITES WITH EASTERN AND PACIFIC OYSTER HEMOCYTES.

# INTRODUCTION

The protozoan *Perkinsus marinus* causes severe mortality in eastern oyster (*Crassostrea virginica*) populations of the Atlantic and Gulf coasts of the United States. Most of the studies conducted on *P. marinus* have described the morphology of this apicomplexan (Perkins, 1991) and reported the epizootiology of the disease (Andrews, 1988). However, little is known about the host defense response to *P. marinus*. Recently a number of studies have investigated the changes of potential host defense activities in eastern oysters infected with *P. marinus* (La Peyre and Chu, 1988; La Peyre et al., 1989; Chintala and Fisher, 1991; Chu and La Peyre, 1993; Chu and La Peyre, In press; Chu et al., In Press). Measurements included both cellular and humoral characteristics such as hemocyte spreading, hemocyte binding, ingestion and chemiluminescent response to foreign particles, plasma agglutinin titer, and lysozyme concentration. No study has yet investigated the direct interaction between host defense activities and *P. marinus*.

Meyers et al. (1991) showed that Pacific oysters developed mostly light infections when challenged with *P. marinus* and that no mortalities could be attributed directly to the parasite. The factors that make *C. gigas* less susceptible to *P. marinus* infections than *C. virginica* are unknown and deserve further investigation. It is possible

that this difference in susceptibility is due to variations in host defense mechanisms.

Oyster hemocytes are believed to play an important role in host defense responses such as inflammation, phagocytosis and encapsulation (Cheng, 1975; Fisher, 1986; Feng 1988). In oysters infected with *P. marinus*, hemocytes accumulate around dividing parasites in several layers attempting to encapsulate them (Perkins, 1976). Moreover, hemocytes of heavily infected oysters contain large numbers of *P. marinus*. Mackin (1951) and Perkins (1976) have suggested that hemocytes play an important role in disseminating the parasite throughout the oyster.

As oyster hemocytes are known to produce antimicrobial compounds such as lysosomal enzymes (Cheng, 1983) and potentially toxic oxygen metabolites (Adema et al., 1991), their inability to control the spread of *P. marinus*, despite their attraction to and attempts at sequestering the parasites, is perplexing. Progression of *P. marinus* infection in eastern oysters occurs when the water temperature exceeds 20° C, with rapid proliferation of the parasite at temperatures above 25° C (Andrews, 1988). At elevated temperature, oysters experience stress that manifests itself through several physiological processes (Quick 1971; Newell et al., 1977), including a reduction in hemocyte motility and phagocytic activity (Fisher 1988;

Fisher et al., 1989; Chu and La Peyre, In Press). It is unknown if intracellular killing of the parasite in hemocytes occurs and what role temperature plays in this process.

The objective of the present study was to determine if oyster hemocytes phagocytose and destroy *P. marinus* cells *in vitro*. Because of reported susceptibility differences, hemocyte/*P. marinus* interactions were compared in the eastern and Pacific oyster.

## MATERIALS AND METHODS

### Oysters

Eastern oysters (*Crassostrea virginica*, 8-12 cm shell size) were collected from the Rappahannock River, VA, at Ross Rock (37° 53′ N, 76° 45′ W), an area free of *P. marinus* in December of 1990. No *P. marinus* infection was detected in 25 of these oysters (Ray 1952, 1966). Pacific oysters (*Crassostrea gigas*, 10-14 cm) were kindly supplied by Dr. Christopher Langdon (Oregon State University). Oysters were held at 15° C to minimize the influence of temperature-induced stress in hemocyte activity. Eastern and Pacific oysters were maintained in separate recirculating water systems filled with 1 μm filtered York River water (YRW, 20

ppt). The water was changed every two weeks. Oysters were fed algal paste, composed of a mixture of *Pavlova lutheri*, *Isochrysis galbana* and Tahitian *Isochrysis galbana*, at a rate of 0.1 g/oyster/day.

*P. marinus* isolation

Merozoites of *P. marinus* were isolated from individual heavily infected oysters according to the method described by La Peyre and Chu (Submitted). Briefly, the excised visceral masses of heavily infected oysters were homogenized in artificial sea water (ASW, 20 ppt, Instant Ocean, Aquarium Systems Inc., Mentor, OH). The homogenates were filtered through a series of Nytex screens (53, 35, 15 $\mu$m mesh size, Tetko Inc., Briarcliff Manor, NY) into a 500 ml beaker and allowed to stand overnight at 4° C. The supernates were gently aspirated with pasteur pipets to avoid disturbing the sediment and centrifuged at 50 *g* for 10 min in order to pellet any remaining oyster cells and tissue. The supernates were then collected and centrifuged at 800 *g* for 20 min to sediment the merozoites. The pellets were washed six times with ASW by repeated centrifugation at 800 *g* for 20 min. Merozoites were resuspended in ASW at the required concentrations for use in phagocytosis and chemiluminescent assays.

Collection of hemolymph

Oyster hemolymph (1-2 ml) was withdrawn from the adductor muscle sinus using a syringe with a 27 gauge needle, through a notch adjacent to the adductor muscle. The sampled hemolymph was transferred immediately into vials on ice to reduce hemocyte clumping. Cell-free hemolymph (plasma) was obtained by centrifugation of hemolymph at 400 *g* for 10 min.

Hemocyte density and percentage of hemocyte types

Hemocyte density and percentage of hemocyte types were determined with a Bright-Line hemacytometer (Reichert, Buffalo, NY). Each hemolymph sample (kept on ice) was mixed thoroughly and withdrawn with a pasteur pipet. The first two drops of each hemolymph sample were discarded, and then both counting chambers of the hemacytometer were filled. The hemocytes were allowed to settle and spread for 5 min before counting. Hemocytes were differentiated based on size, granularity and motility: small hyalinocytes contained few or no granules and showed limited spreading and motility; large hyalinocytes contained few or no granules, showed little motility but spread extensively; and granulocytes generally had many granules and were highly motile.

Phagocytosis assay

The ability of oyster hemocytes to phagocytose isolated *P. marinus* merozoites was determined for individual oysters using hemocyte monolayers. Twenty oysters from each species were sampled. Hemolymph (20 $\mu$l) was added to a slide and hemocytes were allowed to adhere for 30 min. The hemocyte monolayers received 20 $\mu$l of merozoite suspension each ($4x10^7$ cells/ml of ASW) and were incubated for 60 min in humidified chambers. As a positive control, a similar set of slides were simultaneously incubated with 20 $\mu$l of zymosan suspension ($4x10^7$ cells/ml of ASW, Sigma Chemical Company, St. louis, MO). Following incubation, the hemocyte monolayers were washed thoroughly with ASW, fixed with Davidson's AFA (30%[v/v]95% ethanol, 20%[v/v]formalin, 20%[v/v]acetic acid and 20%[v/v]glycerin in distilled water) for 30 min and stained with Hemal I and II staining solution (Hemal Stain Company, Inc, Danbury, CT).

The percentage of phagocytic hemocytes (those binding or ingesting challenge particles) and the number of merozoites or zymosan particles per phagocytic hemocyte were determined microscopically at a magnification of 400 X. An average of 3 fields of view with a minimum of 200 hemocytes was counted for each monolayer. Percentages of phagocytic hemocytes and numbers of particles per phagocytic hemocytes were Arcsin and log10 transformed, respectively.

Chemiluminescence assay

Luminol-enhanced hemocyte chemiluminescence (CL) was measured using a Beckmann LS 150 scintillation counter in the out-of-coincidence mode. Luminol stock solution was prepared according to the method by Scott and Klesius (1981) and diluted 1:500 with ASW prior to use. Hemocyte CL responses to *P. marinus* merozoites were measured in eight hemolymph samples from each oyster species. Each sample consisted of hemolymph pooled from three individual oysters. Luminol working solution (500 µl) was added to 500 µl of hemocyte suspension ($1x10^6$ cells/ml plasma) in microscintillation vials and the CL base activity was measured. The vials received 500 µl of either merozoite suspension ($4x10^7$ cells/ml ASW), zymosan suspension ($4x10^7$ cells/ml ASW, positive control) or ASW (negative control). Vials were counted for 0.5 min each, every 6 min, for 1 hr. Duplicate counts were made for each sample. The CL responses are reported as net counts per minute (CPM): Net CPM = peak CPM of stimulated hemocytes - CPM of negative control hemocytes.

Effect of hemocyte pre-incubation on the enlargement of *P. marinus* in fluid thioglycollate medium

The ability of *P. marinus* merozoites to enlarge in fluid thioglycollate medium (FTM, Ray 1952) after incubation with either hemocytes in plasma or plasma alone was

compared. Hemolymph was collected from nine individuals of each oyster species and pooled by groups of three oysters. Merozoite suspensions (100 $\mu$l, about $5x10^5$ cells/ml of ASW) were first pelleted by centrifugation at 800 *g* for 20 min. Then, either hemocytes in plasma (500 $\mu$l, $1x10^6$ cells/ml) or plasma alone was added to the merozoite pellets and centrifuged at 50 *g* for 10 min to increase contact between hemocytes and merozoites. After an overnight incubation at 15° C, the vials were centrifuged for 20 min at 800 *g* and the supernatant discarded. Fluid thioglycollate medium (1 ml), containing penicillin (50 U/ml) and streptomycin (500 $\mu$g/ml), was added to the pellets. After 5 d of incubation in FTM, the enlarged *P. marinus* cells (hypnospores) were isolated and stained with Lugol's solution according to the method by Gauthier and Fisher (1991). Finally 100 $\mu$l of the stained parasite suspensions (1 ml) were added to microtiter plate wells and the number of cells were counted in 4 fields of view at 200 X with a Nikon inverted microscope. For control, merozoites were directly placed in FTM without pre-incubation with either hemocyte in plasma or plasma alone. Results are reported as the average counts of the four fields of view.

Electron microscopy

To evaluate *P. marinus* merozoite/hemocyte interactions temporally, a mixture of hemocytes ($1x10^6$ cells/ml plasma)

and merozoites was incubated at 15° C, for 15 min at a ratio of 10 parasites to 1 hemocyte and for 12 hr at a ratio of 2 parasites to 1 hemocyte. The high parasite to hemocyte ratio used for 15 minutes increased initial ingestion for easier detection. To eliminate possible ingestion of an overwhelming number of merozoites by hemocytes during the 12 hr incubation period, a low parasite/hemocyte ratio was chosen.

After incubation, the suspensions were then centrifuged at 800 *g* for 20 min. The pellets were fixed for 2 h at room temperature, with 3% glutaraldehyde in ASW buffered with 0.025 M sodium cacodylate (pH 7.4), washed three times with buffered ASW (i.e., 0.025 M sodium cacodylate), post-fixed for 3 h in 1% osmium tetroxide in cacodylate buffer (0.025 M) and then rinsed in buffered ASW. The cells were enrobed in 2% agar in 0.1 M sodium cacodylate, stained with 1% uranyl acetate for 1 h at room temperature, dehydrated through a graded ethanol series and infiltrated and embedded in Spurr's resin (1969). Ultrathin sections were prepared with a Reighert-Jung Ultracut E ultramicrotome (Leica Inc., Deerfield, IL). Sections were stained with uranyl acetate and Reynold's lead citrate, and examined with a Zeiss CEM 902 transmission electron microscope (Carl Zeiss, Inc., Thornwood, NY). The ultrastructure of merozoites with hemocytes, hemocytes alone, and merozoites alone was observed in at least 200 cells for each oyster species.

## Statistical analysis

Data were analysed by one and two factor analysis of variance, followed by SNK's multiple comparisons of means when significant differences ($p<0.05$) were found.

# RESULTS

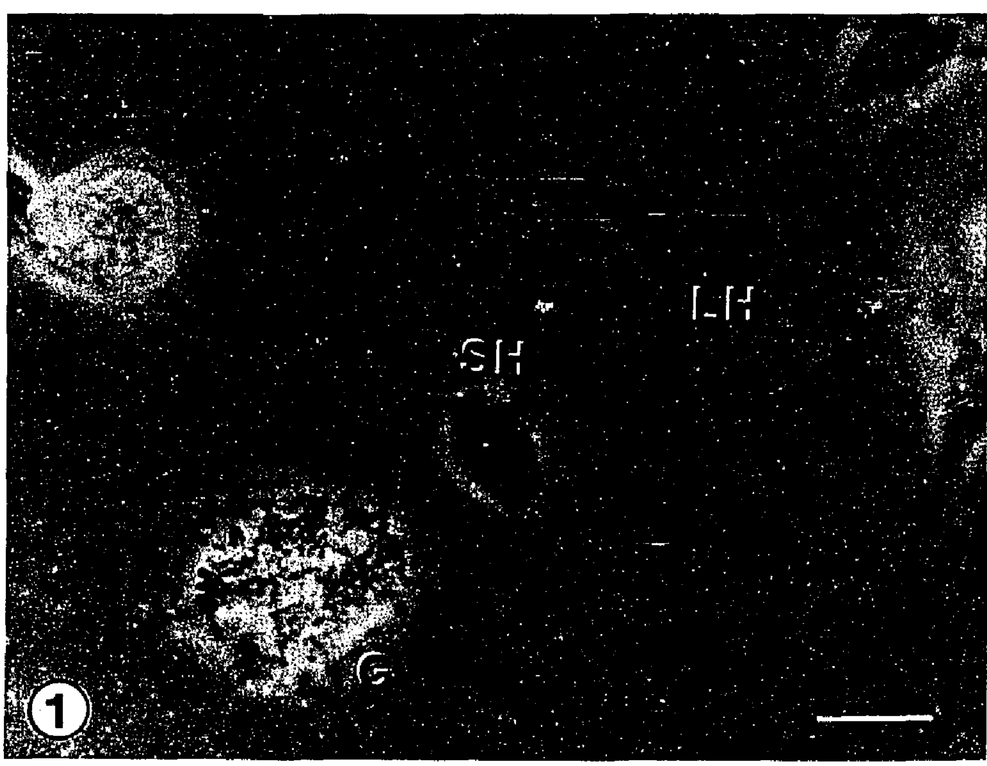
## Hemocyte density and percentage of hemocyte types

No differences in hemocyte density between eastern and Pacific oyster hemolymphs were observed. The three types of hemocytes differentiated in this study are shown in Figure 1. In eastern oysters the granulocyte was the dominant cell (51 ± 12, N=20; 50 ± 10, N=8) while the large hyalinocyte was the least abundant (16 ± 12, N=20; 17 ± 11, N=8). The percentage of large hyalinocytes in Pacific oysters (30 ± 10, N=20; 36 ± 10, N=8) was significantly greater ($p=0.0004$, N=20; $p=0.003$, N=8) than in eastern oysters (16 ± 12, N=20; 17 ± 11, N=8).

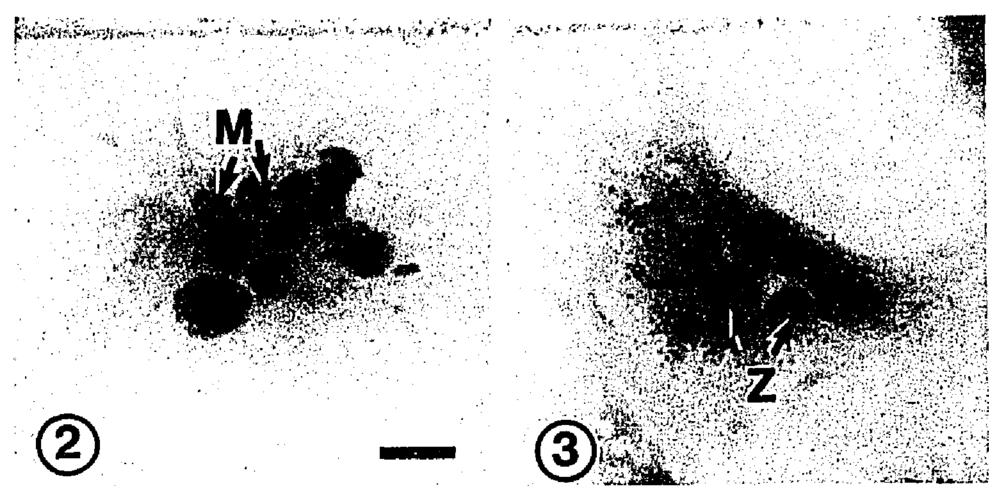
## Phagocytosis

*P. marinus* merozoites were bound to all hemocyte types but in unequal ratios. Most of the large hyalinocytes were associated with few or no merozoites. Unfortunately, it could not be determined precisely, by light microscopy, whether most merozoites were ingested or simply attached to hemocytes (Figure 2).

Figure 1. Light micrograph of eastern oyster hemocytes showing a small hyalinocyte (SH), a large hyalinocyte (LH) and a granulocyte (G). Bar = 10 μm.


LH
SH
G
1

Figures 2-3. Light micrographs of eastern oyster hemocytes associated with *P. marinus* merozoites (Figure 2) and zymosan (Figure 3). Bar = 10 $\mu m$.


M
Z
2
3

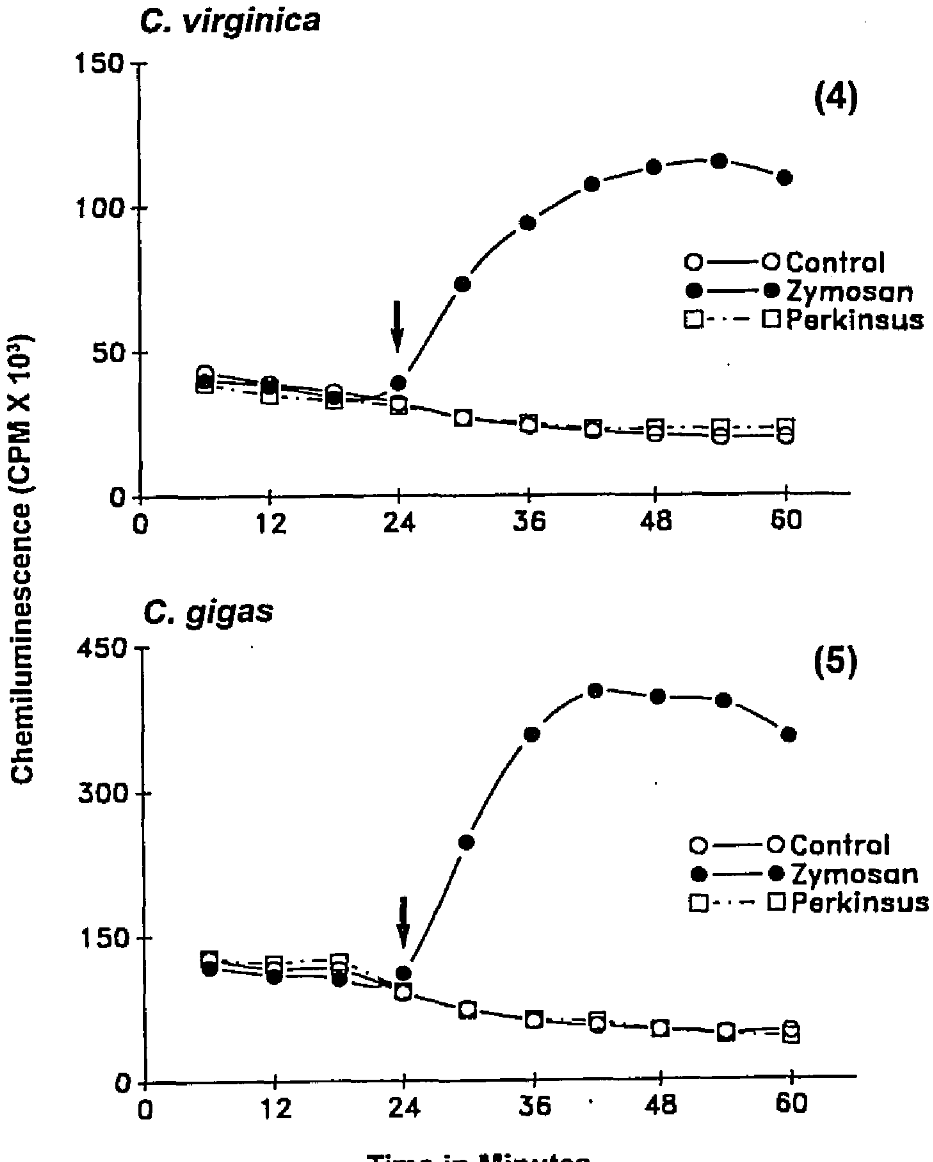
In contrast, most zymosan particles were phagocytosed by granulocytes (Figure 3). The percentage of phagocytic hemocytes in eastern oysters was significantly greater ($p=0.00017$, 2 factor ANOVA) than in Pacific oysters. The percentage of hemocytes associated with merozoites was significantly greater ($p=0.0151$, 2 factor ANOVA) than the percentage of cells associated with zymosan particles.

The number of *P. marinus* merozoites per phagocytic hemocyte tended to be greater (not significantly) than the number of zymosan particles per phagocytic hemocyte. Merozoites, however, were associated with more than one hemocyte type, unlike zymosan. More merozoites than zymosan particles were associated with granulocytes and fewer merozoites were associated with small hyalinocytes than with granulocytes.

Chemiluminescence (CL)

*Perkinsus marinus* merozoites did not elicit any CL responses from hemocytes of either eastern or Pacific oysters. In contrast, zymosan stimulation caused a rapid increase in CL in hemocytes of both *Crassostrea* species (Figures 4 and 5). Generally, Pacific oyster hemocytes reached peak CL responses in about 18-30 min whereas peak CL in eastern oyster hemocytes occurred within 30-42 min. In addition, CL responses to zymosan in hemocytes from Pacific oysters ($419 \pm 294$, $N=8$) were significantly greater

Figures 4-5. Typical chemiluminescent response curves of eastern oyster hemocytes (Figure 4) and Pacific oyster hemocytes (Figure 5) stimulated with *P. marinus* or zymosan. Arrow indicates time at which either zymosan or *P. marinus* was added.


C. virginica
150
100
50
0
0
12
24
36
48
60
(4)
Control
Zymosan
Perkinsus
Chemiluminescence (CPM X 10³)
C. gigas
450
300
150
0
0
12
24
36
48
60
(5)
Control
Zymosan
Perkinsus
Time in Minutes

(p=0.0093) than responses in hemocytes from eastern oysters (98 ± 65, N=8).

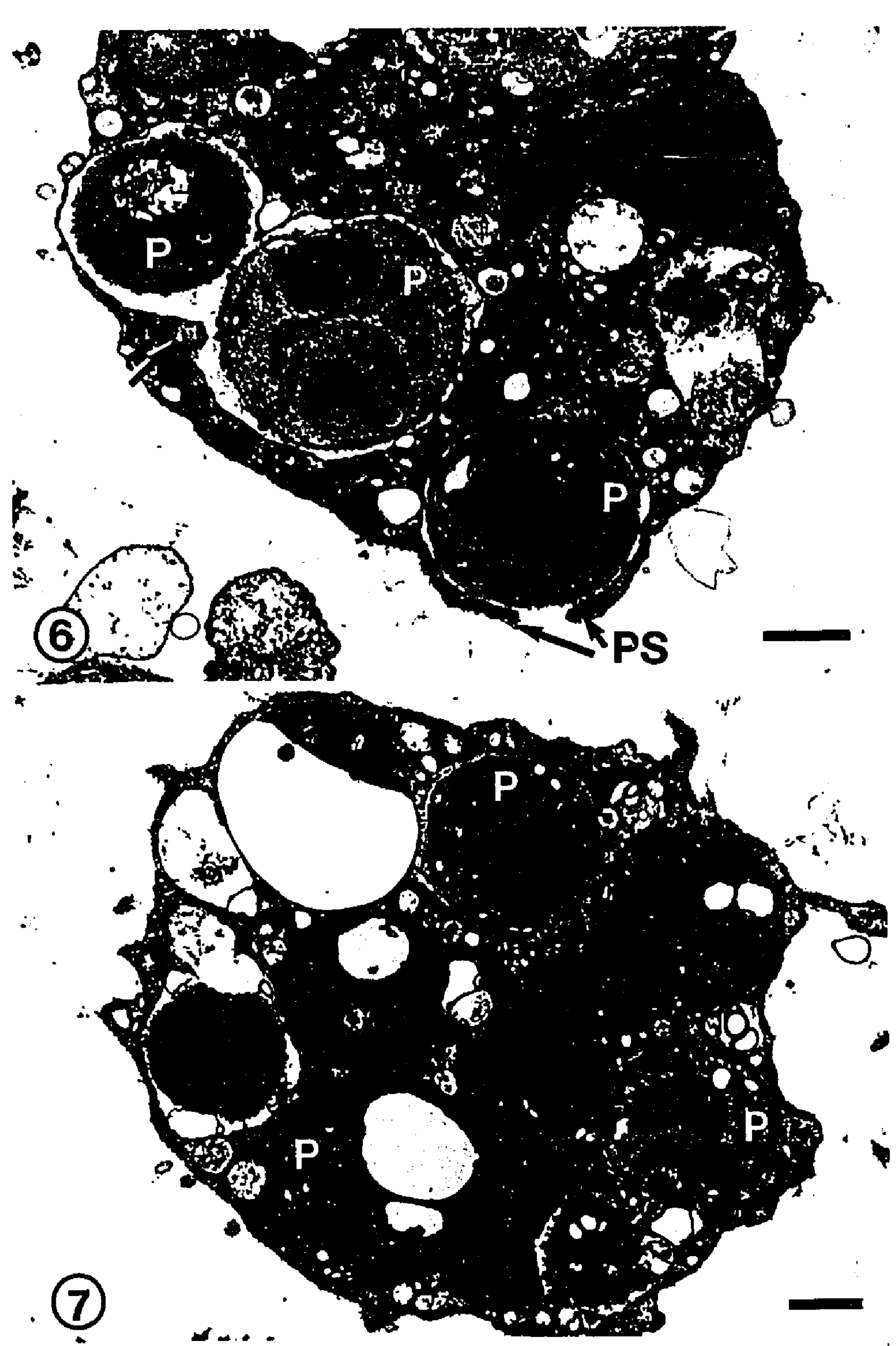
Effect of hemocyte-incubation on merozoite enlargement

The number of merozoites enlarging in FTM after incubation for one day with whole hemolymph (i.e., hemocytes in plasma) from eastern (38 ± 5, N=3) and Pacific (33 ± 4, N=3) oysters was significantly lower (p=0.002, 2 factor ANOVA) than the number enlarging after incubation in plasma alone (eastern: 50 ± 5, N=3; Pacific: 52 ± 6, N=3) oysters. No difference in merozoite enlargement was observed between merozoites placed directly in FTM after isolation (control) and those incubated for one day in plasma alone and then placed in FTM.

Electron microscopy

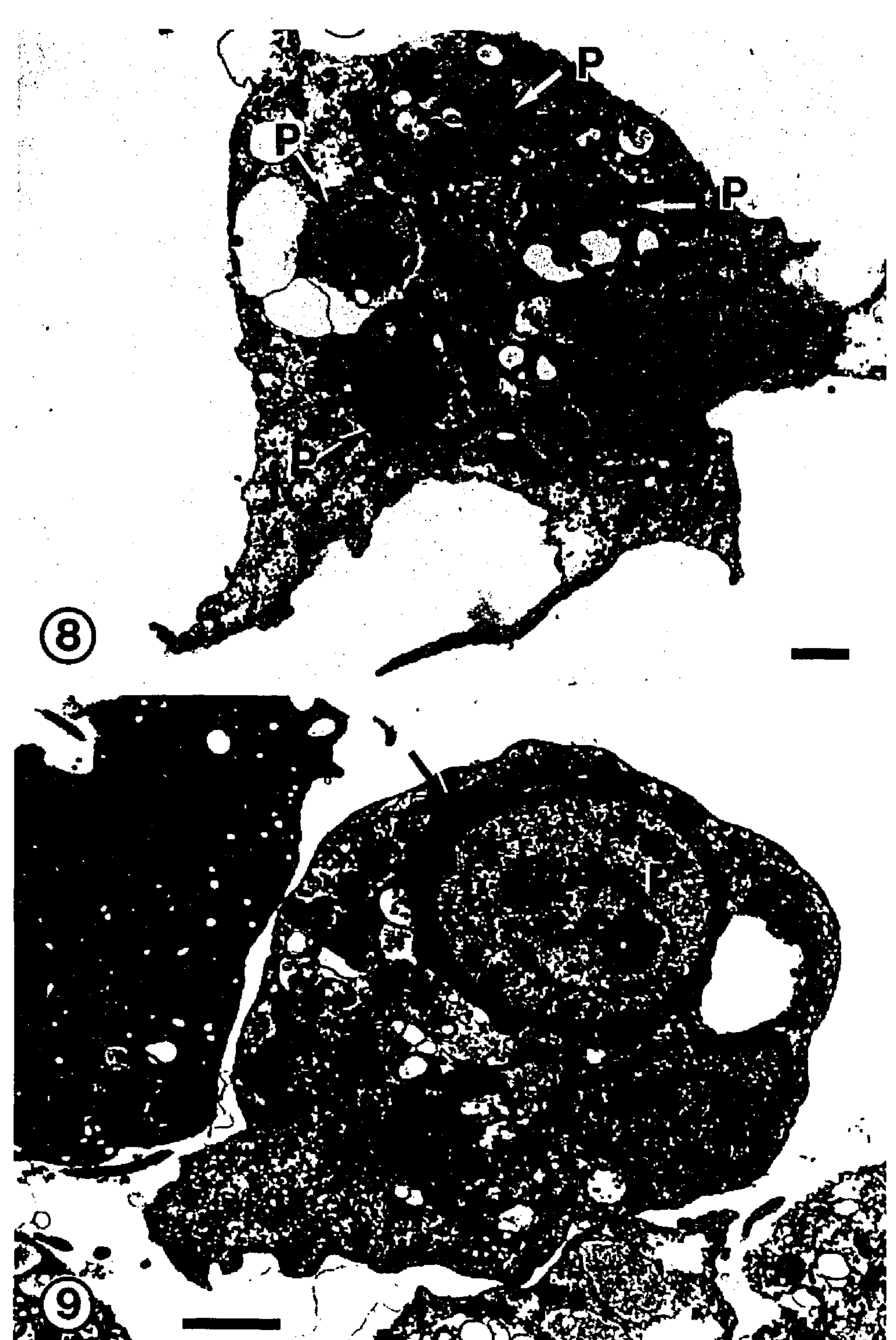
Hemocytes of both species rapidly ingested *P. marinus* merozoites. Within 15 minutes of contact between hemocytes and merozoites, there was invagination of the hemocyte plasma membrane and formation of pseudopods that enclosed parasites (Figure 6). Fusion of phagosomes containing individual merozoites, may have also occurred (Figure 6). Merozoites were bound to all hemocyte types of both oyster species. Merozoites were found, however, only in the phagosomes of the granulocytes. No sign of degeneration was observed after 15 min of incubation.

Figures 6-7. Electron micrographs of *P. marinus* merozoites in eastern oyster granulocytes. 6. Merozoites (P.m.) being ingested, and contained in phagosomes that are possibly fusing (arrow) at 15 min. Bar = 1 μm. 7. Granulocyte filled with merozoites (P.m.) at 12 hr. Bar = 1 μm.


P
P
P
PS
6
P
P
P
7

After 12 hours of incubation, merozoites were found predominantly in the phagosomes of granulocytes of both oyster species, although some merozoites occurred in small and large hyalinocytes. Granulocytes ingested the greatest number of merozoites, with up to 8 parasites per hemocyte (Figure 7). The majority of eastern oyster granulocytes had phagocytosed merozoites and few parasites remained extracellularly. Some eastern oyster hemocytes contained 3-4 degenerating merozoites (Figure 8). Some granulocytes were filled with electron-lucent granules after 12 hr interaction with merozoites, but the number of electron-lucent granules was lower in hemocytes containing several merozoites. In contrast, most electron-dense granules observed in hemocytes of eastern oysters after 15 min were not seen after 12 hr incubation. Merozoites within some phagosomes were surrounded by electron-dense material including some intact granules, suggesting fusion of granules (lysosomes) with the phagosome containing the parasite (Figure 9). It appeared that fewer Pacific oyster hemocytes phagocytosed merozoites than eastern oyster hemocytes and that fewer ingested merozoites showed signs of degeneration. The percentage of control merozoites (i.e., not incubated with hemocytes) showing signs of degradation also appeared much lower than the percentage of merozoites that showed signs of degradation following incubation with either eastern or Pacific hemocytes.

Figures 8-9. Electron micrographs of *P. marinus* merozoites in eastern oyster granulocytes. 8. Degenerating merozoites (arrow) at 12 hr. Bar = 1 $\mu$m. 9. A merozoite (P.m) surrounded by electron-dense granules (arrow), suggesting fusion with the phagosome containing the parasite, at 12 hr. Bar = 1 $\mu$m.



P
P
P
P
8
9

## DISCUSSION

Findings of this study strongly suggest that hemocytes from both eastern and Pacific oysters are capable not only of recognizing and internalizing merozoites of *P. marinus* but also of causing their degeneration. The successful degradation of some merozoites by hemocytes was supported by the 30% reduction in the number of merozoites enlarging in FTM following their incubation with hemolymph (i.e. hemocytes in plasma) compared with plasma alone. The mechanism(s) governing hemocyte-*P. marinus* merozoite interactions and the associated determining factors remains to be investigated.

The involvement of plasma (humoral) factors in hemocyte-*P. marinus* interactions cannot be excluded in this study since whole hemolymph (i.e., hemocytes in plasma) was used. Humoral factors may include soluble lectins that act as opsonins by forming bridges between carbohydrates (i.e., lectin-binding receptors) on the surface of parasites and hemocytes (Hardy at al., 1977; Renwrantz and Stahmer, 1983; Yang and Yoshino 1990). The way in which *P. marinus* merozoites interact with the surface of oyster cells (including hemocytes) has not yet been investigated. It is interesting to note, however, the adherence property of *P. marinus* zoosporangia and merozoites (Perkins, 1966; La Peyre et al., 1993; La Peyre and Chu, Submitted) which might play

a major role in the infection process and in the determination of pathogenicity of *P. marinus*. Cell surface hydrophobicity and lectins of microbes have been implicated in a wide variety of microbial adhesion phenomena and are considered major determinants of virulence in microbial infections (Mirelman, 1986; Doyle and Rosemberg, 1990). The adherence property of *P. marinus* merozoites may have contributed to their attachment to small hyalinocytes and to some large hyalinocytes, in addition to granulocytes.

Killing of *P. marinus* does not appear to be mediated by toxic oxygen metabolites since merozoites did not cause any increase in hemocyte chemiluminescence. Protozoan parasites, especially obligate intracellular parasites use various mechanisms to circumvent the respiratory burst and associated toxic oxygen metabolites produced by the host defense cells. These include: 1) active entry into host cells, 2) entry via non-activating receptors, 3) inhibition of the oxidative burst, and 4) scavenging of oxygen metabolites (Mauel 1984; Moulder, 1985; Bogdan et al., 1990; Hall and Joiner, 1991). For example, certain stages of *Leishmania* spp. can enter macrophages via a receptor that triggers internalization but not the respiratory burst (Wright and Silverstein, 1983; Russell and Talamas-Rohana, 1989). Inhibition of the respiratory burst in monocyte by *Leishmania* promastigotes, possibly through an inhibitory effect on protein kinase C, has also been reported (McNeely

and Turco, 1990).

*P. marinus* is a histozoic parasite, hence, it is unlikely that merozoites use elaborate strategies to escape the effects of toxic oxygen metabolites like typical intracellular parasites. It is possible that merozoites may possess anti-oxidant enzymes that scavenge toxic oxygen metabolites (i.e. catalase, superoxide dismutase, glutathione peroxidase) or inhibit their production (i.e., acid phosphatase). Such enzymes are commonly found on the surface of protozoa (Hassan and Schiavone, 1991).

Interestingly, hemocytes from eastern oysters with advanced *P. marinus* infections exhibit high baseline chemiluminescence, as well as peak chemiluminescence when stimulated by zymosan (Chapter 1). Hemocytes may have been activated by the parasite or parasite-associated oyster tissue damage. Increase in chemiluminescent responses of phagocytic cells by immunostimulants *in vivo* may involve a similar process (Kajita et al., 1990; Sakai et al., 1991).

Although *P. marinus* merozoites apparently avoid the effect of toxic oxygen metabolites, they might not escape nonoxidative killing mechanisms such as degradation by lysosomal hydrolases. In the present study, electron dense granules were observed around phagosomes containing merozoites in some granulocytes. Hemocyte granules, including electron-dense granules, of oyster and other bivalves, contain acid phosphatase, a lysosomal hydrolase

that is often used as a marker for lysosomes (Yoshino and Cheng 1976; Feng et al., 1977; Mohandas and Cheng 1985; Auffret, 1989; Chagot, 1989). Various other lysosomal enzymes have been identified in hemocytes of molluscs (Cheng 1983). The formation of phagolysosomes by discharge or fusion of lysosomes with phagosomes containing merozoites probably occurred.

The susceptibility of *P. marinus* merozoites to various lysosomal enzymes should be investigated. Intracellular parasites such as *Leishmania* spp. can resist lysis, inactivate lysosomal enzyme activities, and replicate in the acidic phagolysosomal environment (Bogdan et al 1990, Hall and Joiner, 1991). It is not known if *P. marinus* merozoites are protected to some extent from lysosomal degradation or what type(s) and concentration(s) of lysosomal enzymes will lyse *P. marinus*.

Phagocytosed merozoites showed evidence of degradation in a minority of eastern oyster hemocytes and in even fewer Pacific oyster hemocytes. There are several reasons which could explain the relatively low percentage of degenerative merozoites observed: 1) some merozoites may have been ingested shortly before the end of the incubation period since the exact time of ingestion of individual merozoites is unknown, 2) hemocyte clumping increased the parasite to hemocyte ratio so that fewer hemocytes were able to ingest a larger number of merozoites thus possibly exceeding the

killing capacity of those hemocytes and, 3) the thick cell wall of *P. marinus* may provide some degree of protection against lysis by lysosomal hydrolases.

In addition to the possible effect of lysosomal enzymes, it is probable that other hemocyte factors not yet identified may be involved in the nonoxidative killing of *P. marinus*. Little is known of these factors except for potentially toxic oxygen metabolites and lysosomal hydrolases. Some of these killing factors (e.g., cationic proteins and nitric oxide) may be similar to those that occur in vertebrate phagocytic cells (Spitznagel, 1984; Liew and Cox, 1991).

It is evident that at 15° C, hemocytes of both oyster species are capable of killing merozoites. The hemocyte's ability to degrade *P. marinus* at 15° C may be one reason the parasite is not usually prevalent in eastern oysters at that temperature. In addition, it is likely that parasite growth is reduced at lower temperatures. In Chesapeake Bay, the number of *P. marinus* cells in eastern oyster tissues does not generally increase below 20° C (Andrews 1988). Hewatt and Andrews (1956) found that eastern oysters injected with minced tissue and held at 15° C did not develop infections.

A number of field studies have reported apparent elimination of *P. marinus* from eastern oysters at low temperature, supporting the role of hemocytes in parasite degradation (Ray, 1954; Andrews and Hewatt, 1957; Mackin,

1962). From late fall to early spring, *P. marinus* prevalence and intensity of infection decline in enzootic areas of the Chesapeake Bay and mortalities are generally low (Andrews and Hewatt, 1957). In addition, hemocytes may play a role in the excretion of the parasite. Oyster hemocytes have been shown to cross epithelia and excrete undigestible biotic and abiotic materials (Stauber, 1950; Tripp, 1960; Alvarez et al., 1992).

Based solely on the data presented in this study, it is premature to define the exact role of hemocytes in the pathogenesis of *P. marinus* since: 1) not all merozoites were degraded *in vitro*, 2) it is unknown if the degradation of merozoites by hemocytes *in vitro* also occurs *in vivo* to the extent of causing their complete destruction and preventing their multiplication, 3) the fate of merozoites in hemocytes at higher temperature (e.g. 25° C) has not yet been determined either *in vitro* or *in vivo*. Likewise the role of hemocytes in the reported difference in susceptibility between eastern and Pacific oysters is still inconclusive since: 1) it appeared that more merozoites were degraded *in vitro* by eastern oyster hemocytes than by Pacific oyster hemocytes and 2) the fate of merozoites in Pacific oysters challenged with *P. marinus* and at high temperature (e.g., 25° C) has not yet been determined.

Recent advancements in techniques to isolate, purify and propagate *P. marinus in vitro* will permit further study

on the interaction of components of the oyster host defense system with *P. marinus* (La Peyre et al., 1993). Evaluation of the mechanisms of killing of *P. marinus* by oyster hemocytes as well as the effects of elevated temperature on hemocyte-parasite interactions is needed and may help greatly in understanding disease resistance in eastern oysters.

# GENERAL DISCUSSION

To become established in a host, a parasite must first contact and gain entrance in the host. Once inside the host, the parasite must overcome host defenses and be able to grow, develop, reproduce and be transmitted (Cheng, 1988; Wakelin, 1988; Coombe, 1991). Each of these processes is dependent on the interaction of multiple host and parasite variables. *P. marinus* is a successful parasite in eastern oysters but not in Pacific oysters. Pacific oysters are infected by *P. marinus* but intensities of infection are generally low and no mortality has been attributed directly to the parasite. This implies no or low net parasite reproduction and transmission. The reasons that Pacific oysters have mostly low intensity of infection by *P. marinus* infection are unknown. Differences in either the entry of merozoites, the interaction of merozoites with host defenses or the ability of merozoites to propagate between Pacific oysters and eastern oysters may be responsible for the differences in susceptibility and are discussed below.

<u>1) Entry</u>

*P. marinus* cells gain access to both eastern and Pacific oysters, but it is unknown if there are differences in the rate of entry of the parasite between species. Detection of *P. marinus* infection in Pacific oysters is delayed compared to eastern oysters (Meyers, 1991) which may

suggest that the rate of entry is lower in Pacific oysters. Unfortunately, very little is known about the mechanism(s) by which *P. marinus* cells enter into oysters. It has been proposed that merozoites can either lyse the basal membrane of the oyster stomach or be carried by hemocytes in the oyster. Possible mechanisms by which the rate of entry of *P. marinus* is greater in eastern oysters than in Pacific oysters need to be investigated and may include: greater attachment of *P. marinus* to the oyster epithelium, greater ability of *P. marinus* to lyse the basement membrane, or greater hemocyte transport of *P. marinus* from outside to inside the host. Much research is needed to understand and characterize the mode of entry of *P. marinus* into the oyster.

2) Propagation

The ability of *P. marinus* to propagate in Pacific oysters has not yet been determined. The findings that some moderate intensity of *P. marinus* infection (Burreson, personal communication) and that one "heavy" intensity of infection has been found in Pacific oysters (Barber and Mann, 1993) suggest, however, that the Pacific oyster is capable of satisfying the physiological needs of *P. marinus*. Nonetheless, the growth rate of *P. marinus* in Pacific oysters may be lower than in eastern oysters. Interestingly, the hemolymph protein concentration in Pacific oysters is lower than in eastern oysters

(chapter 2). Various other nutrients necessary for *P. marinus* growth may also be quantitatively different between the two oyster species. The ability to propagate *P. marinus in vitro* (chapter 4) will allow the study of essential nutritional requirements of the parasite. Further, determination of the level of these essential nutritional requirements in eastern and Pacific oysters might help explain differences in susceptibility between the two oyster species. In addition to nutrients, other compounds such as growth factors or hormones that stimulate increased growth, development and reproduction of *P. marinus* may be greater in eastern oysters than in Pacific oysters.

3) Host Defenses

While *P. marinus* growth rate may be lower in Pacific oysters than in eastern oysters, it is also possible that the host defense in Pacific oysters is better able to control the spread of *P. marinus* than in eastern oysters. For example, the density of circulating hemocytes and the percentage of granulocytes increased in Pacific oysters challenged with *P. marinus* (chapter 2) and may be beneficial since the number of hemocytes capable of ingesting and degrading the parasite may increase; hemocytes from Pacific oysters, maintained at 15° C, can degrade *P. marinus* to some extent (chapter 6). In contrast to Pacific oysters, there is no change in potential host defense activities measured in eastern oysters with light intensity of infection

following challenge with *P. marinus*. Although hemocytes from both eastern and Pacific oysters maintained at 15°C are able to degrade *P. marinus*, the extent and variability of the degradation have not yet been quantified either at 15°C or more importantly at 25°C. In addition, hemocytes from challenged Pacific oysters have not yet been tested for their ability to degrade *P. marinus*. A possible role of humoral defense factors in oysters also cannot be ruled out.

At this time, with the very limited amount of experimental data available, it is not possible to identify the process responsible for the lower susceptibility of Pacific oysters compared to eastern oysters; differences in the rate of entry of the parasite and the suitability of the host or the host defenses may be important.

Differences in potential host defense activities were also observed within and between oysters from different geographical areas. Some of these differences could be attributed to differences in intensities of *P. marinus* infection. Findings of this dissertation provide additional data on the range of values for several cellular and humoral activities in oysters (i.e., density of circulating hemocytes, percentage of hemocyte type, hemocyte activities such as phagocytosis and chemiluminescence, as well as plasma agglutinin titer and lysozyme concentration). The use of potential host defense activities as possible markers

for disease resistance in oysters has been proposed (Fisher and Auffret, 1986) and is discussed below in view of my findings.

1) Density of circulating hemocytes

It is reasonable to expect that a greater density of circulating hemocytes in oysters is advantageous in disease resistance. The density of circulating hemocytes increased and was greater in Pacific oysters than eastern oysters following challenge (chapter 2, experiment 1). However the number of circulating hemocytes also increased in eastern oysters with the intensification of *P. marinus* infection (chapter 1; Chu and La Peyre, 1993). The effects of *P. marinus* infection on the density of circulating hemocytes in eastern and Pacific oysters illustrate some of the difficulties in using this characteristic as a possible marker for disease resistance. A high density of circulating hemocytes may be an indication of better resistance (in the case of Pacific oyster) or of pathological effect (in the case of eastern oysters). Temperature also affects the density of circulating hemocytes; the densities were lower at 15° C (chapter 6) than those reported at 25° C (Chapter 2) in both oyster species. This difference is not surprising since temperature is known to affect hemocyte density (Feng, 1965; Chu and La Peyre, In Press). Feng (1965) demonstrated that

hemocyte density in oyster hemolymph (i.e. vessels and sinuses) was correlated to the oyster heart rate which was dependent on temperature. It is not yet known, however, if in addition, temperature has an effect on the total hemocyte number of an oyster. Changes in density of circulating hemocytes must be interpreted with caution since hemocytes exist not only in the hemolymph but also in oyster interstitial spaces. Hemocytes of oysters and other bivalves can migrate from one compartment to another, for example after the injection of foreign particles or substances (Feng, 1962; Poder, 1980), wounding (Pauley and Sparks 1965, 1967; Ruddell, 1969), feeding (Feng et al., 1971), or gamete resorption (Suresh and Mohandas, 1992). The intensity and timing of these changes are highly variable depending on the circumstances. In addition to cell migration, cell division, (e.g. hemopoiesis) which increases the total number of hemocytes in oysters can be expected to alter the density of circulating hemocytes. For example, there is an increase of hemocytes number in both hemolymph and tissue of oysters infected with the protozoan *Haplosporidium nelsoni* (Ford et al. 1993).

The density of circulating hemocytes does not appear to be a useful marker for disease resistance to *P. marinus* at this time, since: 1) the significance of high hemocyte density has not been demonstrated, 2) the role(s) of the various hemocyte types in host defense against *P. marinus*

are still unknown, 3) the causes for the great variability in the density of circulating hemocytes are not well understood; hemocytes are multifunctional and hence the density of circulating hemocyte is affected by a multitude of factors such as the nutritional and reproductive status of the oyster.

<u>2) Hemocyte composition</u>

The composition of the hemocyte population could be important in disease resistance since it is well known that hemocytes differ in their level of activities. Granulocytes, for example, are the most phagocytic cells and can degrade *P. marinus* to some extent (Chapter 6). Although the percentage of granulocytes in *P. marinus* free oysters was greater for eastern than in Pacific oysters at both 15° C (chapter 6) and 25° C (chapter 2), the percentage of granulocytes in Pacific oysters challenged with *P. marinus* increased compared to control Pacific oysters (Chapter 2, expt. 1). Moreover, heavy infection in eastern oysters was associated with a decrease in the percentage of granulocytes. Previous studies have also revealed that the percentages of hemocyte type in oysters were typically highly variable (Bachere et al., 1991; Chu and La Peyre, 1993, In press). The percentage of granulocytes can comprise as little as 10 % to as high as 90 % of the circulating hemocyte population (La Peyre, unpublished data).

The use of percentages of hemocyte type as a possible marker for disease resistance suffers from the same problems described for hemocyte density. The function(s) of small and large hyalinocytes remain obscure. Relatively little is known about the factors that affect the percentages of hemocyte type, but they probably include the various exogenous and endogenous factors that also affect the density of circulating hemocytes. For example, injection of bacteria (*Bacillus mycoides*) in eastern oysters caused a rapid increase in the percentage of granular cells in hemolymph (Feng, 1971), and infection by *Haplosporidium nelsoni* (MSX) increased the percentage of small hemocytes (small hyalinocytes) (Farley, 1968; Ford et al., 1993). Chagot (1989) also reported an increase in the percentage of small hyalinocytes in edible oysters (*Ostrea edulis*) infected with the protozoan *Bonamia ostrea*.

3) Hemocyte activities: phagocytosis and chemiluminescence

In addition to the number of hemocytes and the percentage of hemocyte types, hemocyte phagocytic activity also needs to be considered. Hemocyte phagocytosis of *P. marinus* merozoites has been demonstrated *in vitro* (chapter 6). Contact between merozoites and hemocytes from both eastern and Pacific oysters was followed by invaginations on the surface of the hemocytes and formation of pseudopods that enclosed the parasite. This uptake mechanism is similar to that described previously, when hemocytes

encounter bacteria (Cheng, 1975; Mohandas, 1985) or the protozoan *Bonamia ostrea* (Chagot et al., 1992).

Both the percentage of phagocytic hemocytes and the number of zymosan particles phagocytosed decreased in eastern oysters at 25° C (both infected and uninfected with *P. marinus*, chapter 1). The decrease in phagocytosis may be the result of temperature stress. Similar results were observed in a related study (Chu and La Peyre, In Press). It is possible that the effect of high temperature on hemocyte activities may in part contribute to the high susceptibility of eastern oysters at elevated temperature. The effect of high temperature on hemocyte activities of Pacific oysters compared to eastern oysters needs to be determined to see whether differences in phagocytosis may account for the differences in susceptibility to *P. marinus*. The phagocytic activity of Pacific oyster hemocytes (i.e., both percentage of phagocytic hemocytes and the number of phagocytosed zymosan particles) at elevated temperature was greater than for eastern oysters (chapter 2, experiment 2); however, most eastern oysters were heavily infected with *P. marinus* which may have also contributed to the decrease in phagocytosis. It is known that many factors affect phagocytosis by oyster and other molluscan hemocytes. These include temperature (Foley and Cheng, 1975; Chu and La Peyre, In press), salinity (Fisher and Newell, 1986; Fisher and Tamplin, 1988) and infection (Abdul-Salam, J.M. and

Michelson, 1980; Riley and Chappell, 1992; Loker et al., 1992) and need to be considered when comparing phagocytosis between groups of oysters. In addition, both the number and the percentage of hemocyte types needs to be evaluated to estimate the "total" phagocytic activity of the hemocyte population per oyster.

There was no increase in chemiluminescence when oyster hemocytes were stimulated with *P. marinus* merozoites (chapter 6). This lack of an increase in hemocyte chemiluminescence indicates that either there is no increase in production of toxic oxygen metabolites or that toxic oxygen metabolites are immediately scavenged by anti-oxidants (e.g., superoxide dismutase, catalase) of *P. marinus*. *P. marinus* is may thus circumvent the effects of potentially toxic oxygen metabolites. The lack of chemiluminescent stimulation in hemocytes in both eastern and Pacific oyster may indirectly suggest that suitability of the host environment is more important than host defense since *P. marinus* appear to resists at least some of the host cytotoxic's factors.

4) Plasma hemagglutinin titer

In this study, the hemagglutinin titer of pacific oysters was increased following challenge with *P. marinus*, whereas hemagglutinin titer tended to decrease in eastern oysters infected with the parasite (chapter 1, chapter 2). In several other studies, higher hemagglutinin titer tended

to be associated with increased survival of oysters infected with *P. marinus* or MSX (La Peyre, unpublished data; Ling, 1990; Chintala and Fisher, 1991; Chu and La Peyre, 1993). The capacity of oyster plasma to agglutinate *P. marinus* and to enhance hemocyte phagocytosis and degradation of the parasite, needs to be measured in order to determine the possible function of hemagglutinin in *P. marinus* infection.

The same caution in interpreting data of hemagglutinin titer needs to be exercised as with other potential host defense activities, since hemagglutinins are involved in physiological processes other than host defense. Lectins, for example, may serve as transport or storage molecules, organize macromolecules or multienzyme complexes and may be involved in nutrition, feeding and reproduction (Yeaton, 1981; Vasta, 1991).

5) Plasma lysozyme

The effect of lysozyme on *P. marinus* has not yet been determined but it has been suggested that lysozyme may play a role in oyster host defense against *P. marinus* (Chu and La Peyre, 1993; Chu et al., 1993). Plasma lysozyme concentration is greater in oysters maintained at low temperature (<20°C, Chu and La Peyre, 1993) and in oysters maintained at low salinity (<10 ppt) (Chu et al., 1993). Low temperature and low salinity are conditions which do not favor *P. marinus* infection. Oysters are poikilothermic and osmoconformers so that the effect of temperature and salt

concentration on lysozyme activity must also be considered when interpreting the possible effect of lysozyme on *P. marinus*. Oyster plasma lysozyme activity decreased when the assay temperature was decreased or when the salt concentrations were increased (La Peyre, unpublished data). Lysozyme activity of Pacific oyster plasma was surprisingly low compared to eastern oyster plasma. Hemocyte lysozyme, however, was not determined.

Although high lysozyme concentration seems to be associated with low *P. marinus* infection in oysters, the exact role of oyster lysozyme in host defenses against the parasite will not be known until the direct effect of oyster lysozyme on the parasite is measured. Lysozymes are antimicrobial enzymes that possess neuraminidase activity. Many lysozymes also exhibit chitinase activity. Unfortunately, the cell wall composition of *P. marinus* has not been determined, but it is interesting to note that the cell wall of *P. marinus* may contain chitin since the cell wall is very resistant and not soluble in alkali.

The use of potential host defense-related activities measured in this study cannot be recommended for markers of disease resistance agaisnt *P. marinus* since there is a basic lack of understanding of the roles of these activities. The direct interaction between *P. marinus* and these activities must first be investigated. In addition, more needs to be

learned about the involvement of potential host defense activities with other physiological processes such as nutrition and reproduction.

The identification of the mechanism(s) of killing of *P. marinus* by oyster hemocytes is urgently needed and is the most relevant approach in view of the problems associated with potential host defense activities. Hemocyte killing activity of *P. marinus* will certainly provide the best marker for selection purposes. In addition, ways might be found to augment the killing activity of hemocytes in eastern oysters. Recent findings suggest that exposure to chemicals can increase the pathogenesis of *P. marinus* in eastern oysters (Chu and Hale, personal communication). If the susceptibility of eastern oysters to *P. marinus* can be increased by exposure to certain chemicals, maybe the susceptibility can also be decreased by exposure to other chemicals. The effect of environmental factors in diseases is often a determining factor in the epizootiology of a disease. In the case of *P. marinus*, temperature is the most important factor. It affects the parasite growth rate as well as the oyster host defense.

The culture of merozoites will provide a much simplified system to study the biology of *P. marinus* independent of the host influences. Information on the effects of environmental factors such as temperature and

salinity that control the growth and multiplication of the parasite may help explain the epizootiology of the disease and provide new ideas for management practices to control the disease. Moreover there is a significant gap in our understanding of the biology of *P. marinus* relating for example to its taxonomy, life cycle and pathobiology. The culture of this parasite has the capacity to produce large quantities of the parasite for research material.

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