THE EFFECTS OF *PERKINSUS MARINUS* INFECTION ON PHYSIOLOGICAL PROCESSES IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

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ABSTRACT   Although *Perkinsus marinus* infections have been associated with high mortalities in populations of the eastern oyster *Crassostrea virginica* for several decades, the pathological mechanism(s) by which death is induced is unclear. Physiological changes in the oyster associated with *P. marinus* infection are not well studied. Infections typically cause significant reductions in growth rate and several studies have shown reductions in condition index as well. The reduction in condition index may be the result of a perturbation in free amino acid metabolism caused by infection. Further, the effects on free amino acid metabolism may be associated with parasite-induced changes in mitochondrial function. A significant acidosis has also been shown to occur in the hemolymph of infected oysters which may affect general tissue functions. However, changes in oxygen consumption and clearance and assimilation rates of whole oysters have not been correlated with increasing infection. Finally, reproductive capacity may be reduced by *P. marinus* infection.

KEY WORDS: *Perkinsus marinus, Crassostrea virginica*, physiology

INTRODUCTION

Most of the research on the sublethal effects of diseases on bivalves has pertained to growth and mortality. Even though physiological data are essential to determine the effects of diseases on oyster populations, very little research regarding the sublethal, physiological effects of diseases has been conducted until recently. However, in the past few years several studies have appeared that provide a beginning to understanding the physiological consequences of *Perkinsus marinus* parasitism in *Crassostrea virginica*.

Oysters are typically stricken with *P. marinus* infections in high-salinity (>15 ppt) waters from the Chesapeake Bay south throughout the Gulf Coast states. However, recently, *Perkinsus* infections have been found in oysters as far north as Massachusetts (Ford 1992) and in Maryland oyster beds at low salinity (<10 ppt) (Smith and Jordan 1992). Shortly after infection oyster growth usually ceases or is greatly reduced (Andrews 1961, Paynter and Burreson 1991) but large-scale mortality in a population typically does not occur until the second summer of infection. Mortality is associated with high summer water temperatures and is greatest in August or September, depending on the latitude. The typical scenario of disease infection, lengthy progression, and eventual death suggests that infection produces significant initial sublethal effects and causes cumulative physiological damage which becomes most acute at high temperatures when metabolic demand in both oyster and parasite is likely highest.

The physiological effects of *P. marinus* infection are most apparent as a reduction in growth rate. However, little is known about the ultimate causes of oyster mortality. Since the protozoan was first described, scientists have observed histological effects which lead to postulation that lytic and cytotoxic agents are produced by the parasite (Mackin 1962, Mackin and Ray 1954), but these results do little to identify the effects on physiological functions of the various host tissues and how they may be impaired. Importantly, Mackin (1962) observed that infected oysters could not maintain valve closure as long as uninfected oysters. The inability to maintain valve closure makes oysters much more vulnerable to predation and limits their ability to tolerate rapid changes in salinity since valve closure is the first defense against osmotic shock. Furthermore, much of the oyster's ability to survive in the estuary is based on cellular adaptation to the environment. How these sophisticated cellular mechanisms are affected has only been recently examined.

Oysters such as *C. virginica* are physiologically complex organisms. They dwell in an estuarine habit that exposes them to a wide range of environmental stresses including high and low temperatures, very little or no oxygen for extended periods of time, and rapid salinity changes of 10 ppt or more. To survive these extremes, *C. virginica* has evolved a sophisticated series of cellular and metabolic mechanisms which either neutralize or avoid potentially fatal environmental changes. One of the most well-studied mechanisms is cellular volume regulation in response to a change in salinity (Lynch and Wood 1966, Paynter et al. 1995). When the ambient salinity increases, oyster cells accumulate free amino acids (FAA) to offset the increasing extracellular osmotic pressure. With osmotic pressures nearly equal on both sides of the cell membrane the cell does not lose water and shrink, and can therefore remain functional. Similarly, when oxygen is depleted from ambient water, the oyster consumes less oxygen, lowering its own metabolic rate (Hammen 1980). It makes the chemical energy necessary for survival through alternative metabolic processes which require less oxygen. In this way the cells can continue to function at a low rate in the absence of oxygen.

Recent research has focused on the possible effects of *P. marinus* on these sophisticated mechanisms and other physiological characteristics of *C. virginica*. In the following section I shall review some of the advances made over the last few years including general effects on growth and condition as well as the effects of infection on FAA concentrations in oysters, differences in mitochondria isolated from infected and noninfected oysters, the acid-base physiology of oyster hemolymph, and the relationship between *P. marinus* infection and physiological energetics. These important studies shed light on the mechanisms through which *Perkinsus* infests and kills oysters and allow us to better understand how to prevent or control infections and mortality.

Growth, Condition and Mortality

Andrews (1961) used an underwater weighing technique to show that individual oysters that acquired *P. marinus* infections exhibited greatly reduced growth. The underwater weighing technique measured shell deposition so that somatic tissue growth, or the effects of infection thereon, could not be assessed. Paynter and Burreson (1991) reported very similar observations on whole populations of oysters; growth of the whole population was greatly reduced even though less than 100% of the population were diagnosed as infected. Similar to the study by Andrews, shell height (a measure of shell production rather than somatic tissue) was measured. The difficulties in assessing effects on somatic growth lie in the seasonal variation of tissue weight in oysters; typically dry tissue weight declines in late summer even without infection. Furthermore, Hilbish (1986) and Borrero and Hilbish (1988) have shown that shell and soft tissue growth in mussels is not always similar and that temporal differences exist between shell and soft tissue growth periods. It is clear, however, that shell deposition is greatly reduced by *P. marinus* infection, subsequently limiting somatic tissue growth.

Most studies measuring the effects of *P. marinus* have used a condition index, dry tissue weight per unit shell volume, as a measure of somatic growth or health (Menzel and Hopkins 1955, Craig et al. 1989, Gauthier et al. 1990, Crosby and Roberts 1990, Burreson 1991, Paynter and Burreson 1991, Chu and La Peyre 1993a, Dittman 1993). This measure typically declines after spawning and during hot summer months in most regions and most studies have shown a negative correlation between condition index and *P. marinus* infection (Gauthier et al. 1990, Crosby and Roberts 1990, Burreson 1991, Paynter and Burreson 1991, Dittman 1993, Volety and Chu 1994). However, other studies (Chu and La Peyre 1993b, Chu et al. 1993, Newell et al. 1994) have revealed no relationship between *P. marinus* infection and condition index. Laboratory exposures of relatively short duration may not allow enough time for condition to become reduced, but they nevertheless reveal that reduction in condition is not always directly associated with increasing infection intensities.

Free Amino Acid Metabolism

C. virginica is a euryhaline species capable of acclimating to wide changes in ambient salinity. Cell volume is controlled by regulating a large, intracellular FAA pool and the quaternary ammonium compound glycine betaine to offset changes in extracellular osmotic pressure, i.e., this oyster is an osmoconformer (Pierce et al. 1992). The time course of amino acid accumulation has been measured in oysters exposed to increased salinity (Paynter et al. 1995) and appears to be typical of other bivalves. In ribbed mussels, for instance, alanine rapidly accumulates and reaches high levels immediately after a hyperosmotic stress. As acclimation proceeds, the glycine concentration rises and within a few days replaces alanine as the major osmotic effector. During the next several days to weeks at high salinity, proline typically appears as a transient peak, beginning to rise slowly after the alanine accumulation peaks and declining as taurine accumulates. Taurine usually becomes the major osmotic effector, often comprising as much as 70% of the FAA pool (Baginski and Pierce 1977). The ability to regulate intracellular amino acids in this way allows *C. virginica* to inhabit estuaries such as Chesapeake Bay.

While the physiological effects of protozoan parasitism have been addressed by a few studies (Newell 1985, Barber et al. 1988a, 1988b, Ford and Figueras 1988, Newell and Barber 1988), none have examined salinity tolerance. Heavily infected oysters appear wasted, watery, and translucent, and the ratio of whole wet tissue weight to dry tissue weight is increased (Paynter and Burreson 1991). In addition, oysters from various locations within Chesapeake Bay had smaller intracellular free amino acid pools, almost no glycine betaine, and reduced salinity tolerances compared with conspecific oysters from several locations along the Atlantic Coast from Georgia to Cape Cod (Pierce et al. 1992). It is likely that all of the Chesapeake Bay oysters in that study were parasitized with *P. marinus*. These observations, together with the observed reductions of morbidity and mortality amongst parasitized oysters in lower salinities (Andrews 1988, Burreson and Andrews 1988, Paynter and Burreson 1991), suggest that *P. marinus* infection may have an impact on the salinity tolerance mechanisms of the oyster, a hypothesis suggested many years ago by Soniat and Koenig (1982).

Paynter et al. (1995) examined the effects of both *P. marinus* parasitism and environmental salinity on intracellular FAA concentrations of oyster tissues during a cycle of infection in the field.
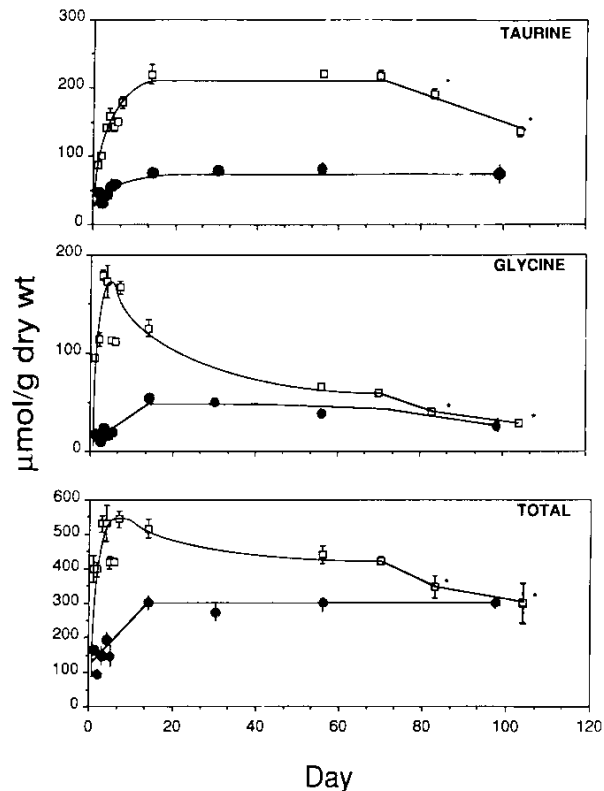

Figure 1. Concentration of taurine, glycine, and total FAA in gill tissues from oysters held at high (20 ppt, open square)- and low (8-12 ppt, closed circle)-salinity sites. Transfer of oysters from low to high salinity occurred on day 0. An increase from 8 to 12 ppt occurred at the low-salinity site between days 10 and 20. Asterisks denote infection of oyster groups by *P. marinus* at high salinity. From Paynter et al. 1995.

In that study, the FAA levels in gill tissues changed after transfer to the high-salinity site (Fig. 1), essentially as predicted by earlier laboratory studies on other bivalve species. Overall, the total FAA increased to a peak around 500 μmol/g dry wt within 3 d and remained constant until the day 85 sample when protozoan infections were first detected and total FAA levels declined. Taurine concentrations at days 85 and 105 were significantly lower than the levels exhibited before infection. Remarkably, glycine and total FAA declined to levels that were not different from those of the low-salinity oysters. The amino acid levels in tissues from the oysters held at low salinity stayed constant after the period between days 10 and 20 when the total FAA level went up, presumably in response to a 4 ppt salinity increase at the site. The mean levels of glycine, taurine, and total FAA remained constant at the low-salinity site for the remainder of the study period.

The levels of FAA in the oysters transferred to high salinity in this study were significantly different among three phases (uninfected, lightly infected, and heavily infected) following salinity acclimation. Mean levels of the major amino acids of the FAA pool—taurine, glutamate, and glycine—and the minor components—threonine, serine, and β-alanine—were all lower in the infected groups compared to the uninfected groups. All of the FAA, except taurine and glutamine, were significantly lower even with light infection intensity, producing a 24% decline in the total FAA pool. Glycine and β-alanine levels declined further as infection intensity increased and taurine levels were substantially reduced in the heavily infected oysters. Both glutamine and alanine levels increased in the heavily infected group. Overall, the total FAA pool in the oysters at the high-salinity field site declined by 40% between the acclimated uninfected phase and the heavily infected phase.

In summary, intracellular FAA levels were much lower in the gills of the groups of high salinity–adapted oysters infected by *P. marinus*. As the infection intensified in the group, the amino acid pool declined by 40% of its original level largely due to taurine decreases in all of the oysters, including those scored as uninfected by our fluid thioglycollate–based diagnostic method. Since taurine levels in oysters or mussels acclimated to a particular salinity remain at the acclimated level unless a subsequent salinity change occurs (Soniat and Koenig 1982, Baginski and Pierce 1977, Bishop et al. 1983, Pierce et al. 1992) and given the lack of a similar decline in taurine in the low-salinity oysters, the reductions in taurine and other FAA in the oysters are likely related to protozoan infection rather than some type of seasonal change. Since oysters are osmoconformers, a reduction of 33% in intracellular FAA must be compensated for by the elevation of other intracellular solutes. At present, these solutes are not known but inorganic ions such as Na^+, K^+, and Cl^- are obvious possibilities. An increase in intracellular ion concentration of this magnitude is likely to produce negative physiological effects (Yancey et al. 1982). The decreased FAA concentration might be caused by perturbations either in the synthesis of FAA important for salinity tolerance or in the membrane characteristics which keep the FAA inside the cell once they are synthesized. The intracellular amino acids that are utilized for salinity tolerance are synthesized largely by the mitochondria (Paynter et al. 1984, Pierce et al. 1992). Once synthesized, the amino acids are transported to the cytosol where their intracellular concentration is regulated by the permeability control mechanisms of the cell membrane (Pierce and Politis 1992). Therefore, the presence of *P. marinus* might affect the

permeability control mechanisms of the cell membrane or synthetic mechanisms in the mitochondria. In addition to the effects on the osmolytes, impaired mitochondrial function could result in a reduction in ATP production, which could account for the reduction of growth observed in the presence of the parasite.

Mitochondrial Metabolism

Pierce et al. (1992) found that oysters from a variety of locales within Chesapeake Bay had salinity tolerances that were more narrow than the tolerances of oyster populations elsewhere along the Atlantic Coast. The basis of this difference was that the FAA pool of Bay oysters was smaller and composed of different amino acids than that of the Atlantic oysters, agreeing with the results reported by Paynter et al. (1995) summarized above. In addition, the Atlantic oysters had substantial intracellular concentrations of glycine betaine not present in the cells of Bay oysters. These differences in osmolyte composition between Chesapeake and Atlantic oysters no doubt account for the salinity tolerance differences. Furthermore, since both the amino acids used in cellular osmoregulation and glycine betaine are synthesized in the mitochondria (Paynter et al. 1984), the differences between Chesapeake and Atlantic oysters may reside in the mitochondria. In addition, since all of the Bay oysters studied have been parasitized with *P. marinus*, it is possible that the differences are due to the parasite rather than to genetics.

The respiratory control ratios (RCRs) of mitochondria from Bay oysters are often higher than those of mitochondria from Atlantic oysters (Fig. 2). In addition, the Bay oyster mitochondria give highest RCRs with malate as a substrate while the Atlantic mitochondria prefer α-ketoglutaric acid. The basis of this coupling ratio difference is not clear at present, but at least suggests that the energy metabolism of Bay and Atlantic animals is different and that the difference may lie with the control of the kinetics of various steps in the Kreb's cycle.

In addition, Pierce et al. (1995) have shown that the uptake of choline, a precursor of the osmotically active compound glycine betaine, by mitochondria isolated from Chesapeake Bay oysters is significantly lower than that by mitochondria from Atlantic conspecifics. While infection levels were not part of this study, all of the Chesapeake oysters tested from the experimental groups were

RCRs from Atlantic and Bay oyster gill mitochondria adapted to 350 mosm
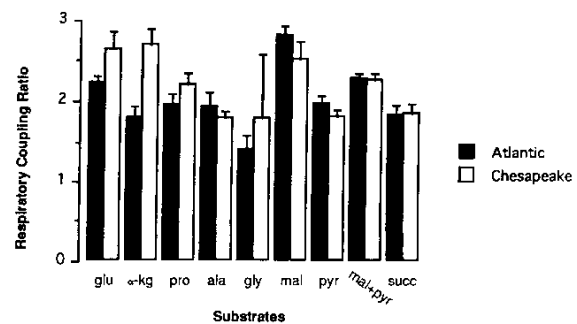

Figure 2. Respiratory coupling ratios of mitochondria isolated from Atlantic and Chesapeake Bay oysters acclimated to low salinity. Histogram bars are the means of at least 10 measurements. Error bars indicate standard errors. From Pierce et al. 1992.

infected with *P. marinus* while most of the Atlantic oysters were not. This suggests that mitochondrial metabolism may be affected by *P. marinus* infection.

The initial accumulation of FAA in response to hyperosmotic shock is the result of a complex biochemical regulatory process that allows oyster cells to route carbon and nitrogen in a very specific way (Bishop et al. 1983). Since the biochemical pathways used to respond to hyperosmotic stress may be the same as, or very similar to, the pathways involved in hypoxic tolerance (Baginski and Pierce 1975), it is possible that the ability of oysters to tolerate hypoxia, which is common in Chesapeake Bay (MacKiernan 1987), might also be diminished by *P. marinus* infection. A reduced tolerance of hypoxia could lead to the large-scale mortalities that occur in Chesapeake Bay oysters during exposure to hypoxia, which occurs frequently during the summer months over many oyster bars.

Hypoxia Tolerance and Acid-Base Balance

Hypoxia or anoxia causes not only the obvious stress associated with the lack of oxygen but also causes a general decrease in tissue pH caused by an increase in CO_2. These changes can have harmful effects on the general well-being of organisms and affect many aspects of normal physiological performance. When oysters are air exposed, they close their valves and the oxygen contained in the water trapped between the valves is quickly exhausted. The oyster tissues then become hypoxic and hypercapnic. Infected oysters cannot hold their valves closed for as long as uninfected oysters when aerobically exposed (Fig. 3). This is thought to be due to the stress induced by infection, but the exact nature of the stress has never been addressed. Dwyer and Burnett (1996) have studied the acid-base physiology of oysters and the effects of *P. marinus* on acid-base balance and discovered important correlations between infection and acidosis in oysters.

Dwyer and Burnett (1996) showed that the normal pH of oyster hemolymph is around 7.7 while oysters infected with *P. marinus* show significant acidosis (pH 7.2; Fig. 4). Furthermore, they showed that minimal hypoxic stress caused a large decline in hemolymph pH in both infected and noninfected oysters but that
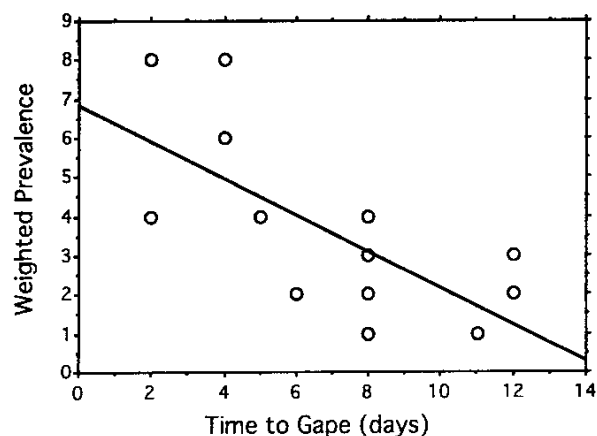
Figure 3. Relationship between weighted prevalence of *P. marinus* infection and time to gape of oysters held undisturbed out of water at room temperature. Regression analysis revealed a strong negative correlation between weighted prevalence and time to gape (P > 0.001).
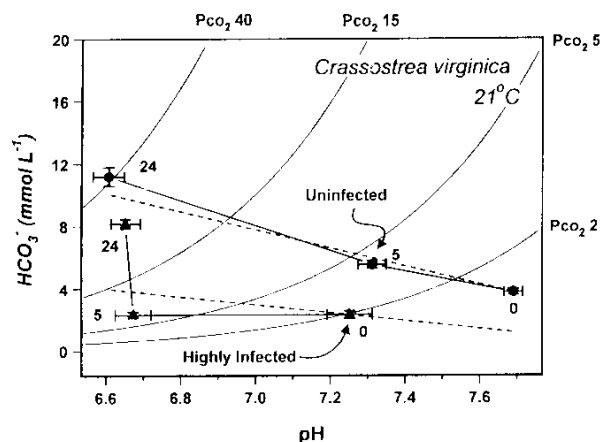
Figure 4. A pH-HCO_3^- diagram showing the acid-base status of oyster hemolymph at 0, 5, and 24 hr of air exposure at 21°C. PCO_2 isopleths (curved lines) are given in torr. In vitro buffer lines are shown as dashed lines. Circles represent uninfected oysters; triangles represent oysters with high infections. Values are means ± SE. N for each experiment ranged from 22 to 56. From Dwyer and Burnett 1996.

infected oysters incurred a steeper decline. The pH of hemolymph of infected oyster dropped to 6.7 after 5 hr of hypoxic stress compared to a decline to 7.3 in healthy oysters. This response could result in large differences in nutrient absorption or retention, blood cell function (see Anderson 1996), oxygen consumption, and metabolic efficiency between infected and uninfected oysters. Indeed, it could be associated with the loss of FAA from cells or an increased rate of parasite growth. It is likely that this acidosis is associated with the inability of infected oysters to remain closed as long as uninfected oysters. In fact, Dwyer and Burnett (1996) have shown that adductor muscles of infected oysters contain significantly lower amounts of glycogen than do uninfected oysters and that heavily infected oysters have less glycogen than lightly infected individuals. This suggests a direct correlation between disease and an oyster's ability to perform ecologically critical tasks such as keeping its valves closed (see Fig. 3). These kinds of studies bring us to a closer understanding of the nature of mortal injury inflicted by the parasite.

Physiological Energetics

Newell (1985) reported that the feeding rates of eastern oysters were significantly reduced when they were infected by the parasite *Haplosporidium nelsoni* (MSX). Such reduced feeding activity resulted in lower amounts of glycogen being sequestered (Barber et al. 1988a), resulting in a reduction in condition index. Energy allocation by MSX-infested oysters to gametogenesis was also disrupted, resulting in significantly inhibited gametogenesis during the spring (Ford et al. 1990). The effects of *P. marinus* infections on the physiological functions of feeding, metabolic energy expenditure, and assimilation efficiency in eastern oysters have only recently been studied.

Using a combination of field and laboratory experiments to study the effects of *Perkinsus* infections on *C. virginica*, Newell et al. (1994) have shown that *P. marinus* infection has a surprisingly small effect on most aspects of feeding physiology and metabolism. In that study, oxygen consumption, clearance rates, condi-

tion index, and assimilation rates were measured over a 2-year cycle of growth and infection at three sites in Chesapeake Bay. When the oysters were transferred from low to higher salinity sites at the initiation of the experiments, feeding rates increased sharply and remained high for 1 to 2 months but declined rapidly in concert with the first detection of *P. marinus* infections in the experimental groups. Surprisingly, however, further declines in the clearance rates of the oysters did not occur in association with progression of disease. In contrast, Mackin and Ray (1954) showed that fecal and psuedofecal production in moderate and heavily infected oysters was less than half that of uninfected oysters.

Other aspects of the physiological energetics were unaffected by *P. marinus* infection. Oxygen consumption did not change between uninfected and infected oysters, even in oysters heavily infected and within weeks of succumbing to the disease. Condition index has been reported to decline with infection (Craig et al. 1989, Gauthier et al. 1990, Crosby and Roberts 1990, Paynter and Burreson 1991, Dittman 1993) but was not associated with infection level in Newell et al. (1994). Most surprisingly, assimilation rates remained unchanged by *P. marinus* infection even though the primary portal of entry by this parasite is thought to be through the intestine. Clearly, the nature of physiological damage caused by *P. marinus* infections cannot yet be fully understood.

Reproductive Capacity

Parasitism by *H. nelsoni* has been shown to significantly affect reproduction in oysters. Gametogenesis is apparently inhibited by infection-induced disruptions in carbohydrate metabolism (Barber et al. 1988b, Ford et al. 1990) which lead to a reduction in fecundity (Barber et al. 1988a). Given these observations one might expect that *P. marinus* infections would also have significant deleterious effects on reproduction in the oyster. However, the effects of *P. marinus* infection on gamete production seem to be much less direct.

Although fecundity or reproductive condition has not been directly studied in relation to *P. marinus* infection as it has with *H. nelsoni* (Barber et al. 1988a), Cox and Mann (1992) have shown that reductions in reproductive activity in oyster populations in the James River, VA, have coincided with increases in *P. marinus* prevalence over the last few years. It also seems likely that *Perkinsus* infection may cause perturbations in gonad development since Ragone Calvo and Burreson (1994) showed that *P. marinus* parasites survive overwintering and can develop into substantial infections soon after temperatures increase, which may coincide with gonadal maturation and spawning.

Kennedy et al. (1995) showed that *P. marinus* infections acquired during the previous year did not have a deleterious effect on reproduction the following year. Similarly, Dittman (1993) showed that oysters with light first-year infections had percent gonad areas that were similar to those of uninfected oysters. However, percent gonad areas in oysters with heavy infections were significantly lower, indicating a significant negative impact of *Perkinsus* infection on reproductive capacity. Ray et al. (1953) and Kennedy et al. (1995) reported significant reductions in reproductive output, in terms of numbers of eggs produced, of oysters heavily infected with *P. marinus*. In contrast, eggs from *P. marinus* infected individuals were not smaller than eggs from uninfected animals and the lipid content of eggs from infected oysters was no different from that of eggs of uninfected oysters (Kennedy et al. 1995). Thus, it appears that heavy *P. marinus* infection may have some deleterious effect on reproduction but that perhaps the oyster can shunt energy from growth (which is reduced even with light infections) to gametogenesis to minimize the effects of infection on egg quality.

SUMMARY

Infection of the eastern oyster by *P. marinus* induces a number of significant changes in the physiology of the oyster. Given the changes in the hemolymph pH associated with infection, one would expect nearly all cell-mediated functions, including ciliary beating, respiration, absorption of nutrients, and excretion of waste products, to be altered. Certainly acidosis could inhibit calcification and shell deposition, accounting for the cessation of shell growth associated with infection. It could also alter membrane characteristics to the point where amino acid uptake was retarded and it may account for changes in blood cell function as described by Anderson (1996). However, given the expected response of general acidosis, the observations of Newell et al. (1994), which show little or no effect on oxygen consumption, clearance rate (a measure of ciliary action of the gills), or food assimilation, are startling. These contradictory observations only serve to demonstrate the need for a better understanding of the biochemical, pharmacological, and physiological effects of *P. marinus* infections on oysters.

La Peyre and Faisal (1996) have shown that *P. marinus* cells in culture produce extracellular proteases. These proteases are thought to play a role in damaging host tissue, protecting the parasite from host immune response, and perhaps enhancing the parasites' ability to replicate within the host. General changes in the host physiology such as a decline in hemolymph pH may enhance the cytotoxic activity of such parasite-produced chemical agents.

It is important to note that the physiological effects of *P. marinus* infection on oysters may differ between physiological races of *C. virginica*. Several studies (Bushek and Allen 1996, Paynter and Burreson 1991, Pierce et al. 1992, Brown et al. 1994) have shown that different intraspecific populations of oysters respond differently to *P. marinus* infections. Therefore, a level of infection that would induce mortality in one population may not induce mortality in another population (Brown et al. 1994). This makes it more important to understand the mechanisms of pathology that result in mortality in oysters.

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