2004

A study of the eastern oyster, Crassostrea virginica: (1) Dermo tolerance, survival, growth, condition and Hsp70 expression in different geographic stocks; (2) Heat tolerance and effects of sublethal heat shock on survival and Hsp70 expression of infected and uninfected oysters

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A STUDY OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*: 1. DERMO TOLERANCE, SURVIVAL, GROWTH, CONDITION AND HSP 70 EXPRESSION IN DIFFERENT GEOGRAPHIC STOCKS; 2. HEAT TOLERANCE AND EFFECTS OF SUBLETHAL HEAT SHOCK ON SURVIVAL AND HSP70 EXPRESSION OF INFECTED AND UNINFECTED OYSTERS.

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

Vincent G. Encomio

APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

Vincent G. Encomio

Approved, September 2004

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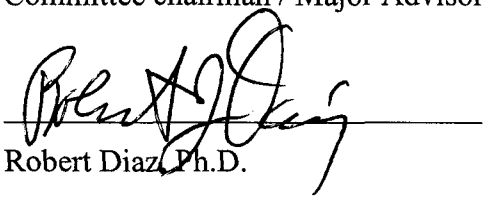

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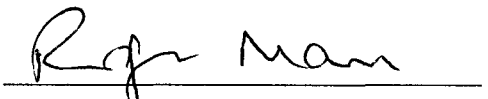

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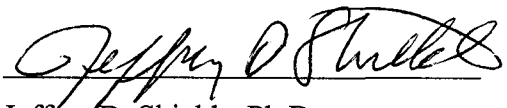

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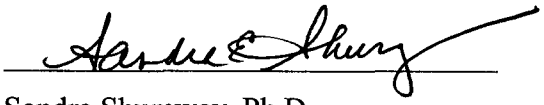

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Dedication

To my father and mother, Gil and Daisy Encomio, for their love and support from both near and afar.

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Acknowledgements

I would first like to thank my advisor Dr. Fu-Lin E. Chu for her tireless efforts in providing all the support, emotional as well as financial, necessary to complete my dissertation. Her energy and perserverance will always be a source of inspiration for me in all my future endeavors. I would also like to thank the rest of my committee: Dr. Roger Mann for his insights in oyster biology, Dr. Robert Diaz for his help in experimental design and statistics, Dr. Sandra S.Shumway for her helpful comments during the whole lengthy process of writing this dissertation, and Dr. Jeffrey D. Shields also for his comments, insights and helpful suggestions on various data analyses.

I would also like to thank Dr. David Evans who served as my moderator for both my qualifying exam and my dissertation defense. Thanks to Dr. Michael Newman for assistance with survival analyses. Dr. Stan Allen kindly provided the facilities and technical support to condition and spawn broodstock. In the EAAH department the support provided by Shirley Sterling was unparalleled. Mary Ann Vogelbein was extremely helpful and gracious in providing and organizing wet lab space for my experiments.

Past and present members of the Chu lab made my experience at VIMS enriching and enjoyable: Georgeta Constantin, Dr. Luis Cruz-Rodriguez, Dr. Yonquin Huang, Dr. Eric Lund, Dr. Philippe Soudant, Lee Steider, the now Dr. Shawn Stickler, who has been a great friend through this whole process, and Adriana Veloza. I would also like to thank the many summer interns that assisted me in my research: Elizabeth Werner, Jennifer Lewis, Aiysha Balbosa, Lara Neece, Alisha Acy, Mariah Love and Calvin Peters.

There are numerous friends at and outside of VIMS that made my life in Gloucester Point a little brighter: Maryse Delaporte, Mike Arendt, Kathleen Apakupakul, Claude O'Brian Jones III (and the rest of the FBSC), Helen Woods, Corinne Audemard, Lawrence Carpenter, Kevin Kiley, Scott Marion, Dave Heroy, Erin Burge, Elizabeth Francis, Jennifer Jawad, Laurent Mezin, Winnie Ryan, Landon Ward, Eric Wooden, Jennifer Cardinal, Alanna MacIntyre, Dave Gauthier, Erica Wescott, Dianne Carter, Jill Meyer, Jamie Smedsmo, David Rudders, Stephanie Wilson and John Carriger to name just a few.

This work was supported in part by a VIMS, College of William and Mary Fellowship, the Craig L. Smith Memorial Scholarship, a College of William and Mary Minor Research Grant and two grants from the NOAA – Oyster Disease Research Program.

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Abstract

The diseases Dermo and MSX have devastated Chesapeake Bay populations of the eastern oyster, *Crassostrea virginica*. The protozoan *Perkinsus marinus*, which causes Dermo, is particularly problematic since it persists over a wide range of salinities and temperatures. An objective of this dissertation was to determine whether specific wild oyster stocks had developed natural resistance to Dermo and if several parameters (survival, growth, condition and energy reserves) were associated with resistance. Another objective was to characterize heat shock protein (hsp70) expression in the eastern oyster. Heat shock proteins such as hsp70 protect organisms from thermal stress and other stressors, and this function may play an important role in disease resistance in oysters.

In field trials a F_0 Chesapeake Bay stock from Tangier Sound (CTS) survived similarly to a disease resistant hatchery strain (XB). A Louisiana stock was also resistant to Dermo, but not MSX. Despite high mortality, a disease-susceptible stock (CRB) reached market size the fastest. Growth and condition index varied between stocks, but did not reflect Dermo resistance. Energy reserves were affected strongly by season, but not disease or stock. Results imply that Dermo resistant strains could be developed from these stocks but criteria for optimal strain selection for aquaculture and restoration may be divergent.

Mortalities of F_1 oysters (CRB, CTS and XB) were similar to F_0 parents, demonstrating a genetic basis to Dermo resistance. Total hsp70 did not correlate with seasonal temperatures, while hsp70 isoforms (hsp69 and hsp72) varied inversely across seasons. Hsp70 did not vary significantly between strains, indicating a stronger environmental influence on hsp70 expression. In lab experiments hsp70 in oyster gills was elevated greater than two weeks after a sub-lethal heat shock. Thermal tolerance, but not hsp70, varied between CTS and Louisiana oyster stocks. Heat shock protected oysters experimentally infected with *P. marinus* and non-infected oysters from lethal heat stress. Infection alone induced expression of hsp70. Observed inherent and induced differences in thermal tolerance suggest that both genotype and phenotype may be manipulated to improve survival in cultured bivalves. The implications of this research for bivalve aquaculture as well as areas for future research are discussed.

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Chapter 1: Introduction

History and Geographic Ranges of Oyster Diseases (Dermo and MSX) in the U.S.

Historical declines in commercial oyster production have been primarily attributed to overharvesting and subsequent habitat loss and degradation (Rothschild, et al. 1994). In recent history, diseases caused by two protozoan pathogens *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) have further depleted remaining oyster stocks (Kennedy 1996). Although losses have been reported beginning in the 1950s, the prevalence of these diseases rose dramatically in the 1980s and 1990s. Disease has since become one of the primary foci in efforts to restore the oyster fishery in the Chesapeake Bay. The range of *P. marinus* extends along the Eastern seaboard of the United States, to parts of New England and along the Gulf of Mexico coast, to Venezuela (Andrews 1988, Craig, et al 1989, Bower, et al. 1994). *Haplosporidium nelsoni* is restricted to high salinity (>15 ppt) areas along the Atlantic coast from Florida to Massachusetts and Maine, with the main enzootic area within Delaware Bay (Haskin and Andrews 1988, Bower, et al. 1994).

Pathology of Dermo and MSX Diseases

Both *P. marinus* and *H. nelsoni* infect oysters via water-borne transmission. Entry into the host, however, differs between the two parasites. *Perkinsus marinus* is transmitted from the disintegrated tissues of dead oysters to adjacent live oysters (Andrews and Hewatt 1957). *Perkinsus marinus* then enters the oyster via the intestinal epithelium and infiltrates the host either actively, via secretion of lytic enzymes, or passively via ingestion by host phagocytes (Perkins 1976). Active infiltration, however, is hypothetical and has not been observed (Chu, pers. comm.). Passive entry via phagocytic hemocytes has been simulated using fluorescent polystyrene beads identical

in size to unicellular parasites (Alvarez et al. 1992). Intense body burdens of *P. marinus* eventually cause occlusion of hemolymph vessels and lysis of infected tissue, resulting in emaciation, decreased growth, poor condition and eventual mortality (Mackin 1951, Ford and Tripp 1996, Bower, et al. 1994).

Perkinsus marinus rapidly proliferates between 20-30 °C (*in vitro* and *in vivo*) and salinities greater than 10 ppt (Chu and Greene 1989, Chu and La Peyre 1993, Ragone and Burreson 1993, Burreson and Ragone-Calvo 1996). The parasite persists in salinities as low as 3 ppt and survives at temperatures as low as 4 °C (Chu, et al.1993, Burreson and Ragone Calvo 1993). Because of its widespread distribution and tolerance to low salinity and temperature, *P. marinus* has become the most prevalent parasite of the eastern oyster, *C. virginica*, in the Chesapeake Bay. Dermo-associated mortalities typically begin in the early summer (June) when water temperature increases (20 °C) and peak between August and September. Mortality occurs rapidly at temperatures above 25 °C (Fisher, et al. 1992). Drought conditions prior to and during the summer can cause environmental conditions (high temperature and salinity) that are especially conducive to disease outbreaks. During the 1980's, three winter-spring droughts (1981, 1985 and 1986) followed by dry, warm summers resulted in high *P. marinus*-induced oyster mortalities (Andrews 1988).

Haplosporidium nelsoni enters the oyster host through the gill or mantle tissue and reside on the basal lamina of epithelial cells. Penetration of the basal lamina initiates the lethal stage of infection (Andrews 1966). Epithelial sloughing by resistant oysters serves as a mechanism to get rid of the parasite (Ford and Haskin 1982, Ford 1985a). Although resistant oysters are able to survive parasitism for an extended period, their

condition eventually deteriorates as a result of infection (Barber et al. 1988). In susceptible oysters, parasites pass through the epithelium rapidly, resulting in mortality within one month (Andrews 1966). Susceptible oysters die rapidly and are usually in good condition, indicative of a possible toxin produced by the parasite (Ford et al. 1988). Pathological signs include recession of the mantle, emaciation, lysis of digestive and connective tissues, and accumulation of hemocytes at the site of infection (Farley 1968, Ford 1985a). Like *P. marinus* the prevalence and intensity of infection increases with temperature and salinity (Ford and Tripp 1996). However, *H. nelsoni* is intolerant of low salinities (<15 ppt) and high temperatures (>20 C) (Ford 1985b, Ford and Haskin 1982, Ford and Tripp 1996).

Physiological and Biochemical Condition in Natural Dermo Resistant Oysters

Parasitic Effects on Growth and Condition Index

Parasitism can deleteriously affect oyster growth. The ectoparasitic snail *Boonea impressa* reduced growth rates in *C. virginica* by directly interfering with the ability of the oyster to feed (Ward and Langdon 1986, Wilson et al. 1988). Growth rates, as change in shell heights, in *C. virginica* decreased as the prevalence of *P. marinus* infection increased in moderate and high salinity sites (Paynter and Burreson 1991). Although direct growth is easily measured, shell heights do not fully describe the condition of the animal. Dry weight and condition index may give a more reliable estimate of the general physiological state of an organism. Condition index is generally determined as the ratio of dry tissue weight to shell volume or shell weight. The condition index serves as a static indicator of the nutritive state of the animal. One of the systemic effects of diseases such as Dermo and MSX is a reduction in soft tissue growth, resulting in a decreased

condition index (Crosby and Roberts 1990, Gauthier et al. 1990, Paynter and Burreson 1991, Barber et al 1988a, Newell 1985, Ford et al 1988). Oysters infected with *P. marinus* had a decreased condition index and lower serum protein compared to non-infected oysters (Volety and Chu 1994). Because the reduction of the dry weight condition index is directly correlated with energy reserves (Widdows 1985), biochemical composition may also indicate the extent of disease effects on the host and help identify differences among individuals or groups that exhibit variable responses to disease.

Effects of Disease on Energy Reserves

Glycogen content can be a significant proportion of the dry weight of the oyster and is often associated with changes in condition index (Galtsoff 1964). Glycogen is an important energy source for gametogenesis. (Engle 1951). Additionally, glycogen is the major metabolic substrate during periods of hypoxic stress, such as tidal emersion (De Zwaan 1977). Triglycerides are the major lipid reserve in bivalves (Swift, et al. 1980) and their consumption can increase under stress (Reddy and Rao 1989). In adult oysters, high protein catabolism may be an indicator of stress. During winter, when food availability was low, protein contributed the most energy to starved Pacific oysters (*C. gigas*) (Whyte et al. 1990). As carbohydrate and lipid stores are exhausted, protein will be catabolized to support overall metabolism (Gabbot and Bayne 1973). Measurement of metabolic reserves has been used to characterize the effects of parasitic stress on oysters. Biochemical composition was altered in oysters parasitized by *H. nelsoni* (Barber et al. 1988b). Glycogen, lipid and protein were reduced as the intensity and duration of MSX infections increased. Parasitism by the trematode *Bucephalus* sp. reduced total fats and glycogen content in *C. virginica* (Cheng 1965, Cheng and Burton 1966).

Although *P. marinus* is hypothesized to cause deterioration of oyster condition by energy depletion (Choi, et al 1989, Newell 1994), the effects of *P. marinus* on oyster energy reserves is still unclear. Glycogen content was reduced in eastern oysters infected with *P. marinus* (Stein and Mackin 1957, Soniat et al. 1989). Conversely, *P. marinus* infection increased in oysters previously parasitized by the snail *Boonea impressa*, with glycogen content increasing in those oysters versus unparasitized controls (White et al. 1988). White et al. (1988) speculated that gluconeogenesis may have been stimulated by *P. marinus*. It is unknown how parasitism by *B. impressa* may have influenced this result. White et al. (1988) showed that glycogen increased in large (6-8 cm) oysters, but not in small (2-4 cm) oysters, and that the larger oysters had higher prevalences and intensities of *P. marinus* infection. The larger oysters, however, may have been increasing glycogen stores for reproduction during the period of experimentation. Lipid content and free fatty acids increased with *P. marinus* infections (Wilson et al. 1988, White et al. 1988). Infections, however, were light to moderate in the study of Wilson et al. (1988), and growth rates were not retarded by infection. In the study of White et al. (1988), lipid contents increased in large oysters in the fall and may still have been the result of continued growth versus any effects of *P. marinus*. *Perkinsus marinus* also caused a reduction in the free amino acid content of infected oysters, which could affect the ability of the oyster to adapt to changing salinities (Soniat and Koenig 1982, Paynter et al. 1995).

The energetic cost of parasitism likely impacts the allocation of energy for growth and reproduction. Infection by *P. marinus* impaired reproductive development of gametes (Choi et al 1994, Dittman 1993, Dittman et al. 2001). Mackin (1962) found that *P.*

marinus inhibited reproduction in eastern oysters, especially during the early stages of gametogenesis. Reproductive condition was reduced in an oyster population located within a sub-estuary (Little Choptank River, MD) of Chesapeake Bay and may have had some relation to *P. marinus* infection (Kennedy et al. 1995). However, these results were confounded by the fact that these oysters may have spawned, which may have explained the reduced reproductive index for that period. Whether Dermo disease affected levels of energy reserves prior to the reproductive period was not determined.

Allocation of energy reserves to support reproduction causes a substantial physiological stress on the organism. The presence of an additional stressor such as disease may deplete the availability of stored nutrients required to support normal metabolic processes. Measurement of metabolic reserves in *P. marinus*-infected oysters is particularly important since depletion of the energy reserves of the host may be one of the mechanisms by which *P. marinus* degrades oyster condition. Choi et al. (1989) quantified the number of *P. marinus* hypnospores within an oyster host and calculated the energy requirements of various infection levels. Reduced growth and reproduction were attributed to the metabolic demand of *P. marinus,* resulting in a negative energy balance for the host. Newell et al. (1994) measured several physiological rates (metabolic rate, clearance rate, and assimilation efficiency) of *C. virginica* in response to *P. marinus* infection, but did not find any differences between infected and uninfected oysters. The lack of correlation between metabolic rate and assimilation efficiency in *P. marinus*-infected oysters was attributed to the ability of the parasite to out compete the host for absorbed nutrients, more so than any effect on energy metabolism (Newell et al. 1994).

Genetic and Physiological Variation in Disease Resistance

All eastern oysters are prone to infection by *P. marinus* and *H. nelsoni*, but variation in survival to these pathogens is known to occur. Since the 1950's, oysters from some areas were observed to be more resistant to MSX, although the basis of this resistance is unknown (Andrews and Hewatt 1957, Mackin and Sparks 1962, Valiulis 1973). Selective breeding for MSX resistance has been successful (Haskin and Ford 1979, Mathiessen et al. 1990), and these oyster lines remain integral to selective breeding programs today. Evidence of heritable disease resistance in oysters has been tested and effectively demonstrated with regards to *H. nelsoni* (MSX) (Haskin and Ford 1979). Evidence of heritable resistance to Dermo is less clear.

There had been early observations of individuals and populations that appeared resistant to *P. marinus* (Andrews and McHugh 1956, Andrews and Hewatt 1957). Cumulative mortalities in Delaware Bay native stocks were lower than Mobjack Bay and James River stocks at sites in the Chesapeake Bay with high Dermo prevalence, indicative of intraspecific variation in susceptibility to *P. marinus* (Burreson 1991). All of the stocks in the study of Burreson (1991) were highly susceptible to *P. marinus*, however. Bushek and Allen (1996) also found that oysters from different geographical regions show distinct differences in their response to infection with *P. marinus*. Following challenge with *P. marinus*, oysters from Texas had lower intensities of infection than other populations from Virginia, New Jersey and Maine (Bushek and Allen 1996). In this study, the degree of resistance to *P. marinus* corresponded to the exposure history of each population, indicating that natural selection for resistance had developed in these populations. The study of Bushek and Allen (1996) was performed in the

laboratory and field trials demonstrating heritable natural resistance to Dermo are still necessary.

Physiological variation of the oyster host may affect response to disease (Gaffney and Bushek 1996). Growth patterns of eastern oyster strains reared in a common environment show persistent differences over several generations, implying a genetic basis for these differences (Dittman et al. 1998). Evidence of intraspecific genetic variation in the timing of the reproductive cycle also exists (Loosanoff and Nomejko 1951, Loosanoff 1969, Barber et al. 1991), which may affect response to disease (Barber et al. 1991). For example, a group of oysters from Long Island, raised in Delaware Bay for 6 generations, retained the reproductive timing and developmental characteristics of their native population (Barber et al. 1991). Temperature-dependent differences in reproductive timing between transplanted northern and southern populations of the eastern oyster correlated with the effects of MSX on gonadal development. The southern strains experienced greater inhibition since they were still in early stages of gametogenesis when the period of increased prevalence occurred (Ford et al. 1990). In the southern strain, *H. nelsoni* may have depleted energy reserves of the host when energy requirements for high (Ford et al. 1990).

A model simultaneously incorporating the physiological energetics of the host (the eastern oyster) and the parasite (*P. marinus*) with environmental variation in food supply and temperature was developed to test the effects of these variables on oyster production. Simulations of the coupled model (oyster population-*Perkinsus marinus*) showed that oysters may overcome lethal effects of *P. marinus* parasitism by outgrowing the disease (Hoffman et al. 1995). If oysters are able to outgrow the parasite and build up

sufficient energy reserves, bouts of parasitism may be tolerated until seasonal periods of disease quiescence (winter) occur. Whether variation in production and disease response can be attributed to genetic differences between populations remains to be determined. Significant variation exists among individual oysters and between populations in defense-related hemocyte activities and humoral factors (Chu and La Peyre 1993a, Chu and LaPeyre 1993b, Chu et al. 1993, Cheng et al. 1993, 1995, Fisher et al. 1996, Oliver and Fisher 1996). These observations imply that oysters surviving outbreaks of Dermo disease must possess physiological factors that support continued growth during these epizootic events. The mechanisms that contribute to Dermo resistance, however, are presently unknown.

Research Objectives

This study compares presumptive Dermo-resistant and non-resistant oysters from two geographic regions (Chesapeake Bay and the Gulf of Mexico). The objective of this portion of the dissertation is to characterize the effect of *P. marinus* parasitism on mortaliy, growth, condition and energy reserves (glycogen, protein and lipid) in several stocks of the eastern oyster, *C. virginica*, that vary in their tolerance to Dermo disease. To date, there have been no studies on the effects of Dermo on biochemical reserves between genetically distinct oyster stocks. More disease tolerant stocks should possess higher levels of energy reserves than non-resistant stocks and thus withstand parasitic stress. Comparisons of mortality, growth, condition and energy reserves among natural Dermo-resistant oyster stocks comprises chapter II of this dissertation.

Heat Shock Proteins in Eastern Oysters - Implications for Thermal Tolerance and Disease Resistance

Heat Shock Proteins

Temperature is one of the most important factors governing the growth, metabolism, and distribution of all organisms. Distributions of species are strongly tied to their optimal thermal ranges. Temperature is a strong selective force on speciation of organisms that determining much of their distribution. At the biochemical level, temperature adaptation reflects, in large part, the degree of thermal stability in structural and metabolic proteins (Somero 1995). When organisms are stressed at or beyond the upper thermal limits that their proteins are capable of withstanding, denaturation of proteins can occur, and may result in death if heat stress is chronic. One of the primary biochemical adaptations counteracting thermal stress is the activity of stress proteins, or heat shock proteins (hsps). Heat shock proteins are one of the most important agents involved in maintaining cellular homeostasis and undoing protein damage that occurs during thermal stress. They are highly conserved proteins, occurring in virtually all organisms, from bacteria to mammals (Schlesinger et al. 1982, Feder and Hofmann 1999). They are essential for housekeeping and adaptive functions (Lindquist and Craig 1988, Welch et al. 1989, Angelidis et al. 1991). Heat shock proteins aid in the proper protein folding, assembly and intracellular transport of proteins, thus protecting organisms from thermal or other stress-induced damage (Lindquist and Craig 1988, Morimoto et al. 1990, Gething and Sambrook 1992, Gupta and Golding 1993). Heat shock proteins also aid in protecting cells from microbial or other pathological stressors and are involved in immune functions and in host-pathogen interactions (Parsell and

Lindquist 1994, Polla 1991, Healy et al. 1992, Murray and Young 1992, Young et al. 1993). The synthesis of these proteins is activated by a variety of inducers, such as ischemia, xenobiotics, and viral infection, in addition to increased temperatures (Nover 1984, Sanders and Dyer 1994). Thermal and pollutant stresses have increased the stress protein expression in many aquatic species including fish, and several bivalve molluscs (e.g., mussel, clam and two oyster species), (Sanders et al. 1993, Bradley et al. 1998, Shamseldin et al. 1997, Clegg et al. 1998, Cruz-Rodriguez et al. 2000). The ecological and evolutionary significance of hsps in contributing to the thermal limits and hence the distribution of species has also been investigated (review by Feder and Hofmann 1999, Tomanek and Somero 1999, Tomanek and Somero 2000). These studies found that interspecific differences in thermal-tolerance were reflected by patterns of hsp expression.

The 70 kilodalton (kDa) family of hsp70 functions as molecular chaperones, playing an essential role in stress tolerance in many biological systems (Lindquist 1986, Sanders et al. 1991, Xue and Grossfeld 1993, Baler et al. 1996, Dilorio et al. 1996, Ellis 1996, Kohler et al. 1996, Feder and Hofmann 1999) and accounts for much of the translational activity in cells responding to environmental stress. The hsp 70 family shows consistent induction and conservation of reaction across species including mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas* and *C. virginica*) (Sanders 1990, 1993, Stegeman et al. 1992, Shamseldin et al. 1997, Clegg et al. 1998, Cruz-Rodriguez et al. 2000). Increased synthesis of hsp 70 has been shown to promote thermal-tolerance in many organisms including oysters (Parsell and Lindquist 1994, Shamseldin et al. 1997, Clegg et al. 1988). Hsp70 also played a protective role in pre-heat-treated mussels (*M.*

edulis) exposed to cadmium (Bradley 1998). The two oyster species (*C. gigas* and *C. virginica*) showed persistent elevated levels of hsp70 (>2 weeks) (Clegg et al. 1998, Shamseldin et al. 1997). Acquired thermal tolerance persisted up to 10-14 days in *C. gigas* (Shamseldin et al 1997, Clegg et al. 1998).

Regulation of Hsp 70 Expression

The persistence of increased hsp 70 expression could be due to repeated translation of hsp 70 mRNA or decreased metabolism (and therefore decreased protein turnover) following initial heat shock. The stability of hsp70 mRNA increases after heat shock, thus making repeated translation possible (Chang and Gellie 1997). Though the half –life of the hsp protein itself may only be several days (Hofmann 1999), the induced expression could recur when the organism is further subjected to heat stress. Increased endogenous levels of hsp 70 following heat shock may protect the organism from further heat stress (Parsell and Lindquist 1994). Additionally, increased levels of hsp 70 in the cell may regulate transcription of the hsp gene (Parsell and Lindquist 1994, Morimoto 1998).

Transcription of the hsp gene is initiated by binding of Heat Shock Factor One (HSF1) to the promoter region (heat shock element or HSE) of the hsp gene. Trimerization of HSF1 monomers is necessary for binding to the HSE. In the absence of stress, the hsp 70 protein is believed to complex with HSF1 monomers (along with hsp 90). As levels of misfolded or denatured proteins increase due to stress, hsp 70 proteins bind to the aberrant proteins releasing HSF1 monomers, which are free to trimerize and initiate transcription of the hsp gene (Morimoto 1993). Transcriptional regulation of hsp synthesis in this manner is known as the 'cellular thermometer' model of hsp gene

regulation (Hofmann 1999). Increased levels of hsp70 in the cell may increase the threshold of hsp gene activation by binding and decreasing levels of free HSF1 (Morimoto 1998). Shifts in the induction temperature of hsp genes may be one of the important mechanisms involved in thermal acclimation or seasonal acclimatization of organisms (Hofmann 1999).

Heat Shock Proteins and Parasitic Diseases

As previously mentioned, heat shock proteins are not only induced by elevated temperatures, but also by other environmental stressors, such as pollutants. Disease can be an important environmental stress (Harvell, et al. 1999). Therefore, parasitic disease is a potential stressor that may induce heat shock protein expression (Feder and Hofmann 1999). Levels of hsp70 increased in livers and kidneys of coho salmon when artificially infected with *Renibacterium salmonarum*, the agent responsible for bacterial kidney disease in salmonid fish (Forsyth et al. 1997). House martins parasitized by triatoniid bugs and trypanosomes had increased levels of hsp 60 in their blood (Merino et al. 1998). Levels of hsp70 were correlated with *P. marinus* infection in *C. virginica* at high salinity sites (Brown et al. 1993).

Heat shock proteins may also play a defensive role for the host during host-pathogen interactions (Garbe 1992). The nematode *Trichinella spiralis* induced elevated expression of Hsp 25 and 70 in rats (Martinez, et al. 1999). Heat shock proteins appeared to prevent hepatocyte injury by mediating host signal transduction and stabilizing cellular microfilaments (Martinez et al. 1999), protected macrophages infected with *Salmonella choleraesius* (Yoshikai 1998), and induced T-cell production of factors responsible for regulating inflammation in the immune response (Yoshikai 1998, Zugel and Kaufmann

1997, 1999b). These studies show that exposure to pathogens not only induces hsp expression, but elevated levels of hsps may serve as a protective mechanism against the effects of disease.

Conversely, induced hsp expression in a parasite may serve as a thermal-protective mechanism that allows it to exploit the host environment. An ectothermic parasite entering a mammalian or avian host encounters an extreme change in temperature. Increased hsp expression may be necessary to withstand the abrupt increase in temperature (Sing, et al. 1997). Also, hsps play an integral role in the life cycle of parasites during the transition from a free living stage or an ectothermic intermediate host to an endothermic host. *Trypanosoma brucei*, the parasite responsible for sleeping sickness, increases transcription of hsp 70 and hsp 83 genes as it leaves the tsetse fly for a mammalian host (Van der Ploeg et al. 1985). The hsps of microbial pathogens and parasites can also be antigenic targets of host immune response. Production of antibodies against parasite hsp70 was found in humans exposed to *Plasmodium falciparum* (Kumar, et al. 1990). *Perkinsus marinus* also expresses heat shock proteins. Molecular sizes and immunospecificity differs between *P. marinus* and the oyster (Tirard et al. 1995). The induction temperature for hsps in *P. marinus* is higher (46 °C) than the oyster (39-41 °C), possibly allowing normal function of *P. marinus* under thermal conditions that may be stressful to the host (Tirard et al. 1995).

Induced Thermal Tolerance and Hsp70 in Oysters

Thermal stress has caused mass mortalities during the summer in Pacific oyster (*C. gigas*) populations in Northern California. Recent studies on the West Coast demonstrate that exposure to elevated, but sublethal temperatures (37 °C) significantly

reduced the mortality of oysters exposed to a subsequent lethal heat treatment (43-44 °C) in two Pacific oyster populations (Shamseldin et al. 1997). Further, this induced thermal tolerance differed in two oyster populations. The Humboldt Bay population from California showed higher induced thermal-tolerance than oysters from Washington State, providing some evidence that intraspecific variation in thermal-tolerance existed between populations. The induced thermal-tolerance in oysters appeared to be associated with the enhanced expression of the heat shock proteins of the 70 kDa family (hsp 70). Clegg et al. (1998) reported enhanced production of two constitutive isoforms of the 70kDa family stress proteins (77 and 72 kDa) and induction (*de novo* synthesis) of a 69 kDa protein in the gills of Pacific oysters. In addition, the heat-treated oysters not only tolerated immediate transfer to a temperature 25 °C higher than their holding temperature, but also acquired a thermal tolerance that remained for at least two weeks (Clegg et al. 1998). Enhanced production of hsp70 has also been shown in eastern oysters. *Crassostrea virginica* produced hsps after heat shock similarly to *C. gigas*, although the degree of induced thermal tolerance was lower (Friedman et al. 1999). Using an antibody raised against human hsp70 (Affinity Bioreagent, MA3-006), Tirard et al. (1995) detected an increased synthesis of hsps in the 70 kDa family (constitutive isoforms, 71 and 70 kDa) for several days in the hemocytes of the Eastern oyster after a one hour heat treatment at 46 °C. Using the same antibody probe, our laboratory also found that a one hour heat shock at 37 °C enhanced the synthesis of two constitutive isoforms (69 and 71 kDa hsps, Cruz-Rodriguez et al. 2000) in the gill tissues of eastern oysters. No inducible isoform of hsp 70 kDa family was detected, however, in either our lab or the study by Tirard, et al. (1995). Also, a preliminary study in our laboratory showed that hsp70 expression

increased and persisted for at least two weeks. The different observations between *C. gigas* and *C. virginica* could be a result of the different antibodies used (Affinity BioReagent, MA3-001, Golden, CO) in each study or the differences in hsp response and induced thermal-tolerance between oyster populations and species. A direct relationship between thermal-tolerance and increased hsp70 expression in oysters and the role of hsp70 in disease resistance remains to be verified.

While summer mortalities in eastern oysters are most likely attributed to the advanced *P. marinus* infection in oysters, there is also the possibility that mortality is related to the temperature-associated depression of host defense mechanisms and physiological condition. Reduction of phagocytic activity in hemocytes occurred at ~25 °C (Chu and La Peyre 1993). Although Brown et al. (1993) found that hsp70 in eastern oysters correlates with *P. marinus* infection levels, environmental temperature and *P. marinus* intensity were also correlated. Thus, although a link between hsp70 levels and Dermo infection seems to exist, the functional significance of hsp70 in tolerating oyster disease remains to be demonstrated.

The role of heat shock proteins in enhancing thermal and stress tolerance in oysters is especially important with regards to pathogens as disease may decrease the ability of oysters to tolerate high temperatures. Acute elevation of temperature from 20 °C to 30 °C increased oxygen consumption and ammonia production in MSX-infected oysters compared to uninfected oysters (Littlewood and Ford 1990). Challenge with the bacterium *Nocardia,* a suspected co-factor in summer mortalities of Pacific oysters, reduced thermal tolerance in *C. gigas* (Cheney et al. 2000, Friedman et al. 1999). High summer temperatures, although usually below the critical thermal limit of oysters, may

still impose a thermal stress on oysters. Intertidal eastern oysters can experience temperatures from 46-49 °C and even up to 55 °C during low tide exposure (Galtsoff 1964, Wilson and Burnett 2000). This natural range of temperatures can denature proteins in the oyster, as has been found in intertidal populations of *Mytilus* species (Buckley et al. 2001, Hofmann and Somero 1995, Hofmann and Somero 1996). Within the range of their distribution, eastern oysters can experience aquatic temperatures up to 37 °C (Paynter and Dimichele 1990). Oysters grown at field sites for the current study were exposed to aquatic temperatures near 30 °C. Although eastern oysters can tolerate high temperatures, the interactive effect of parasitism by *P. marinus* or *H. nelsoni* with temperature could be intense, resulting in death.

There is some evidence of intraspecific variation in hsp70 and thermal tolerance in bivalves. Intraspecific variation in hsp70 was found in populations of *Mytilus edulis* and linked to their variable response to toxicants and thermal stress (Bradley et al. 1998, Tedengren et al. 1999). As indicated earlier, induced thermal tolerance differed in two *C. gigas* populations (Clegg et al. 1998). Therefore, to identify whether existing oyster populations vary in their inherent thermal-tolerance and whether there is an interaction with disease resistance, intraspecific variation in hsp expression among several oyster stocks will be examined. This study is the first attempt examining the role of induced and inherent thermal tolerance, and heat shock proteins in response to oyster disease.

The role of heat shock proteins in thermal tolerance in oysters has been studied in other oyster species, but interactions with disease have not been thoroughly examined. Estuaries are highly variable environments, in which oysters are particularly suited. Biochemical mechanisms such as hsp function likely play an important role in

acclimation and adaptation to this environment. Oyster diseases show significant interactions with temperature and salinity and may adversely affect mechanisms that allow the oyster to respond to changes in temperature or salinity. Enhanced thermal tolerance may contribute to survival in oysters in the face of disease, particularly in disease resistant oysters.

Research Objectives

The objective of this section of the dissertation is to evaluate the role of induced and inherent thermal-tolerance and associated hsp expression in conferring tolerance to disease and thermal stress in oysters. Seasonal and intraspecific variation in hsp70 expression between oyster stocks exhibiting variation in response to diseases (Dermo and MSX) was compared. The heat shock response was characterized and its contribution to thermal tolerance in oysters was also examined. Finally, the effects of *P. marinus* infection on induced thermal tolerance and hsp70 expression were characterized.

Dissertation description

This dissertation focuses on a problem endemic to both the Atlantic and Gulf coasts: oyster disease. The dissertation consists of three main studies: (1). A comparison of presumptive disease-resistant and disease-susceptible oyster stocks, (2) Comparison of seasonal variation in hsp70 among disease resistant and susceptible oyster strains and (3) Determination of the role of heat shock proteins (hsp70 family) and thermal tolerance in resistance to *Perkinsus marinus*. In Chapter II, growth and survival among oyster stocks presumed to possess natural resistance and susceptibility to Dermo disease were compared. Additionally, condition and energy reserves were compared among stocks to determine whether sublethal effects of disease vary along patterns of resistance. This may

determine whether resistant oysters maintain superior growth characteristics, which is important in determining the suitability of specific strains for culture. Identification of disease-resistant oyster populations is extremely important to restoring oysters in the Chesapeake Bay for commercial and restoration purposes. Understanding how oysters may combat the disease will aid in selective breeding and management efforts and contribute to understanding the nature of this host-pathogen relationship.

The interaction of disease and thermal stress may be intense, resulting in mortality in even disease resistant strains. Improved tolerance to thermal stress may be an important trait to improve survival in domesticated oysters. Heat shock proteins play an important role in acclimation and acclimatization to changes in the thermal environment. In chapter III, seasonal variation in heat shock proteins among disease resistant and susceptible oyster strains were described. Variation in heat shock protein levels may infer thermal tolerance capacities of these strains. Growth, survival, and *P. marinus* infections are also compared and correlated to Dermo resistance. In chapter IV, the role of heat shock proteins in conferring thermal tolerance to oysters and its interaction with disease resistance was tested. The duration of the heat shock response was characterized. The relationship between thermal tolerance and heat shock proteins was compared among oyster stocks. Thermal tolerance, heat shock proteins, and survival were compared between uninfected oysters and oysters experimentally infected with *P. marinus*. Mechanisms of thermal tolerance may have additive effects on improving disease resistance in eastern oysters. Chapter V discusses the implications of the results of chapters II through IV and directions for future research.

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Chapter 2: Performance of "natural Dermo-resistant" oyster stocks: Survival, disease, growth, condition and energy reserves.

(submitted to Journal of Shellfish Research)

Performance of "natural Dermo-resistant" oyster stocks: Survival, disease, growth, condition and energy reserves.

Short running title: Dermo resistance in oyster stocks.

Key words: *Crassostrea virginica*, *Perkinsus marinus*, Dermo, disease resistance, condition index, energy reserves, oyster

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(cover page for submission to Journal of Shellfish Research)

Note: References for chapters 2-5 are formatted according to the Journal of Shellfish Research

Abstract

To determine if natural populations of the eastern oyster possess resistance to *Perkinsus marinus*, progeny representing several oyster stocks from the Chesapeake Bay and the Gulf of Mexico were deployed at two sites within the Chesapeake Bay. Mortality, *P. marinus* infection (prevalence and intensity), shell height, condition index, and energy reserves (glycogen, protein, and lipid) were compared between these stocks. Oyster stocks from the Chesapeake Bay had higher intensities of Dermo infection than Louisiana stocks, with differences among individual stocks. Throughout the two-year study, a natural Dermo-resistant stock from Tangier Sound (CTS), was identified. Despite infection intensities approaching those of a susceptible Rappahannock River stock (CRB) and higher than a Gulf of Mexico stock (LOB), CTS consistently had lower mortality for the two-year grow out, and was comparable to a hatchery disease-resistant strain (XB). At a site (Port Kinsale) where only *P. marinus* occurs, the LOB stock had low mortality and significantly lower intensities of infection. Shell heights were highest overall in the CRB stock at another site (Regent Point), despite high susceptibility to disease. At Port Kinsale, the LOB stock grew to the largest shell heights, while maintaining the lowest infection levels, implying resistance to Dermo disease. Condition index varied between stocks, although not necessarily along trends of disease resistance since condition was highest in both the CRB and XB stocks. Variations in energy reserves were strongly influenced by season, but not disease, or stock origin. The present study shows that differences between stocks contain an underlying genetic component. Differences seen between deployed stocks in mortality, growth, and condition have

strong implications for development of selective criteria for an aquaculture-based industry.

Introduction

Diseases caused by two protozoan pathogens, *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX) have devastated oyster populations along the east coast of the USA, particularly the Chesapeake Bay region. Extensive annual mortalities have hampered efforts to enhance oyster populations for both commercial and restoration purposes. Efforts to circumvent the effects of disease have mostly focused on selective breeding to produce more disease-resistant strains, achieving the most success with MSX resistance (Haskin and Ford 1979, Ford and Haskin 1987, Ford 1988). Selection for resistance to Dermo, however, has been more difficult to achieve (Ford and Tripp 1996).

Dermo disease is chronic and disease-associated mortality does not typically occur until the second year of growth, or when oysters are at or near market size (75mm) (Andrews and Hewatt 1957, Andrews and Ray 1988). Individuals that grow beyond market size, and have survived more than two seasons of exposure to *P. marinus,* are thought to be resistant to Dermo. However, these resistant individuals are often continually removed from most populations during commercial harvests, thus preventing long term establishment of native resistant populations (Andrews and Ray 1988, Ray 1954, Kennedy 1996.). Some native populations, however, are suspected to possess inherent resistance or increased tolerance to *P. marinus* (Andrews 1954, Mackin and Sparks 1962).

Past field studies have noted differences in resistance of translocated oyster stocks (Andrews and Hewatt 1957). Lab experiments indicated varying resistance among eastern oyster populations from different regions, supportive of natural selection for Dermo resistance (Bushek and Allen 1996). Native populations naturally selected for

Dermo resistance could provide the basis for development of more resistant strains, but until recently very few of these populations have been identified in the Chesapeake Bay and none had been reported from the Gulf of Mexico (Ford and Tripp 1996).

In 1996, Virginia oystermen discovered substantial numbers of large eastern oysters (lengths >110mm), in Tangier Sound of the Chesapeake Bay (Chesapeake Bay Journal 1997). Similarly, populations from the Gulf of Mexico in Louisiana (Grande Terre and Oyster Bayou) were found that were characterized by predominantly large-sized individuals in areas of high Dermo prevalence (Dr. J. Supan, Louisiana State University, pers. comm.). Because these populations possessed large and presumably long-lived (≥ 2-3 years) individuals in an enzootic area for Dermo, it was presumed that these populations may possess some natural Dermo resistance (Dr. J. Wesson, Virginia Marine Resources Commission, pers. comm.).

The Dermo resistance of a particular oyster strain may be a function of its ability to withstand the pathogenic effects of *P. marinus*. Secretion of proteases by *P. marinus* may be a virulence factor in causing mortality (La Peyre et al. 1995, Oliver et al. 2000). Mortality associated with disease is due to depletion of the energetic reserves of the oyster (Choi et al. 1989), resulting in impaired physiological function and degraded condition. One of the systemic effects of diseases such as Dermo and MSX is a reduction in soft tissue growth, resulting in a decreased condition index and changes to the biochemical composition of the host (Stein and Mackin 1957, Crosby and Roberts 1990, Gauthier et al. 1990, Paynter and Burreson 1991, Barber et al 1988a, Barber et al. 1998b, Newell 1985, Ford et al 1988). Changes in condition and biochemical composition may indicate differences among individuals or groups that exhibit variable response to disease

stress. Few studies have examined the effects of infection by *P. marinus* on biochemical composition, particularly in relation to the progression of disease. Moreover, no study has compared condition and energy reserves among disease-resistant and susceptible oyster strains.

To determine if there are native Dermo resistant oyster populations we collected oysters from several Chesapeake Bay and Gulf of Mexico populations. We compared disease resistance in F_0 progeny from presumptive "natural Dermo-resistant " (NDR) and non-resistant populations deployed in Chesapeake Bay. We determined 1) whether some stocks showed natural resistance to disease and 2) whether alterations in static physiological indices reflect response to disease. To achieve these objectives, mortality, disease, growth, condition, and energy reserves among several presumptive Dermo-resistant oyster stocks were compared in a common-garden experiment at two Chesapeake Bay sites. Oysters were naturally exposed to *P. marinus* for two years (2000-2001) at these sites. Dermo resistance was defined in this study as lower prevalence or intensity of *P. marinus*, and lower disease-associated mortality (Valiulis 1973; Bushek and Allen 1996). Resistant populations may maintain lower prevalences and intensities of infections, in addition to improved survival. (Gaffney and Bushek 1996).

Materials and Methods

Stock and Site Selection

Oysters from representative populations from the Chesapeake Bay (Tangier Sound -CTS, lower Rappahannock River - CRB, Choptank River - CCR), the Gulf of Mexico (Louisiana region) (Grande Terre - LGT, Oyster Bayou - LOB, Hackberry Bay - LHB)

(Fig. 1A. and 1B.), and one disease-resistant hatchery strain (CROSBreed - XB, bred for MSX resistance 9 generations, Dermo resistance 4 generations) were spawned in the Virginia Institute of Marine Science (VIMS) oyster hatchery and reared from larval stage to settlement. Natural oyster stocks were selected for testing based upon the preponderance of large sized individuals within those populations, and designated NDR, or "natural Dermo resistant" stocks (CRB, CTS, LGT, LOB). Parents from these stocks were greater than market size (75 mm shell height) and many were larger than 100 mm. For spawning, only individuals >100mm were collected. Susceptible stocks (CCR and LHB) were characterized by a history of low exposure to *P. marinus*. The progenies from these seven stocks were transferred to mesh bags at 3-5 mm shell height and grown in Taylor floats (Luckenbach et al. 1997). Oyster stocks were placed in separate floats containing 800 individuals per float. Two replicate floats per stock were deployed at two sites (Regent Point Marina - Rappahannock River and Port Kinsale - Yeocomico River, see Fig. 1A.) within the Chesapeake Bay from September 1999 to December 2001. Salinity ranges for both sites were 10-16 ppt (Fig. 2). Sites were selected primarily because salinity ranges would be favorable for *P. marinus* exposure, but not *H. nelsoni*. Initial surveys of wild oysters at these sites showed that MSX was not present at either site. Ease of access and availability of space were also factors in the choice of these sites.

Field Sampling

Sampling was conducted every 4-5 weeks during each of two growing seasons from spring to late fall. These seasons coincide with the peak disease periods. Temperature and salinity were measured on each sampling day by thermometer and refractometer. Oysters from each float were counted to assess mortalities. Oysters from

each float were randomly sampled and returned to the lab to measure shell heights, dry weights, energy reserves, and infection levels of *P. marinus*. Ten oysters were sampled for shell heights, dry weights, and *P. marinus* infections. Five to 10 oysters/float were used for individual protein and glycogen measurements and 3 oysters/float were sampled for lipid analysis.

Analyses were performed on four stocks (CRB, CTS, LOB, and XB). This was based on variation in survival and Dermo infection of these stocks after one year of growth. As the study progressed, the CRB stock exhibited high mortalities and high *P. marinus* infection intensities at Regent Point in the first year (2000). We re-classified the CRB stock as disease-susceptible, despite it matching our initial criteria for an NDR stock. All Gulf oysters exhibited a similar pattern of mortality at Regent Point, therefore one stock (LOB) was selected to represent the Gulf of Mexico populations. Thus, the remaining stocks represented the range of characteristics (CRB disease-susceptible, Chesapeake NDR - CTS, Gulf NDR - LOB, XB) pertinent to our general hypothesis that natural resistance to Dermo exists in distinct oyster populations.

Mortality

Live and dead oysters were counted at each sampling. Data was expressed as percent cumulative mortality [(# dead oysters at time *t* (current period sampled) + # total dead over previous sampling(s)) ÷ (total # live oysters at t=0 - # removed for sampling)]. Cumulative mortalities were compared between each group at each site.

Shell Height

Shell height was measured from the anterior (shell hinge) to posterior (edge at the highest point) using vernier calipers. Shell heights were reported in mm.

Tissue Sampling

After shell height measurement, oysters were shucked, and tissues removed. Total wet weight of oyster tissue was recorded and whole tissue was divided in two fractions. To ensure equal organ representation between tissue fractions, tissues were sectioned in half along the left and right valve axes. One fraction was used to determine the intensity of infection by *P. marinus* and the other was used for analysis of energy reserves.

Diagnosis of *P. marinus* (Dermo) infection

Prevalence and intensity of *P. marinus* infection in experimental oysters were determined using total body burden assessment (Choi et al. 1989, Nickens et al. 2002). Oyster tissues were removed from the shell. The tissue fraction used for *P. marinus* diagnosis was homogenized in 10 ml of 0.2 M phosphate buffer and weighed. One ml of tissue slurry was added to 10 ml alternative fluid thioglycollate medium (AFTM, Sigma Biochemicals) containing the antibiotic chloramphenicol and the anti-fungal agent nystatin. The homogenate was reweighed after tissue removal to estimate the wet weight of tissue used to assess counts of *P. marinus*. The tissue aliquot added to AFTM was incubated in the dark at room temperature for 5-7 days. Tissues were then pelleted by centrifugation, resuspended in 2M NaOH and incubated overnight at 60 °C. Tissue pellets were washed 2-3 times with distilled water (dH_2O) and resuspended in 0.1 M phosphate buffer containing 0.2% sodium azide. Tissue suspensions were diluted if necessary, to facilitate counting and 100 µl aliquots were added to a 96 well plate. One to two drops of Lugol's solution (1:9 dilution with dH_2O) was added to each well, and *P. marinus* cells were counted using an inverted microscope. *Perkinsus* infection intensity was expressed as number (#) of *P. marinus* cells /g wet tissue weight (ww). Presence or

absence of *P. marinus* was used to calculate prevalence data and expressed as percentage (%) of infected oysters.

Diagnosis of *H. nelsoni* (MSX) infection

Periodically, oysters were examined histologically for *H. nelsoni* infection (Burreson et al. 1988). A transverse tissue section across gill, stomach, intestine, and digestive diverticula was removed from each oyster. Tissues were fixed in Davidson's solution, dehydrated, cleared, and embedded in paraffin. Sections 5 μm thick, were cut, mounted on glass slides and stained with iron hematoxylin and eosin. Intensities of infection were categorized as high, medium, or low

Dry weight and Condition index

Tissue fractions, except the 1.0 ml aliquot of tissue used to diagnose *P. marinus*, were freeze dried for 48 hrs and weighed. The relationship between wet weight and dry weight of the *P. marinus* tissue fraction was calculated so as to estimate dry weight in tissue slurries added to AFTM. Total dry weights of soft tissues were measured by adding all aforementioned dry weight fractions together. Shells were dried in an oven at 60 °C for 48 hours. Total dry weights and dry shell weights were used to calculate condition index (tissue dry weight/ dry shell weight *100) (Walne and Mann 1975, Lucas and Beninger 1985). This method is comparable to, but varies from the shell capacity method recommended by Crosby and Gale (1990), and is more amenable to rapid processing of numerous samples (Rainier and Mann 1992).

Energy Reserve Measurements

Glycogen Analysis

Freeze-dried tissues were analyzed for glycogen content using the anthrone reagent method (Van Handel 1965). Tissues were homogenized in phosphate buffer (50 mM sodium phosphate, 1 mM EDTA and 0.5 mM PMSF, pH 7.2), digested in boiling 30% potassium hydroxide and precipitated with 95% ethanol and saturated sodium sulfate. The glycogen precipitate was dried overnight at 60 °C. Anthrone reagent (0.15 % in 72% sulfuric acid) was added to the precipitate and incubated at 90 °C for 20 min and then cooled in ice. Positive reaction with the anthrone reagent produced a blue color, directly proportional to the amount of glycogen contained within the sample. Samples were added to a 96-well microplate and read on a microplate spectrophotometer at 620 nm. Commercially purified oyster glycogen (Sigma Biochemicals) was used to generate a standard curve for each set of microplates. Glycogen content was expressed as mg glycogen/g dry weight of tissue.

Total Protein Analysis

Total protein concentration was determined by a modified Lowry protocol (DC BioRad assay, Lowry et al., 1951). An aliquot of the homogenate prepared for glycogen analysis was removed and diluted 1:3 in 1.0 M NaOH. Diluted samples were boiled in water for 5 min and assayed for protein content on 96 well microplates at 690nm. Protein amounts were expressed in mg/g dry weight.

Lipid Analysis

Total lipids were extracted from freeze-dried oyster tissue (~50 mg dry weight) with chloroform: methanol: water (2:2:1) according to the procedure of Bligh and Dyer (1959). The extracted lipids were evaporated under N_2 at 40 °C and resuspended in chloroform to 10-12 mg/ml total lipid, capped under N_2 and stored at -20 °C until

analysis. Lipid contents and lipid class composition were analyzed by thin layer chromatography and flame ionization detection (TLC/FID) using an Iatroscan TH-10, MK-III analyzer (Iatron Laboratories, Japan) (Chu and Ozkizilick 1995). Briefly, silica coated glass rods (S-III chromarods, Iatron Laboratories Inc., Japan) were activated and cleaned by flame ionization on the Iatroscan. One µl of the lipid extract was then spotted on to each chromarod using a Hamilton syringe. Neutral (steryl esters, triacylglycerol, free fatty acids, and cholesterol) and polar (total phospholipid content only) lipid classes were separated on the chromarods after development in hexane:diethyl ether:formic acid (85:15:0.04). Following development, lipid contents and lipid class composition were quantified by FID on the Iatroscan. Operating conditions were 2000 ml/min air flow, 0.73 kg/cm^3 hydrogen pressure, and a scan speed of 3.1 mm/sec. Peak area integrations were performed using PeakSimple software (SRI Inc.). Peak areas corresponded to the amount of ionized lipid in each separated component. Lipid class concentrations were determined by comparison to a standard curve for each lipid class component. Lipid class standards were cholesteryl palmitate (steryl ester), triolein (triacylglycerol), oleic acid (free fatty acid), cholesterol, and phosphatidylcholine (phospholipid). Units were expressed in mg lipid/g dry tissue weight.

Statistics

Cumulative mortalities were compared by contingency table analysis (Zar 1996), using stock as row variables and live and dead numbers as column variables. Comparisons were controlled for site. Prevalence of *P. marinus* was also compared by contingency table analysis with stock as row variables and infected and uninfected as

column variables. Prevalence comparisons were controlled for by date and by site. For both contingency table analyses, stock replicates were pooled.

For infection intensities, shell height, condition index, and energy reserves, oyster stocks were compared by an unreplicated repeated-measures ANOVA (Underwood 1997) with stock and sampling period as main effects. Replicate floats were nested within stock as a within subjects random factor. Analyses were separated by year (2000 and 2001) and by site. Stock differences and monthly trends were mainly uniform across both sites. Cases where there were interactions between stock and site were described qualitatively. In several comparisons, month x rep (stock) interactions were significant, making interpretation difficult and precluding the application of this statistical design (Underwood 1997). In those cases, variables were compared separately by month with alpha (probability of Type I error) levels adjusted by Bonferonni correction (alpha (α) / # samplings: Year 2000 $\alpha = 0.05/6$, Year 2001 $\alpha = 0.05/5$) (Underwood 1997). Application of this procedure also allowed comparisons of stocks in months where the CRB stock died, resulting in an unbalanced design. Shell height, dry weight, condition index, and energy reserves (glycogen, lipid, and protein) were analyzed separately. All variables were transformed to meet assumptions of normality and homogeneity of variance when necessary. Pair-wise comparisons from ANOVA analyses were determined by Student Newman Keul's procedure. Statistical analyses were conducted using Statistical Analysis System, Version 8 (SAS Institute 1999).

Results

Temperature and Salinity

Temperatures were similar at both Port Kinsale and Regent Point, and salinities were generally, although slightly, higher at Regent Point (Fig. 2A. and 2B.). Seasonal variation in temperature was apparent with maximum temperatures in the summer months (28-30 °C) of both years and near freezing-to-freezing temperatures in January, 2001 (3 °C at Port Kinsale and 0 °C at Regent Point). Salinities ranged from 10-17 ppt. Differences in salinities between the sites were most apparent in September, 2000 and April, 2001. During these months salinity at Regent Point was 5-6 ppt higher than Port Kinsale. Mean salinity was ~2 ppt higher at Regent Point (14.4 ppt) than at Port Kinsale (12.5 ppt).

Mortality

Significant differences in cumulative mortalities were found among stocks at each site ($p < 0.0001$). High mortalities were seen in the CRB ($53.8 \pm 3.1\%$, n=2) and LOB ($45.1 \pm 1.0\%$, n=2) stocks by November, 2000 (Fig. 3). The LOB stock and all other Gulf oysters deployed at Regent Point experienced significant mortalities in September, 2000 (LOB - $33.5 \pm 2.2\%$). Overall, patterns of cumulative mortality were consistent at both Regent Point and Kinsale. Mortality was highest in the CRB group with 100% mortality at Regent Point by July 2001 and $82.8 \pm 1.4\%$ at Kinsale. Both the CTS and XB stocks showed lower (average of both sites: 46.6% and 52.6%, respectively) cumulative mortalities. Mortalities were higher at Regent Point (CTS - 72.4%, XB-75.0%) than Port Kinsale (CTS - 20.2%, XB - 31.0%). Cumulative mortalities for the LOB stock (47.4%) were intermediate between the CRB and XB/CTS groups at Port Kinsale. High

mortalities in the LOB stock at Port Kinsale, however, were observed in the March, 2001 sampling. Mortality of LOB oysters reached 100% at Regent Point, by the end of the study.

Perkinsus marinus infections

Prevalence

Perkinsus marinus infections were detected at Regent Point in June of 2000 and at Port Kinsale in August, 2000 (Fig. 4A.). Infections progressed more rapidly at Regent Point, resulting in higher prevalences compared to Port Kinsale. Prevalence at Kinsale was lower (8.8 ± 3.0%) in May, 2001 compared to Regent Point (70.0 ± 10.0%), suggesting that infections may have cleared in oysters at Port Kinsale during the overwintering period. The prevalence remained high at Regent Point during May 2001, indicating that oysters were exposed to *P. marinus* earlier and at higher levels than at Port Kinsale. Significant variation in prevalence between stocks occurred from 2000 to June, 2001 (Fig. 4B and 4C). Within each month, prevalence fluctuated between stocks. Despite inter-stock variation in prevalence, across months and sites there remained a significant effect of stock on prevalence ($p=0.0002$). Prevalence was lower in LOB and CTS stocks (70.0% and 74.8%), while prevalences in CRB and XB stocks were higher (88.8% and 81.9% respectively). By July, 2001, prevalences were at or near 100% for all stocks through the remaining sampling periods.

Infection intensities

Stocks were compared for differences in intensities of infection (# of *P. marinus* cells/g ww) by month for each site. In 2000, *Perkinsus* infections varied significantly by month ($p<0.0001$) at both sites. At Port Kinsale, intensities showed no significant effects

between stocks. Differences between stocks were most apparent at Regent Point. Mean intensities for all stocks were $8.4 \times 10^6 \pm 8.3 \times 10^6$ cells/g ww at Regent Point and $1.2 \times 10^3 \pm 1.0 \times 10^3$ cells/g ww at Port Kinsale. Because of significant interactions between replicates and months at Regent Point, data were analyzed separately by month. Nevertheless, differences in infection between stocks were apparent and significant starting in June, 2000 ($p<0.01$), as overall mean infection levels were low (87.0 ± 0.6 cells/g ww) and fluctuated from June to August 2000. By November 2000, infections at Regent Point were highest in the CRB stock ($1.8 \times 10^7 \pm 2.1$ cells/g ww) ($p<0.01$). The CTS and LOB stocks had intermediate mean intensities and the XB oysters had the lowest intensities of *P. marinus* (Fig. 5). At both sites and across all stocks, *P. marinus* infections increased over time and were highest at Regent Point. Intensity decreased from Nov, 2000 to May, 2001.

From May to October, 2001, intensities increased (Fig. 5) coincident with increased mortalities (Fig. 3). Significant month by stock interactions reflected high variability in infections among replicate floats at both Kinsale and Regent. Despite this, there were significant differences in infection levels among stocks. Consistent with the previous year, the CRB stock had the highest infections, and was significantly higher than the other stocks at Regent Point, before reaching 100% mortality by May-July, 2001 (Fig. 3A.). At Port Kinsale, LOB infections remained below 1.0×10^6 cells/g ww throughout October, with maximum infections reaching 5.0×10^5 cells/g ww. Infection levels in the LOB stock were significantly lower at both Kinsale and Regent. Overall, infections at Regent were higher than at Kinsale and mortality increased as infections

reached or exceeded 1.0 x10^6 cells/g ww at both sites. By October, 2001 all oysters at Regent Point had infections above 1.0 x10^7 cells/g ww.

MSX infections

At Regent Point, oysters examined between July and November, 2000 showed presence of *H. nelsoni*. At Regent Point in 2001, MSX was detected in 48/124 animals examined in May, July and October. No significant differences between stocks were observed, but MSX was not detected in any XB oysters examined. *Haplosporidium nelsoni* was not detected at Port Kinsale in 129 animals examined.

Shell Heights

In 2000, shell heights increased over time and were significantly different between stocks ($p<0.0001$). Comparisons between stocks showed that CRB grew significantly more than CTS, LOB, and XB stocks at both sites (Fig. 6). At Port Kinsale, the CTS stocks had significantly lower shell heights than the other three stocks, while the LOB and XB stocks were not significantly different. Shell heights were larger at Regent Point than at Port Kinsale, while shell height increased over time at both sites. Interactive effects were significant as well, but as with *P. marinus* infections, overall trends in shell height across stock, site, and month were consistent.

In 2001, increases in shell height over time and among stocks were significant at Port Kinsale ($p<0.0001$). At Port Kinsale, the CRB and LOB stocks grew to the largest average shell heights. Final shell heights measured in October, 2001 were CRB: 88.1 ± 0.2 mm, LOB: 95.8 ± 1.4 mm (mean ± standard error of the mean, or s.e.m., n=20 oysters from each stock). The CTS and XB shell heights were lower (76.4 ± 0.5 mm and 79.3 ± 0.7 mm). From May to July, shell heights at Regent Point were significantly different

between stocks ($p<0.0001$). At Regent Point the CRB stock grew to the largest sizes but reached 100% mortality by July 2001. Shell heights at Regent Point were similar among the CTS, LOB, and XB stocks (73.9± 0.8, 74.3 ± 1.3, 76.8 ± 2.7 mm respectively) from May to July. During September to October, XB oysters grew to significantly greater sizes ($p=0.0053$ for main effects of stock only) than the LOB group. The LOB shell heights were the lowest at Regent Point, but highest at Port Kinsale, indicating an interactive effect of site on this stock. Within the other stocks, shell heights were greater at Regent Point than at Port Kinsale.

Dry weights

Dry weights varied significantly among stocks and months at both sites ($p<0.0001$) in 2000. Dry weight differences among stocks reflected differences in shell height (CRB>LOB, XB> CTS). Increases in dry weight were highest from September to November sampling dates, particularly in the CTS, LOB and XB stocks (Fig.7). Differences in dry weight among stocks showed consistent trends from month to month, although stock x month effects were significant ($p<0.0001$). At Regent Point, the CTS stock grew rapidly from September to November and had the highest dry weights compared to the other stocks. At both sites, growth was greatest during the fall with dry weights increasing from September to November by 1.5 and 2.0 times at Regent Point and Port Kinsale.

Tissue dry weights in 2001 were characterized by a sharp decrease in June from peak dry weights in May (Fig. 7), and no longer reflected changes in shell height. Significant differences between stocks were seen at both sites. At Port Kinsale, dry weights were highest in the LOB stock. At Regent Point, differences among stocks were

similar to 2000, with the CRB stock having the highest dry weights from May to July, although the CRB stock was not statistically significant from the LOB and XB stocks. The CTS stocks had the lowest dry weights in the fall. As in the previous year, dry weights were higher at Regent Point than at Port Kinsale. This was uniform across all stocks except for the LOB stock, which had lower dry weights at Regent Point than at Port Kinsale.

Condition Index

In 2000, condition index (CI) varied significantly between stocks and months ($p<0.0001$), and reflected changes in dry weight. Condition index decreased from May to July and increased in all stocks at both sites from September to November (Fig. 8). Condition index increased from the fall to winter, coinciding with an accumulation of glycogen and TAG (see following sections).

In 2001 the highest condition index was during the May sampling with decreases in the subsequent month. Variation among stocks in condition index was consistent with the previous year, the XB strain maintained the highest condition over the other stocks at both sites ($p<0.0001$). At Regent Point, changes in LOB condition reflected changes in shell height and dry weight. Prior to 100% mortality, CRB oysters also had a significantly higher condition index than CTS and LOB stocks at both sites.

Glycogen

Glycogen content varied seasonally with maximum values seen during the winter-spring periods (Fig. 9). Decreases in glycogen occurred during the summer months. Patterns in glycogen content were consistent across both sites. No significant differences were noted.

Protein

Protein content did not vary by stock but seasonal variation was seen with minimum levels observed in the spring (Fig. 10). Changes in weight-specific protein amounts (mg/g dry weight) decreased during periods of increased glycogen.

Lipid

Lipid class composition (steryl esters, triacylglycerol, free fatty acids, cholesterol, and phospholipids) was compared among oyster stocks in August and November, 2000 and May-July and September-October, 2001 (Fig. 11). Significant differences were found in triacylglycerol (TAG) content due to month and stock. Interaction between month and stock were significant at Port Kinsale, but not at Regent Point. Seasonal variation in TAG was most apparent at Kinsale, with an increase in TAG in all stocks except the CRB stock, between November 2000 and May 2001 (Fig 11A). At both sites TAG decreased in June and July 2001 (Fig 11A and 11B). TAG levels were highest in LOB and CRB stocks at both sites.

Discussion

Natural Dermo resistance

Our results showed that two native stocks (CTS and LOB) possess resistance to Dermo disease, comparable to that of a hatchery strain (XB) selected for resistance to both Dermo and MSX. Performance of these two stocks verified the assumption that individuals from these populations were Dermo-resistant, and that resistance is genetically based. LOB oysters at Kinsale, grew beyond market size (>100 mm), had lower levels of infection and lower mortality rates during the period of *P. marinus* exposure, providing evidence for Dermo resistance in this stock. Although LOB

cumulative mortality was higher than the CTS or XB, at Kinsale most of that mortality was attributed to deaths that occurred just prior to the March, 2001 sampling. This mortality event was believed to be caused by exposure to freezing temperatures during extreme low tides that occurred during the weeks before sampling. These mortalities only occurred in the exposed floats that contained Louisiana oysters. Chesapeake and XB floats that were similarly exposed did not contain noticeable mortalities. After this mortality event, monthly mortality rates were low in the LOB stock, and lower than all other groups.

Dermo disease is generally characterized as causing mortality in oysters in their second year of growth, as they approach or attain market size and accumulate *P. marinus* to a critical infection level (Burreson 1991, Paynter and Burreson 1991), generally at 10^6 cells/g ww (Choi et al. 1989, Bushek et al. 1994). The CTS and XB oysters were at or near market size (75 mm shell height) and had infections exceeding 1.0×10^6 cells/g ww, when mortalities began to increase. Cumulative mortalities in the CTS and XB groups, however, remained lower than the CRB stock, despite comparable infection levels. While mortalities did increase rapidly in those stocks at 1.0×10^6 cells/g ww, mortality was delayed, implying an ability to resist infections for longer periods. Delayed mortality in disease-resistant oysters was also demonstrated in strains of *O. edulis* exposed to *Bonamia ostreae*, a protistan parasite that causes mortality after chronic infection, a similar characteristic of Dermo disease (Naciri-Graven et al. 1998). Stock differences in survival, growth and condition were consistent between grow-out sites, further suggesting a genetic basis for their differences.

Previous studies have shown that disease resistance among eastern oysters contains a genetic component (Haskin and Ford 1979, Burreson 1991, Bushek and Allen 1996). Bushek and Allen (1996) found that Gulf of Mexico oysters were uniformly resistant to a range of geographic strains of *P. marinus*, but these relationships were not tested in the field. Field comparisons have shown variation in survival between groups of oysters (Andrews and Hewatt 1957, Burreson 1991, Ragone-Calvo et al. 2003) exposed to Dermo. Those studies differed from the present study in several ways. Andrews and Hewatt (1957) compared mortalities in translocated oysters, rather than stocks spawned and reared under identical conditions. Burreson (1991) examined differences between native stocks similarly to our study, but primarily focused on comparisons with MSX resistant strains and response to both Dermo and MSX. Further, stocks were deployed in disease enzootic sites at different ages, making stock comparisons difficult.

Ragone-Calvo et al. (2003) examined a Delaware Bay strain (DEBY) that exhibited improved resistance to both Dermo and MSX over successive generations. Comparisons with native stocks (F_0 Tangier Sound and Mobjack Bay) showed superior survival in the DEBY strain. The F_0 Tangier Sound stock used in their comparisons experienced high mortalities (~80%) in high salinity environments (15-25 ppt during periods of high Dermo prevalence). In contrast, the Tangier Sound stock used in our study survived better than a XB strain bred for multiple generations (9 MSX, 4 Dermo) for disease resistance in two moderate salinity environments (8-15 ppt). Clearly, the Tangier Sound population requires further examination, particularly with regards to genotype-environment interactions. However, comparisons of the F_0 CTS and CRB

oysters indeed demonstrate intra-regional variability in Dermo resistance between Chesapeake Bay stocks.

Site variation in disease dynamics

Site differences in *P. marinus* prevalence and intensity, although not directly compared, were apparent. Regent Point was characterized as a site of higher disease exposure, as prevalence and intensity of *P. marinus* occurred earlier and at higher levels than at Kinsale. High prevalence and intensity of Dermo at the end of 2000 at Regent Point may have affected the number of overwintering infections and subsequent infection rates in 2001. Overwintering infections generally decrease with decreasing temperature and salinity (Ragone-Calvo and Burreson 1994). Evidence of a winter decline in prevalence was seen at Port Kinsale, but not at Regent Point, where prevalences remained high (70%) during the spring. Salinity was lower at Port Kinsale (8-12 ppt) than at Regent Point (15 ppt) during the early spring (March and April, 2001), and may have contributed to differences in prevalence. The high prevalence at Regent Point suggests that individuals retained high infections over the winter to initiate transmission of *P. marinus* when environmental conditions were favorable for development of the parasite. At Port Kinsale, prevalences and intensities of *P. marinus* infections were lower than at Regent Point in the spring, suggesting a lower number of overwintering infections. However, frequency distribution of infection intensities from May, 2001 indicates that there were individuals with high infection intensities at Port Kinsale (Fig. 12). Such individuals may be responsible for initiating an epizootic, even when mean infection levels are low (Ford et al. 1999).

The presence of MSX disease also may explain differences in mortality between sites. MSX was initially detected in September 2000 at Regent Point and in the summer months of 2001, but was not detected at Port Kinsale throughout the study. Overall cumulative mortalities were higher at Regent Point than at Port Kinsale. Mortalities in the LOB stock were higher at Regent Point, and similar to the CRB stock. MSX does not occur in the Gulf of Mexico, and so the LOB stock would have had no resistance to *H. nelsoni*. Response to selection for resistance to *H. nelsoni* is apparently high, however, and can be attained in 1-2 generations (Ford and Haskin 1987). It would be expected that MSX resistance could be readily accomplished in Louisiana stocks through selective breeding. Crossing with known MSX resistant strains would probably also render dual disease-resistant strains.

Growth

In addition to increased survival, it was expected that disease-resistant stocks would exhibit improved growth since they would be better able to withstand the chronic effects of disease. Oyster strains that were MSX-resistant displayed an energetic advantage over susceptible strains (Barber et al. 1991a). The CRB stock, although highly susceptible to disease, reached market size (75 mm shell height) earlier than the other stocks. At Regent Point the CRB oysters reached market size in August, 2000 at Regent Point and in November, 2000 at Port Kinsale, before infection intensities became lethal. Comparisons of time to market size and cumulative mortalities at market size show that the CRB stock reached market size the fastest, and had the lowest mortalities at the time market size was attained (Fig.13A and 13B). The rapid growth of the CRB stock indicates that this stock may be a useful aquaculture strain, particularly in areas where

MSX intensities are low or absent. Fast growing oyster strains can be used to avoid the effects of Dermo disease, even in strains with no developed resistance to *P. marinus* (Allen 1993). Disease resistance in this stock may also be improved through further selection, as has been the case with some MSX-resistant strains, which now grow faster than unselected strains (Mathiessen et al. 1990, Ragone-Calvo et al. 2003).

Dry weights declined in June, 2001 while shell heights continued to increase, making growth comparisons difficult. In the present study we used shell height and dry weight as indices of overall growth. From a practical perspective, shell height is the most convenient method to compare performance among oyster strains. Determination of market size is based on shell height, and so remains an important metric to oyster culturists. Additionally, despite the seasonal decrease in dry weight, log mean dry weights among all stocks correlated strongly with log mean shell heights at both sites from deployment to the end of the experiment (Fig. 14).

Condition index

Demonstration of sublethal effects of disease on condition and energy reserves that can be ascribed to differences in disease resistance, remain equivocal. Differences in condition did not reflect patterns of survival (and presumably disease resistance), as condition index was highest in both resistant (XB) and susceptible (CRB) stocks (Fig 8). In previous studies, condition index was reduced by *P. marinus* infection (Craig et al. 1989, Crosby and Roberts 1990, Paynter and Burreson 1991). However, high site-specific variation, attributed to differences in salinity, made it difficult to distinguish among several Gulf coast sites (Craig et al. 1989). Although statistically significant, intensity of infection explained less than 10% of the variablility in condition index in South Carolina

oyster populations (Crosby and Roberts 1990). Paynter and Burreson (1991) compared the condition index between infected and uninfected oysters, not the relationship between intensity of infection and condition index. In their study, condition indices remained high during the months of increasing Dermo infections, in contrast to the present study.

In the present study, environmental influences (e.g. changes in temperature and food availability) on condition were likely greater than effects of disease. Seasonal variation in oyster condition exhibits remarkable inter-annual and intra-river consistency in the Chesapeake Bay (Austin et al. 1993). This may be related to consistency in the timing of phytoplankton blooms and food availability (Deslous-Paoli and Heral 1988). Because seasonal variation in condition index can be consistent from year to year, identifying effects of disease on condition may be difficult or unique to specific regions within the Chesapeake Bay.

Sublethal effects of Dermo disease may be important at critical periods of the eastern oyster's reproductive cycle. Condition and gonadal indices decreased with infection during periods prior to and during gametogenesis (Dittman et al. 2001). These relationships may be more of a reflection of prior exposure to *Perkinsus*, as negative effects on condition were only seen during periods when the parasite would be expected to be quiescent (winter to early spring). Their study implies that increased parasite loads hinder the ability of the oyster to undergo reproductive maturation. In the present study, effects of disease on condition may be more relevant during similar periods (late fall-winter and early spring), when condition index is high. During periods of low condition (summer, presumably post spawning) effects are not discernible because seasonal effects on condition mask any effects of disease. Spawning periods in the Virginia portion of the

Chesapeake Bay are typically from June to September, with two spawning periods in the summer and fall (Andrews 1979, Hayes and Menzel 1981). A critical period may be during maturation prior to the second spawning in the fall, when oysters are still heavily infected. During this period, increases in dry weight were seen in the XB and LOB stocks at Kinsale, implying that these stocks were able to recover from the dual stresses of spawning and disease.

Despite the difficulty in detecting effects of *P. marinus* on physiological condition, the observation that condition index varies among stocks grown under a common environment has important implications for aquaculture. Condition index is used as an indicator of meat quality (Lucas and Beninger 1985). Indicators of condition may be important criteria, along with growth and survival, in choosing a suitable strain for grow out. Stocks showed significant differences in condition. These differences were most significant at specific months. If these differences are consistent over successive growth seasons, then months when condition is highest may be targeted as optimal harvest periods, a typical practice of oyster growers (Brown and Hartwick 1988). Consistent differences in condition among stocks or strains may be useful indicators of performance and criteria for selective breeding. Growth and reproductive patterns of genetically distinct eastern oyster strains can remain fixed over multiple generations (Loosanoff 1969, Barber et al. 1991b, Dittman et al. 1998), improving the predictability of optimizing strain selection. Condition index alone, however, may not be solely indicative of meat quality. In the first year of growth, condition index of all stocks decreased during the summer and was attributed to increases in shell weight, as dry weights continued to increase during this period. At Kinsale, condition index was

comparatively low in the LOB stock, but their dry weights were highest among all stocks. It was more likely that changes in condition index of the LOB stock reflected differences in shell weight, as all Louisiana oysters grown at Kinsale developed noticeably thicker shells.

Energy reserves

Glycogen and TAG values decreased during the summer months in a similar manner to the condition index, suggesting mobilization of nutrient reserves during gametogenesis and spawning (Engle 1951, Trider and Castell 1980). Seasonal influences on energy reserves may have masked effects of disease. Infection by *H. nelsoni* reduced condition index and energy reserves in *C. virginica* (Barber et al. 1988a, Barber et al. 1988b). In earlier studies, effects of *P. marinus* on biochemical composition were variable. Stein and Mackin (1957) showed in histochemical assays that glycogen was depleted in infected oysters. Glycogen, however, was higher in infected oysters than in uninfected oysters in studies by Wilson et al. (1988) and White et al. (1988). In these studies, oysters were also parasitized by the snail, *Boonea impressa,* so glycogen levels may have been affected by this parasite, as well. In Galveston Bay, glycogen concentrations were negatively correlated with infections that were greater than light (Soniat et al. 1989). Glycogen, however, also decreased with salinity, so environmental effects on glycogen could not be ruled out. Increases in lipid phosphate and fatty acids were observed in *P. marinus* infected oysters (Wilson et al. 1988), but no other recent studies, besides this one, have examined effects of Dermo disease on lipid content of the oyster. In the present study, effects of *P. marinus* on TAG were not apparent. As with glycogen, seasonal variation in TAG may make it difficult to detect any effects *P.*

marinus might have on lipid stores. Individual variation in both TAG and glycogen was also high. *Perkinsus marinus* was demonstrated to reduce reproductive output and gametogenic development (Kennedy et al. 1995, Dittman et al. 2001). Demonstration of other physiological effects has been less clear. Oxygen consumption in oysters was not reduced by Dermo infection (Newell 1994, Willson and Burnett 2000). Seasonal cycles in energy reserves strongly influence the physiological state of both disease resistant and susceptible oysters and must be taken into account when examining effects of disease in the field. As shown by Dittman et al. (2001), specific periods related to gametogenic phases of the oyster may be when effects of disease are most crucial. Processes of nutrient assimilation and storage must be examined in the context of these periods to determine the physiological effects of Dermo disease.

Conclusions

In conclusion, we have identified native stocks possessing resistance to *P. marinus*. This study is the first field test demonstrating variation in Dermo-resistance of native stocks within (Chesapeake Bay – CRB and CTS) and between (Gulf of Mexico vs. Chesapeake Bay) regions. The identification of Louisiana stocks with low Dermo infection and mortality, and the CTS stock as Dermo-resistant is an important step in developing disease–resistant hatchery strains. Based on these findings, the VIMS Aquaculture Genetics and Breeding Technology Center crossed Louisiana oysters with the Delaware Bay (DEBY) hatchery strain in 2001 with the intent of adding increased Dermo resistance to a stock that already has proven MSX resistance. Such a stock would be of great importance in the burgeoning oyster aquaculture industry in the mid-Atlantic region where both diseases can cause considerable mortality. Should a correlation

between specific defense activities and disease resistance be established, it could further accelerate efforts in these breeding programs. The development of disease-resistant strains is a paramount objective in the Chesapeake Bay, for developing commercial aquaculture, and for providing seed for oyster reef restoration. In addition to survival and disease resistance, performance related traits such as growth and condition must be considered when selecting suitable strains for aquaculture since parameters related to disease resistance and performance are not necessarily correlated.

Acknowledgements

This research was supported by the NOAA-Sea Grant Oyster Disease Research Program (Project # VA-OD-99-3). We thank personnel at the VIMS-ABC hatchery for assistance in broodstock conditioning, spawning, larval care, and nursery of oysters. We also thank members of the Chu and Allen labs for assistance in field and lab sampling. We thank members of Dr. Eugene M. Burreson's lab for assistance with MSX diagnosis. Special thanks are given to Drs. Jeff Shields, Sandy Shumway and Bob Diaz for constructive reviews of earlier drafts of this manuscript.

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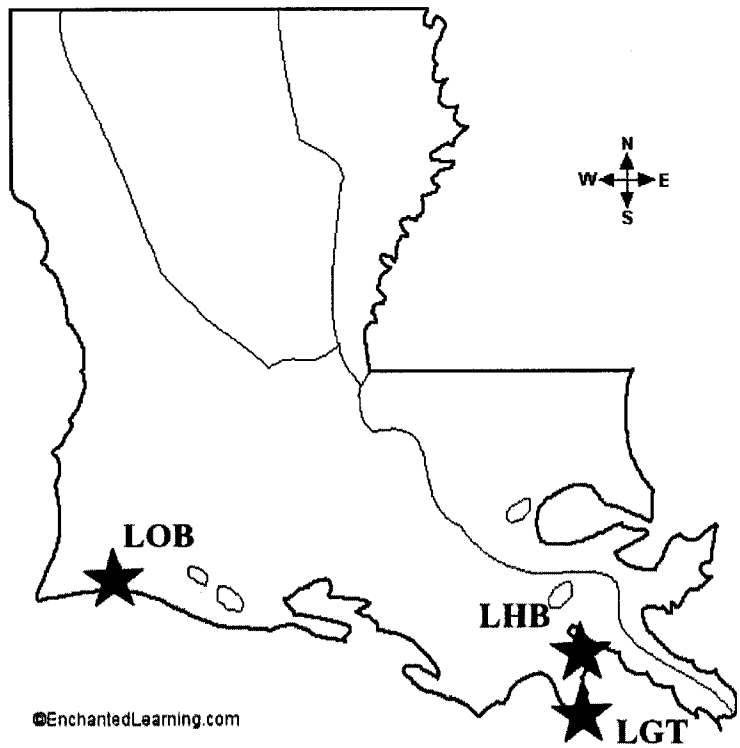
Figure 1 (A.) Chesapeake Bay and (B.) Louisiana maps showing locations of natural oyster stocks. Location of oyster stocks are indicated by a star (★). Stock acronyms are CCR – Chesapeake Bay Choptank River, CRB – Chesapeake Bay Rappahannock River, CTS- Chesapeake Bay Tangier Sound, LGT- Louisiana Grande Terre, LHB- Louisiana Hackberry Bay, LOB- Louisiana Oyster Bayou. Open circles (O) indicate sites where oyster stocks were grown (Port Kinsale – Yeocomico River, Regent Point – Rappahannock River)

A.



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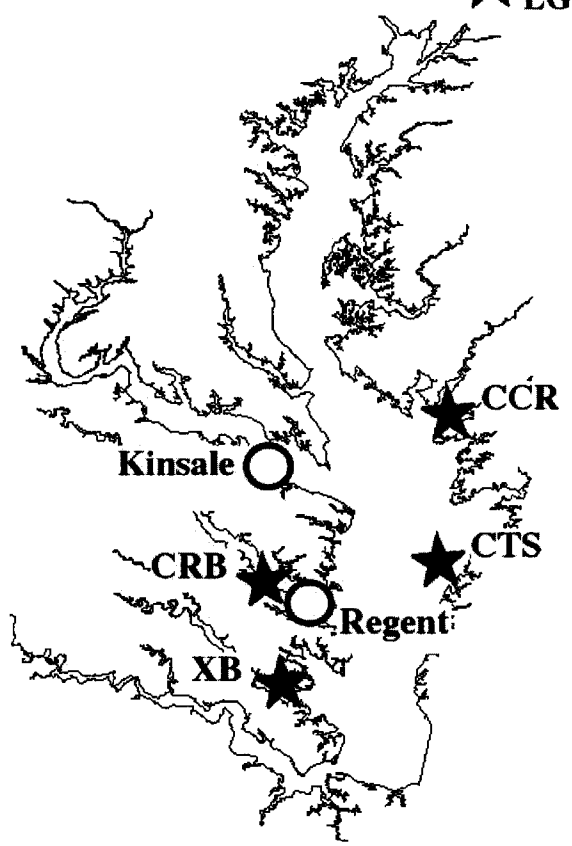
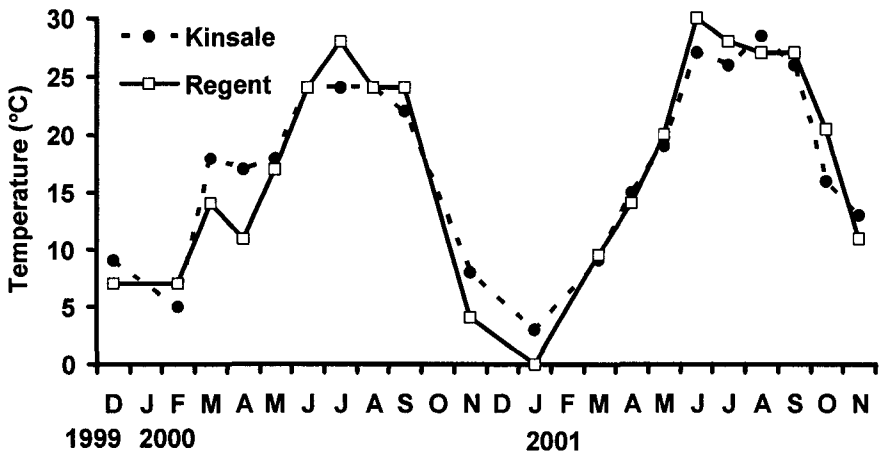


Figure 2. (A.) Water temperatures (°C) and (B.) salinities (parts per thousand, or ppt) at two sites in the Chesapeake Bay: Port Kinsale (Yeocomico River) and Regent Point (Rappahannock River) from 1999-2001.

A.



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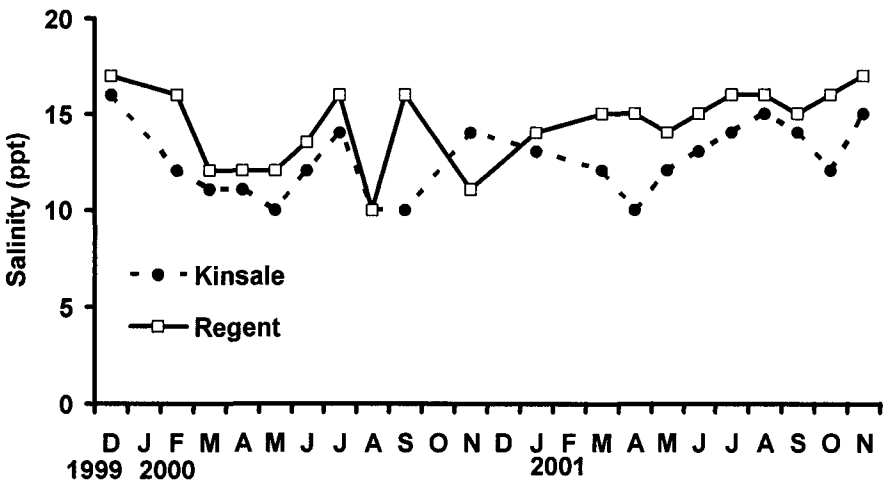
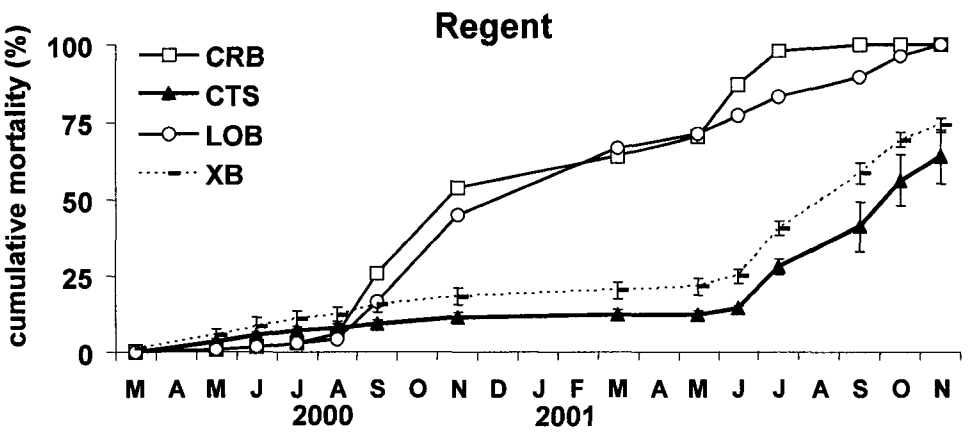


Figure 3. Cumulative mortality (%) data of three native oyster stocks and one hatchery disease-resistant strain at (A.) Port Kinsale and (B.) Regent Point. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain. Data was collected every 4-5 weeks in two seasonal periods (May-November 2000 and May-November 2001). Sampling is not continuous between November 2000 and May 2001. Data are presented as mean percentage (%) mortality ± standard

A.



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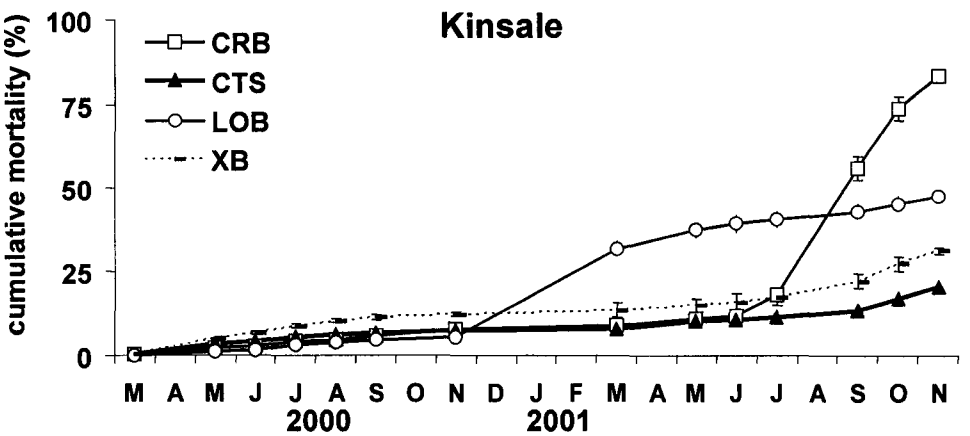
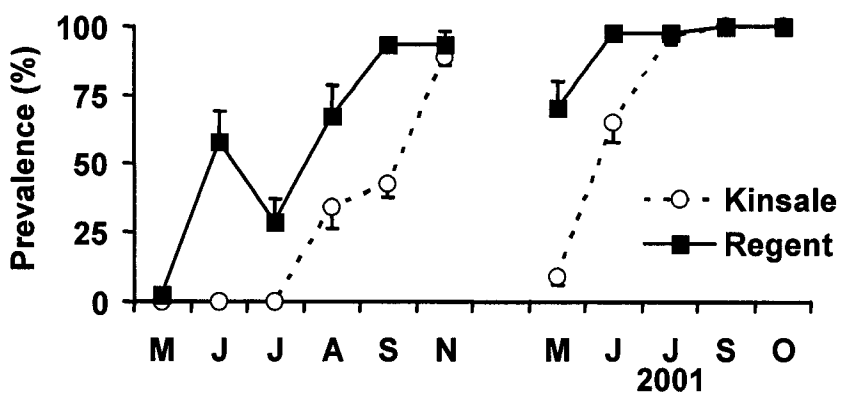


Figure 4. Prevalences of *Perkinsus marinus* at Port Kinsale and Regent Point from 2000-2001. Prevalence is percentage (%) of infected oysters. Data presented in the top figure (A.) are mean prevalence ± s.e.m. (n=4 oyster stocks (20 oysters/stock) for each site).Prevalences of individual stocks (CRB, CTS, LOB and XB) at each site (B. and C.) are shown separately. Prevalences of each stock are collapsed across two replicate floats/stock. At Regent Point, prevalence data for the CRB stock from September to October 2001 is absent due to 100% mortality in that stock. Note that data are presented for each month sampled. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

A.



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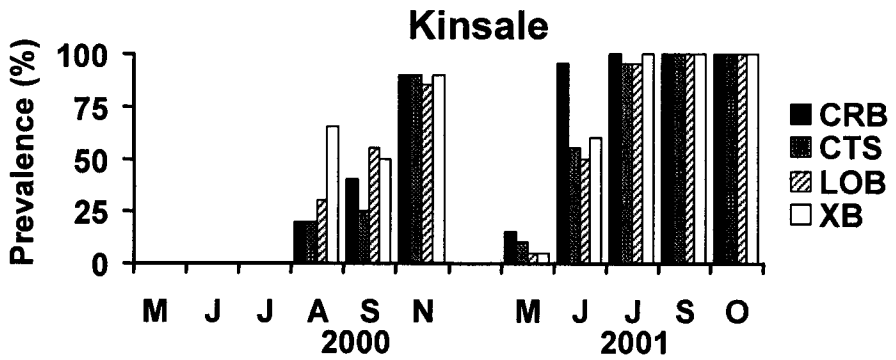

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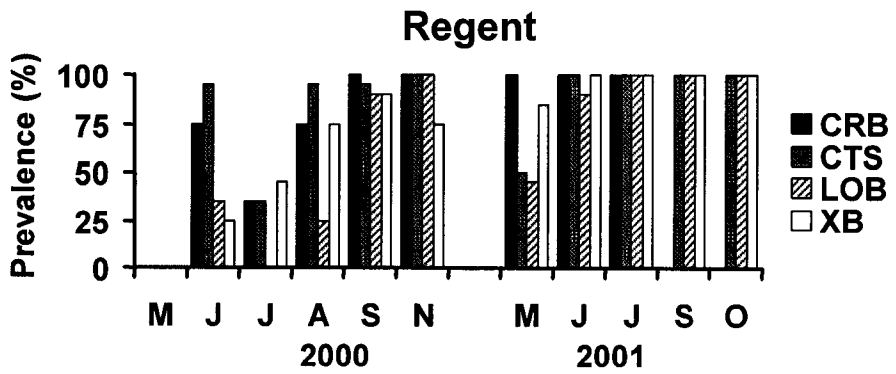


Figure 5. Infection intensities of CRB, CTS, LOB and XB oysters at (A.) Port Kinsale and (B.) Regent Point from 2000-2001. Data are presented as mean number (#) of *P. marinus* cells (enlarged hypnospores)/g wet weight (ww) ± s.e.m. (n=2 replicates of 10 animals for each stock). Numbers of *P. marinus* cells/g ww are graphed on a logarithmic scale. Note break in sampling periods between November 2000 and May 2001. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

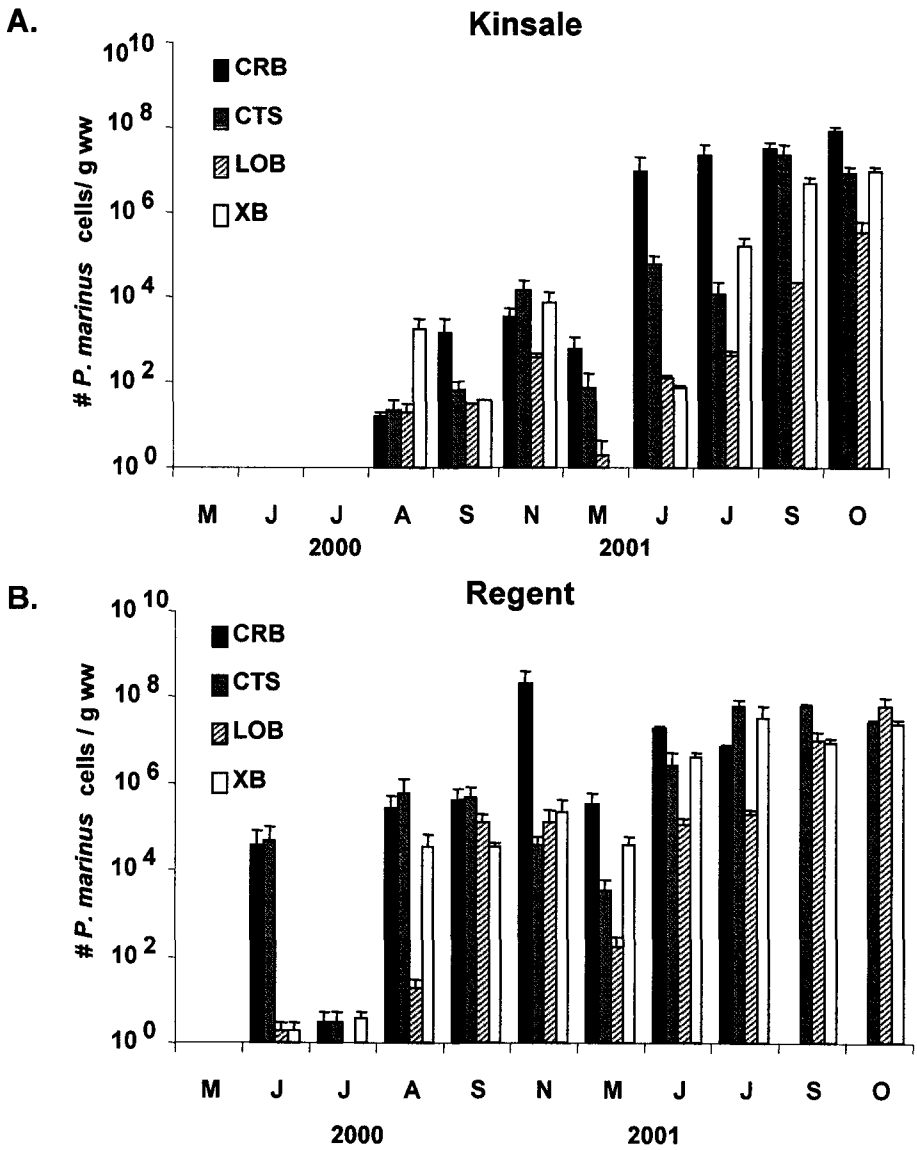
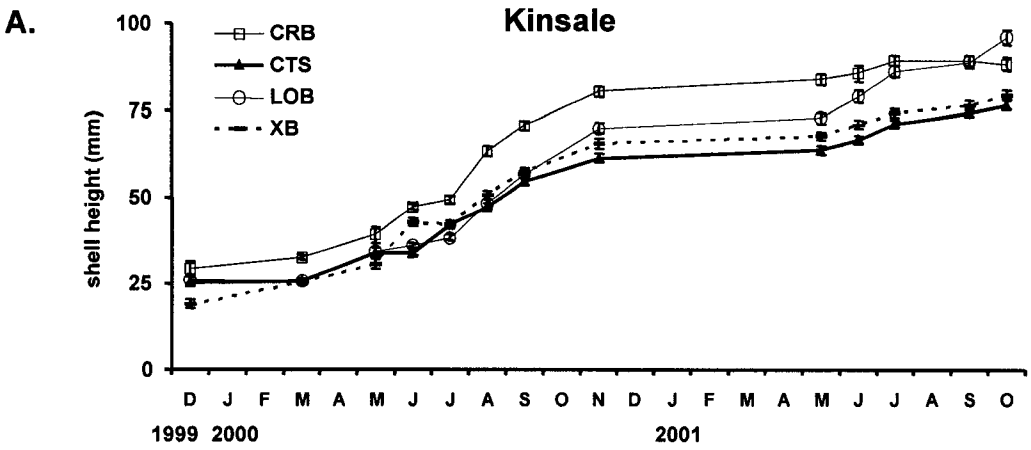


Figure 6. Comparisons of shell heights among CRB, CTS, LOB and XB oysters at (A.) Port Kinsale and (B.) Regent Point during periods (shown as months) sampled in 2000-2001. Data are mean shell heights (mm) ± s.e.m. (n=2 replicates of 10 oysters for each stock). CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.



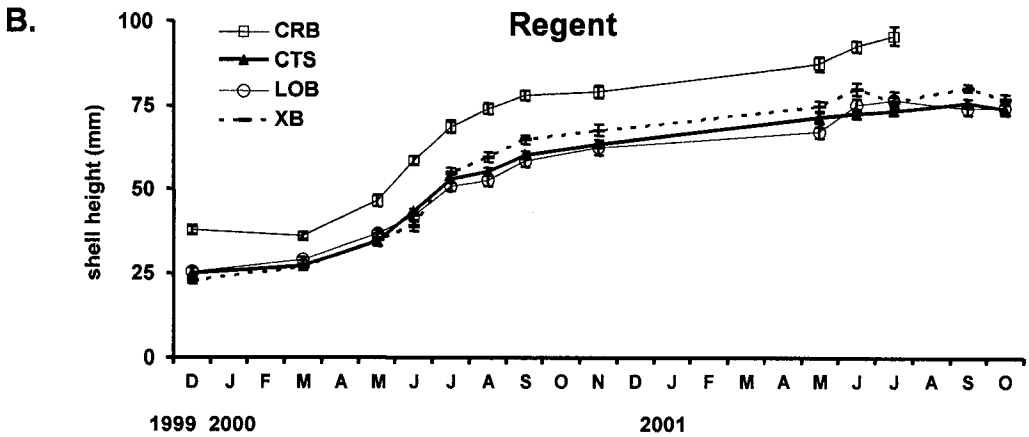


Figure 7. Dry tissue weight measurements of CRB,CTS, LOB, and XB oysters at (A.) Port Kinsale and (B.) Regent Point during periods (shown as months) sampled in 2000-2001. Data are mean dry weights (g) ± s.e.m. (n=2 replicates, 10 oysters/replicate/stock). CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

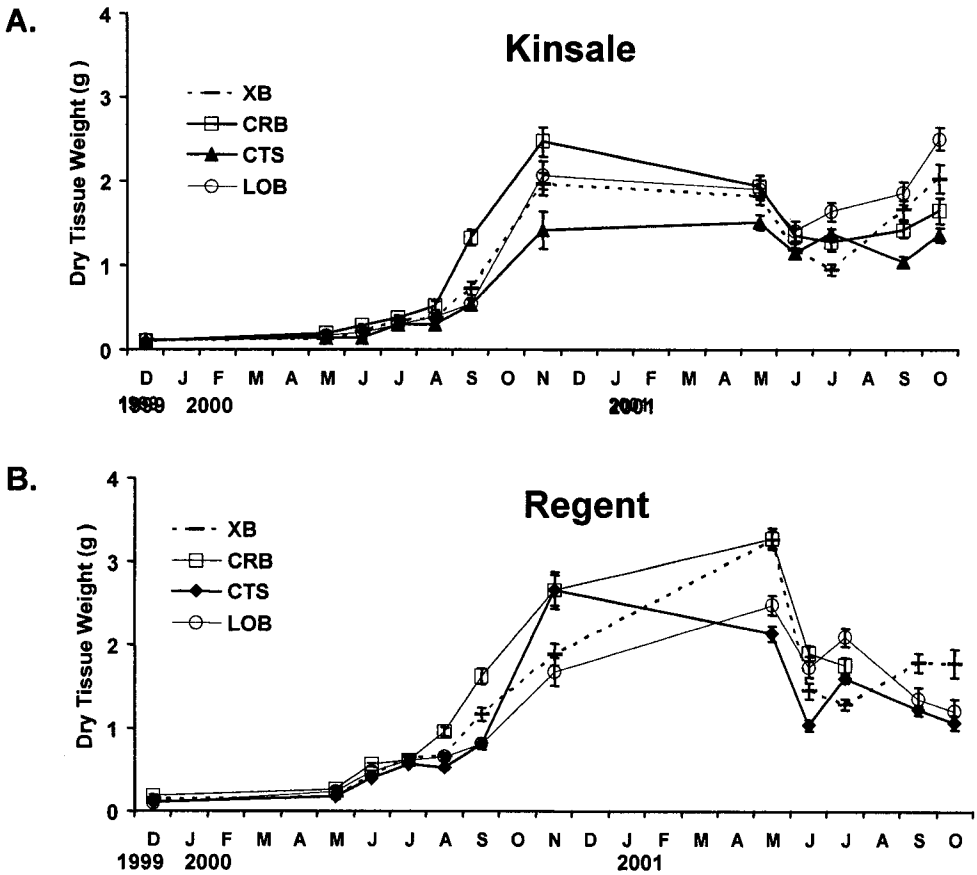


Figure 8. Condition index (C.I.)values of CRB, CTS, LOB and XB oysters at (A.) Port Kinsale and (B.) Regent Point during periods (shown as months) sampled in 2000-2001. Condition index was measured according to Lucas and Beninger (1985) as (dry tissue weight/dry shell weight)*100. Data are presented as mean C.I.± s.e.m. (n=2 replicates of 10 oysters/rep/stock). CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

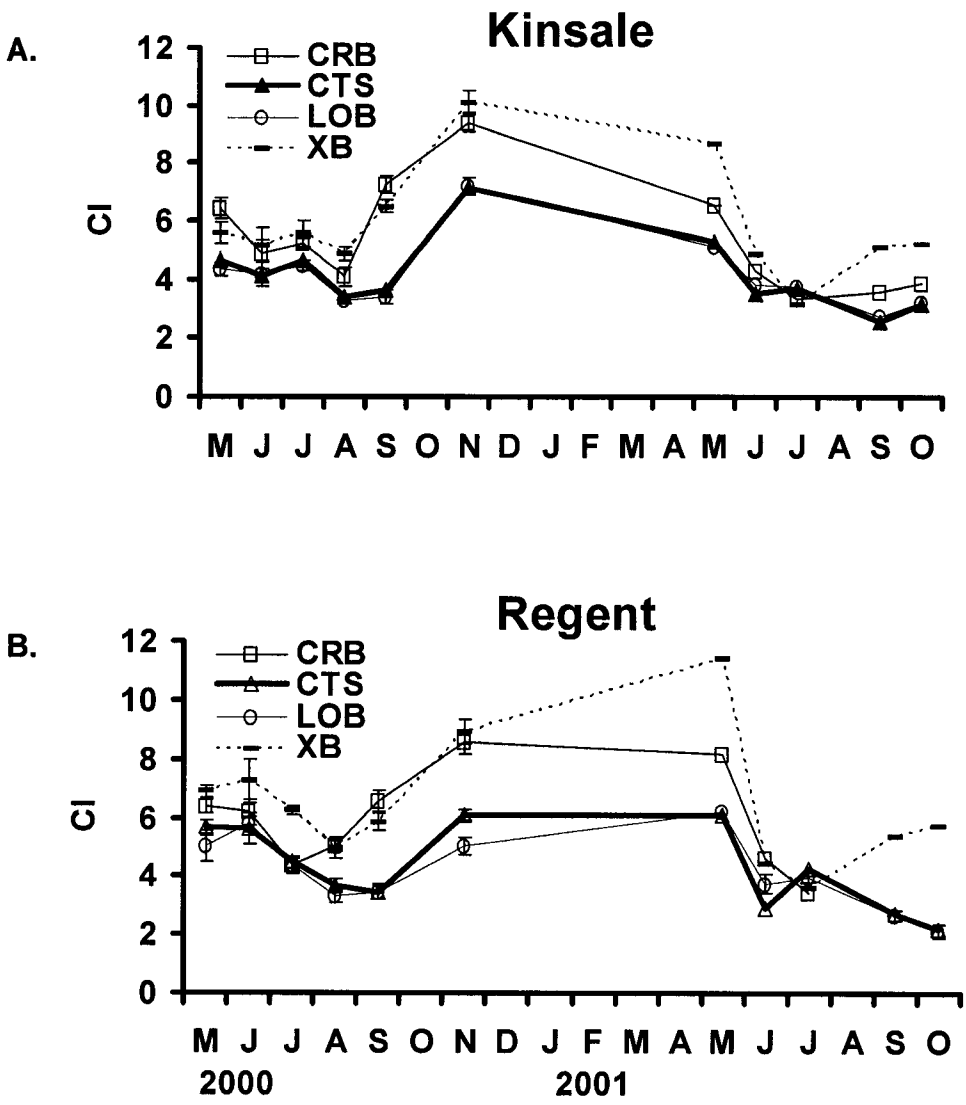


Figure 9. Glycogen contents of CRB, CTS, LOB and XB oysters at (A.) Port Kinsale and (B.) Regent Point during periods (shown as months) sampled in 2000-2001. Data presented are mean glycogen (mg/g dry weight, or dw) ± s.e.m. n=2 replicates of 6-10 oysters per replicate per stock. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

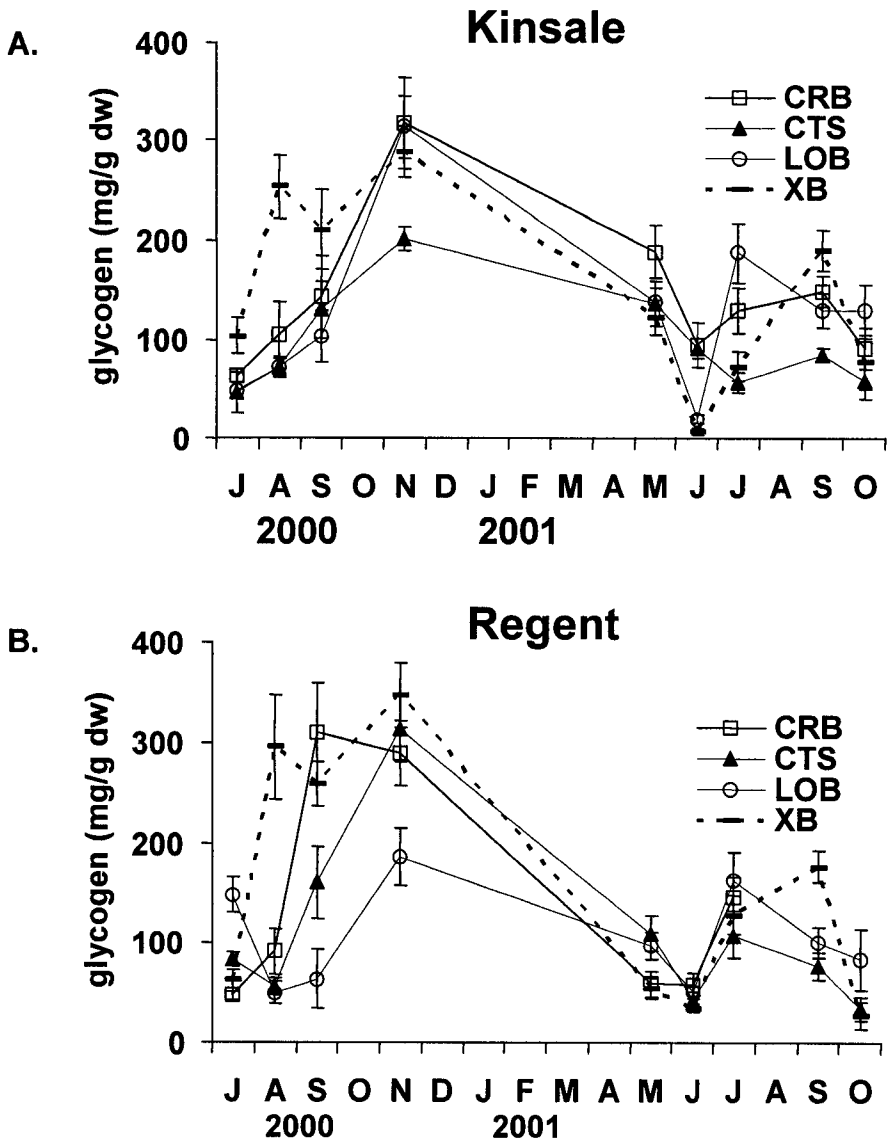
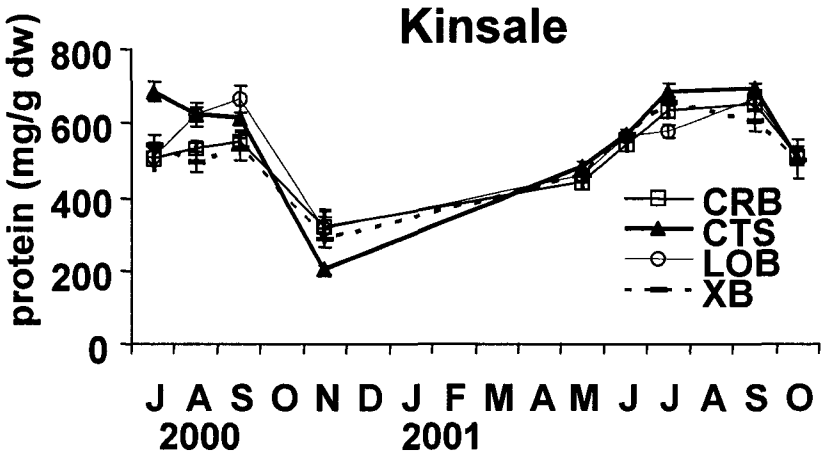


Figure 10. Comparisons of protein contents among CRB, CTS, LOB and XB oysters at (A.) Port Kinsale and (B.) Regent Point during periods (shown as months) sampled in 2000-2001. Data presented are mean protein (mg/g dry weight, or dw) ± s.e.m., n=2 replicates of 6-10 oysters each. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

A.



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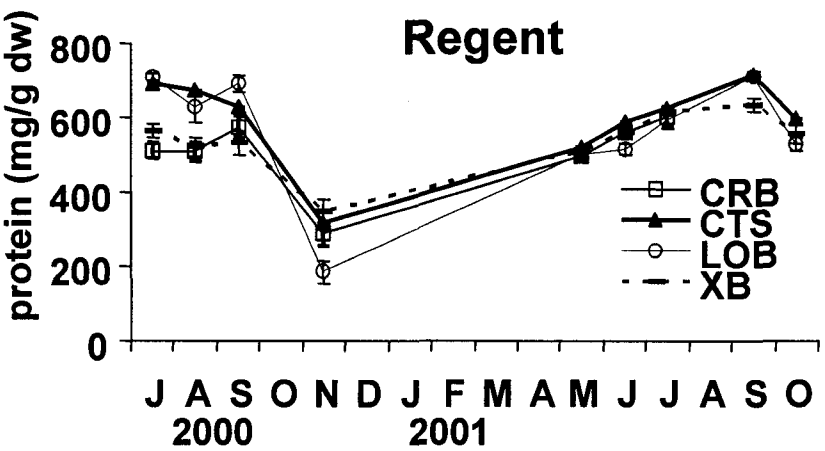


Figure 11. Triacylglycerol (TAG) contents in CRB, CTS, LOB and XB oysters at (A.) Port Kinsale and (B.) Regent Point during periods (shown as months) sampled in 2000-2001. Mean TAG (mg/g dry weight, or dw) ± s.e.m. n=2 replicates of 3-8 oysters/replicate/stock are presented. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

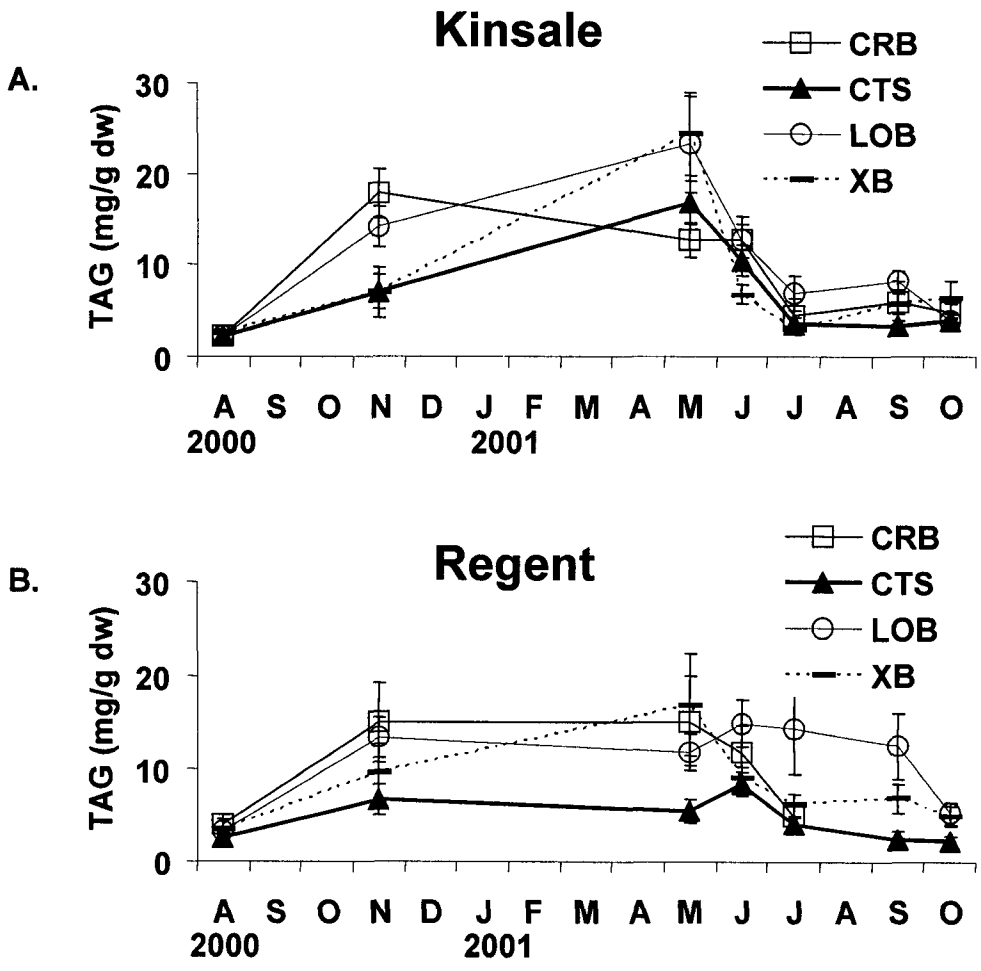


Figure 12. Frequency distribution of infection intensities of *P. marinus* during May, 2001 at Port Kinsale. Distribution of infection intensities among the CRB, CTS, LOB and XB oysters illustrate the presence of individuals with high intensities of infection during a period when mean infection intensities are low. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

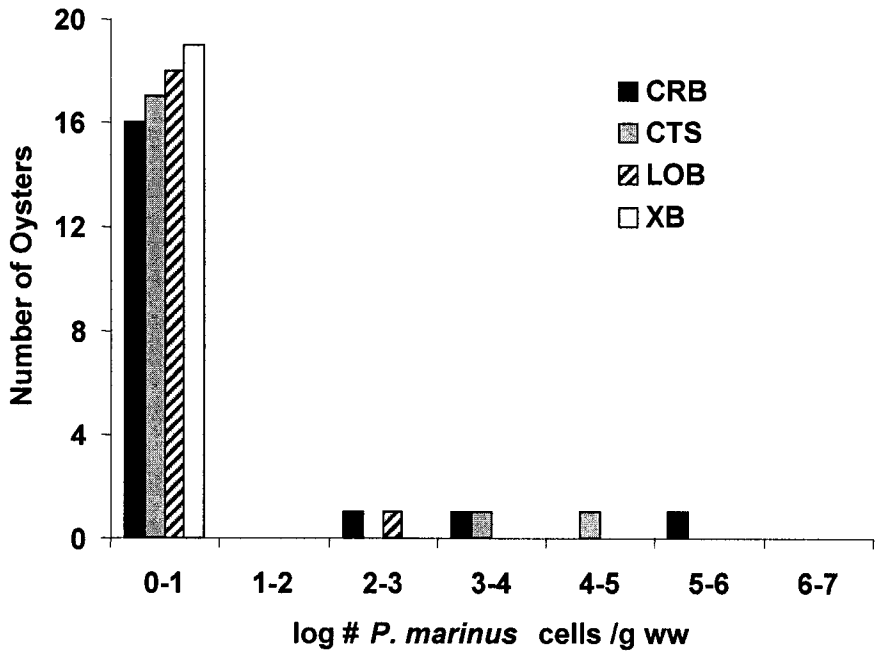


Figure 13. (A.) Months to market size among natural oyster stocks (CRB, CTS, LOB) and one hatchery strain (XB) at Port Kinsale and Regent Point. (B.) Cumulative mortalities (%) of oyster stocks at market size (75 mm) at Port Kinsale and Regent Point. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

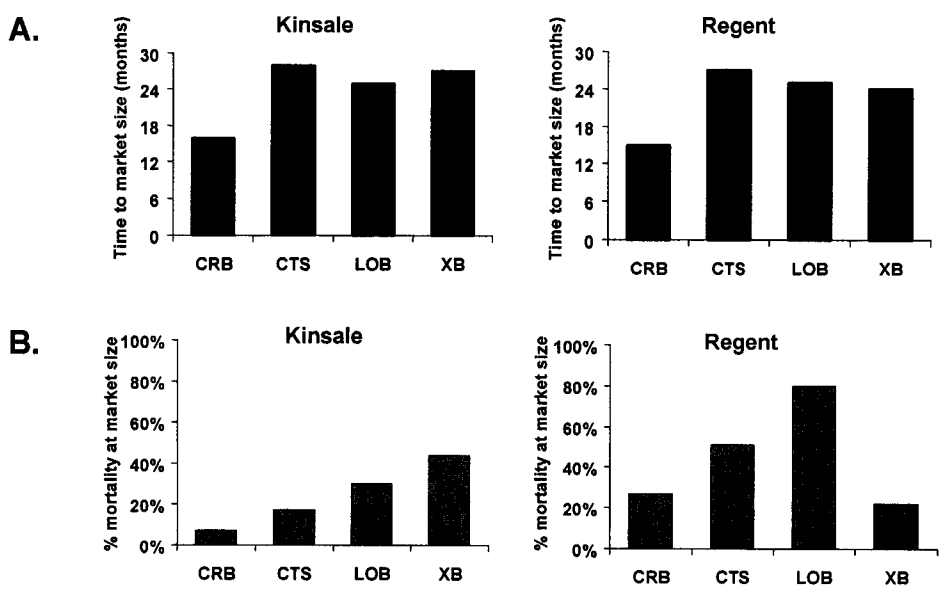
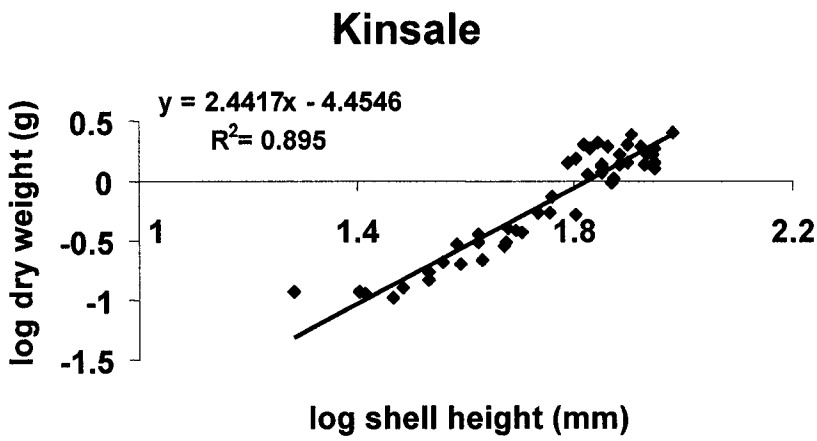


Figure 14. Comparisons of log shell height (mm) and log dry weight (g) at (A.) Port Kinsale and (B.) Regent Point. Shell height and dry weight data are the log of mean shell height and dry weight at each period sampled. The means are collapsed across oyster stocks. CRB – Chesapeake Bay, Rappahannock River, CTS – Chesapeake Bay, Tangier Sound, LOB – Louisiana, Oyster Bayou, XB – CROSBreed strain.

A.



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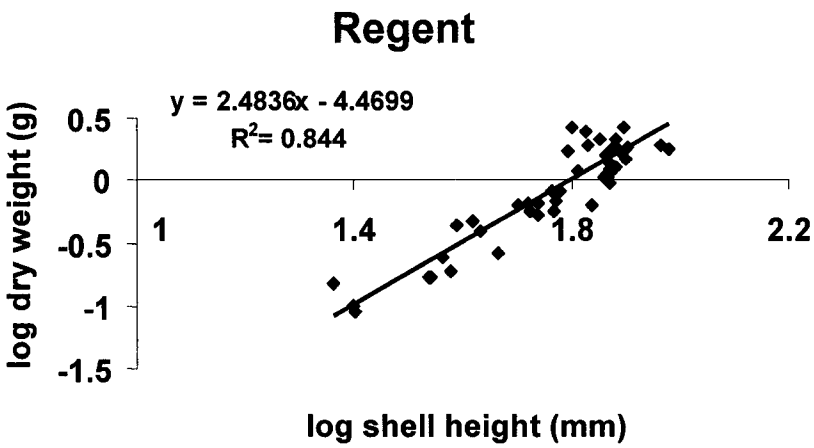

Figure 14.

Chapter 3: Seasonal variation in hsp70 in eastern oyster (*Crassostrea virginica*) stocks resistant and susceptible to *Perkinsus marinus* (Dermo).

(submitted to Journal of Shellfish Research)

Seasonal variation in hsp70 in eastern oyster (*Crassostrea virginica*) stocks resistant and susceptible to *Perkinsus marinus* (Dermo).

Short running title: Hsp70 in eastern oysters

Key words: *Crassostrea virginica*, heat shock protein, hsp70, *Perkinsus marinus,* seasonal variation

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Abstract

Eastern oysters (*Crassostrea virginica*) inhabit highly variable environments and are exposed to large seasonal shifts in temperature. Prevalence and intensity of oyster diseases, particularly *Perkinsus marinus* (Dermo), increases during thermally stressful periods, thus posing additional stress on the oyster host. Heat shock proteins (hsps) are important in protecting organisms from thermal and overall environmental stress. Additionally, heat shock proteins may play protective roles for both the host and parasite during infection. The interactive effects of temperature and disease on hsp expression in oysters, however, are unknown. Disease resistant oysters may express different patterns and amounts of heat shock proteins than susceptible ones, affecting their comparative abilities to adapt to environmental change. In the present study, using slot and western blotting assays, seasonal and intraspecific variation in heat shock protein 70 (hsp70) expression was compared among stocks of *C. virginica* known to be resistant or susceptible to Dermo at two sites in the Chesapeake Bay. Mortalities, shell heights, condition and *P. marinus* infections were also compared among stocks to examine relationships between hsp70 and these variables. Hsp70 was analyzed at 4 seasonal samplings (fall, winter, spring, and summer months), while all other variables were measured bimonthly. Stock mortalities were consistent with the patterns of disease resistance exhibited by their stock parentage, implying existence of a strong genetic component to resistance to Dermo disease. Differences in shell heights, condition index and *P. marinus* infection differences showed significant associations among stock, site and time. Patterns and amounts of hsp70 expression varied significantly across different seasons, but did not correspond with seasonal temperature. Total amounts of hsp70 were

significantly highest in the fall, but with no apparent variation with seasonal temperature. Seasonal variation in specific isoforms of hsp 70 (69 kDa and 72 kDa) was observed. Highest amounts of each were expressed in the spring and fall, respectively, and they were inversely proportional to each other. Differential expression was observed during the winter and spring, with several individuals expressing only hsp72 in the winter and only hsp69 in the spring. Although hsp72 changed concurrently with seasonal changes in infection, both hsp72 and hsp69 did not vary significantly between stocks or with levels of *P. marinus* infection. The present study reveals that measuring total levels of hsp70 do not sufficiently describe the effect of seasonal temperatures on hsp70 expression. Variation in hsp70 did not reflect differences in infection among oyster stocks, indicating that hsp70 may not be a useful indicator to distinguish the effects of pathogenic stress between resistant and susceptible oyster stocks. Differences in expression between hsp69 and hsp72 suggest that seasonal patterns of specific hsp70 isoforms must be understood to determine the role of hsp70 proteins in stress and disease resistance in oysters.

Introduction

Since the 1950's diseases caused by two protozoan pathogens, *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX), have been identified as the causes of intense seasonal mortalities of the eastern oyster, *Crassostrea virginica*, in the Chesapeake Bay (Andrews 1988). Together, these diseases have caused mass mortalities and have hampered efforts to restore oyster stocks to levels that can sustain a viable fishery. Presently, *P. marinus* is considered the most prevalent oyster parasite in the Chesapeake Bay due to its persistence over a wide range of temperatures and salinities (Burreson et al. 1994).

Temperature is one of the most important factors regulating interactions between *P. marinus* and the oyster host (Andrews 1965, Chu and LaPeyre 1993, Chu and Volety 1997). *Perkinsus marinus* rapidly proliferates and develops between 20-30 ° C (*in vitro* and *in vivo*) and salinities greater than 10 ppt (Chu and Greene 1989, Chu and La Peyre 1993, Burreson and Ragone-Calvo, 1996). Dermo-associated mortality usually begins in early summer (June) when water temperature increases (~20 °C) and peaks (27-30 °C) between August and September (Andrews and Hewatt 1957; Andrews 1988). Mortalities can be particularly intense above 25 °C (Mackin 1951, Fisher et al. 1992, Chu and LaPeyre 1993). These increases in seasonal temperature intensify overwintered and newly acquired infections (Ragone-Calvo and Burreson 1994).

Increased temperature itself also poses an additional stress on eastern oysters. Defense-associated factors such as lysozyme activity were lowest during summer months (Chu et al. 1995) and phagocytic activity of hemocytes decreased at temperatures greater

than 25 °C (Chu and LaPeyre 1993). Decreases in oyster condition occur after spawning in the late spring/early summer, coinciding with increases in seasonal temperatures and disease (Galtsoff 1964, Austin et al. 1993, Dittman et al. 2001). Summer mortalities of Pacific oysters (*Crassostrea gigas*) on the west coasts of the U.S. and France are associated with elevated seasonal temperatures and multiple interacting factors (Cheney et al. 2000, La Coste et al. 2001, Soletchnik 1999). Increased levels of microbial pathogens are one of the factors that may contribute to summer mortality when temperatures increase, and the incidence of *Vibrio* spp. in moribund *C. gigas* increases in association with elevated temperatures (LaCoste et al. 2001). Further, additive effects of thermal and parasitic stress may contribute to oyster mortalities during the summer months. Thermal stress increased respiration rates in oysters infected with MSX, compared to uninfected oysters (Littlewood and Ford 1990). Thermal stress alone is enough to cause deleterious physiological effects. Acute thermal stress during emersion was shown to cause reversible and irreversible protein denaturation in intertidal mussels (Hofmann and Somero 1995).

Molecular chaperones, or heat shock proteins, are among the major cellular factors that counteract the effects of thermal stress. Heat shock proteins maintain proper conformation of proteins in the face of thermal stress. Induction of heat shock proteins in response to other environmental stressors has also been observed, suggesting their use as general biomarkers for stress (Sanders et al. 1988; Pyza et al. 1997; Cruz-Rodriguez and Chu 2002). Additionally heat shock proteins play protective roles for both the host and parasite during infection (Merino et al. 1998; Robert, 2003) and as a stimulant of the host immune response (Tamura et al. 1997; Zugel and Kaufmann 1999). Coho salmon with

bacterial kidney disease had higher levels of hsp70 than uninfected fish (Forsyth et al. 1997).

Among the major families of heat shock proteins, the hsp70 family (heat shock proteins in the 70 kDa MW range) is the most responsive to environmental perturbations (Parsell and Lindquist 1993, Feder and Hofmann 1999, Lewis et al. 1999). In most species, hsp70 is a multi-gene family encoding several distinct protein isoforms (Lindquist 1986). The hsp70 genes are largely recognized as being highly conserved both across organisms and within these multi-gene families (Favatier et al. 1997).

Efforts to revitalize the oyster fishery have been directed at producing oysters that are resistant to Dermo and MSX (Allen et al. 1993, Calvo et al. 2003). Several strains and stocks resistant to Dermo and MSX have been produced (Allen et al. 1993, Calvo et al. 2003, Encomio et al., submitted). The mechanisms, however, underlying increased resistance to Dermo and MSX are poorly understood. These mechanisms are likely influenced by fluctuating environmental conditions. Other than disease resistance, little attention has been paid to the enhancement of other potentially selectable traits. Furthermore, it is unclear what traits are linked to, or associated with, disease resistance. Improving general stress tolerance, particularly thermal stress, may reduce the additive effects of stress and disease. While mechanisms of disease resistance in oysters are still unclear, mechanisms associated with resistance to thermal stress, such as heat shock proteins, have been characterized in many organisms, including oysters (Sorenson et al. 2003; Feder and Hofmann 1999, Shamseldin et al. 1997, Clegg et al. 1998; Cruz-Rodriguez and Chu 2002; Piano et al. 2002, Boutet et al. 2003, Hamdoun et al. 2003).

Increased levels of heat shock proteins have been associated with enhanced thermal tolerance in oysters (Clegg et al. 1998, Piano et al. 2002).

The range of intraspecific variation in hsp70 expression among eastern oysters is currently unknown. Variation in hsp70 expression may indicate differences in thermal-tolerance capacities and tolerance to other stressors such as disease. The objective of this study was to characterize seasonal and intraspecific variation in hsp70 among hatchery-produced strains of eastern oysters. We hypothesized that heat shock proteins would follow seasonal patterns of temperature with the highest levels of heat shock proteins expressed during periods of highest seasonal temperatures. The hatchery-produced oysters used in this study were progeny of F_0 stocks that displayed variation in survival patterns consistent with disease resistant strains (Encomio et al., submitted). Additionally, growth, mortality, condition and *P. marinus* infections among these F_1 strains were compared to confirm if differences between parental stocks were genetic. Oysters from a disease-susceptible strain were also deployed for comparative purposes. This strain was hypothesized to be under greater stress, and therefore would express higher levels of heat shock proteins than resistant stocks. Intra-strain variation in hsp70 expression during seasonal acclimatization would suggest a genetically based response to environmental stress, as strains were reared in a common environment. Changes in hsp70 were also compared with seasonal variations in condition and infection to determine whether changes in hsp70 were associated with changes in physiological state and disease.

Materials and Methods

Oyster growout and experimental design

The F_1 progeny of F_0 Rappahannock River (CRB), Tangier Sound (CTS), and CrosBreed (XB) stocks were used in this study. Oysters were supplied as spat from the Aquaculture Genetics and Breeding Technology Center (ABC), VIMS, and deployed at two sites within the Chesapeake Bay (Regent Point Marina - Rappahannock River and Port Kinsale - Yeocomico River). The parental stocks (F_0) were produced in a previous study testing the existence of Dermo resistance among native Chesapeake and Gulf of Mexico oyster stocks. Six native stocks (3 Chesapeake and 3 Gulf) and one hatchery strain (XB) were compared in this study. Extensive variation in survival, growth, condition, and Dermo infection was seen among stocks grown in a common environment (Encomio et al., submitted). Based on survival patterns of F_0 stocks, F_1 CRB oysters were identified as disease susceptible, F_1 CTS oysters were disease resistant, and XB oysters were disease resistant controls (Encomio et al., submitted). Each stock was grown in mesh bags placed in four replicate floats. Sampling was conducted approximately bimonthly from August 2002 to July 2003. During each sampling, oysters were counted to assess mortalities and 3 oysters were randomly sampled from each replicate bag (n=4; 12 oysters/stock/sampling), placed on ice and brought to the laboratory. Water temperature and salinity were also recorded for each sampling period. In the laboratory, shell heights were measured with vernier calipers. Gill tissues were removed for hsp70 measurement and the remaining oyster tissue was processed for *P. marinus* diagnosis. Gill and whole tissue homogenates were freeze-dried for 48 hours and weighed to estimate total dry weights for condition index. Condition index was determined according to Lucas and Beninger (1985).

Diagnosis of *P. marinus* (Dermo) infection

Prevalence and intensity of *P. marinus* infection in experimental oysters was determined using total body burden assessment (Bushek et al. 1994, Choi et al. 1989). Oyster tissue was weighed and mechanically homogenized in 0.1 M sodium phosphate buffer. A 1.0 ml tissue aliquot was incubated in alternative fluid thioglycollate medium (AFTM) (Sigma Biochemicals) for 5-7 days at room temperature. Tissue suspensions were then centrifuged at 800 x g for 10 min. Tissue pellets were resuspended in 2M NaOH and incubated overnight at 60 °C. Tissue pellets were washed, centrifuged, and resuspended in distilled water. One hundred µl aliquots of each sample were added to a 96-well plate and stained with 1-2 drops of Lugol's solution (1:10 dilution). Stained *P. marinus* cells were counted under an inverted microscope at 400x magnification. Results are expressed as number (#) of *P. marinus* cells/g wet tissue weight (ww).

Detection of Hsp70

Gill samples from four periods (November 2002, February 2003, May 2003 and July 2003) were chosen to represent fall, winter, spring, and summer periods. Both slot blot (Lewis et al. 1999, Cruz-Rodriguez and Chu 2002) and western blotting techniques were used to detect hsp70. Slot blot analysis only examines total amounts of hsp70, as the primary antibody used recognizes multiple isoforms of hsp70. Western blot analysis was performed, to determine if variation in isoform expression was responsible for changes in total hsp70 amounts. For both slot and western blot analyses, one individual gill sample was selected from replicate floats of each stock (n=3-4 per stock at each site and sampling).

Gill tissues were excised, freeze-dried for 48 hours, weighed and stored at -80 °C. Gill tissues were then homogenized on ice in 2 ml of buffer (66 mM Tris pH 7.2, 3% Nonidet, 0.1 mM PMSF). The homogenate was centrifuged at 10,000 x g for 30 minutes at 4 ^{0}C, and the supernatant (gill extract) collected. Total protein concentration was determined using a modified version of the Lowry assay (Biorad DC Protein Assay, Lowry et al. 1951).

Slot Blot Detection

Total hsp70 isoforms in oyster gills were detected with a monoclonal antibody raised against human hsp70 (Affinity Bioreagent, 3A3) and total hsp70 quantified using the slot blot assay (Cruz-Rodriguez and Chu 2002, Lewis et al. 1999). Samples were loaded in triplicates. Unknown samples were diluted to 1.5 μg total protein and vacuumed onto nitrocellulose. A standard concentration gradient (0.25, 0.5, 1, 1.5, 2, and 2.5 μg total protein) from a "reference sample", obtained by exposing an oyster to 1 hour heat shock at 40 °C, was also added to each blot. The blot was removed and blocked with 5% bovine serum albumin (BSA) in Tween-Tris-buffered saline (TTBS - 0.05% Tween, 30 mM NaCl, 24 mM Tris pH 7.5) for 30 minutes followed by two washes in Tris-buffered saline (TBS - 30 mM NaCl, 24 mM Tris pH 7.5) for 5 minutes each. Primary monoclonal antibody against Hsp70 (clone 3A3 - catalog # MA3-006, Affinity Bioreagents) was applied for 90 minutes (1:5000 dilution), followed by two 5 minute washes with TBS. A secondary antibody (Goat Anti-Mouse AP conjugated) was applied for 90 minutes (1:1000 dilution), washed once in TBS, and placed in a developing solution containing NBT (*p*-Nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). Bands developed between 30-60 minutes. The blot was

stored in deionized water until analysis. Densitometric analysis of developed slot blots was performed using Enprotech scanner and software. The areas of the samples were recorded and normalized to the area of the reference sample in each blot to account for inter-blot variation.

SDS-PAGE and Western Blot Detection

A portion of the gill extract was diluted 1:2 in Laemmli sample buffer (BioRad) for SDS-PAGE. Samples were boiled for 5 minutes and 10 μg total protein per sample was electrophoresed on 8% polyacrylamide gels (150 V, 90 minutes). Separated proteins were then transferred onto nitrocellulose membrane at 100 V for 1 hour in transfer buffer (192 mM glycine, 24 mM Tris base and 20% methanol). After transfer, nitrocellulose blots were processed for immunodetection of hsp70 isoforms as previously mentioned. Blots were scanned and analyzed as previously described. Relative amounts of hsp70 were obtained by normalizing band densities to the "reference" sample described previously. As with the unknown samples, 10 μg of the reference sample was added to each gel.

Specificities of commercial Hsp70 antibodies

To confirm differences in antibody specificity we performed additional western blot analysis using the clone 7.10 (catalog # MA3-001, Affinity Bioreagents). Other studies of oysters have used this antibody (Clegg et al. 1998, Piano et al. 2002, Hamdoun et al. 2003, Brown et al. 2004). The 3A3 antibody was used in the present study and others (Tirard et al. 1995, Shamseldin et al. 1997, Cruz-Rodriguez and Chu 2002). Both antibodies recognize recombinant human hsp70, although both recognize different amino

acid sequences. The 7.10 antibody recognizes amino acids 473-479 of human hsp70 and 3A3 recognizes amino acids 504-617 of human hsp70 (Affinity Bioreagents). They both recognize hsp70 in a wide variety of organisms.

Statistics

Data were analyzed by repeated-measures ANOVA for effects of sampling time, site, and stock. The Tukey test was used for multiple comparisons of significant ANOVA effects. Data were transformed when necessary to meet assumptions of normality and homogeneity of variance. Pearson's correlation was used to compare hsp70 and other parameters (temperature, salinity, mortality, growth, and condition and *P. marinus* infection). Data are presented as mean ± standard error of the mean (s.e.m.).

Results

Temperature and salinity

Seasonal variation in temperature and salinity are shown in figure 1. Temperatures were lowest at both sites in February 2003. The highest temperatures were during July 2003 (28-29 °C). Salinities at both Regent Point and Port Kinsale remained moderately high from August 2002 to February 2003 (16-24 ppt) and then decreased during May and July 2003 (fig 1). Overall, salinities were relatively higher at Regent Point than at Port Kinsale.

Mortality, growth and condition

Mortality was highest in the CRB strain at both sites compared to the XB and CTS ($p=0.03$). The CRB strain also showed higher mortality at Regent Point than at Port Kinsale ($p=0.027$) (Figure 2). Shell heights were significantly different among sampling

periods, site, and stock ($p<0.0001$ for each). Shell heights increased over time (Figure 3A) albeit changes in shell height were relatively small (57.8 ± 1.0 mm to 64.8 ± 1.5 mm). Shell heights at Regent Point were greater than at Port Kinsale (Figure 3B). Shell height in the CTS group was significantly lower than both CRB and XB strains ($p<0.05$) (Figure 3C).

Condition index (CI) varied significantly with sampling time and strain ($p<0.0001$ and $p=0.018$, respectively). Condition index was lowest in July 2003 (4.53 ± 0.13) and largely accounted for the significant differences in CI by month (Figure 4). The CI was highest in the CRB stock compared to both XB and CTS stocks ($p<0.05$). Differences in CI between sites were not significantly different.

***Perkinsus marinus* infection**

Intensities of infection by *P. marinus* varied significantly among month, site, and strain ($p<0.0001$ each) (Figure 5). *Perkinsus* infections remained high (~6-9 x 10^6 cells/g wet weight) from August-November 2002. Infections, though relatively high for the winter, decreased significantly in December 2002 (2.66 ± 0.82 x 10^6 cells/g wet weight) and decreased further in February and May 2003 (1.55 ± 0.49 x 10^6 cells/g wet weight and 0.256 ± 0.24 x 10^6 cells/g wet weight respectively). Infections increased in July (2.11 ± 0.76 x 10^6 cells/g wet weight) from May. Overall, *P. marinus* infections were higher at Regent Point than at Port Kinsale. Infections were the highest in the CRB stock compared to the CTS and XB stocks (Tukey test $p<0.0001$) (Figure 5B). Interactions between site and month were significant ($p=0.001$) and were attributed to an increase in

infection at Kinsale during November, 2002 and the increase in infection intensity in July, 2003. (Fig. 5A)

Hsp70 analyses

Data for hsp70 showed no significant differences among stocks or sites. The data was therefore pooled for analysis of sampling time. Although there were seasonal differences in hsp70 (hsp 69, hsp72, and total hsp70), there was no correlation between changes in heat shock proteins and other measured parameters (temperature, salinity, oyster mortality, growth, and condition, and *P. marinus* infection).

Slot blot analysis

Slot blot analysis showed a significant ($p=0.001$) sequential decrease in total hsp70 from fall through summer periods (Figure 6). Fall and winter hsp70 levels were significantly greater than spring and summer levels. Changes in total hsp70 did not correspond with changes in seasonal temperatures.

Western blot analysis

Immunological detection of hsp70 isoforms by western blot (using 3A3 antibody) found two isoforms of hsp70 expressed in the gills (Figure 7A). The higher molecular weight (MW) isoform was estimated to be 72 kilodaltons (kDa) and the lower MW isoform was 69 kDa. Separate statistical analyses were performed for each isoform. Total amounts of hsp70 were calculated by adding relative amounts of each isoform. Western blots probed with the 7.10 antibody detected three isoforms of hsp70 (69, 72, and 77 kDa) in heat-shocked and non-heat-shocked oysters, similar to those reported by Clegg et al. (1998) in

C. gigas. The 72 and 69 kDa bands corresponded, although not precisely, with the 72 kDa and 69 kDa bands detected by the 3A3 antibody (Figure 7D).

Similar to slot blot results, western blot analyses of total hsp70, hsp69 and hsp72 isoforms, showed significant variation with month (Fig. 8A). Levels of total hsp70 in the fall were significantly higher than in the winter, spring and summer ($p<0.0001$). Amounts of total hsp70 from the spring and summer were higher, but not significantly different from winter values.

Patterns of seasonal variation, however, differed between the two isoforms. Hsp72 was predominantly expressed in the fall and winter and decreased significantly ($p<0.0001$) from fall through the spring, and increased again in the summer (Figure 8B). Hsp69 decreased from fall to winter and increased in spring ($p<0.05$), with a number of individuals (7 out of 16 total oysters in the spring) only expressing this isoform. Levels of hsp69 decreased in the summer to levels similar to those of the fall and winter (Figure 8B). A switch in expression patterns was also seen in the summer sampling. Hsp72 increased as hsp69 decreased, with some individuals only expressing hsp72 (see Figure 7B and 7C for examples). Changes in hsp69 followed changes in temperature from fall to spring, but decreased in the summer, during the periods of highest temperature (Figure 8B). An increase in hsp72 coincided with the increase in summer temperatures but did not increase with spring temperatures. Slot blot results and total hsp70 from western blotting were positively correlated ($r =0.54$, $p=0.007$) (Figure 9).

Discussion

In laboratory-based disease challenges, Bushek and Allen (1996) showed that resistance to Dermo is present in distinct populations of eastern oysters. Our previous

field studies of oyster stocks from the Gulf of Mexico and the Chesapeake Bay confirm this (Encomio et al., submitted). Specific stocks of eastern oysters from the Chesapeake Bay and the Gulf of Mexico exhibit varied resistance to Dermo. Survival during periods of elevated exposure to *P. marinus* was high in one Chesapeake stock (Tangier Sound, or CTS) and several Gulf stocks from Louisiana. Comparisons of mortalities to a Dermo-susceptible Chesapeake stock (Rappahannock River) and a hatchery strain selected for disease resistance (CrosBreed or XB) confirmed that the Tangier and Gulf stocks were indeed disease resistant. Comparisons of mortality, growth, condition, and *P. marinus* infection show that results are similar to those from the F_0 parental stocks (Encomio et al., submitted). The consistency among stocks exhibited in the F_0 and F_1 generations implies that a strong genetic component underlies these differences. Most significantly, these apparent genetic differences are maintained in their resistance to *P. marinus*.

Increases in hsp70 with seasonal temperature have been observed in mussels and marine snails (Hofmann and Somero 1995, Chapple et al. 1998, Tomanek and Somero 1999, Minier et al. 2000). It has not, however, been unequivocally demonstrated that seasonal variation in hsp70 corresponds directly to seasonal temperatures. Subtidal mussels (*Mytilus* spp.) expressed higher levels of hsp 70 in the winter than in the summer (Roberts et al. 1997). Seasonal patterns of hsp70 expression varied from year to year in eastern oysters (Cruz-Rodriguez 2001) and did not show consistent increases with seasonal temperatures. Total hsp70 was higher in the spring than the summer among four species of stream fish (Fader et al. 1994). In this study, hsp70 was analyzed by ELISA, which like the slot blot assay, does not distinguish between specific isoforms. In the present study, slot blots and western blots produced similar results, showing that total

amounts of hsp70 did not correlate positively with seasonal variation in temperature. This implies that total amounts of hsp70 may not be a good indicator of thermal exposure, or other factors, such as salinity or disease may alter expression of hsp70. In the Asian clam, *Potamocorbula amurensis*, hsp70 expression increased with salinity (Werner et al. 2000). Variable salinity could have a significant effect on the heat shock response in oysters, because temperature effects on oyster metabolism can be significantly altered by salinity. In oysters acclimated to lower salinities, temperature effects on VO_2 were more pronounced than in oysters acclimated to higher salinities (Shumway and Koehn 1982). How salinity affects thermal acclimation of hsp70 expression in oysters, however, is still unknown and warrants further study.

Our results show that seasonal variation in hsp70 is isoform specific. Interestingly, several animals expressed only the low MW isoform (hsp69) in the spring, implying that expression of hsp69 and hsp72 may be regulated differently. A distinct switch in isoform expression may have been necessary in response to a high relative increase in seasonal temperature from winter to spring. In several bivalve species 2-3 isoforms of hsp70 have been observed. In the mussel, *Mytilus edulis*, the lower MW isoform (70kDa) was largely absent in the winter months (Chapple et al. 1998). In contrast, a 72 kDa hsp was continually present in *M. galloprovincialis* specimens and increased with seasonal temperatures (Minier et al. 2000). In other studies of oysters (*C. gigas, O. edulis*) the intermediate (72 kDa) and high (77 kDa) MW isoforms are always present (Clegg et al. 1998, Piano et al. 2002, Hamdoun et al. 2003). In *C. gigas*, variable isoform expression was mainly attributed to expression of a third low molecular weight isoform (hsp69) after acute heat shock (Hamdoun et al. 2003). The expression of the

heat-inducible hsp69 has also been observed in other studies of *C. gigas*, and in the oyster species *Ostrea edulis* and *Ostreola conchaphila* (Clegg et al. 1998, Piano et al. 2003, Brown et al. 2004). Expression of hsp69 in these studies was only seen after heat-shock in the laboratory. All the preceding studies suggest that the lower MW isoforms are more responsive to heat shock than higher molecular weight isoforms. Using the 3A3 antibody we detected only two isoforms of 69 and 72 kDa MW, but not a third, higher MW isoform, such as the 77 or 78 kDa heat shock proteins found in the studies of Clegg et al. (1998) and Chapple et al. (1998). This could be due to differences in specificity of the 3A3 antibody compared to the 7.10 antibody, which was used to detect hsp70 in several species of oysters (Clegg et al. 1998, Hamdoun et al. 2003, Piano et al. 2003, Brown et al. 2004). Using the 3A3 antibody, Tirard et al. (1995) found expression of two distinct hsp70 MW isoforms in heat-shocked hemocytes of *C. virginica,* similar to our results. In the study of Tirard et al. (1995), the low MW isoform was shown to be heat-inducible. In contrast, we found that the low MW hsp70 isoform, hsp69, was endogenously expressed in field-collected oysters that were not heat-shocked. Similarly, laboratory heat-shock treatments did not result in *de novo* synthesis of an "inducible" hsp70 in *C. virginica* (Cruz-Rodriguez 2001). It is possible that more hsp70 isoforms may exist than recognized by either antibody alone. If this is the case, antibodies specific to the oyster hsp70 family would need to be produced to fully characterize hsp70 expression patterns. In a previous study, expression of hsp70 increased with *P. marinus* infection in *C. virginica* (Brown et al. 1993). However, effects of increased salinity on heat shock protein expression could not be differentiated from infection, as they are both highly correlated. Stress caused by disease could retard the heat shock response. Decreased

levels of hsp70 in *M. edulis/M. galloprovincialis* hybrids were associated with higher infections of the protistan parasite *Marteilia refringens* (Fuentes et al. 2002). We did not observe differences in hsp70 among strains, although there were significant differences in survival and infections with *P. marinus*. Intraspecific differences in thermal tolerances have been demonstrated in Pacific oysters. Oysters from California exhibited a higher degree of induced thermotolerance than those from Washington State (Shamseldin et al. 1997). However, hsp70 expression was not compared between the two populations, so it was unknown whether hsp70 was associated with differences in thermal tolerance. Intraspecific variation in hsp70 expression was not dramatic in fruit fly strains representing populations with variable thermal histories, and was attributed to a high degree of gene flow between natural populations (Garbuz et al. 2003). This could be the case in eastern oyster populations within the Chesapeake Bay, or that natural selection on thermal tolerance is uniform across populations within the Bay. Homogeneous allozyme frequencies among Atlantic and Gulf oyster populations were thought to be the result of similar selective pressures (Karl and Avise 1992). It would be hypothesized that selection on thermal tolerance and disease resistance is independent, resulting in similar heat shock protein expression among oyster strains, even though differences in disease resistance are apparent

The pathogenicity of oyster diseases is significantly affected by natural stress. Resistance to natural stress has largely been ignored in selective breeding of *C. virginica*. Improved survival is mainly attributed to disease resistance, but environmental interactions can decrease performance and survival, even when disease is not a factor. Selection for thermal tolerance could be readily incorporated into selection criteria for

improved performance in oysters. Quantitative trait loci associated with thermal tolerance have been identified in trout (Perry et al. 2001) and could be applied to shellfish. Further study may help to establish whether heat shock proteins could serve as a readily measurable marker for thermal tolerance. Improving thermotolerance in oyster strains already selected for disease resistance could enhance survival through increased resistance natural stress, making culture successful across a wider range of estuarine conditions.

In conclusion we have shown that endogenous hsp70 levels in strains of the eastern oyster exhibit seasonal variability, and that this variability does not directly correspond with seasonal temperature. Changes in hsp70 did not vary intraspecifically or across levels of *P. marinus* infection. Variation in hsp70 expression is partly explained by changes in expression of specific high and low molecular weight isoforms of hsp70. When compared to total levels of hsp70, relative changes in these isoforms are compensatory and inversely proportional, suggesting that expression of each is regulated in a different manner, as opposed to both increasing with temperature. Although the significance of variable isoform expression and their individual function remains to be investigated, measuring total hsp70 alone may not provide a complete representation of how oysters cope with seasonal changes in temperature. Moreover, factors other than temperature can modulate the expression of hsp70. Understanding the mechanisms regulating heat shock protein expression is necessary to determine whether or not heat shock proteins can be used as a marker of increased stress tolerance in oysters.

Acknowledgements

This research was supported by the NOAA-Sea Grant Oyster Disease Research Program (Project # VA-OD-01-05). The authors would like to thank Stan K. Allen, Jr. and the VIMS-ABC for providing oyster strains. Thanks to Eric Lund and Shawn Stickler for assistance with field deployment and sampling. We would also like to thank Georgeta Constantin for assistance with tissue collection and *P. marinus* diagnosis. Special thanks are given to Drs. Jeff Shields and Sandy Shumway for constructive reviews of earlier drafts of this manuscript.

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Figure 1. Seasonal (A.) temperatures and (B.) salinities from 2002-2003 at two Chesapeake Bay sites (Port Kinsale-Yeocomico River, and Regent Point-Rappahannock River).

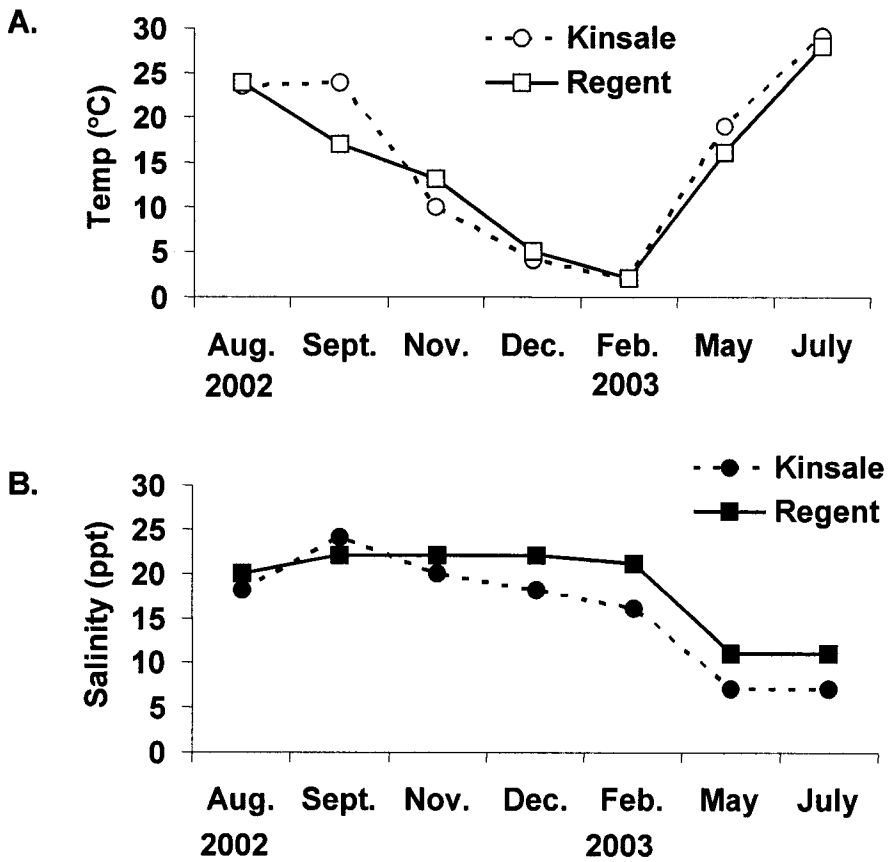


Figure 2. Cumulative mortalities of three deployed oyster stocks from 2002-2003 at (A.) Port Kinsale and (B.) Regent Point. CRB = Rappahannock River, CTS = Tangier Sound, XB = CrosBred hatchery strain. The CRB and CTS groups are progeny of F_0 stocks originally spawned in 1999. Data are means and standard error of the mean (± s.e.m.) of 4 replicate bags/stock/site.

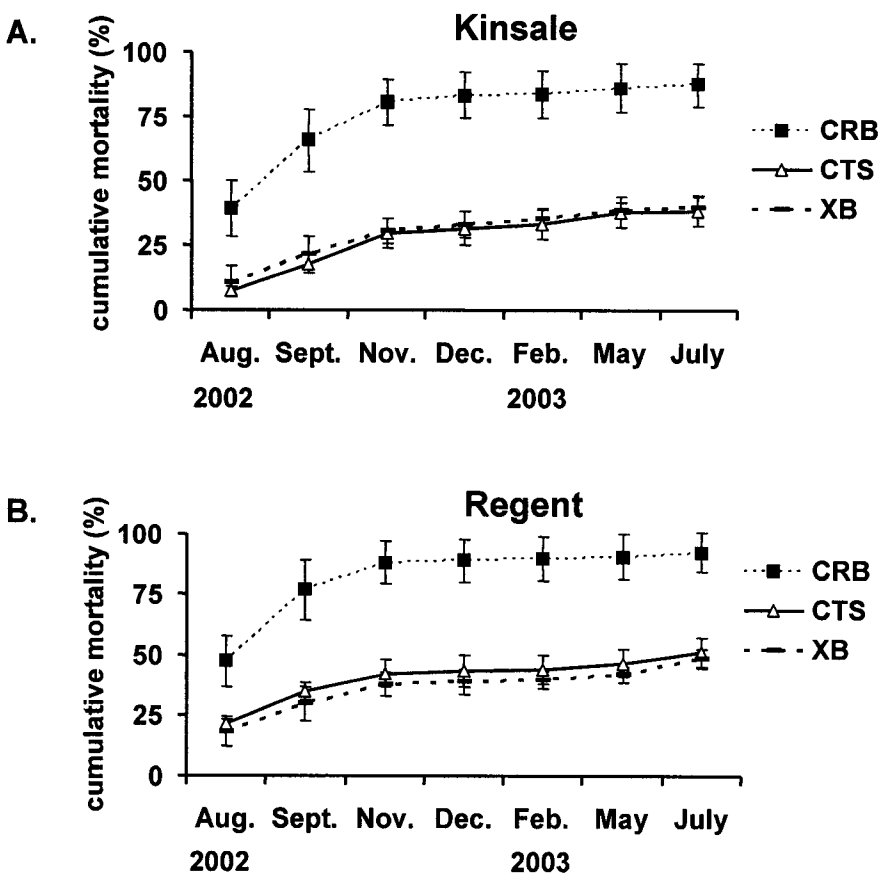
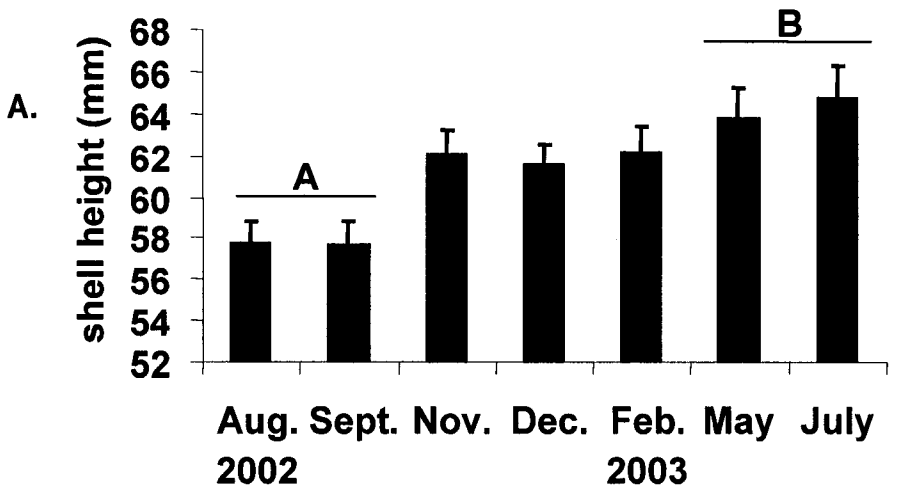
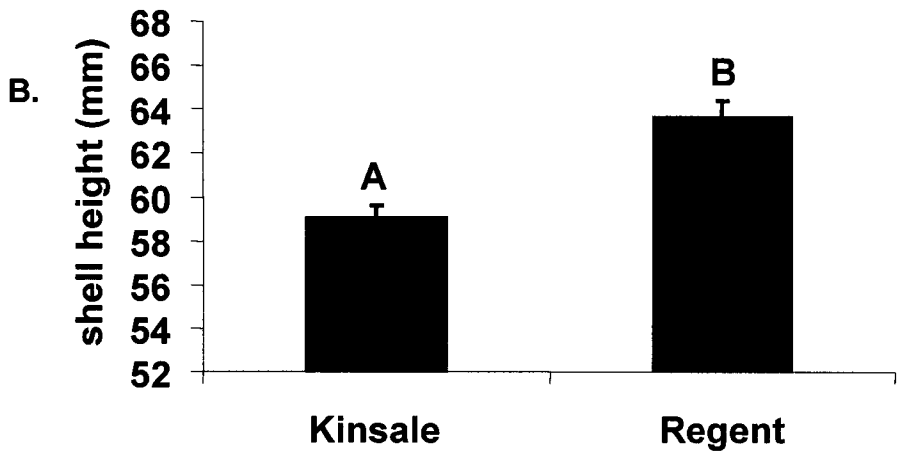


Figure 3. (A.) Changes in shell height over sampling month across treatments of stock and site. Differences in shell height due to month were significant at $p<0.0001$. Means are pooled across replicates of stock and site (n=24/month, except July 2003 (n=21)) (B.) Differences in mean shell height between sites ($p<0.0001$). Data are pooled across replicates of stock and month (n=83 at Kinsale; n=82 at Regent). (C.) Differences in mean shell heights among stocks ($p<0.0001$). Means are pooled across month and site. Mean CTS shell heights were significantly lower than CRB and XB shell heights ($p<0.05$, n=53 CRB, n=56 CTS and XB). CRB=Rappahannock River, CTS=Tangier Sound, XB=CrosBreed. All data are presented as mean ± s.e.m. Significantly different means ($p<0.05$) are indicated by different letters. Lines under each letter designate means that are not significantly different.





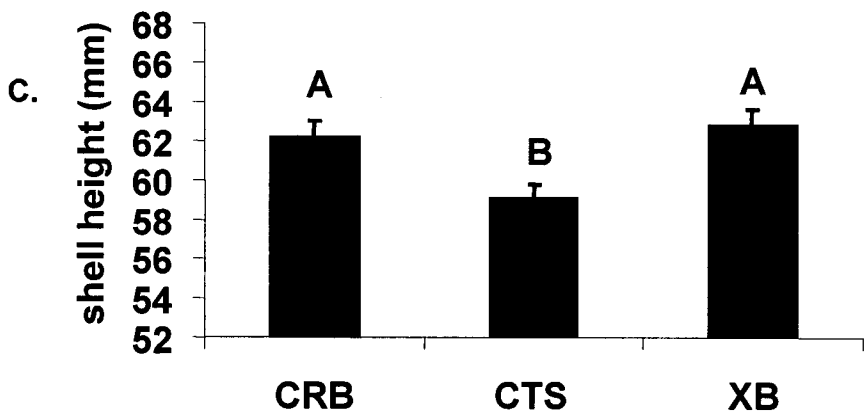


Figure 4. Changes in condtion index (CI) at sampling periods across stock and site ($p<0.0001$). Data are mean ± s.e.m. of 4 replicates (3 individuals measured per replicate)/stock/site). July 2003 CI values were significantly different from all other months, and indicated by an asterisk (*) ($p<0.05$).

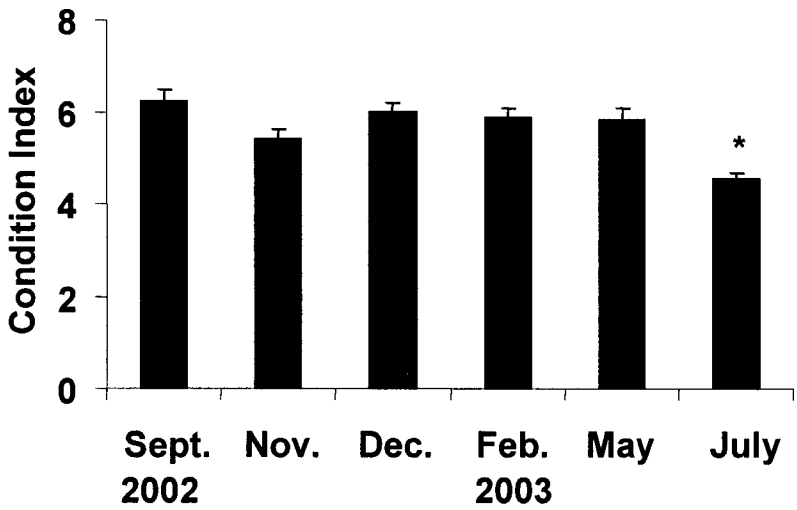
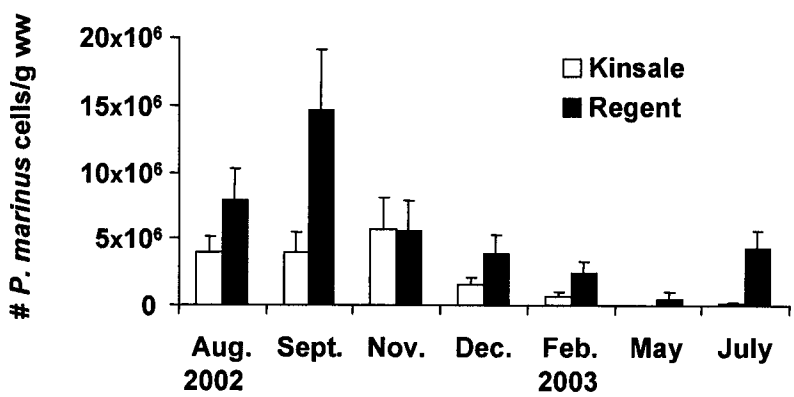


Figure 5. (A.) *Perkinsus marinus* infection intensities (number (#) of *P. marinus* cells / g wet weight (ww)) in *C. virginica* as a function of sampling month and site. Mean differences due to sampling period (month), site, and their interactive effects (month x site) were all significant ($p<0.05$). (B.) *P. marinus* infections as a function of stock. Means shown are pooled across month and site. Interactions between stock and month or site were not significant. CRB infections were the highest among the three stocks ($p<0.05$). CRB=Rappahannock River, CTS=Tangier Sound, XB=CrosBreed. All data are presented as means ± s.e.m. Significant differences ($p<0.05$) among means are indicated by different letters. Lines under each letter designate means that are not significantly different.

A.



B.

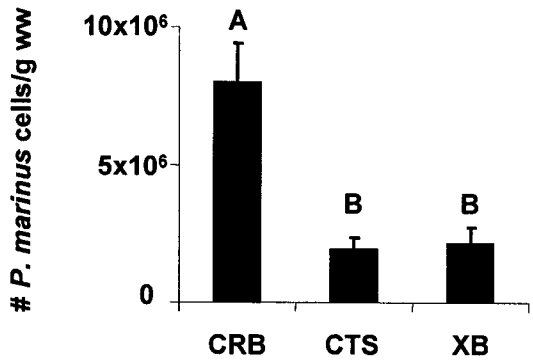


Figure 6. Levels of hsp70 measured using the slot blot assay. Data are mean hsp70 ± s.e.m. at four sampling periods (November 2002, February 2003, May 2003 and July 2003). Significantly different ($p<0.05$) means are indicated by different letters. Lines under each letter designate means that are not significantly different.

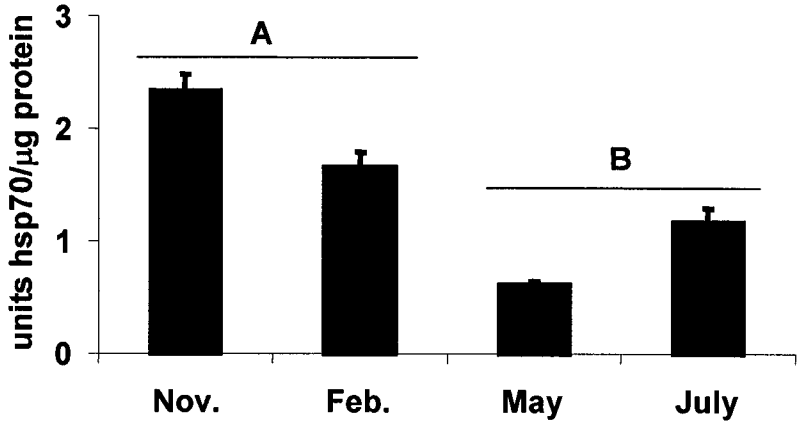


Figure 7. (A.) Representative western blot of *C. virginica* gill samples. Molecular weights (MW) of standards in kilodaltons (kDa) are shown on the left. (B &C.) Hsp70 in oyster gills from three *C. virginica* stocks. (CRB=Rappahannock River, CTS=Tangier Sound, XB=CrosBred) The reference sample is pooled gill tissue from 5 oysters heat shocked for 1 hour at 40 °C. The reference sample was run on each SDS-PAGE gel/blot to normalize band densities. 10 μg total protein of each sample and reference was run on each gel. Samples from different seasons are indicated below each blot. Note variation in expression of low MW (69 kDa) and high MW (kDa) bands between seasons. Purified hsp70 (50 ng) is also shown on each blot, indicating antibody specificity.
(D.) Comparison of specificities of two commercially available antibodies (clones 7.10 and 3A3, Affinity Bioreagents) for hsp70 isoforms in gill tissue of *C. virginica*. MW designations in kDa are indicated. Total protein (μg) of each sample is indicated below each sample. 7.10 detected 3 isoforms of 77, 72, and 69 kDa. 3A3 detected 2 isoforms of 72 and 69 kDa.

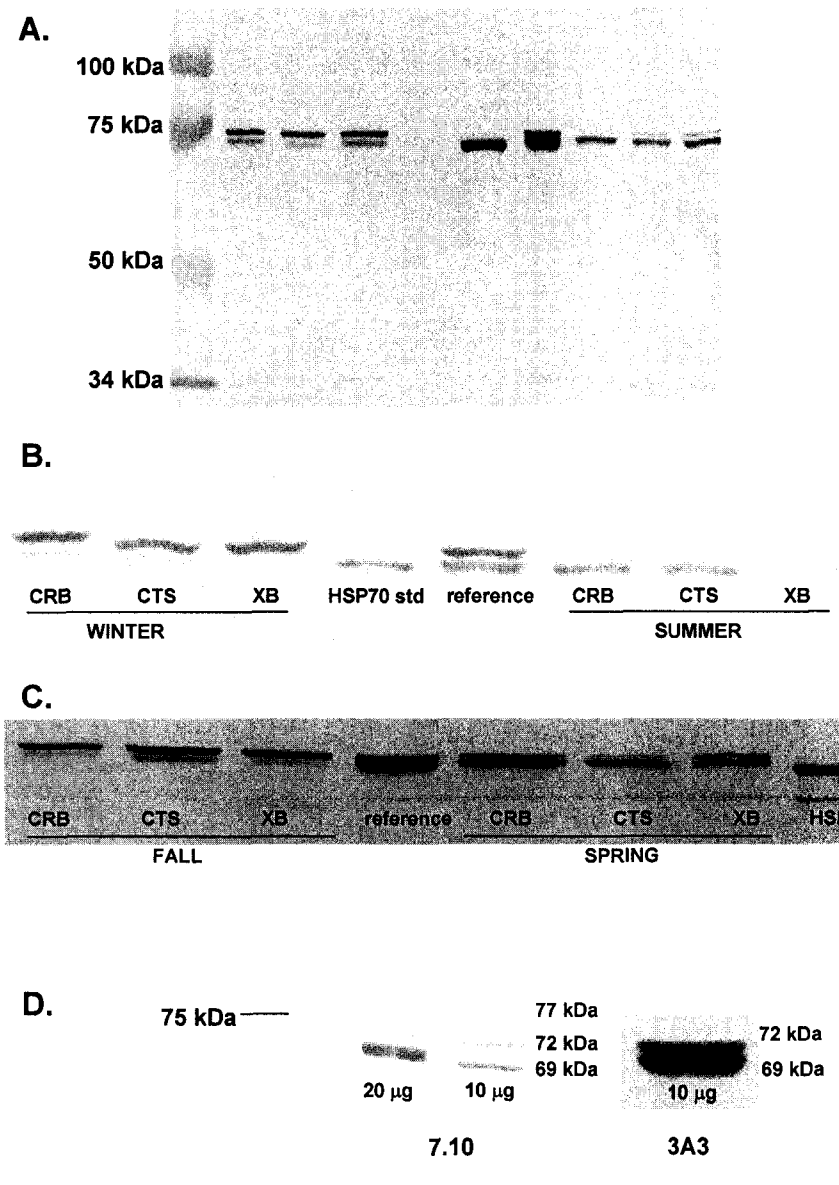
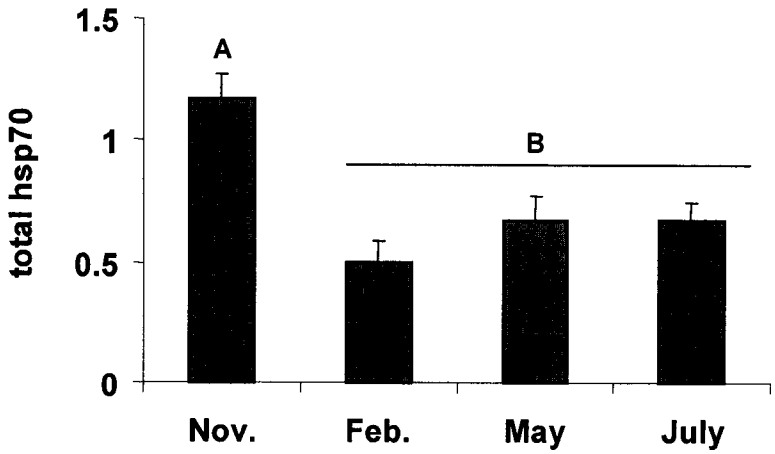


Figure 8. (A.) Total relative amounts of hsp70. Different letters denote means that are significantly different ($p<0.05$). Different letters indicate significantly different means. Lines under each letter designate means that are not significantly different. (B.) Levels of hsp70 isoforms (hsp69 and hsp72). Isoforms were compared independently. Different letters represent significantly different means ($p<0.05$).Lowercase letters indicate significantly different means among hsp69 levels. Uppercase letters represent significantly different means among hsp72 values. All data are means ± s.e.m. of 3-4 individuals/sampling/stock/site.

A.



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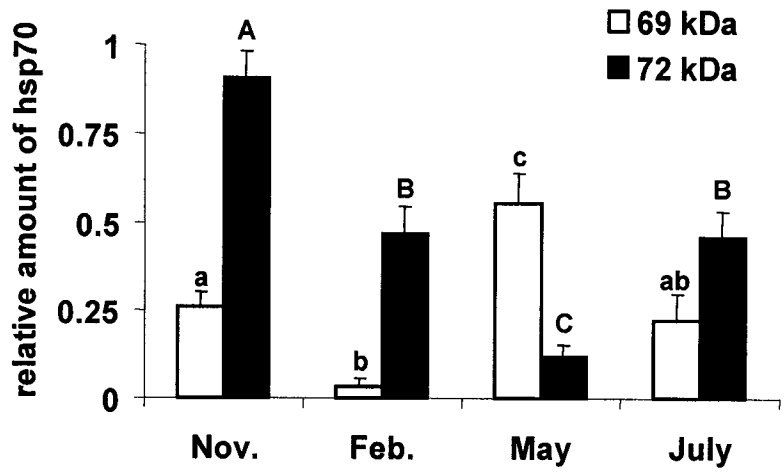
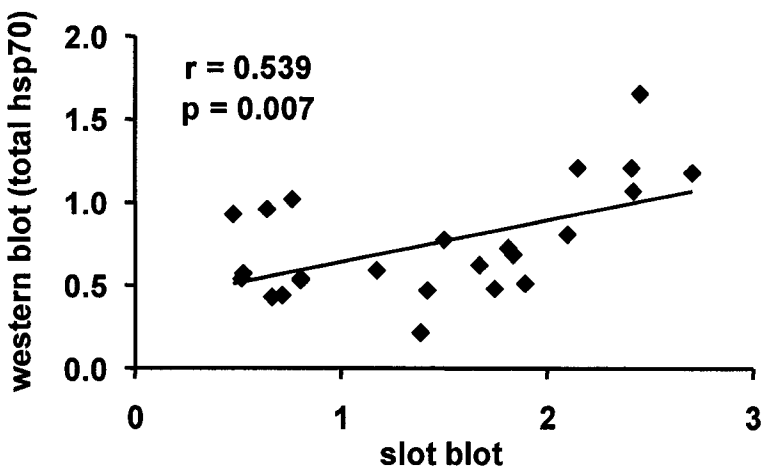


Figure 9. Comparison of slot blot hsp70 values and total hsp70 values calculated from western blot results. Means across month, site and stock were compared.



Chapter 4: The role of heat shock proteins (hsp70) in tolerance to disease (Dermo) in the eastern oyster, *Crassostrea virginica*.

Abstract

Over-expression of heat shock proteins is associated with increased tolerance to thermal stress and can confer protection to other stressors, such as pathogens. The role of heat shock proteins in protecting oysters from Dermo disease, caused by the protozoan pathogen *Perkinsus marinus,* was tested. Enhanced levels of heat shock protein 70 (hsp70) were induced experimentally by heat shock (37 °C, 1 hour) in the eastern oyster *Crassostrea virginica*. After heat shock, levels of hsp70 increased over time and remained elevated for up to two weeks. In addition, thermal tolerance was determined in two stocks of the eastern oyster, F0 progeny from Tangier Sound in the Chesapeake Bay (CTS) and F0 progeny from Oyster Bayou in Louisiana (LOB). In previous studies, both F0 and F1 generations from these two stocks were found to be Dermo-resistant. Lethal temperatures occurred at 43 and 44 °C. After a one hour heat shock of 43 °C, the CTS stock had lower mortality (30%) than the LOB stock (87% mortality), indicating that thermal tolerance differs between stocks. Levels of total hsp70, however, were not significantly different among heat shock temperatures, but total hsp70 was higher in the CTS stock at 43 °C than the LOB stock two days after the heat shock. To examine the interaction between the heat shock response and oyster disease, oysters were experimentally infected with *P. marinus*. When infections progressed to 10^4-10^5 cells/g wet weight, oysters were treated with a sub-lethal heat shock (40 °C, one hour) to determine the effects of infection on the heat shock response, and whether sub-lethal heat shock improved survival of infected and uninfected oysters to lethal heat stress (44 °C, one hour). Other treatments included heat shocked, uninfected oysters and non-heat shocked treatments (uninfected and infected). Acquired thermal tolerance was compared

among these treatments by administering a lethal heat treatment (44 °C, 1 hour). Sub-lethal heat shock enhanced survival to lethal heat treatment in both infected and uninfected oysters. Heat shock protein expression changed significantly over time. Although levels of hsp70 isoforms (hsp69 and hsp72) did not vary significantly by heat shock or infection status, responses due to these treatments were apparent when comparing hsp70 within infected and uninfected treatments. Infection induced expression of hsp69, regardless of whether oysters were heat shocked or not. In uninfected oysters, hsp72 increased due to heat shock 2 and 7 days post heat shock. This result demonstrates that heat shock can improve survival in oysters, even in oysters infected with *P. marinus*.

Introduction

Dermo disease, caused by *Perkinsus marinus* has devastated populations of the eastern oyster since the 1950's (Andrews 1988). Because *P. marinus* persists over a broad range of temperatures and salinities along the Atlantic and Gulf of Mexico coasts, the disease is particularly problematic (Burreson et al. 1994, Chu and LaPeyre 1993). Mortalities caused by *P. marinus* increase at temperatures above 25 °C as the parasite proliferates rapidly, causing extensive damage to host cells and tissues (Andrews 1965, Fisher et al. 1992, La Peyre et al. 1996). Although eastern oysters can tolerate high temperatures, the interactive effect of disease and thermal stress may be intense, leading to death. Dermo-resistant oysters have been identified and selectively bred, but mechanisms contributing to Dermo resistance are currently unknown (Allen et al. 1993, Calvo et al. 2003, Encomio et al., submitted – Chapter 2). Mechanisms such as heat shock proteins, which provide protection to cells and tissues, may be important in counteracting the dual stresses of temperature and disease in oysters.

Heat shock proteins act as molecular chaperones, facilitating proper folding, assembly and intracellular transport of proteins. They are highly conserved, occurring in virtually all organisms (Feder and Hofmann 1999). Heat shock proteins in the 70 kilodalton (kDa) family are the most commonly expressed heat shock proteins in response to stress (Lindquist 1986). Their activity aids in protecting organisms from thermal or other stress-induced damage (Lindquist and Craig 1988, Morimoto et al. 1990, Gething and Sambrook 1992, Gupta and Golding 1993). Enhanced tolerance to stress, resulting in improved survival and condition, has been correlated with increased levels of heat shock proteins (Bosch et al. 1988, Sanders et al. 1988).

In a wide variety of organisms, thermal tolerance can be enhanced experimentally via exposure to a sub-lethal heat shock (Lindquist 1986). Over-expression of heat shock proteins is associated with this phenomenon, known as acquired, or induced thermal tolerance (Sanchez and Lindquist 1990). Furthermore, application of sub-lethal heat shock has been shown to increase resistance to other stressors besides high temperature. For example, sub-lethal heat shock increased hsp70 and cadmium resistance in mussels (Tedengren et al. 1999). Increased expression of heat shock proteins due to sub-lethal heat shock and the acquisition of enhanced tolerance to stress, be it thermal or otherwise, are distinct characteristics of the heat shock response.

Oysters are commercially important estuarine species that are impacted by a variety of stressors (disease, summer mortality and toxicants). They are particularly subject to rapid and wide fluctuations in temperature. An understanding of the stress response in oysters may offer insights on how to improve survival and production of these species. The heat shock response has been studied in several species of oysters (Boutet, et al. 2002; Brown et al. 2004; Clegg et al. 1998; Hamdoun et al. 2003; Piano et al. 2003; Shamseldin et al. 1997). Increased levels of heat shock proteins were associated with improved thermal tolerance in Pacific oysters (Clegg et al. 1998). Levels of hsp70 increased after heat shock treatments of eastern oyster hemocytes, leading Tirard et al. (1995) to propose a potential mechanism by which immune function in oysters could be improved (Tirard et al. 1995). But, to our knowledge, no study has examined how the heat shock response (elevated expression of heat shock proteins and acquired thermal tolerance) in eastern oysters varies between oyster stocks from varying geographic origin or whether the heat shock response is affected by infection with *P. marinus*.

The objectives of the present study were to characterize the heat shock response in eastern oysters and to evaluate whether or not induced thermal tolerance and enhanced hsp70 expression in oysters was affected by *P. marinus* infection. Specifically, we first examined the duration of hsp70 expression after a sub-lethal heat shock. Secondly, we compared thermal tolerances in two stocks of the eastern oyster to determine if oyster stocks from different geographic areas varied in their thermal tolerances, and if hsp70 corresponded with those differences. Finally, we examined the effects of sub-lethal heat shock and *P. marinus* infection on induced thermal tolerance and expression of hsp70 in experimentally infected eastern oysters.

Materials and Methods

Duration of the heat shock response

Two experiments were conducted to test the duration of hsp70 expression in eastern oysters. Eastern oysters used in these experiments originated from the Damariscotta River, Maine (Pemaquid Oyster Company, Waldoboro, ME USA). Although *P. marinus* has been detected in oysters from Maine (Kleinshuster and Parent 1995), based on our experience, oysters from this area are rarely infected by it.

In the first experiment, oysters were acclimated for two weeks in a flow-through seawater flume. Temperature and salinity were recorded daily. During the course of the experiment water temperatures were 9-10 °C and salinities were 18-19 parts per thousand (ppt). Oysters were subjected to a sub-lethal heat treatment at 37 °C for 1 hr and returned to a flow-through seawater flume. This heat shock temperature was shown to cause an increase in hsp70 expression in preliminary experiments that we performed and in Pacific oysters (*C. gigas*) (Clegg et al. 1998). Corresponding control treatments of non-heat

shocked oysters were not included in this initial experiment. Oysters were sampled at 0, 1, 2, 4, 8, 10 and 15 days post heat shock (n = 6-7 / day sampled). Gill tissues were then analyzed for hsp70 by slot blot analysis (see following sections for methodology). A one-way Analysis of Variance (ANOVA) was performed to determine the effect of time (days post heat shock) on expression of hsp70.

In the second experiment, oysters from Maine (n = 150) were acclimated for two weeks in tanks containing 1.0 μm filtered York River water (YRW, salinity = 15-18 ppt) at 20-22 °C (ambient conditions), and fed daily (0.2g / oyster) with algal paste (Reed Mariculture). The water was changed three times a week. Temperature and salinity were recorded daily. After acclimation, oysters were subjected to a sub-lethal heat shock at 37 °C for 1 hr, and sampled at 0, 2, 7, 14, 21 and 30 days post heat shock (n=4-6 individuals sampled/day), and analyzed for hsp 70 levels. Non-heat shocked oysters served as controls. Three replicate tanks were used for each treatment. Gill tissues were dissected from individual oysters and stored at -80 °C until hsp 70 analysis. Data were analyzed by ANOVA for effects of heat shock (control vs. heat shock), time (days post heat shock) and their interactions. The Tukey's test for multiple comparisons was used to compare means when ANOVA was statistically significant. Data for both experiments are expressed as mean units of hsp70/μg protein ± the standard error of the mean (s.e.m.).

Comparisons of thermal tolerance in oysters from two geographic areas

This experiment determined if there was variation in thermal tolerance between stocks and if hsp70 expression corresponds to variation in thermal tolerance. Thermal tolerance and hsp70 expression over a range of temperatures (41-45 °C) was compared between two geographically disparate oyster stocks (Chesapeake Bay vs. Gulf of

Mexico). Oysters were collected from Port Kinsale in the Yeocomico River of the Chesapeake Bay during the early spring of 2002. Only *P. marinus* had been detected and not *H. nelsoni* at this site (Encomio et al., submitted – Chapter 2). Oysters were exposed to a range of temperatures (41, 42, 43, 44 and 45 °C) for one hour, and mortality was assessed one week after returning oysters to ambient conditions (20-21 °C, 24 ppt). At 44 °C and 45 °C mortality was 100% within 2 days. There were three replicate tanks for both stock and temperature treatments, each containing 10-13 oysters. The arcsine square root transformation was applied to proportional mortality data (Zar 1996). Data were analyzed by ANOVA for temperature treatments of 42, 43, and 44 °C, but not for the 41 and 45 °C, as mortalities were identical to the 42 and 44 °C treatments.

To assess hsp70 levels for each heat shock treatment and between stocks, one oyster was collected from each replicate tank at 0, 2 and 7 days post heat shock (n=3/day sampled). Hsp70 was assayed by slot blot in the gill tissues of these oysters. Data were log transformed to meet ANOVA assumptions of normality and homogeneity of variance. Factors compared by ANOVA were time, temperature, stock and their interactive effects. Multiple comparisons between treatments were analyzed by Student-Newman-Keuls test when ANOVA was significant. Data are presented as mean units hsp70/μg protein ± s.e.m.

Induced thermal tolerance and tolerance to *P. marinus* infection

This experiment determined the influence of parasitism by *P. marinus* on induced thermal tolerance and hsp70 expression. Oysters were subjected to a sub-lethal heat shock (40 °C, 1 hour) after challenge with *P. marinus* (~10^6 cells/oyster). A higher temperature was used than in the first two experiments (37 °C), because it was observed

that temperatures of 40 °C did not kill oysters. This temperature was chosen to ensure a maximal heat shock response.

Meront and merozoite stages of *P. marinus* were freshly isolated from infected oysters as described by Chu and Volety (1997). Briefly, infected oyster tissue was mechanically homogenized in 0.22 μm filtered York River water (YRW). The suspension was then passed through a series of filters (100, 50, 35 and 20 μm mesh sizes). The filtrate was then centrifuged and washed several times to remove any remaining tissue debris. Meronts were counted in a hemacytometer, the cell density adjusted with YRW (10 x 10^6 cells/ml), and oysters were inoculated (100 μl volume = 1 x 10^6 cells/oyster). Inoculation was performed by shell cavity injection through a notch made on the dorsal axis of the oyster shell. Uninfected oysters were inoculated with a sham treatment of 100 μl YRW (Chu and Volety 1997).

Four experimental treatments were arranged in a 2 x 2 factorial design with 3-4 replicate holding tanks for each of the treatments (18-20 oysters/tank). Treatments were sub-lethal heat shocked + infected (HS+I), non-heat shocked + infected (N+I), sub-lethal heat shocked + uninfected (HS+U), and non-heat shocked + uninfected (N+U) (Table 1).

To monitor progression of infection three oysters from two tanks, each containing 24 oysters, were sampled weekly for *P. marinus*. Sub-lethal heat shock to enhance hsp70 and induce thermal tolerance was administered to experimentally infected and non-infected oysters 8 weeks after challenge with *P. marinus*, when oysters attained moderate to high infections (~10^4-10^5 cells/g wet weight). Moderate to high densities of

infection can be obtained typically 60 days post challenge with 2.5 x 10^4 cells (meront stage) per oyster (Chu and Volety 1997).

Hsp70 and *P. marinus* infection in all treatments (HS+I, N+I, HS+U, N+U) were sampled in oysters 0, 2, and 7 days post-heat shock and analyzed to determine the effect of heat shock on hsp70. Hsp70 was analyzed by western blot. Three oysters from each tank were removed at each sampling.

After oysters were sampled on day 7, all treatments were subjected to a lethal heat treatment (LHT) of 44 °C for 1 hour and mortality was monitored for 1 week. The number of oysters remaining in each tank at the time of the LHT ranged between 8-11 individuals per tank. The chosen temperature for the LHT was based on the thermal tolerance experiment described previously. Surviving oysters were sampled for hsp70 and *P. marinus* one week after LHT (14 days post sub-lethal HS). *Perkinsus* infections were assessed by the body burden enumeration technique (Choi et al. 1989, Bushek et al. 1994).

Proportional survival data were arcsine-square root transformed and analyzed by ANOVA for effects of heat treatment (heat shocked vs. non-heat shocked oysters), infection status (infected vs. uninfected oysters) and their interaction. In addition to ANOVA, mortalities were compared by survival analysis using the product-limit (Kaplan-Meier) method (Cox and Oakes 1980, Newman 1995). Analysis was performed using the LIFETEST procedure in SAS. This procedure calculates log-rank and Wilcoxon statistics, which test if time-to-death is the same for each treatment.

Relative amounts of hsp70 isoforms and *P. marinus* data were log transformed to meet assumptions of normality and homogeneity of variance. Data were analyzed by repeated

measures ANOVA for factors of treatment (HS - heat shocked or N - non-heat shocked), infection status (I - infected or U - uninfected), day (0, 2, 7 and 14 days post sub-lethal heat shock) and their interactions. Aquaria sampled over time in each treatment were treated as a within subjects random factor.

Detection of Hsp70

Both slot blot and western blotting techniques were used to detect hsp70. The slot blot method (Lewis et al. 1999, Cruz-Rodriguez and Chu 2002) was used for the experiments characterizing the duration of hsp70 expression and the comparisons of thermal tolerance between oyster stocks. Gill tissues were excised, freeze-dried for 48 hours, weighed and stored at -80 °C. Gill tissues were then homogenized on ice in 2 ml of buffer (66 mM Tris pH 7.2, 3% Nonidet, 0.1 mM PMSF). The homogenate was centrifuged at 10,000 x g for 30 minutes at 4 °C, and the supernatant (gill extract) collected. Total protein concentration was determined using a modified version of the Lowry assay (Biorad DC Protein Assay, Lowry et al. 1951).

Slot blot detection of hsp70

Total hsp70 isoforms in oyster gills were detected with a monoclonal antibody raised against human hsp70 (Affinity Bioreagent, 3A3) and total hsp70 quantified using the slot blot assay (Cruz-Rodriguez and Chu 2002, Lewis et al. 1999). Samples were loaded in triplicates. Unknown samples were diluted to 1.5 μg total protein and vacuumed onto nitrocellulose. A standard concentration gradient (0.25, 0.5, 1, 1.5, 2, and 2.5 μg total protein) from a "reference sample", obtained by exposing an oyster to a 1 hour heat shock at 40 °C, was also added to each blot. The blot was removed and blocked with 5% bovine serum albumin (BSA) in Tween-Tris-buffered saline (TTBS - 0.05%

Tween, 30 mM NaCl, 24 mM Tris pH 7.5) for 30 minutes followed by two washes in Tris-buffered saline (TBS - 30 mM NaCl, 24 mM Tris pH 7.5) for 5 minutes each. Primary monoclonal antibody against Hsp70 (clone 3A3 - catalog # MA3-006, Affinity Bioreagents) was applied for 90 minutes (1:5000 dilution), followed by two 5 minute washes with TBS. A secondary antibody (Goat Anti-Mouse AP conjugated) was applied for 90 minutes (1:1000 dilution), washed once in TBS, and placed in a developing solution containing NBT (*p*-Nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). Bands developed between 30-60 minutes. The blot was stored in deionized water until analysis. Densitometric analysis of developed slot blots was performed using Enprotech scanner and software. The areas of the samples were recorded and normalized to the area of the reference sample in each blot to account for inter-blot variation.

SDS-PAGE and Western Blot Detection

A portion of the gill extract was diluted 1:2 in Laemmli sample buffer (BioRad) for SDS-PAGE. Samples were boiled for 5 minutes and 10 μg total protein per sample was electrophoresed on 8% polyacrylamide gels (150 V, 90 minutes). Separated proteins were then transferred onto nitrocellulose membrane at 100 V for 1 hour in transfer buffer (192 mM glycine, 24 mM Tris base and 20% methanol). After transfer, nitrocellulose blots were processed for immunodetection of hsp70 isoforms as previously mentioned. Blots were scanned and analyzed as previously described. Relative amounts of hsp70 were obtained by normalizing band densities to the "reference" sample described previously. Relative amounts of two isoforms of 69 and 72 kilodaltons (kDa) were

normalized to the corresponding isoform in the reference sample. As with the unknown samples, 10 μg of the reference sample was added to each gel.

Diagnosis of P. marinus (Dermo) infection

In the first three experiments (Duration of heat shock response 1 and 2, and thermal tolerance experiments) presence and intensity of *P. marinus* infection was checked by fluid thioglycollate medium (FTM) diagnosis according to Ray (1954). Briefly, rectal tissue from an oyster was incubated in FTM for 5-7 days in the dark. Squash preps of the tissues were prepared on glass slides and stained with 1-2 drops of Lugol's solution. Diagnosis of infection was performed by examining for presence of stained, enlarged *P. marinus* hypnospores. Intensities of infection were classified as Light, Moderate, or Heavy (Mackin 1962).

In the induced thermal tolerance experiment prevalence and intensity of *P. marinus* infection in oysters was determined using total body burden assessment (Choi et al. 1989, Bushek et al. 1994). Oyster tissue was weighed and mechanically homogenized in 0.1 M sodium phosphate buffer (Ultraturrax). A 1.0 ml tissue aliquot was incubated in alternative fluid thioglycollate medium (AFTM) for 5-7 days at room temperature. Tissue suspensions were then centrifuged at 800 x g for 10 min. Tissue pellets were re-suspended in 2M NaOH and incubated overnight at 60 °C. Tissue pellets were washed, centrifuged, and re-suspended in distilled water. Aliquots of 100 μl from each sample were added to a 96-well plate and stained with 1-2 drops of Lugol's solution (1:9 dilution). Stained *P. marinus* cells were counted under an inverted microscope at 400x magnification. Results are expressed as log (number (#) of *P. marinus* cells + 1)/g wet tissue weight (ww).

Results

Duration of hsp70 expression

Results of the first experiment showed that elevated expression of hsp70 were significantly higher ($p < 0.0001$) at 8 days post-heat shock and remained so for up to 15 days (see Fig. 1). In the second experiment, levels of total hsp70 were significantly affected by time ($p < 0.0001$) and heat shock ($p = 0.031$). Interactive effects (time x heat shock) were also significant ($p < 0.0001$). Both heat shocked and non-heat shocked oysters exhibited elevated levels of hsp70 in gill tissues at 7, 21, and 30 days post-heat shock (Fig. 2). The mean hsp70 levels were higher in heat shocked than non-heat shocked oysters at 7, 14, and 30 days post-heat shock.

Thermal tolerance experiments

Thermal tolerance comparisons between CTS and LOB oysters showed significant effects due to stock ($p = 0.002$), temperature ($p < 0.0001$) and their interaction ($p = 0.003$). No mortality in the CTS and LOB stocks was observed after heat shocks at 41 and 42 °C. At 43 °C, mortality was seen in both groups with the LOB stock exhibiting significantly higher mortality (86.7 ± 8.8 %, $n = 3$ replicate tanks) than the CTS stock (30.0 ± 5.8 %, $n = 3$). At 44 °C, mortality was 96.7 ± 3.3 % for the CTS stock and 100 % for the LOB stock. Mortality was 100% for both groups at 45 °C (Fig. 3).

Levels of hsp70 were significantly affected by time ($p = 0.012$) and interactive effects between time and temperature ($p = 0.017$). Hsp70 significantly increased at 7 days post heat shock at 41 and 42 °C and decreased at 43 °C in both CTS and LOB stocks (Fig 4A). Effects of stock, temperature, and all interactive effects, except time x temperature effects, were not significant. Although the effect of stock was not

significant, CTS oysters contained higher amounts of hsp70 (0.694 ± 0.243 units hsp70/μg protein, n=3) than the LOB oysters (0.483 ± 0.084 units hsp70/μg protein, n=3) two days after a heat shock of 43 °C (Fig. 4B and 4C). Using the standard FTM assay (Ray 1954), *Perkinsus marinus* infections were not detected in any oysters.

Interaction between induced thermal tolerance and Dermo disease

Oysters experimentally infected with *P. marinus* had mean infection densities of $7.76 \times 10^3 \pm 0.70$ cells/g wet weight (ww) 60 days after inoculation. *Perkinsus marinus* was also detected in presumably uninfected oysters, but infections were significantly lower than experimentally infected oysters ($p < 0.0001$, $n = 93$) (Fig. 5). Further, these oysters were unaffected by heat shock treatment (Fig. 6). Prevalence of infections among oysters that were not experimentally infected was 32.7% and mean densities of infection were 9.23 ± 0.84 cells/g ww over the course of the experiment. Prevalence among the experimentally infected oysters was 100% and mean densities were $6.38 \times 10^4 \pm 3.85$ cells/g ww.

Induced thermal tolerance was observed in oysters subjected to a sub-lethal heat shock (40 °C) prior to a lethal heat stress of 44 °C. Survival was significantly elevated in HS treatments compared to non-heat shocked treatments (ANOVA: $p = 0.013$, $n = 3$-4 replicate tanks of 8-11 oysters each). Effects of infection were not significant and survival was similar between infected and uninfected oysters (Fig. 7).

Survival analysis also showed that sub-lethal heat shock significantly improved survival compared to oysters that were not heat shocked (Log-rank test: $p<0.0001$, Wilcoxon test: $p<0.0001$) (Fig. 8). Infection status did not affect survival after lethal heat shock. Within infection status, effects of heat shock on survival were significantly higher

in oysters not subject to heat shock (Log-rank test: p=0.0002, Wilcoxon test: p=0.0003). Heat shock also improved survival within uninfected oysters (Log-rank test: p=0.033, Wilcoxon test: p=0.035). The effect of infection within heat shocked and non-heat shocked oysters was also compared within heat treatment. The effect of infection was not significant, although a trend was apparent in heat-shocked oysters (p=0.054 for both log-rank and Wilcoxon tests). In that comparison, infected oysters exhibited higher survival than uninfected oysters (100% vs. 90.3 ± 5.8 %, n=4 tanks for infected oysters, n=3 tanks for uninfected oysters).

Western blot analyses showed variation in expression of hsp70 isoforms. Two molecular weight isoforms were detected at 69 kDa and 72 kDa as previously described (Encomio and Chu 2004, submitted; Chapter 3). The 69 kDa isoform (hsp69) varied significantly by day ($p<0.0001$) but not by heat treatment or infection status. Levels of hsp69 increased significantly from day 0 to day 2, 7 and 14 ($p<0.05$, Fig. 9A). Interactions between heat treatment, infection status and day (HS/N x U/I x day) were significant (p=0.020) in the 72 kDa (hsp72) isoform, but main effects (HS/N, U/I, and day separately) were not significant. Total relative amounts of hsp70, assessed by western blot, increased significantly over time (p=0.007). Interactions between heat treatment, infection status and day were significant (p=0.043). Significant interaction effects in hsp72 and total hsp70 were likely attributed to high variability between individuals. The mean total hsp70, as assessed by western blot, were higher in heat-shocked oysters, but not statistically significant (p=0.057, Fig. 9B). Effects of infection status on total hsp70 were not significant.

Western blotting showed that there was an increase in hsp69 over time, which accounted for the change in total hsp70. Although ANOVA did not detect significant differences due to heat shock or infection, there were apparent differences in isoform expression of hsp70 when examining infected (HS and non-heat shocked - N) and uninfected (HS and N) treatments separately. Among infected oysters hsp69 increased over time in both heat shocked and non-heat shocked oysters (Fig. 10A). Infection alone (N+I treatment) appeared to induce expression of hsp69, similar to those that were heat shocked (HS+I). In uninfected oysters hsp69 was higher over time in heat shocked individuals (Fig. 10B). Infection alone appeared to have a greater effect on hsp72 levels than combined effects of heat shock and infection. Hsp72 decreased after lethal heat treatment in both treatments (Fig. 11A and 11B). In uninfected oysters, heat shock increased expression of hsp72.

Discussion

A sustained, elevated expression of heat shock proteins after exposure to acute thermal stress is an almost universally characteristic of the heat shock response (Feder and Hofmann 1999, Lindquist 1986, Lindquist and Craig 1988). The length of the response may be dependent on the severity of the heat stress (Lindquist 1986). In *C. virginica*, we observed that total hsp70 remained elevated in gill tissues for 2-4 weeks after heat shock. This also occurred in *C. gigas* and the flat oyster *Ostrea edulis*, in which elevated levels of hsp70 persisted for 2 weeks (Clegg et al. 1998, Piano et al. 2002). In several bivalve species increased levels of heat shock proteins are seen after a distinct period of time (~48-96 hours after heat shock). This has been observed in mussels - *Mytilus edulis*, and several species of oysters - *C. gigas*, *Ostrea edulis* and *C.*

virginica (Chapple et al. 1997, Clegg et al. 1998, Piano et al. 2002, Cruz-Rodriguez 2001, Tirard et al. 1995). During this period thermal tolerance is enhanced. Pre-treatment of *M. edulis* with high temperature resulted in increased heat resistance after 3 days, and induced thermal tolerance was highest 2 days after heat shock in *C. gigas* (Huppert and Laudien 1980, Clegg et al. 1998). The elevated expression of hsp70 in *C. gigas* was associated with an enhanced thermal tolerance that lasted up to 14 days (Clegg et al. 1998). Although thermal tolerance was not tested in the first two heat shock experiments, we showed in our later experiment that a sub-lethal heat shock improved resistance to thermal stress in *C. virginica* for at least 7 days. The implication for elevation of hsp70 and thermal tolerance is that both eastern and Pacific oysters possess the capacity to resist multiple bouts of thermal stress over prolonged periods. The reasons for sustained elevation of heat shock proteins are unknown, but it is speculated that heat shock may disrupt protein synthesis and the mechanisms controlling the regulation of hsp synthesis, resulting in reduced turnover of heat shock proteins (Hochachka and Somero 2002). Additionally, hsp70 mRNA may be stable for several days (Hoffman 1999).

Our results suggest that the LT_{50} (temperature at 50% survival) lies between 43 and 44°C, similar to that of *C. gigas* (Shamseldin et al. 1997). Interestingly, there was significant variation in thermal tolerance between the Tangier Sound (CTS) and Louisiana (LOB) stocks. Total hsp70 was not significantly different between stocks, but the CTS had higher amounts of hsp70 two days after exposure to 43 °C, when mortalities were significantly different between the two stocks. These stocks originate from latitudinally distinct populations, and so their thermal tolerance ranges might vary. Given its range, the more southern LOB stock would be more thermotolerant than the CTS

stock. Oysters from these stocks had been spawned and reared under common conditions, however, and thus had identical thermal histories. Because of this, differences in survival suggest a genetic component to their thermal tolerance ranges, which could lead to development of strains more tolerant to thermal stress. Development of improved thermal tolerance in aquaculture species is being developed in other species such as trout (Danzmann et al. 1999). Because the heat shock response results in increased tolerance to other stressors, more thermotolerant strains may be resistant to other stresses as well.

During low tide exposure, the rate of heating would be expected to be much slower than the heat shocks applied in our experiments. Although heat shock is not a simulation of natural conditions, the absolute temperatures (>40 °C) and the length of exposure (≥1 hour) tested in the present experiment are within the range of conditions that eastern oysters might experience in their native habitats. Intertidal eastern oysters can experience temperatures from 46-49 °C and even up to 55 °C during low tide exposure (Galtsoff 1964; Willson and Burnett 2000). Thus, tolerance ranges seen in our experiments are reflective of the thermal tolerances of these stocks in the field.

Chesapeake Bay oysters occupy a wider thermal range than oysters from the Gulf of Mexico and may have a higher tolerance to overall stress than oysters from a more thermally stable environment. During growout, Louisiana oysters experienced high mortalities during cold aerial exposures, while Chesapeake stocks did not, suggesting a reduced capacity to resist more extreme environmental conditions (Encomio et al. 2004, submitted; Chapter 2). Differences in size may have also affected differences in thermal tolerances. The LOB oysters were larger than the CTS oysters and grew to significantly greater shell heights (Encomio et al. 2004 - Chapter 2). As shown in mussels, large

animals may also lose heat more slowly; due to greater tissue mass (Helmuth 1998). If, like mussels, larger oysters lose heat at a slower rate, equilibration of tissues to recovery temperatures would be slower compared to smaller oysters. Although oysters gaped after heat shock, comparative rates of heat loss should still be slower in larger oysters. Larger oysters also may have had a greater metabolic demand for repair of proteins during recovery from heat shock. Size effects on thermal resistance in oysters must be examined further, as this may affect survival of fast-growing oyster strains during periods of high thermal stress.

We demonstrated that sub-lethal heat shock improved thermal tolerance in *C. virgnica*, in a manner similar to *C. gigas* (Shamseldin et al. 1997, Clegg et al. 1998, Hamdoun et al. 2003). We expected that *P. marinus* infections would increase susceptibility to lethal heat stress. In MSX-infected oysters acute elevation of temperature from 20 °C to 30 °C increased oxygen consumption and ammonia production compared to uninfected oysters (Littlewood and Ford 1990). Challenge with the bacterium *Nocardia* reduced thermal tolerance in *C. gigas* (Friedman et al. 1999). Other parasites, such as trematodes, reduce the resistance of gastropods to extreme changes in temperature and salinity (Lauckner 1980). In the present study, however, infection did not reduce thermal tolerance. The level of protection conferred by heat shock may have overridden any deleterious effects of infection on thermal tolerance. The heat shock temperature also may have affected function in *P. marinus*. Heat treatments of 40 °C and higher have been found to reduce the viability of cultured *P. marinus* (Soudant, pers. comm.). High temperatures that oysters are capable of withstanding may limit the growth of *P. marinus*. Infection did not reduce condition, as there were no differences in

condition index between infected and uninfected oysters. Physiological condition in oysters is only significantly reduced in advanced infections (Dittman et al. 2001). Infection intensities did reach levels (10^4-10^5 cells/g wet weight) that would be expected to affect oyster physiology. However, physiological effects of *P. marinus* infection have been difficult to demonstrate, and are strongly influenced by season, environment and reproductive state (Newell 1994, Craig et al. 1989, Dittman et al. 2001, Encomio et al., submitted – chapter 2).

Infection with *P. marinus* may have also contributed to the increase in hsp69. Pathogens have been shown to induce expression of heat shock proteins in various host species. Levels of hsp70 increased in livers and kidneys of coho salmon when artificially infected with *Renibacterium salmonarum* (Forsyth et al. 1997). House martins parasitized by triatonid bugs and trypanosomes had increased levels of hsp 60 in their blood (Merino et al. 1998). The nematode *Trichinella spiralis* induced elevated expression of hsp 25 and 70 in rats (Martinez, et al. 1999). The increase in hsp69, however, was not indicative of an increase in thermal tolerance, as non-heat shocked oysters experienced greater mortalities than those that were heat shocked, regardless of differences in heat shock protein expression. The increase in hsp69 may not substantially contribute to thermal tolerance and may be an indicator of stress, but not stress resistance. Additionally, heat shock protein expression and thermal tolerance are not always tightly coupled (Easton et al. 1987). Other heat shock proteins and other mechanisms, such as changes in membrane stability may contribute to thermal tolerance.

As shown previously (Encomio and Chu 2004, submitted – chapter 3), isoform expression varied and was not identified strictly as inducible (hsp69) or constitutive

(hsp72), as shown in previous studies (Clegg et al. 1998, Brown et al. 2004, Piano et al. 2003, Boutet et al. 2002, Hamdoun et al. 2003). In our experiments hsp69 was expressed in a constitutive manner, and present in almost all samples and treatments. Differences in hsp69 between treatments were due to an increase in levels, and not induction of expression, which implies *de novo* synthesis of this isoform. Furthermore, hsp69 was predominantly expressed over hsp72 (Fig. 12). These differences may be due to differences in antibody specificity. The antibody clone (7.10, Affinity Bioreagents Boulder, CO) used in other studies recognized three isoforms of hsp70 (69, 72 and 77 kDa), with the 69 kDa isoform characterized as inducible upon heat shock (Clegg et al. 1998, Boutet et al. 2002, Hamdoun et al. 2003, Piano et al. 2003, Brown et al. 2004). Although the antibody we used (clone 3A3 Affinity Bioreagents) recognized a 69 kDa isoform, it is possible this isoform, is still different from the inducible isoform identified in the preceding studies. Although similar in molecular weight, isoforms may have different isoelectric points, resolvable only by two-dimensional electrophoresis. If hsp69 is more heat sensitive, predominant expression may be a result of using Maine oysters in our experiments, which would be expected to be more sensitive to temperature than Chesapeake Bay oysters. Control temperatures in our experiments (~20 °C) approach the maximal aquatic temperatures (25 °C, C. Davis, Pemaquid Oysters personal communication) that Maine oysters experience in their native habitat.

In the present study we have shown that the heat shock response can be sustained for greater than 2 weeks. Prolonged over-expression of heat shock proteins may be indicative of how long oysters can remain tolerant to stress, and whether that resistance can be artificially enhanced. We also show that thermal tolerance varies between two

oyster stocks and may be an important selectable trait in breeding new strains. Lastly, we show that sub-lethal heat shock improved survival in oysters. Thermal tolerance was enhanced even in oysters infected with *P. marinus*, showing that the heat shock response was not negatively affected by disease. This is important as heat shock could be utilized as a way to improve survival in diseased oysters. Techniques to harden animals, such as intertidal culture, are often employed to improve survival of oysters and reduce fouling (Ventilla 1984, Handley and Bergquist 1997). Selective breeding may result in strains with higher stress tolerance, which may be more amenable to these methods of culture. An understanding of the heat shock response and its role in thermal and pathogenic stress is important to improve survival in cultured bivalves.

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Table 1. Treatment combinations for experiment comparing the effects of *P. marinus* infection on acquired thermal tolerance and hsp70 in gill tissues of the eastern oyster *Crassostrea virginica*. The four treatments were: heat shocked + infected (HS + I), heat shocked + uninfected (HS + U), non-heat shocked + infected (N+I) and non-heat shocked + uninfected (N+U).

		Heat treatment	
		Heat shocked (HS)	Non-heat shocked (N)
Experimental infection	Infected (I)	**HS + I**	**N + I**
	Uninfected (U)	**HS + U**	**N + U**

Figure 1. Levels of gill hsp70 over time in *C. virginica* after a one hour heat shock of 37 °C delivered on day 0. Data shown are mean units of hsp70/μg protein ± standard error of the mean (s.e.m.), n=6-7 oysters sampled/day. Lines over bars indicate means that are not significantly different. The letters A and B over the lines indicate groups of means that are significantly different ($p<0.05$).

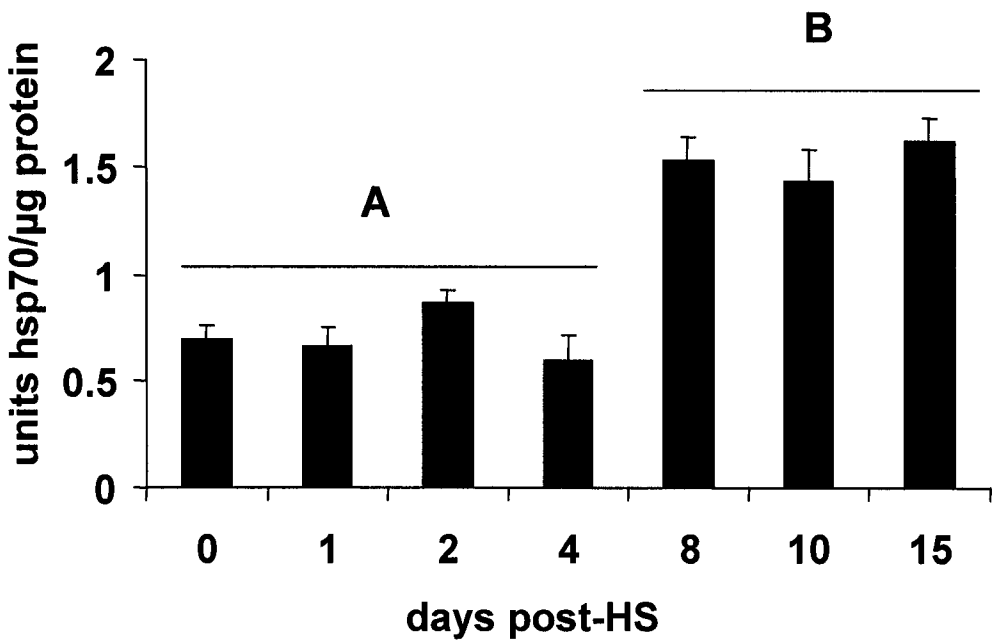


Figure 2. Second experiment showing levels of hsp70 over time in relation to heat shock. Non-heat shocked controls were also included in the experimental design. Data are mean units hsp70/μg protein ± s.e.m. n=6 oysters/treatment.

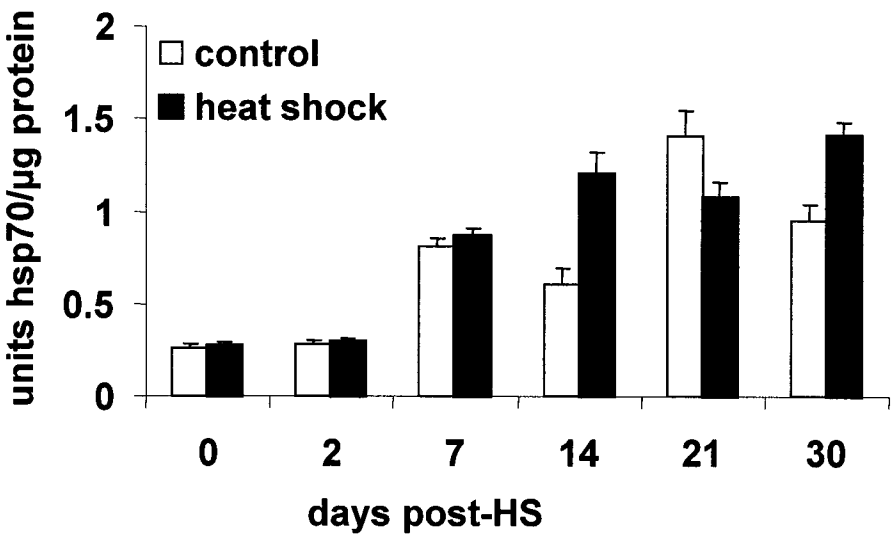


Figure 3. Comparison of thermal tolerances for two oyster stocks (Tangier Sound, Chesapeake Bay - CTS and Oyster Bayou, Louisiana - LOB). Data shown are mean % mortalities ± s.e.m. of three replicate tanks of 10 oysters.

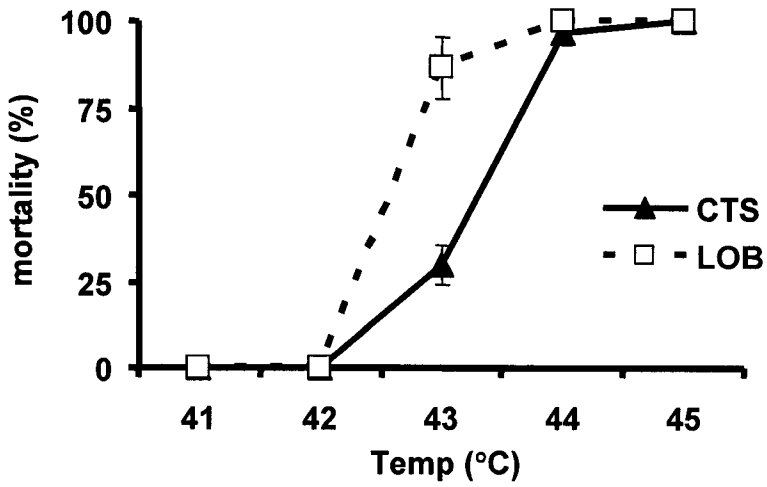
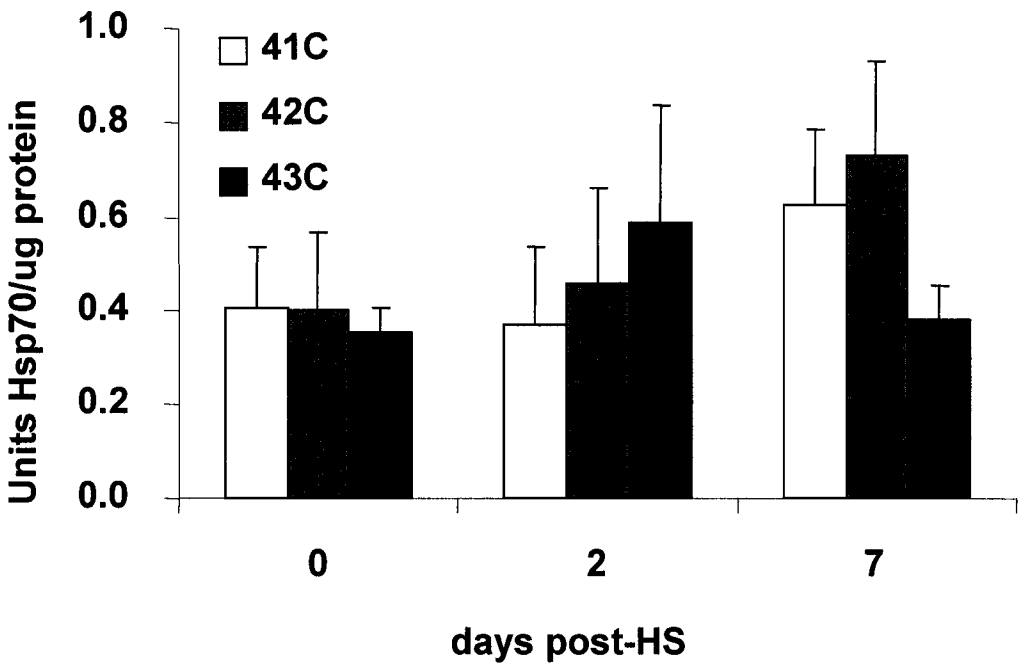
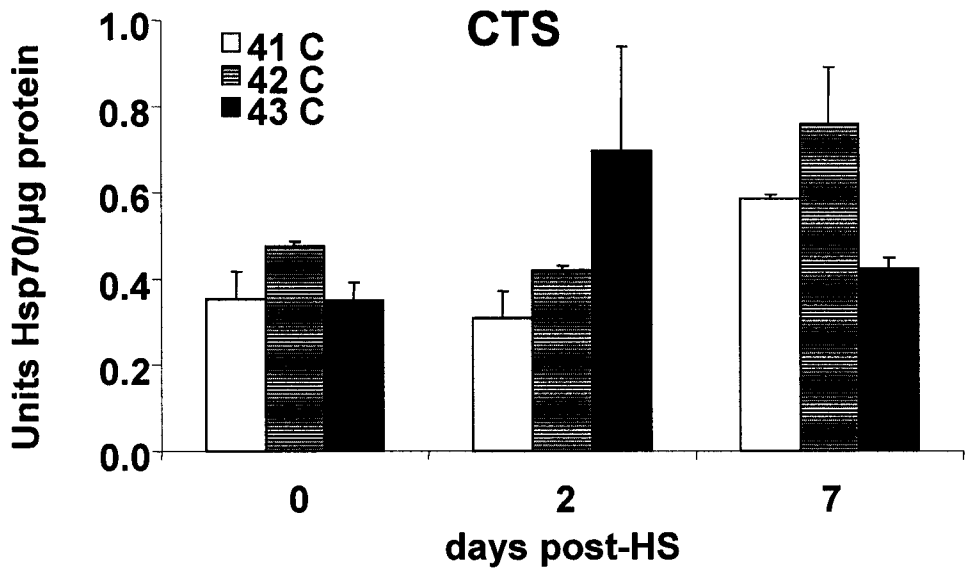


Figure 4. (A) Levels of hsp70 over time in gill tissues after exposure to heat shock at 41, 42 and 43 °C for one hour. Levels of total hsp70 in gills from CTS (4B) and LOB (4C) oysters after one hour heat shocks at 41, 42 and 43 °C. Data are mean units of hsp70/μg protein ± s.e.m (n=3).

A.



B.



C.

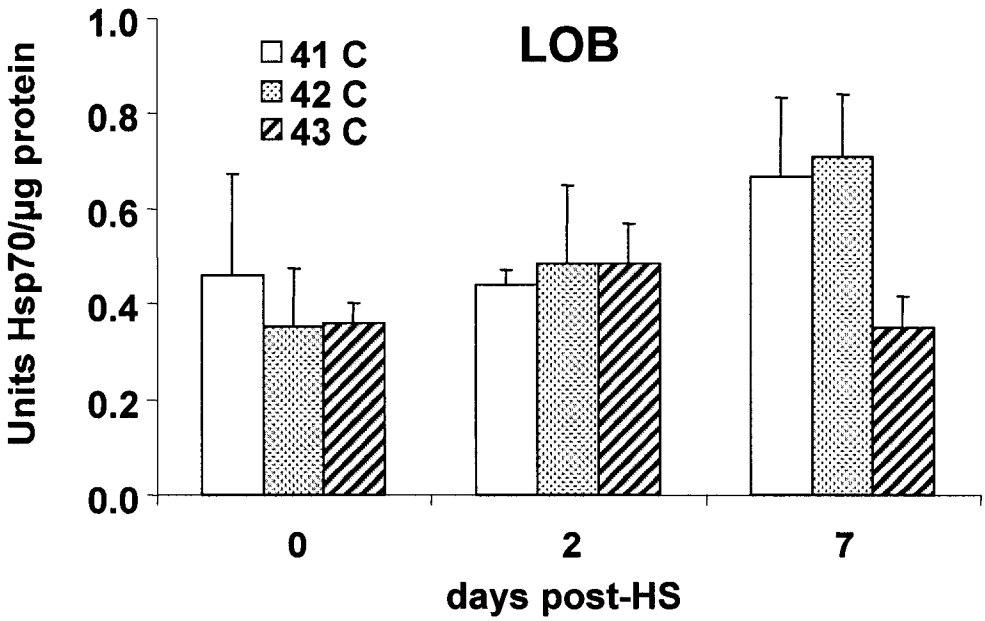


Figure 5. Densities of infection by *Perkinsus marinus* in experimentally infected oysters and non-infected controls in relation to induced thermal tolerance. Data are presented as log # *P. marinus* cells/ g wet weight (ww) ± s.e.m. (n= 93 oysters for each infected and uninfected treatments). Oysters were subjected to a lethal heat treatment (LHT) for one hour at 44 °C) just after the day 7 sampling. Infected oysters were evident in the controls, albeit at significantly lower levels than experimentally infected oysters ($p < 0.0001$).

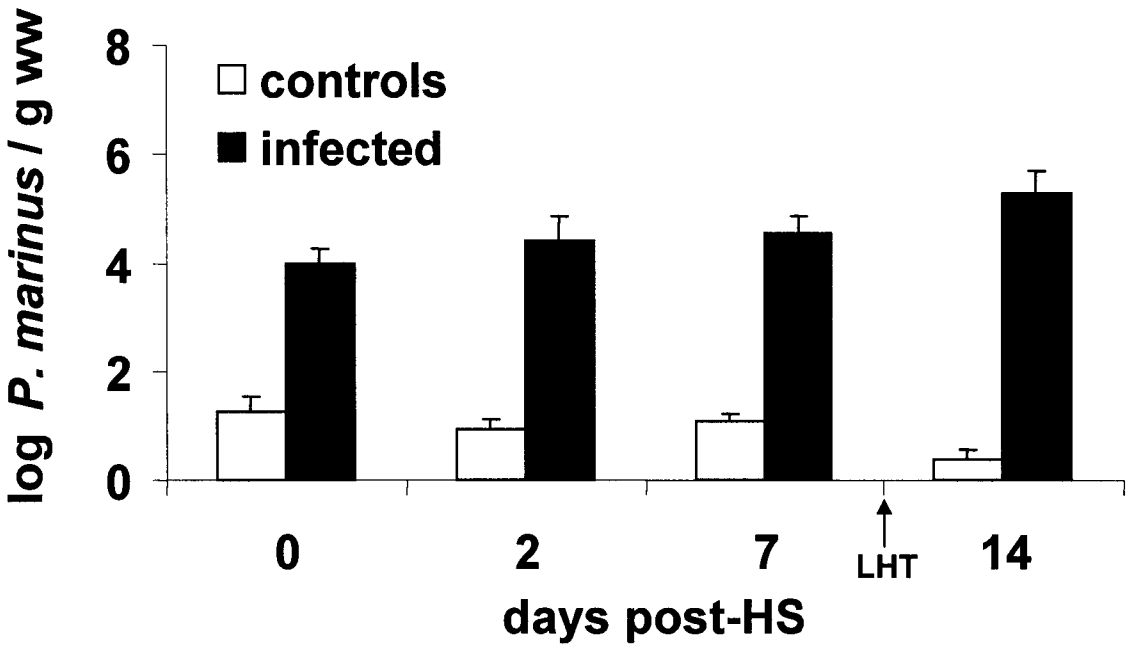


Figure 6. Densities of *P. marinus* in heat shocked and non-heat shocked oysters. Infection intensities are shown as log # *P. marinus* cells/ g wet weight (ww) ± s.e.m. (n = 12). A lethal heat treatment (LHT) was applied for one hour at 44 °C.

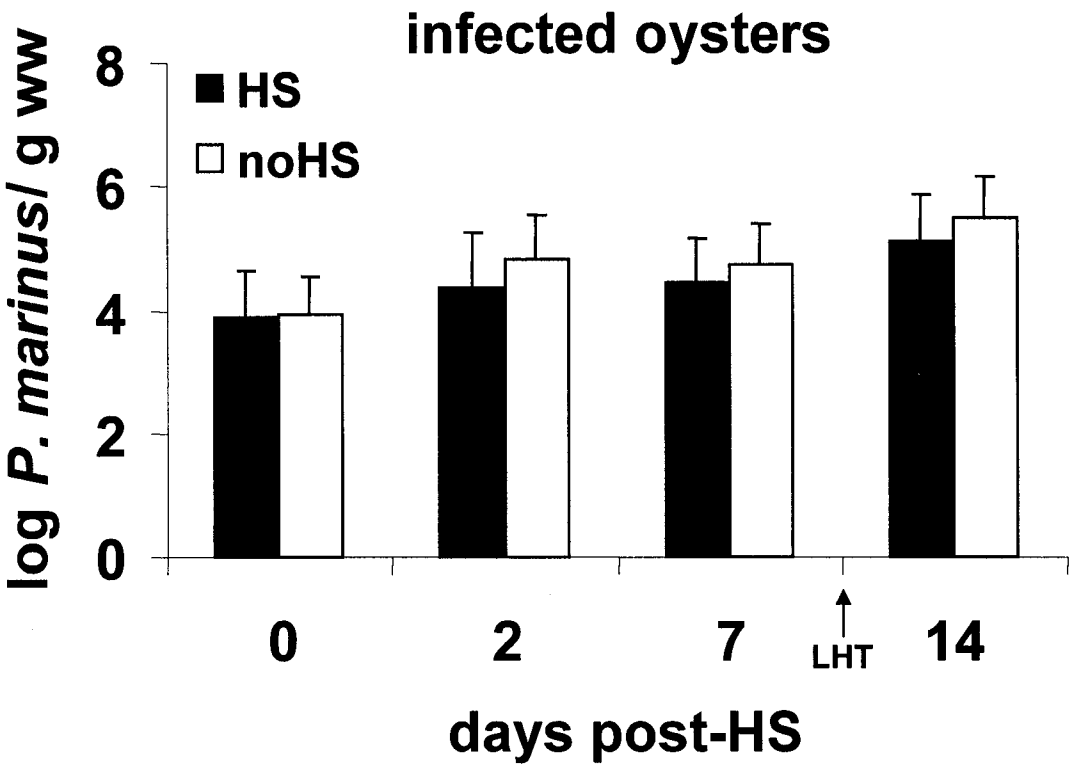


Figure 7. Survival (%) of oysters over four treatments. The four treatments were: heat shocked (HS) + infected, heat shocked + uninfected, non-heat shocked (non-HS) + infected and non-heat shocked + uninfected. Data shown are percent survival ± s.e.m. of 3-4 tanks per treatment with each tank containing 8-11oysters/tank. Effects of heat shock significantly enhanced survival (ANOVA, $p = 0.013$). Infection status did not significantly affect survival.

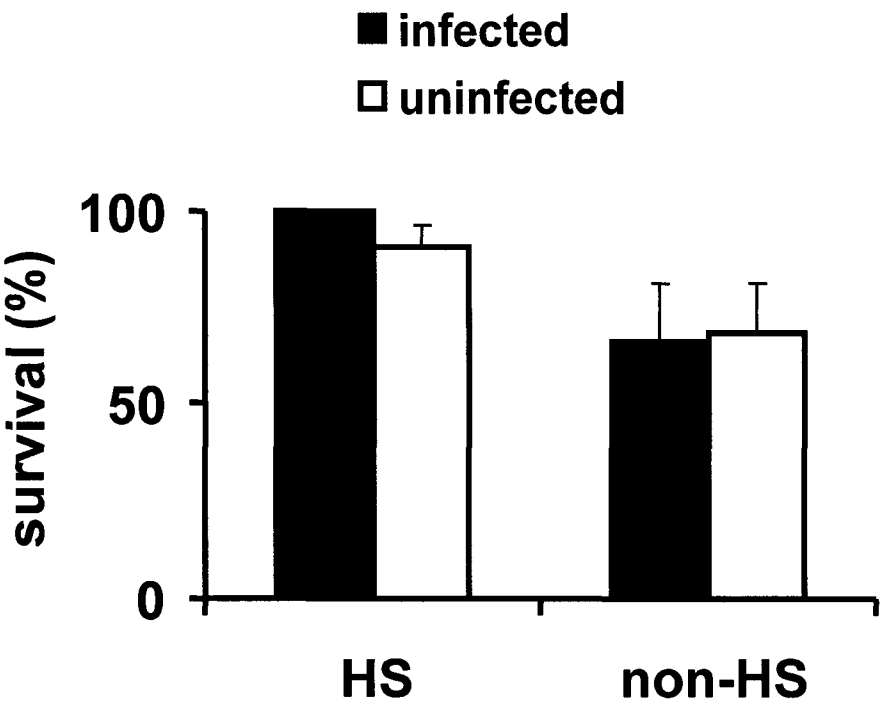


Figure 8. Survival (%) over 7 days in oysters subjected to a lethal heat treatment (LHT) of 44 °C for one hour. Treatments shown are: heat shocked (HS) + infected, HS + uninfected, non-heat shocked (non-HS) + infected and non-HS + uninfected. (Time-to-death analyses: $p < 0.0001$ for both Log-rank and Wilcoxon tests). Data shown are mean % survival/treatment ± s.e.m. at each day. N=3-4 tanks per treatment with 8-11 oysters/tank

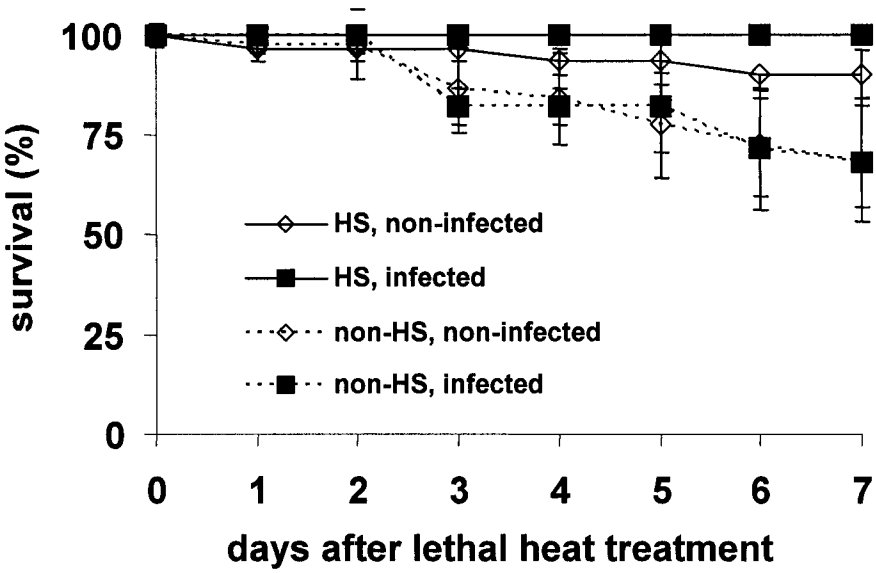
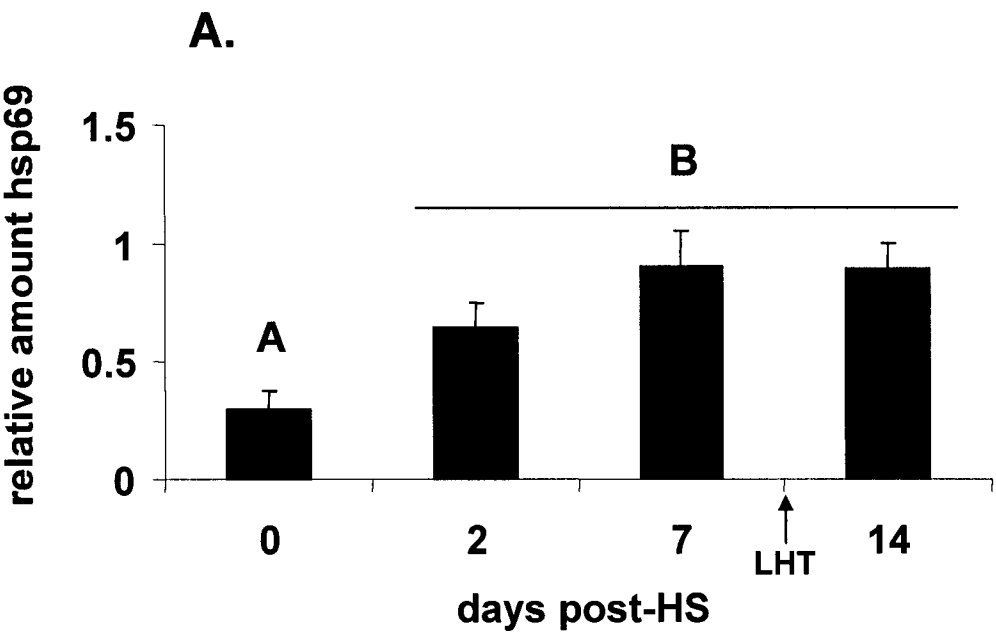


Figure 9. (A) Changes in the hsp69 isoform 0, 2, 7 and 14 days post-heat shock (post-HS). Significantly different means ($p < 0.05$) are indicated by different letters. Data are presented as mean relative amounts hsp69 ± s.e.m. (n = 13-14) collapsed across factors of heat shock treatment and infection status. (B) Total amounts of hsp70 (hsp69 + hsp72) as measured by western blot. Data presented are means ± s.e.m. (n = 30 for HS and n = 24 for N treatments) collapsed across effects of day and infection status. A lethal heat treatment (LHT) of one hour at 44 °C was administered just after the day 7 sampling, as shown on figure A and B.



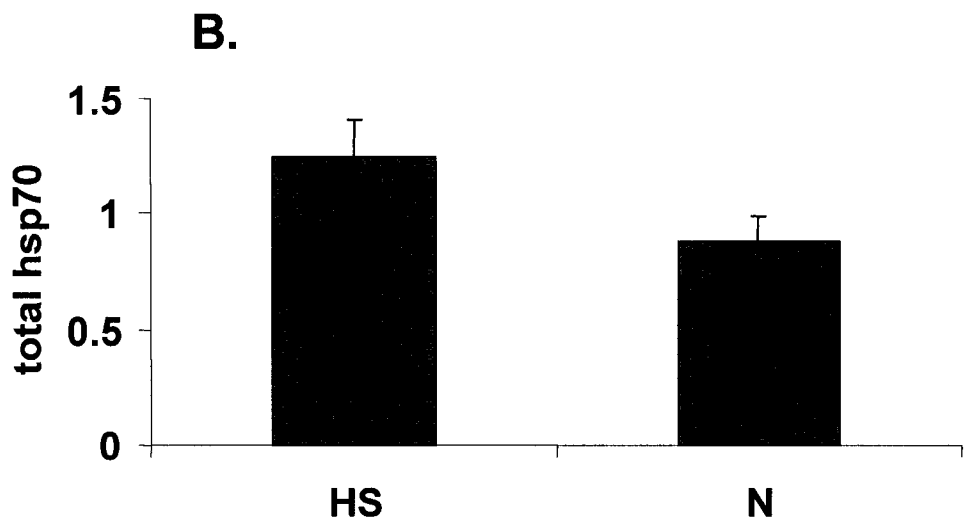


Figure 10. (A) Changes in hsp69 0, 2, 7 and 14 days post-heat shock (post-HS) in oysters experimentally infected with *P. marinus*. Relative amounts of hsp69 in heat shocked, infected (HS + I) and non-heat shocked, infected (N + I) oysters are shown. (B) Levels of hsp69 in heat shocked, uninfected (HS + U) and non-heat shocked, uninfected (N + U) oysters. Data are presented as mean relative amounts of hsp69 ± s.em. (n = 3). A lethal heat treatment (LHT) of one hour at 44 °C was administered just after the day 7 sampling.

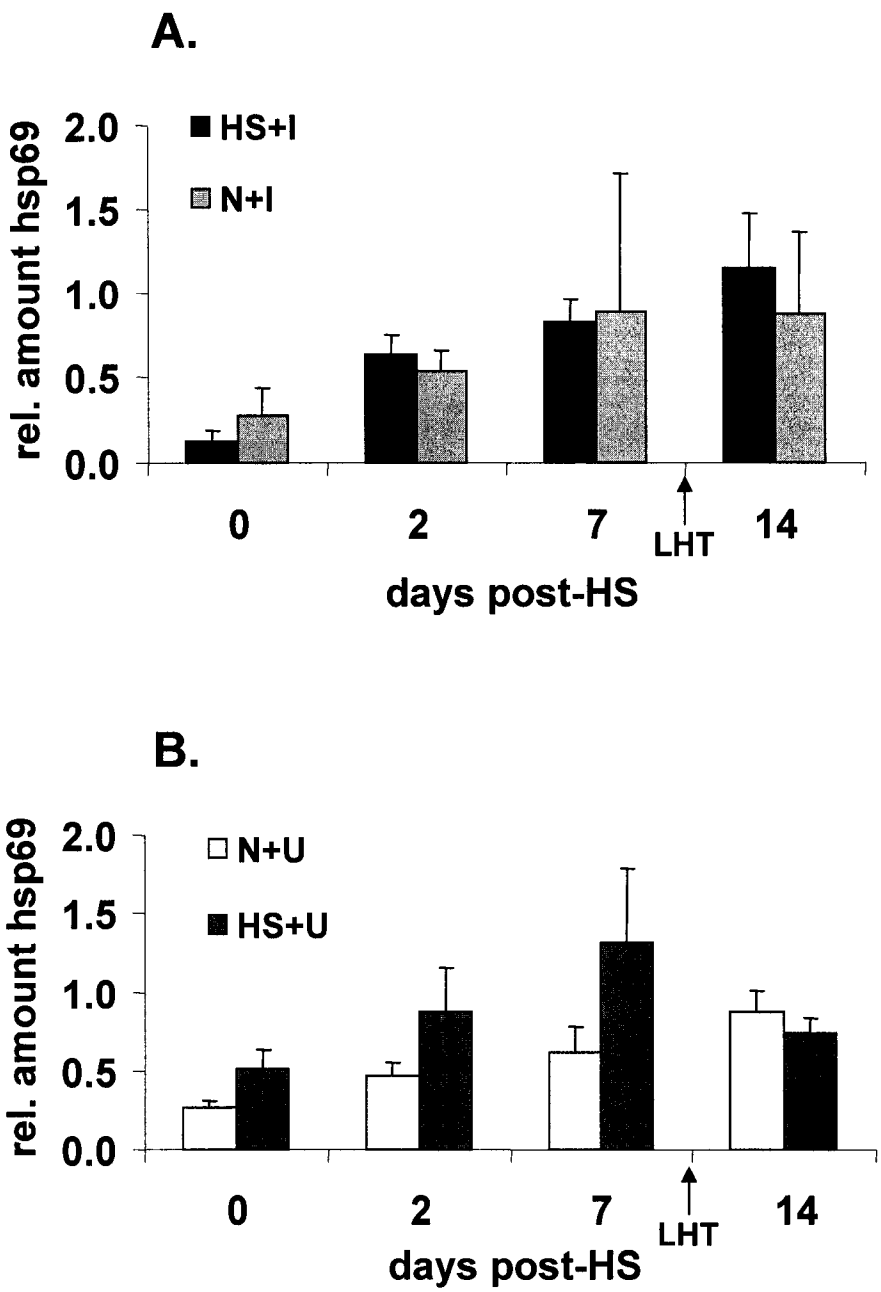


Figure 11 (A) Relative levels of hsp72 as analyzed by western blot. (B) Relative amounts of hsp72 due to heat shock in uninfected oysters. Data are presented as mean relative amounts of hsp72 ± s.em. (n = 3). A lethal heat treatment (LHT) of one hour at 44 °C was administered just after the day 7 sampling, as shown on both figures.

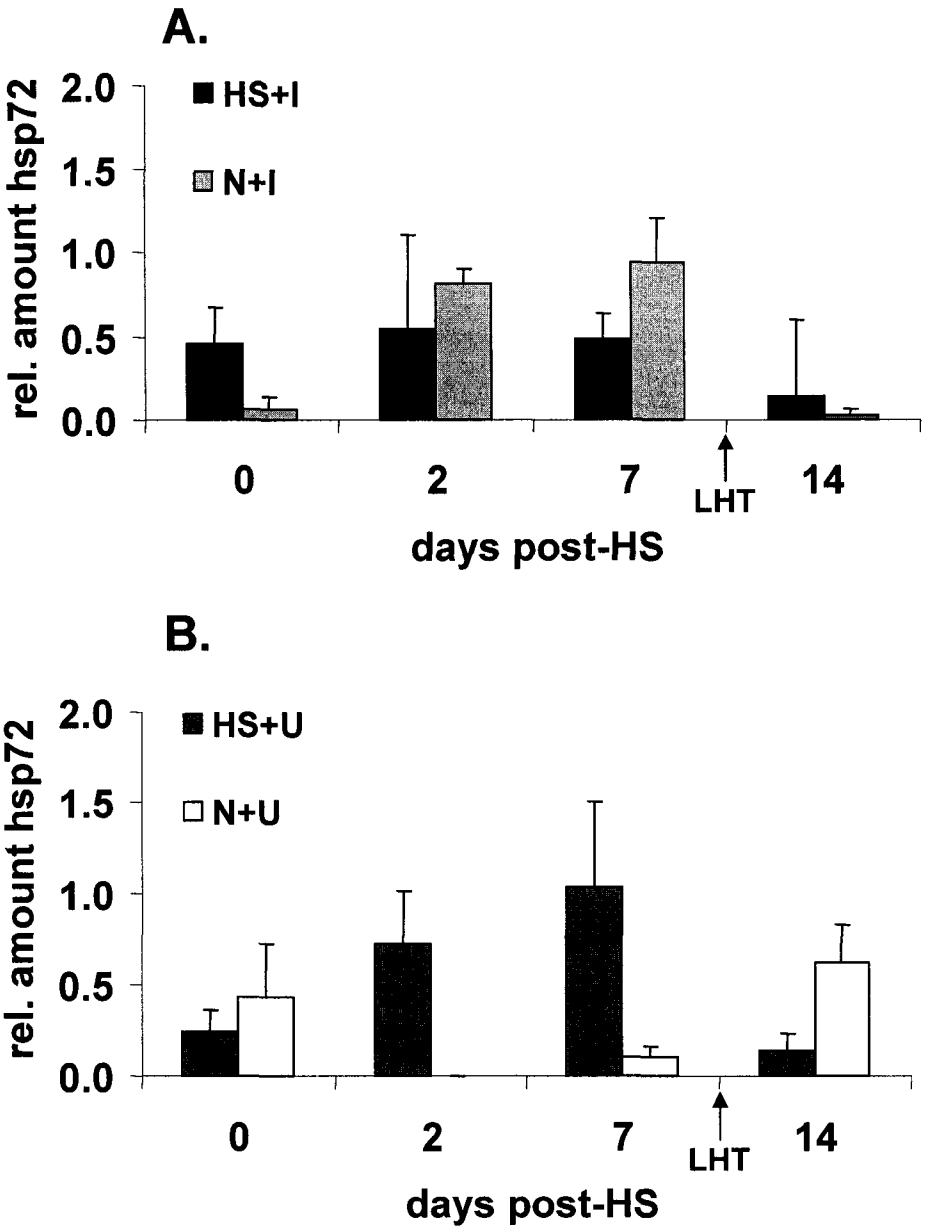
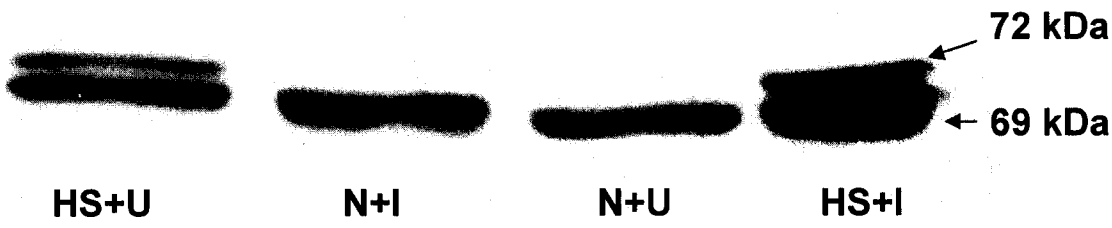


Figure 12. Western blot showing expression of hsp70 isoforms (hsp69 and hsp72). Samples shown are representative of four different treatments: HS+U - heat shocked + uninfected, N+I – non-heat shocked + infected, N+U - non-heat shocked + uninfected, HS+I – heat shocked + infected.



Chapter 5: Conclusions – Implications of Research Findings and Future Research

Implications of Research Findings

Recent efforts to restore oyster populations within the Chesapeake Bay have largely focused on minimizing the effects of disease. Discovery of wild oysters resistant to Dermo and MSX has been shown in this dissertation (Chapter 2) and in other recent studies (Calvo et al. 2003, Sorabella 2002). In chapter 3, a subsequent generation of the CTS stock exhibited the same pattern of survival as its parents, demonstrating a genetic basis to Dermo-resistance in addition to an apparent resistance to MSX. If oyster populations are developing resistance to Dermo via natural selection, then consideration must be given to keeping areas within the Bay free from commercial harvest to allow the spread of disease-resistant genotypes throughout the oyster population and to allow recovery of existing and restored oyster beds as these oysters reproduce. The genetic diversity of these wild disease-resistant populations are likely greater than hatchery strains. Therefore, these populations would serve as valuable broodstock to increase diversity in existing strains and to develop new strains. Hatchery produced oysters are now being used for both aquaculture and reef restoration. As these efforts continue to develop, more attention will need to be given to enhancing traits other than survival to optimally match oyster strain selection to habitats, methods of culture and whether oysters are grown for restoration or food. Fast growth and improved meat quality are traits that are as important as survival when considering growing oysters for consumption. Differences in growth, dry meat weight and condition between stocks show that survival is not the only trait to consider, particularly in commercial aquaculture.

The demonstration of induced thermal tolerance in oysters shows that mechanisms of stress resistance may improve survival in disease-resistant oysters. Because heat shock proteins confer tolerance to other stressors besides temperature, heat shock proteins may protect and repair proteins and tissues damaged by *P. marinus* proteases. It has been shown that hsp70 is involved in conferring cytoprotection, possibly by blocking pathways that induce inflammation or apoptosis (Gabai et al. 1997, Hightower et al. 2000). Enhanced levels of heat shock proteins may function similarly in oysters by repairing damaged proteins and protecting oysters from subsequent stress. Although hsp70 may provide protection from multiple stressors, tests of thermal tolerance may be the clearest way to assess the capacity of an organism to synthesize hsp70, and therefore resist stress.

Heat shock to improve survival can be applied in several ways. Aquatic sub-lethal thermal shock could be administered to oyster seed in the hatchery. Natural exposure at low tide can also be exploited as a way to harden oysters to stress and has been incorporated in several culture methods (Ventilla 1984). Improved survival of oysters under intermediate levels of tidal exposure has been observed (Littlewood et al. 1992, O'Beirn et al. 1994, Bartol et al. 1999). Placement of oysters on reefs and the design of reefs must consider how variations in thermal exposure can influence survival on these reefs. Whether resistance to intertidal conditions also protects oysters from disease remains to be seen, but intertidal populations in the southeastern U.S. maintain sub-lethal levels of *P. marinus* infections (Bushek et al. 2002). This may due to lower exposure, but simulated hypercapnia, which commonly occurs during aerial exposure, can decrease the growth of *P. marinus* (Burnett and Milardo 2002). Growth of *P.*

marinus was also reduced at temperatures >40 °C, a temperature frequently occurring in the intertidal zone (P. Soudant, pers. comm., unpublished data).

Inherent expression of hsp70 does not differ between different oyster strains, but it does appear that variation in thermal tolerance between oyster stocks is reflected by differences in hsp70 expression. Differences in survival to heat stress between the CTS and LOB stocks suggest that there is a genetic basis to these differences in thermal tolerance, although size effects cannot be ruled out at this time. If further comparisons show that these differences are truly inherent, then traits related to stress tolerance must be considered in selective breeding approaches.

Attempts are being made to genetically improve thermal tolerance in trout (Danzmann et al. 1999). Pacific oyster strains that are resistant to the summer mortality phenomenon have been developed (Samain et al. 2004). The basis of this resistance is yet undefined, but variation in the heat shock response may be a phenotypic marker of resistance. Recent studies have shown that strains resistant to summer mortality had a higher temperature threshold for induction of hsp70 than susceptible strains, which were more temperature-sensitive (L. Degremont, personal comm.). Thus, genetically improving thermal tolerance in oysters may enhance their capacity to acclimate to thermal stress and may also improve resistance to disease.

Mechanisms of thermal adaptation in oysters are especially important in the context of climate change. Climate change has been implicated in the northward expansion of *P. marinus* and in increases in marine diseases as a whole (Ford 1996, Harvell et al. 1999). The ability of oysters to respond to these dual stressors is essential. Oyster or other bivalve growers may be able to improve survival either by manipulating

the stress response (via heat shock) or selective breeding of more disease and stress resistant strains.

Future Research

Obvious differences in survival and growth were apparent between the oyster stocks compared in this study. The physiological effects of disease, however, were less clear. Seasonal effects may supersede any potential effects of Dermo infection on condition index or energy reserves. The long time (4-5 weeks) between sampling periods in Chapter 1 may have missed the sublethal effects of parasitism by *P. marinus*. Future studies should sample oysters over a shorter period, particularly at the initial periods of infection and during early stages of gametogenesis, when effects of infection may reduce the condition index (Dittman et al. 2001). Because there were significant differences in growth in this study, measurements of energy metabolism (oxygen consumption, filtration and excretion rates) may determine what physiological processes contribute to differences in growth between genetically distinct oyster stocks. Finally, the reproductive output of these stocks needs to be assessed to assess their comparative genetic contributions to oyster populations in the Chesapeake Bay.

Intertidal conditions may potentially protect oysters from lethal effects of disease. Whether intertidal conditions retard the lethal effects of disease remains to be tested. Comparative effects of tidal exposure on oyster strains should be examined to determine which strains are suitable for aquaculture or deployment on reefs. Other environmental effects, such as salinity, on the heat shock response need to be evaluated as well. Reduced salinities due to freshwater discharges caused increased mortalities in oysters

during the summer months (La Peyre et al. 2003). Reduced salinity is known to decrease thermal tolerance in oysters (Loosanoff 1953).

Expression of hsp70 was seasonally dependent, but specific isoforms did not uniformly change with seasonal temperatures. How seasonal changes in hsp70 expression affect induction of hsp70 and acquisition of thermal tolerance in the eastern oyster are unknown. Because commercial antibodies that recognize human hsp70 were used to detect hsp70 in oysters in the present study and other studies, the complete patterns of hsp70 expression are unknown. Development of monoclonal antibodies specific to oyster hsp70 will likely reveal previously undetected isoforms of hsp70 and should allow more detailed characterization of the heat shock response in oysters. The present study examined how overall stress tolerance could be improved in oysters. The specific activity of hsp70 in the innate immune response of the oyster is yet to be examined. Effects of heat shock on hemocyte activities such as phagocytosis and the production of reactive oxygen intermediates will determine if elevated levels of heat shock proteins are involved in enhancing these functions in oysters.

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