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VG Encomio

FLE Chu

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SEASONAL VARIATION OF HEAT SHOCK PROTEIN 70 IN EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) INFECTED WITH *PERKINSUS MARINUS* (DERMO)

VINCENT G. ENCOMIO[1,2] AND FU-LIN. E. CHU[1]*

[1]Virginia Institute of Marine Science, 1208 Greate Road Gloucester Point, Virginia 23062; [2]Florida Gulf Coast University 10501 FGCU Blvd. South, Modular Building #7, Fort Myers, Florida 33965

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), increase 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, hsps may play protective roles for both the host and parasite during infection. The interactive effects of temperature and disease on heat shock protein expression in oysters, however, are unknown. In this 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. 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. 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. This study reveals that measuring total levels of hsp70 do not sufficiently describe the effect of seasonal temperatures on hsp70 expression. 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. 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.

KEY WORDS: *Crassostrea virginica*, heat shock protein, hsp70, *Perkinsus marinus,* seasonal variation

INTRODUCTION

Since the 1950s diseases caused by 2 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 & LaPeyre 1993, Chu & Volety 1997). *Perkinsus marinus* rapidly proliferates and develops between 20°C to 30°C (in vitro and in vivo) and salinities greater than 10 ppt (Chu & Greene 1989, Chu & La Peyre 1993, Burreson & Ragone-Calvo, 1996). Dermo-associated mortality usually begins in early summer (June) when water temperature increases (~20°C) and peaks (27°C to 30°C) between August and September (Andrews & Hewatt 1957, Andrews 1988). Mortalities can be particularly intense above 25°C (Mackin 1951, Fisher et al. 1992, Chu & LaPeyre 1993). These increases in seasonal temperature intensify overwintered and newly acquired infections (Ragone-Calvo & Burreson 1994).

Increased temperature 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 & 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 United States and France are associated with elevated seasonal temperatures and multiple interacting factors (Cheney et al. 2000, Lacoste et al. 2001, Soletchnik et al. 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 with uninfected oysters (Littlewood & Ford 1990). Thermal stress alone is enough to cause deleterious physiologic effects. Acute thermal stress during emersion was shown to cause reversible and irreversible protein denaturation in intertidal mussels (Hofmann & Somero 1995).

Molecular chaperones, or heat shock proteins (hsps), 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 hsps in response to other environmental stressors has also been observed, suggesting their use as general biomarkers for stress (Sanders 1988, Pyza et al.

*Corresponding author. E-mail: chu@vims.edu

1997, Cruz-Rodriguez & Chu 2002). Additionally hsps play protective roles for the host and parasite during infection (Merino et al. 1998, Robert 2003) and act as a stimulant of the host immune response (Tamura et al. 1997, Zugel & 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 hsps, the hsp70 family (hsps in the 70 kDa MW range) is the most responsive to environmental perturbations (Parsell & Lindquist 1993, Feder & Hofmann 1999, Lewis et al. 1999). In most species, hsp70 is a multigene family encoding several distinct protein isoforms (Lindquist 1986). The hsp70 genes are largely recognized as being highly conserved across organisms and within these multigene families (Favatier et al. 1997).

Mechanisms associated with resistance to thermal stress, such as hsps, have been characterized in many organisms, including oysters (Sorenson et al. 2003, Feder & Hofmann 1999, Shamseldin et al. 1997, Clegg et al. 1998, Cruz-Rodriguez & Chu 2002, Piano et al. 2002, Boutet et al. 2003, Hamdoun et al. 2003). Increased levels of hsps 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 hsps would follow seasonal patterns of temperature with the highest levels of hsps 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 2004). 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 hsps than resistant stocks. Intrastrain variation in hsp70 expression during seasonal acclimatization would suggest a genetically based response to environmental stress, because 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 physiologic state and disease.

MATERIALS AND METHODS

Oyster Grow-out 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 2 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 between 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 2004). 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 2004). 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 per 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 h and weighed to estimate total dry weights for condition index (CI). 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 tissue aliquot (1.0 mL) was incubated in alternative fluid thioglycollate medium (AFTM) (Sigma Biochemicals) for 5–7 days at room temperature. Tissue suspensions were then centrifuged at 800 *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 ×400 magnification. Results are expressed as number (#) of *P. marinus* cells/g wet tissue weight (ww).

Detection of Heat Shock Protein 70

Gill samples from 4 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 & Chu 2002) and western blotting techniques were used to detect hsp70. Slot blot analysis only examines total amounts of hsp70, because 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 h, 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 *g* for 30 min 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

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 & Chu 2002, Lewis et al. 1999). Samples were loaded in triplicates. Unknown samples were diluted to 1.5 μg total pro-

tein 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 control," obtained by exposing an oyster to 1 h 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 min followed by two washes in Tris-buffered saline (TBS—30 mM NaCl, 24 mM Tris pH 7.5) for 5 min each. Primary monoclonal antibody against Hsp70 (clone 3A3—catalog # MA3-006, Affinity Bioreagents) was applied for 90 min (1:5000 dilution), followed by two 5 min washes with TBS. A secondary antibody (Goat AntiMouse AP conjugated) was applied for 90 min (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 min. 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 control" in each blot to account for interblot 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 min, and 10-μg total protein per sample was electrophoresed on 8% polyacrylamide gels (150 V, 90 min). Separated proteins were then transferred onto nitrocellulose membrane at 100 V for 1 h 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 control" described previously. As with the unknown samples, 10 μg of the reference control was added to each gel.

Specificities of Commercial Heat Shock Protein 70 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 this study and others (Tirard et al. 1995, Shamseldin et al. 1997, Cruz-Rodriguez & 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 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 means (SEM).

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°C to 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 with the XB and CTS ($P = 0.03$). The CRB strain also showed higher mortality at Regent Point than at Port Kinsale ($P = 0.027$) (Fig. 2). Shell heights were significantly different among sampling periods, site, and stock ($P < 0.0001$ for each). Shell heights increased over time (Fig. 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 (Fig. 3B). Shell height in the CTS group was significantly lower than both CRB and XB strains ($P < 0.05$) (Fig. 3C).

Condition index 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 (Fig. 4). The CI was highest in the CRB stock compared with 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) (Fig. 5). *Perkinsus* infections remained high (~6–9 × 10^6 cells/g ww) from August to November 2002. Infections, though relatively high for the winter, decreased significantly in December 2002 (2.66 ± 0.82

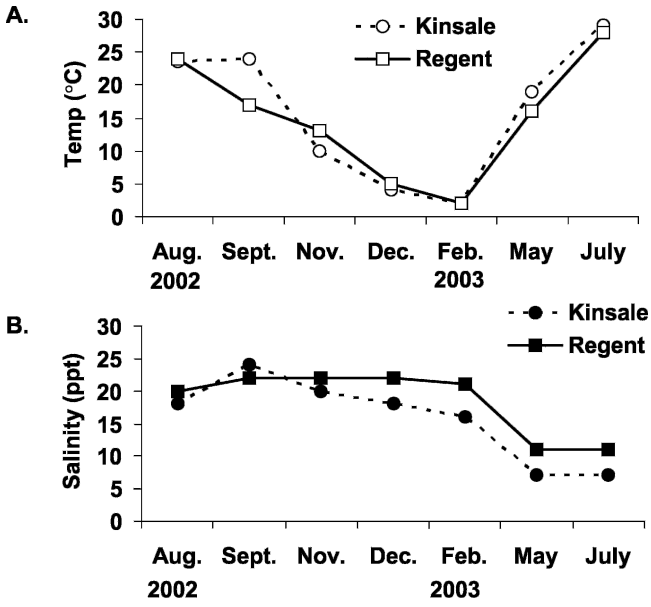

Figure 1. Seasonal temperatures (A) and salinities (B) from 2002 to 2003 at two Chesapeake Bay sites (Port Kinsale-Yeocomico River, and Regent Point-Rappahannock River).

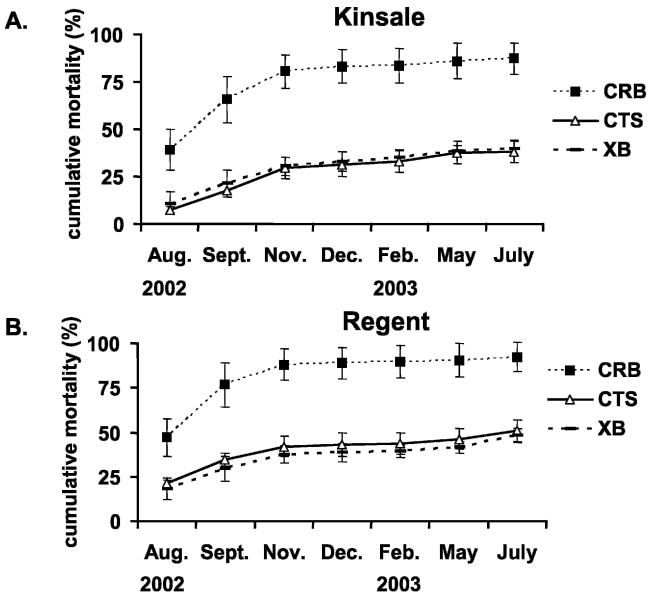

Figure 2. Cumulative mortalities of three deployed oyster stocks from 2002 to 2003 at (A) Port Kinsale and (B) Regent Point. CRB = Rappahannock River, CTS = Tangier Sound, XB = CrosBred hatchery strain. The CRB and CTS groups are progeny of F_0 stocks originally spawned in 1999. Data are means and standard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

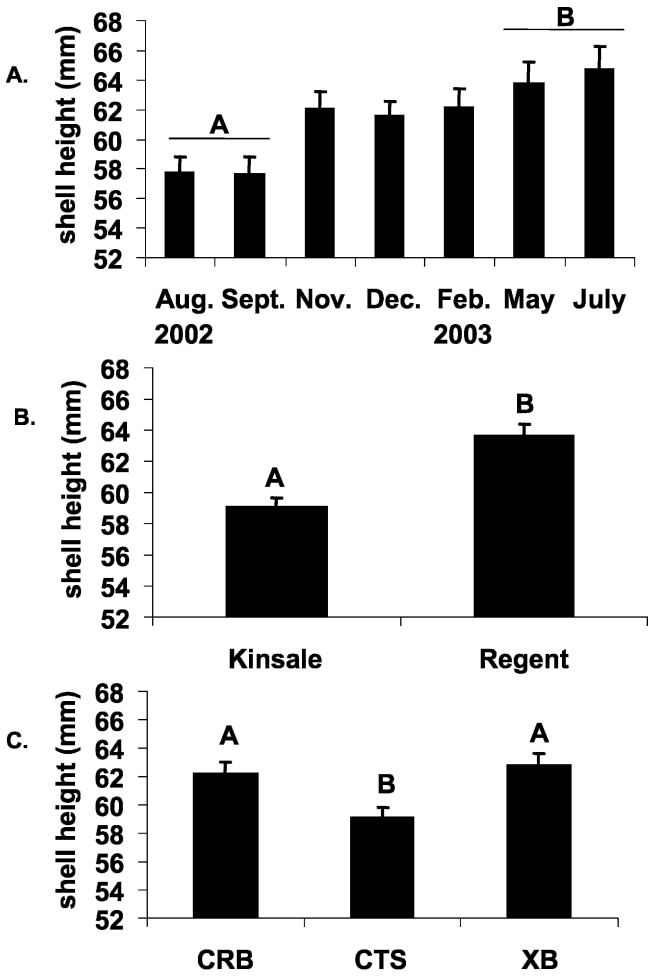

Figure 3. A. Changes in shell height over sampling month across treatments of stock and site. Differences in shell height due to month were significant at $P < 0.0001$. Means are pooled across replicates of stock and site (bditn = 24/month, except July 2003 [$n = 21$]) B. Differences in mean shell height 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 ± SEM. Significantly different means ($P < 0.05$) are indicated by different letters. Lines under each letter designate means that are not significantly different.

$\times 10^6$ cells/g wet weight) and decreased further in February and May 2003 ($1.55 \pm 0.49 \times 10^6$ cells/g ww and $0.256 \pm 0.24 \times 10^6$ cells/g ww respectively). Infections increased in July ($2.11 \pm 0.76 \times 10^6$ cells/g ww) from May. Overall, *P. marinus* infections were higher at Regent Point than at Port Kinsale. Infections were the highest in the CRB stock compared with the CTS and XB stocks (Tukey test $P < 0.0001$) (Fig. 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).

Heat Shock Protein 70 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 hsps 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 (Fig. 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

Immunologic detection of hsp70 isoforms by western blot (using 3A3 antibody) found two isoforms of hsp70 expressed in the gills (Fig. 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 3 isoforms of hsp70 (69, 72, and 77 kDa) in heat-shocked and nonheat-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 (Fig. 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

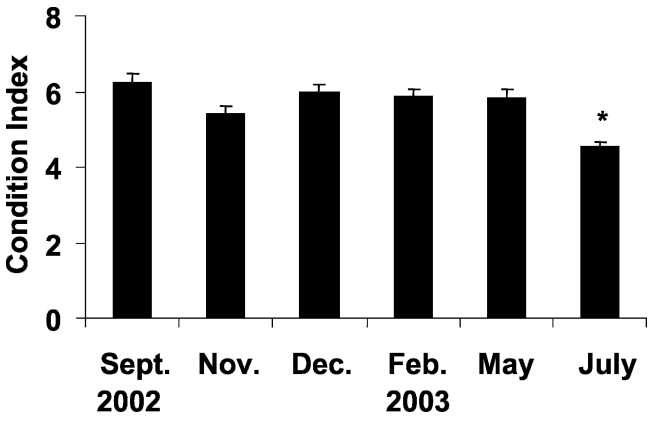

Figure 4. Changes in condition index (CI) at sampling periods across stock and site ($P < 0.0001$). Data are mean ± SEM of 4 replicates (3 individuals measured per replicate/stock per site). July 2003 CI values were significantly different from all other months, and indicated by an asterisk (*) ($P 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

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 (Fig. 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 (Fig. 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 (Fig. 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 ($r = 0.54$, $P = 0.007$) (Fig. 9).

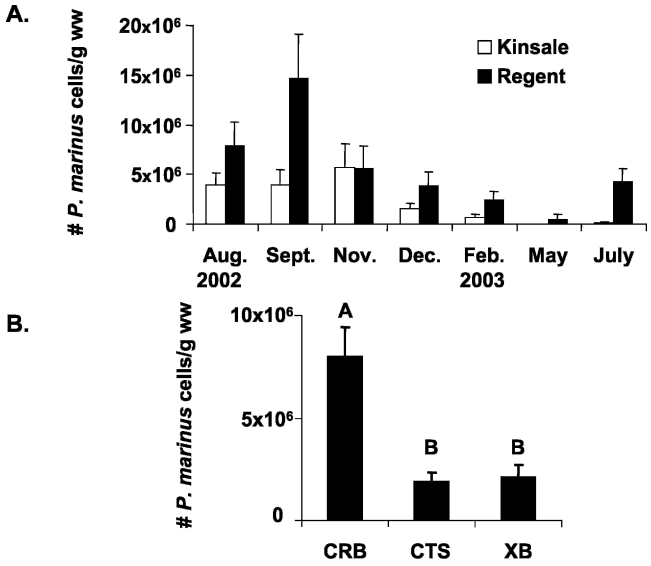

Figure 5. A. *Perkinsus marinus* infection intensities (number [#] of *P. marinus* cells/g wet weight [ww]) in *C. virginica* as a function of sampling month and site. Mean differences due to sampling period (month), site, and their interactive effects (month × site) were all significant ($P < 0.05$). 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 3 stocks ($P < 0.05$). CRB = Rappahannock River, CTS = Tangier Sound, XB = CrosBreed. All data are presented as means ± SEM. Significant differences ($P < 0.05$) among means are indicated by different letters. Lines under each letter designate means that are not significantly different.

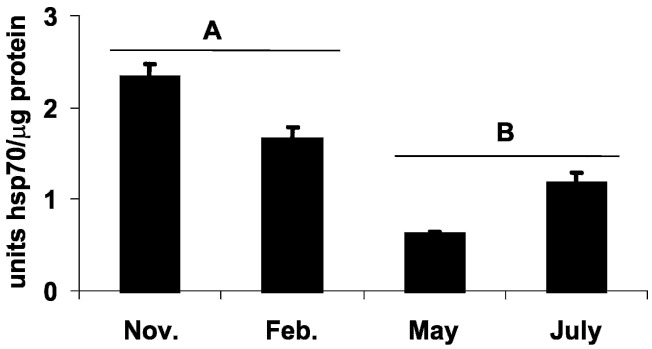

Figure 6. Levels of hsp70 measured using the slot blot assay. Data are mean hsp70 ± SEM at 4 sampling periods (November 2002, February 2003, May 2003, and July 2003). Significantly different ($P < 0.05$) means are indicated by different letters. Lines under each letter designate means that are not significantly different.

DISCUSSION

Increases in hsp70 with seasonal temperature have been observed in mussels and marine snails (Hofmann & Somero 1995, Chapple et al. 1998, Tomanek & 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 their 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 & Hinton 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 & 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

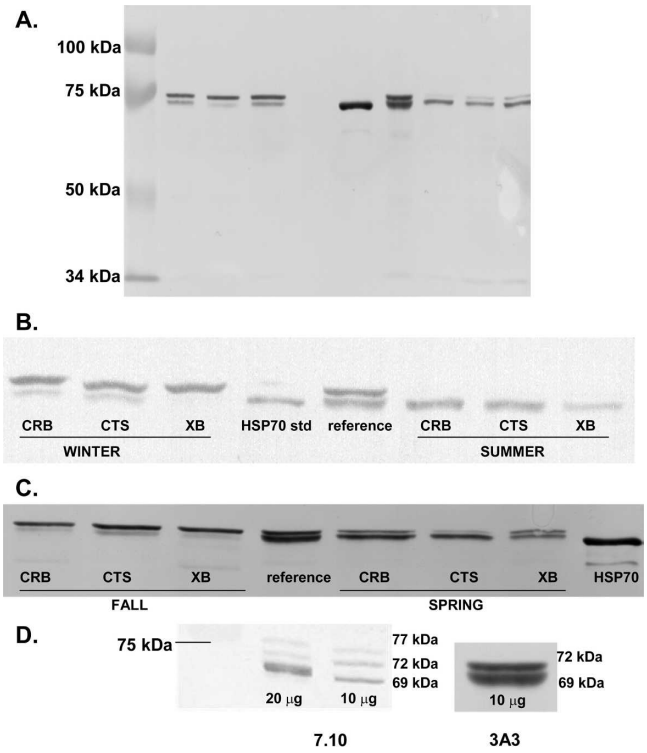

Figure 7. A. Representative western blot of *C. virginica* gill samples. Molecular weights (MW) of standards in kilodaltons (kDa) are shown on the left. B & C. Hsp70 in oyster gills from 3 *C. virginica* stocks. (CRB = Rappahannock River, CTS = Tangier Sound, XB = CrosBred) The reference control is pooled gill tissue from 5 oysters heat shocked for 1 h at 40°C. The reference control 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 2 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.

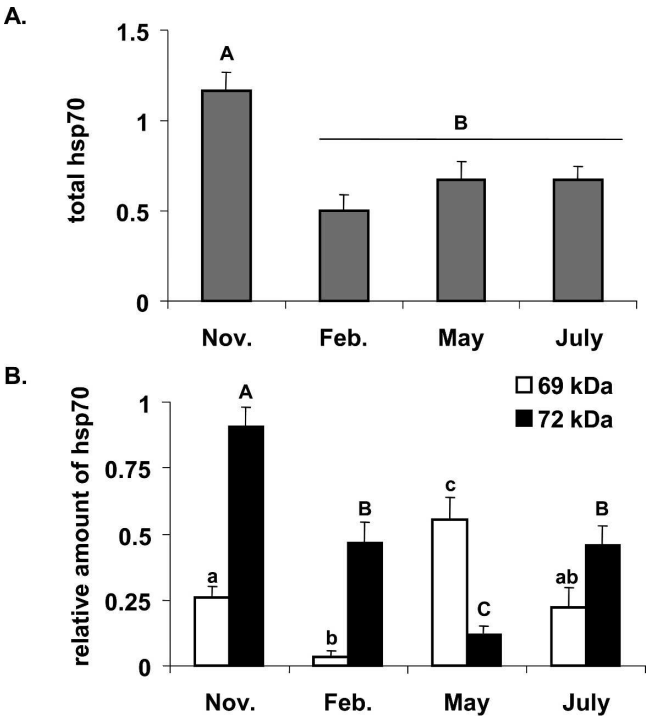

Figure 8. A. Total relative amounts of hsp70. Different letters denote means that are significantly different ($P < 0.05$). Different letters indicate significantly different means. Lines under each letter designate means that are not significantly different. B. Levels of hsp70 isoforms (hsp69 and hsp72). 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 ± SEM of 3–4 individuals/sampling per stock/site.

several bivalve species 2–3 isoforms of hsp70 have been observed. In the mussel, *Mytilus edulis*, the lower MW isoform (70 kDa) 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. 2002, 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 2 isoforms of 69 and 72 kDa MW, but not a third, higher MW isoform, such as the 77 or 78 kDa hsps 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 with 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. 2002, 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

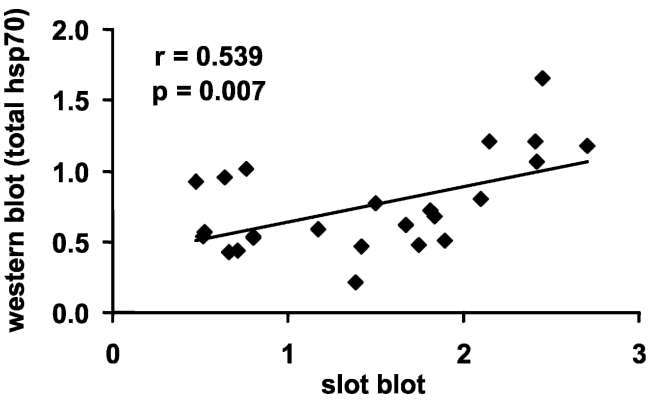

Figure 9. Comparison of slot blot hsp70 values and total hsp70 values calculated from western blot results. Means across month, site, and stock were compared.

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, because 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).

Specific stocks of eastern oysters from the Chesapeake Bay and the Gulf of Mexico exhibit varied resistance to Dermo (Encomio 2004). Survival during periods of elevated exposure to *P. marinus* was high in one Chesapeake stock (Tangier Sound, or CTS). Comparisons of mortalities to a Dermo-susceptible Chesapeake stock (Rappahannock River) and a hatchery strain selected for disease resistance (CrosBreed or XB) suggested that the Tangier stock were indeed disease resistant. Comparisons of mortality, growth and condition, and *P. marinus* infection of the F_1 CRB and CTS oysters in this study show that results are similar to those from the F_0 parental stocks (Encomio 2004). We did not, however, 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 believed to be the result of similar selective pressures (Karl & 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 hsps 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 with 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 hsps can be used as a marker of increased stress tolerance in oysters.

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