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# HEAT SHOCK PROTEIN 70 EXPRESSION IN JUVENILE EASTERN OYSTERS, *CRASSOSTREA VIRGINICA* (GMELIN, 1791), EXPOSED TO ANOXIC CONDITIONS

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***ABSTRACT*** Anoxic water events in conjunction with summer high temperatures are thought to be one of the causes of declines in natural oyster reefs on the eastern shore of Mobile Bay. Work is underway to determine whether tolerance to low oxygen can be selected for in hatchery-produced oysters. As a component of this work, the expression of heat shock protein 70 (HSP 70) was examined in control (normoxia) and anoxia-challenged juvenile oysters. Parental Eastern oysters, *Crassostrea virginica* were collected from 2 sites, Cedar Point Reef (CP), an area considered to have normoxic conditions, and White House Reef (WH), an area suspected to experience periodic anoxia. F1 generation oysters were produced from CP and WH parents that survived an anoxic exposure of 96 h. Control F1 generation oysters from both parental stocks not exposed to anoxia were also produced. The F1 generation oysters were subsequently exposed to anoxia or control normoxic conditions, and differences in expression of HSP 70 were examined. Nitrogen was used to create the anoxic conditions for both the parental and F1 generations. Three HSP 70 isoforms—2 constitutive forms (HSC 77 and HSC 72) and 1 inducible form (HSP 69)—were expressed in both anoxia- and normoxia-exposed oysters from all groups. Although there were differences among groups of oysters from the 2 sites, there were no differences in the expression of HSC 77 and HSC 72 between the control and anoxia-treated oysters within a group. Interestingly, the expression of HSP 69 was higher in oysters exposed to normoxia than the ones from anoxia treatments. These differences are thought to reflect a combination of responses to nutritional stress in the controls and facultative anaerobiosis and metabolic arrest in the anoxia groups.

***KEY WORDS:*** eastern oyster, heat shock protein 70, anoxia, Jubilee

## INTRODUCTION

Anoxic water events in estuaries have been reported from all over the world, including the coasts of the United States (May 1973, Officer et al. 1984, Balls et al. 1996, Garnier et al. 2001, Rabalais et al. 2002, Yasuhara & Yamazaki 2005). Benthic organisms in those estuaries have shown negative impacts, including decreased population sizes, mass mortality, failure of larval recruitment, decreased growth rates, and lowered propagation of fish, crustaceans, molluscs, and phytoplankton (May 1973, Officer et al. 1984, Osman et al. 1991, Osman & Abbe 1995, Lenihan & Peterson 1998). As studies suggest that fish and crustaceans are able to avoid anoxic water because they are motile (Pavela et al. 1983), anoxic water events may have greater impacts on sessile organisms, leading to deterioration of benthic ecosystems and challenging restoration attempts.

The anoxic water events that frequently occur during summer months in Mobile Bay, AL, are locally known as ''Jubilees'' (May 1973, Saoud et al. 2000). During a Jubilee, anoxic water from the deeper bay bottom enters the shallow shore areas and can remain there for up to a week. These events have been observed in large areas of the eastern shore and some areas of the western shore of Mobile Bay (May 1973). Reports suggesting a role of anoxia in the declines of natural oyster reefs in Mobile Bay (May 1973, Saoud et al. 2000) have been supported by a recent field study in which oysters placed at a historic reef site suffered 100% mortality during several multiday low-oxygen events (Rikard, pers. obs.)

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Researchers from Auburn University Shellfish Laboratory have been attempting to produce hypoxia- and anoxia-tolerant Eastern oysters, *Crassostrea virginica* (Gmelin, 1791), by challenging oysters with anoxia and breeding the survivors. Similar methods have shown successful results for improving growth rate, disease resistance, and survival rate (Lannan 1980, Sheridan 1997, Langdon et al. 2003). Current interests of that group are to assess improvement of anoxia tolerance for oysters by comparing median lethal tolerances (LT-50s) in long-term anoxia exposure for selected and nonselected offspring.

To assist in evaluating tolerance to anoxia as a general stress response, heat shock protein 70 (HSP 70) was selected as a potential biomarker. HSPs are a group of molecular chaperones that assist in intracellular folding and transportation of newly synthesized polypeptides and proteins, and the correct folding of denatured proteins in cells (Gething & Sambrook 1992). There are 2 types of HSPs, cognate forms (HSC), which are constitutively expressed or can be increased by stresses in the cells, and inducible forms (HSP), which are expressed in the cells only in response to stress. As a general rule, the more severe the stress, the greater the expression of HSPs in the cells. Therefore HSP expression has been used as a biomarker to detect stress levels (see reviews in Sanders [1993], Feder and Hofmann [1999], and Dahlhoff [2004]). Classification of HSPs is based on their molecular weight—for example, those in the 70-kDa size range are called HSP 70. Numerous studies have been conducted for HSP 70 because of its prominent expression in response to stresses, including hypoxia and anoxia (Heads et al. 1995, Ma & Haddad 1997).

Changes in expression of HSP 70 are known to occur in the Eastern oyster, *C. virginica*, in response to thermal stress, dissolved metal ions, polynuclear aromatic hydrocarbons, and season (Tirard et al. 1995, Ringwood et al. 1999, Cruz-Rodriguez & Chu 2002, Encomio & Chu 2005). Changes in HSP 70 levels have also been found to occur in other oyster species in response to thermal stress (Clegg et al. 1998, Piano et al. 2002, Hamdoun et al. 2003, Brown et al. 2004). Those studies identified 3 isoforms in HSP 70: 2 constitutive forms (HSC 77 and HSC 72) and 1 inducible form (HSP 69). However, expression of HSP 70 in response to hypoxic stress has not examined in any oyster species. Because upregulation of HSP 70 gene expression has been reported in fruit flies and rats in response to hypoxia (Heads et al. 1995, Ma & Haddad 1997), HSP 70 may serve as a potential low-oxygen biomarker. Moreover, if HSP 70 plays an important role in protecting oysters against anoxia stress, differences in the magnitude of HSP 70 expression may be observed between nonselected and anoxia exposure-selected groups. The objective of this study was to examine differences in HSP 70 expression in the F1 generation of normoxia- and anoxia-exposed oysters from 2 sites: a control site that is not known to experience hypoxia and one known to experience hypoxia.

## MATERIALS AND METHODS

### *Parental Oyster Collections*

Parental wild oysters were collected in 2004 from 2 sites—Cedar Point (CP) and White House (WH) in Mobile Bay, AL—to compare the differences in survival of offspring. It is thought that the oysters from CP have not experienced hypoxic/anoxic events whereas the oysters from WH are suspected to have experienced periodic hypoxic/anoxic events.

### *Parental Oyster Selections*

Oysters collected from the 2 sites were exposed to anoxic conditions (<0.05 mg/L) for at least 96 h and survivors were designated as Cedar Point experimental (CPE) and White House experimental (WHE). Anoxic conditions were produced by bubbling the holding system with nitrogen gas. Control oysters, not exposed to anoxia, were designated as Cedar Point control (CPC) and White House control (WHC). Both control and treatment oysters were used to produce F1 generation oysters for use in further anoxia exposure experiments.

### *Hatchery and Culture Conditions*

Spawning of selected parental oysters for each group (CPC, CPE, WHC, and WHE) was induced by thermal shock at Auburn University Shellfish Laboratory, Dauphin Island, AL. Fertilized eggs and larvae were cultured in 900 L aerated seawater for each group. Larvae were fed twice a day with algal paste (Reed Mariculture, Inc., San Jose, CA). Seawater was pumped directly from the Gulf of Mexico and filtered (1 μm) and sterilized via ultraviolet light. Water in the tanks was changed every other day. When larvae reached a size of approximately 300 μm, they were transferred to downweller settlement tanks and set on microcultch (250 μm) (Ford 2005). Spat of F1 generations were subsequently cultured in upwellers for 2 mo in flow-through unfiltered seawater until they were 7–15 mm in height (Ford 2005).

### *Anoxia Exposure*

One hundred twelve-liter water tanks were filled with filtered seawater (17‰) for anoxic and normoxic treatments. Anoxic conditions were maintained by bubbling nitrogen gas; normoxic conditions were maintained with aeration. Prior to the experiment, F1 generation oysters were acclimated to 28°C for 2 days from ambient condition (26.5°C, 17.0‰, and 5.5 mg/L). Then the oysters were transferred to either an anoxic tank (< 0.05 mg/L) or a normoxic tank (6.0–6.5 mg/L). Conditions of temperature and salinity used for acclimation were maintained in each tank for the duration of the treatment. Temperature, salinity, and oxygen concentrations were monitored with Hydrolab MiniSonde 4 (Hydrolab Corporation, Austin, TX) throughout the experiment.

Prior to the experiment, spat were sampled for each group. Spat for experiments were placed in approximately 10 × 10-cm size mesh bags and transferred to either the anoxic treatment or the normoxic treatment tank. During the experiment, neither anoxic nor normoxic groups were fed. Samples were taken from both the anoxic and normoxic treatment tanks at 48 h and 96 h. Five oysters per replicate with 3 replicates per treatment were sampled. Samples were flash-frozen in liquid nitrogen and then placed into 50-mL tubes. The tubes were kept on dry ice, brought back to the laboratory at the University of South Alabama, and held at –80°C for further analysis.

### *Protein Extraction and Western Blot*

Modifications of techniques used by Sarkis et al. (2005) were used to examine protein expression. A mixture of gill, mantle, and adductor muscle tissues was used for protein extractions. Tissue sample weights were recorded and the tissue was homogenized in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris; pH, 6.8; 10% glycerol, and 2% SDS) at a 1:4 ratio of sample weight to buffer volume. The homogenates were incubated in boiling water for 3 min, and then centrifuged at 14,000 *g* for 10 min. The supernatant was recentrifuged for an additional 10 min. From the resulting supernatant, subsamples for total protein analysis were diluted with SDS sample buffer at a 1:10 ratio (sample to buffer). The subsamples and the remaining supernatants were stored at –80°C.

Protein assays were conducted for the diluted subsamples using the Bio-Rad DC assay (BioRad, Hercules, CA) with a bovine serum albumin standard. An 8% SDS–polyacrylamide resolving gel, with a 4% stacking gel, was used for protein separation (Laemmli 1970). All samples were loaded at 20 μg total protein. A reducing agent of dithiothreitol (DTT, 1:5 to sample protein load) and a marker dye of bromophenol blue (1:10 of the combined DTT and protein load volume) were added to each sample based. The mixture was boiled for 3 min, then centrifuged (relative centrifugal force [RCF] = 14,000 *g*) for 1 min. To normalize for inherent variations in protein expression among gels, each gel contained a previously collected adult oyster mantle tissue as a standard sample. The standard sample, which had all 3 HSP 70 isoforms, was prepared as described earlier. Gels were run at 100 V for 150 min. After protein separation, the gels were stained with a fluorescent protein stain (Sypro Tangerine protein stain; Molecular Probes, Eugene, OR) for 45 min and scanned for later protein load analyses.

The proteins were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Piscataway, NJ) at 100 V for 60 min in buffer containing 25 mM Tris base, 192 mM glycine, and 15% ethanol (Towbin et al. 1979). The membrane was probed with a mouse anti-HSP 70 antibody (Sigma-Aldrich, Saint Louis, MO) at a 1:10,000 dilution in 5% nonfat dried milk in Tris-buffered saline (TBS) (137 mM NaCl and 20 mM Tris solution; pH, 7.6) and 0.1% Tween 20 solution (MTTBS), and a goat antimouse horseradish peroxidase (HRP) secondary antibody (Bio-Rad, Richmond, CA) at a 1:3000 dilution in MTTBS. To detect the expression levels of proteins, an HRP chemiluminescent detection system (Lumi-Glo and peroxide; Cell Signaling Technology, Beverly, MA) was used. Membranes were exposed to either film (Kodak X-OMAT, Rochester, NY) for 3 min or developed by LAS-1000 Image Reader (Fuji film, Tokyo, Japan) to capture the image.

### *Data Analysis and Statistics*

HSP 70 expression was quantified from digital images of tangerine-stained gels and images from the film and LAS-1000 Image Reader (Fugi Film, Tokyo, Japan). Relative intensities of protein bands were determined using Adobe Photoshop Elements 2 (San Jose, CA).

Relative intensities of each HSP 70 isoform—HSC 77, HSC 72, and HSP 69—were normalized to corresponding bands of the standard sample on the same gel. Arcsine transformations were used to normalize intensities prior to analysis (Zar 1984). Levine's homogeneity of variance tests were run to ensure equal variance. Normalized values of band intensities for each isoform were analyzed individually. Three-factor ANOVAs were used to compare differences among groups (CPC, CPE, WHC, and WHE), between exposure times (48 h and 96 h), and between treatments (normoxia and anoxia) for each isoform. A Tukey multiple comparison post hoc test was used to examine differences among group samples. An alpha value of 0.05 was used for all statistical analyses (Sokal and Rohlf 1995, Zar 1984).

## RESULTS

Three HSP 70 isoforms—HSC 77, HSC 72, and HSP 69—were detected in all samples from both sites (see sample blot for CPE samples, Fig. 1). There were significant differences in both HSC 77 and HSC 72 expression among groups ($P = 0.032$ and $P = 0.012$, respectively; Fig. 2). The expression of HSC 77 in CPC and of HSC 72 in CPE was significantly higher than that of WHC ($P = 0.041$ and $P = 0.007$, respectively; Fig. 2). Significant differences in HSP 69 expression were found for group, time, and treatment ($P = 0.049$, $P = 0.023$, and $P = 0.010$, respectively; Fig. 2). Because the $P$ value for groups was close to 0.05, multiple comparison tests among groups were not run for HSP 69 (Sokal & Rohlf 1995). The expression of HSP 69 showed a significant increase between the 48-h and 96-h time points for all sites and treatments, except for the normoxic WHE samples, for which a decrease was seen. Interestingly, except for the WHE 96-h samples, the normoxia samples showed higher expression of HSP 69 than the anoxia-exposed samples. There were no interactions between or among factors for any of the 3 HSP 70 isoforms analyzed.

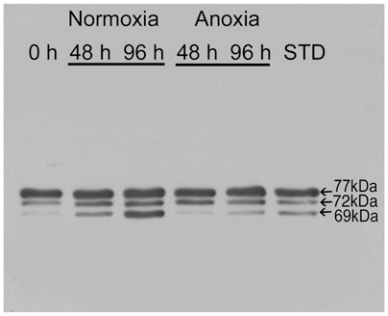


**Figure 1. A representative Western blot for HSP 70 expression in juvenile Eastern oyster, *Crassostrea virginica*. Samples shown are siblings of parental oysters from Cedar Point that survived anoxia. Three isoforms—HSC 77, HSC 72, and HSP 69—were expressed in all samples. Oysters were exposed to normoxia and anoxia. Samples were collected prior to the experiment (0 h), and at 48 h and 96 h of exposure. For the standard sample, a protein sample extracted from mantle tissue of a previously collected adult oyster was used.**

## DISCUSSION

As has been shown previously, there are 3 HSP 70 isoforms—2 constitutive (HSC 77 and HSC 72) and 1 inducible HSP 69—expressed by juvenile Eastern oysters, *C. virginica* (Encomio & Chu 2005) (Fig. 1). Although there were significant differences in both HSC 77 and HSC 72 expression among groups (CPC, CPE, WHC, and WHE), the expression levels of HSC 77 and HSC 72 were not significantly affected by anoxia treatment or duration of exposure (Fig. 2). Except for the WHE samples, the expression of HSP 69 significantly increased over time for both normoxic and anoxic treatments, with lower expression for anoxia-exposed samples than normoxia samples at both time points (Fig. 2). There were also significant differences in HSP 69 expression among groups (CPC, CPE, WHC, and WHE).

Prior to running the experiment, it was hypothesized that the expression of cognate forms of HSP 70 would be the highest for the WHE oysters, because they were selected for anoxia resistance and exposed to anoxia. However, there were no consistent patterns in expression of HSC 77 or HSC 72 among groups (Fig. 2). This suggests that, as has been seen for HSP 16.2 in the worm *Caenorhabditis elegans*, expression of HSP 70 in *C. virginica* may not be heritable (Rea et al. 2002). Alternatively, HSP 70 may not play a significant role in responses to anoxia, and so could not be selected for through anoxia-linked breeding. It is interesting to note that Ford (2005) also found surprising results for LT-50 studies using the same batch of oysters. Although the LT-50 of CPE was significantly higher than the CPC, the WHE LT-50 was significantly lower than that of WHC (Ford, 2005).

In this study, expression of HSP 69 after anoxia exposure, at least that created by nitrogen exposure, was, in general, lower than expression under normoxic conditions (Figs. 1 and 2). Although some studies have shown induction of HSP 70 messenger RNA after exposure to anoxia or hypoxia, others have shown little or no response of HSP 70 to hypoxia (Chiral et al. 2004, Cara et al. 2005, Sarkis et al. 2005). Facultative anaerobiosis and metabolic arrest during anoxia or hypoxia in molluscs including *Crassostrea* species is a well-known strategy for survival (Hochachka & Mustafa 1973, Storey 1993), and

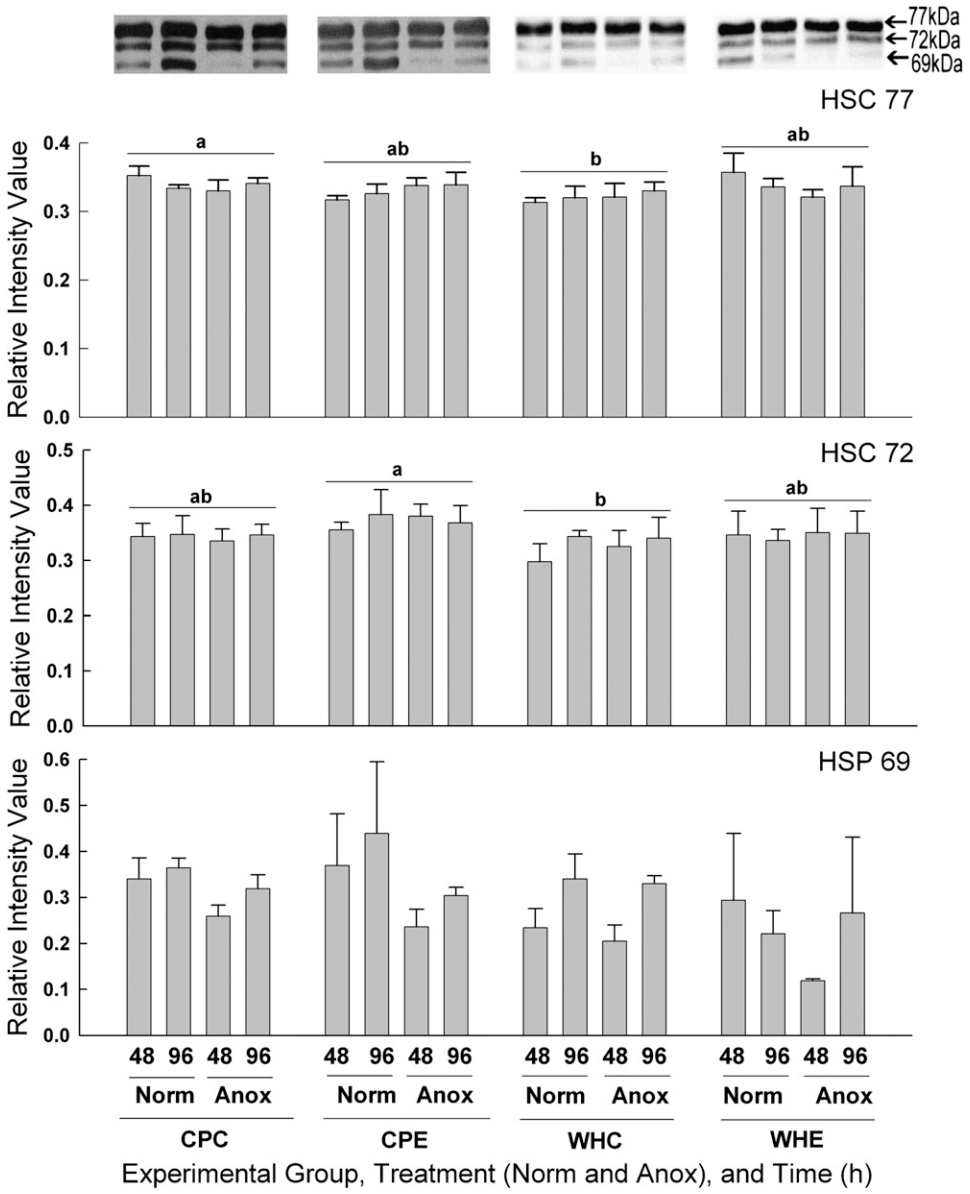


**Figure 2. Comparisons of relative intensities (mean ± SD) of expression of HSC 77, HSC 72, and HSP 69 in juvenile Eastern oyster, *Crassostrea virginica*, at 48 h and 96 h exposure to normoxia (Norm) and anoxia (Anox). Samples were siblings of parental oysters from 2 different sites (Cedar Point and White House) that either survived anoxia exposure (CPE and WHE) or were held under normoxic conditions (CPC and WHC). Each isoform was analyzed by a 3-factor ANOVA (group [CPC, CPE, WHC, and WHE], treatment, and exposure time). A model Western blot of protein expression for each group is shown at the top of the figure. Letters (a, b) above bars indicate significant differences among groups for HSC 77 and HSC 72. There were no time or treatment differences for these isoforms. For HSP 69, there were significant differences in exposure time ($P = 0.023$), treatment ($P = 0.010$), and groups ($P = 0.049$). Except for the WHE normoxia, the 96-h samples showed higher expression than the 48-h samples. Except for the WHE 96-h anoxia sample, the normoxia samples showed higher expression than the anoxia samples. Multiple comparisons among groups for HSP 69 could not be conducted.**

limited induction of HSP 69 in this study is possibly related to a shift toward anaerobic metabolism. The metabolic rate of *C. virginica* (30–50 mm) under anoxia at 25°C is 75% of the normoxic rate at 25°C (Stickle et al. 1989). In juvenile oyster (approximately 16 mm), it decreases to 3% of the normoxic rate at 22°C (Widdows et al. 1989). As a response to metabolic arrest, protein synthesis is suppressed (Storey & Storey 2004). Because synthesis of stress proteins is thought to be energetically costly (Creighton 1993) and functional activities of HSP 70 are driven by adenosine triphosphate (Hightower et al. 1994), it is reasonable that significant suppression of stress protein synthesis also occurs under the metabolic arrest. Similar studies with Eastern oysters have also shown that there is no significant increase of HSP 69 expression in oysters exposed to hypoxic conditions (2.8 mg/L) for 12 h (Ueda 2006). In the Calico Scallop, *Argopecten gibbus*, aerial exposure for 24 h does not increase HSP 70 expression (Sarkis et al. 2005).

An unexpected finding in this experiment was that the expression level of HSP 69 in oysters exposed to normoxia was higher than the ones exposed to anoxia. Because the

expression of HSP 69 was upregulated above 0 h samples in all cases, induction of HSP 69 must have occurred during the experiment. One possible explanation for induction of HSP 69 under normoxic conditions is lack of food. Induction of HSP 70 and HSP 90 by food deprivation has been reported in larval gilthead sea bream and rainbow trout (Cara et al. 2005). According to that report, HSP 70 and HSP 90 expression was significantly induced by 12-h food deprivation in larval sea bream and 7-day food deprivation for larval rainbow trout. The authors of that study hypothesized that accelerated proteolysis during food deprivation is the cause of induction of HSP 70 and HSP 90. Spat in the size range used in the current study (7–15 mm) exhibit rapid growth and may parallel the larval fish responses. The differences in the expression patterns between the normoxia and anoxia groups suggest that aerobic metabolism may allow for increased HSP 69 synthesis. Therefore, cellular defense capacity in juvenile Eastern oysters may be weakened during exposure to anoxia because there may be reduced production of these protective molecular chaperones.

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