Differences in heat shock protein 70 expression during larval and early spat development in the Eastern oyster, *Crassostrea virginica* (Gmelin, 1791)

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Abstract For a variety of species, changes in the expression of heat shock proteins (HSP) have been linked to key developmental changes, i.e., gametogenesis, embryogenesis, and metamorphosis. Many marine invertebrates are known to have a biphasic life cycle where pelagic larvae go through settlement and metamorphosis as they transition to the benthic life stage. A series of experiments were run to examine the expression of heat shock protein 70 (HSP 70) during larval and early spat (initial benthic phase) development in the Eastern oyster, *Crassostrea virginica*. In addition, the impact of thermal stress on HSP 70 expression during these early stages was studied. *C. virginica* larvae and spat expressed three HSP 70 isoforms, two constitutive, HSC 77 and HSC 72, and one inducible, HSP 69. We found differences in the expression of both the constitutive and inducible forms of HSP 70 among larval and early juvenile stages and in response to thermal stress. Low expression of HSP 69 during early larval and spat development may be associated with the susceptibility of these stages to environmental stress. Although developmental regulation of HSP 70 expression has been widely recognized, changes in its expression during settlement and metamorphosis of marine invertebrates are still unknown. The results of the current study demonstrated a reduction of HSP 70 expression during settlement and metamorphosis in the Eastern oyster, *C. virginica*.

Keywords Eastern oyster · Heat shock protein 70 · Larvae · Settlement and metamorphosis · Spat

Abbreviations

DNA Deoxyribonucleic acid
HSC Heat shock cognate/constitutive
HSP Heat shock protein
PCR Polymerase chain reaction
SDS Sodium dodecyl sulfate

Introduction

Heat shock proteins (HSPs) serve as regulators of normal cell function, assisting in proper folding, assembly, and transport of nascent proteins (Gething and Sambrook 1992). They also function as cellular defenses, aiding in the refolding and removal of proteins denatured by biotic and abiotic stress, increasing in expression when organisms are exposed to environmental stresses (Sanders 1993; Feder and Hofmann 1999). Among HSP families, the group in the 70-kDa size range (HSP 70) is the most extensively studied because of its prominent response to stresses (see reviews in Sanders 1993; Feder and Hofmann 1999). Heat shock protein 70 isoforms include those that are stress-inducible (HSP) as well as constitutive (HSC) forms.

Interestingly, in a wide range of organisms, the expression of HSP 70 has also been shown to vary during development (Dix 1997; Feder and Hofmann 1999; Giudice et al. 1999; Wood et al. 1999; Yeh and Hsu 2002; Karouna-Renier et al. 2003; Chang 2005; Mahroof et al. 2005). The differential expression has been studied during events that involve morphological changes in organisms such as gametogenesis, embryogenesis, and metamorphosis (Feder and Hofmann

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1999). Although the roles of HSP 70 during developmental stages are still obscure, they may function both as chaperones for new protein production as well as protectants during stress-sensitive developmental periods (Feder and Hofmann 1999). Currently, little is known about developmental regulation of HSP 70 in marine invertebrates, although studies have shown that HSP 90 plays a role as a metamorphosis regulator (Giudice et al. 1999; Bishop and Brandhorst 2001; Bishop et al. 2001; Brown et al. 2004; Chang 2005; Gunter and Degnan 2007).

Many marine invertebrates have biphasic life stages, often with pelagic larvae that are microscopic and benthic juvenile/adults that are morphologically different from the larvae (McEdward and Janies 1993). It is common that such marine invertebrates go through settlement followed by metamorphosis at the end of the larval stage to transform to benthic post larvae. Not only do morphological changes occur, but cellular changes from the larval to adult form can also be pronounced, as is the case for the Eastern oyster *Crassostrea virginica*.

After settlement of *C. virginica*, larval structures, including the velum, velar muscle, foot, eyespot, and anterior adductor muscle, are resorbed by the spat and permanent reorganization of other structures occurs (Kennedy 1996). Work on veliger larvae of the California native oyster, *Ostreola conchaphila*, showed constitutive expression of HSC 77 and HSC 72 and induction of HSP 69 by thermal stress (Brown et al. 2004). Similarly, increases in all three isoforms were seen in the Pacific oyster, *Crassostrea gigas*, in response to thermal stress (Clegg et al. 1998; Hamdoun et al. 2003). Three HSP 70 isoforms (HSC 77, HSC 72, and HSP 69) were identified in adult Eastern oyster (Encomio and Chu 2005); however, the expression of HSP 70 during settlement and metamorphosis was not addressed. Preliminary work in our lab comparing expression of HSP 70 in pediveliger larvae, i.e., those immediately pre-settlement, and spat 2 days post-settlement indicated that there was a drop in expression of HSP 70 in early spat (data not shown). To better understand when changes in HSP 70 expression occur during *C. virginica* development, differences in the expression of HSP 70 in larvae from 2 to 14 days post-fertilization and in spat 17 to 30 days post-fertilization (3 to 14 days post-settlement) were examined. The impact of thermal stress during development was also examined.

Results and discussion

This is the first report of the expression series of HSP 70 during settlement and metamorphosis of a marine invertebrate. In an experiment looking only at developmental changes (experiment 1), HSC 77 and HSC 72 were detected in all larval and spat samples; however, HSP 69 was not detected in any of the samples (Figs. 1A and 2). There were significant

Fig. 1 Representative Western blots for two experiments examining HSP 70 expression in larval and spat samples of the Eastern oyster, *C. virginica*. **a** Experiment 1: Expression of HSP 70 in samples held at ambient conditions. Larvae (~0.06, 0.1, 0.2, and 0.3 mm) and spat (0.6–0.7 and 2 mm) were collected at 2, 6, 11, 14, 18, and 30 days post-fertilization. Spat sized 0.6–0.7 and 2 mm were collected 5 and 14 days post-settlement. Two constitutive isoforms, HSC 77 (*top band*) and HSC 72 (*bottom band*), were expressed in all samples. **b** Experiment 2: Expression of HSP 70 held at ambient conditions (*top blot*) and those exposed to thermal stress (*bottom blot*). For both conditions, larvae (~0.1, 0.2, and 0.3 mm) and spat (~0.6 and 1.8 mm) were collected, which correspond to 6, 11, 14, 17, and 27 days post-fertilization. In the ambient samples, two constitutive isoforms, HSC 77 (*top band*) and HSC 72 (*bottom band*), were expressed in all samples. In the thermal stress samples, two constitutive isoforms, HSC 77 (*top band*) and HSC 72 (*middle band*), and one inducible isoform, HSP 69 (*bottom band*), were expressed in all samples. All larval and spat samples were provided by the Auburn University Shellfish Laboratory, Dauphin Island, AL and were collected under ambient culture conditions. Spawning of *C. virginica* was induced by thermal shock. Fertilized eggs and larvae were cultured in 900 L of aerated seawater. Larvae were fed twice a day with algal paste (Reed Mariculture, Inc. San Jose, CA, USA). Seawater was pumped directly from the Gulf of Mexico, filtered to 1 µm, and changed every other day. Samples to be exposed to thermal stress were transferred to 250-ml glass beakers in a water bath held at a temperature 10°C higher than the culture temperature. They were exposed to this thermal stress for 1 h and returned to the culture temperature for 24 h of recovery. Three replicates of each size sample were collected, flash frozen in liquid nitrogen, and held on liquid nitrogen until return to the laboratory at the University of South Alabama. Samples were stored at –80°C for further analysis. Methods for immunoblotting follow those used by Sarkis et al. (2005) with minor modifications. Frozen larval and spat samples were homogenized in sodium dodecyl sulfate sample buffer (SDS sample buffer, 125 mM Tris, 10% glycerol, and 2% SDS) at a ratio of 1:4 sample weight to SDS sample buffer volume. The homogenates were placed in boiling water for 3 min then centrifuged at 14,000×g for 10 min. Supernatants were collected and re-centrifuged for an additional 10 min. Samples were stored at –80°C for further analysis. Total protein concentrations of diluted subsamples (1:10) were determined using the Bio-Rad DC assay (BioRad, Hercules, CA, USA) with a bovine serum albumin standard. Samples of equal total protein content (20 µg) were prepared with the addition of the reducing agent, dithiothreitol. Samples were placed in boiling water for 3 min prior to separation by SDS–polyacrylamide gel electrophoresis (8%; Laemmli 1970). A standard sample was prepared from previously collected adult oyster gill tissue and used for normalization of inherent variations in protein load and intensity among gels. The standard sample was prepared as described above and used in all gels run. Gels were run at 100 V for 150 min followed by staining with Sypro Tangerine protein stain (Molecular Probes, Eugene, OR, USA) and scanned for later protein load analyses. The proteins were then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences) at 100 V for 60 min (Towbin et al. 1979), analyzed by immunoblotting with monoclonal anti-HSP 70 clone BRM-22 antibodies (1:10,000 dilution, Sigma-Aldrich, Saint Louis, MO, USA), and visualized by chemiluminescence (LumiGlo; Cell Signaling Technology, Beverly, MA, USA). The protein images were captured by LAS-1000 Image Reader (Fuji Film, Tokyo)

differences in both HSC 77 and HSC 72 expression ($P < 0.001$ and $P = 0.003$, respectively) among developmental stages (Fig. 2). The expression patterns of the two isoforms were similar (Fig. 1). Through larval development, the expression

of HSC 77 and HSC 72 decreased. Decreases in expression of HSC 77 and HSC 72 were observed in 0.2-mm larval samples with subsequent increases in the 0.7-mm spat sample. HSC 77 expression in 0.2- and 0.3-mm larvae and 0.7-mm spat were significantly lower than that in 0.06- and 0.1-mm larvae and 2-mm spat (Fig. 2). HSC 72 expression in 0.3-mm larvae was significantly lower than in all other larval or spat samples (Fig. 2). In a second experiment (experiment 2) looking at both changes during development and in response to thermal stress, HSC 77 and HSC 72 were detected in all larval and spat samples of both the control and thermal stress groups, while the stress-inducible isoform, HSP 69, was detected in all larval and spat samples of the thermal stress group (Figs. 1B and 3). There were no statistically significant differences among developmental stages for HSC 77 and HSC 72 (Fig. 3). The expression of HSP 69 varied with developmental stage (Fig. 3). The 1.8-mm size treatment group had significantly higher HSP 69 expression than all other sizes in this group ($P < 0.001$; Fig. 3).

Interestingly, Mahroof et al. (2005) saw similar developmental changes in studies with the red flour beetle, *Tribolium castaneum*. In the red flour beetle, the lowest levels of a constitutively expressed HSP 70 gene were seen in pupae just prior to metamorphosis. While increases in expression of HSP 70 can be explained as a form of cell level defense during stress-sensitive phases of development, detrimental effects of HSP overexpression in the cells are likely to account for the lower expression of this protein during other phases of development (Krebs and Feder 1998; Feder and Hofmann 1999). Feder and Hofmann (1999) proposed that one of the possible explanations for the negative effects of HSPs is the reduction in overall cellular energy budget because of high energetic costs of synthesis/degradation of HSPs. As a corollary to this

explanation, we hypothesize that high cellular metabolism required during settlement and metamorphosis may lead to a suppression of synthesis of HSC 77 and HSC 72 in the pediveliger stage of the Eastern oyster.

Although there were significant differences in the expression of HSP 72 and 77 in experiment 1, while looking only at developmental changes (Fig. 2), no significant differences in these isoforms were found in experiment 2 examining both developmental and thermal

Fig. 2 Comparisons of relative intensities (mean $\pm$ SD) of HSC 77 and HSC 72 bands in larval (0.06, 0.1, 0.2, and 0.3 mm) and spat (0.7 and 2.0 mm) samples of the Eastern oyster, *C. virginica*, from experiment 1 looking at difference associated with development. Two constitutive isoforms, HSC 77 and HSC 72, were expressed in all samples. To quantify HSP 70 expression, the HSP 70 bands from the immunoblots were digitized and analyzed for intensity with Adobe Photoshop Elements 2.0. These relative intensities were then normalized to corresponding bands of the standard sample on the same gel. Arcsine transformations were utilized to normalize intensity data prior to analyses (Zar 1984). Levine's homogeneity of variance tests was run to assure equal variance among samples. The transformed values of band intensities were analyzed by separate one-factor analyses of variance (ANOVAs; factor = oyster size) for the developmental series in 2005. A Tukey multiple comparison test was used to examine differences among samples. An α value of 0.05 was used for all analyses (Zar 1984). Letters above error bars indicate significant differences at $p < 0.05$; $n = 3$

Fig. 3 Comparisons of relative intensities (mean and $\pm$ S.D.) of HSC 77 and HSC 72 larval (0.06, 0.1, 0.2, and 0.3 mm) and spat (0.6 and 1.8 mm) samples of the Eastern oyster, *C. virginica*, from experiment 2 looking at difference associated with development and thermal stress. The quantification of protein band intensities was described as Fig. 2. The band intensities were analyzed by separate one-factor ANOVAs (factor = oyster size) for the ambient and thermal stress series. A Tukey multiple comparison test was used to examine differences among samples. An α value of 0.05 was used for all analyses (Zar 1984). Letters above error bars indicate significant differences at $p < 0.05$; $n = 3$. N.E. no expression

stress-related changes (Fig. 3). The differences between experiments are thought to be associated with differences in environmental conditions between experiments and among collection times within an experiment. Due to laboratory constraints, oysters in both experiments were cultured under ambient conditions. During the first experiment, these ambient conditions remained relatively uniform. However, during the second experiment, there were greater variations in environmental conditions, particularly in salinity, which varied from 11.8 to 30.0 over the course of the study (i.e., across collection points). These fluctuations may have impacted the expression across collection dates and, therefore, across age.

The low expression of HSP 69 in response to thermal stress among the larval stages and early spat stage of *C. virginica* was surprising in light of studies done with the

California native oyster, *O. conchaphila* (Brown et al. 2004). In this species, veliger larvae showed significant increases (fourfold) in HSP 69 expression after a 1 h heat shock. Embryonic *O. conchaphila* did not express HSP 69. Differences in larval HSP 69 expression levels between the two oyster species may relate to differences in their early life history strategies. *O. conchaphila* is larviparous, nurturing embryos and early larval stages that develop in the mantle cavity of female individuals. This species also has a longer pelagic stage (3–8 weeks) and lower fecundity (250,000–300,000/single brood; Hopkins 1936; Baker 1995; Brown et al. 2004) than *C. virginica*. The Eastern oyster is oviparous, with females releasing 23–86 million eggs in a single spawn (Davis and Chanley 1956), and the duration between fertilization and settlement is only about 2 weeks (Langdon and Newell 1996). Differences in level

of parental care and in potential exposure to unfavorable environmental conditions due to these differences in parental care may act as selection factors contributing to differential abilities of larval stages to express HSPs, particularly the inducible forms. These differences may help explain or predict phases during which organisms may be more at risk to environmental stress.

Advancement of the current molecular/cellular techniques (quantitative PCR, DNA microarray, and proteomics) will allow us to further investigate whether differences in HSP 70 expression linked to both developmental processes and environmental stress reflect diversity in early life history strategies. Understanding these differences may provide a clearer understanding of larval survival and dispersal patterns (Sorte and Hofmann 2004), which, in turn, can be used in management plans and in examinations of the effects of environmental change caused by coastal development and climate disruption.

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