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Induced Expression of Ovarian Heat Shock Proteins with Response to Thermal Stress in an Endemic Freshwater Crab, *Sartoriana spinigera* (Wood-mason, 1871) from East Coast of India

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A B S T R A C T

The Heat shock proteins (HSP) play an important role in controlling the apoptosis and inflammatory response. These proteins are released into the extracellular environment and act as mediators of inflammation. Protein folding accuracy is ensured by chaperone activity, which also prevents the accumulation of abnormal isoforms. In the current work, *Sartoriana spinigera*, an endemic freshwater crab, is examined for its survival and Hsps expression patterns in ovarian tissues in response to short-term heat stress. Crabs maintained at 25 ^{o}C served as control. Expression of Hsp genes were analyzed using semi-quantitative and quantitative PCR in both groups to see whether these crabs have lost the Hsp gene plasticity. Hsp gene expression analysis showed up-regulation of *hsp90* (2.1-fold) and *hsp47* (2.5-fold) in heat stressed crabs, whereas there was no alteration in expression of other Hsps. Up-regulation of all the Hsps was observed in the heat shocked crabs, whereas expression of all Hsps were down regulated to the basal level in crabs maintained at 34 ^{o}C/24 hour. These results indicate that Hsp90 and Hsp60 play important role in survival of *Sartoriana spinigera* under thermal stress.

Introduction

The most difficult environmental issue influencing the reproductive physiology of various species, including mammals, fish, and birds, is the rise in ambient temperature brought on by global warming. In order to mitigate this negative consequence, organisms create a variety of different survival methods. In this regard, the heat shock response (HSR) is a highly conserved cellular process

involved in preserving an organism's maximum temperature tolerance by synthesizing a family of proteins known as the heat shock protein (Hsps). Although Hsps are reportedly a key factor in thermal adaptation, little is known about how they manifest themselves in the crab *Sartoriana spinigera*. (Bo Zhang *et al.,* 2016). Hsps are profoundly preserved and can be categorized into a few families such as, Hsp110, Hsp90, Hsp70, Hsp60 and some small Hsps indicated by their molecular weights (Lindquist and Craig, 1988; Gupta, 1995; Csermely *et al.,* 1998; Mohanty *et al.,* 2018). In general, Hsp90, Hsp70 and Hsp60 are commonly found in invertebrates, including birds, fish and mammals (Salway *et al.,* 2011). The synthesis of various Hsps was once thought to be triggered by heat, but further research has shown that Hsp production is also triggered by other stresses, including ethanol (Beck, 1995), heavy metals (Lindquist, 1986), pollutants (Feder and Hofmann, 1999), and chemicals (Mica *et al.,* 2013).In order to transport proteins to tertiary structures, Hsp90 typically works as part of a complex with other chaperones and co-chaperones (Matthews, 1992; Kosmaoglou *et al.,* 2008). It has also been linked to hypothermia resistance, cell proliferation, and controlling the cell cycle in various vertebrates (Herring and Gawlik, 2007). The Hsp70 family of essential chaperones controls protein folding in conjunction with other proteins to maintain the structure of proteins in both the cytosol and mitochondrial matrix (Kriegenburg *et al.,* 2012). Hsp70 has been recognized as a key stress biomarker because it has been found to be more responsive to environmental changes in some vertebrates than Hsp90 and Hsp60 (Dehbi *et al.,* 2010; Lewis *et al.,* 2000). According to Kosmaoglou *et al.,* (2008), the classical chaperone family Hsp60 generates ring crystals, aids in protein folding, and may be necessary for cold tolerance (Herring and Gawlik, 2007). Additionally, it has been reported to control immune system hormones in vertebrates (Young, 1990). Although, different Hsps are reported to respond temperature stress in wild vertebrates, still, little is known about their mechanism of action (Clark and Peck, 2009; Ryan and Hightower, 1996). Therefore, the present study is designed to identify the principal Hsps as an indicator of thermal stress in crabs by measuring the variations in-circulating Hsps level by exposing the crabs to instant and short-term heat stress.

Materials and Methods

Reagents

RNA stabilizer was obtained from Sigma-Aldrich, St Louis, MO, USA. RNA extraction kit was purchased from Himedia, India. DNase I and cDNA synthesis kit were purchased from New England BioLab, UK. Taq DNA polymerase and DNTPs were purchases from MP Biomedicals, India. Oligos were obtained from IDT, USA. iQsyber green super mix was purchased from Bio-Rad, UK.

Collection of Crab

The experiments were carried out with crabs *Sp. Sartoriana spinigera* having length 5.31±1.11 cm and weighing 16.25±1.20 gm. Crabs were collected from a local farm nearby Cuttack, Odisha, India and were acclimatized under a control laboratory condition for 30 days. During acclimatization, holding condition was maintained with temperature of 26±2^{o}C and alternation of light and dark cycle of 12 hr.

Experimental conditions

The crabs were randomly assigned into control and experimental groups and divided into three groups having 12 numbers of crabs in each. Group 1 was served as untreated control and kept at holding temperature of 25±2^{o}C. Group 2 and 3 were treated as experimental groups and were maintained at a temperature of 34 ^{o}C for a period of 1hr (instant) and 24hr (short term) heat shock, respectively.

Collection of crab ovary tissue

Collection of crab ovary tissues were made after the completion of the respective exposure time periods as mentioned earlier. The crabs were washed

properly with PBS prior to dissection and ovary tissues were collected and stored in RNA stabilizer at -80 ^{o}C for transcriptomics analysis.

RNA isolation and cDNA synthesis

Total RNA was extracted from the crab ovarian tissues using the RNA extraction kit following the manufacturer's instructions (Himedia, India). At first, the genomic DNA was removed by DNase I digestion and its quality were assessed using agarose gel electrophoresis. Next, RNA concentrations were measured using Nanodrop and 1 µg of RNA was taken from each sample for the synthesis of cDNA using the fast stand cDNA synthesis kit following the manufacturer's protocol (NEB, UK) (Purohit *et al.,* 2014).

Semi quantitative reverse transcription-PCR analysis

Reverse transcription PCR was conducted to compare the HSP gene expression in control and heat shocked treatment groups, according to manufacturer's protocol (Purohit *et al.,* 2012) using specific primers (see Table1). In brief, 50ng of first strand cDNA was added in the reaction mixture from each sample and a thermal cycler system (Eppendorf, Germany) was used for the PCR analysis. 30 cycles of amplification were followed for each candidate genes and β-Actin was used as an internal control for normalizing the expression to the candidate genes.

Quantitative real-time PCR analysis

CFX connect 96 well PCR detection system (Bio-Rad, UK) was used for the quantitative real-time PCR analysis to compare the HSP gene expression in control and heat shocked treatment groups. According to manufacturer's protocol (NEB, UK) using 100 ng of cDNA from each sample using specific primers (see Table1) (Purohit *et al.,* 2014). Gene expression differences between a control and treated sample were calculated using the comparative Cq (ΔCq) method. A standard curve was plotted in triplicate for each primer pair using six-fold dilutions of a standard batch of cDNA for the quantitative real-time PCR for determining the its efficiency (Pfaffl, 2001). Moreover, the qPCR efficiency of one cycle for each gene was calculated by applying the slope of a linear regression equation to the equation E=10 [-1/slope].

Statistical analysis

Data presented in this manuscript are representative of three independent experiments. ΔCt was used to determine the gene expression levels. Values are expressed as the mean ± SD. Prism software was used to analyze the results using one-way analysis of variance (ANOVA). Data were statistically significant when *$p \leq 0.05$.

Results and Discussion

Heat shock increases the expression of ovarian HSPs in *Sartoriana spinigera*

To study the expression of ovarian Hsps in response to heat shock in *Sartoriana spinigera*, the average temperature was optimized for laboratory conditions and 34 ^{o}C was chosen for the heat shock condition. Semi-quantitative gene expression analysis showed a time dependent increase of band intensity for Hsp70, Hsp60 and Hsp90 in response to heat shock with respect to control (Figure 1). The relative mRNA expression of Hsp70 (3.9-fold) and Hsp90 (3.7-fold) were found to be significantly higher than Hsp60 (0.8-fold) expression in 24 hr of heat treatment, indicating its major role play in adverse condition. Likely, the expression of Hsp70 (3.3-fold) was observed highest in 1 hr of heat shock treatment in compared to Hsp60 (0.4-fold) and Hsp90 (0.8-fold) suggesting its preliminary and rapid action of defense against heat shock in *Sartoriana spinigera* (Figure1 and figure2).

Environmental stress threatens the functional integrity of an organism and is associated with the expression of a series of genes and proteins as a defensive mechanism against adverse conditions

(Tomanek, 2010; Parsell, 1993; Feder, 1999). All cellular organisms, whether they are stressed or not, produce a suite of highly conserved stress proteins known as HSPs having varying molecular weights (Park and Kwak, 2008). A number of studies have documented the expression of HSPs in response to environmental stresses (Feder, 1999; Qari, 2004; Lockwood, 2010; Schoville 2012). For example, Mingyan Bei *et al.,* has revealed that heat stress reduces the competence of oocyte, and is responsible for lower fertility in animals (Mingyan Bei *et al.,* 2020). Similarly, high environmental temperaturesaffect the fertility by changing animal physiology along with performance (Takahashi, 2012). In pig, heat stress induced ovarian autophagy during follicular development had no effect on follicle size and number **(**Hale *et al.,* 2017**)**. In livestocks like dairy cows, heat stress decreased the estradiol production and reduced the viability of granulosa cells by changingthe levels of glucose and non-esterified fatty acids in the blood (Miller-Cushon *et al.,* 2019). Thorough research has revealed that small Hsp genes were induced more quickly as compared to the other groups and alsotheir expression declines at 3 and 6 weeks. This suggeststhat they regulated the early responses to heat stress (Mingyan Bei *et al.,* 2020). In some of the studies, Hsp levels have been shown to vary over hours as a result of thermal stress (Malev, 2010; Tomanek, 2002, Purohit *et al.,* 2014). The stress-induced appearance of denatured proteins with exposed hydrophobic residues is thought to be the trigger for the production of Hsps of several different families. In addition to providing protection from further damage, the Hsps also help to restore function once the stress has passed (Parsell and Lindquist, 1993; Willmer, 2004).The molecular chaperones or proteases within stressed cells can refold or cleave denatured proteins in stressed cells, or they can break them down (Wickner, 1999). Studies showed that Hsp70 is responsive to a wide variety of environmental and physiological stressors, including bacteria, high temperatures, chemicals, and environmental variables. There are two main genes within the Hsp70 family, heat-shock cognate protein 70 kDa (Hsc70) and caspase-3. In normal conditions, Hsp70 was not expressed or expressed only slightly under stress conditions, while inducible Hsps70 kDa was not expressed or little expressed under stress conditions (Deane and Woo, 2005; Francisellitti and Fabbri, 2005; Li *et al.,* 2015). The crabs exposed to high temperatures for 1hr heat shock showed dramatic up-regulation of Hsp70 (3- folds) and during the 24 hours of heat shock, HSP70 levels were increased (4-folds) in comparison with control. Molecular chaperones such as Hsp90 assist cellular proteins in folding properly (Mahanty *et al.,* 2017a, b; Mahanty *et al.,* 2019). As a co-chaperone, it mediates conformational regulation of several client proteins, including folding, cytoprotection, and proteasome degradation. They associate with steroid hormone receptors and maintain them in a non-functional state until hormone binding and interact with cellular signaling proteins (Bohen, 1995; Luan, 2009). Hsp90 genes have been isolated from various crustaceans including lobsters (Chang, 2005), shrimps (Wu and Chu, 2008; Jiang 2009) and crabs (Li, 2009; Zhang, 2009). Hsp90 mediates estrogen signal transduction, which regulates the synthesis of vitellogenin (Wu and Chu, 2008). Hsp90 may be involved in the defense system or the stress response of the blue crab, *Callinectes sapidus*. Hsp90 is involved in protein folding, cyto-protection and proteosomal degradation and teams up with a large set of co-chaperones to mediate the conformational regulation of several client proteins. Hsp90 showed a similar expression pattern as Hsc70. Expression of Hsp90 gene was up-regulated at 1, 0.61 and 1.56 folds in crabs was exposed to 1 hr and 24 hr of heat shock. Hsp60 plays an important role in health, in particular in the development of inflammation and the specific and nonspecific responses to bacterial and viral infections. Intensity of the Hsp60 response is not only dependent on the induction, but also organ specific (Xu *et al.,* 2011). No significant change was observed in expression of Hsp60 relative to the reference site.

Table.1 Primer sets used for semi-quantitative and quantitative RT-PCR analysis of the genes in *Sartoriana spinigera.*

Primer Name	Primer Sequence (5'-3')	Annealing temperature	Amplicon size (bp)	Gene bank Accession No.
Beta actin	F: TCAGAGATATTTCGCCACCT R: TGTTCTTGATGGCTCTCTCC	55°C	115	KU375113
hsp70	F: TTTGTGTGCTTAAAGGGACC R: AAGGCCAGTAATTTCACCAG	57°C	101	KU375112
hsp60	F: AGGAGCGAATGGCACGACT R:ATCCAGGGCAGGCAAGCAG	62°C	92	Bao *et al* 2014
hsp90	F:CTATCCCATCAGGCTCCTTGTT R:CACCTACATCCTCAATCTTTGGCTT	59°C	102	Bao *et al* 2014

Fig.1 Transcript analysis of gonadal hsp70, hsp60 and hsp90 genes of the *Sartoriana spinigera* exposed experimental groups and were maintained at a temperature of 34 °C for a period of 1hr (instant) and 24hr (short term) heat shock.

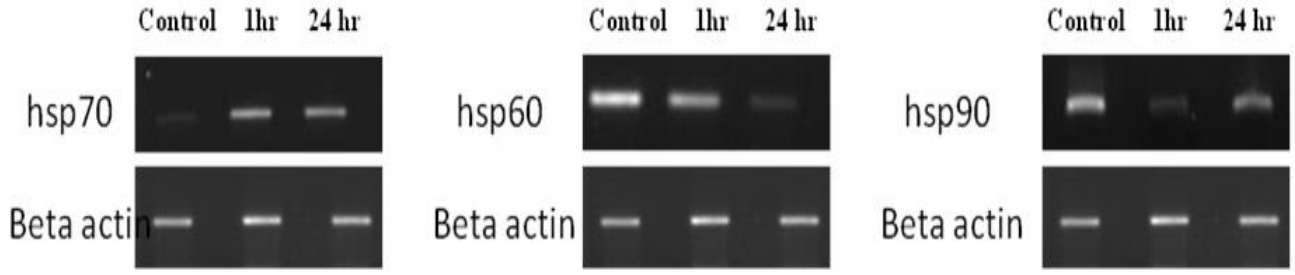
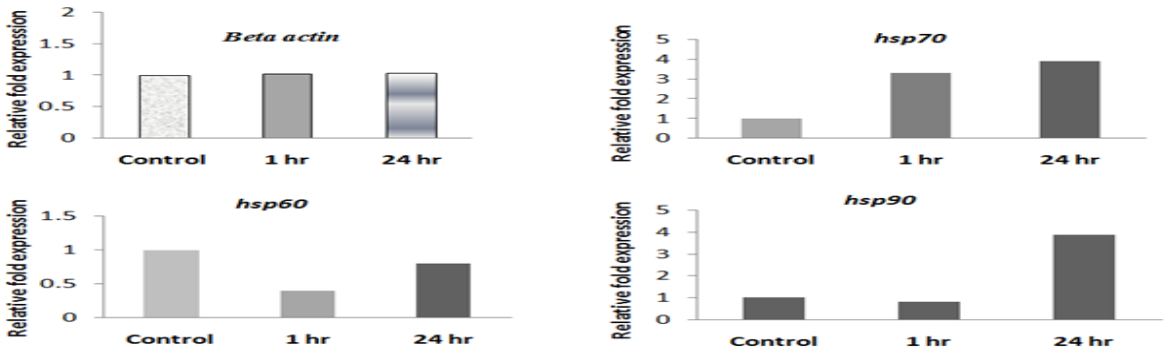


Fig.2 Relative fold expression of three Hsp genes (hsp70, hsp60, hsp90) were determined by qPCR from gonadal samples of *Channa striatus* exposed 1hr (instant) and 24hr (short term) at 36°C.



Hsps serve as molecular chaperones and are involved in organisms' heat acclimation and environmental plasticity (Hong and Vierling, 2000). According to Sorensen *et al.*, (2003), HSPs are essential for the ability to resist environmental stress. The results of our study have provided new insights into the behavior of crabs under heat stress. Both Hsp70 and Hsp90 transcripts are important for *Sartoriana spinigera* survival as an adaptation to environmental changes as a result of rapid climate change.

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