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HSP70, HSP90A, and HSP90B are Differentially Regulated in Response to Thermal, Osmotic and Hypoxic Stressors

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Abstract

The Heat shock proteins, Hsp70 and Hsp90 are highly conserved and play a significant role in cellular response to a variety of stressors. In response to stressors, cellular expression levels of these heat shock proteins are increased to stabilize degrading proteins and to initiate thermotolerance among other complex functions. Maintenance of physiological temperature in all organisms is imperative to ensure that biological systems function normally. This is especially critical for cold-blooded organisms whose internal temperature is subject to their environmental conditions. High temperatures and other stressors can deleteriously affect animal motility, its ability to avoid predators, neuronal activity and affect neuropeptide populations. However, the effect of stressors on muscle and on neuronal activity are not always equal. Here we use the important commercially important Jonah crab, Cancer borealis as a model to assess the effect of different stressors on transcript levels of the major heat shock proteins, HSP70, HSP90A and HSP90B. Since these genes have not been identified or sequenced in C borealis, we cloned each gene and we demonstrate that a) these genes have different expression profiles in thermal, osmotic and hypoxic stresses and that b) these genes are differentially expressed in muscle and brain cells.

Keywords: Crab; Heat shock protein; Hsp90; Hsp70; Stress response; HSPs; Cancer borealis; Jonah crab

Abbreviations: STG: Stomatogastric Ganglion; HSPs: Heat Shock Proteins; BP: Base Pairs; AA: Amino Acid; NT: Nucleotides.

Introduction

Temperature fluctuations and thermal stress can affect physiological homeostasis and normal biological processes. This is especially significant for cold-blooded organisms. The lobster Homerusamericanus, reacts negatively in response to increase thermal levels and with significant impact on some neural circuits [1,2]. In response to a thermal gradient, the Jonah crab Cancer borealis will also behaviourally thermoregulate its movements toward a preferential temperature [3]. Acute high temperatures above 23 degrees Celsius are debilitating to C borealis, and mass spectrometric studies during thermal stress have identified consequential changes in neuropeptide populations [4-6]. *C borealis* contains a relatively small number of cells in its

stomatogastric ganglion (STG), and consequently it has become a model system for understanding neural control of motility and behavior. To date, there have been few genetic or molecular studies that establish how neuronal systems in invertebrates regulate metabolism and we are limited in our understanding of how these systems respond when subjected to external stressors.

Heat shock proteins (HSPs) are a family of proteins that are major regulators of cellular and organismal stress, including thermal stress. HSPs are highly conserved and are found in nearly all organisms from bacteria to mammals. HSPs were identified for the significant increase in their expression levels in response to thermal stress in the fruitfly, Drosophila melanogaster and were subsequently named heat shock proteins [7-9]. In addition to thermal stress, other environmental challenges such as hypoxia, osmotic stress, heavy metals and ultraviolet damage, as well as physical trauma such as infection, oncogenesis, inflammation, ischemia and reperfusion can stimulate the expression of HSPs [10-12]. These essential cellular chaperones also assist in protein folding, inhibit protein aggregation, control of transcription and protein kinases, aid in transporting signals across membranes, maintenance of cellular homeostasis by protein-protein interactions and potentially aid in the invasion of hosts by infectious parasites [13-15].

HSPs participate in most metabolic functions as molecular chaperones and are classified into 6 major groups based on their molecular mass. These are comprised of Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and a small 8-KDa ubiquitin, which functions in protein degradation [16]. Hsp70 and Hsp90 are the most well-characterized of the HSP family and have diverse roles that are still being identified. They have been reported to function to protect trigeminal ganglion neurons from thermal stress and to play role in neuronal polarization, as well as a role in neuronal disease [17]. Although both Hsp70 and Hsp90 are important stress-responsive proteins, the genes encoding neither of these proteins have been cloned, nor have their expression profiles been characterized in *C. borealis*. Here we report the full-length cDNA sequence of the C borealisHSP70(CbHSP70) and two HSP90 isolates identified from *C. borealis* CbHSP90A and CbHSP90B, which we will simply refer to as HSP70, HSP90A and HSP90B. We also show that HSP70 and HSP90are differentially regulated in response to multiple environmental stresses in both muscle and in nerve cells. These data provide novel insights into the neuron regulation mechanisms in *C. borealis* and open new questions for understanding a role for heat shock proteins in neuronal maintenance and regulatory control.

Materials and Methods

Animal Preparation

Adult male *C. borealis* were obtained from Commercial Lobster (Boston, MA), and maintained in artificial, aerated 100% seawater. They were acclimated to 10°C, 32ppt salinity and 7mg/L dissolved oxygen (DO), in individual aquariums (60cm x 36cm x 31cm) for at least 1 week prior to experiment to minimize non-specific stress. Five animals were sacrificed immediately after the each experiment and the dissection of muscle and thoracic ganglion was done in chilled *C.borealis* physiological saline (440mM NaCl, 26mM $MgCl_2$, 13mM $CaCl_2$, 11mM KCl, 10mM Trizma base, and 5mM maleic acid, with pH 7.4-7.6). Five unstressed control animals were also sacrificed and dissected as described above.

Stressors

Heat shock: Animals received an acute thermal stress (25°C) for 2 hours, in individual aquariums. Before the treatment, a heater (TECO, CA200) was placed into the aquarium to increase the temperature from 10°C to 25°C.
Osmotic stress: 60cm x 36cm x 31cm aquarium was fitted with air stones and filled with 50% seawater (16 ppt). Salinity was determined by a refractometer (Cole Parmer). Five animals were placed in low salinity aquarium for 2 hours, individually.
Hypoxic stress: To impose hypoxia, DO levels at 10°C and 32ppt saline sea water in a 60cm x 36cm x 31cm aquarium were brought down to 2mg/L by flushing with nitrogen. Five animals were placed in hypoxic conditions in individual aquaria for 17 hours. During the treatment time, hypoxic conditions was maintained with a continuous flow of nitrogen. The DO levels were determined using a handheld dissolved oxygen meter YSI Model 55 (Yellow Springs).

RNA Extraction

C.borealismuscle and nerve tissue under normal, heatshock, hypoxic and hyperosmotic conditions were dissected and frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent in addition with PureLink RNA Mini Kit (Invitrogen) from the above tissues as described in the manufacture's protocol. The concentration of isolated RNA was quantified using a NanoDrop8000 (Thermo Scientific).

Gene Sequencing

Oligonucleotides for amplifying both 5' and 3' end partial complementary DNA (cDNA) of *C. borealis* HSP90isoform1 (HSP90A), HSP90 isoform2 (HSP90B), HSP70 (HSP70) and β-actin (CbACTB) genes from crab

mRNA, were designed by comparing conserved expressed sequence tags (ESTs) among orthologs in other species [18,19]. The primers sequences were as follows (5, 5'-rapid amplification of cDNA ends (RACE); 3, 3'-RACE): HSP90A-5: 5'-CTTTTTCTTCTTCTCACCTTCCTTCTTGTCAGCATC-3'; CbHSP90A-3: 5'-CARTTIYATTGGCTAYCCMATCAAG-3'; CbHSP90B-5: 5'-TTCAGCTCCTCATCTTCACTGTAC-3'; CbHSP90B-3: 5'-GACGTCGAGCAAGCTAAGAAGTTT-3';CbHSP70-5: 5'-CTTGGTGGGGATGGTAGTGTTGCG-3'; CbHSP70-3: 5'-CTGCAGGACTTCTTCAATGGNAAG-3'; CbACTB-5: 5'-GAGGTAAGCAGTCAGGTCACGGCC-3'; CbACTB-3: 5'-AACTGGGACGAYATGGARAAGATC-3'. RACE experiments were performed using the SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instruction. PCR amplicons of predicted length were gel purified by WizardSV Gel and PCR Clean-Up System (Promega) and subcloned into pCR2.1-TOPO vector (Invitrogen). Vectors were transformed and propagated in One Shot Top10 Competent Cells (Invitrogen). Amplified vectors were harvested by NucleoSpin Plasmid (Macherey-Nagel) and inserted gene fragments were sequenced on bi-direction using primers M13 Forward -20 (5'- GTA AAA CGA CGG CCA GT -3') and M13 Reverse (5'-GGA AAC AGC TAT GAC CAT G-3') by fluorescence-labelled dideoxy nucleoside terminators system (Elim Biopharmaceuticals). The 5' and 3' RACEs sequences of each EST were manually aligned and spliced together into an entire gene according to the overlapping DNA sequence.

In Silico Gene Sequences Analysis

The open reading frames (ORF) as well as the translated peptides sequences of HSP90A, HSP90B,CbHSP70, and CbACTB cDNA were predicted using the NCBI translate tool (National Center for Biotechnology Information) from the cDNA sequence. We used NCBI BLAST programs to comparison the amino acid sequences against the database to verify that the proteins had signatures for heat shock proteins 70, 90 and Actin. We then compared Hsp70 and Hsp90 protein sequences against other similar sequences (other Hsp70 and 90 protein sequences, respectively) using BLAST. A phylogenetic tree square cladogram was made in BLAST Tree View using Fast minimum evolution, 0.85 maximum sequence difference and Grishin protein distance.

Quantitative Polymerase Chain Reaction (qPCR)

Two-step Real Time (RT) PCR (SYBR Green) was performed to produce cDNA by using MultiScribe Reverse Transcriptase (Applied Biosystems) with1.2µg total RNA isolated from crabs nerve tissue from animals maintained under normal, heat-shocked, hypoxic and hyperosmotic conditions. After reverse transcription, reaction mixtures were diluted to 120µl with double distilled water (ddH2O) as a concentration of cDNA was approximate to 10ng/µl. 90ng diluted cDNA was then used as a template in a 60µlreaction mix with 1× SYBR Green PCR Master Mix and 90nM of each of the forward and reverse primers. The following gene-specific primers for RT-PCRofHSP90A, HSP90B, HSP70 and ACTB were designed by Primer3 software (F, forward, R, reverse) Rozen, et al. : HSP90a-F: 5'- CTG ACC ATG GAG AGT TTT CCT -3';HSP90A-R: 5'- CTC CTC TTC CTC ATC ATC AGA C -3'; HSP90B_F: 5'- GAC AGA GGG GAT CCA ATT CA -3'; HSP90B-R: 5'- CTC CTC GCC TTC CTT CTC TT -3'; HSP70-F: 5'- ATC AAC CCT GAC GAA GCT GT -3'; HSP70-R: 5'- TTG GTG GGG ATG GTA GTG TT -3'; ACTB-F: 5'- GAG CGT GGC TAC TCC TTC AC -3'; ACTB-R: 5'- AGG AAG GAA GGC TGG AAG AG-3'. The 60µl PCR reaction was divided into three 20µl triplicates. The PCR was carried out and analyzed in StepOnePlus Real-Time PCR System (Applied Biosystems) using 45 cycles as follows: 30s at 95ºC, 30s at 60ºC and 30s at 72ºC. Melting curves reaction showed that only one specific PCR product had been amplified by any performed reactions. The SYBR Green fluorescent signals were detected at each end of 72ºC steps. The relative gene expression was compared by the $\Delta\Delta C_t$ method using CbACTB as an endogenous control. The gene expression level was calculated by compared to the control sample using the formula RATE=2 $^{-\Delta\Delta Ct}$. Experimental data were than verified by Student's t-test using Excel (Microsoft) and p-value less than 0.05 was considered to be statistically significant. The same experiment was repeated by using RNA extracted from crab muscle tissue.

Results

Identification and Cloning of *C borealis* Heat Shock Proteins

We were interested in understanding the expression profile of HSP70 and HSP90 in *C borealis* under stress conditions. We previously described the effect of thermal stress on HSPs in the crayfish *P. clarkii*, which are highly adaptable to heat stress [20]. However, when we looked to identify the HSPs in C borealis, we found that there were no transcript sequences in the available databases for these genes. To identify and to clone HSPs from *C. borealis*, we used sequences for HSP70and HSP90genes from other species and designed degenerate primers to clone C borealisHSP70 (CbHSP70) and HSP90 (CbHSP90) using 3' and 5' RACE as described in Materials and Methods.

In like manner, we did not have an effective standard control to assess transcript levels using comparative qPCR. In order to have an acceptable standard for quantitative PCR, we used the same approach to identify and to clone the *C borealis* beta Actin homolog which we named (CbACTB).These genes were subsequently sequenced and deposited into Genbank with the following accession numbers: MH706758 (CbHSP90A), MH706759 (CbHSP90B), MH706760(CbHSP70), and MH706761(CbACTB).

CbHSP90Aand CbHSP90Bhave2160basepairs (bp) and 2181 base-pair (bp) coding regions respectively and encode for 719 amino acid (aa) and 726aa polypeptides. The two proteins are 85% identical and 92% similar as determined by NCBI BLAST pair wise alignment using default parameters (data not shown). CbHSP70 is 1962 nucleotides (nt) and encodes for a 653aaprotein.CbACTB is 1131nt and encodes for a 376aa protein. Thus, we identified, cloned and sequenced three HSPs (CbHSP70, CbHSP90A, CbHSP90B) and SmACTB as a quantitative PCR control.

Phylogenetic Trees Analysis

To validate whether CbHsp70, CbHsp90A, and CbHsp90B encode proteins that are representative of Hsp70 and Hsp90 proteins, we built two phylogenetic trees for comparative and evolutionary analysis. CbHsp70 (highlighted in yellow) tightly clusters with two other crab species, the mudcrabScylla paramamosain and the Japanese blue crab Portunus trituberculatus Figure 1.

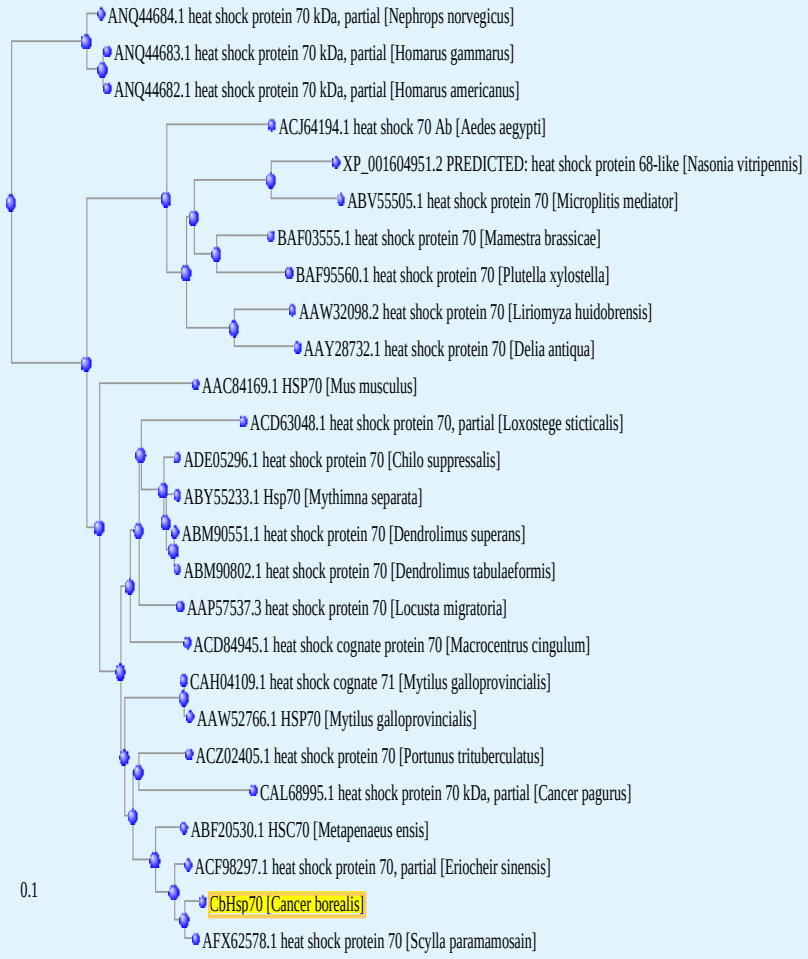


Figure 1: Phylogenetic tree of CbHsp70 and other Hsp70 protein sequences with accession numbers. The tree was constructed by fast minimum evolution in NCBI's Tree View. CbHsp70 is highlighted in yellow near a cluster of Hsp70 proteins from the mudcrab*Scylla paramamosain* and the Japanese blue crab *Portunus trituberculatus.* The scale bar represents 0.1 amino acid substitutions per site.

With the exception of the prawn Metapanaeus.ensis, a broader crab cluster also includes Cancerpagurus and Portunus trituberculatus. However, this class is distinct from the cluster of lobster Hsp70 sequences, Nephrops norvegicus, Homarusgammarus, and Homarusamericanus. In like manner, we built a phylogenetic tree that included both Hsp90 proteins CbHsp90A and CbHsp90B, highlighted in yellow Figure 2. The same series of crab species cluster with Hsp90A and Hsp90B clusters with Hsp90-2 from *P.trituberculatus*. Thus, based on molecular taxonomic data, our designation of it as a second or Hsp90B protein is supported.

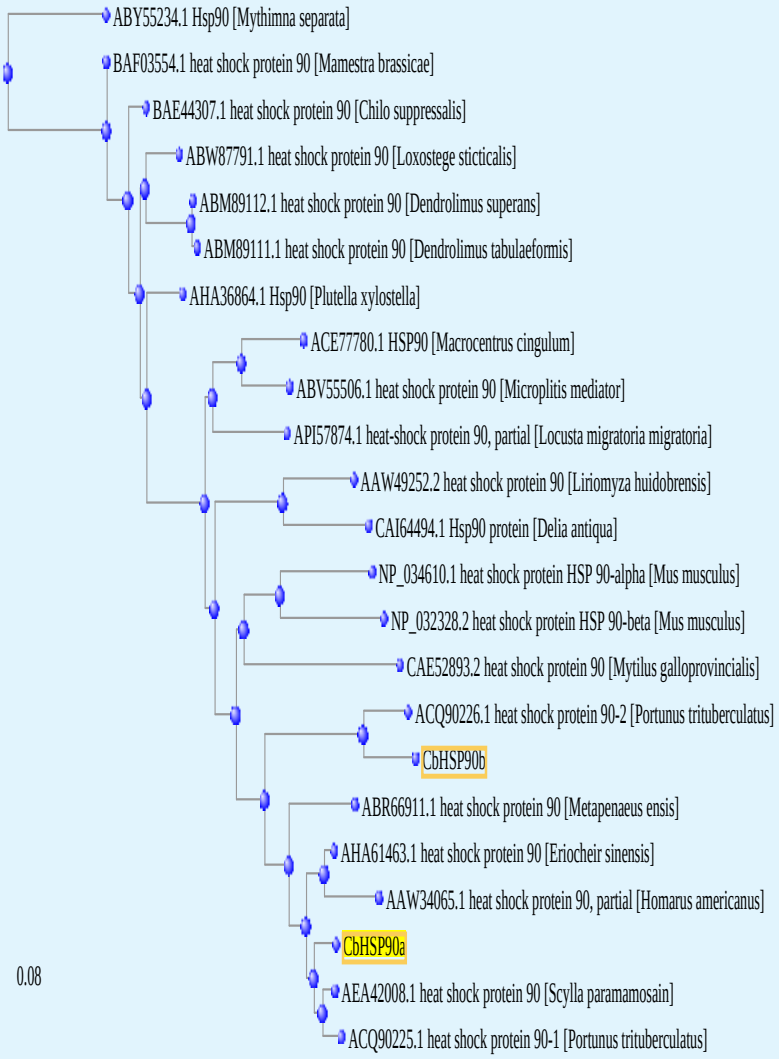
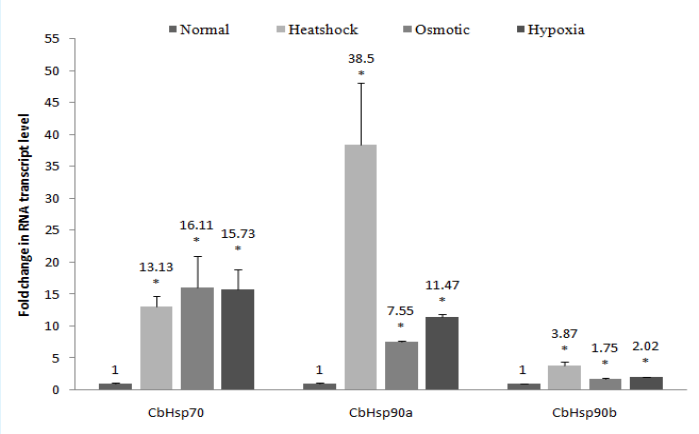


Figure 2: Phylogenetic tree of CbHsp90A, CbHsp90B, and other Hsp90 protein sequences with accession numbers. The tree was constructed by fast minimum evolution in NCBI's Tree View. Both CbHsp90 proteins are highlighted in yellow. The scale bar represents 0.08 amino acid substitutions per site.

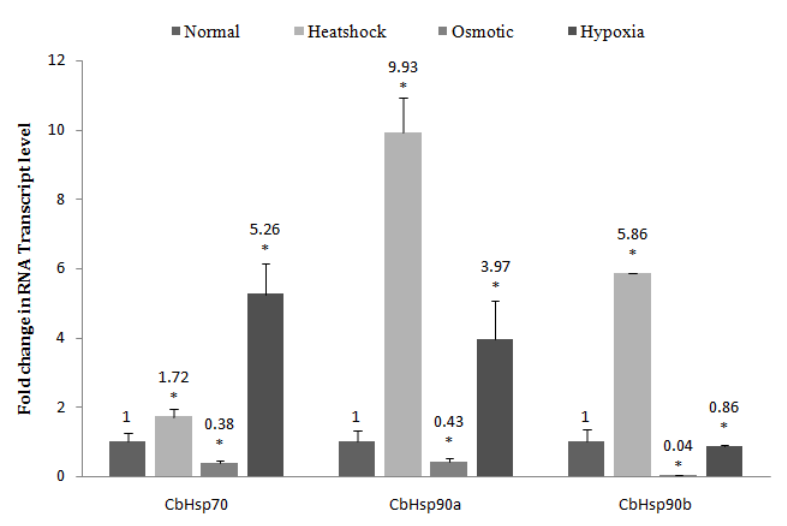
HSPs are Differentially Elevated in Response to Different External Stress

We wanted to determine the response of conserved HSPsCbHSP70, CbHSP90A, and CbHSP90B to three types of stress, thermal stress, osmotic stress, and to hypoxic stress; and measured expression of these stressors in both neurons and in muscle cells (Figures 3 & 4).



Nervous Tissue

Figure 3: CbHSP70, CbHSP90A and CbHSP90Btranscript levels in nerve tissue exposed to heat shock, hyperosmotic and hypoxic stresses and measured by quantitative PCR. Crab nervous tissue extracted under normal conditions is used as control and is the default for qPCR set to 1-fold. Results are calculated using the $\Delta\Delta C_t$ method with CbACTB serving as the endogenous control. Values above are verified by Student's t-test and all p-value are less than 0.05.



Muscle Tissue

Figure 4: CbHSP70, CbHSP90A and CbHSP90Btranscript levels in muscle tissue exposed to heat shock, hyperosmotic and hypoxic stresses and measured by quantitative PCR. Crab muscle tissue extracted under normal conditions was used as control and is the default for qPCR set to 1-foldResults are calculated by $\Delta\Delta C_t$ method using CbACTB as the endogenous control. Values above are verified by Student's t-test and all p-value are less than 0.05.

CbHSP70 levels were upregulated 13-16 fold under all stress conditions in neural tissue. However, in muscle tissue, CbHSP70 was upregulated only ~2-fold under thermal stress and ~5-fold under hypoxic conditions. In contrast, when subjected to high osmolarity, CbHSP70 levels were reduced more than 2-fold.

CbHSP90A transcript levels were upregulated in response to all stressors in neural tissue, albeit with significant prejudice. Under thermal stress in neurons, CbHSP90. A levels increased 39-fold, but 8-fold and 11-fold in osmotic and hypoxic conditions, respectively. In muscle tissue, CbHSP90A was elevated 10-fold in thermal stress and 4-fold in hypoxic stress. CbHSP90 levels were

reduced more than 2-fold under osmotic stress. The response of CbHSP90B to all of the stressor was modest relative to CbHSP70 and CbHSP90A. Transcript levels were elevated under all stressors in nervous tissue some 2-4 fold. In muscle tissue, CbHSP90B levels were elevated 6-fold in response to thermal stress and were moderately down regulated under hypoxic conditions but not expressed under osmotic stress.

Overall, gene expression was elevated for CbHSP70, CbHSP90A, and CbHSP90B in response to all stressors in nervous tissue. In muscle tissue, gene expression was elevated under thermal stress and down regulated under osmotic stress. With the exception of CbHSP90B, both CbHSP70 and CbHSP90A were upregulated in response to hypoxic stress.

Discussion and Conclusion

The Jonah crab, *C borealis* has a broad range of habitats across the North American East coast and its natural range extends from Southern Canada to Florida in the United States, and the crab has been observed to travel as far as 400m below sea level [21]. Consequently, it is exposed to various natural stressors including thermal stress, osmotic stress, and hypoxic stress. HSPs are highly conserved and play a major role in maintaining cellular stability and viability in response numerous stressors. Hsp70 and Hsp90 are two of the most well characterized of this family of proteins. Here, we characterized the role of heat shock proteins in the commercially important Jonah crab, Cancer borealis. The genome for *C borealis* has not been sequenced. In the absence of available genomic sequence data, cloning genes from *C borealis* was more of a challenge. In order to clone HSP70 and HSP90 from C borealis, we designed degenerate primers based on available DNA sequences to heat shock proteins from other organisms. We identified and cloned three genes, which we namedCbHSP70, CbHSP90A and CbHSP90B. These genes were sequenced and deposited into Genbank with accession numbers MH706758 (CbHSP90A), MH706759 (CbHSP90B), and MH706760 (CbHSP70). In addition, we cloned beta actin (CbACTB) to be used as a standard control for expression analysis of the HSPs by quantitative PCR. The CbACTB sequence was deposited into Genbank (MH706761).

CbHsp70 and CbHsp90 proteins have all the signatures and motifs that are structurally conserved in Hsp70 and Hsp90 proteins (sequence data not shown). We looked for these characteristic signatures in the cloned sequences. We found three characteristic signatures normally found in Hsp70 proteins in the CbHsp70 (DLGTTYSCV, IFDLGGGTFDVSIL, IVLVGGSTRIPKIQKL) and the nuclear localization signal KRALRRLRTACERAKRTL (Cui, Liu et al. 2010). For CbHsp90A and CbHsp90B, we found the highly conserved Hsp90 family sequence motifs (NKEIFLRELISN [S/A] SDALDKIR, LGTIA [K/R] SGT, IGQFGVGFYSA[Y/F] LVA [E/D], IKLYVRRVFI, GVVDS [E/D] DLPL N [I/V] SRE) as well as the consensus amino acids MEEVD at the C-terminus which is the cytoplasmic localization signal of the Hsp90 [22,23]. We also identified other major functional motifs typically in HSP90 such as classical nuclear localization sequence (KKEGEKKKKK in CbHsp90Aand KDVEQAKKFK in CbHsp90B) [24]. The sequence data alone indicates that CbHSP70 is a member of Hsp70 family and that both CbHSP90A and CbHSP90B encodecytosolic Hsp90 proteins.

We previously reported that in the crayfish, *P clarkii*, HSP70 and HSP90 are differentially regulated in muscle and nerve tissue after thermal stress. We decided to take this a initial study further in our analysis of the crab, *C borealis* by looking at three stressors- thermal, osmotic and hypoxic stress, in both muscle and in nerve tissue. We found that there are clear distinctions in the expression profile between muscle and nervous tissue. Under all stress conditions, each HSP was elevated in neural tissue (Figure 3). However HSP90A had the most robust response to thermal stress with a 39-fold increase after stress induction. HSP90B had the most modest increase relative to all the HSPs in response to these stressors in nerve tissue, and maximally at 4-fold under thermal stress.

For muscle cells, HSP90A had the highest levels in response to thermal stress. However, for all of HSPs tested, transcript levels were down regulated in response to osmotic stress (Figures 3 & 4). This is in contrast to what we would have predicted, suggesting that for the C borealis, HSPs 70 and 90 may not be the important regulators during osmotic stress in muscle tissue, but that *C borealis* may have evolved alternative mechanisms for this regulation. In response to high osmolarity, the yeast Saccharomyces cerevisiae has evolved an alternative gene pathway that involves the high-osmolarity glycerol (HOG) and Mitogen Activated Protein Kinase Pathway (MAP Kinase pathway) which result in classes of gene expression modifications to safeguard against yeast lethality under osmotic stress [25-27]. In mammalian neural tissue, there are multiple pathways for managing osmotic stress which, in addition to HSPs, include Fos and Jun, and the early growth response protein-a (EGR-1 or NGFI-A). In mice and human, there are two HSP70 genes, and only one is induced in response to osmotic stress [28]. Taken together, these results could simply suggest that other regulators other that CbHsp70 and CbHsp90A

or CbHsp90 could be responsible for stress response in high osmolarity. We identified only one Hsp70 protein in C borealis. Without more available genomic or transcript data, we cannot rule out the possibility of another Hsp70 gene in C borealis.

In crayfish, as observed for P clarkia, HSP70 and HSP90 expression was up regulated in both muscle and nerve tissue [20]. However, in crayfish HSPs 70 and 90 were significantly more elevated in muscle tissue relative to nerve tissue, which is contrast to our observations for C borealis. In like manner, for the crab Portunus trituberculatus, thermal stress resulted in differential responses of ptHSP90-1 and ptHSP90-2 depending on what organ (ovary, gill, muscle) was tested [19]. It is not clear why these distinctions are observed, but it is of interest how crustaceans may have evolved these distinctions in response to their environments.

Crustaceans are cold-blooded animals and have had to evolve to adapt to varying aquatic stressors. The regulation of stress in crustaceans and other organisms is complex, and it clear that our understanding of gene regulatory networks regulating this potential environmental challenges is limited. Our work in the crab *C borealis* shows clear distinctions in the response of heat shock proteins to different stressors and in different tissue could aid in strategies for commercial maintenance of the crab for a food source. In addition, further investigation of the genetic and molecular distinctions observed between species, even closely related species during stress could increase our understanding of how animals have evolved to survive and thrive in their specific ecological niches.

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