**Characterizing the effects of heavy metal and pathogen exposure on hsp70 expression in *Crassostrea gigas*.**

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Abstract:

*Introduction*

Stressors in the environment can alter gene expression and these changes can help assess stress levels of indicator species (Veldhoen et al., 2011). A common method for measuring an organisms response to environmental change is by characterizing gene expression leves, as this is often thought to be the primary process regulating expression of stress response proteins (Bierkens, 2000). However, protein expression does not always correlate with messenger RNA (mRNA) levels (Gygi et al., 1999; Pina et al., 2007). Consequently, analysis of stress response pathways that do not account for both transcriptional and translational mechanisms may not accurately represent organism’s response (Greenbaum et al., 2003).

Heat shock proteins are a common focus in molecular assays used to establish measurements of physiological and environmental stress (Franzellitti et al., 2010; Roberts et al., 2010; Pina et al., 2007; Downs, 2001). Heat shock proteins are involved in a variety of stress related processes including thermal stress, immune response, and apoptosis (Roberts et al., 2010). The most highly conserved family of heat shock proteins is hsp70 (Sanders 1993). Hsp70 is a molecular chaperone involved in the folding and unfolding of newly translated proteins and proteins that have been damaged from various forms of cellular stress (Feder & Hofmann, 1999). While studies have documented hsp70 accumulation in response to individual stressors, combinations of environmental contaminants are of increasing concern (Anderson et al., 1998; Gupta et al., 2010), particularly their impacts on resistance and tolerance to naturally occurring infectious disease (Morley, 2010, Paul-Pont et al., 2010)

Oysters, like other bivalves, are sessile organisms that bioaccumulate pollutants making them ideal candidates for monitoring and studying the impacts of environmental contaminants (Rittschof et al., 2005; Fabbri et al., 2008; Morley, 2010). Hsp70 was originally characterized in *C. gigas* by Gourdon et al. in 2000, followed by further sequence identification and characterization by Boutet et al. (2003). Hsp70 is one of the best characterized targets for studying the stress response in shellfish (Fabbri et al., 2008).

One of the primary objective of the current study was to characterize the hsp70 stress response in *C.gigas* when exposed to two environmental stressors; copper, an environmental contaminant commonly found in urban and agricultural runoff (REF) and *Vibrio tubiashii* (*Vt*), a re-emergent bacterial pathogen of shellfish on the west coast of North America (Elston et al., 2008). In addition we set out to characterize similarities and differences between regulation of hsp70 transcription (mRNA) and translation (protein) when exposed to these environmental stressors.

**Materials and Methods**

*Experimental design*

Pacific oysters (*C. gigas*) were collected from the University of Washington’s field station at Big Beef Creek, WA in November 2010. A total of 32 oysters were collected with a mean length of 112.3mm. Following four days of acclimation 5L seawater (12oC, pH8.0) Oysters were randomly assigned in to four treatment groups (n=8), one receiving a dose of copper(II) sulfate (33mg/L) for 72 hours, one challenged with *Vibrio tubiashhi* ATTC 19106 (7.5x105 CFU/ml), for 24 hours, a third receiving a 72 hour exposure to copper(II) sulfate combined with a 24 hour exposure to *V. tubiashii,* and a fourth as a control held in seawater for the duration for the experiment. *Vt* was cultured overnight at 37°C in 1 liter LB broth + an additional 1% NaCl. Cells were harvested by centrifugation at 4,200 rpm for 20min. Pelleted bacteria were then re-suspended in 0.22um filter sterilized seawater. Immediately following treatments, gill tissue was removed using sterile techniques and stored at -80°C for subsequent RNA extractions.

*cDNA synthesis and Quantitative PCR analysis*

RNA from all gill tissue samples (~25mg) was extracted using TRI Reagent (Molecular Research Center, Inc.) following the manufacturer’s protocol. Total RNA was quantified using a spectrophotometer (Nanodrop). Samples were treated with the Turbo DNA-*free* Kit (Ambion) per manufacturer’s recommended standard protocol to remove genomic DNA carryover. RNA (1 ug) was reverse transcribed using M-MLV reverse transcriptase (Promega), 0.25ug oligo-dT primer (Promega), 1.25 uL 10mM dNTPs (Bioline) in 25uL total volume. For gene expression analysis, primers were designed for heat shock protein 70 (AJ318882) (5’-TGGCAACCAATCGCAAGGTGAG; 3’-CCTGAGAGCTTGAGGACAAGGT) using Primer3 software (Rozen & Skaletsky, 2000). Quantitative PCR reactions were carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each 25-µl reaction contained 1X Immomix Master Mix (Bioline USA Inc., Boston, MA), 0.2 µM of each primer, 0.2µM Syto-13 (Invitrogen), 2 µl of cDNA, and sterile water. Reactions were carried out as follows: 10 min at 95ºC, 40 cycles of 15 sec at 95ºC, 15 sec at 55ºC, and 30 sec at 72ºC, with fluorescence measured at the end of annealing and extension steps. Following qPCR cycling, melting curve analysis was performed by increasing the temperature from 65ºC to 95ºC at a rate of 0.2ºC sec-1, measuring fluorescence every 0.5ºC. All samples were run in duplicate. Analysis of qPCR data was carried out based upon the kinetics of qPCR reactions (1/(1+efficiency)Ct (Sheng et al., 2005)) and normalized to elongation factor 1 alpha (5’-AAGGAAGCTGCTGAGATGGG; 3’-CAGCACAGTCAGCCTGTGAAGT (AB122066) total RNA loaded. Data are expressed as fold increases over the minimum. Two-way ANOVA were carried out using SPSS statistical software to determine significant differences in expression (p<0.05).

*Protein Extractions and Western Blot*

Protein was extracted using CellLytic Cell Lysis Reagent tissue (Sigma, St Louis, MO, USA) containing protease inhibitor cocktail (Sigma) at a ratio of 1:20 (1·g tissue/20·ml reagent). The protein quantity of each sample was determined using Coomassie Plus (Pierce, Rockford, IL, USA).

The levels of HSP were assessed by western analysis using a primary antibody produced by Western Breeze Manufacturer (product#?). 17000 μg of gill tissue protein extract were electrophoresed on two SDS-PAGE gels %?Gradient? (Pierce). Gels were transferred to nitrocellulose membranes, blocked homemade or bought? If brought whats is called and from who? Hsp70 antibody was diluted 1:3000 in TBS-T ? and incubated for 1hr. Blots were rinsed in TBS-T(what kind of membranes?) and incubated with a secondary antibody solution any more info on this? (Western Breeze Manufacturer). Visualization was achieved by application of Chromogenic Substrate (What is this? Concentration?) until purple bands appeared. Blots were photographed using\_\_\_\_\_\_ and integrated density values were calculated using Image J. Values of background densities on SDS-PAGE gels were calculated and used to normalize values based on protein density. Background values were also used to normalize densities between gels, to account for differences in lighting and protein densities.

Results:

*Hsp70 mRNA and protein expression in V. tubiashii exposed oysters*

Expression of hsp70 mRNA in gill tissue of *C. gigas* was not significantly different (p>.05) from that of control tissues with an average mRNA expression of ~5 fold +/-1SE over the minimum expression value for *V. tubiashii* exposed animals compared to 2.7 fold +/-0.8SE over the minimum for control animals. Hsp70 protein was observed at levels 35 fold +/- 4.2SE and 32 fold +/- 6.1SE above the minimum observed values in control and *Vt* exposed oyster respectively. Differences in hsp70 protein levels were not statistically different between control and *Vt* exposed oysters (p>.05).

*Hsp70 mRNA and protein expression in Vt and Cu(II) exposed oysters*

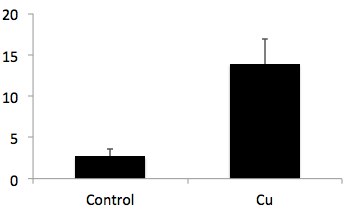
Significant up regulation (p<.05) of hsp70 mRNA (~15.3 fold +/- 3.7SE over the minimum) was observed in the CuSO4 treatment in combination with *V. tubiashii* compared to control or *V. tubiashii* alone. Total levels of hsp70 protein were significantly lower in Cu(II) + *V.tubiashii,* treatment compared to both control or *V.tubiashii* exposed oysters (p<.05). There was no detectable effect of *V.tubiashii* on both hsp70 mRNA and protein expression (p>0.05) in combination with exposure to copper. Expression values for both mRNA and protein in oysters exposed to either copper or copper and *V.*tubiashii were pooled for subsequent copper exposure analysis.

*Hsp70 mRNA and protein expression in Cu(II) exposed oysters*

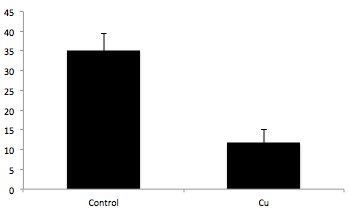
Copper exposed oysters had significantly higher hsp70 mRNA (~14fold +/- 3.0SE) expression in their gill tissue compared to controls (p<.01). Protein levels for hsp70 were significantly lower **(fold?)** in Cu(II) oysters compared to either control oysters (p<.01).

Figures: Sorry about formatting, will work on more finalized figure format including axis titles etc. once the paper moves to a more format friendly medium.

*Gene expression figure fold/min +/- Standard Error*



*Protein expression figure fold/min +/- Standard Error*



Discussion

While there have been numerous studies examining hsp70 activity in oysters, there are limited studies that characterize both gene and protein expression. This study demonstrates that exposure to copper influences hsp70 gene and protein expression in oysters. Furthermore, exposure to copper resulted in discordant regulation of hsp70 mRNA and protein.

Oysters used in this study were exposed to 33mg/L of copper and this resulted in a significant increase in hsp70 gene expression. This represents the highest exposure of copper tested in a laboratory in which changes in gene expression have been documented. Other studies in shellfish exposed to copper have documented increases in hsp70 mRNA, albeit at lower concentrations of copper. In a study by Zapata et al. (2009), *Argopecten purpuratus* exposed to 2.5 ug/L, 5ug/L, and 10ug/L Cu for eight days showed significant increases in hsp70 mRNA at 4 days in the 2.5ug/L treatment, and 2 days in the 10ug/L treatments only. A study in *Fenneropenaeus chinensis* (Luan et al., 2010) showed a significant increase in hsp70 mRNA in shrimp exposed to 50uM CuSO4 after 24 hours of exposure and a significant decrease after 72 hours of exposure. *Driessena polymorpha* exposed to Cu (20ug/L) had significantly elevated levels of hsp70 mRNA in gill tissue after 24 hours of exposure that returned to control levels after 7 days (Navarro et al., 2011). Transcripts for hsp70 mRNA in copepods were significantly elevated after 96 hours of exposure to 0.1, 0.2, 0.5 μg/L CuCl2 compared to controls (Rhee et al., 2009). While these studies represent copper exposure levels that are an order of magnitude lower than the one used in, the documentation of an increase in hsp70 mRNA under these conditions is suggestive of the robustness of these organisms to respond to extreme changes in their environment.

Increases in gene expression are often associated with concordant increases in protein concentration, however, under these experimental conditions, a significant decrease in hsp70 protein concentration was observed. These results are consistent with other studies in *C. gigas*  in which oysters were exposed to 0.4 and 4uM copper, resulting in a decrease in hsp70 protein concentration for both treatments (Boutet et al., 2003). Copper exposure in other species however, observed increases in hsp70 protein concentration. Zebra mussels exposed to 100-500ug/L Cu showed increased hsp70 protein levels while no change was detected in copper exposures between 30-75ug/L (Clayton et al., 2000). Protein levels for hsp70 were also elevated in *Mytilus edulis* after 7 days exposure to 1, 3.2, 10, 32, and 100ug/L Cu (Sanders, 1994). In *Chamelea gallina*, a concentration dependent regulation of protein expression was observed in which protein concentrations increased in the presence of low copper concentrations (<1mg/ml) and decreased in high copper treatments (>5mg/ml) (Rodriguez-Ortega et al., 2003). The regulation of hsp70 protein levels in shellfish exposed to copper appears to depend on copper concentration with lower concentrations inducing hsp70 protein expression and higher levels reducing hsp70 protein levels. This pattern suggests that copper is affecting the animal’s ability to synthesize protein, and/or maintain protein integrity.

The observed discordant expression of hsp70 mRNA and protein in this study may be caused by the rate of protein degradation exceeding that of mRNA production and protein translation. Copper is known to cause cytopathological damage (Sanders, 1994; Pawert et al., 1996; Triesbskorn and Kohler, 1996; Quig, 1998) as the result of peroxidation reactions that produce free radicals that damage lipids and proteins (Donato,1981). Consequently, copper can directly impact the integrity of hsp70 protein resulting in observed decreases in hsp70 protein concentrations, or it could impact the translational machinery (Lewis et al., 2001), which would also result in an observed decrease in hsp70 protein concentration.

The minimum concentration of copper that results in the lack of correlation between hsp70 mRNA and protein levels in adult oysters is unknown. The concentration of copper used in this study was higher than those previously described to illustrate the extreme changes to the molecular landscape that can be caused by copper exposure. Further analysis is needed in order identify the minimum exposure level of copper that can disrupt the correlation between hsp70 mRNA and protein concentrations. It is reasonable to expect that sensitivity to copper may change with the addition of multiple stressors and can vary with the life history stage of the organism, different populations, and genetic variation. Due to coppers ability to denature proteins necessary for transcription and translation (Lewis et al., 2001), the disruption of the mRNA and protein correlation may not be specific to only hsp70 regulation. Other genes and pathways should therefore be fully characterized at both the mRNA and protein levels when conducting toxicological experiments containing cytotoxic compounds.

Induction of hsp70 stress response can be caused by a variety of environmental stressors including thermal stress, heavy metals, xenobiotics (Boutet et al., 2003), oxidative stress (Monari et al., 2011) and changes in pH (Cummings et al., 2011; Hernroth et al., 2011). However, current studies regarding hsp70 stress response in oysters primarily focus on either the transcriptional (mRNA) or translational (protein) response. As illustrated by the present study, for proper characterization of molecular stress response pathways, it is necessary to consider regulation of stress response proteins at both the transcription (mRNA) and translation (protein) level. These observations will be particularly important when studying organisms from highly contaminated areas, as it has been shown here that *C. gigas* is capable of inducing gene expression in extremely unfavorable conditions which fail to coincide with changes in protein levels.

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Table 1

Primer and probe sequences for qRT-PCR, and GenBank accession #.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  |  |  |
| Gene | Abbreviation | Sequences 5’-3’ | GenBank |
| Heat shock protein 70 | Hsp70 Fwd  Hsp70 2a Rev | TGGCAACCAATCGCAAGGTGAG  CCTGAGAGCTTGAGGACAAGGT | **AJ318882** |
| Heat shock protein 70 | Hsp70 2b Fwd  Hsp70 2b Rev | ATCTTTGACGCCAAGAGGCTGATA  TCAGCACCATTGAGCTGATTTCTTC | **AB122063** |