Abstract

The Olympia oyster, *Ostrea lurida*, is the focus of many rehabilitation efforts within the Puget Sound. These efforts are ongoing but have had limited success in transplanting portions of populations to distressed areas. Many factors are involved in this problem, this study investigates the phenomenon of local adaptation as being the primary cause of transplantation issues. A significant biomarker for stress is HSP70, a common protein used to maintain protein function during stressful environmental events. Oysters are known to express HSP70 in the face of many stressors. In this study relative levels of HSP70 mRNA transcripts are used to determine the response to mild heat stress in three distinct populations from the Puget Sound. Using primers for HSP70 and Actin for PCR and qPCR this study was unable to find significant differences between populations possibly due to primer design and inadequate stressing. Future studies are suggested to determine the effects of heat stress on the many different biochemical pathways in *O. lurida* and are encouraged to use primers for not just HSP70 but any of the processes involved in homeostasis.

Introduction

The Olympia oyster, *Ostrea lurida*, has undergone many challenges in the last 100 years. Stressors from anthropogenic development of the Puget Sound, industrial waste, habitat loss, and climate change have caused population decline among Olympia oysters within the Sound (Steele et al, 1957; Rumrill, 2013). Future threats to Olympia oysters include ocean acidification and warming of surface waters in near shore areas (Miller et al., 2009; Feely et al., 2010; Hettinger et al., 2012). To combat long term dissolution of native populations in the Puget Sound, restoration efforts by government and non-profit led groups have had limited success in rehabilitating populations in distressed areas of the Sound. This study is an effort to account for these limited successes by determining biologically relevant causes for transplanted oysters’ inability to succeed in nonnative environments. A likely major phenomena to cause this is the heritable adaptation the individual oyster populations have in accordance with their specific habitat and its oceanographic properties.

Local adaptation is a phenomena in which geographically separated populations of a native species are better adapted to the habitat parameters of their specific location (Kawecki and Ebert, 2004). Many species across all taxa have shown evidence for local adaptation such as pine trees in Scandinavia (Savolainen, 2007), Brown trout in Denmark (Jensen, 2008), and copepods along the Pacific coasts (Edmands and Diemler, 2004). In each of these cases there were significant phenotypic and genomic biomarkers for adaptation such as differences in nutrient uptake and usage efficiency, lipid storage, and thermal tolerance respectively. Local adaptation allows for multiple generations of a population to have a survival advantage under local conditions versus invasive populations which may not have undergone similar natural selection (Ruesink et al, 2005). Other benefits of local adaptation are clade survival in the face of region wide habitat change such as conditions that occur during extinction events like the Paleocene-Eocene climate change event (Kitchell et al, 1986). Jablonski et al. (1986) have shown that the wider range of local adaptation that a species undergoes the higher the likelihood that a subpopulation of the species will have adapted to survive conditions similar to those during extinction events. Oyster species have shown similar effects from local adaptation (Murray and Hare, 2006). Efforts to transplant oysters from areas of high salinity to low salinity or from lighter pollution to heavier pollution have limited success. With other oyster species showing possible local adaptation it is of great interest to rehabilitation efforts for Olympia oysters to determine whether local adaptation is occurring in Puget Sound populations for both long term survival of planted oysters as well as that of native populations.

Olympia oysters have historically been harvested throughout the Puget Sound until periods of increased pollution and population mismanagement lead to depletion of the resource (Rumrill, 2013; White et al., 2009). O. Lurida is the only native oyster to the West Coast with its habitat range as far south as the Baja of Mexico and as far north as Alaska (Rumrill, 2013). From the mid 1800’s until the 1940’s they were heavily harvested for their unique flavor and texture being considered a delicacy. Severe population declines in the Puget Sound have lead their once comprehensive beds to be limited to a few bays around the Sound (White et al., 2009). O. Lurida is a cold water oyster species with most populations experiencing average temperatures between 10-20 Celsius. Within the sound average temperatures can vary between 5 degrees Celsius of each as well as experience a variety of differences in dissolved oxygen, pCO2, nutrient content, pollution, salinity, etc. Most populations have various macro and micro scale responses to stressors caused by swings in any of the conditions previously listed. Animals can close up during periods of low oxygenation and high pCO2 as well increased feeding efforts to offset energy usage for maintaining homeostasis (Timmins-Shciffman, 2013). On the micro scale these animals have developed an impressive array of molecular mechanisms to combat stressors. In other species there is extensive evidence for changes in these molecular mechanisms to adapt to location specific stressor profiles that may or may not be effective when transplanted to new areas.

Many cellular mechanisms are modified through selection events in native populations which lead to organisms that survive stressor conditions in specific locations. Animals such as Copepod ss. have developed differences in isoforms of the HSP70 protein in relation to whether they live in tidal pools versus nearshore waters. Brown algae have shown differences in expression in copper toxicity related enzymes due to adaptation to local conditions (Ritter et al., 2009) Other mechanistic adaptations include changes to phosphoglucose isomerase in California montane beetles in response to variations in regional differences in seasonal temperatures and food availability (Dahlhoff, 1999) Bivalves have shown similar acclimation events. The Mytilus sp. have developed several phenotypic and genetic differences along the Atlantic coastlines leading to possible speciation events (Riginos and Cunningham, 2004) Possible adaptions for Olympia oysters could be related to being able to handle the relatively warmer water of Southern bays versus those in cooler temps in the areas around the Strait of Juan de Fuca.

Heat shock proteins are homeostatic molecular mechanisms that help cells maintain proper protein folding under thermal stress (Feder and Hofman, 1999). HSP70 is considered the most conserved form and has functions far beyond just protein refolding such as heat shock priming and cell growth and development (Sorenson, 2003). Many species have presented with heat shock proteins such as copepods (Edmands and Deimler, 2004), mussels (Riginos and Cunningham, 2005), killifish (Fangue et al., 2006; Schulte, 2007), and oysters (Elise, 2005; Timmins-Schiffman, 2013) Heat shock priming is of high importance in the face of thermal shock as primed cells have higher survival rates than those with no priming. The priming functions of HSP70 including creating Heat Shock Factors that act as targets for transcription on necessary cell maintenance genes as well as increasing production of more HSP70 proteins. Studies have shown that native species will be better primed for thermal stress than non native species of kelp (Henkel and Hofmann, 2008). Increased concentration of HSP70 proteins can also prime the cell by being more available to deal with denaturation events that occur during thermal shock. The priming effects of HSP70 have been shown to occur at sub lethal temperatures and could be used as indicators for local adaptation differences between populations (Fangue et al, 2006). If a population is better adapted to warmer temperatures, slight temperature (<10 C increase) increases should illicit very little response from HSP70 mRNA transcripts. Whereas populations less tolerant to warmer temperatures might have a disproportionate response to slight temperature increases.

Using previously verified RNA isolation techniques, this study will use in situ mRNA concentrations for HSP70 transcripts to determine whether slight increases in temperature illicit strong or weak reactions from offspring of populations from 3 geographically distinct locations within the Puget Sound. mRNA transcripts will be converted to cDNA through the use of reverse transcriptase. cDNA will then be used to perform PCR tests to determine presence of HSP70 transcripts and qPCR tests will determine the relative abundance of HSP70 transcripts between populations and time points. From this information we will be able to deduce whether geographically distinct populations have significantly different reactions to sub lethal heat stress caused by selection leading to local adaptation amongst these populations.

Materials/Methods

Olympia oysters bred over summer 2013 from populations in Fidalgo Bay (Northern Puget Sound), Dabob Bay (Hood Canal or Middle Sound), Oyster Bay (South Sound) were collected at Port Gamble, WA on November 1st. 10 Samples from each population were then acclimated to 12 C temperatures for 1 week in cold storage and fed 15-30 ml Commercial Shellfish Diet once every 24 hours. After one week 5 animals were randomly removed from each population for initiation time point samples. The temperature was quickly raised to 24 C in the tank and the remaining oysters were fed a final time. At roughly 24 hours, the final 5 animals from each population were sampled.

Sample preparation was done as follows. Animals were patted dry and then measures for length. Under sterile conditions and with the use of a flame sterilizer, the animals were opened and a small portion of whole body tissue was dissected out into 1.5 ul RNase free tubes. Samples were placed on dry ice for flash freezing. When sampling was completed, tubes were weighed to determine approximate weight of dissected tissue. Finally 500 ul of TriReagent was added to the tissue samples and they were homogenized with a plastic pestle for 30 seconds to 1 minute. Samples were stored at -80 C until RNA isolation could be performed. All following protocols and can be found online in Roberts Lab wikia and Fish 441 Lab Wikia (2013).

RNA isolation protocol was as follows. Samples were allowed to thaw to room temperature and another 500 ul of TriReagent was added to each tube then vortexed. 200 ul of Chloroform was added to each tube followed by more vortexing and a 5 minute incubation at room temperature. Samples were centrifuged at 4C for 15 minutes at 16.1 rcf. The clear portion on top was then extracted and placed in fresh RNase Free tubes with 500 ul of Isopropanol followed by vortexing and a 10 minute incubation at room temperature. Samples were then centrifuged at 4C for 8 minutes at 16.1 rcf and supernatant wastes were removed from the pelleted material. One ml of 75% EtOH was added to the sample for salt cleansing followed by vortexing and being centrifuged at 4C for 5 minutes at 7.5 rcf. The supernatant waste was again removed and eliminated. Samples were then briefly centrifuged to pool excess EtOH remaining in the sample which was drawn out by pipetting. Samples were then air dried for 5 minutes under a hood and resuspended using 100 ul 0.1% DEPC H2O, incubated at 55 C for 5 minutes, vortexed, and then stored at -80C.

Samples were quantified using a Nanodrop with sample concentrations ranging between 3600-4000 ng/ul. Using the isolated RNA, cDNA was created to do PCR and qPCR with. cDNA was created using the following protocol. In 0.5 ml tubes 4 ul Nuclease Free cDNA, 1 ul oligo DT, and 5 ul of RNA were vortexed together and incubated at 70 C for 5 minutes then transferred immediately to ice. A master max of the following reagents was created; 5 ul MMLV 5X Reaction Buffer, 5 ul dNTP, 1 ul MMLV Reverse Transcriptase, and 4 ul Nuclease Free Water. 15 ul of the Master Mix was added to each 0.5 ml tube. Samples were then incubated at 42C for 60 minutes followed by a temperature increase to 70 C for 3 minutes. Finally samples were spun down and stored at -80 C for later use.

Following the creation of cDNA a series of PCR and qPCR tests were used to determine the presence and abundance to HSP70 mRNA transcripts using a previously validated HSP70 primer from Crassostrea gigas and a previously validated primer for Actin in O. lurida which is used as a normalizing gene. A test PCR to determine presence of target and functionality of primer was completed using the following protocol. A Master Mix of 12.5 ul goTaq Green, 1 ul 1 nM Forward Primer, 1 ul 1 nM Reverse Primer was created and then added in 14.5 ul volumes to PCR tubes. 1 ul of cDNA was added as well as 9.5 ul of Nuclease free water. PCR program was as follows: 2 minutes at 95 C for initial denaturation, then for duplication steps 45 seconds at 95 C, 45 seconds at 54 C, and 2 minutes at 73 C for 31 cycles, and a final extension step at 74 C for 5 minutes. Samples were held overnight at 4 C until an agarose gel could be run.

The agarose gel electrophoresis protocol was as follows. 7 ml of 25X TAE was added to 175 ml Nanopure H2O. 2 g of standard agarose was mixed into the TAE and microwaved for 2 minutes 30 seconds with a 30 second swirl, then another 30 seconds in the microwave. Gel was allowed to cool and had 17.5 ul EtBr added to the solution then poured quickly into a gel casting tray with 20 well comb. Running gel was cooled to room temperature, placed in electrophoresis box, and covered with 750 ml 1X TAE. The wells were filled with either 5 ul ladder or 12.5 ul PCR samples. It was then ran at 100v for 40 minutes and checked frequently to make sure the loading dies separated evenly across the gel. After completing the gel run, the gel was then illuminated on a transilluminator in a darkened room to determine whether replication had occurred.

Following verification of primer function and presence of mRNA transcripts a qPCR was completed using the following protocols. Wells of a qPCR tray were loaded with 10 ul Ssofast Evagreen MM, 8 ul Nuclease Free H20, 0.5 ul Forward Primer, 0.5 ul Reverse Primer, and 1 ul cDNA. qPCR tray was loaded into the BioRad Opticon 2 qPCR machine and used the program SYBR cDNA 55 melt 2 read protocol that read the wells twice per replication to determine the cycles in which replication allowed for fluorescence to appear upon the reader. After 2 hours 10 minutes the reads were finalized and replication curves were designated by the opticon programming.

Results

The standard PCR products had strong banding at low bp markers on the ladder (<200 bp) for both the HSP70 and Actin Primers as seen in Figure 1 and 2. While these products were low the banding was strong enough with no smearing that a qPCR was run to verify target production and relative quantity of target mRNA sequences.

qPCR results were not as significant with both primers appearing to replicate late in the cycle period (cycle 25-30 out of 39 for most samples). While there seemed to be some difference between time points in each population it was not statistically significant. Results may have been swayed due to relative RNA/cDNA concentrations which were not normalized for these tests. A normalized value was generated for the qPCR results by dividing a crude value for HSP70 sequence replications created by a pre-determined algorithm by the same crude valued created for Actin. This normalization compares HSP70 concentration within a sample to Actin concentration and determines the relative concentration. These were graphed in Figure 3.

Discussion

Since little was gained from the final results of the qPCR there is little about local adaptation that can be stated from these experiments. Some populations did seem to have different starting concentrations of HSP70 transcripts and were affected by the mild heat stress treatment though Actin replication also seemed to have similar responses and thus could indicate that the transcript quantity was less affected by heat stress treatment and moreso by non normalized cDNA concentration in the samples that were run through qPCR treatments. From the Actin normalized values we can see there was an issue with HSP70 replication in Time point 0 sample from the Fidalgo population. The other values show a small increase in HSP70 transcripts between time point 0 and time point 24 in the Dabob and Oyster Bay samples. These numbers would be greatly improved if more replicates could have been run in the qPCR but due to limited resources they were not run.

Using mRNA transcripts for markers of local adaption are somewhat limited and can be easily swayed by concentration issues or processing problems. Final results from this study should not be considered as positive or negative results for local adaption but instead be used to better understand the short falls of using such sequences so that future work may benefit from the mistakes and missteps made here. If samples from this experiment are re-used in the future there are two definite issues that need to be resolved. 1) Better primers need to be generated and validated for future work as these primers were over a year old and the HSP70 primer had not been properly stored during that period possibly leading to long term degradation. 2) cDNA normalization needs to occur at both the PCR and qPCR steps to ensure that effective concentrations for replication are maintained and that primers are not overwhelmed by the shear excess of transcripts found within each sample. While I think that sublethal and mild heat shock are key in elucidating possible regulation differences among populations it is possible that temperature change in this study was too low to elucidate a significant response in any population and in future studies will need to be more excessive in the future.

This pilot study is part of a larger study to determine the effects of localization on Olympia oysters. While this study didn’t not work due to many different issues it is hoped that the samples can be re-used in the future with more specific primers to generate data about how disparate populations deal with subtle changes in the environment. It is suggested that future primers include other biochemical pathways such as those for glycogen store processing, growth, cell damage, and cell death to help bolster any findings from future genomic and epigenomic work on oysters.

Figures/Tables

Figure 1. HSP70 primer PCR products in 1.3% agarose gel with EtBr. 5 ul of Ladder, 12.5 ul of PCR sample in each well. To the left of the ladder is the negative control, to the right samples are as follows: NO1, N241, SO1, S241, HO1, H241. Gel run at 100v for 40 minutes. Visualized using Transilluminator.



Figure 2. Actin primer PCR products in 1.3% agarose gel with EtBr. 5 ul of Ladder, 12.5 ul of PCR sample in each well. To the left of the ladder is the negative control, to the right samples are as follows: NO1, N241, SO1, S241, HO1, H241. Gel run at 100v for 40 minutes. Visualized using transilluminator.

Figure 3. Normalized HSP70 values for each population at each time point.

References

Dahlhoff, Elizabeth P., and Nathan E. Rank. “Functional and Physiological Consequences of Genetic Variation at Phosphoglucose Isomerase: Heat Shock Protein Expression Is Related to Enzyme Genotype in a Montane Beetle.” *Proceedings of the National Academy of Sciences* 97, no. 18 (August 29, 2000): 10056–10061. doi:10.1073/pnas.160277697.

Edmands, Suzanne, and Julie K. Deimler. “Local Adaptation, Intrinsic Coadaptation and the Effects of Environmental Stress on Interpopulation Hybrids in the Copepod Tigriopus Californicus.” *Journal of Experimental Marine Biology and Ecology* 303, no. 2 (June 8, 2004): 183–196. doi:10.1016/j.jembe.2003.11.012.

Fangue, Nann A., Myriam Hofmeister, and Patricia M. Schulte. “Intraspecific Variation in Thermal Tolerance and Heat Shock Protein Gene Expression in Common Killifish, Fundulus Heteroclitus.” *Journal of Experimental Biology* 209, no. 15 (August 1, 2006): 2859–2872. doi:10.1242/jeb.02260.

Feder, Martin E., and Gretchen E. Hofmann. “HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology.” *Annual Review of Physiology* 61, no. 1 (1999): 243–282. doi:10.1146/annurev.physiol.61.1.243.

Feely, Richard A., Simone R. Alin, Jan Newton, Christopher L. Sabine, Mark Warner, Allan Devol, Christopher Krembs, and Carol Maloy. “The Combined Effects of Ocean Acidification, Mixing, and Respiration on pH and Carbonate Saturation in an Urbanized Estuary.” *Estuarine, Coastal and Shelf Science* 88, no. 4 (August 10, 2010): 442–449. doi:10.1016/j.ecss.2010.05.004.

Henkel, Sarah K., and Gretchen E. Hofmann. “Differing Patterns of hsp70 Gene Expression in Invasive and Native Kelp Species: Evidence for Acclimation-Induced Variation.” *Journal of Applied Phycology* 20, no. 5 (October 1, 2008): 915–924. doi:10.1007/s10811-007-9275-3.

Hettinger, Annaliese, Eric Sanford, Tessa M. Hill, Ann D. Russell, Kirk N. S. Sato, Jennifer Hoey, Margaux Forsch, Heather N. Page, and Brian Gaylord. “Persistent Carry-over Effects of Planktonic Exposure to Ocean Acidification in the Olympia Oyster.” *Ecology* 93, no. 12 (June 14, 2012): 2758–2768. doi:10.1890/12-0567.1.

Jablonski, David. “Background and Mass Extinctions: The Alternation of Macroevolutionary Regimes.” *Science* 231, no. 4734 (January 10, 1986): 129–133. doi:10.1126/science.231.4734.129.

Jensen, Lasse Fast, Michael M. Hansen, Cino Pertoldi, Gert Holdensgaard, Karen-Lise Dons Mensberg, and Volker Loeschcke. “Local Adaptation in Brown Trout Early Life-History Traits: Implications for Climate Change Adaptability.” *Proceedings of the Royal Society B: Biological Sciences* 275, no. 1653 (December 22, 2008): 2859–2868. doi:10.1098/rspb.2008.0870.

Kawecki, Tadeusz J., and Dieter Ebert. “Conceptual Issues in Local Adaptation.” *Ecology Letters* 7, no. 12 (2004): 1225–1241. doi:10.1111/j.1461-0248.2004.00684.x.

Kitchell, Jennifer A., David L. Clark, and Andrew M. Gombos. “Biological Selectivity of Extinction: A Link between Background and Mass Extinction.” *PALAIOS* 1, no. 5 (October 1, 1986): 504–511. doi:10.2307/3514632.

Miller, A. Whitman, Amanda C. Reynolds, Cristina Sobrino, and Gerhardt F. Riedel. “Shellfish Face Uncertain Future in High CO2 World: Influence of Acidification on Oyster Larvae Calcification and Growth in Estuaries.” *PLoS ONE* 4, no. 5 (May 27, 2009): e5661. doi:10.1371/journal.pone.0005661.

Riginos, C, and C W Cunningham. “Local Adaptation and Species Segregation in Two Mussel (Mytilus Edulis X Mytilus Trossulus) Hybrid Zones.” *Molecular Ecology* 14, no. 2 (February 2005): 381–400. doi:10.1111/j.1365-294X.2004.02379.x.

Ritter, Andrés, Martin Ubertini, Sarah Romac, Fanny Gaillard, Ludovic Delage, Aaron Mann, J. Mark Cock, Thierry Tonon, Juan A. Correa, and Philippe Potin. “Copper Stress Proteomics Highlights Local Adaptation of Two Strains of the Model Brown Alga Ectocarpus Siliculosus.” *PROTEOMICS* 10, no. 11 (2010): 2074–2088. doi:10.1002/pmic.200900004.

Ruesink, Jennifer L., Hunter S. Lenihan, Alan C. Trimble, Kimberly W. Heiman, Fiorenza Micheli, James E. Byers, and Matthew C. Kay. “Introduction of Non-Native Oysters: Ecosystem Effects and Restoration Implications.” *Annual Review of Ecology, Evolution, and Systematics* 36 (December 31, 2005): 643–689.

Rumrill, Steve. “Natural History, Geographic Distribution, Status, and Life-Cycle of Olympia Oysters (Ostrealurida).” Oregon Institute of Marine Biology, September 2013.

Savolainen, Outi, Martin Lascoux, and Juha Merilä. “Ecological Genomics of Local Adaptation.” *Nature Reviews Genetics* 14, no. 11 (November 2013): 807–820. doi:10.1038/nrg3522.

Savolainen, Outi, Tanja Pyhäjärvi, and Timo Knürr. “Gene Flow and Local Adaptation in Trees.” *Annual Review of Ecology, Evolution, and Systematics* 38, no. 1 (2007): 595–619. doi:10.1146/annurev.ecolsys.38.091206.095646.

Schulte, Patricia M. “Responses to Environmental Stressors in an Estuarine Fish: Interacting Stressors and the Impacts of Local Adaptation.” *Journal of Thermal Biology* 32, no. 3 (April 2007): 152–161. doi:10.1016/j.jtherbio.2007.01.012.

Sørensen, Jesper Givskov, Torsten Nygaard Kristensen, and Volker Loeschcke. “The Evolutionary and Ecological Role of Heat Shock Proteins.” *Ecology Letters* 6, no. 11 (2003): 1025–1037. doi:10.1046/j.1461-0248.2003.00528.x.

Timmins-Schiffman, Emma B., Carolyn S. Friedman, Dave C. Metzger, Samuel J. White, and Steven B. Roberts. “Genomic Resource Development for Shellfish of Conservation Concern.” *Molecular Ecology Resources* 13, no. 2 (2013): 295–305. doi:10.1111/1755-0998.12052.

Timmins-Schiffman, Emma, Brook L. Nunn, David R. Goodlett, and Steven B. Roberts. “Shotgun Proteomics as a Viable Approach for Biological Discovery in the Pacific Oyster.” *Conservation Physiology* 1, no. 1 (January 1, 2013): cot009. doi:10.1093/conphys/cot009.

White, J., J. L. Ruesink, and A. C. Trimble. “The Nearly Forgotten Oyster: Ostrea Lurida Carpenter 1864 (Olympia Oyster) History and Management in Washington State.” *Journal of Shellfish Research* 28, no. 1 (2009): 43–49. doi:10.2983/035.028.0109.