Evidence for Thermotolerance in Pre-Heat Stressed Olympia Oysters

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Abstract:

 Temperature is the leading factor influencing biodiversity in natural ecosystems. Pacific oysters have been shown to exhibit some forms of thermotolerance with previous exposure to heat, but no research has been done on heat affects on Olympia oysters. To determine if Olympia oysters exhibit an induced heat tolerance three treatments of oysters, a control, an initial heat stress, and a pre-heat stress were created. The gene expression levels of the stress response gene hsp70 and the antioxidant gene glutathione peroxidase were measured. Animals from the pre-heat stressed treatment responded with the highest levels of expression in both genes. In hsp70, the initial heat stress treatment also showed higher levels of expression than the controls. Glutathione peroxidase levels in the initial heat-stress and the control treatments showed no difference. These results indicate that the pre-heat stressed animals are responding differently to the secondary heat stress then the animals experiencing the stress for the first time. In the future these results could be applied to treating animals to provide a buffering capacity against natural disasters.

Introduction:

 Natural environments exhibit many season changes and disaster events that any form of life living within that environment must adapt to. These changes come by form of light availability, food resources, and often most important in the aquatic environment, temperature. In many systems, changes in thermal niches are the primary factor in predicting the future distributions and diversity of life within ecosystems (Feeley et al. 2012, Lewandowska et al. 2012). Temperature is an important factor to consider because it often affects many systems of the body, ranging from metabolism to reproductive timing (Elisio et al. 2012). Temperature ranges in oceanic organisms are of even greater focus now due to predictions of rising ocean temperatures with global warming.

 Shellfish, which tend to be tolerant to many temperature ranges, can be extremely useful in experiments looking at the affects of increased temperature on animals. Shellfish respond to these stresses in many different ways. Behaviorally, they can clamp together their shells, limiting either the influx of a stress into their shell or preventing the outflow of important resources such as water. Heat stresses are a common occurrence for oysters in nature and could be an important indicator of the ability to adapt to these stresses for future survival of these species. An example of this was found in Pacific Oysters when individuals pretreated with heat were shown to develop an induced heat tolerance that better prepared them for future heat stresses (Zhang et al. 2012). The Olympia oyster, the species of interest in this study, is less researched but also commercially important. Variation in responses could be of interest for future conservation efforts for both species.

 Temperature tolerance in oysters can be measured by mortality rates or by feeding rates, inferred to be metabolism. Gene expression, however, can give an indication and comparable measurement of how the oysters are responding on a molecular level. Two genes were measured for expression in this study, that of heat shock protein 70 (hsp70) and glutathione peroxidase (gpx). Hsp70 was chosen for this study because this gene is known for expression under conditions of general stress, and has been used in previous studies of thermotolerance (Clegg et al. 2012). GPX is an antioxidant gene expressed when cells are under oxidative stress (Hermes-Lima et a. 2012). Oxidation is a side effect of temperature stress.

 Three different treatments were designed for this study. A control, initial heat stress and pre-heat stress treatments were used to determine if Olympia oysters exposed to a previous heat stress would exhibit an induced thermal tolerance, as has been shown in Pacific oysters (Zhang et al. 2012). We hypothesized that hsp70 gene expression would be lowest in the control treatment and highest in the initial heat stressed animals, with pre-stressed expression coming in at levels in between the two. As for GPX, I hypothesize the same trend as with hsp70 expression.

Methods:

 Experiment:

 The *Ostreola conchaphila*, or the Olympia Oysterused in this experiment were harvested from Puget Sound. Before experimentation, the oysters were held in aerated tanks filled with filtered seawater in a cold room. These were later used as control tanks. The ten oysters reserved for the pre-stress treatment were placed in aerated beakers filled with seawater at 40 degrees Celsius for one hour. The animals were then removed and immediately placed into the control tank. About 26 hours later, the pre-stress animals along with the animals reserved for the initial heat stress treatment were all placed in an aerated seawater tank at 35 degrees Celsius. The animals were held at these temperatures for one hour and then were immediately dissected. The gill tissue was collected and frozen at -80 degrees Celsius to prevent degradation of RNA.

 RNA Isolation:

 Samples tissues were homogenized individually used sterile equipment and TriReagent. Chloroform was added and after incubation the samples were centrifuged in a refrigerator. The clear aqueous phase, the liquefied RNA, was transferred into a new vial and isopropanol was added. After another incubation period at room temperature, the tubes were again centrifuged in the refrigerator until a pellet of RNA had formed. The supernatant was again removed and 75%EtOH was added. After the tube was spun in the centrifuge, the EtOH supernatant was removed and the pellets were air dried for a few minutes to allow excess ethanol to evaporate. The pellet was then dissolved in 0.1% DEPC-H2O, incubated at 55 degrees Celsius and nanodroped to ensure the RNA was present.

 Reverse Transciption:

 In order to make cDNA, each RNA sample was mixed with oligo dT and nuclease free water. The tubes containing each solution were incubated at 70 degrees Celsius in the thermocycler then immediately transferred to ice. After remixing the sample tubes, M-MLV 5X Reaction Buffer, dNTPs, M-MLV RT and more nuclease free water were added. This mixture was then incubated at 42 degrees Celsius and then heated at 70 degrees Celsius again on the thermocycler. These samples were then stored at -20 degrees Celsius until needed for qPCR.

 qPCR:

 Master Mix was prepared according to the concentrations in Table 1, using primers for the genes hsp70 (fw GGCAAATCCAACCGAATCACC, rv TGTCGCCATTTTCCTCGCTT) and glutathione peroxidase (fw GTCTCCCAAAACAGCCTCCA, rv GAGGTTGGCAAAAGCACAGG) created by Primer-BLAST. Master mix was added to qPCR wells, one for each sample and each primer set along with two negative controls to test for Master Mix contamination. cDNA was then added to each well, with nuclease free water added to the negative controls. After caps were secured and all plates were labeled, the qPCR was run under the conditions outlined in Table 2.

 Data Analysis:

 Data analysis was performed by first downloading the qPCR data into Microsoft Excel. PCR Miner was used to give CT values and average gene efficiency for each experimental treatment by gene. This allowed for the calculation of individual gene expression by the following formula (1/(1+avg gene efficiency))^CT value. Individual gene expression was then normalized by dividing the gene expression value by the individually calculated normalizing coefficient. Means and standard deviations were calculated for each treatment and two-tailed t-tests were performed to compare gene expression across treatments.

Results:

 Gene expression of hsp70 showed that individuals within the three treatments were responding to the stresses differently (Figure 1). Even though only the relationship between the control and initial heat shock treatment was significantly different, there exists a large difference in the mean levels of expression between all of the treatments. The pre-heat stressed animals showed the highest level of expression, with a mean value of 106. Initial heat stressed animals had a mean expression level of 20, and mean expression in the control animals was 0.59. These results and p-values are provided in Table 3.

 Expression for the gene glutathione peroxidase showed different trends from hsp70 in that the initial heat stress and control treatments appeared to have no difference in expression (Figure 2). The mean expression for the control treatment was 0.57 and 0.6 for the initial heat stress treatment. The animals in the pre-heat stress treatment showed a mean expression level of nearly twice those previous, being 0.92 (Table 3).

Discussion:

 The experimental hypothesis that hsp70 would be highest for the initial heat stress, lowest for the control, and in the middle for the pre-heat stress treatment. This hypothesis was proven wrong in that we saw the highest level of gene expression in the pre-heat stress treatment (Figure 1). This is interesting because it is showing that the animals are responding with higher levels of gene expression, which could still be an indication of induced thermal tolerance. Though the hypothesis suggested that heat tolerance in these oysters would be shown by lower expression levels indicating they were not as stressed as during the initial heat stress, the data could be showing us a second form of induced heat tolerance. This second form of induced heat tolerance could be that animals that have experienced a previous heat stress are responding quicker to the stress and therefore elevated their levels of hsp70 far more rapidly then those animals experiencing the stress for the first time.

 In expression of the gene GPX there were also surprising results. The hypothesis regarding GPX that the control and initial heat stress oysters would have different levels of gene expression was proven incorrect because the oysters in the control and initial heat stress exhibited very similar levels of gene expression. Interestingly enough, the highest levels of expression were seen in the pre-heat stressed condition. This could be an indication that the animals exposed to the heat treatments experience long-term cellular oxidation that is not initially identified and so hasn’t yet been responded to in the initial heat stressed animals, but is early identified in the pre-heat stressed animals. On the other hand, the fact that the pre-stress individuals have such high levels of gene expression could be a left over from the previous treatment in that these animals are repairing oxidative stress from the initial shock.

 This experiment was effective in showing a difference in gene expression between the treatments, but many future studies could stem from this work. For instance, more significant differences between the expression in the treatments probably could most likely have been obtained with larger sample sizes. Also, pre-heat stressing the animals for a longer period of time might have allowed for a more defined development of induced thermotolerance, as has been seen in Pacific oysters (Jackson et al. 2011). A third way to extend this study would be to look at these and other gene expression levels in other types of tissues (Jackson et al 2011). This would allow for a more in-depth analysis of the tissues affected by heat shock and would potentially allow for the prediction of the ecosystem effects of increased heat. For example, if increasing temperatures were to increase oyster metabolism, higher feeding and clearing rates of water would be a potential consequence of this changing environmental factor.

 This experiment, along with any that may follow are important in today’s society, where global warming and loss of biodiversity are very real concerns. Mass mortality of oysters and other commercially important species are all too familiar, making sustainable harvest even more difficult. Studies like this that may provide a way to buffer species against some environmental factors such as rising temperatures are invaluable. But this method of approaching mass mortality is not only applicable to rising oceanic temperatures, but also to things like increased contaminant levels. By discovering a way to buffer species against their rapidly changing environments, it might be possible to preserve much of the original biodiversity.

Bibliography:

Clegg, J.S., Uhlinger, K.R., Jackson, S.A., Cherr, G.N., Rifkin, E., and Freidman, C.S. (2012) Induced thermotolerance and heat shock protein-70 family in the Pacific oyster Crassostrea gigas. *Molecular Marine Biology and Biotechnology*. **7**, 21-30.

Elisio, M., Chalde, T. and Miranda, L.A (2012) Effects of short periods of warm water fluctuations on reproductive endocrine axis of the pejerrey (Odontesthes bonariensis) spawning. *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology*. **163**, 47-55.

Hermes-Lima, M., Carreiro, C., Moreira, D.C., Polcheira, C., Machado, D.P. and Campos, E.G. (2012) Glutathione status and antioxidant enzymes in a crocodilian species from the swamps of the Brazilian Pantanal. *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology*. **163**, 189-198.

Feeley, K.J., Malhi, Y., Zelazowski, P., and Silman, M.R. (2012) The relative importance of deforestation, precipitation chance, and temperature sensitivity in determining the future distributions and diversity of Amazonian plant species. *Global Change Biology*. **18**, 2636-2647.

Jackson, S.A., Uhlinger, K.R. and Clegg, J.S. (2011) Duration of induced thermal tolerance and tissue specific expression of hsp/hsc70 in the eastern oyster, Crassostrea virginica and the pacific oyster, Crassostrea gigas. *Aquaculture*. **317**, 168-174.

Lewandowska, A.M., Breithaupt, P., Hillebrand, H., Hoppe, H.G., Jurgens, K., and Sommer, U. (2012) Responses of primary productivity to increased temperature and phytoplankton diversity. *Journal of Sea Research*. **72**, 87-93.

Metzger, D.C., Pratt, P. and Roberts, S.B. (2012) Characterizing the effects of heavy metal and vibrio exposure on HSP70 expression in *Crassostrea gigas* gill tissue. *Journal of Shellfish Research*. **31**: 627-630.

Zhang, L.L, Hou, R., Su, H.L., Hu, X.L., Wang, S. and Bao, Z.M. (2012) Network Analysis of Oyster Transcriptome Revealed a Cascade of Cellular Responses during Recovery after Heat Shock. *PLOS ONE*. **7**:e35484

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | 1 Reaction (uL) | 1. of Reactions (plus 1)
 | Total in Master Mix (uL) |
| 2X Immomix (Master Mix) | 12.5 | 31 | 387.5 |
| 10uM Forward Primer | 1.25 | 38.75 |
| 10uM Reverse Primer | 1.25 | 38.75 |
| SYBR Green (50uM) | 1 | 31 |
| PCR Water | 7 | 217 |

Tables and Figures:

Table 1. Master Mix preparation table for qPCR reactions. Made once for each primer set.

Table 2. Conditions for qPCR run.

PCR conditions:

1. 95°C for 10 minutes

2. 95°C for 15s

3. 55 °C for 15 s

4. 72°C for 30 s (+ plate read)

5. Return to step 2 39 more times

6. 95°C for 10s

7. Melt curve from 65°C to 95°C, at 0.5°C for 5s (+plate read)

Table 3. Means and standard deviations of control, initial heat stress, and pre-heat stressed treatments. Also listed is the p-values by the treatments compared, significant values are highlighted in red.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | Average | Standard Deviation |  |  | p-value |
| HSP70 | Control | 0.595338349 | 0.438052559 |  | Control/Heat | 0.049459654 |
|  | Heat Stress | 20.98203076 | 30.57204658 |  | Control/Pre | 0.112892111 |
|  | Pre-Stress | 106.9144598 | 201.7818607 |  | Heat/Pre | 0.224619625 |
|  |  |  |  |  |  |  |
|  |  | Average | Standard Deviation |  |  | p-value |
| GPX | Control | 0.571498969 | 0.39197108 |  | Control/Heat | 0.887779472 |
|  | Heat Stress | 0.6019879 | 0.532119043 |  | Control/Pre | 0.17194842 |
|  | Pre-Stress | 0.916070765 | 0.64404753 |  | Heat/Pre | 0.276009918 |

Figure 1. Hsp70 gene expression as a function of treatment in Olympia Oysters.

Figure 2. Glutathione peroxidase expression as a function of treatment in Olympia Oysters.