Differential response to stress in *Ostrea lurida* as measured by gene expression.

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Abstract

Olympia oysters are the only native oyster to the west coast of North America. The population within Puget Sound, WA has been decreasing significantly since the early 1900’s. Current restoration efforts are focused on supplementing local populations with hatchery bred oysters. A recent study by Heare et al. (2015) has shown that there appears to be differences in stress response in oysters from different locations in Puget Sound. However, nothing is known about the underlying mechanisms associated with these observed differences. In this study, expression of genes associated with growth, immune function, and gene regulatory activity in oysters from Oyster Bay, Dabob Bay, and Fidalgo Bay were characterized following temperature and mechanical stress. We found that heat stress and mechanical stress significantly changed expression in molecular regulatory activity and immune response, respectively. We also found that oysters from Oyster Bay had the most dramatic response to stress at the gene expression level. These data provide important baseline information on the physiological response of Ostrea lurida to stress and provide clues to underlying performance differences in the three populations examined.

Keywords: Ostrea lurida, gene expression, stress response, mechanical stress, heat stress
Olympia oysters, *Ostrea lurida*, are the only native oyster species on the west coast of North America. The species inhabits bays and estuaries within Puget Sound, WA. *O. lurida* is typically smaller than the invasive Pacific oyster, *Crassostrea gigas*, with adults attaining an average size between 40 – 60 mm (Hopkins, 1937; Baker, 1995). As protandric hermaphrodites, Olympia oysters usually spawn as both male and female within the first year (Coe, 1932; Hopkins, 1937; Baker, 1995). Unlike the *Crassostrea*, *O. lurida* does not release its eggs into the water column and instead females collect planktonic sperm balls and larvae are brooded for approximately two weeks before being released into the water column. Temperature tolerance range for Olympia oysters is between 5°C – 39°C (Hopkins, 1937; Brown et al., 2004) with notable mortalities occurring below freezing (Davis, 1955) and above 40°C (Brown et al., 2004). The species is also sessile once larvae settle and are typically moved via predator interactions or wave action. Colonizing lower intertidal habitats, *O. lurida* typically can be found in the inner portions of bays or estuaries where dynamic conditions can shape the phenotypes of local populations (Baker, 1995; White et al., 2009). Freshwater influx, tidal exchange, shifts in water temperature, and food availability produce a myriad of stressors which affect long term survival of *O. lurida* populations (Hopkins, 1937; Baker, 1995). Coupled with the loss of habitat due to invasive species, overharvest, and pollution research is needed to understand how this species interacts with its environment and responds to stress.

Thermal stress has been widely studied in mollusks, especially bivalves. It is suspected that mass summer mortalities of *C. gigas* may be linked to the effects of heat stress during spawning events (Li et al., 2007). The California mussel, *Mytilus californianus*, has been found to divert resources to physiological defense during thermal stress events (Petes, Menge, and Harris, 2008;
Expression of homeostasis related genes such as HSP70, Glutamine synthetase, Citrate synthase in *C. gigas* have been shown to fluctuate under prolonged heat stress at 25°C for 24 days (Meistertzheim et al. 2007). Temperature stress has been shown to induce a variety of up and down regulation of genes to maintain homeostasis (Tomanek, 2010). In oysters, there has been a significant amount of work examining the change in heat shock protein family gene expression. Specifically seasonal variation of HSPs and HSCs, heat shock cognates, in response to ambient temperatures for *C. gigas* (Hamdoun, Cheney, and Cherr, 2003; Farcy et al., 2009), induction of HSP70 and HSP69 in *Ostrea edulis* at temperatures greater than 38°C (Piano et al., 2005), and characterization of HSP70 in Antarctic clams (*Laternula elliptica*) (Park, Ahn, and Lee, 2007). Tolerance to heat shock has also been shown to be a heritable trait in oyster species (Lang et al., 2009).

The response of bivalves to mechanical stress has also received considerable attention. One reason for this is that researchers have shown mechanical stress elicits a classical stress response. With respect to the endocrine response of mechanical stress in oysters, it has been shown to increase of catecholamines present in hemolymph (Qu et al., 2009; Lacoste et al., 2001c). Upon centrifugation, researchers have found adrenocorticotropic hormone (ACTH), a hormone that induces production of noradrenaline and dopamine, increases (Lacoste et al. 2001a; Lacoste et al., 2001b; Lacoste et al., 2001c). Mechanical stress has also been shown to activate inflammation factors that are also observed during bacterial challenges (Lacoste et al., 2001c; Lacoste et al., 2001d; Aladaileh, Nair, and Raftos, 2008; Roberts et al., 2011). Studies in Pearl oysters (*Imbricata pinctada*) and abalone (*Halliotus tuberculata*) have found significant decreases in phagocytosis and phenoloxidase activity due to mechanical stress (Kuchel, Raftos, and Nair, 2010; Malham et al., 2003). Exposure to mechanical stress also has ecological
relevance as oysters are exposed to this in the intertidal environment and in aquaculture production practices (ie culling).

Here we set out to examine the response to temperature and mechanical stress in *Ostrea lurida*, while comparing differences in three local populations (Heare et al., 2015). Each of the three populations come from distinct bays within Puget Sound, WA. From north to south there is Fidalgo Bay, Dabob Bay, and Oyster Bay. Fidalgo Bay is the furthest northern population, located in a bay that is directly fed from the Salish Sea and the Strait of Juan de Fuca, has the coldest average year round temperatures. Typically, this population does not experience strong fluctuation in temperatures due to the fact that it resides in the lower part of the intertidal area and is submerged for most of the time. Olympia oysters from Fidalgo Bay experience significant growth when placed in warmer habitats but otherwise express little other observable phenotypes. Dabob Bay is a large bay at the northern most portion of Hood Canal with the population of Olympia oysters residing near the inner most portions of the bay such as Tarboo creek. This area experiences extreme temperature fluctuations throughout the year and is often partially or completely exposed during low tide events. The previous study has shown that during tidal changes, temperatures can be as high as 29°C during summer or as low as -3°C during winter (Heare et al., 2015). Oysters from Dabob Bay have been shown to experience high survival when faced with temperature challenges possibly due to adaptive structure of the local population (Heare et al., 2015). Oyster Bay is the southernmost bay which sustains a healthy population of *O. lurida*. The conditions here are on average the warmest throughout the year though due to their intertidal placement the animals are mostly submerged during tidal changes. The bay has
extensive food resources and appears to more energy resources into reproductive activity, based
on our prior field studies.

In this study, we investigated differences between these populations based on mRNA expression
of select target genes as measured by quantitative PCR. A suite of genes was selected based on
their predicted function (gene regulation, immune response, and growth). Given the field
performance of these populations, the hypothesis is that oysters from Dabob Bay will
demonstrate a more effective response to stress that could be evident by greater changes in
expression of immune response genes.

Materials and Methods

Experimental Design

Adult oysters from three founder populations (Dabob Bay, Fidalgo Bay, and Oyster Bay (Figure
1)) grown for 19 months at Clam Bay, WA were used for this experiment. All oysters were held
at 8°C for two weeks at the University of Washington prior to the experiment. Oysters from each
population were subjected to acute temperature stress (38°C water for 1 hour; n=14 per
population), mechanical stress (1000 rpm x 5 min; n=14) or served as controls (maintained at
8°C; n=14). Oysters were placed back in 8°C seawater and sampled at 1 hour post stress (n=8).
Six oysters were also monitored daily for 14 days to assess survival. Ctenidia and mantle tissue
was dissected and stored in RNAzol RT (Molecular Research Center, Inc.) at -80°C for later
analysis.

RNA Isolation
RNA was isolated according to the manufacturer's protocol for total RNA isolation (RNAzol RT (Molecular Research Center, Inc.)). Briefly, 400μL of 0.1% DEPC-H2O was added to the homogenized ctenidia tissue (~1mg), vortexed for 15 seconds, and incubated at room temperature for 15 minutes. The samples were centrifuged for 15 minutes, 16,000g, at room temperature. After centrifugation, 750μL of the supernatant was transferred to a clean tube, added an equal volume of isopropanol (750μL), mixed, and incubated at room temperature for 15 minutes. The samples were centrifuged at 12,000g for 10 minutes at room temperature. The supernatant was discarded and the pellets were washed with 500μL of 75% ethanol (made with 0.1% DEPC-H2O) and centrifuged at 4,000g for 3 minutes at room temperature. This wash step was then repeated. Ethanol was removed and pellets were resuspended in 100μL of 0.1% DEPC-H2O. Samples were quantified using a NanoDrop1000 (ThermoFisher) and stored at -80C.

**DNase Treatment and Reverse Transcription**

Total RNA was treated with DNase to remove residual genomic DNA (gDNA) using the Turbo DNA-free Kit (Ambion/Life Technologies). The manufacturer's rigorous protocol was followed. Briefly, 1.5μg of total RNA was treated in 0.5mL tubes in a reaction volume of 50μL. The samples were incubated with 1μL of DNase for 30 minutes at 37°C. An additional 1μL of DNase was added to each sample and incubated at 37°C for an additional 30 minutes. The DNase was inactivated with 0.2 volumes of the inactivation reagent according to the manufacturer's protocol. Samples were quantified using a NanoDrop1000 (ThermoFisher) and verified to be free of gDNA via quantitative PCR (qPCR).

Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega) with oligo dT primers (Promega), using 500ng of DNased RNA. The RNA was combined with 0.25ug of primers in a volume of 74.75uL, incubated at 70°C for 5 minutes in a thermal cycler without a
heated lid (PTC-200; MJ Research), and immediately placed on ice. Added 25.25μL to each RNA-primer mix of a master mix of 5x Reverse Transcriptase Buffer (Promega), 10mM each of dNTPs (Promega), and M-MMLV Reverse Transcriptase (50U/reactions). Samples were incubated at 42°C for 1hr and then at 95°C for 3 minutes in a thermal cycler without a heated lid (PTC-200; MJ Research) and then stored at -20°C.

Quantitative PCR

Quantitative PCR was completed using primers developed from an *O. lurida* Transcriptome (version 3) which can be found at Heare et al. (2015b). This transcriptome was annotated using SwissPro and Gene Ontology Databases. Based on function related to stress resilience and homeostasis, gene targets were selected for characterization. Sequence contigs were then pulled from the transcriptome using the seqinR package (Charif and Lobry, 2007). NCBI primer blast was used to develop primers sequences for qPCR using the following parameters: Sequence size 100-400 bp, GC content 55-60%, Melt temperatures ~60 °C and with 0.5 °C of each other, self and 3’ complementarity was limited to 4.00 or less with smallest values being selected, primer sequence 19-21 bp in length. List of primers can be viewed in Table 1.
### Table 1. Table of qPCR Primers for genes of interest. Full sequences for primer creation are available. (Heare et al., 2015b).

<table>
<thead>
<tr>
<th>Gene Abbr</th>
<th>Uniprot ID</th>
<th>Annotation</th>
<th>Function</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>comp7220_c0_seq2</td>
<td>Q6DC04</td>
<td>CARM1_DANRE</td>
<td>Histone arginine methyltransferase</td>
<td>TGGITATCCAGCCCGCCGAC</td>
<td>TGGTGTTACCCGAGGGAG</td>
</tr>
<tr>
<td>comp23747_c0_seq1</td>
<td>Q9ID78</td>
<td>TLR21_CHICK</td>
<td>Toll-like receptor 2 type 1</td>
<td>ACAAAGATTCCACCGGGAAC</td>
<td>ACAACAAAGCAACAGGAAGTG</td>
</tr>
<tr>
<td>comp25000_c0_seq1</td>
<td>Q9DD78</td>
<td>H2A_motif</td>
<td>Histone H2A V</td>
<td>H2A</td>
<td>2.00E-64</td>
</tr>
<tr>
<td>comp24065_c0_seq1</td>
<td>Q9DD78</td>
<td>TLR21_CHICK</td>
<td>Toll-like receptor 2 type 1</td>
<td>TGGTTATCAA</td>
<td>CAGCCCCGAC</td>
</tr>
<tr>
<td>comp24273_c0_seq2</td>
<td>Q6DC04</td>
<td>CARM1_DANRE</td>
<td>Histone arginine methyltransferase</td>
<td>CAGCCCGCCGAC</td>
<td>TGGTGTTACCCGAGGGAG</td>
</tr>
<tr>
<td>comp7183_c0_seq1</td>
<td>Q6DC04</td>
<td>CARM1_DANRE</td>
<td>Histone arginine methyltransferase</td>
<td>CAGCCCGCCGAC</td>
<td>TGGTGTTACCCGAGGGAG</td>
</tr>
<tr>
<td>comp10127_c0_seq1</td>
<td>P62994</td>
<td>GRB2_RAT</td>
<td>Growth factor receptor-bound protein 2</td>
<td>AACTTTGTCCA</td>
<td>CCCAGACGG</td>
</tr>
<tr>
<td>comp6939_c0_seq1</td>
<td>P32240</td>
<td>PE2R4_MOUSE</td>
<td>Prostaglandin E2 receptor EP4 subtype</td>
<td>ACAGCGACGG</td>
<td>AACTTTGTCCA</td>
</tr>
<tr>
<td>comp25313_c0_seq1</td>
<td>Q60803</td>
<td>TRAF3_MOUSE</td>
<td>Tumor Necrosis Factor receptor-associated factor 3</td>
<td>ACAGCGACGG</td>
<td>AACTTTGTCCA</td>
</tr>
<tr>
<td>comp30443_c0_seq2</td>
<td>Q8TA69</td>
<td>Q8TA69_CRAGI</td>
<td>Actin</td>
<td>ACTGGTATCG</td>
<td>AACTTTGTCCA</td>
</tr>
</tbody>
</table>

The Ssofast evagreen supermix (BioRad, USA) was used with forward and reverse primers (100 mM each) (Integrated DNA Technologies) to prime samples for qPCR. Sample cDNA was diluted (1:9) with 9μL added. Samples were run on white qPCR plates (Gennessee Scientific, USA) with optically clear lids (Gennessee Scientific, USA) in a BioRad CFX Real Time Thermocycler (BioRad, USA) and DNA Engine Opticon 2 System (BioRad, USA). The program run was for 40 cycles with reads occurring before and after the termination step (95°C for 10 min, 95°C for 30 sec, 60°C for 1 min, 72°C for 30 sec, repeat 40 times, termination 95°C for 1 minute).

**Statistical Analysis**
To determine original RNA copy number for each gene, cycle threshold (Ct) values were calculated by the BioRad CFX Manager 3.1 (version 3.1.1517.0823, Windows 8.1) and Opticon Manager 3 (Windows 8.1). This was accomplished by subtracting global minimum fluorescence from samples and determining the point in the cycle which amplification reached exponential amplification phase. To standardize the Ct values between runs, default settings were accepted for each program to ensure reproducibility. The BioRad CFX Manager used default settings of single threshold for Cq determination and baseline subtracted curved fit for each run. The Opticon Manager used default settings of subtract baseline via global minimum which estimated the threshold as being between 0.019 and 0.028. Log scale for these programs were checked to determine and correct any issue the default settings may have caused. Ct values for technical replicates (>\(\geq\)=2) were averaged. Gene expression values were determined as normalized mRNA levels \(2^{(\text{mean Ct housekeeping genes}-\text{mean Ct target})}\) as described by Pfaffl (2001). The dCt was then log transformed (\(\log_{10}\text{dCt}\)) for statistical analysis. Two way ANOVA followed by Tukey’s Honestly Significant Difference post hoc test ([base](https://github.com/rstudio), R Core Team, 2014) were performed on \(\log_{10}\text{dCt}\) for each target (p<0.05).

**Results**

**Mortality**

There were no significant mortality differences between mechanical and heat stress treated oysters with both groups dead by Day 6. There were no mortality differences between populations. There were no mortalities in the control group.
Gene Expression Analysis

Without considering separate populations, acute heat shock resulted in increase in expression of CARM (ANOVA, df=2, Tukey’s HSD p=0.00007) (Figure 2) and H2AV (ANOVA, df=2, Tukey’s HSD p=0.001) (Figure 3). An increase in expression of CRAF (ANOVA, df=2, Tukey’s HSD p=0.008) (Figure 4) occurred upon exposure to mechanical stress.

There was a clear difference in response to stress in oysters from Oyster Bay as compared to oysters from Dabob and Fidalgo Bays. Specifically, upon heat shock H2AV expression was increased (ANOVA, df=4, Tukey’s HSD = 0.05) (Figure 3) when compared to the control. When exposed to mechanical stress BMP2 (ANOVA, df=4, Tukey’s HSD p=0.03) (Figure 5) and GRB2 (ANOVA, 5df=4, Tukey’s HSD p=0.03) (Figure 5) expression was decreased in the Oyster Bay population, whereas there was no response in the other populations.

There was no difference in expression in PGRP, TLR, and PGEEP4 (Figure 7, 8, 9). HSP70 gene expression was only different when comparing temperature and mechanical stress (ANOVA, df=4, Tukey’s HSD p=0.006) (Figure 10) that was driven primarily by changes in expression in the Oyster Bay population.

Discussion

This work provides the first gene expression study with Ostrea lurida focused on multiple stressors and multiple functional gene targets. Some limitations in this study confound findings because we were unable to determine sex or reproductive stage of the animals prior to running this experiment. Both of which may have had an effect on mortality or gene expression. Regardless, these data provide important information on the response of O. lurida to stress as
well as differences in populations found in Puget Sound. Contrary to expectations, both stressors
(1000 rpm x 5 min and 38°C water for 1 hour) caused total mortality in all populations after 6
days under ambient conditions. Brown et al. (2004) only observed 100% mortality after 1 hour
exposure to 39°C in *O. lurida* after 6 days but it took 20 days for 100% mortality in oysters
exposed to 38.5°C. Oysters from this experiment experienced total mortality after 6 days from a 1
hour exposure to 38°C. This is possibly due to the populations being from a more northern
latitude as compared to the *O. lurida* Brown et al. (2004) used from California. Most studies that
examine mechanical stress do not assess mortality however Lacoste et al. (2001b) found that less
than 10% of mechanically stressed *C. gigas* died within 7 days. One explanation of why oysters
in the current study experience 100% mortality is because they were vulnerable due to an innate
secondary stressor, such as illness, which induced higher mortality similar to the findings of
Lacoste et al. (2001b).

Response to Temperature Stress

The response of *Ostrea lurida* to acute heat stress appears to include an alteration in gene
regulatory activity, likely in an effort to conserve energy resources. This is based on an increase
of CARM and H2AV gene expression 1 hour following temperature stress. Histone-arginine
methyltransferase 1, CARM, is involved in methylation of histones, which regulates binding of
DNA in chromatin (Biel, Wascholowski, and Giannis, 2005). This in turn affects the ability of
transcription factors to bind and transcription to proceed. Increase in CARM expression could
indicate that overall gene regulatory activity is decreased (via transcription factor binding
inhibition) to conserve energy resources necessary to effectively respond to temperature stress.
These results are similar to those of Wang et al. (2011) where researchers described an increase
in expression of Histone-arginine methyltransferase in the sea cucumber, *Apostichus japonicus*, after experiencing 25 °C temperatures for 7 days. They suggested that this was due to an induced dormancy and lower metabolic rate, to provide resources for stress resilience. Histone H2A.V, H2AV, is a variant of the Histone H2A protein. This variant has been shown to act as a transcription promoter agent as well as assist with heterochromatin formation. Truebano et al. (2010) characterized changes in transcription in Antarctic clams, *L. elliptica*, and found that gene modifiers including an H2A variant were significantly upregulated under heat stress conditions (3 °C for 12 hours). As indicated here, they also suggested that changes in gene regulation were important in maintaining cell function during stress conditions.

Heat shock proteins are often found in response to overt stress but this study only found a significant difference of mRNA expression of HSP70 in the Oyster Bay population between mechanical and heat stress. Brown et al. (2004) found the maximum heat shock protein expression in *O. lurida* occurred 24-48 hours post exposure to 39 °C. The absence of a strong response of HSP70 could be related to temporal changes in expression or an isoform specific response, as there are many genes in this gene family, particularly in oysters (Clegg et al., 1998; Piano et al., 2005). Mediterranean mussels, *Mytilus galloprovincialis*, have shown different isoforms of heat shock proteins and cognates that have differential expression patterns caused by heat, mercury exposure, and chromium exposures stressors suggesting that the isoforms have slightly different functions (Franzellitti and Fabbri, 2005).

**Response to Mechanical Stress**
Mechanical stress increased expression of an inflammation related target genes likely associated with tissue damaging ademas. In all populations, there was a significant increase in immune system related responses seen via the expression of tumor necrosis factor receptor type 3, CRAF, which is involved in internal tissue damage recognition and apoptosis. The main function of CRAF is to assist in cell death initiation caused by stress conditions within tissues (Arch, Gedrich, and Thompson, 1998). Upregulation in relation to mechanical stress could be akin to inflammation occurring due to edema from the mechanical stress and used to remove damaged cells as suggested by Roberts et al. (2011) when C. gigas were exposed to mechanical stress. Other immune system targets such as PGRP, TLR, and PGEEP4 did not show any significant difference in expression but other studies have found that the time scale for expression may vary (Meistertzheim et al., 2007; Farcy et al., 2009).

Population differences

Contrary to our hypothesis, oysters from Oyster Bay demonstrated the greatest difference in response to stress compared to the other populations. Specifically oyster originally from Oyster Bay, had an increase in H2AV expression during heat stress as compared to control, a decrease in BMP2 and GRB2 upon mechanical stress, and differences in HSP70 expression between the two treatments. As previously mentioned, changes to H2AV could be indicative of epigenetic silencing of non-essential genes to promote expression of important resilience genes. Bone morphogenic protein 2, BMP2, and Growth-factor receptor bound protein 2, GRB2, were significantly decreased in expression which could be indicative of growth inhibition, similarly
related to energy conservation. Both genes are related to growth and development of tissues with BMP2 being a pre-cursor to osteoblastic cells that produce shell (Pereira Mouries et al., 2002) and GRB2 is used for signal transduction between cells during growth phases (Oda et al., 2005). By downregulating these targets, this may be in an effort to reduce energetically costly processes in favor of processes that promote survival during stress events. Organisms faced with overt stress are often required to reallocate energy resources to homeostasis related functions in an effort to improve longterm survival of the species (Sokolova et al., 2012). This change in expression coupled with the upregulation of H2AV strengthens the idea of shifting priorities for stress resilience.

While only speculation at this point, the gene expression pattern differences observed here with oysters from Oyster Bay coupled with corresponding field-based observation that this population has the greatest reproductive activity, could indicate this population has a greater ability to effectively respond to energy demands. Another way to consider this is that the Oyster Bay population has a relatively higher degree of phenotypic plasticity, or more specifically, the rate at which an organism can change its phenotype is perhaps elevated (Angilletta et al., 2003). The gene expression data indicates a clear population response, and lack of differential response in other populations, to stress that suggests shifts in energy balance. Some possible explanations for this relatively rapid response include a more sensitive cell-signaling system (ie cytokines) or a more robust transcription initiation process. Yao and Somero (2012) observed higher heat stress tolerance in M. galloprovincialis than M. californius likely due to their ability to maintain cell signaling through the production of phosphor-p38-MAPK kinases, which may be how the Oyster Bay population is able to quickly respond to stress. Ultimately, this ability to respond to change
or stress would have likely been selected upon due to increased fitness. Based on earlier field work, this could be directly linked to increased larval production, and processes allocating limited resources into reproduction (Heare et al., 2015). This trait could certainly be perceived as advantageous for restoration purposes. On the other hand, the results of the gene expression analysis does suggest population differences, many of which we do not completely understand. Caution should be used in using non-local stocks when structure exists, as it is possible to have supplemented oysters outcompete the native population or to create hybrids that are ultimately less fit than the native counterparts (Camara and Vadopalas, 2009). Both such phenomena decrease overall genetic diversity leaving the remaining population to be less robust for future challenges and possibly leading to local extirpation.

The other consideration is to what degree does phenotypic plasticity (or the rate of obtaining a new phenotype, in this case) have in restoration. At one level the ability to achieve a number of phenotypes with a given genotype could be advantageous, particularly in a rapidly the changing environment. There is a paradox in the fact that too much plasticity negates the ability of natural selection to function. Populations with high phenotypic plasticity become deprived of negative selection and thus are often able to survive in rapidly changing environments as long as the changes are consistent and somewhat predictable. However, with this increased adaptive ability, genetic diversity and adaptation become limited within a population that may be unable to properly respond to novel challenges in the future (Crispo, 2008). Alternatively, the Baldwin effect may enhance longterm genetic diversity by allowing species to colonize novel habitats and, with phenotypic plasticity, and eventually genetically diverge from the source population through induced genetic adaptations (Crispo, 2007). For longterm restoration of *O. lurida*
populations in Puget Sound, understanding the phenotypic plasticity of individual populations will help determine proper supplementation procedures for existing and historic habitats.

Citations


Figure 1. Map of Puget Sound with *Ostrea lurida* broodstock and outplant sites. Conditioning site was Port Gamble (G). Broodstock collected from Fidalgo Bay (F), Dabob Bay (D), and Oyster Bay (O).
Figure 2. Expression of CARM mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences (p<0.05) between treatments within a population. Capital letters indicate significant differences (p<0.05) between overall treatment groups.
Figure 3. Expression of H2AV mRNA. Mean delta Ct indicated by line in middle of box plot.

Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers.

Asterisks indicate significant differences (p<0.05) between treatments within a population.

Capital letters indicate significant differences (p<0.05) between overall treatment groups.
Figure 4. Expression of CRAFT mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences (p<0.05) between treatments within a population. Capital letters indicate significant differences (p<0.05) between overall treatment groups.
Figure 5. Expression of BMP2 mRNA. Mean delta Ct indicated by line in middle of box plot.

Shaded boxes are 2\textsuperscript{nd} and 3\textsuperscript{rd} quartile. Lines are 1\textsuperscript{st} and 4\textsuperscript{th} quartiles. Dots indicate outliers.

Asterisks indicate significant differences (\(p<0.05\)) between treatments within a population.

Capital letters indicate significant differences (\(p<0.05\)) between overall treatment groups.
Figure 6. Expression of GRB2 mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences (p<0.05) between treatments within a population. Capital letters indicate significant differences (p<0.05) between overall treatment groups.
Figure 7. Expression of PGRP mRNA. Mean delta Ct indicated by line in middle of box plot.  
Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers.  
Asterisks indicate significant differences (p<0.05) between treatments within a population.  
Capital letters indicate significant differences (p<0.05) between overall treatment groups.
Figure 8. Expression of TLR mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences (p<0.05) between treatments within a population. Capital letters indicate significant differences (p<0.05) between overall treatment groups.
Figure 9. Expression of PGEEP4 mRNA. Mean delta Ct indicated by line in middle of box plot.

Shaded boxes are 2\textsuperscript{nd} and 3\textsuperscript{rd} quartile. Lines are 1\textsuperscript{st} and 4\textsuperscript{th} quartiles. Dots indicate outliers.

Asterisks indicate significant differences (p<0.05) between treatments within a population.

Capital letters indicate significant differences (p<0.05) between overall treatment groups.
Figure 10. Expression of HSP70 mRNA. Mean delta Ct indicated by line in middle of box plot.

Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers.

Asterisks indicate significant differences (p<0.05) between treatments within a population.

Capital letters indicate significant differences (p<0.05) between overall treatment groups.