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

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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 lurid*a to stress and provide clues to underlying performance differences in the three populations examined.

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Abstract

Olympia oysters are the only oyster native 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 lurid*a 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

Introduction

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. *Ostrea 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 *C. gigas*, *O. lurida* does not release its eggs into the water column. Instead females collect planktonic sperm balls and larvae are brooded for approximately two weeks before being released into the water column. The adults are sessile 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).

Loss of habitat due to invasive species, overharvest, and pollution have greatly reduced the native Olympia oyster population. Although restoration efforts are underway, basic research is needed to understand how this species interacts with its environment and responds to stress. Freshwater influx, tidal exchange, food availability, shifts in water temperature, and physical stresses from water flow and predation are examples of a myriad of stressors which affect long term survival of *O. lurida* populations (Hopkins, 1937; Baker, 1995).

Thermal stress has been widely studied in mollusks, especially bivalves. *Ostrea lurida* has a temperature tolerance range between 5℃ – 39℃ (Hopkins, 1937; Brown et al., 2004), with notable mortalities occurring below freezing (Davis, 1955) and above 40℃ (Brown et al., 2004). 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; Fitzgerald-Dehoog, Browning and Allen, 2012). Expression of homeostasis-related genes, such as HSP70, glutamine synthetase, and citrate synthase in *C. gigas* has been shown to fluctuate under prolonged heat stress at 25℃ 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 (HSP) family gene expression. Specifically, seasonal variation of HSPs and heat shock cognates (HSCs) levels 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).

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. Mechanical stress in oysters has been shown to increase catecholamines present in hemolymph (Qu et al., 2009; Lacoste et al., 2001c). Upon mechanical stress, researchers have found increases in adrenocorticotropic hormone (ACTH), a hormone that induces production of noradrenaline and dopamine (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*) have found significant decreases in phagocytosis and phenoloxidase activity due to mechanical stress (Kuchel, Raftos, and Nair, 2010;). Exposure to mechanical stress also has ecological relevance as oysters are exposed to this in the intertidal environment and in aquaculture production practices (i.e. 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 comes from distinct bays within Puget Sound, WA: Fidalgo Bay, Dabob Bay, and Oyster Bay. Fidalgo Bay, the furthest northern population (48°28'31.1"N 122°34'48.6"W), is directly fed from the Salish Sea and the Strait of Juan de Fuca, and has the coldest average year-round temperatures of the three locations. Typically, this population does not experience strong fluctuations 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 lack other observable phenotypes (Heare et al., 2015). Dabob Bay (47°49'27.4"N 122°48'37.9"W) 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 (e.g. Tarboo Creek). This area experiences extreme temperature fluctuations throughout the year and this population of *O.lurida* is often partially, or completely, exposed during low tide events. 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 (47°06'21.2"N 123°04'32.8"W) is the southernmost bay which sustains a healthy population of *O. lurida*. The conditions here are, on average, the warmest of the three locations throughout the year. Due to their intertidal place ment, the animals are mostly submerged during tidal changes. The bay has extensive food resources and oysters appears to allocate

more energy resources into reproductive activity compared to the other populations, based on our prior field studies (Heare et al. 2015).

In this study, we investigated differences between these populations in their responses to mechanical and temperature stresses, 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 pronounced response to stress via changes in gene expression.

Materials and Methods

Experimental Design

Adult oysters from three 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 (submerged in 500mL 38°C sea water for 1 hour; n=14 per population), mechanical stress (1000 rpm x 5 min; Sorvall T21, ST-H750 rotor; 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 remaining from each group (temperature stress, mechanical stress, & control) 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 using RNAzol RT (Molecular Research Center, Inc.) according to the manufacturer's protocol for total RNA isolation. Briefly, ctenidia tissue was homogenized in RNAzol RT and incubated at room temperature (RT) for 10 minutes. 400µL of 0.1% DEPC-H2O was added to the homogenized ctenidia tissue, vortexed for 15 seconds, and incubated at RT for 15 minutes. The samples were centrifuged for 15 minutes, 16,000g, at RT. After centrifugation, 750µL of the supernatant was transferred to a clean tube, added an equal volume of isopropanol, vortexed for 10 seconds, and incubated at RT for 15 minutes. The samples were centrifuged at 12,000g for 10 minutes at RT. 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 37C. 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 qPCR (data not shown).

Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega) with oligo dT primers (Promega), using 250ng 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 25.25uL to each RNA-primer mix of a master mix of 5x Reverse Transcriptase Buffer (Promega), 10mM each of dNTPs (Promega), and M-MLV Reverse Transcriptase (50U/reactions). Samples were incubated at 42°C for 1hr, 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 (qPCR) was completed using primers developed from an *O. lurida* transcriptome (version 3) which can be found at Heare and Roberts (2015). This transcriptome was annotated using SwissProt and Gene Ontology Databases. Gene targets were selected based on annotations related to stress resilience and homeostasis. Corresponding contigs were then selected from the transcriptome using the seqinR package (Charif and Lobry, 2007). NCBI Primer Blast was used to develop primers for qPCR using the following parameters: amplicon size 100-400 bp, GC content 55-60%, melt temperatures ~60 ℃ and within 0.5 ℃ 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.

Full Sequence	Uniprot ID	Uniprot ID	Annotation	Function	Gene Abbr	evalue	FWD	REV
comp7220_c0_seq2	Q6DC04	CARM1_DANRE	Histone-arginine methyltransferase	Transfers methyl groups to Histone 3 and 4 to change how DNA is bound up in chromatin	CARM	0	TGGTTATCAACAGCCCCGAC	GTTGTTGACCCCAGGAGGAG
comp23747_c0_seq1	Q9DD78	TLR21_CHICK	Toll-like receptor 2 type 1	Assists with recognition of foreign pathogens and endogenous materials for consumptions by phagocytes in early stages of inflammation	TLR	8.00E-29	ACAAAGATTCCACCCGGCAA	ACACCAACGACAGGAAGTGG
comp25000_c0_seq1	P08991	H2AV_STRPU	Histone H2A.V	One of 5 main Histone Proteins involved in the structure of chromatin and the open reading frame of DNA	H2AV	5.00E-64	TGCTTTCTGTGTGCCCTTCT	TATCACACCCCGTCACTTGC
comp24065_c0_seq1	O75594	PGRP1_HUMAN	Peptidoglycan recognition protein 1	Assists with recognition of bacteria in an immune response	PGRP	2.00E-42	GAGACTTCACCTCGCACCAA	AACTGGTTTGCCCGACATCA
comp44273_c0_seq2	Q8MWP4	Q8MWP4_OSTED	Heat Shock Protein 70kDa	Molecular chaperone and protein preservation in heat response	HSP70	0	TTGTCGCCATTTTCCTCGCT	GTTCCGATTTGTTCCGTGCC
comp7183_c0_seq1	P12643	BMP2_HUMAN	Bone morphogenetic protein 2	Directs calcification in shell creation	BMP2	2.00E-93	TGAAGGAACGACCAAAGCCA	TCCGGTTGAAGAACCTCGTG
comp10127_c0_seq1	P62994	GRB2_RAT	Growth factor receptor-bound protein 2	Assists in signal transduction/cell communication	GRB2	1.00E-83	AACTTTGTCCACCCAGACGG	CCAGTTGCAGTCCACTTCCT
comp6939_c0_seq1	P32240	PE2R4_MOUSE	Prostaglandin E2 receptor EP4 subtype	Receptor for Prostaglandin E2 which suppresses inflammation due to injury	PGEEP4	1.00E-50	ACAGCGACGGACGATTTTCT	ATGGCAGACGTTACCCAACA
comp25313_c0_seq1	Q60803	TRAF3_MOUSE	Tumor Necrosis Factor receptor-associated factor 3	Related to immune response specifically cell death initiation	CRAF	3.00E-145	AGCAGGGCATCAAACTCTCC	ACAAGTCGCACTGGCTACAA
comp30443_c0_seq2	Q8TA69	Q8TA69_CRAGI	Actin	Housekeeping gene used for baseline	Actin	0	GACCAGCCAAATCCAGACGA	CGGTCGTACCACTGGTATCG

Table 1. Table of qPCR Primers for genes of interest. Full sequences for primer creation are available. (Heare and Roberts 2015).

Quantitative PCR reactions were carried out using Ssofast Evagreen Supermix (BioRad, USA). Forward and reverse primers (Integrated DNA Technologies) were used at a final concentration of 0.25uM each. Sample cDNA was diluted (1:9) with molecular-grade water. Nine microliters of diluted cDNA was used as template. Reactions were run in low-profile, non-skirted, white qPCR plates (USA Scientific) with optically clear lids (USA Scientific) in a BioRad CFX Real Time Thermocycler (BioRad, USA) and DNA Engine Opticon 2 System (BioRad, USA).

185 Cycling conditions were: one cycle of 95°C for 10 min; 40 cycles of 95°C for 30 sec, 60°C for 1
186 min, 72°C for 30 sec.

187 **Statistical Analysis**

188 To calculate relative expression levels for each gene, cycle threshold (Ct) or cycle quantity (Cq)
189 values were calculated by the BioRad CFX Manager 3.1 (version 3.1.1517.0823, Windows 8.1)
190 and Opticon Manager 3 (Windows 8.1). This was accomplished by subtracting global minimum
191 fluorescence from samples and determining the point in the cycle which amplification reached
192 exponential amplification phase. To standardize the Ct values between runs, default settings were
193 accepted for each program to ensure reproducibility. The BioRad CFX Manager used default
194 settings of single threshold for Cq determination and baseline subtracted curved fit for each run.
195 The Opticon Manager used default settings of subtract baseline via global minimum which
196 estimated the threshold as being between 0.019 and 0.028. Gene expression values were
197 determined as normalized mRNA levels using the following equation: $2^{-\Delta Ct}$; where ΔCt is: target
198 Ct – actin Ct) (Schmittgen and Livak, 2008). The delta Ct was log transformed (logdCt) for
199 statistical analysis. Two-way analysis of variance (ANOVA) followed by Tukey's Honestly
200 Significant Difference post hoc test (*base*, R Core Team, 2014) were performed on logdCt for
201 each target ($p<0.05$).

202

203 **Results**

204 **Mortality**

205 There were no significant mortality differences between mechanical and heat stress treated
206 oysters. All oysters in both groups were dead by Day 6. There were no mortality differences
207 between populations. There were no mortalities in the control group (data not shown).

208

209 **Gene Expression Analysis**

210 Without considering separate populations, acute heat shock resulted in statistically significant
211 increases in expression of CARM (ANOVA, df=2, Tukey's HSD p=0.00007) (Figure 2) and
212 H2AV (ANOVA, df=2, Tukey's HSD p=0.001)(Figure 3). A statistically significant increase in
213 expression of CRAF (ANOVA, df=2, Tukey's HSD p=0.008) (Figure 4) occurred upon exposure
214 to mechanical stress.

215 There was a clear difference in response to mechanical stress in oysters from Oyster Bay as
216 compared to oysters from Dabob and Fidalgo Bays. ~~Specifically, upon heat shock H2AV~~
217 ~~expression was increased (ANOVA, df=4, Tukey's HSD = 0.05) (Figure 3) when compared to~~
218 ~~the control.~~ When exposed to mechanical stress BMP2 (ANOVA, df=4, Tukey's HSD p=0.03)
219 (Figure 5) and GRB2 (ANOVA, df=4, Tukey's HSD p=0.03)(Figure 6) expression was
220 decreased in the Oyster Bay population, whereas there was no response in the other populations.

221 There was no statistical difference in expression in PGRP, TLR, and PGEEP4 (Figure 7, 8, 9).
222 HSP70 gene expression was statistically different between temperature and mechanical stress
223 (ANOVA, df=4, Tukey's HSD p=0.006) (Figure 10).

224

225 **Discussion**

This work provides the first gene expression study with *Ostrea lurida* focused on multiple stressors and multiple functional gene targets. 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℃ in *O. lurida* after 6 days but it took 20 days for 100% mortality in oysters exposed to 38.5℃. Oysters from this experiment experienced total mortality after 6 days from a 1 hour exposure to 38℃. This difference 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. (2001d) found that less than 10% of mechanically stressed *C. gigas* died within 7 days. One explanation of why oysters in the current study experienced 100% mortality is because they were vulnerable due to an innate secondary stressor, which induced higher mortality similar to the findings of Lacoste et al. (2001d).

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 one hour following temperature stress. Coactivator-associated 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℃ 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 2AV, 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 ℃ for 12 hours).

Increases in HSPs are often observed in response to stress, but this study only found a significant difference of mRNA expression of HSP70 in the Oyster Bay population between mechanical and heat stresses (Figure 10). Brown et al. (2004) found the maximum HSP expression in *O. lurida* occurred 24-48 hours post exposure to 39℃. The absence of a strong response of HSP70, relative to the control group, 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 inflammation-related target genes, likely associated with tissue-damaging edemas. In all populations, there was a significant increase in immune system-related responses seen via the expression of CD40 receptor-associated factor, (tumor necrosis factor receptor-associated factor3))CRAF (TRAF3), 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. virginica* 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, oysters 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 an effort to reduce energetically costly processes in favor of processes that promote survival during stress events. Organisms faced with stress are often required to reallocate energy resources to homeostasis-related functions in an effort to improve long-term 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

CONCLUSIONS

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 stress. 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

315 respond to stress. Ultimately, this ability to respond to stress would have likely been selected
316 upon due to increased fitness. Based on earlier field work, this could be directly linked to
317 increased larval production, and processes allocating limited resources into reproduction (Heare
318 et al., 2015). This trait could certainly be perceived as advantageous for restoration purposes.
319 Caution should be used in using non-local stocks when structure exists, as it is possible to have
320 supplemented oysters out-compete the native population or to create hybrids that are ultimately
321 less fit than the native counter parts (Camara and Vadopalas, 2009). Both such phenomena
322 decrease overall genetic diversity leaving the remaining population to be less robust for future
323 challenges and possibly leading to local extirpation.

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

338

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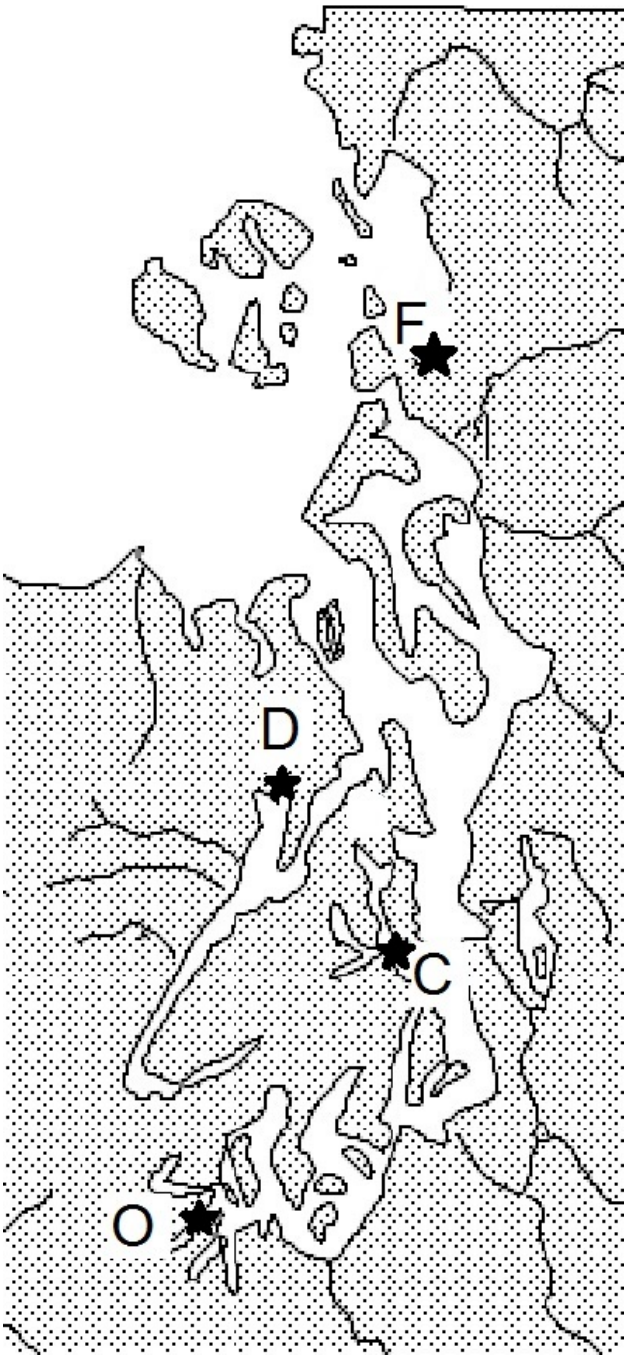
457 **Figures**



458

459 Figure 1. Map of Puget Sound with *Ostrea lurida* broodstock locations. Broodstock collected

460 from Fidalgo Bay (F), Dabob Bay (D), and Oyster Bay (O) and held at Clam Bay (C).

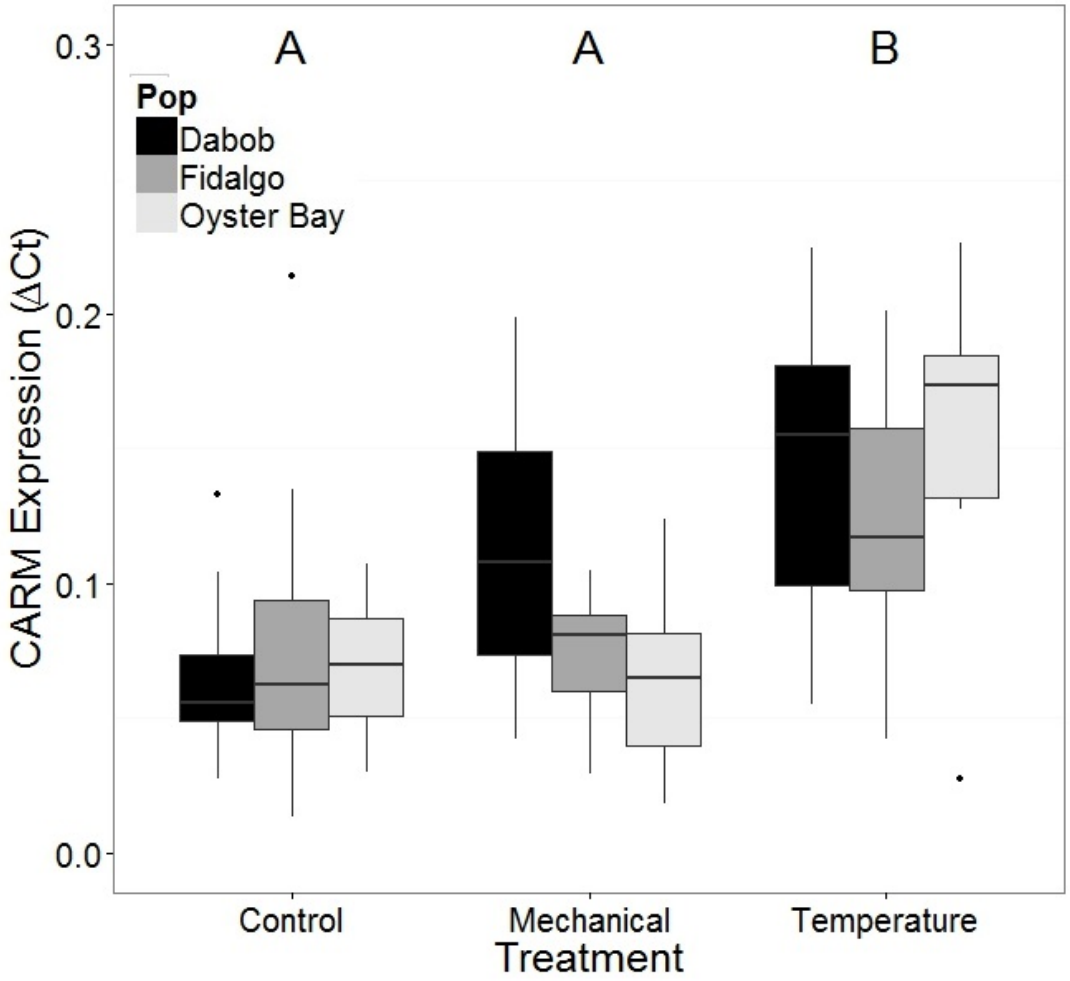


Figure 2. Expression of CARM mRNA. Median delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. Capital letters indicate significant differences ($p<0.05$) between overall treatment groups.

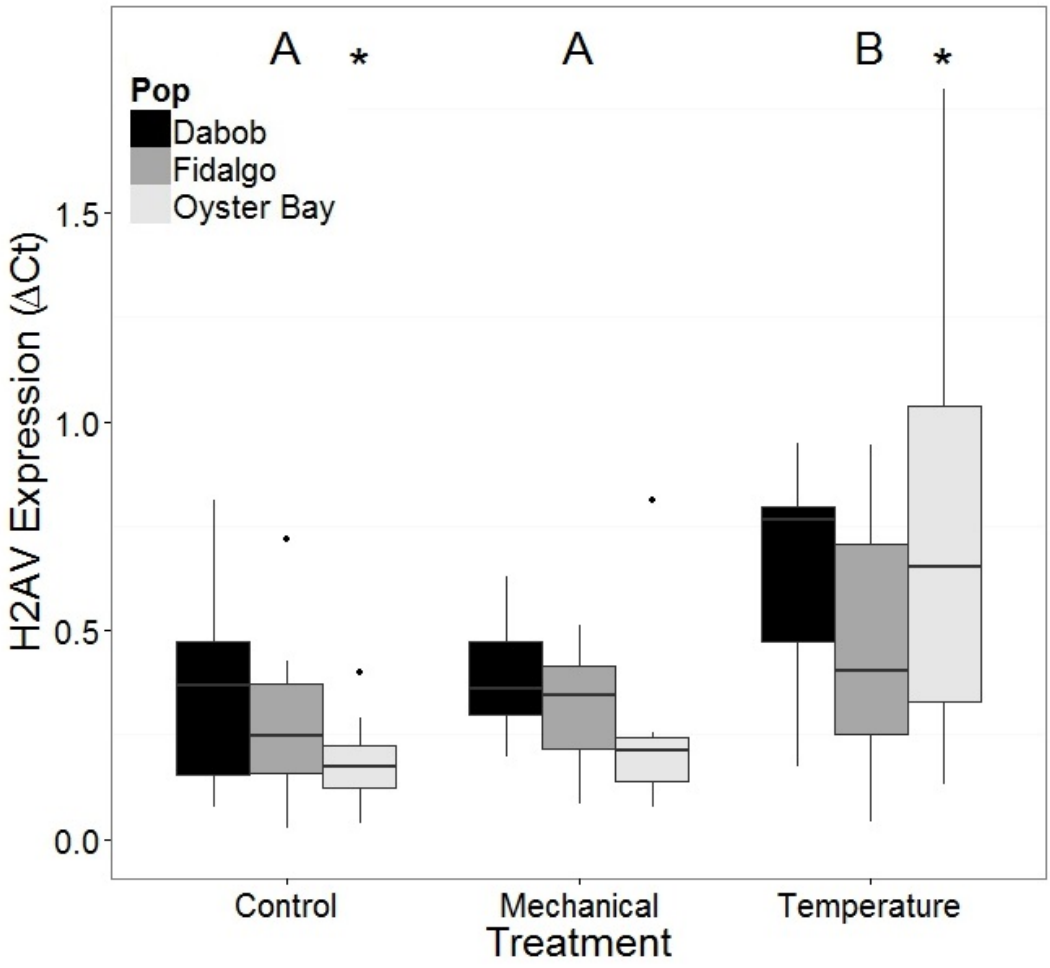


Figure 3. Expression of H2AV mRNA. Median delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. Asterisks indicate significant differences ($p<0.05$) between treatments within a population. Capital letters indicate significant differences ($p<0.05$) between overall treatment groups.

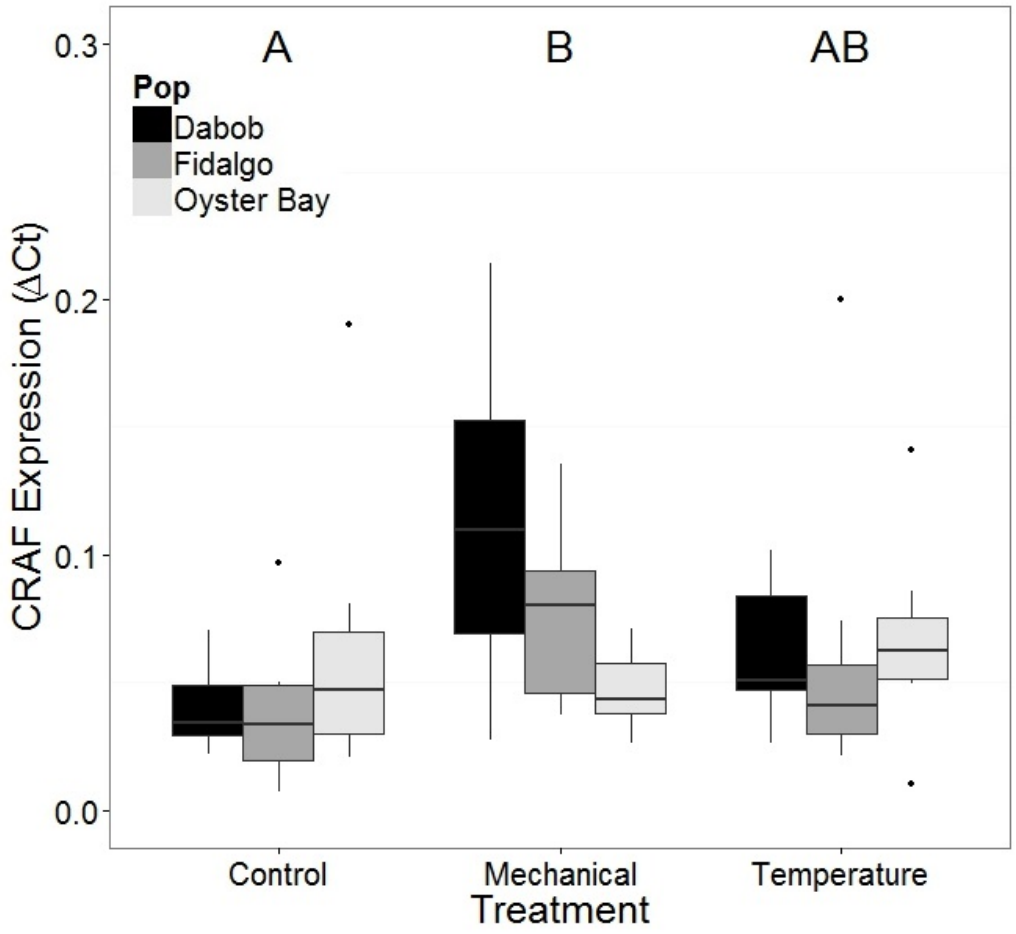
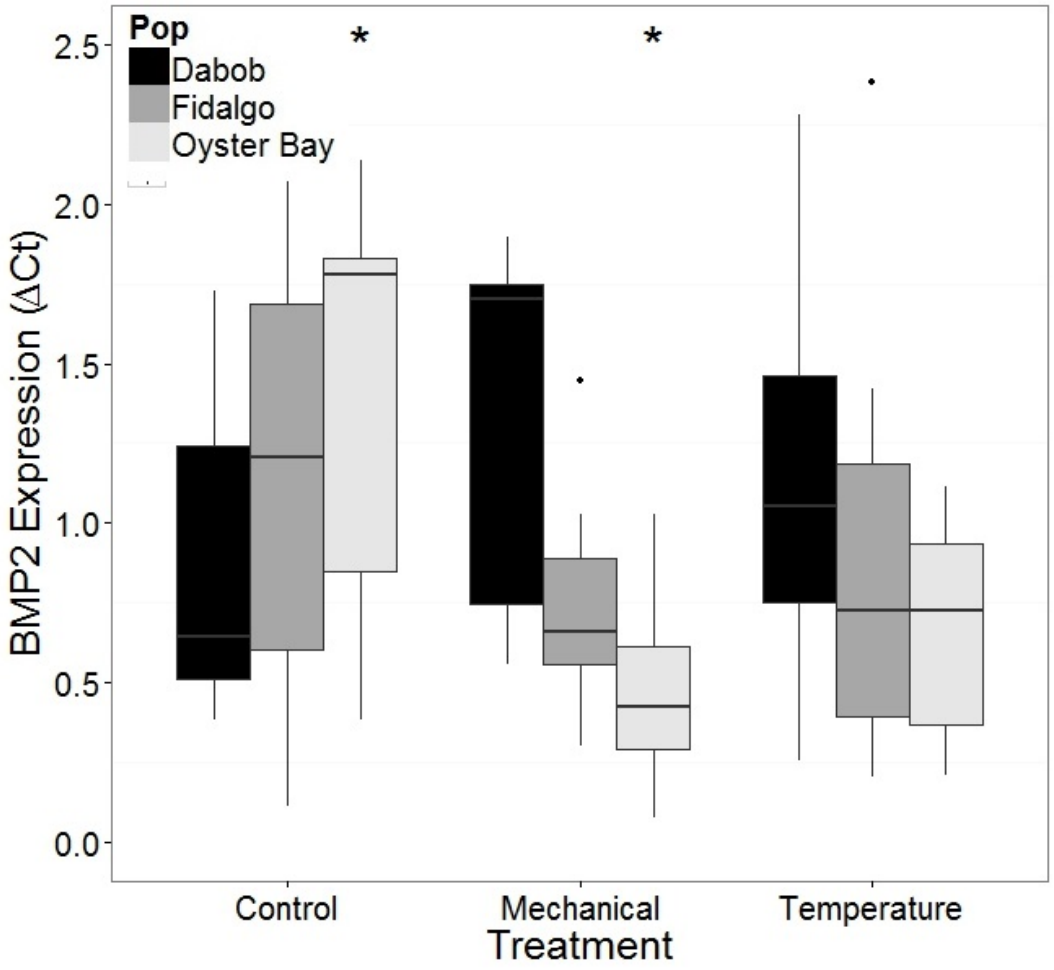


Figure 4. Expression of CRAF mRNA. Median delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. Capital letters indicate significant differences ($p<0.05$) between overall treatment groups.



480

481 Figure 5. Expression of BMP2 mRNA. Median delta Ct indicated by line in middle of box plot.
482 Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate
483 outside values. Asterisks indicate significant differences ($p<0.05$) between treatments within a
484 population.

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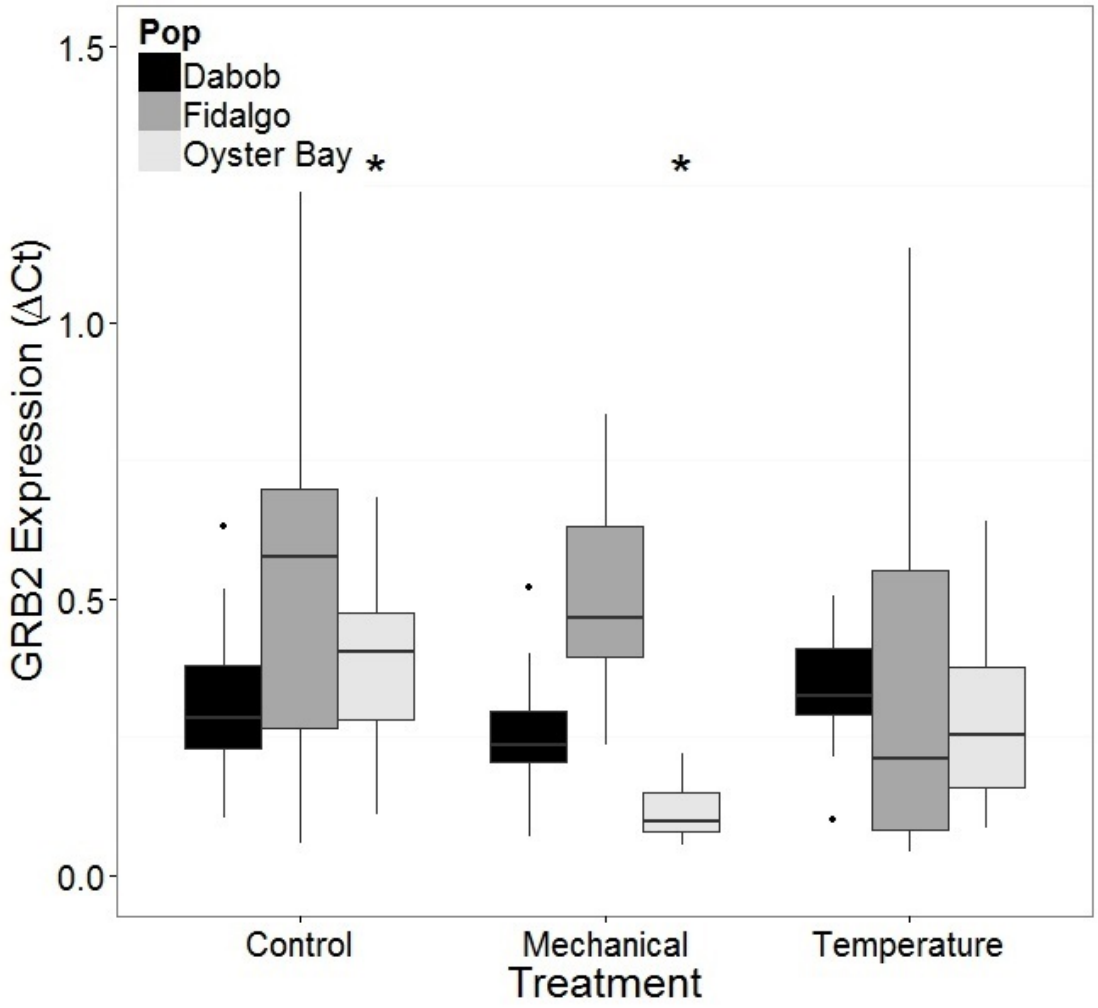
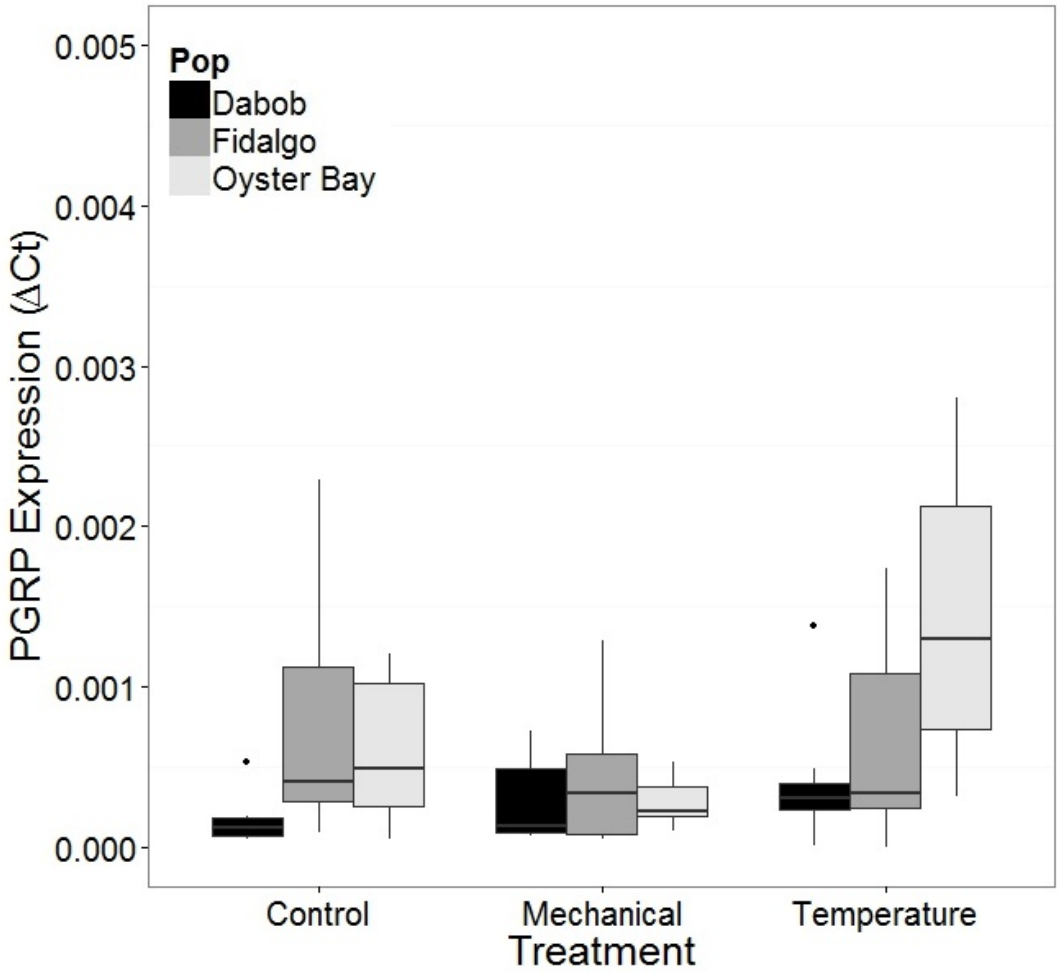


Figure 6. Expression of GRB2 mRNA. Median delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. Asterisks indicate significant differences ($p<0.05$) between treatments within a population.



492

493 Figure 7. Expression of PGRP mRNA. No statistical difference observed between treatments,
494 nor between populations. Median delta Ct indicated by line in middle of box plot. Shaded boxes
495 are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values.

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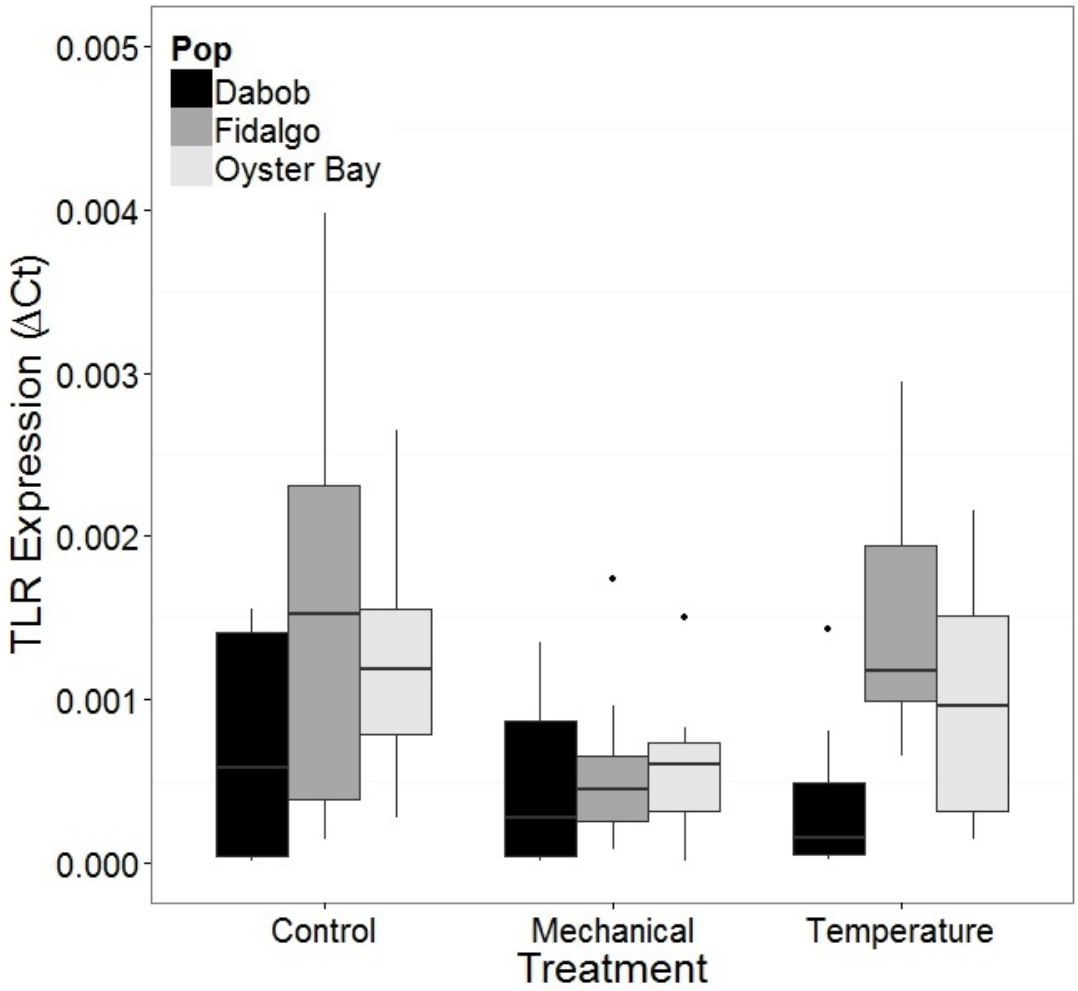

497

498 Figure 8. Expression of TLR mRNA. No statistical difference observed between treatments, nor
499 between populations. Median delta Ct indicated by line in middle of box plot. Shaded boxes are
500 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values.

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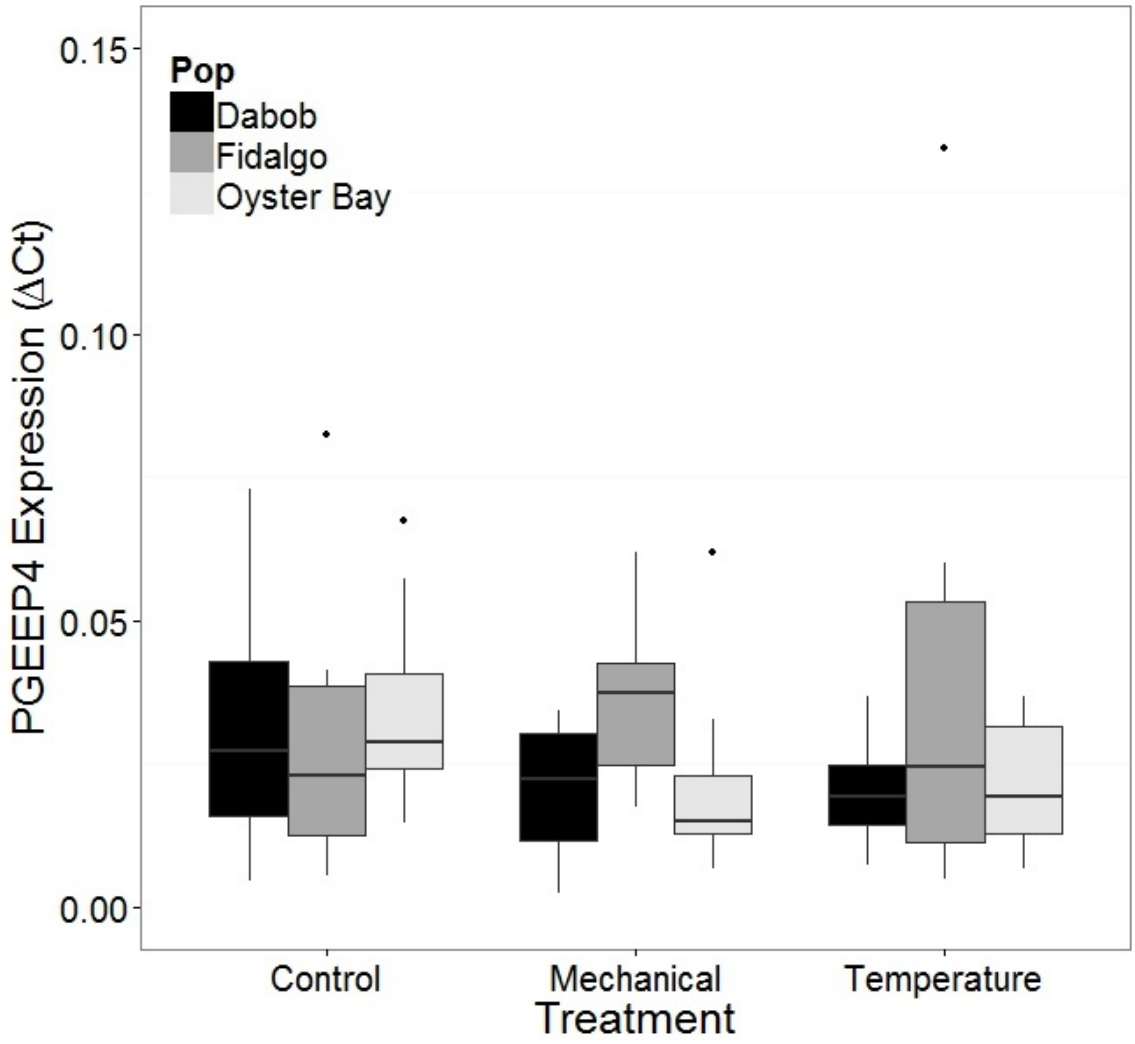

Figure 9. Expression of PGEEP4 mRNA. No statistical difference observed between treatments, nor between populations. Median delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values.

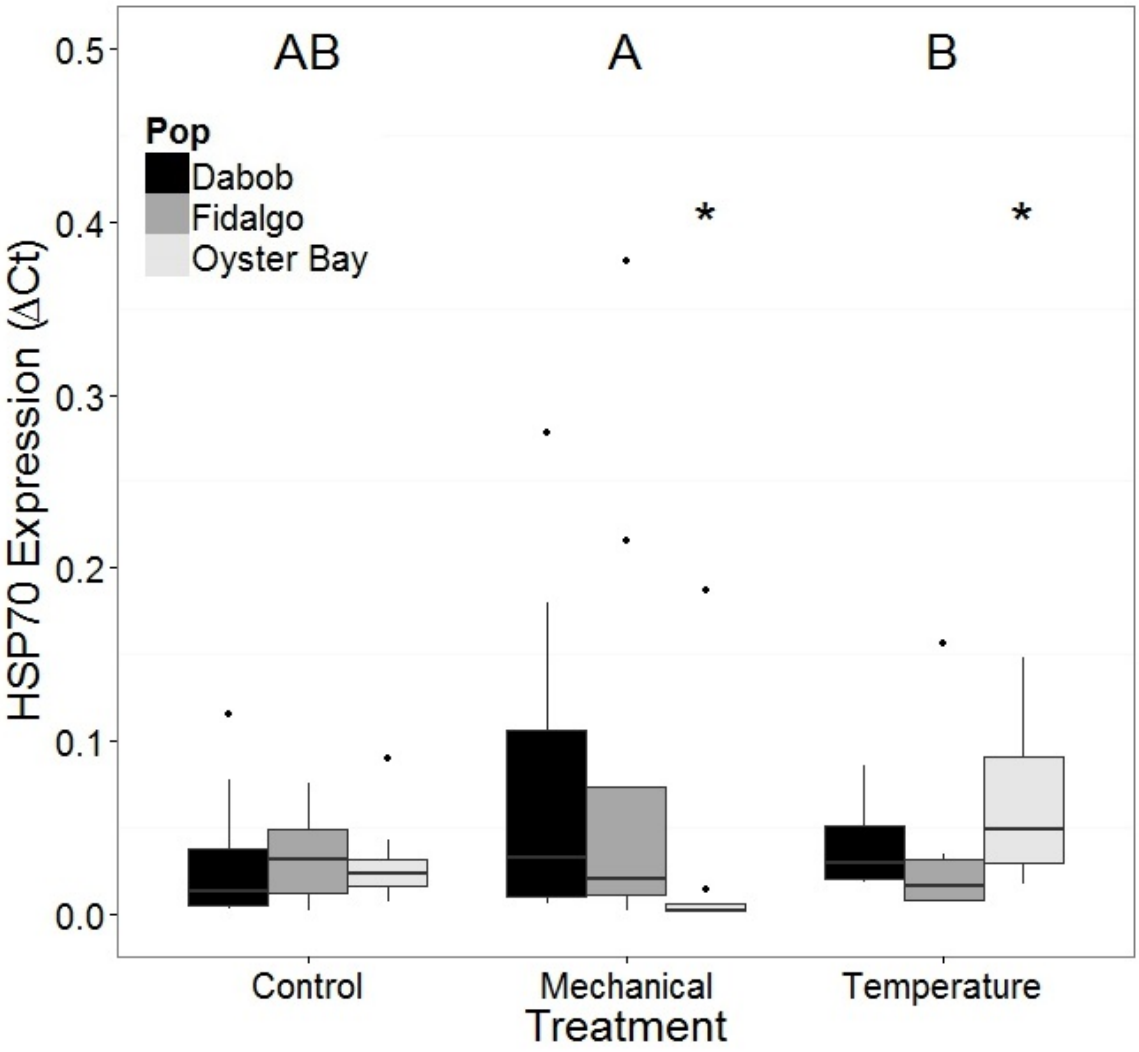

Figure 10. Expression of HSP70 mRNA. Median delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. Asterisks indicate significant differences ($p<0.05$) between treatments within a population. Capital letters indicate significant differences ($p<0.05$) between overall treatment groups.