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

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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 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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12   **Abstract**

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

Introduction

27 Olympia oysters, *Ostrea lurida*, are the only native oyster species on the west coast of North
28 America. The species inhabits bays and estuaries within Puget Sound, WA. *Ostrea lurida* is
29 typically smaller than the introduced Pacific oyster, *Crassostrea gigas*, with adults attaining an
30 average size between 40 – 60 mm (Hopkins, 1937; Baker, 1995). As protandric hermaphrodites,
31 Olympia oysters usually spawn as both male and female within the first year (Coe, 1932;
32 Hopkins, 1937; Baker, 1995). Unlike *C. gigas*, *O. lurida* does not release its eggs into the water
33 column. Instead females collect planktonic sperm balls and larvae are brooded for approximately
34 two weeks before being released into the water column. The adults are sessile and are typically
35 moved via predator interactions or wave action. Colonizing lower intertidal habitats, *O. lurida*
36 typically can be found in the inner portions of bays or estuaries where dynamic conditions can
37 shape the phenotypes of local populations (Baker, 1995; White et al., 2009).

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

44 Thermal stress has been widely studied in mollusks, especially bivalves. *Ostrea lurida* has a
45 temperature tolerance range between 5℃ – 39℃ (Hopkins, 1937; Brown et al., 2004). It is
46 suspected that mass summer mortalities of *C. gigas* may be linked to the effects of heat stress
47 during spawning events (Li et al., 2007b). The California mussel, *Mytilus californianus*, has been
48 found to divert resources to physiological defense during thermal stress events (Petes, Menge,
49 and Harris, 2008; Fitzgerald-Dehoog, Browning and Allen, 2012). Expression of homeostasis-
50 related genes, such as HSP70, glutamine synthetase, and citrate synthase in *C. gigas* has been
51 shown to fluctuate under prolonged heat stress at 25℃ for 24 days (Meistertzheim et al. 2007).
52 Temperature stress has been shown to induce a variety of up and down regulation of genes to
53 maintain homeostasis (Tomanek, 2010). In oysters, there has been a significant amount of work
54 examining the change in heat shock protein (HSP) family gene expression. Seasonal variation of
55 HSPs and heat shock cognates (HSCs) levels have been characterized in response to ambient
56 temperatures for *C. gigas* (Hamdoun, Cheney, and Cherr, 2003; Farcy et al., 2009). Additionally,
57 induction of HSP70 and HSP69 in *Ostrea edulis* at temperatures greater than 38˚C have been
58 reported (Piano et al., 2005).

59 The response of bivalves to mechanical stress has also received considerable attention. One
60 reason for this is that researchers have shown mechanical stress elicits a classical stress response,
61 providing a simple method to allow for investigation of fundamental physiological stress
62 responses. Additionally, most oyster restoration and aquaculture practices do involve handling
63 and movement which would be a form of mechanical stress. Mechanical stress in oysters has
64 been shown to increase catecholamines present in hemolymph (Qu et al., 2009; Lacoste et al.,
65 2001c). Upon mechanical stress, researchers have found increases in adrenocorticotropic
66 hormone (ACTH), a hormone that induces production of noradrenaline and dopamine (Lacoste et
67 al. 2001a; Lacoste et al., 2001b; Lacoste et al., 2001c). Mechanical stress has also been shown to
68 activate inflammation factors that are also observed during bacterial challenges (Lacoste et al.,
69 2001c; Lacoste et al., 2001d; Aladaileh, Nair, and Raftos, 2008; Roberts et al., 2011). Studies in
70 Pearl oysters (*Imbricata pinctada*) have found significant decreases in phagocytosis and
71 phenoloxidase activity due to mechanical stress (Kuchel, Raftos, and Nair, 2010;).

72 Here we set out to examine the effects of temperature and mechanical stress on *Ostrea lurida*, by
73 comparing differences in gene expression among three local populations (Heare et al., 2015).
74 Each of the three populations comes from distinct bays within Puget Sound, WA: Fidalgo Bay,
75 Dabob Bay, and Oyster Bay. Fidalgo Bay, the furthest northern population (48°28'31.1"N
76 122°34'48.6"W), is directly fed from the Salish Sea and the Strait of Juan de Fuca, and has the
77 coldest average year-round temperatures of the three locations. Typically, this population does not
78 experience strong fluctuations in temperatures due to the fact that it resides in the lower part of
79 the intertidal area and is submerged for most of the time. Olympia oysters from Fidalgo Bay
80 experience significant growth when placed in warmer habitats, but otherwise lack other
81 observable phenotypes (Heare et al., 2015). Dabob Bay (47°49'27.4"N 122°48'37.9"W) is a large
82 bay at the northern most portion of Hood Canal with the population of Olympia oysters residing
83 near the innermost portions of the bay (e.g. Tarboo Creek). This area experiences extreme
84 temperature fluctuations throughout the year and this population of *O.lurida* is often partially, or
85 completely, exposed during low tide events. During tidal changes, temperatures can be as high as
86 29℃ during summer or as low as -3℃ during winter (Heare et al., 2015). Oysters from Dabob
87 Bay have been shown to experience high survival when faced with temperature challenges,
88 possibly due to adaptive structure of the local population (Heare et al., 2015). Oyster Bay
89 (47°06'21.2"N 123°04'32.8"W) is the southernmost bay which sustains a healthy population of
90 *O. lurida*. The conditions here are, on average, the warmest of the three locations throughout the
91 year. The bay has extensive food resources and oysters appear to allocate more energy resources
92 into reproductive activity compared to the other populations, based on our prior field studies
93 (Heare et al. 2015).

94 For long-term restoration of *O. lurida* populations in Puget Sound, understanding the phenotypic
95 plasticity of individual populations will help determine proper supplementation procedures for
96 existing and historic habitats. To this end, and to attempt reveal relationship of gene expression
97 response with stress exposure, we investigated differences between these populations in their
98 responses to mechanical and temperature stresses, based on mRNA expression of select target
99 genes as measured by quantitative PCR (qPCR). A suite of genes was selected based on their
100 predicted functions related to gene regulation, immune response, and growth. Given the field
101 performance of these populations, we hypothesized we would see differences in response that
102 could be indicative of underlying genetic population differences. A specific hypothesis is that
103 oysters from Dabob Bay will demonstrate a more pronounced response to stress via changes in
104 gene expression.

105 **Materials and Methods**

106 **Experimental Design**

107 Adult, hatchery produced oysters from three wild source populations (Dabob Bay, Fidalgo Bay,
108 and Oyster Bay grown for 19 months at Clam Bay, WA were used for this experiment. All oysters
109 were held at 8°C for two weeks at the University of Washington prior to the experiment. Oysters
110 from each population (n=8 per population) were subjected to acute temperature stress (submerged
111 in 500mL 38°C sea water for 1 hour), mechanical stress (120g x 5 min; Sorvall T21, ST-H750
112 rotor) or served as controls (maintained at 8°C). After the stress treatments, oysters were returned
113 to 8°C seawater and sampled at 1 hour post stress (n=72). Ctenidia tissue was resected from each
114 individual and stored separately in 500μL RNAzol RT (Molecular Research Center, Inc.), frozen
115 on dry ice. All samples were stored 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, volume was brought up to 1mL with RNAzol RT, vortexed vigorously for 15 seconds, 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, an equal volume of isopropanol added, 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). Treated RNA was verified to be free of gDNA via qPCR using actin primers (see Primer Design section below) known to amplify gDNA.

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 primers (0.25ug) 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. A master mix of 5x Reverse Transcriptase Buffer (1x final concentration; Promega), 10mM each of dNTPs (0.5mM final concentration of each dNTP; Promega), and M-MLV Reverse Transcriptase (50U/reaction) was made and 25.25μL of the mix was added to each sample (final reaction volume 100μL). Samples were incubated at 42°C for 1hr, followed by 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

Primer Design

Primers for qPCR analysis were developed from an *O. lurida* transcriptome (version 3) which can find in the repository associated with this manuscript (Roberts 2017). This transcriptome was annotated using SwissProt and Gene Ontology Databases. Specifically, gene function annotations were based on the protein in the UniProt/SwissProt database that had highest homology with the Olympia oyster sequence (i.e. top Blastp hit).. Gene targets were selected based on annotations related to gene regulation, immune response, and growth (Table 1). 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

159   size 100-400 bp, GC content 55-60%, melt temperatures ~60 ℃ and within 0.5 ℃ of each other,
160   self and 3' complementarity was limited to 4.00 or less with smallest values being selected,
161   primer sequence 19-21 bp in length.

162   Primer binding sites were assessed for the presence of single nucleotide polymorphisms (SNPs)
163   via Sanger sequencing. The majority of primer binding sites did not contain any SNPs. Those that
164   did, had only a single SNP and did not appear to impact qPCR data, as there were no noticeable
165   difference in qPCR efficiencies in individuals having a SNP within a primer binding site for a
166   given target.

167   List of primers can be viewed in Table 2.

168   **Transcriptome Contig Name**

169   **Biological Category**

170   **Uniprot Accession**
171   **Uniprot Entry Name**

172   **Uniprot Annotation**
173   **Function**
174   **Gene Abbr**

175   **BLASTX evalue**
176   comp7220_c0_seq2

177   Gene Regulation
178   Q6DC04
179   CARM1_DANRE
180   Histone-arginine methyltransferase
181   Transfers methyl groups to Histone 3 for chromatin remodeling

182   CARM1
183   0
184   comp23747_c0_seq1

185   Immune Response
186   Q9DD78
187   TLR21_CHICK
188   Toll-like receptor 2 type 1
189   Assists with recognition of foreign pathogens and endogenous materials for consumptions by phagocytes in early stages of inflammation
190   TLR
191   8.00E-29
192   comp25000_c0_seq1
193   Gene Regulation
194   P08991
195   H2AV_STRPU
196   Histone H2A.V
197   One of 5 main Histone Proteins involved in the structure of chromatin and the open reading frame of DNA

198   H2AV
199   5.00E-64
200   comp24065_c0_seq1

201   Immune Response
202   O75594
203   PGRP1_HUMAN
204   Peptidoglycan recognition protein 1
205   Assists with recognition of bacteria in an immune response
206   PGRP
207   2.00E-42
208   comp44273_c0_seq2

209   Immune Response
210   Q8MWP4
211   Q8MWP4_OSTED
212   Heat Shock Protein 70kDa
213   Molecular chaperone and protein preservation in heat response
214   HSP70
215   0
216   comp7183_c0_seq1

217   Growth
218   P12643
219   BMP2_HUMAN
220   Bone morphogenetic protein 2
221   Directs calcification in shell creation
222   BMP2
223   2.00E-93
224   comp10127_c0_seq1

225   Growth
226   P62994
227   GRB2_RAT
228   Growth factor receptor-bound protein 2
229   Assists in signal transduction/cell communication
230   GRB2
231   1.00E-83
232   comp6939_c0_seq1

233   Immune Response
234   P32240
235   PE2R4_MOUSE
236   Prostaglandin E2 receptor EP4 subtype
237   Receptor for Prostaglandin E2 which suppresses inflammation due to injury
238   PGEEP4
239   1.00E-50
240   comp25313_c0_seq1

241   Immune Response
242   Q60803
243   TRAF3_MOUSE
244   Tumor Necrosis Factor receptor-associated factor 3
245   Related to immune response specifically cell death initiation

246   TRAF3
247   3.00E-145
248   comp30443_c0_seq2

249   Q8TA69
250   Q8TA69_CRAGI
251   Actin

252   Cytoskeletal formation.

253   Used as a normalizing gene for qPCR analysis.
254   Actin
255   0

Transcriptome Contig Name	Biological Category	Uniprot Accession	Uniprot Entry Name	Uniprot Annotation	Function	Gene Abbr	BLASTX evalue
comp7220_c0_seq2	Immune Response, Gene Regulation	Q6DC04	CARM1_DANRE	Histone-arginine methyltransferase	Transfers methyl groups to Histone 3 for chromatin remodeling	CARM1	0
comp23747_c0_seq1	Immune Response	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
comp25000_c0_seq1	Immune Response, Gene Regulation	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
comp24065_c0_seq1	Immune Response	O75594	PGRP1_HUMAN	Peptidoglycan recognition protein 1	Assists with recognition of bacteria in an immune response	PGRP	2.00E-42
comp44273_c0_seq2	Immune Response	Q8MWP4	Q8MWP4_OSTED	Heat Shock Protein 70kDa	Molecular chaperone and protein preservation in heat response	HSP70	0
comp7183_c0_seq1	Growth	P12643	BMP2_HUMAN	Bone morphogenetic protein 2	Directs calcification in shell creation	BMP2	2.00E-93
comp10127_c0_seq1	Gene Regulation	P62994	GRB2_RAT	Growth factor receptor-bound protein 2	Assists in signal transduction/cell communication	GRB2	1.00E-83
comp6939_c0_seq1	Immune Response	P32240	PE2R4_MOUSE	Prostaglandin E2 receptor EP4 subtype	Receptor for Prostaglandin E2 which suppresses inflammation due to injury	PGEEP4	1.00E-50
comp25313_c0_seq1	Immune Response	Q60803	TRAF3_MOUSE	Tumor Necrosis Factor receptor-associated factor 3	Related to immune response specifically cell death initiation	TRAF3	3.00E-145
comp30443_c0_seq2	Growth	Q8TA69	Q8TA69_CRAGI	Actin	Cytoskeletal formation. Used as a normalizing gene for qPCR analysis.	Actin	0

256 **Table 1.** Table of genes of interest. The table lists the source transcriptome contigs (annotated by
257 BLASTx against the Uniprot database), as well as the biological categorization, the Uniprot
258 Accession, Uniprot Entry Name, Uniprot Annotation, a brief description of the proteins'
259 functions, and the BLASTx e-values.

260 **Gene Abbreviation**

261 **FWD**

262 **REV**

263 CARM1

264 TGGTTATCAACAGCCCCGAC

265 GTTGTTGACCCCAGGAGGAG

266 TLR

267 ACAAAGATTCCACCCGGCAA

268 ACACCAACGACAGGAAGTGG

269 H2AV

270 TGCTTTCTGTGTGCCCTTCT

271 TATCACACCCCGTCACTTGC

272 PGRP

273 GAGACTTCACCTCGCACCAA

274 AACTGGTTTGCCCGACATCA

275 HSP70

276 TTGTCGCCATTTTCCTCGCT

277 GTTCCGATTTGTTCCGTGCC

278 BMP2

279 TGAAGGAACGACCAAAGCCA

280 TCCGGTTGAAGAACCTCGTG

281 GRB2

282 AACTTTGTCCACCCAGACGG

283 CCAGTTGCAGTCCACTTCCT

284 PGEEP4

285 ACAGCGACGGACGATTTTCT

286 ATGGCAGACGTTACCCAACA

287 TRAF3

288 AGCAGGGCATCAAACTCTCC

289 ACAAGTCGCACTGGCTACAA

290 Actin

291 GACCAGCCAAATCCAGACGA

292 CGGTCGTACCACTGGTATCG

Gene Abbreviation	FWD	REV
CARM1	TGGTTATCAACAGCCCCGAC	GTTGTTGACCCCAGGAGGAG
TLR	ACAAAGATTCCACCCGGCAA	ACACCAACGACAGGAAGTGG
H2AV	TGCTTTCTGTGTGCCCTTCT	TATCACACCCCGTCACTTGC
PGRP	GAGACTTCACCTCGCACCAA	AACTGGTTTGCCCGACATCA
HSP70	TTGTCGCCATTTTCCTCGCT	GTTCCGATTTGTTCCGTGCC
BMP2	TGAAGGAACGACCAAAGCCA	TCCGGTTGAAGAACCTCGTG
GRB2	AACTTTGTCCACCCAGACGG	CCAGTTGCAGTCCACTTCCT
PGEEP4	ACAGCGACGGACGATTTTCT	ATGGCAGACGTTACCCAACA
TRAF3	AGCAGGGCATCAAACTCTCC	ACAAGTCGCACTGGCTACAA
Actin	GACCAGCCAAATCCAGACGA	CGGTCGTACCACTGGTATCG

293   **Table 2.** Table of qPCR Primers for genes of interest. Includes the Uniprot Entry Name, the Gene
294   Abbreviation used throughout this manuscript, and the forward (FWD) and reverse (REV) primer
295   sequences. Full sequences utilized for primer creation are available. (Heare and Roberts 2015).

296   **Quantitative PCR**

297   Quantitative PCR reactions were carried out using Ssofast Evagreen Supermix (BioRad, USA).
298   Forward and reverse primers (Integrated DNA Technologies) were used at a final concentration
299   of 0.25uM each. Sample cDNA was diluted (1:9) with molecular-grade water. Nine microliters of
300   diluted cDNA was used as template. Reaction volumes were 20µL and were run in low-profile,
301   non-skirted, white qPCR plates (USA Scientific) with optically clear lids (USA Scientific) in a
302   BioRad CFX Real Time Thermocycler (BioRad, USA) and DNA Engine Opticon 2 System
303   (BioRad, USA). Cycling conditions were: one cycle of 95°C for 10 min; 40 cycles of 95°C for 30
304   sec, 60°C for 1 min, 72°C for 30 sec. Two qPCR replicates were run for each sample, for each
305   primer set.

306   **Statistical Analysis**

307   To calculate relative expression levels for each gene, cycle quantity (Cq) or cycle threshold (Ct)
308   values were calculated using BioRad CFX Manager 3.1 (version 3.1.1517.0823, Windows 8.1)
309   and Opticon Manager 3 (Windows 8.1), respectively. This was accomplished by subtracting
310   global minimum fluorescence from samples and determining the point in the cycle which
311   amplification reached exponential amplification phase. Default settings were accepted for each
312   program to ensure reproducibility. The BioRad CFX Manager used default settings of single
313   threshold for Cq determination and baseline subtracted curved fit for each run. The Opticon
314   Manager used default settings of subtract baseline via global minimum, which estimated the
315   threshold as being between 0.019 and 0.028. Gene expression values were determined as
316   normalized mRNA levels using the following equation (ΔCt): $2^{-\Delta Ct}$; where ΔCt is: (target Ct –
317   actin Ct) (Schmittgen and Livak, 2008). Actin expression levels were determined to be consistent
318   across all samples and served as an internal amplification control to use for expression
319   normalization. Data from ΔCt did not exhibit normal distributions, so were log transformed
320   (logΔCt), to establish normal data distributions for statistical analysis. Two-way analysis of
321   variance (ANOVA) followed by Tukey's Honestly Significant Difference post hoc test (*base*, R
322   Core Team, 2014) were performed on logΔCt for each target ($p<0.05$).

323   **Results**

324   **Gene Expression Analysis**

325   Without considering separate populations, acute heat shock resulted in statistically significant
326   increases in expression of coactivator-associated arginine methyltransferase 1 (CARM1) (n=24
327   oysters per treatment, ANOVA, df=2, Tukey's HSD p=0.00007) (Figure 1) and Histone 2AV
328   (H2AV) (n=24 oysters per treatment, ANOVA, df=2, Tukey's HSD p=0.001)(Figure 2). A
329   statistically significant increase in expression of tumor necrosis factor receptor-associated factor
330   3 (TRAF3) (n=24 oysters per treatment, ANOVA, df=2, Tukey's HSD p=0.008) (Figure 3)
331   occurred upon exposure to mechanical stress.

332   There was a clear difference in response to mechanical stress in oysters from Oyster Bay as
333   compared to oysters from Dabob and Fidalgo Bays. Specifically, upon heat shock, H2AV
334   expression in oysters from Oyster Bay increased (n=8 oysters per population, ANOVA, df=4,
335   Tukey's HSD = 0.05) (Figure 2) when compared to the control. When exposed to mechanical
336   stress, bone morphogenic protein 2 (BMP2) (n=8 oysters per population, ANOVA, df=4, Tukey's
337   HSD p=0.03) (Figure 4) and growth-factor receptor bound protein 2 (GRB2) (n=8 oysters per
338   population, ANOVA, df=4, Tukey's HSD p=0.03)(Figure 5) expression was decreased in the
339   Oyster Bay population, whereas there was no significant differences in responses in the other
340   populations. Additionally, significant interactions were identified between population and
341   treatment in both BMP2 and GRB2 (p<0.05).

342   There was no statistical difference in expression in Peptidoglycan recognition protein 1 (PGRP),
343   toll-like receptor 2 type 1 (TLR), and prostaglandin E2 receptor EP4 subtype (PGEEP4) (Figures
344   6, 7, & 8, respectively) within any comparison. Heat shock protein 70 gene expression was
345   significantly different between temperature and mechanical stress (n=24 oysters per treatment,
346   ANOVA, df=4, Tukey's HSD p=0.006) (Figure 9).

347   **Discussion**

348   **Response to Temperature Stress**

349   The response of *Ostrea lurida* to acute heat stress appears to include an alteration in gene
350   regulatory activity and the innate immune response, as indicated by significant increases of
351   H2AV (Figure 2) and CARM1 (Figure 2) gene expression one hour post-temperature stress.

352   Histone 2AV, H2AV, is a variant of the histone H2A protein. This variant has been shown to act as
353   a transcription promoter agent as well as assist with heterochromatin formation. Truebano et al.
354   (2010) characterized changes in transcription in Antarctic clams, *L. elliptica*, and found that an
355   H2A variant was significantly upregulated under heat stress conditions (3 ℃ for 12 hours). In
356   addition to involvement in the heat stress response, histone H2A has been shown to exhibit
357   antimicrobial properties in three invertebrates: two marine invertebrates (Pacific white shrimp
358   and scallops; Patat et al., 2004, Li et al., 2007a), as well as in a freshwater shrimp (Arockiaraj et
359   al., 2013). In *D. melanogaster,* H2Av is phosphorylated in response to DNA damage (Madigan et
360   al., 2002) to inhibit apoptosis, suggesting an additional role in in cellular survival.

361   Coactivator-associated arginine methyltransferase 1, CARM1, is involved transcriptional
362   activation via methylation of histones (Chen et al., 1999, Lee et al., 2005). This in turn affects the
363   ability of transcription factors to bind and transcription to proceed. It is possible that increases in
364   CARM1 expression could indicate that overall gene regulatory activity is increased in response to
365   temperature stress. Our results are similar to those of Wang et al. (2011) where researchers
366   described an increase in expression of Histone-arginine methyltransferase in the sea cucumber,
367   *Apostichus japonicus*, after experiencing 25℃ temperatures for 7 days. The authors suggested
368   that this was due to an induced dormancy and lower metabolic rate to provide resources for stress
369   resilience. CARM1 is also a component of the cellular immune response, as it has been identified
370   as a regulator of NF-kB (Covic et al., 2005). Thus another explanation is that acute heat could
371   possible impact the immune response, likely in a negative manner. Future work, that would be

372 relevant to restoration activities, should increase the number of stressors examined in oyster to
373 include pathogens.

374 Increases in HSPs are often observed in response to stress, but this study only found a significant
375 difference of mRNA expression of HSP70 in the Oyster Bay population between mechanical and
376 heat stresses (Figure 9). Brown et al. (2004) found the maximum HSP expression in *O. lurida*
377 occurred 24-48 hours post exposure to 39℃. The absence of a strong response of HSP70, relative
378 to the control group, could be related to temporal changes in expression or an isoform-specific
379 response, as there are many genes in this gene family, particularly in oysters (Clegg et al., 1998;
380 Piano et al., 2005). Mediterranean mussels, *Mytilus galloprovincialis*, have shown different
381 isoforms of heat shock proteins and cognates that have differential expression patterns caused by
382 heat, mercury exposure, and chromium exposures stressors suggesting that the isoforms have
383 slightly different functions (Franzellitti and Fabbri, 2005). Additionally, there are members of the
384 HSP70 gene family that are constitutively expressed and do not exhibit increases in mRNA in
385 response to heat stress (Sorger & Pelham, 1987; Somji et al., 1999). Without a sequenced genome
386 for *Ostrea lurida*, combined with utilizing an incomplete transcriptome, it is difficult to ascertain
387 how many isoforms might exist, as well as the number of alternatively spliced products. Upon
388 addition of new genomic resources the entire family of molecular chaperones could be examined
389 and compared across populations.

390 **Response to Mechanical Stress**

391 Mechanical stress increased expression of inflammation-related target genes. In all populations,
392 there was a significant increase in immune system-related responses seen via the expression of
393 tumor necrosis factor receptor-associated factor 3, TRAF3 (Figure 3), which is involved in
394 internal tissue damage recognition and apoptosis. The main function of TRAF3 is to assist in cell
395 death initiation caused by stress conditions within tissues (Arch, Gedrich, and Thompson, 1998).
396 Upregulation in relation to mechanical stress could be akin to inflammation occurring due to
397 edema from the mechanical stress and used to remove damaged cells as suggested by Roberts et
398 al. (2012) when *C. virginica* were exposed to mechanical stress. Significant differences in
399 expression of other immune system targets such as PGRP, TLR, and PGEEP4 were not
400 detected(Figures 6, 7, & 8, respectively), but other studies have found that the time scale for
401 expression may vary (Meistertzheim et al.,2007; Farcy et al., 2009).

402 **Population differences**

403 We suspected that the Dabob Bay population would have demonstrated a more pronounced
404 response to stress as this population is subjected to greater environmental fluctuations with
405 respect to salinity and temperature (Heare et al 2015).Contrary to our hypothesis, oysters from
406 Oyster Bay were the only population that exhibited a difference in gene expression in response to
407 mechanical or heat stress. Oysters from Oyster Bay parents showed an increase in H2AV
408 expression during heat stress as compared to control (Figure 2), a decrease in BMP2 and GRB2
409 upon mechanical stress (Figures 4 & 5, respectively), and differences in HSP70 expression
410 between heat and mechanical stresses (Figure 9). Given the putative function of H2AV in

411   transcriptional regulation (Table 1), the increase in expression could be indicative of the role of
412   this protein in controlling the molecular response to stress . Bone morphogenic protein 2, BMP2,
413   and growth-factor receptor bound protein 2, GRB2, were significantly decreased in expression
414   which could be indicative of growth inhibition. Both genes are related to growth and
415   development of tissues, with BMP2 being a pre-cursor to osteoblastic cells that produce shell
416   (Pereira Mouries et al., 2002) and GRB2 is used for signal transduction between cells during
417   growth phases (Oda et al., 2005). By down-regulating these targets, this may be an effort to
418   reduce energetically costly processes in favor of processes that promote survival during stress
419   events. Organisms faced with stress are often required to reallocate energy resources to
420   homeostasis-related functions in an effort to improve long-term survival of the species (Sokolova
421   et al., 2012). This change in expression coupled with the up-regulation of H2AV (Figure 2) is in
422   accord with the idea of shifting priorities for stress resilience.

423   Interactions were identified between population and treatment for both BMP2 and GRB2.
424   Differences between gene expression in control and mechanical stress in the Oyster Bay
425   population are driving this interaction for both genes. Although statistical interactions of this
426   nature are difficult to interpret, it could be related to fact the Oyster Bay population is from a
427   relatively "low-stress" environment (i.e. abundant food and less-pronounced temperature
428   fluctuations).

429   CONCLUSIONS

430   The gene expression pattern differences observed here with oysters from Oyster Bay coupled
431   with corresponding field-based observation that this population has the greatest reproductive
432   activity (Heare et al., 2015), could indicate this population has a greater ability to effectively
433   respond to stress. Another way to consider this is that the Oyster Bay population has a relatively
434   higher degree of phenotypic plasticity, or more specifically, an elevated rate of phenotypic change
435   (Angilletta et al., 2003). The gene expression data indicates a clear population-level stress
436   response, and lack of differential response in other populations that suggests shifts in energy
437   balance. Some possible explanations for this relatively rapid response include a more sensitive
438   cell-signaling system (ie cytokines) or a more robust transcription initiation process. Yao and
439   Somero (2012) observed higher heat stress tolerance in *M. galloprovincialis* than *M. californius*
440   likely due to their ability to maintain cell signaling through the production of phosphor-p38-
441   MAPK kinases, which may be how the Oyster Bay population is able to quickly respond to
442   stress. This ability to quickly respond to stress may be due to increased fitness in Oyster Bay,
443   however more research is needed to identify the link between gene expression and performance.
444   Based on earlier field work, this could be directly linked to increased larval production, and
445   processes allocating limited resources into reproduction (Heare et al., 2015). This trait could
446   certainly be perceived as advantageous for restoration purposes. Caution should be used in using
447   non-local stocks when structure exists, as it is possible to have supplemented oysters out-compete
448   the native population or to create hybrids that are ultimately less fit than the native counter parts
449   (Camara and Vadopalas, 2009). Both such phenomena decrease overall genetic diversity leaving
450   the remaining population to be less robust for future challenges and possibly leading to local
451   extirpation.

452 Another interpretation of gene expression patterns in the Oyster Bay population is that the
453 differences observed upon stress exposure are not indicative of an effective response that has
454 been selected for, but rather indicative of plasticity. In other words, the change in gene expression
455 upon stress is representative of a phenotype that is tolerable to a wide range of pressure. At one
456 level the ability to achieve a number of phenotypes with a given genotype could be advantageous,
457 particularly in a rapidly the changing environment. There is a paradox in the fact that too much
458 plasticity negates the ability of natural selection to function. Populations with high phenotypic
459 plasticity become deprived of negative selection and thus are often able to survive in rapidly
460 changing environments as long as the changes are consistent and somewhat predictable.
461 However, with this increased adaptive ability, genetic diversity and adaptation become limited
462 within a population that may be unable to properly respond to novel challenges in the future
463 (Crispo, 2008). Alternatively, the Baldwin effect may enhance longterm genetic diversity by
464 allowing species to colonize novel habitats and, with phenotypic plasticity, and eventually
465 genetically diverge from the source population through induced genetic adaptations (Crispo,
466 2007). For longterm restoration of *O. lurida* populations in Puget Sound, understanding the
467 genetic differences and phenotypic plasticity of individual populations will help determine proper
468 supplementation procedures for existing and historic habitats.

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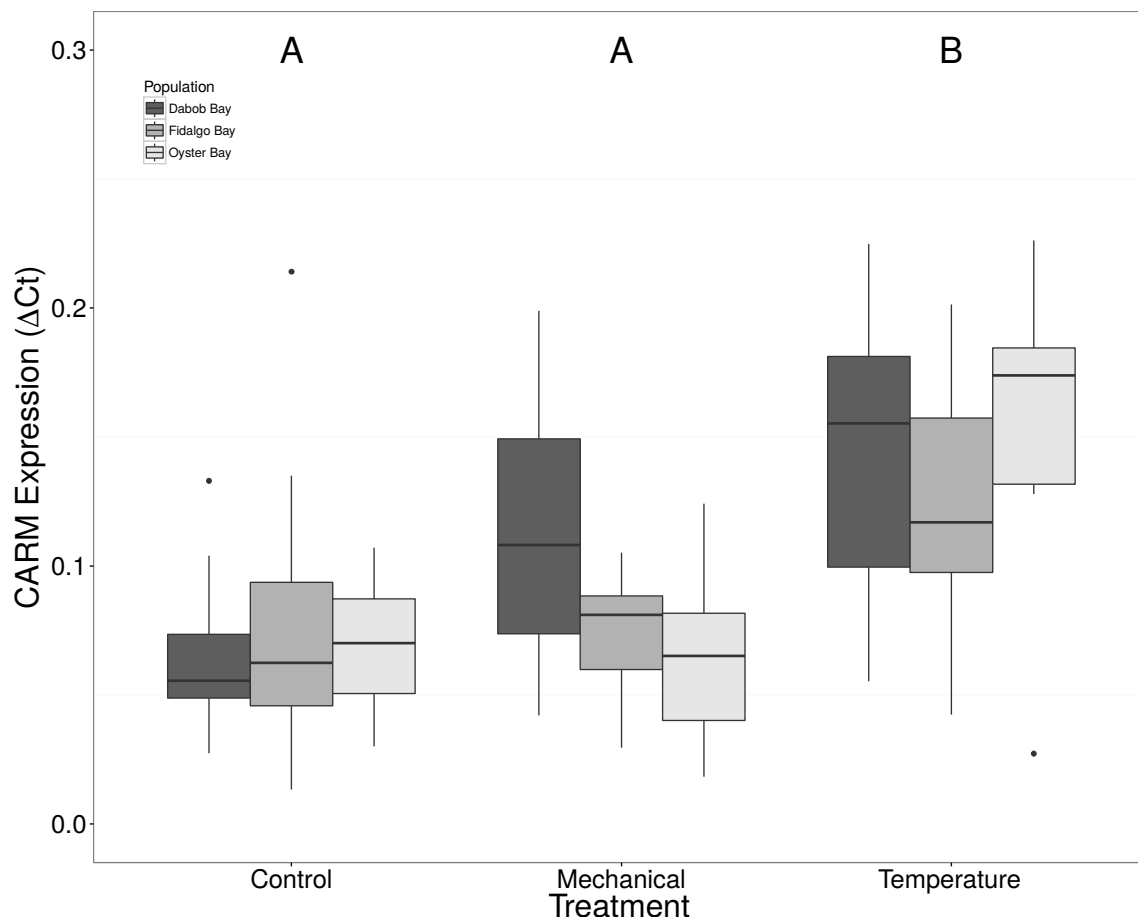
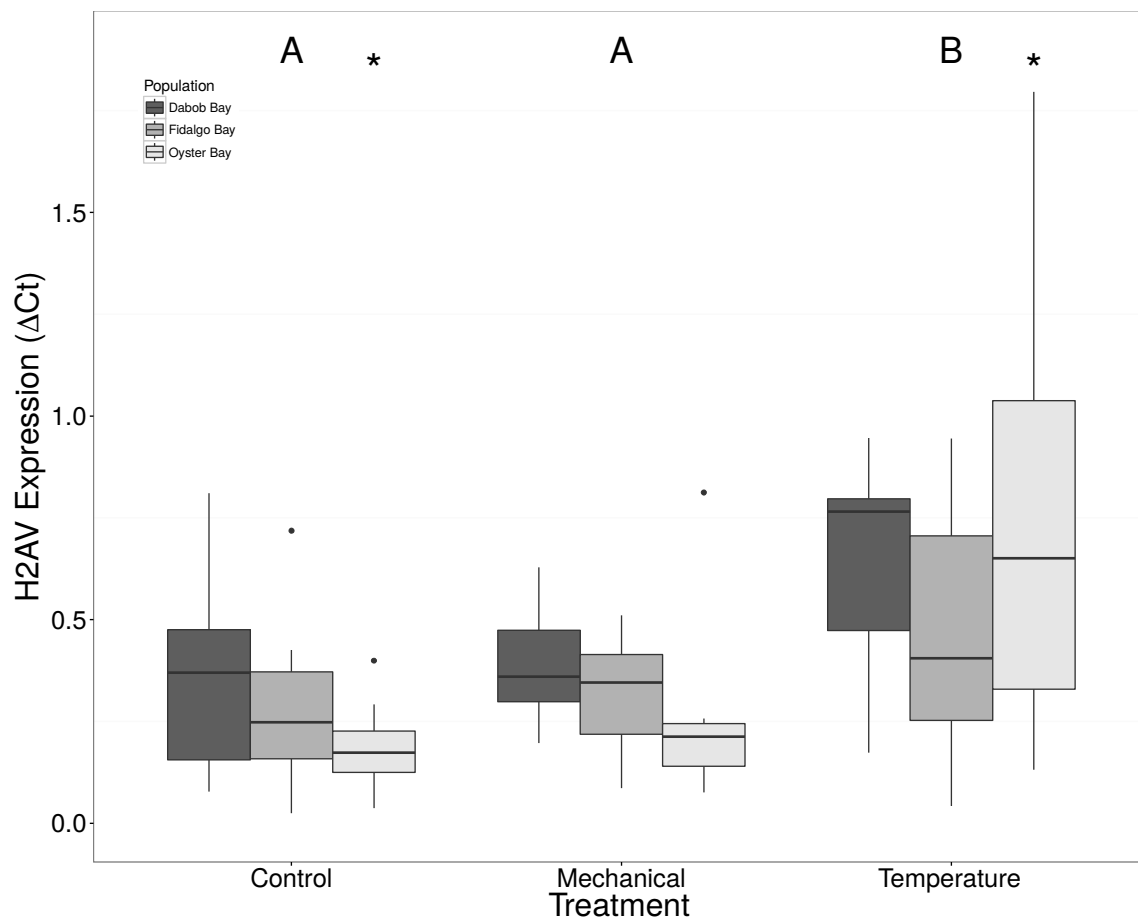
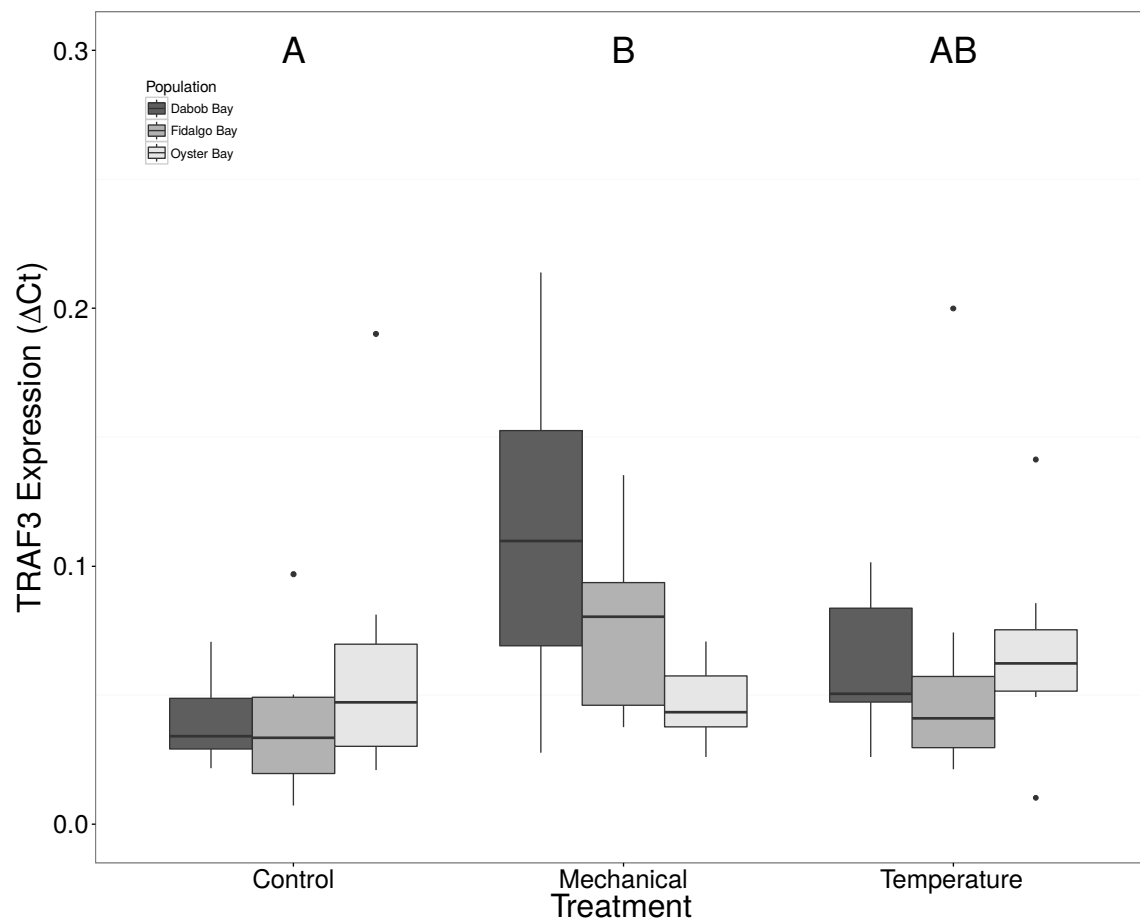
614   **Figures**

615   Figure 1. Expression of CARM1 mRNA. Median ΔCt indicated by line in middle of box plot.
616   Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate
617   outside values. Capital letters indicate significant differences (p<0.05) between overall treatment
618   groups (n=24 animals per treatment). No statistical differences (p>0.05) were observed between
619   populations (n=24 animals per population), nor within a given population (n = 8 animals per
620   treatment).

621   Figure 2. Expression of H2AV mRNA. Median ΔCt indicated by line in middle of box plot.
622   Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate
623   outside values. Asterisks indicate significant differences (p<0.05) between treatments within a
624   population (n=8 animals per treatment). Capital letters indicate significant differences (p<0.05)
625   between overall treatment groups (n=24 animals per treatment). No statistical differences
626   (p>0.05) were observed between populations (n=24 animals per population).

627   Figure 3. Expression of TRAF3 mRNA. Median ΔCt indicated by line in middle of box plot.
628   Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate
629   outside values. Capital letters indicate significant differences (p<0.05) between overall treatment
630   groups (n=24 animals per treatment). No statistical differences (p>0.05) were observed between
631   populations (n=24 animals per population), nor within a given population (n = 8 animals per
632   treatment).

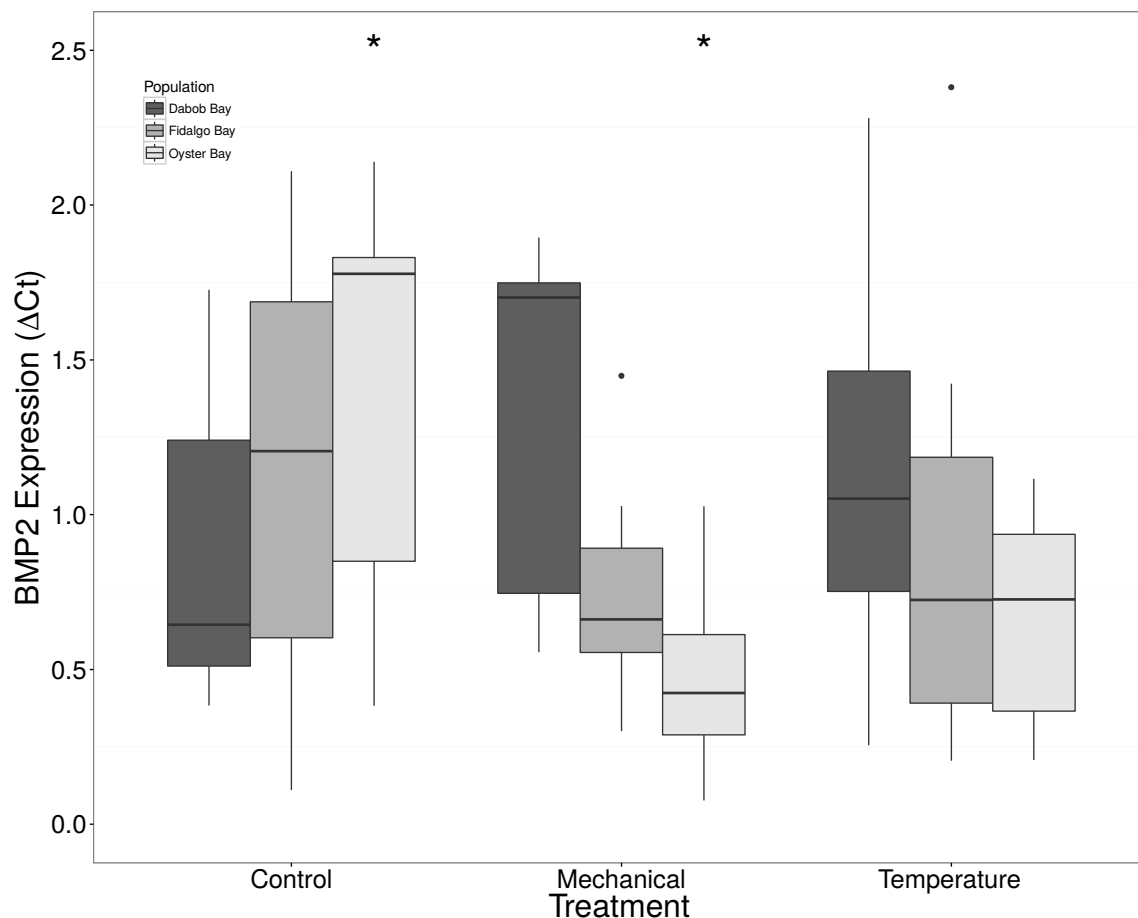
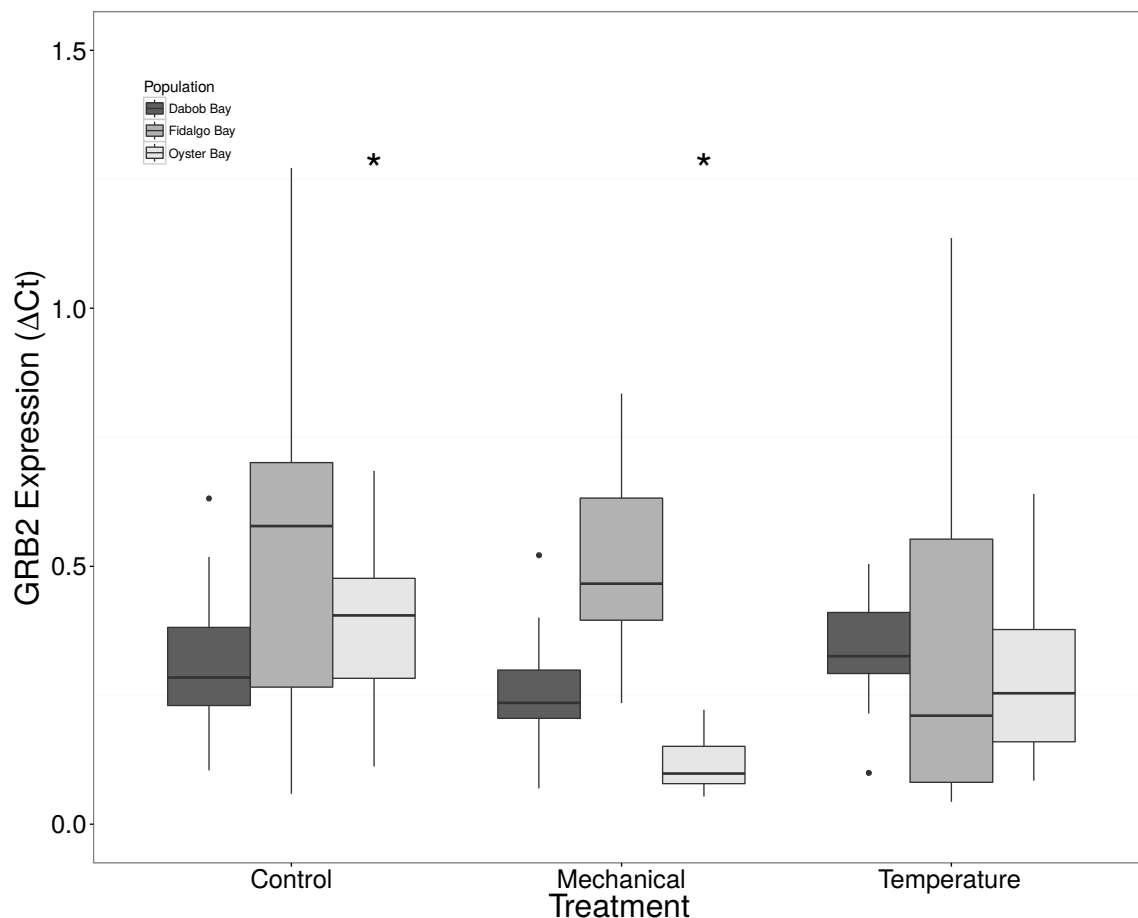
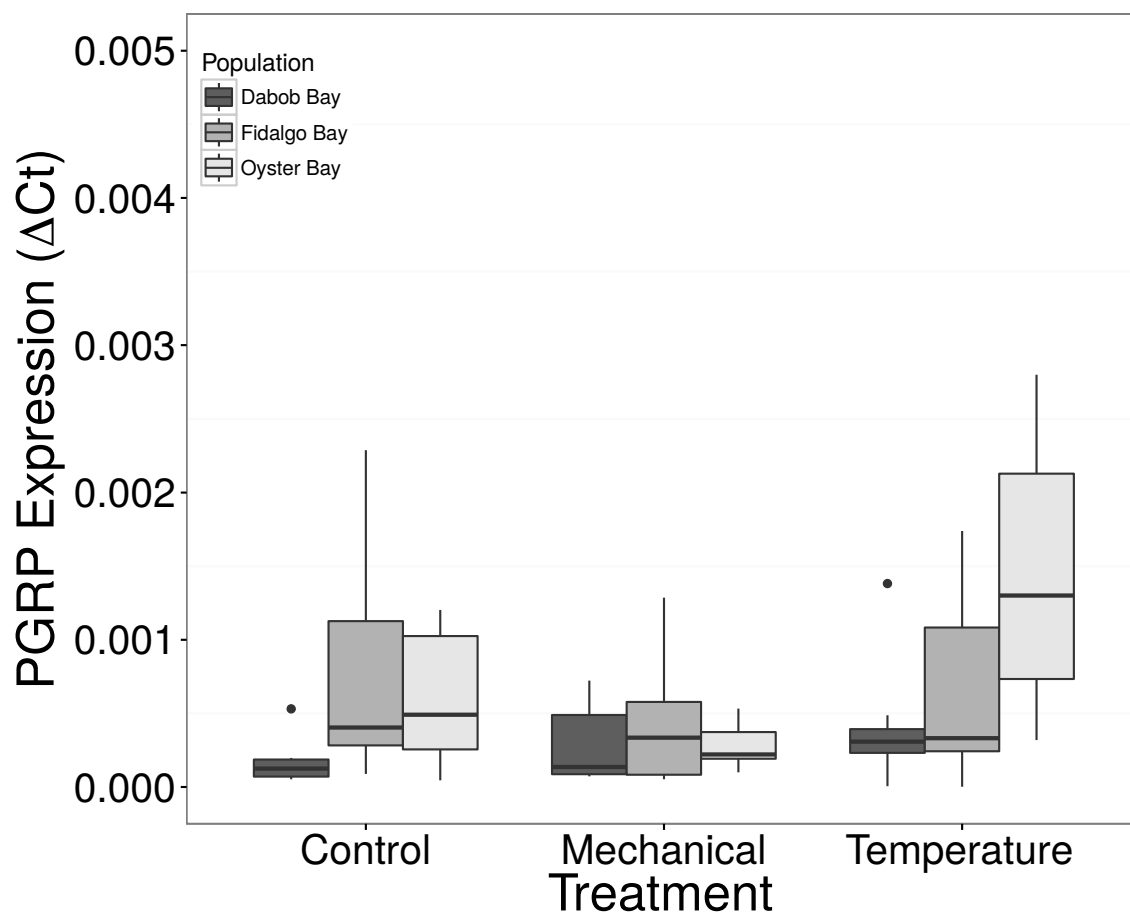
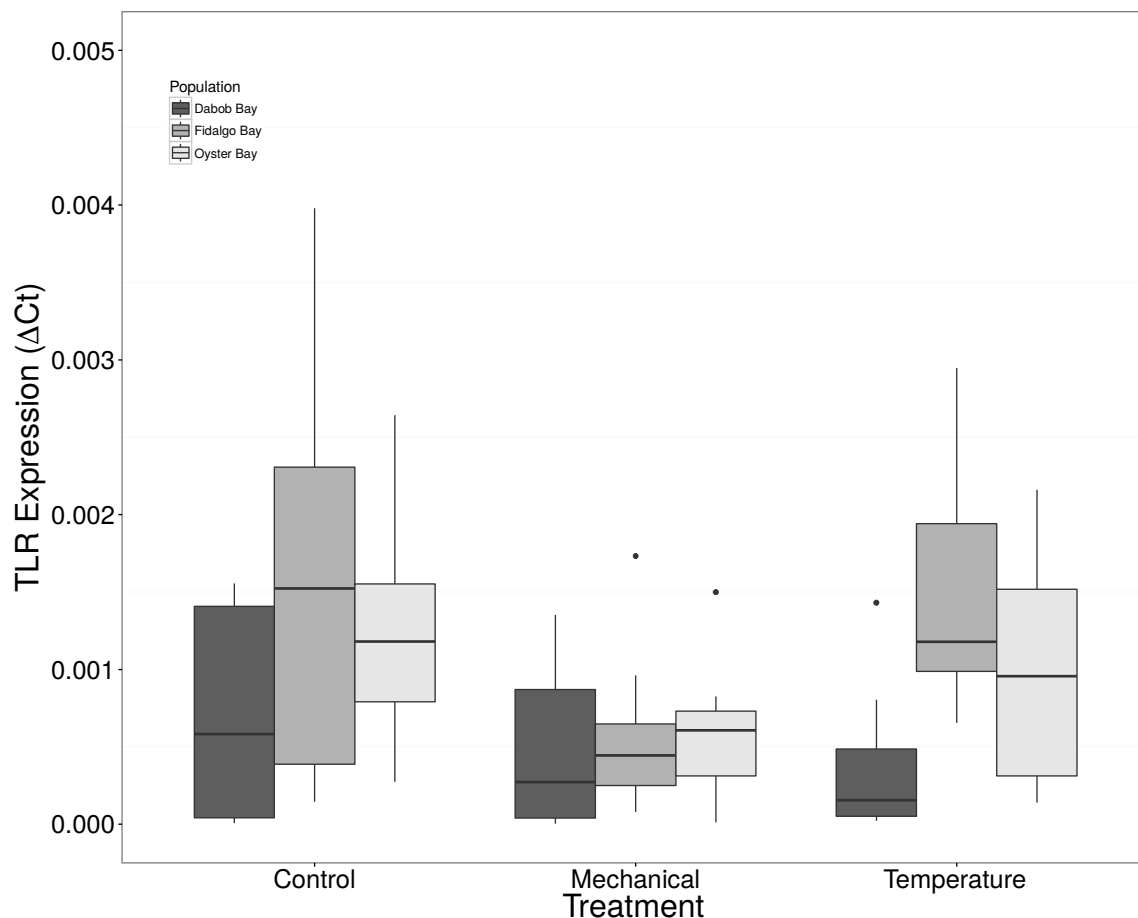
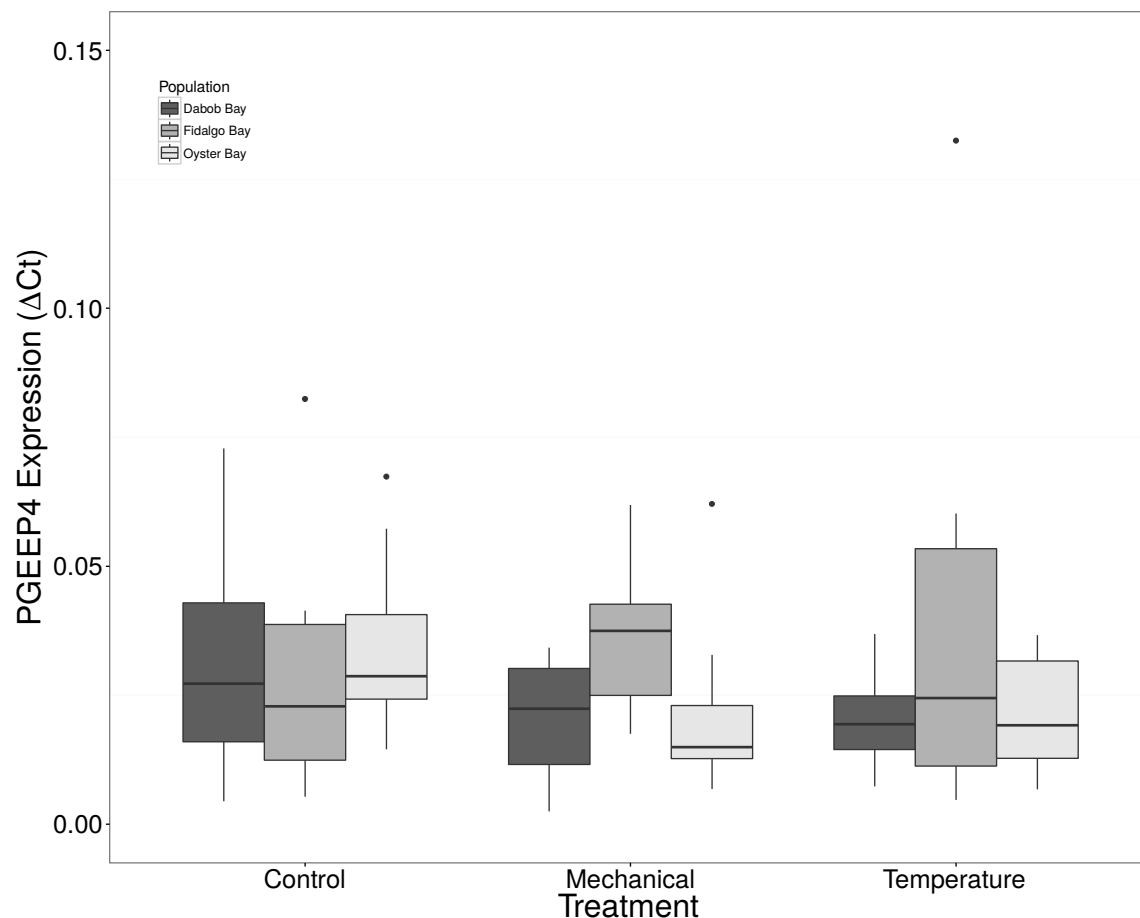
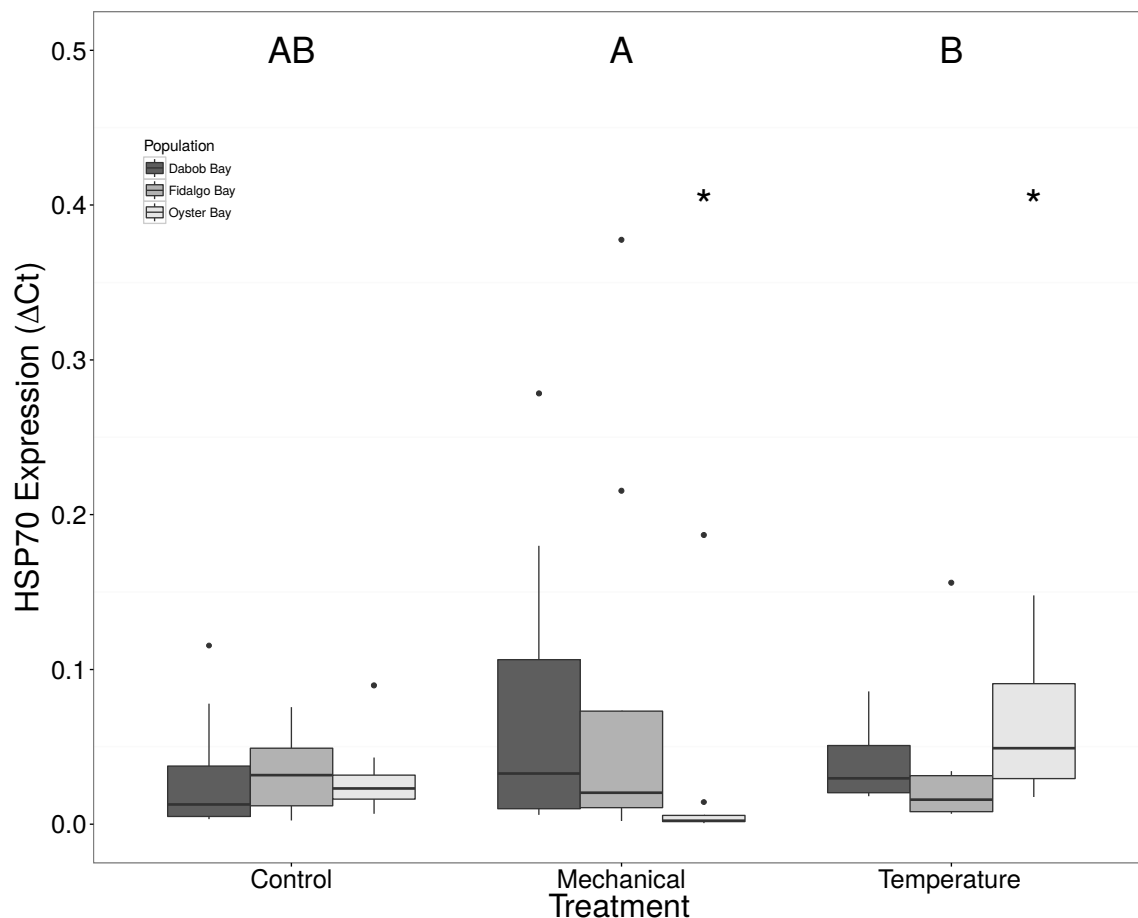



Figure 4. Expression of BMP2 mRNA. Median ΔCt indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. Asterisks indicate significant differences (p<0.05) between treatments within a population (n=8 animals per treatment). No statistical differences (p>0.05) were observed between populations (n=24 animals per population), nor between treatments (n = 24 animals per treatment).

639   Figure 5. Expression of GRB2 mRNA. Median ΔCt indicated by line in middle of box plot.
640   Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate
641   outside values. Asterisks indicate significant differences (p<0.05) between treatments within a
642   population (n=8 animals per treatment). No statistical differences (p>0.05) were observed
643   between populations (n=24 animals per population), nor between treatments (n = 24 animals per
644   treatment).

645 Figure 6. Expression of PGRP mRNA. No statistical difference observed between treatments, nor
646 between populations. Median ΔCt indicated by line in middle of box plot. Shaded boxes are 2nd
647 and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. No
648 statistical differences ($p > 0.05$) were observed within populations between treatments (n=8
649 animals per treatment), between populations (n=24 animals per population), or between
650 treatments (n = 24 animals per treatment).

651   Figure 7. Expression of TLR mRNA. No statistical difference observed between treatments, nor
652   between populations. Median ΔCt indicated by line in middle of box plot. Shaded boxes are 2nd
653   and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. No
654   statistical differences ($p > 0.05$) were observed within populations between treatments (n=8
655   animals per treatment), between populations (n=24 animals per population), or between
656   treatments (n = 24 animals per treatment).

657   Figure 8. Expression of PGEEP4 mRNA. No statistical difference observed between treatments,
658   nor between populations. Median ΔCt indicated by line in middle of box plot. Shaded boxes are
659   2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. No
660   statistical differences ($p > 0.05$) were observed within populations between treatments (n=8
661   animals per treatment), between populations (n=24 animals per population), or between
662   treatments (n = 24 animals per treatment).

663   Figure 9. Expression of HSP70 mRNA. Median ΔCt indicated by line in middle of box plot.
664   Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate
665   outside values. Asterisks indicate significant differences (p<0.05) between treatments within a
666   population (n=8 animals per population). Capital letters indicate significant differences (p<0.05)
667   between overall treatment groups (n=24 animals per treatment). No statistical differences
668   (p>0.05) were observed between populations (n=24 animals per population).