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

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19Abstract

20Olympia oysters are the only oyster native to the west coast of North America. The population
21within Puget Sound, WA has been decreasing significantly since the early 1900's. Current
22restoration efforts are focused on supplementing local populations with hatchery bred oysters. A
23recent study by Heare et al. (2015) has shown differences in stress response in oysters from
24different locations in Puget Sound however, nothing is known about the underlying mechanisms
25associated with these observed differences. In this study, expression of genes associated with
26growth, immune function, and gene regulatory activity in oysters from Oyster Bay, Dabob Bay,
27and Fidalgo Bay were characterized following temperature and mechanical stress. We found that
28heat stress and mechanical stress significantly changed expression in molecular regulatory
29activity and immune response, respectively. We also found that oysters from Oyster Bay had the
30most dramatic response to stress at the gene expression level. These data provide important
31baseline information on the physiological response of *Ostrea lurida* to stress and provide clues to
32underlying performance differences in the three populations examined.

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37Introduction

38Olympia oysters, *Ostrea lurida*, are the only native oyster species on the west coast of North
39America. The species inhabits bays and estuaries within Puget Sound, WA. *Ostrea lurida* is
40typically smaller than the introduced Pacific oyster, *Crassostrea gigas*, with adults attaining an
41average size between 40 – 60 mm (Hopkins, 1937; Baker, 1995). As protandric hermaphrodites,
42Olympia oysters usually spawn as both male and female within the first year (Coe, 1932;
43Hopkins, 1937; Baker, 1995). Unlike *C. gigas*, *O. lurida* does not release its eggs into the water
44column. Instead females collect planktonic sperm balls and larvae are brooded for approximately
45two weeks before being released into the water column. The adults are sessile and are typically
46moved via predator interactions or wave action. Colonizing lower intertidal habitats, *O. lurida*
47typically can be found in the inner portions of bays or estuaries where dynamic conditions can
48shape the phenotypes of local populations (Baker, 1995; White et al., 2009).

49Loss of habitat due to invasive species, overharvest, and pollution have greatly reduced the
50native Olympia oyster population. Although restoration efforts are underway, basic research is
51needed to understand how this species interacts with its environment and responds to stress.
52Freshwater influx, tidal exchange, food availability, shifts in water temperature, and physical
53stresses from water flow and predation are examples of a myriad of stressors which affect long
54term survival of *O. lurida* populations (Hopkins, 1937; Baker, 1995).

55Thermal stress has been widely studied in mollusks, especially bivalves. *Ostrea lurida* has a
56temperature tolerance range between 5°C – 39°C (Hopkins, 1937; Brown et al., 2004). It is
57suspected that mass summer mortalities of *C. gigas* may be linked to the effects of heat stress

58during spawning events (Li et al., 2007b). The California mussel, *Mytilus californianus*, has been
59found to divert resources to physiological defense during thermal stress events (Petes, Menge,
60and Harris, 2008; Fitzgerald-Dehoog, Browning and Allen, 2012). Expression of homeostasis-
61related genes, such as HSP70, glutamine synthetase, and citrate synthase in *C. gigas* has been
62shown to fluctuate under prolonged heat stress at 25°C for 24 days (Meistertzheim et al. 2007).
63Temperature stress has been shown to induce a variety of up and down regulation of genes to
64maintain homeostasis (Tomanek, 2010). In oysters, there has been a significant amount of work
65examining the change in heat shock protein (HSP) family gene expression. Seasonal variation of
66HSPs and heat shock cognates (HSCs) levels have been characterized in response to ambient
67temperatures for *C. gigas* (Hamdoun, Cheney, and Cherr, 2003; Farcy et al., 2009). Additionally,
68induction of HSP70 and HSP69 in *Ostrea edulis* at temperatures greater than 38° C have been
69reported (Piano et al., 2005).

70The response of bivalves to mechanical stress has also received considerable attention. One
71reason for this is that researchers have shown mechanical stress elicits a classical stress response,
72providing a simple method to allow for investigation of fundamental physiological stress
73responses. Additionally, most oyster restoration and aquaculture practices do involve handling
74and movement which would be a form of mechanical stress. Mechanical stress in oysters has
75been shown to increase catecholamines present in hemolymph (Qu et al., 2009; Lacoste et al.,
762001c). Upon mechanical stress, researchers have found increases in adrenocorticotrophic
77hormone (ACTH), a hormone that induces production of noradrenaline and dopamine (Lacoste et
78al. 2001a; Lacoste et al., 2001b; Lacoste et al., 2001c). Mechanical stress has also been shown to
79activate inflammation factors that are also observed during bacterial challenges (Lacoste et al.,
802001c; Lacoste et al., 2001d; Aladaileh, Nair, and Raftos, 2008; Roberts et al., 2011). Studies in

81 Pearl oysters (*Imbricata pinctada*) have found significant decreases in phagocytosis and
 82 phenoloxidase activity due to mechanical stress (Kuchel, Raftos, and Nair, 2010;).

83 Here we set out to examine the effects of temperature and mechanical stress on *Ostrea lurida*, by
 84 comparing differences in gene expression among three local populations (Heare et al., 2015).
 85 Each of the three populations comes from distinct bays within Puget Sound, WA: Fidalgo Bay,
 86 Dabob Bay, and Oyster Bay (Figure 1). Fidalgo Bay, the furthest northern population
 87 (48°28'31.1"N 122°34'48.6"W), is directly fed from the Salish Sea and the Strait of Juan de Fuca,
 88 and has the coldest average year-round temperatures of the three locations. Typically, this
 89 population does not experience strong fluctuations in temperatures due to the fact that it resides
 90 in the lower part of the intertidal area and is submerged for most of the time. Olympia oysters
 91 from Fidalgo Bay experience significant growth when placed in warmer habitats, but otherwise
 92 lack other observable phenotypes (Heare et al., 2015). Dabob Bay (47°49'27.4"N
 93 122°48'37.9"W) is a large bay at the northern most portion of Hood Canal with the population of
 94 Olympia oysters residing near the inner-most portions of the bay (e.g. Tarboo Creek). This area
 95 experiences extreme temperature fluctuations throughout the year and this population of
 96 *O. lurida* is often partially, or completely, exposed during low tide events. During tidal changes,
 97 temperatures can be as high as 29°C during summer or as low as -3°C during winter (Heare et al.,
 98 2015). Oysters from Dabob Bay have been shown to experience high survival when faced with
 99 temperature challenges, possibly due to adaptive structure of the local population (Heare et al.,
 100 2015). Oyster Bay (47°06'21.2"N 123°04'32.8"W) is the southernmost bay which sustains a
 101 healthy population of *O. lurida*. The conditions here are, on average, the warmest of the three
 102 locations throughout the year. The bay has extensive food resources and oysters appear to

103allocate more energy resources into reproductive activity compared to the other populations,
104based on our prior field studies (Heare et al. 2015).

105For long-term restoration of *O. lurida* populations in Puget Sound, understanding the phenotypic
106plasticity of individual populations will help determine proper supplementation procedures for
107existing and historic habitats. To this end, and to attempt reveal relationship of gene expression
108response with stress exposure, we investigated differences between these populations in their
109responses to mechanical and temperature stresses, based on mRNA expression of select target
110genes as measured by quantitative PCR (qPCR). A suite of genes was selected based on their
111predicted functions related to gene regulation, immune response, and growth. Given the field
112performance of these populations, we hypothesized we would see differences in response that
113could be indicative of underlying genetic population differences. A specific hypothesis is that
114oysters from Dabob Bay will demonstrate a more pronounced response to stress via changes in
115gene expression.

116

117Materials and Methods

118Experimental Design

119Adult, hatchery produced oysters from three wild source populations (Dabob Bay, Fidalgo Bay,
120and Oyster Bay (Figure 1)) grown for 19 months at Clam Bay, WA were used for this
121experiment. All oysters were held at 8°C for two weeks at the University of Washington prior to
122the experiment. Oysters from each population (n=8 per population) were subjected to acute
123temperature stress (submerged in 500mL 38°C sea water for 1 hour), mechanical stress (120g x 5
124min; Sorvall T21, ST-H750 rotor) or served as controls (maintained at 8°C). After the stress

125 treatments, oysters were returned to 8°C seawater and sampled at 1 hour post stress (n=72).
 126 Ctenidia tissue was resected from each individual and stored separately in 500µL RNazol RT
 127 (Molecular Research Center, Inc.), frozen on dry ice. All samples were stored at -80°C for later
 128 analysis.

129 RNA Isolation

130 RNA was isolated using RNazol RT (Molecular Research Center, Inc.) according to the
 131 manufacturer's protocol for total RNA isolation. Briefly, ctenidia tissue was homogenized in
 132 RNazol RT, volume was brought up to 1mL with RNazol RT, vortexed vigorously for 15
 133 seconds, and incubated at room temperature (RT) for 10 minutes. 400µL of 0.1% DEPC-H₂O
 134 was added to the homogenized ctenidia tissue, vortexed for 15 seconds, and incubated at RT for
 135 15 minutes. The samples were centrifuged for 15 minutes, 16,000g, at RT. After centrifugation,
 136 750µL of the supernatant was transferred to a clean tube, an equal volume of isopropanol added,
 137 vortexed for 10 seconds, and incubated at RT for 15 minutes. The samples were centrifuged at
 138 12,000g for 10 minutes at RT. The supernatant was discarded and the pellets were washed with
 139 500µL of 75% ethanol (made with 0.1% DEPC-H₂O) and centrifuged at 4,000g for 3 minutes at
 140 room temperature. This wash step was then repeated. Ethanol was removed and pellets were
 141 resuspended in 100µL of 0.1% DEPC-H₂O. Samples were quantified using a NanoDrop1000
 142 (ThermoFisher) and stored at -80C.

143 DNase Treatment and Reverse Transcription

144 Total RNA was treated with DNase to remove residual genomic DNA (gDNA) using the Turbo
 145 DNA-free Kit (Ambion/Life Technologies). The manufacturer's rigorous protocol was followed.
 146 Briefly, 1.5µg of total RNA was treated in 0.5mL tubes in a reaction volume of 50µL. The

147 samples were incubated with 1 μ L of DNase for 30 minutes at 37°C. An additional 1 μ L of DNase
 148 was added to each sample and incubated at 37°C for an additional 30 minutes. The DNase was
 149 inactivated with 0.2 volumes of the inactivation reagent according to the manufacturer's protocol.
 150 Samples were quantified using a NanoDrop1000 (ThermoFisher). Treated RNA was verified to
 151 be free of gDNA via qPCR using actin primers (see Primer Design section below) known to
 152 amplify gDNA.

153 Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega) with oligo
 154 dT primers (Promega), using 250 ng of DNased RNA. The RNA was combined with primers
 155 (0.25 μ g) in a volume of 74.75 μ L, incubated at 70°C for 5 minutes in a thermal cycler without a
 156 heated lid (PTC-200; MJ Research), and immediately placed on ice. A master mix of 5x Reverse
 157 Transcriptase Buffer (1x final concentration; Promega), 10 mM each of dNTPs (0.5 mM final
 158 concentration of each dNTP; Promega), and M-MLV Reverse Transcriptase (50 U/reaction) was
 159 made and 25.25 μ L of the mix was added to each sample (final reaction volume 100 μ L). Samples
 160 were incubated at 42°C for 1 hr, followed by 95°C for 3 minutes in a thermal cycler without a
 161 heated lid (PTC-200; MJ Research), and then stored at -20°C.

162

163 Quantitative PCR

164 Primer Design

165 Primers for qPCR analysis were developed from an *O. lurida* transcriptome (version 3) which
 166 can find in the repository associated with this manuscript (Roberts 2017). This transcriptome
 167 was annotated using SwissProt and Gene Ontology Databases. Specifically, gene function
 168 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. 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 °C and within 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.

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

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List of primers can be viewed in Table 2.

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Transcriptome Contig Name	Biological Category	Uniprot Accession	Uniprot Entry Name	Uniprot Annotation	Function	Gene Abbr	BLAST X value
comp7220_c0_seq2	Gene Regulation	Q6DC04	CARM1_DANRE	Histone-arginine methyltransferase	Transfers methyl groups to Histone 3 for chromatin remodeling	CARM01	
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	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	Growth	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		Q8TA69	Q8TA69_CRAGI	Actin	Cytoskeletal formation.	Actin	0

					Used as a normalizing gene for qPCR analysis.		
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190

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

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Gene Abbreviat ion	FWD	REV
CARM1	TGGTTATCAACAGCCCC GAC	GTTGTTGACCCCAGGA GGAG
TLR	ACAAAGATTCCACCCG GCAA	ACACCAACGACAGGAA GTGG
H2AV	TGCTTTCTGTGTGCCCT TCT	TATCACACCCCGTCACT TGC
PGRP	GAGACTTCACCTCGCA CCAA	AACTGGTTTGCCCGAC ATCA
HSP70	TTGTCGCCATTTTCCTC	GTTCCGATTGTTCCTG

	GCT	GCC
BMP2	TGAAGGAACGACCAAA GCCA	TCCGGTTGAAGAACCT CGTG
GRB2	AACTTTGTCCACCCAG ACGG	CCAGTTGCAGTCCACTT CCT
PGEEP4	ACAGCGACGGACGATT TTCT	ATGGCAGACGTTACCC AACA
TRAF3	AGCAGGGCATCAAACCT CTCC	ACAAGTCGCACTGGCT ACAA
Actin	GACCAGCCAAATCCAG ACGA	CGGTCGTACCACTGGTA TCG

198

199**Table 2.** Table of qPCR Primers for genes of interest. Includes the Uniprot Entry Name, the
200Gene Abbreviation used throughout this manuscript, and the forward (FWD) and reverse (REV)
201primer sequences. Full sequences utilized for primer creation are available. (Heare and Roberts
2022015).

203

204Quantitative PCR

205Quantitative PCR reactions were carried out using Ssofast Evagreen Supermix (BioRad, USA).
206Forward and reverse primers (Integrated DNA Technologies) were used at a final concentration
207of 0.25uM each. Sample cDNA was diluted (1:9) with molecular-grade water. Nine microliters of
208diluted cDNA was used as template. Reaction volumes were 20µL and were run in low-profile,
209non-skirted, white qPCR plates (USA Scientific) with optically clear lids (USA Scientific) in a

210 BioRad CFX Real Time Thermocycler (BioRad, USA) and DNA Engine Opticon 2 System
 211 (BioRad, USA). Cycling conditions were: one cycle of 95°C for 10 min; 40 cycles of 95°C for
 212 30 sec, 60°C for 1 min, 72°C for 30 sec. Two qPCR replicates were run for each sample, for each
 213 primer set.

214 Statistical Analysis

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

231

232Results

233Gene Expression Analysis

234Without considering separate populations, acute heat shock resulted in statistically significant
 235increases in expression of coactivator-associated arginine methyltransferase 1 (CARM1) (n=24
 236oysters per treatment, ANOVA, df=2, Tukey's HSD p=0.00007) (Figure 2) and Histone 2AV
 237(H2AV) (n=24 oysters per treatment, ANOVA, df=2, Tukey's HSD p=0.001)(Figure 3). A
 238statistically significant increase in expression of tumor necrosis factor receptor-associated factor
 2393 (TRAF3) (n=24 oysters per treatment, ANOVA, df=2, Tukey's HSD p=0.008) (Figure 4)
 240occurred upon exposure to mechanical stress.

241There was a clear difference in response to mechanical stress in oysters from Oyster Bay as
 242compared to oysters from Dabob and Fidalgo Bays. Specifically, upon heat shock, H2AV
 243expression in oysters from Oyster Bay increased (n=8 oysters per population, ANOVA, df=4,
 244Tukey's HSD = 0.05) (Figure 3) when compared to the control. When exposed to mechanical
 245stress, bone morphogenic protein 2 (BMP2) (n=8 oysters per population, ANOVA, df=4, Tukey's
 246HSD p=0.03) (Figure 5) and growth-factor receptor bound protein 2 (GRB2) (n=8 oysters per
 247population, ANOVA, df=4, Tukey's HSD p=0.03)(Figure 6) expression was decreased in the
 248Oyster Bay population, whereas there was no significant differences in responses in the other
 249populations. Additionally, significant interactions were identified between population and
 250treatment in both BMP2 and GRB2 (p<0.05).

251There was no statistical difference in expression in Peptidoglycan recognition protein 1 (PGRP),
 252toll-like receptor 2 type 1 (TLR), and prostaglandin E2 receptor EP4 subtype (PGEEP4) (Figures
 2537, 8, & 9, respectively) within any comparison. Heat shock protein 70 gene expression was

significantly different between temperature and mechanical stress (n=24 oysters per treatment, ANOVA, df=4, Tukey's HSD p=0.006) (Figure 10).

256

257 Discussion

258

259 Response to Temperature Stress

The response of *Ostrea lurida* to acute heat stress appears to include an alteration in gene regulatory activity and the innate immune response, as indicated by significant increases of H2AV (Figure 3) and CARM1 (Figure 2) gene expression one hour post-temperature stress.

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 an H2A variant was significantly upregulated under heat stress conditions (3 °C for 12 hours). In addition to involvement in the heat stress response, histone H2A has been shown to exhibit antimicrobial properties in three invertebrates: two marine invertebrates (Pacific white shrimp and scallops; Patat et al., 2004, Li et al., 2007a), as well as in a freshwater shrimp (Arockiaraj et al., 2013). In *D. melanogaster*, H2Av is phosphorylated in response to DNA damage (Madigan et al., 2002) to inhibit apoptosis, suggesting an additional role in cellular survival.

272

Coactivator-associated arginine methyltransferase 1, CARM1, is involved transcriptional activation via methylation of histones (Chen et al., 1999, Lee et al., 2005). This in turn affects the

275ability of transcription factors to bind and transcription to proceed. It is possible that increases in
 276CARM1 expression could indicate that overall gene regulatory activity is increased in response
 277to temperature stress. Our results are similar to those of Wang et al. (2011) where researchers
 278described an increase in expression of Histone-arginine methyltransferase in the sea cucumber,
 279*Apostichus japonicus*, after experiencing 25°C temperatures for 7 days. The authors suggested
 280that this was due to an induced dormancy and lower metabolic rate to provide resources for stress
 281resilience. CARM1 is also a component of the cellular immune response, as it has been identified
 282as a regulator of NF-κB (Covic et al., 2005). Thus another explanation is that acute heat could
 283possibly impact the immune response, likely in a negative manner. Future work, that would be
 284relevant to restoration activities, should increase the number of stressors examined in oyster to
 285include pathogens.

286

287Increases in HSPs are often observed in response to stress, but this study only found a significant
 288difference of mRNA expression of HSP70 in the Oyster Bay population between mechanical and
 289heat stresses (Figure 10). Brown et al. (2004) found the maximum HSP expression in *O. lurida*
 290occurred 24-48 hours post exposure to 39°C. The absence of a strong response of HSP70, relative
 291to the control group, could be related to temporal changes in expression or an isoform-specific
 292response, as there are many genes in this gene family, particularly in oysters (Clegg et al., 1998;
 293Piano et al., 2005). Mediterranean mussels, *Mytilus galloprovincialis*, have shown different
 294isoforms of heat shock proteins and cognates that have differential expression patterns caused by
 295heat, mercury exposure, and chromium exposures stressors suggesting that the isoforms have
 296slightly different functions (Franzellitti and Fabbri, 2005). Additionally, there are members of the
 297HSP70 gene family that are constitutively expressed and do not exhibit increases in mRNA in

298response to heat stress (Sorger & Pelham, 1987; Somji et al., 1999). Without a sequenced
 299genome for *Ostrea lurida*, combined with utilizing an incomplete transcriptome, it is difficult to
 300ascertain how many isoforms might exist, as well as the number of alternatively spliced products.
 301Upon addition of new genomic resources the entire family of molecular chaperones could be
 302examined and compared across populations.

303

304Response to Mechanical Stress

305Mechanical stress increased expression of inflammation-related target genes. In all populations,
 306there was a significant increase in immune system-related responses seen via the expression of
 307tumor necrosis factor receptor-associated factor 3, TRAF3 (Figure 4), which is involved in
 308internal tissue damage recognition and apoptosis. The main function of TRAF3 is to assist in cell
 309death initiation caused by stress conditions within tissues (Arch, Gedrich, and Thompson, 1998).
 310Upregulation in relation to mechanical stress could be akin to inflammation occurring due to
 311edema from the mechanical stress and used to remove damaged cells as suggested by Roberts et
 312al. (2012) when *C. virginica* were exposed to mechanical stress. Significant differences in
 313expression of other immune system targets such as PGRP, TLR, and PGEEP4 were not
 314detected(Figures 7, 8, & 9, respectively), but other studies have found that the time scale for
 315expression may vary (Meistertzheim et al.,2007; Farcy et al., 2009).

316

317

318Population differences

319 We suspected that the Dabob Bay population would have demonstrated a more pronounced
 320 response to stress as this population is subjected to greater environmental fluctuations with
 321 respect to salinity and temperature (Heare et al 2015). Contrary to our hypothesis, oysters from
 322 Oyster Bay were the only population that exhibited a difference in gene expression in response to
 323 mechanical or heat stress. Oysters from Oyster Bay parents showed an increase in H2AV
 324 expression during heat stress as compared to control (Figure 3), a decrease in BMP2 and GRB2
 325 upon mechanical stress (Figures 5 & 6, respectively), and differences in HSP70 expression
 326 between heat and mechanical stresses (Figure 10). Given the putative function of H2AV in
 327 transcriptional regulation (Table 1), the increase in expression could be indicative of the role of
 328 this protein in controlling the molecular response to stress. Bone morphogenic protein 2, BMP2,
 329 and growth-factor receptor bound protein 2, GRB2, were significantly decreased in expression
 330 which could be indicative of growth inhibition. Both genes are related to growth and
 331 development of tissues, with BMP2 being a pre-cursor to osteoblastic cells that produce shell
 332 (Pereira Mouries et al., 2002) and GRB2 is used for signal transduction between cells during
 333 growth phases (Oda et al., 2005). By down-regulating these targets, this may be an effort to
 334 reduce energetically costly processes in favor of processes that promote survival during stress
 335 events. Organisms faced with stress are often required to reallocate energy resources to
 336 homeostasis-related functions in an effort to improve long-term survival of the species (Sokolova
 337 et al., 2012). This change in expression coupled with the up-regulation of H2AV (Figure 3) is in
 338 accord with the idea of shifting priorities for stress resilience.

339 Interactions were identified between population and treatment for both BMP2 and GRB2.
 340 Differences between gene expression in control and mechanical stress in the Oyster Bay
 341 population are driving this interaction for both genes. Although statistical interactions of this

342nature are difficult to interpret, it could be related to fact the Oyster Bay population is from a
343relatively “low-stress” environment (i.e. abundant food and less-pronounced temperature
344fluctuations).

345

346

347CONCLUSIONS

348

349The gene expression pattern differences observed here with oysters from Oyster Bay coupled
350with corresponding field-based observation that this population has the greatest reproductive
351activity (Heare et al., 2015), could indicate this population has a greater ability to effectively
352respond to stress. Another way to consider this is that the Oyster Bay population has a relatively
353higher degree of phenotypic plasticity, or more specifically, an elevated rate of phenotypic
354change (Angilletta et al., 2003). The gene expression data indicates a clear population-level
355stress response, and lack of differential response in other populations that suggests shifts in
356energy balance. Some possible explanations for this relatively rapid response include a more
357sensitive cell-signaling system (ie cytokines) or a more robust transcription initiation process.
358Yao and Somero (2012) observed higher heat stress tolerance in *M. galloprovincialis* than *M.*
359*californius* likely due to their ability to maintain cell signaling through the production of
360phosphor-p38-MAPK kinases, which may be how the Oyster Bay population is able to quickly
361respond to stress. This ability to quickly respond to stress may be due to increased fitness in
362Oyster Bay, however more research is needed to identify the link between gene expression and
363performance. Based on earlier field work, this could be directly linked to increased larval

364production, and processes allocating limited resources into reproduction (Heare et al., 2015).

365This trait could certainly be perceived as advantageous for restoration purposes. Caution should
366be used in using non-local stocks when structure exists, as it is possible to have supplemented
367oysters out-compete the native population or to create hybrids that are ultimately less fit than the
368native counter parts (Camara and Vadopalas, 2009). Both such phenomena decrease overall
369genetic diversity leaving the remaining population to be less robust for future challenges and
370possibly leading to local extirpation.

371Another interpretation of gene expression patterns in the Oyster Bay population is that the
372differences observed upon stress exposure are not indicative of an effective response that has
373been selected for, but rather indicative of plasticity. In other words, the change in gene
374expression upon stress is representative of a phenotype that is tolerable to a wide range of
375pressure. At one level the ability to achieve a number of phenotypes with a given genotype could
376be advantageous, particularly in a rapidly the changing environment. There is a paradox in the
377fact that too much plasticity negates the ability of natural selection to function. Populations with
378high phenotypic plasticity become deprived of negative selection and thus are often able to
379survive in rapidly changing environments as long as the changes are consistent and somewhat
380predictable. However, with this increased adaptive ability, genetic diversity and adaptation
381become limited within a population that may be unable to properly respond to novel challenges
382in the future (Crispo, 2008). Alternatively, the Baldwin effect may enhance longterm genetic
383diversity by allowing species to colonize novel habitats and, with phenotypic plasticity, and
384eventually genetically diverge from the source population through induced genetic adaptations
385(Crispo, 2007). For longterm restoration of *O. lurida* populations in Puget Sound, understanding

the genetic differences and phenotypic plasticity of individual populations will help determine proper supplementation procedures for existing and historic habitats.

388

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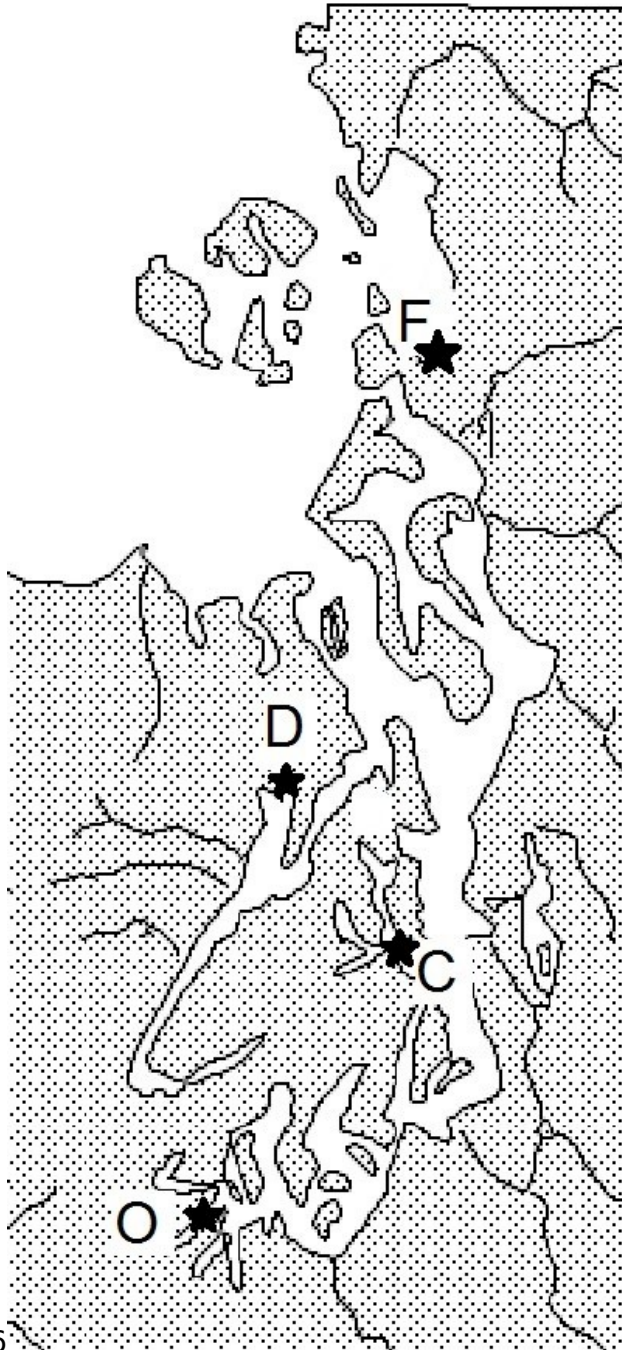
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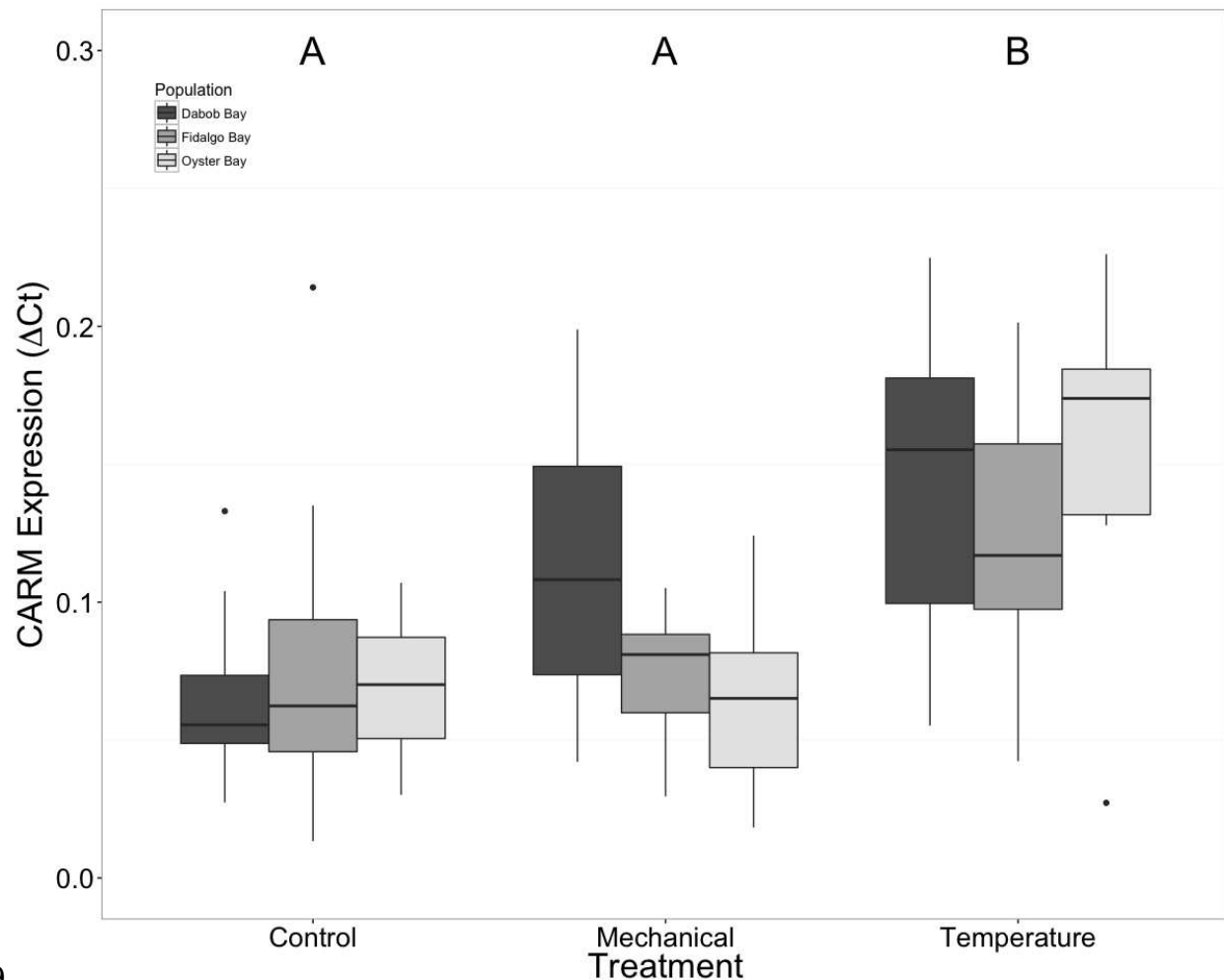
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535 **Figures**



536

537 Figure 1. Map of Puget Sound with *Ostrea lurida* broodstock locations. Broodstock collected
538 from Fidalgo Bay (F), Dabob Bay (D), and Oyster Bay (O) and held at Clam Bay (C).



539

540 Figure 2. Expression of CARM1 mRNA. Median ΔC_t indicated by line in middle of box plot.

541 Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate

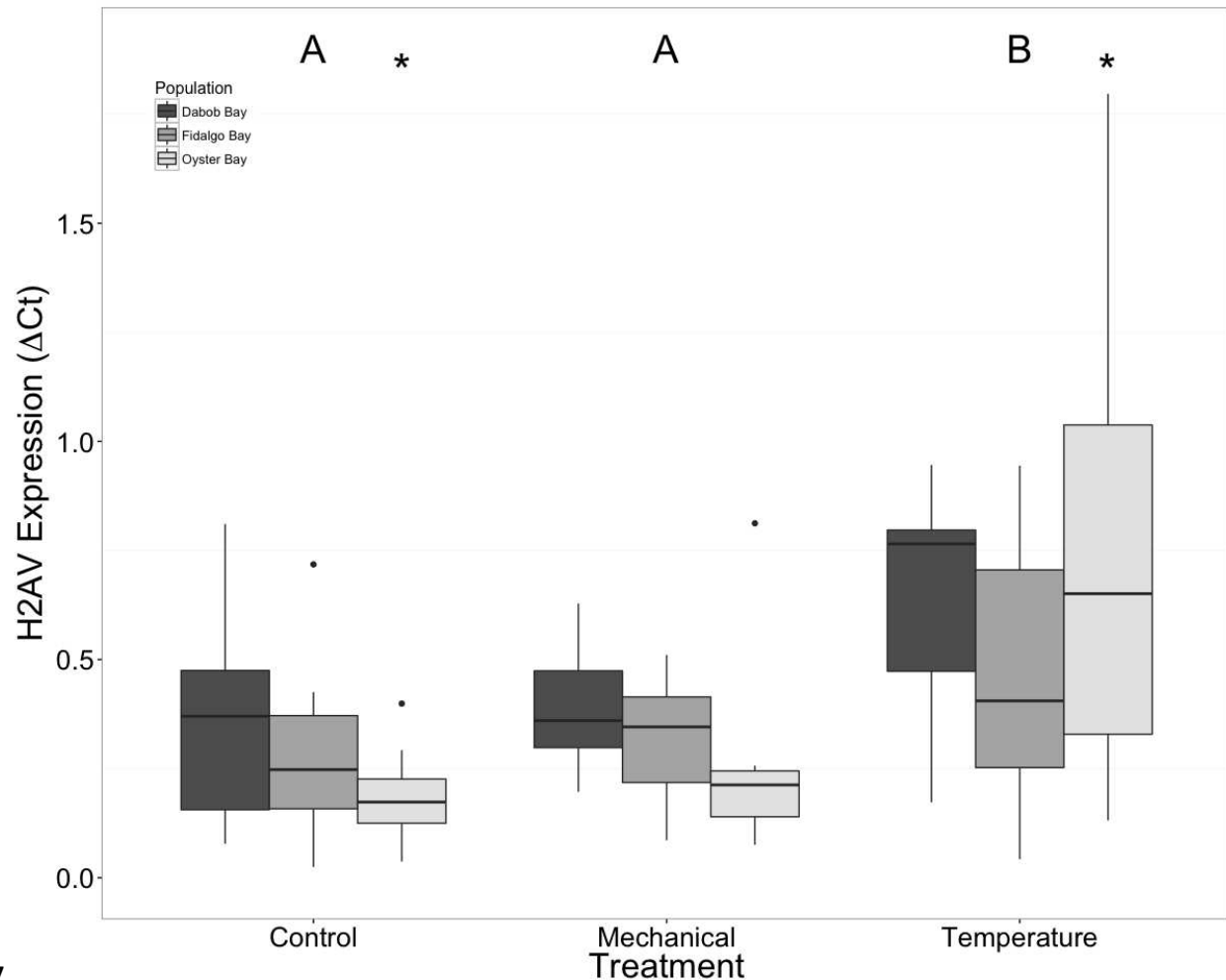
542 outside values. Capital letters indicate significant differences ($p < 0.05$) between overall treatment

543 groups ($n = 24$ animals per treatment). No statistical differences ($p > 0.05$) were observed between

544 populations ($n = 24$ animals per population), nor within a given population ($n = 8$ animals per

545 treatment).

546



547

548Figure 3. Expression of H2AV mRNA. Median ΔCt indicated by line in middle of box plot.

549Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate

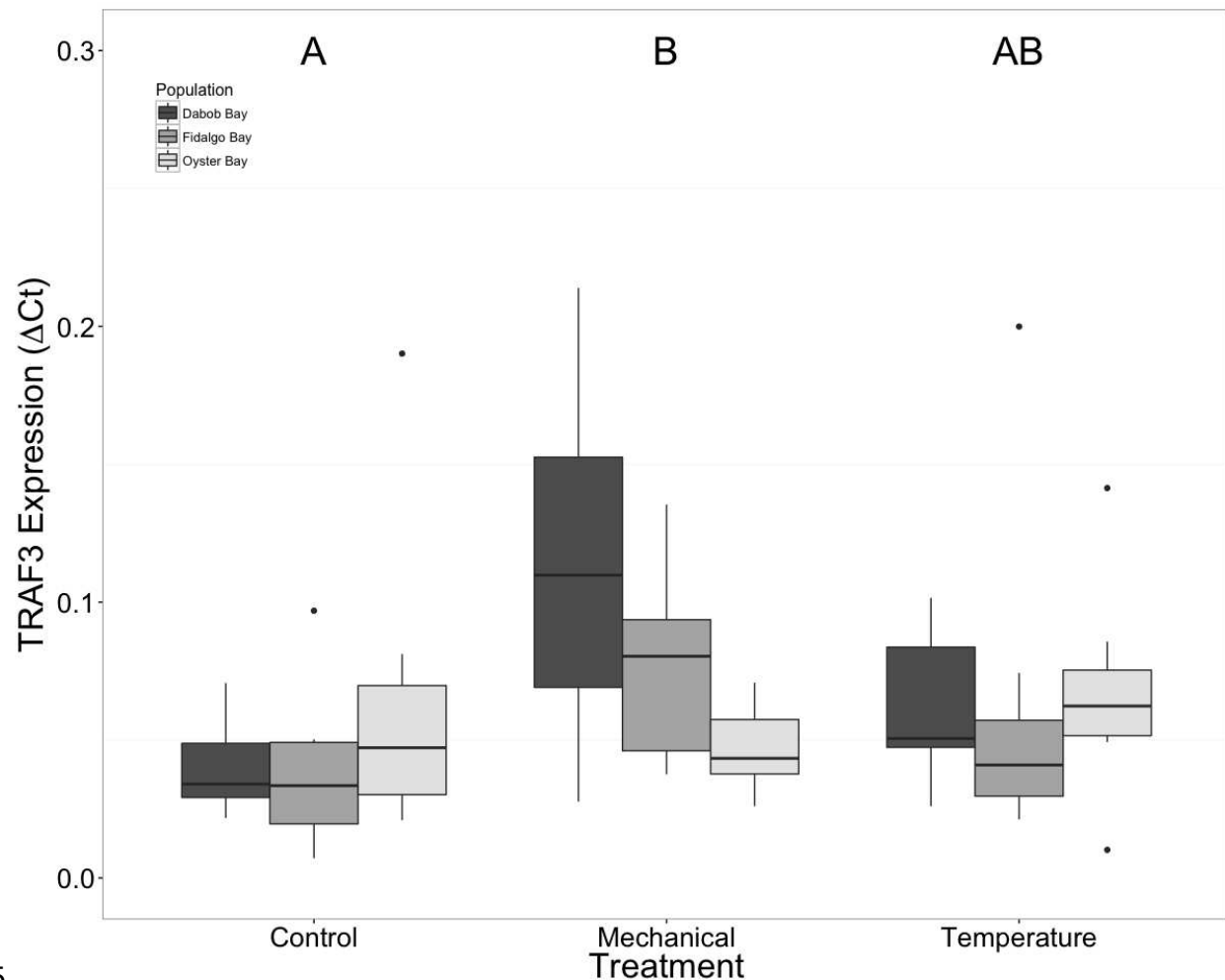
550outside values. Asterisks indicate significant differences ($p < 0.05$) between treatments within a

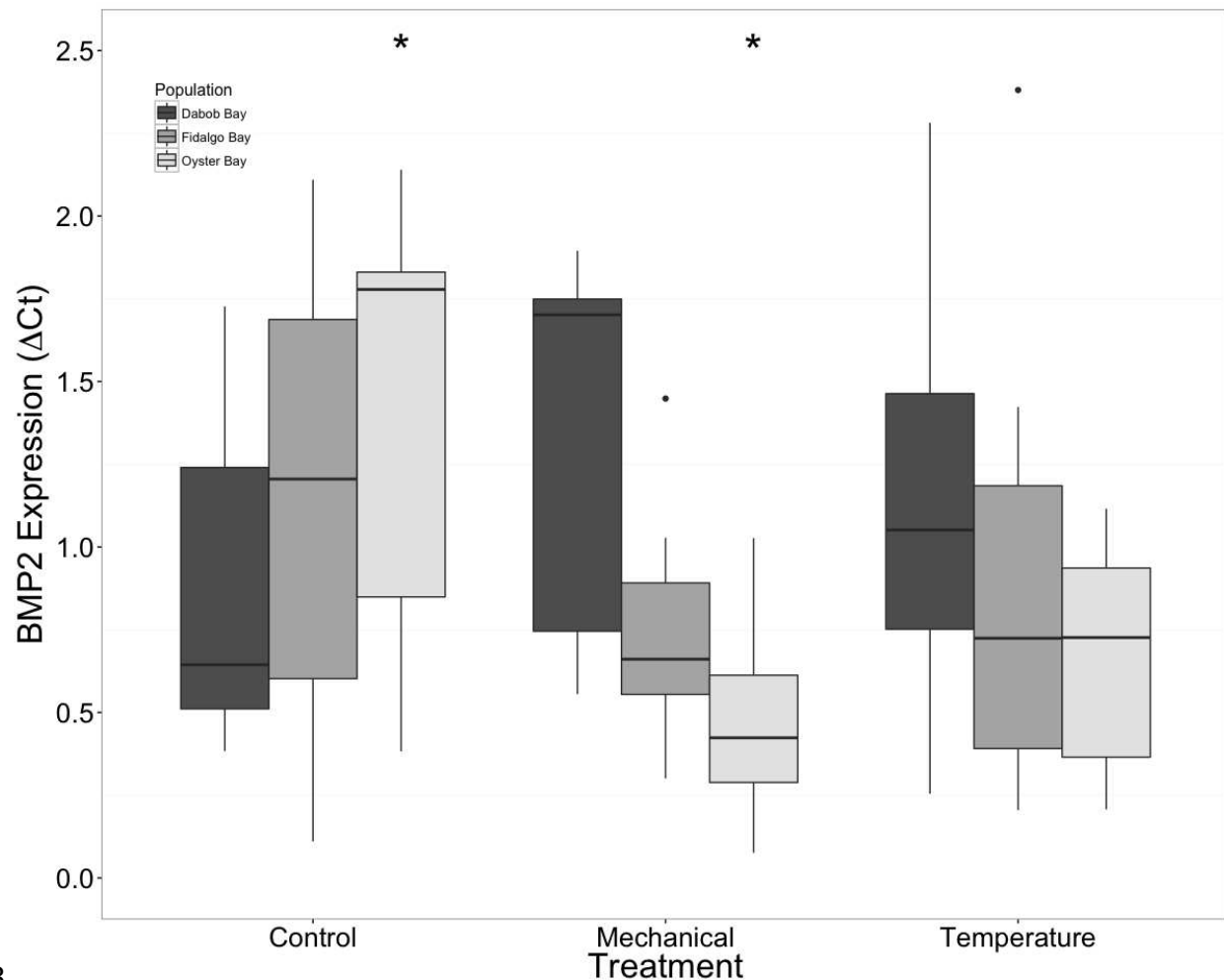
551population ($n = 8$ animals per treatment). Capital letters indicate significant differences ($p < 0.05$)

552between overall treatment groups ($n = 24$ animals per treatment). No statistical differences

553($p > 0.05$) were observed between populations ($n = 24$ animals per population).

554





563

564 Figure 5. Expression of BMP2 mRNA. Median ΔC_t indicated by line in middle of box plot.

565 Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate

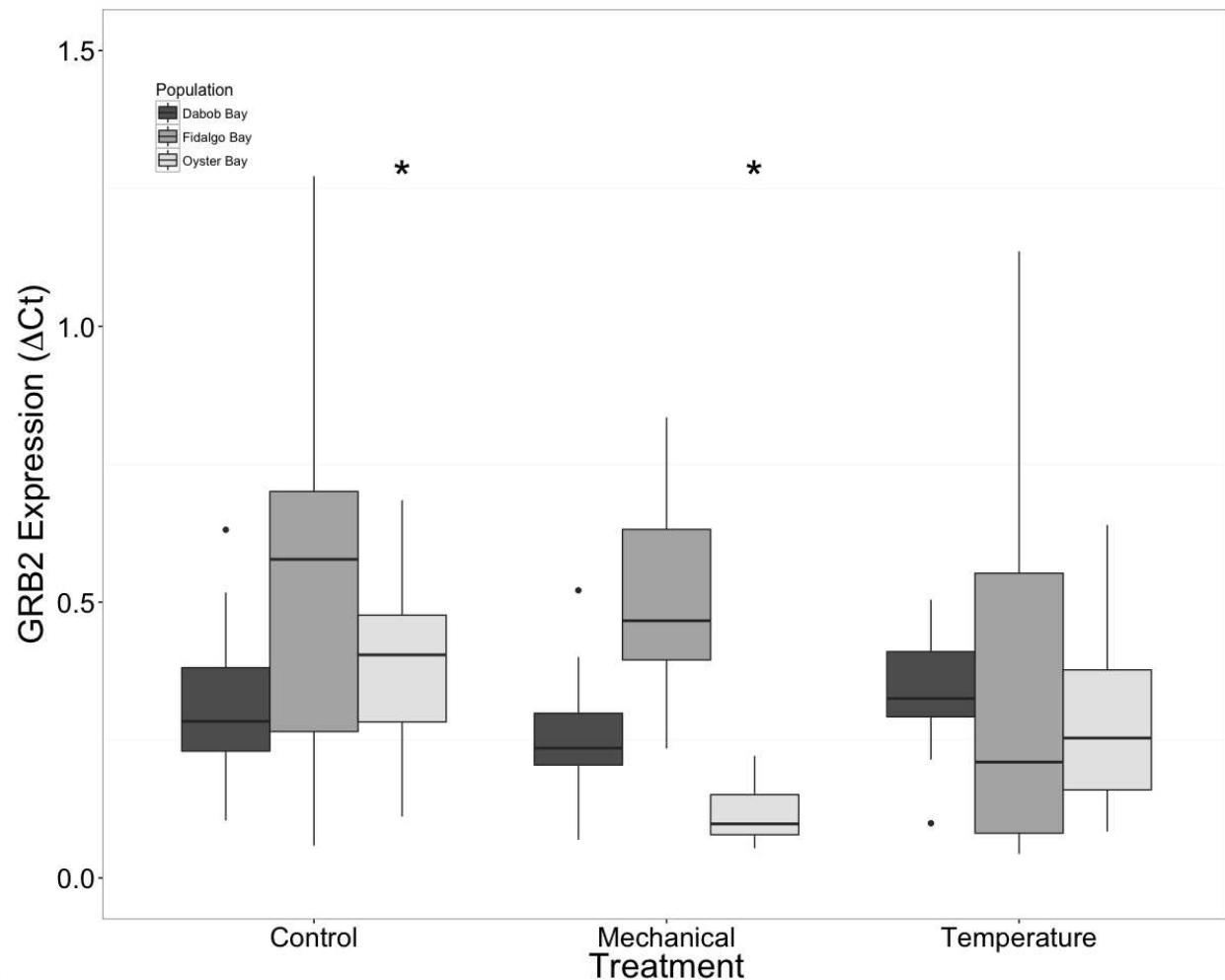
566 outside values. Asterisks indicate significant differences ($p < 0.05$) between treatments within a

567 population ($n = 8$ animals per treatment). No statistical differences ($p > 0.05$) were observed

568 between populations ($n = 24$ animals per population), nor between treatments ($n = 24$ animals per

569 treatment).

570



571

572 Figure 6. Expression of GRB2 mRNA. Median ΔCt indicated by line in middle of box plot.

573 Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate

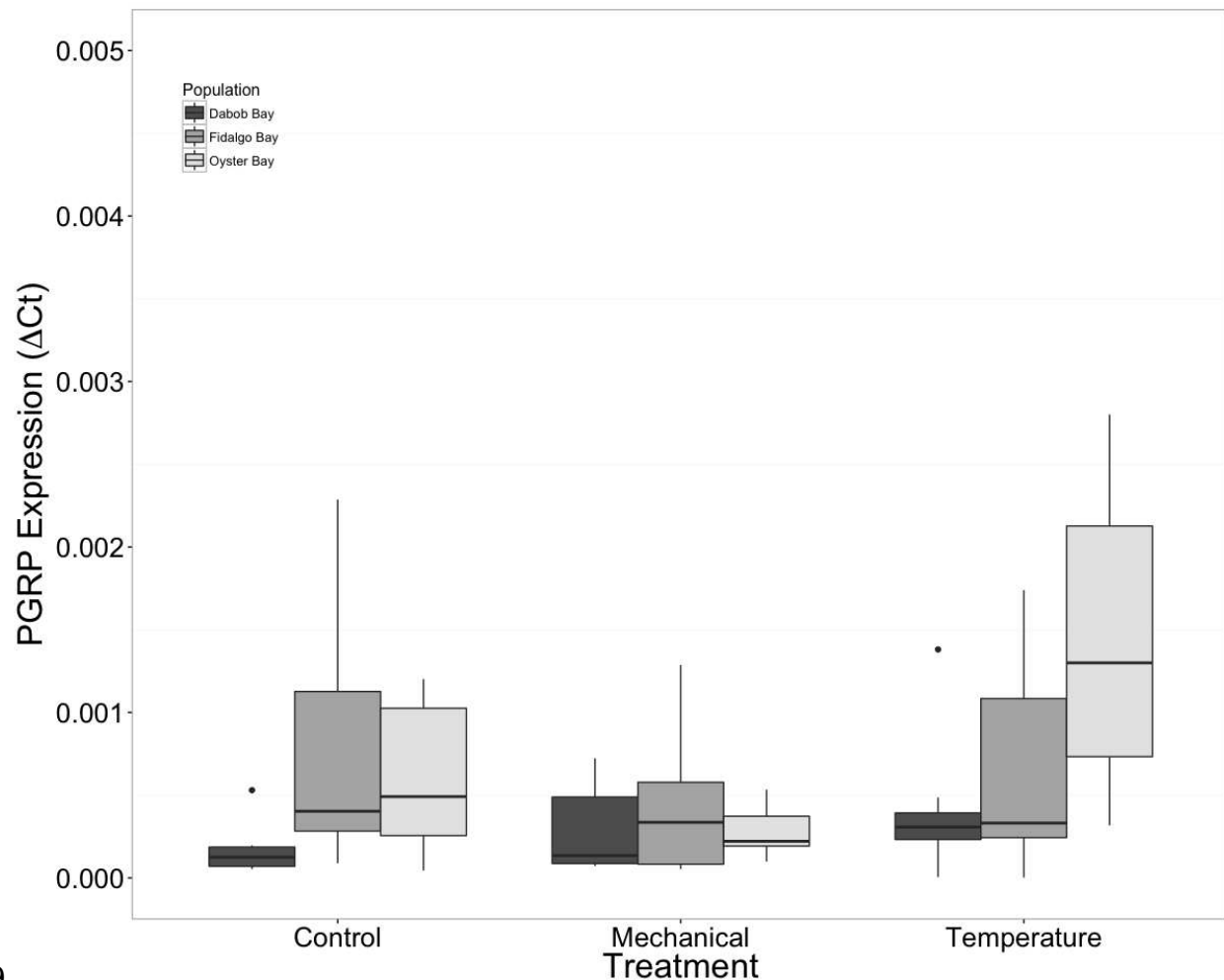
574 outside values. Asterisks indicate significant differences ($p < 0.05$) between treatments within a

575 population ($n = 8$ animals per treatment). No statistical differences ($p > 0.05$) were observed

576 between populations ($n = 24$ animals per population), nor between treatments ($n = 24$ animals per

577 treatment).

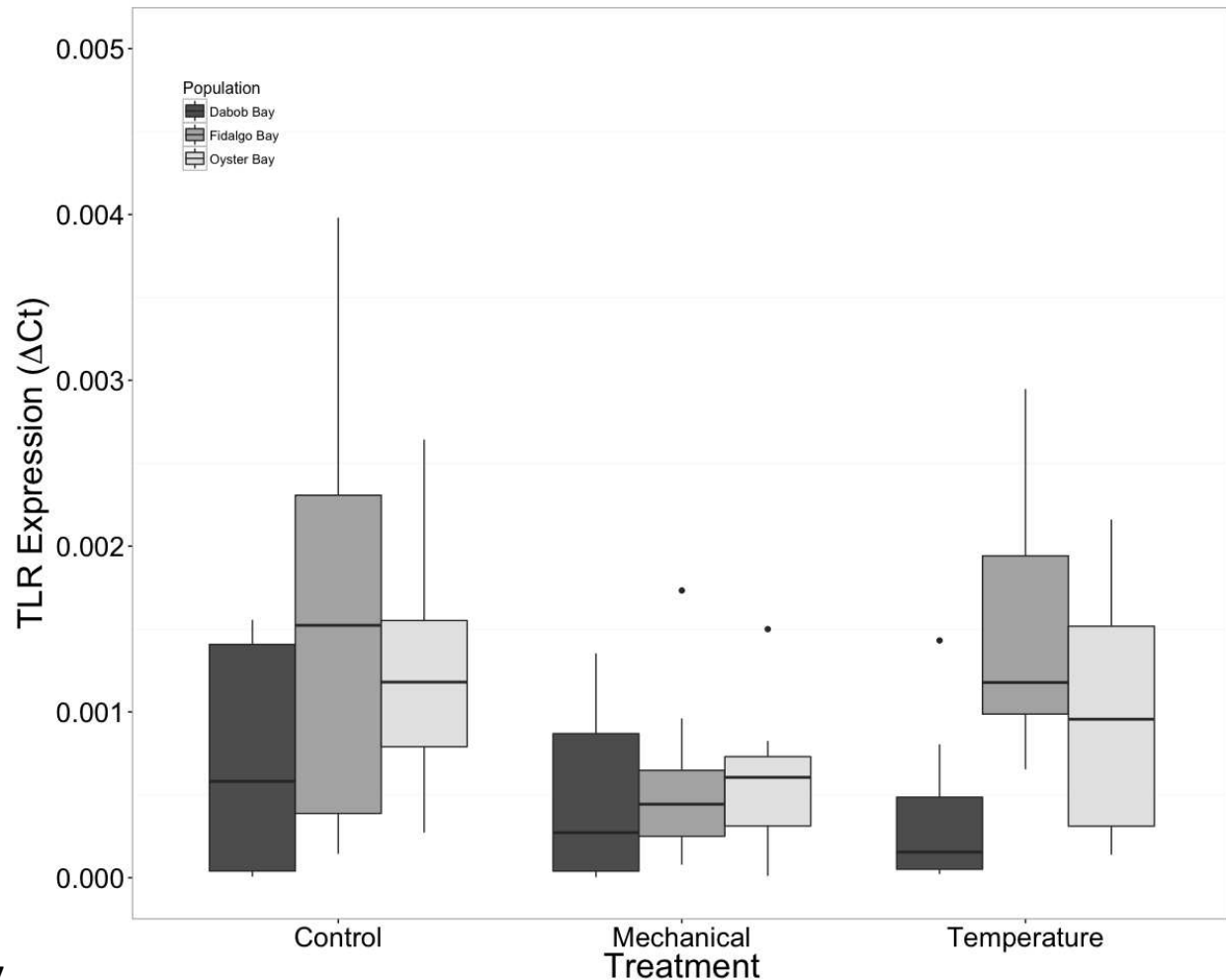
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579

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

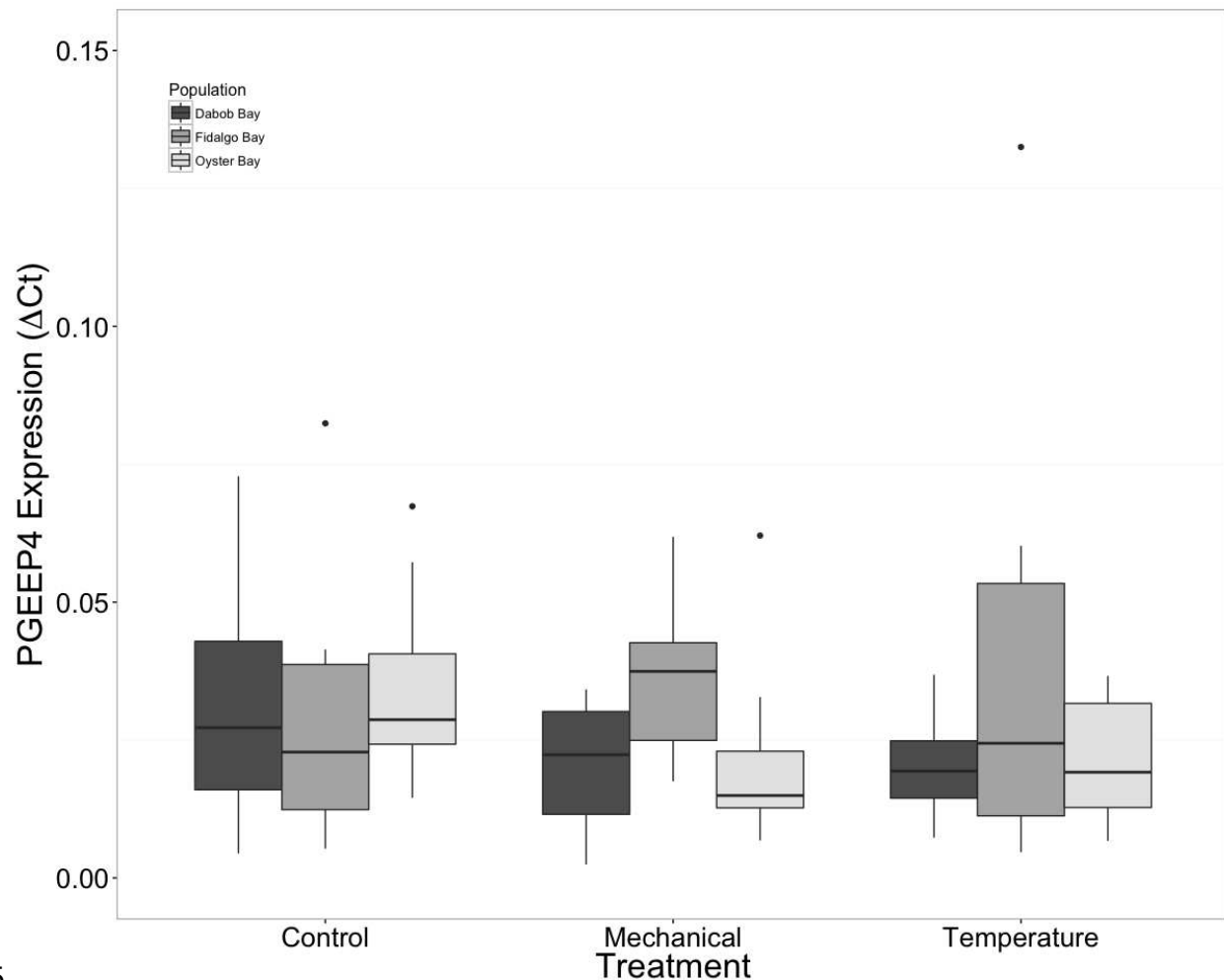
586



587

588Figure 8. Expression of TLR mRNA. No statistical difference observed between treatments, nor
 589between populations. Median ΔCt indicated by line in middle of box plot. Shaded boxes are 2nd
 590and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. No
 591statistical differences ($p > 0.05$) were observed within populations between treatments ($n = 8$
 592animals per treatment), between populations ($n = 24$ animals per population), or between
 593treatments ($n = 24$ animals per treatment).

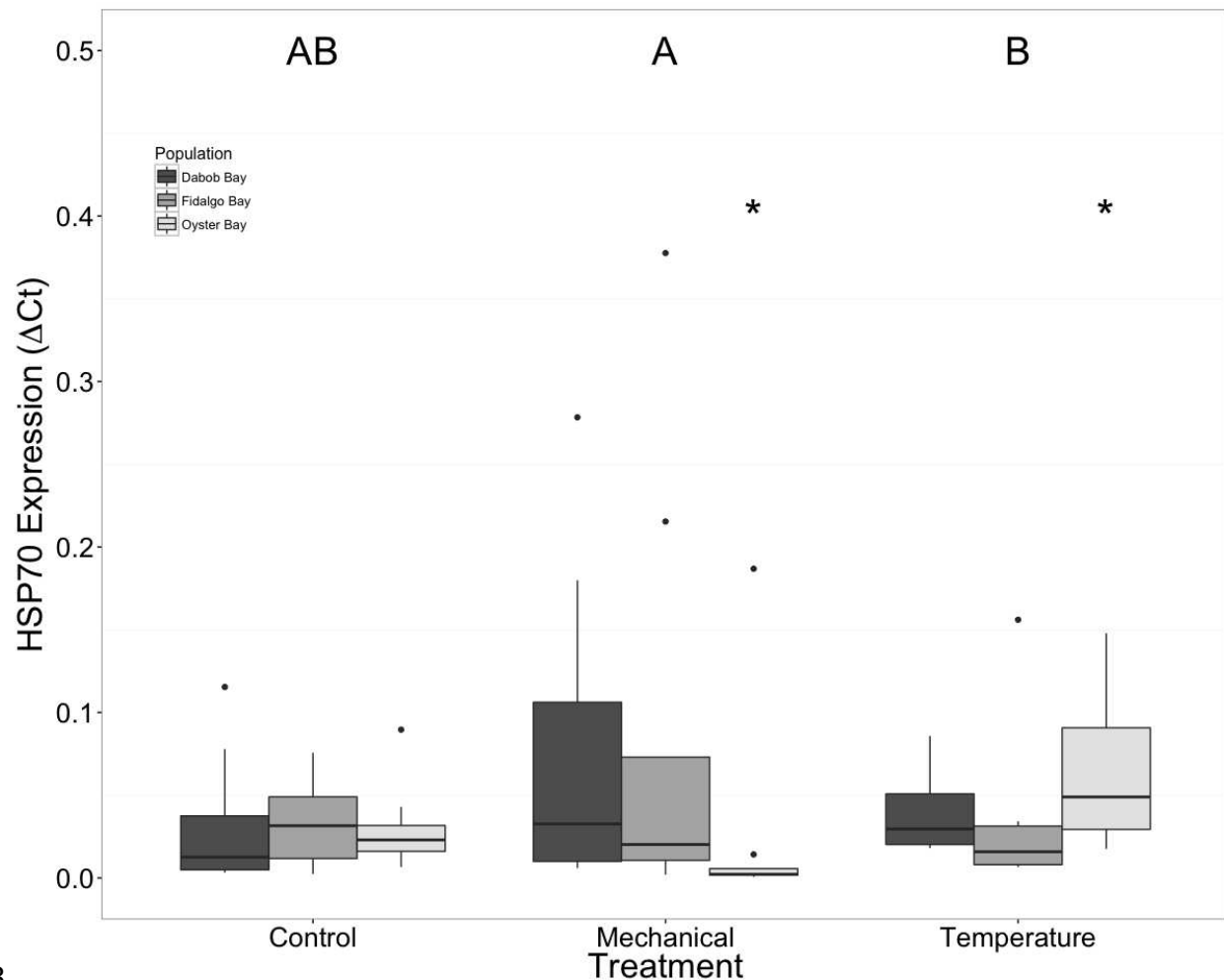
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595

596 Figure 9. Expression of PGEEP4 mRNA. No statistical difference observed between treatments,
 597 nor between populations. Median ΔC_t indicated by line in middle of box plot. Shaded boxes are
 598 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate outside values. No
 599 statistical differences ($p > 0.05$) were observed within populations between treatments ($n = 8$
 600 animals per treatment), between populations ($n = 24$ animals per population), or between
 601 treatments ($n = 24$ animals per treatment).

602



603

604Figure 10. Expression of HSP70 mRNA. Median ΔCt indicated by line in middle of box plot.

605Shaded boxes are 2nd and 3rd quartile groups. Lines are 1st and 3rd quartiles. Dots indicate

606outside values. Asterisks indicate significant differences ($p < 0.05$) between treatments within a

607population ($n = 8$ animals per population). Capital letters indicate significant differences ($p < 0.05$)

608between overall treatment groups ($n = 24$ animals per treatment). No statistical differences

609($p > 0.05$) were observed between populations ($n = 24$ animals per population).

610