

Response of Olympia oysters (*Ostrea lurida*) to changing environmental conditions

Jake Heare

A thesis submitted
In partial fulfillment of the
Requirements for the degree of

Master of Science

University of Washington

2015

Committee:

Steven B. Roberts, Chair

Kerry Naish

Bonnie J. Becker

Program Authorized to Offer Degree:

Aquatic and Fishery Sciences

Copyright 2015
J. Emerson Heare

University of Washington

Abstract

Response of Olympia oysters (*Ostrea lurida*) to changing environmental conditions

J. Emerson Heare

Chair of Supervisory Committee:
Assistant Professor Dr. Steven B. Roberts
School of Aquatic and Fishery Sciences

The Olympia oyster is an iconic oyster species in the Pacific Northwest with special significance in Puget Sound, WA. Oyster populations in the region were decimated to historic lows during the 20th century due to a number of factors including overharvest, habitat loss, and invasive species. Restoration projects have seen limited success, likely due to the limited information on stock structure within Puget Sound, especially in regards to adaptive abilities and habitat suitability. Chapter one of this study investigates population related fitness measures (ie. mortality, growth, reproduction) within three resident populations from geographically isolated locations in Puget Sound. Using a reciprocal transplant experiment with *Ostrea lurida* populations from Fidalgo Bay, Dabob Bay, and Oyster Bay, we found that two of the three populations (Dabob Bay and Oyster Bay) express significant phenotypic signatures related to the population. Using this information we offer restoration strategies catered to population phenotypes in an effort to improve restoration projects in the Puget Sound. In Chapter two, we ran a thermal and mechanical stress experiment due to differences in mortality between populations observed in Chapter 1 to investigate expression of genes (via qPCR) related to survival. We found differences in expression related to gene transcription, which indicates possible phenotypic plasticity previously unknown in the study populations though further investigation is required.

Table of Contents

List of Figures.....	5
List of Tables.....	6
Chapter I: Evidence of <i>Ostrea lurida</i> (Carpenter 1864) population structure in Puget Sound, WA.....	9
Abstract.....	9
Introduction.....	9
Methods.....	11
Results.....	15
Discussion.....	19
References.....	27
Figures.....	30
Chapter II: Differential response to stress in <i>Ostrea lurida</i> as measured by gene expression.....	43
Abstract.....	43
Introduction.....	43
Methods.....	47
Results.....	51
Discussion.....	52
Citations.....	57
Figures.....	61
Appendix A. Annotated Bibliography for <i>Ostrea lurida</i>	71
Appendix B. Data Analysis R Codes	79

List of Figures

- I. 1. Map of Puget Sound with *Ostrea lurida* broodstock and outplant sites.
- I. 2. Minimum observed daily temperatures for all sites.
- I. 3. Maximum observed daily temperatures for all sites.
- I. 4. Proportion survival for three *Ostrea lurida* populations at four locations.
- I. 5. *Ostrea lurida* shell length in September 2014 at Southern site.
- I. 6. *Ostrea lurida* shell length in October 2014 at Northern site.
- I. 7. *Ostrea lurida* shell length in October 2014 at Central site.
- I. 8. Growth rate of mean shell length in *Ostrea lurida* outplanted at Northern site.
- I. 9. Growth rate of mean shell length in *Ostrea lurida* outplanted at Central site.
- I. 10. Growth rate of mean shell length in *Ostrea lurida* outplanted at Southern site.
- I. 11. Percent *Ostrea lurida* brooding females from each population at each sample date at Southern site.
- I. 12. Percent *Ostrea lurida* brooding females from each population at each sample date at Northern site.
- I. 13. Percent *Ostrea lurida* brooding females from each population at each sample date at Central site.
- I. 14. *Ostrea lurida* brooding female shell length comparison among sites.
- II. 1. Map of Puget Sound with *Ostrea lurida* broodstock and outplant sites.
- II. 2. Expression of CARM mRNA.
- II. 3. Expression of H2AV mRNA.
- II. 4. Expression of CRAF mRNA.
- II. 5. Expression of BMP2 mRNA.
- II. 6. Expression of GRB2 mRNA.
- II. 7. Expression of PGRP mRNA.
- II. 8. Expression of TLR mRNA.
- II. 9. Expression of PGEEP4 mRNA.
- II. 10. Expression of HSP70 mRNA.

List of Tables

II. 1. Table of qPCR Primers for genes of interest.

Acknowledgements

It is with great pleasure that I thank Dr. Steven B. Roberts, my graduate advisor and chair of my thesis committee for his support and guidance. He saw potential in a young guy working in Southern Louisiana and offered him a life changing opportunity. His patience and care for his students has been evident throughout my time in his lab. His promotion of open science through online notebooks and social media has exposed my work to the world. I would also like to thank the other members of my committee, Dr. Kerry Naish and Dr. Bonnie J. Becker, for their wisdom and insight that has opened my mind to larger concepts in restoration, conservation, and shellfish.

This work would not have been possible without funding provided by Washington Sea Grant, University of Washington, pursuant to National Oceanographic and Atmospheric Administration Award No. NA10OAR4170057 Projects R/LME/N-3.

I am grateful for the collaborative support from Puget Sound Restoration Fund, particularly Ryan Crim and Brian Allen for their work in collecting, spawning, conditioning, and various other support work without which this project would never have happened. I am also thankful for the support of The Fagergren Family, Rock Point Oyster Company, Crab Fresh Inc. (especially Brendan Mahaffey and Jeff Koenings), Fidalgo Marina (especially Harbor Master Einer Sortun), the NOAA Dive Team, and Taylor Shellfish (especially Gordon King, Jeremy, and the mussel raft crew).

I want to extend a huge THANK YOU to the members of the Roberts Lab for all the support. Brent Vadopalas for his help on the often difficult statistics and fieldwork. Sam J. White for processing and assisting with laboratory procedures. Graduate students Mackenzie Gavery, Claire Olson, Emma Timmins-Schiffman, Andy Jasonowicz, Doug Immerman, Jay Dimond, and Megan Hintz for their help and support. Undergraduates Katie Jackson, Hannah Wear, Etileit Maipei, Joelle Blais, Jonathon Allen, Sean Bennet, and Grace Crandall for assistance with field work and data processing.

Finally I want to thank my beautiful wife, L. Christine Savolainen, for moving with me to Seattle and starting a new life together. She has seen me at my highest highs and lowest lows, never wavering in her support and encouragement. She been an extra hand when I needed one, a warm embrace after a hard day, and a constant reminder that there are more things to life.

Dedication

This thesis is dedicated to the three most important mentors in my life so far.

To Dr. John W. Lynn, who encouraged independent thinking, exploration, use of old techniques to solve current problems, and the joy of hardwork. Without his mentorship and assistance, I would not have made it to graduate school nor the Pacific Northwest.

To the late Dr. Sammy Ray, who inspired me to research oysters with his deep love of the little guys. His patience and kindness helped mold me into the scientist I am today. He is deeply loved and will not be forgotten.

Finally, To my mom, Lorna Heare, who encouraged me to follow whatever path I wanted in life.. She took me to the sea when I wanted to be a marine biologist. When I wanted to go to college 600 miles away from home, she made every effort to get me there. Without her, none of this would be possible.

Chapter 1: Evidence of *Ostrea lurida* (Carpenter 1864) population structure in Puget Sound, WA

Abstract

For long-term persistence of species, population structure is important. Traits that hold adaptive advantage such as reproductive timing and stress resilience may differ among locales.

Knowledge and consideration of these traits should be integrated into conservation efforts. To test for adaptive differences between Olympia oyster populations a reciprocal transplant experiment was carried out monitoring survival, growth, and reproduction using three established populations of *Ostrea lurida* within Puget Sound, Washington. Performance differed for each population. *Ostrea lurida* from Dabob Bay had higher survival at all sites but lower reproductive activity and growth. Oysters from Oyster Bay demonstrated greater proportion of brooding females at a majority of sites with moderate growth and survival. Together these data suggest the existence of *O. lurida* population structure within Puget Sound and provide information on how broodstock should be selected for restoration purposes.

Introduction

Restoration of native oysters is of increasing importance due to ongoing habitat degradation, loss of ecosystem services, and global climate change (Anderson, 1995; Lotze et al., 2011). Resource managers and restoration groups place viable animals into habitats to supplement dwindling populations and encourage persistence. Success of these efforts is highly dependent on the fitness of the transplanted individuals (McKay et al., 2005).

The Olympia oyster, *Ostrea lurida* Carpenter, 1864, is the only native oyster to the west coast of North America and have received considerable attention with respect to restoration. Olympia oysters exist in a variety of habitats within its range from Baja California, Mexico to British Columbia, Canada (Hopkins, 1937). In Puget Sound, oysters experience temperatures ranging from 5 °C to 20 °C (Hopkins, 1937). They have increased mortality in freezing temperatures (0 °C) (Davis, 1955; Baker, 1995) or prolonged exposure to temperatures above 39 °C (LT50) (Brown et al., 2004). *Ostrea lurida* are rhythmical consecutive hermaphrodites (Coe, 1932b), spawning first as males followed by cycling between male and female within a spawning season. Hopkins (1937) observed in south Puget Sound that a maximum of 10-15% of *O. lurida* are brooding at any given time during a spawning season (1932). Peak larval settlement, roughly correlated with peak spawning, occurs twice annually within south Puget Sound (Hopkins, 1937). Even with the body of information presented by previous research (see Appendix A for annotated bibliography) on *O. lurida*, little is known about existing stock structure.

In the marine environment, the assumption of broad-scale fitness among marine invertebrates has been challenged. Palumbi (1997) demonstrated that geographic morphology affected sea urchin population structure and Burford et al. (2014) recently demonstrated a fitness cline in the eastern oyster, *Crassostrea virginica*, along the Atlantic coast. Findings such as these indicate that many similar species have unknown population structures that could hinder restoration efforts.

Despite several studies on Olympia oyster ecology and life history traits in Puget Sound, WA, information on population structure is limited and nothing is known about adaptive divergence of populations within Puget Sound (Camara and Vadopalas, 2009). Given the size, hydrologic features, and diverse environments of Puget Sound, it is possible that certain populations are

adapted to local conditions. Among methods testing for local adaptation, reciprocal transplant experiments are considered robust (Sanford and Kelly, 2011) for investigating fitness. These experiments involve using parent populations from environmentally diverse locales to produce offspring that are placed reciprocally in their home and foreign environments. Population differences in key metrics for fitness can provide evidence of adaptive divergence (Burford et al., 2014). Alternatively, there are other phenomena such as balanced polymorphism or low effective population size that can present variation phenotypic features that may falsely be attributed to local adaptation (Camara, 2008; Camara and Vadopalas, 2009).

The main objective of this study was to use a reciprocal transplant experiment to determine whether *O. lurida* populations from geographically diverse areas of Puget Sound, WA exhibit population-level differences in survival, reproduction, and growth in different environments. We predict that *O. lurida* populations within the Puget Sound exhibit significant variation in phenotypes that persist under different environmental conditions.

Material and Methods

Broodstock Conditioning and Outplanting

Adult oysters (n=600) were collected from three locations in Puget Sound; Fidalgo Bay, Dabob Bay, and Oyster Bay (Figure 1) during November and December 2012. Oysters were held for 5 months in common conditions in Port Gamble, Washington and spawned in June 2013. To ensure genetic diversity, each population from each site was subsequently spawned in 24 groups of 20-25 oysters. This spawning procedure is based on the findings from previous work within the Roberts lab suggesting that this technique maintains genetic diversity. Larvae produced from

each population were reared in tanks based on spawning group and settled on microcultch. Post-settlement spat were grown in four replicate screened silos and fed ad libitum until attaining the minimum outplant size (shell length (SL) = 5 mm).

In August 2013, 480 juvenile oysters (5-10 mm) from each population were placed at Fidalgo, Oyster, Dabob, and Clam Bays (Figure 1). For simplicity, we will call these sites Northern site (Fidalgo Bay), Southern site (Oyster Bay), Hood Canal site (Dabob Bay), and Central site (Clam Bay). At each site, oysters were placed into four 0.61 x 0.61 m grow out trays per population with 12 trays total outplanted. In each tray, oysters (120) were equally distributed in four 10 x 7.5cm mesh (1475 micron) bags containing 30 oysters each. Size out outplant was similar for all sites except the Central site where the Fidalgo Bay population was larger (see results). Trays were anchored into substrate using rebar stakes. In late autumn 2013, trays at Northern, Southern, and Central sites were subsequently suspended from floating structures to reduce exposure to extreme temperatures during tidal exchanges and oysters were removed from mesh bags. Trays remained anchored to the substrate submerged in a perched lagoon in the Hood Canal site as no suitable floating structure was available and oysters were removed from mesh bags.

Temperature Monitoring

At each site, temperature loggers (HOBOLogger, OnSet, USA) were deployed. Data from temperature loggers were collected at regular intervals and used for minimum and maximum observed temperature for each day using the statistical analysis programming language R (R 3.0.3, R Core Team, 2014) and package *plyr* (Wickham, 2014). The number of days above 20 °C

and below 5 °C was calculated for the duration of the project. Degree days (D) was calculated by adding the cumulative difference between the daily minimum temperature and the 2014 winter average minimum of 8 °C to determine the amount of energy needed to produce peak brooding activity. Raw temperature data and analysis procedures used are available (Heare et al., 2015). In addition, analysis procedures used (R code) can be found in Appendix B.

Mortality

Mortality was determined by counts of live and dead oysters during visits to each site. Survival rates were assessed at all sites in December 2013, January (Hood Canal site only due to mortality in December), February, April (Hood Canal and Central sites only), May (Northern and Southern sites only), and June 2014. At Hood Canal, evidence of oyster drill mortalities was observed and accounted for by counting number of shells with holes in them. Differences in mortality within sites were determined through a Mantel-Haenszel test comparing categorical live/dead counts at each sample point in each site for significant differences in the patterns of survival performed with the R package *survival* (Therneau, 2014). To account for oyster drill, *Ocenebrellus* sp. and *Urosalpinx* sp., mortalities we incorporated a general linear model with binomial distribution and corrected for overdispersion with the *dispmod* package (Scrucca, 2012) which corrects P-values based on chisquare values divided by degrees of freedom times the standard error for the factor. Mortality and drill predation data and analysis procedures used are available (Heare et al., 2015). In addition, analysis procedures used (R code) can be found in Appendix B.

Growth

Size was determined using ImageJ analysis (Rasband, 2010) of digitized images taken in August 2013 (all sites), March (Northern, Central, and Southern sites), April (Hood Canal site), May (Northern, Central, and Southern sites), September (Southern site), and October 2014 (Northern and Central sites). For each image, a size reference was measured along with all oysters. For all oysters, shell length (SL) was determined via a linear measurement of the longest distance from umbo to valve margin. Descriptive statistics (maximum size, minimum size, quartiles, standard deviation) were produced by the R package *pastecs* (Grosjean and Ibanez, 2014). Size distributions were tested for normality using the Shapiro-Wilkes test (*stats* package, R Core Team, 2014). To investigate significant differences between populations, sites, and population/site interaction we used a linear effects model with fixed effects being population and site and random effects being population by tray using the R package *lme4* (Bates et al., 2014) and P-values provided by the mixed function of the *afex* R package (Singmann, Bolker, & Walker, 2015). Shell length data from end of year one was compared using Kruskal-Wallis assuming non-normal distribution based on findings from Shapiro-Wilkes test (*stats* package, R Core Team, 2014). Pairwise comparisons for population by site were performed using the Nemenyi Post Hoc test, a joint rank sum test using information from Kruskal-Wallis to determine significant differences in rank, using Tukey assumptions (*PMCMR* package, Pohlert, 2014). Size data and analysis procedures used are available (Heare et al., 2015). In addition, analysis procedures used (R code) can be found in Appendix B.

Reproductive Activity

To assess reproductive activity, individual trays of oysters were anesthetized and each oyster was visually inspected for presence of brooding larvae in the mantle chamber. Specifically, trays were removed from water and exposed to air for 45 minutes then immersed in 0.3M magnesium

sulfate (heptahydrate sulfate mineral epsomite ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)) (also known as Epsom salt) dissolved in a 50/50 mix freshwater/sea water for 45 minutes. The counts of brooding oysters were determined on weekly basis over three months (May 14th - August 15th, 2014) for a total of 15 time point observations for each site with each brooding female recorded for the day and then measured using calipers. A different tray was checked for each population at each site in a rotation until all four trays for that population at that site had been checked. This was true for all sites except for the Southern site in which several trays were missing, the same tray was checked several weeks in a row until the missing trays were recovered at which point the tray rotation resumed. Following Hopkins (1937) observation of the daily minimum temperature spawning threshold for *O. lurida* of 12.5°C , we counted the number of days from the first date which reached this threshold to the date of the first brooding females observed and the maximum proportion of brooding females. The proportion of brooding females per site per visit were arcsine transformed to improve normality of proportions and analyzed via Two Way ANOVA (*base* package, R Core Team, 2014). Significant differences among sites, populations, and site/population pairwise comparisons were determined using TukeyHSD (*base* package, R Core Team, 2014). Sizes at brooding were likewise compared via Two Way ANOVA and TukeyHSD to explore population, site, and population by site differences (*base* package, R Core Team, 2014). Female brooding data and analysis procedures used are available (Heare et al., 2015). In addition, analysis procedures used (R code) can be found in Appendix B.

Results

Site Characteristics

The Southern site had the highest daily minimum temperature (18.43°C) (Figure 2) in August 2014 while the Hood Canal site had the lowest daily minimum temperature (-3.32°C) during

February 2014 (Figure 2). The Hood Canal site experienced the highest amount of temperature variability due to the intertidal placement of samples and the extreme cold weather during low tide events (Figures 2 & 3). From June to August 2014, the Southern site experienced warmer daily temperatures as compared to all other sites (Figures 2 & 3).

Survival

Differences in mortality per population were observed at three of the four sites. Dabob Bay oysters had significantly less mortality by the end of the study period at Hood Canal ($X^2=141$, $df=2$, $P<0.0001$), Southern ($X^2=76.3$, $df=2$, $P<0.0001$), and Central sites ($X^2=13.7$, $df=2$, $P=0.00105$) (Figures 4A, 4B, & 4C) than other populations.

The Hood Canal site location experienced unexpected elevated mortality, necessitating the premature termination of the Hood Canal site trial in April 2014. Evidence of high oyster drill related mortalities was observed at Hood Canal and it was found that the Fidalgo population experienced significantly more drill related mortalities (~48% of Fidalgo population as compared to ~28% of the Dabob population and ~29% of the Oyster Bay population) (GLM, $X^2 =6.2$, $df=6$, $P<0.0165$). There were significant differences in mortality among populations ($X^2=141$, $df=2$, $P=0$), with the Fidalgo Bay oysters having the lowest survival (21.2% +/- 2.1SD %) (Figure 4C). Limited mortality was observed at both the Central and Northern site where at least 80% of oysters remained after 11 months (July 2014) (Figures 4B & 4D).

Growth

Oyster mean size at outplant was 11.4 (+/-3.2SD) mm and with no differences in size among population except for the Central site where the Fidalgo population was larger (Figure 9). At the end of the experiment the size of oysters among sites were significantly different (LME $F=268.29$, $df=2$, $P<0.0001$ & Kruskal-Wallis, $X^2=383.4$, $df=2$, $P<0.0001$), with the Southern site producing the largest oysters (Figure 5: Figure 10) and Central site producing the smallest (Figure 7: Figure 9). Oyster size also differed among populations ((LME $F=86.42$, $df=2$, $P=0.007$ & Kruskal-Wallis, $X^2=196.1$, $df=2$, $P<0.0001$). The linear model also indicated that the interaction between populations and sites was significant (LME $F=23.34$, $df=4$, $P<0.0001$). At the Southern site, Fidalgo Bay oysters were larger than Dabob (Nemenyi Post-Hoc, $P<0.0001$) and Oyster Bay (Nemenyi Post-Hoc, $P<0.0001$) oysters (Figure 5). Based on integrated size data from throughout the experiment it is apparent this difference arose during Summer months (Figure 10). At the Northern site, oysters from Dabob Bay broodstock were smaller than Fidalgo Bay (Nemenyi Post-Hoc, $P<0.0001$) and Oyster Bay (Nemenyi Post-Hoc, $P<0.0001$) oysters at the end of the experiment (Figures 6: Figure 8). Similar results were also observed at the Central site, however as indicated outplant size was different. At the Central site, while the Oyster Bay and Dabob oysters started at the same size, the Oyster Bay oysters did end up larger than the Dabob oysters by the end of the experiment (Nemenyi Post-Hoc, $P=0.00028$) (Figure 7: Figure 9).

Brooding Females

The proportions of brooding females varied among populations (ANOVA, $F=9.1$, $df=2$, $P=0.0002$) and among sites (ANOVA, $F=11.4$, $df=2$, $P<0.0001$). The greatest proportion of total brooding females present was at the Southern site (Figure 11) compared to the Northern

($P=0.007$) and Central sites ($P<0.0001$). The smallest proportion of brooding females was documented at the Central site (Figure 13). The Oyster Bay population produced significantly more brooding females than Fidalgo Bay (Tukey's HSD, $P=0.001$) or Dabob Bay (Tukey's HSD, $P=0.0005$) populations. The Fidalgo and Dabob Bay populations were not different from one another at all sites (Tukey's HSD, $P=0.942$).

The Southern site reached the spawning temperature threshold of 12.5°C (as defined by Hopkins, 1937) on May 14th and the first brooding female was observed 15 days later on May 29th (Figure 11). Ambient water temperatures in the Southern site rose steadily from late winter reaching the spawning threshold and continuing to increase to the summer maximum of 18.43°C (Figure 11). At the Southern site, Oyster Bay oysters reached the maximum percentage of brooding females on June 19th, 36 days post 12.5°C , equating to 308 °D. At this location, Dabob Bay and Fidalgo Bay oyster populations reached the maximum percentage of brooding females on July 10th, 57 days post 12.5°C , 453 °D (Figure 11).

At the Northern site, the 12.5°C temperature was also reached on May 14th and the first brooding female was observed on June 6th (Figure 12), 23 days later. The Northern site exhibited a slower, less steady temperature increase throughout the spring season with ambient water temperatures reaching 12.5°C in mid-May but then dipping into the $10\text{-}11^{\circ}\text{C}$ range until early June, after which the site remained above the threshold for the remainder of the summer (Figure 12). The Oyster Bay oysters in the Northern site reached maximum percentage brooding females by July 11th, 58 days later or 354 °D. Fidalgo Bay and Dabob Bay oysters' populations did not reach maximum percentage brooding females observed until August 8th (Figure 12), 87 days later or 513 °D.

The Central site reached 12.5°C on June 8th and brooding females were observed on June 18th from the Oyster Bay population (Figure 13), 10 days later. Temperatures in the Central site

reached 12.5 °C in early June but varied above and below this temperature for several days at a time throughout most of summer (Figure 13). Peak spawning could not be determined due to low number of brooding individuals observed at the Central site.

Size at brooding varied significantly among populations (ANOVA, $F=18.2$, $df=2$, $P<0.0001$) and sites (ANOVA, $F=33.1$, $df=2$, $P<0.0001$) with the smallest brooding females observed at the Central site (Figure 14). Size at brooding by population was significantly different between all populations. Size was significantly smaller at the Central site compared the other sites (Northern site (Tukey's HSD, $P<0.0001$), Southern site (Tukey's HSD, $P<0.0001$)). No differences in size of brooding females was observed between Southern site and Northern site ($P=0.8$). The average minimum size at brooding of the ten smallest oysters was 19.1(+/-3.7SD) mm. Two brooding females of 15.0 mm were observed at the Central site from the Dabob Bay population. The overall average size of brooding females was 27.1 (+/- 4.5SD) mm.

Discussion

A primary objective for this study was to evaluate population performance in relation to stock structure of *Olympia* oysters in Puget Sound, WA. Findings from this study provided new information about *Ostrea lurida* life history as well as distinct phenotypes associated with populations. At the population level, we found some populations favor survival over other traits and some populations favor reproduction suggesting the existence of adaptive structure within Puget Sound, WA. In the remainder of this section, findings from this study are discussed in terms of differences in sites, differences in population performance, and implications of these findings with respect to restoration efforts.

Site Differences

Mortality

Mortality rates were different across sites, and likely influenced by temperature and predation. The Hood Canal site experienced the highest mortality rates, experiencing temperature extremes and predation as evidenced by prevalent holes likely caused by oyster drills, *Urosalpinx* sp. and *Ocenebrellus* sp., and direct observations of these gastropod. Interestingly there was a difference in susceptibility in the population to drill predation (see below). The Hood Canal site exceeded the temperature range reported by Baker (1995) on 35% of the total days (85 out of 242 days) with two subfreezing events of -0.78°C and -3.3°C in December 2013 and February 2014 respectively (Figure 2). The Southern site, which also experienced moderate mortality, had a total of 39 days (9% of 398 days) outside of the $5\text{-}20^{\circ}\text{C}$ range. The majority (34 days) were above the upper limit (20°C) but not near the lethal temperature (LT50) of 39°C reported by Brown et al. (2004). The Northern and Central site had fewer days outside of the range (24 days and 0 days respectively) and had low mortality. The role of temperature as a primary determinant of survival when oysters are transplanted outside of their broodstock populations range is similar to its role as found by Burford et al. (2014).

Growth

In the present study, Olympia oysters attained an average size of $35.8 (+/-6.4\text{SD})$ mm during the first year of growth. Some individuals attained sizes >45 mm. These observations differ from the 2-3 years needed to attain this size in *O. lurida* reported by Hopkins (1937). This discrepancy

could be due to changes in environmental conditions present at the site or differences of the population sampled.

A difference in size occurred in relation to site. Oysters from all populations at the Southern site grew to the largest size and experienced the warmest temperatures year round. This finding is in accord with other studies (e.g. Malouf and Breese, 1977; Brown and Hartwick, 1988; Shpigel et al., 1992) that demonstrate that warm temperatures improve oyster growth as long as the temperatures are within the tolerable range.

Reproduction

Oysters reproduced as females in Puget Sound at a mean size of 27.1 (+/- 4.5SD) mm. This result contrasts with results of previous research (Hopkins, 1937; Coe, 1932 a&b) that describe *O. lurida* as only reproductive at sizes of 30 mm or greater. The ability to reproduce at smaller sizes is important because it may provide reproductive advantage.

It has been generally accepted that *O. lurida* begin spawning at relatively low temperatures (13 °C Coe, 1931a; 12.5 °C Baker, 1995). Hopkins (1937) suggested that this temperature threshold must occur during high tide, which is related to the daily minimum temperature. In accordance with these earlier studies, we found at all sites brooding only occurred after daily minimum temperatures increased above 12.5 °C. The steady increase in temperature as observed in the present study in the Southern site may have allowed *O. lurida* to spawn much earlier in the season than at other sites (Figures 11, 12, & 13).

By comparing the reproductive initiation and peak brooding observed to observations by Hopkins (1937) in the same area, it appears that the reproductive period occurred approximately

two weeks later in 2014 than in 1932-1933. Further investigation is required to determine if this is simple natural variation or an important change to the spawn timing in the region.

Population Differences

Mortality

Survival differed among populations within 3 out of 4 sites. The population derived from Dabob Bay broodstock exhibited better survival than the other two populations (Figure 4). The observed temperature variability (Figures 2 & 3) at the Hood Canal site in the present study may be indicative of historic temperature trends to which the parent populations were exposed. If so, the significantly greater survival of the Dabob Bay population at three of the four sites could be a function of increased stress resilience of offspring in response to prevalent temperature extremes. Previous studies on thermal tolerance, (e.g. bay scallops, *Argopecten irradians*, (Brun et al., 2008) and Mediterranean mussels, *Mytilus galloprovincialis*, (Dutton and Hofman, 2009)) demonstrate more frequent exposure to temperature extremes result in elevated heat shock proteins (HSP) and HSP mRNA transcripts. In addition, Sørensen et al. (2004) found that many species exhibit heritable heat shock protein production patterns. The higher survival rates observed in the Dabob Bay population may likewise be related to heritable traits and warrants investigation.

Predation was also a factor in population specific survival, at least at Hood Canal where oyster drills were prevalent. Interestingly the Fidalgo Bay population had higher mortality attributed to oyster drills at this location. This may be indicative of the population being free of drill predation

at their homesite. Related, populations from Dabob and Oyster Bay may have been selected for less susceptibility having persisted in environments with oyster drills. The mechanism associated with susceptibility is not known though could be related to shell thickness or metabolic signatures.

Growth

At all transplant sites, the population derived from Dabob Bay parents exhibited the lowest growth. This observation is likely related to the fact the Dabob Bay population also had the highest survival. Applebaum et al. (2014) found energetic tradeoffs may improve survival over growth in the Pacific oyster, *C. gigas*. Arendt (1997) suggested that “stress tolerators” exhibit slower intrinsic growth that is relatively unresponsive to improved conditions. Further investigation is required to determine the links between growth, energetic tradeoffs, and environmental variables affecting *O. lurida*.

Reproduction

The Oyster Bay population had a greater proportion of brooding females and reached peak spawning earlier than the other populations, across all sites. One explanation for this is that the relatively rapid water temperature increase and higher temperatures in south Puget Sound may have selected for early spawning oysters in the Oyster Bay population. Evidence for this includes the fact that it took 150 fewer °D for the Oyster Bay population to reach peak spawning compared to the other two populations at two sites. The general rate of temperature increase at a particular locale may influence spawn timing (Lawrence and Soame, 2004). Chávez-Villalba et al. (2002) found place of origin for *C. gigas* broodstock affected the rate of gametogenesis under

different temperatures with some populations becoming reproductively active sooner than others do. Barber et al. (1991) found gametogenesis and spawn timing were heritable traits within populations of *C. virginica*.

Conclusions

Differences in life history traits among *Ostrea lurida* populations grown in different locations within Puget Sound, WA suggest adaptations possibly linked with environmental cues. High survival, low growth, and low reproductive activity of the Dabob Bay population is likely due to extreme environmental variation at their home site leading to improved stress resilience. The greater proportion of brooding females in the Oyster Bay population and reduced environmental energy (D) needed to induce peak spawning may be related to positive selection pressure for early spawners due to warmer temperature trends at their home site. Findings from this study indicate possible local adaptation in two of the three populations observed but there may be other factors dictating observed phenotypes.

While findings from this study certainly could be indicative of local adaptation, it should be pointed out that there could be other explanations for our observations. Given the nature of larval dispersal, for one, we do not know that the oysters used as broodstock were from parents from that environment. Thus the traits could be a result of selection in a different habitat. Along the same lines of assuming larval dispersion from a separate source population, negative selection could have taken place. For example, barnacle species have shown significant differences in stress tolerance phenotypes related to settlement upon either upper or lower intertidal but this is due to nascent stress tolerance within an individual and not representative of the population as a

whole (Sanford and Kelly, 2011). Another possible explanation of the different traits observed for each population is that this could be a result of limited effective population size, or number of successful pairings during spawning. In other words, if too few parents existed there could be a significant family effect and/or inbreeding depression, a phenomenon that has been reported in aquaculture of *C. gigas* (Camara et al., 2008) and discussed as a potential issue for *O. lurida* restoration by Camara and Vadopalas (2009). Future genotyping and parentage analysis will be able to answer this question.

While we cannot conclusively demonstrate a mechanism of local adaptation in this study, our results certainly have important implications for restoration of *O. lurida* within Puget Sound, WA. There are a number of ways that these findings could be used in generating restoration strategies specific to Puget Sound and in the face of climate change. Based on the fact that Dabob Bay oysters had the lowest mortality, use of the most robust population for broodstock may increase chances for outplant survival. Generally, this approach would dictate organisms should be used from home environments that experience persistent stressful conditions. An alternative approach managers might take given the current findings is to take the population with the greatest reproductive output (Oyster Bay) and use it as a source of broodstock. This would increase the likelihood of juvenile recruitment and ultimate restoration of the species, while also producing more offspring for outplant. Interestingly, at this time habitats are facing environmental shifts imposed by climate change and ocean acidification. Having a strong understanding of population related phenotypes creates another option for restoration efforts. An assisted gene flow strategy that incorporates the outplanting of populations known to contain phenotypes fit for the new environmental parameter and have them interbreed with resident populations (Aitken and Whitlock, 2013). It is highly debated whether such a strategy would

have benefits that outweigh the drawbacks, such as possible outbreeding depression, but should be considered for restoration efforts facing a variety of climate change scenarios. It should also be pointed out, regardless of the process resulting in the different phenotypes, we do not know whether phenotypes are firmly held in each population. Due to factors including plasticity and epigenetic phenomena, these traits could be lost over time.

Ultimately, what this study demonstrates is that population structure can and does exist on a relatively small geographic scale and thus moving oyster populations to locations where remnant stocks exist could be disadvantageous. When population structure exists, there should be concern with respect to moving populations as: 1) transplanted populations could overwhelm locally adapted remnant resident populations, and possibly not persist themselves, 2) transplanted populations might not survive in new location and thus wasting resources required for restoration, and 3) transplanted populations could interbreed with remnant population and thus result in overall reduced fitness through outbreeding depression. Many of these implications make assumptions regarding plasticity and adaptive potential, though we still know little about this in marine invertebrates, particularly on the temporal and geographic scales involved.

Acknowledgements

The authors thank Puget Sound Restoration Fund for collecting, conditioning, broodstock, settling larvae, and seed husbandry prior to outplant for this experiment. The authors appreciate field assistance provided by L. Christine Savolainen, J. Allen, S. Bennett, J. Blais, S. Adams, C. Olson, S. White, M. Gavery, H. Wear, K. Jackson, D. Immerman, A. Jasonowicz, A. Godersky, J. Stevick, E. Maipi, J. Richards, and M. Hedberg. The authors also thank the following for

allowing them to deploy equipment on their property: Crab Fresh Inc., The Fagergren Family, Fidalgo Marina, Rock Point Oyster Company, and Taylor Shellfish.

This work was funded from Washington Sea Grant, University of Washington, pursuant to National Oceanographic and Atmospheric Administration Award No. NA10OAR4170057 Projects R/LME/N-3. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub-agencies.

References

- Aitken SN., & MC. Whitlock. 2013. Assisted Gene Flow to Facilitate Local Adaptation to Climate Change. *Annual Rev. of Eco., Evol., and Sys.* 44:367–388.
- Anderson, P. 1995. Ecological restoration and creation: a review. *Biol. J. of the Linnaean Soc.* 56: 187–211. doi:10.1111/j.1095-8312.1995.tb01133.x
- Applebaum, S.L., Pan, T.-C.F., Hedgecock, D., & D.T. Manahan. 2014. Separating the nature and nurture of the allocation of energy in response to global change. *Integr. Comp. Biol.* icu062. doi:10.1093/icb/icu062
- Arendt, J.D. 1997. Adaptive intrinsic growth rates: An integration across taxa. *The Q. Rev. of Biol.* 72: 149–177.
- Baker, P. 1995. Review of ecology and fishery of the Olympia oyster, *Ostrea lurida*, with annotated bibliography. *J. of Shellfish Res.* 14: 503–518.
- Barber, B.J., Ford, S.E., & R.N. Wargo. 1991. Genetic variation in the timing of gonadal maturation and spawning of the eastern oyster, *Crassostrea virginica* (Gmelin 1791). *Biol. Bull.* 181: 216–221.
- Bates, D., Maechler, M., Bolker, B., & S. Walker. 2014. lme4: Linear mixed-effects models using Eigen and S4_. R package version 1.1-7, <URL:http://CRAN.R-project.org/package=lme4>.
- Brown, H. M., A. Briden, T. Stokell, F. J. Griffin, & G. N. Cherr. 2004. Thermotolerance and Hsp70 profiles in adult and embryonic California native oysters, *Ostreola conchaphila* (Carpenter, 1857). *J. of Shellfish Res.* 23: 135-141.
- Brown, J.R. & E.B. Hartwick. 1988. Influences of temperature, salinity and available food upon suspended culture of the Pacific oyster, *Crassostrea gigas*: I. Absolute and allometric growth. *Aquaculture* 70, 231–251. doi:10.1016/0044-8486(88)90099-3

- Brun, N.T., Bricelj, V.M., MacRae, T.H., & N.W. Ross. 2008. Heat shock protein responses in thermally stressed bay scallops, *Argopecten irradians*, and sea scallops, *Placopecten magellanicus*. *J. Exp. Mar. Biol. Ecol.* 358: 151–162 doi:10.1016/j.jembe.2008.02.006
- Burford, M.O., Scarpa, J., Cook, B.J., & M.P. Hare. 2014. Local adaptation of a marine invertebrate with a high dispersal potential: evidence from a reciprocal transplant experiment of the eastern oyster *Crassostrea virginica*. *Mar. Ecol. Prog. Ser.* 505: 161–175. doi:10.3354/meps10796.
- Camara MD., Evans S., & CJ. Langdon. 2008. Parental Relatedness and Survival of Pacific Oysters from a Naturalized Population. *J. of Shellfish Res.* 27:323–336.
- Camara, M.D. & B. Vadopalas. 2009. Genetic aspects of restoring Olympia oysters and other native bivalves: Balancing the need for action, good intentions, and the risks of making things worse. *J. of Shellfish Res.* 28: 121–145 doi:10.2983/035.028.0104
- Chávez-Villalba, J., Pommier, J., Andriamiseza, J., Pouvreau, S., Barret, J., Cochard, J.-C., & M. Le Penec. 2002. Broodstock conditioning of the oyster *Crassostrea gigas*: origin and temperature effect. *Aquaculture* 214: 115–130. doi:10.1016/S0044-8486(01)00898-5
- Coe, W.R. 1932a. Season of attachment and rate of growth of sedentary marine organisms at the pier of the Scripps Institution of Oceanography, La Jolla, California. *Scripps Inst. Oceanog. Tech. Ser.* 3: 37-86.
- Coe, W.R., 1932b. Development of the gonads and the sequence of the sexual phases in the California oyster (*Ostrea lurida*). *Bull. Scripps Instn. Oceanogr. Tech.* 3: 119-144.
- Davis, H.C. 1955. Mortality of Olympia oysters at low temperatures. *Biol. Bulletin* 109: 404–406. doi:10.2307/1539172
- Dutton, J.M. & G.E. Hofmann. 2009. Biogeographic variation in *Mytilus galloprovincialis* heat shock gene expression across the eastern Pacific range. *J. of Exp. Mar. Biol. and Ecol.* 376: 37–42. doi:10.1016/j.jembe.2009.06.001
- Grosjean, P. & F. Ibanez. 2014. pastecs: Package for Analysis of Space-Time Ecological Series. R package version 1.3-18. <http://CRAN.R-project.org/package=pastecs>
- Heare, J., Vadopalas, B. & S.B. Roberts. 2015. OluridaSurvey2014: UW Manuscript. Zenodo. doi:10.5281/zenodo.34143
- Hopkins, A.E. 1937. Ecological observations on spawning and early larval development in the Olympia oyster (*Ostrea lurida*). *Ecology* 17: 551–566. doi:10.2307/1932760
- Kawecki, T.J. & D. Ebert. 2004. Conceptual issues in local adaptation. *Ecology Ltrs.* 7:1225–1241.
- Lawrence, A.J. & J.M. Soame. 2004. The effects of climate change on the reproduction of coastal invertebrates. *Ibis* 146: 29–39. doi:10.1111/j.1474-919X.2004.00325.x

- Lotze, H.K., Coll, M., Magera, A.M., Ward-Paige, C., & L. Airoidi. 2011. Recovery of marine animal populations and ecosystems. *Trends Ecol. Evol.* 26: 595–605. doi:10.1016/j.tree.2011.07.008
- Malouf, R.E. & W.P. Breese. 1977. Seasonal changes in the effects of temperature and water flow rate on the growth of juvenile Pacific oysters, *Crassostrea gigas* (Thunberg). *Aquaculture* 12: 1–13. doi:10.1016/0044-8486(77)90042-4
- McKay, J.K., Christian, C.E., Harrison, S., & K.J. Rice. 2005. “How Local Is Local?”—A review of practical and conceptual issues in the genetics of restoration. *Restoration Ecol.* 13: 432–440. doi:10.1111/j.1526-100X.2005.00058.x
- Palumbi, S.R., Grabowsky, G., Duda, T., Geyer, L., & N. Tachino. 1997. Speciation and Population Genetic Structure in Tropical Pacific Sea Urchins. *Evolution* 51: 1506–1517. doi:10.2307/2411203
- Pohlert, T. 2014. PMCMR: Calculate Pairwise Multiple Comparisons of Mean Rank Sums. R package.
- R Core Team. 2014. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Rasband, W. 2010. ImageJ Image Processing Program. National Institute of Health, MD, USA.
- Sanford, E. & M.W. Kelly. 2011. Local adaptation in marine invertebrates. *Annu. Rev. of Mar. Sci.* 3: 509–535. doi:10.1146/annurev-marine-120709-142756
- Scrucca, L. 2012. dispmod: Dispersion models. R package version 1.1. <http://CRAN.R-project.org/package=dispmod>
- Shpigel, M., Barber, B.J., & R. Mann. 1992. Effects of elevated temperature on growth, gametogenesis, physiology, and biochemical composition in diploid and triploid Pacific oysters, *Crassostrea gigas* Thunberg. *J. of Exp. Mar. Biol. and Ecol.* 161: 15–25. doi:10.1016/0022-0981(92)90186-E
- Singmann H., Bolker B., & J. Westfall. 2015. afex: Analysis of Factorial Experiments. R package version 0.13-145. <http://CRAN.R-project.org/package=afex>
- Sørensen, J.G., Kristensen, T.N., & V. Loeschcke. 2003. The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* 6: 1025–1037. doi:10.1046/j.1461-0248.2003.00528.x
- Therneau, T. 2014. A Package for Survival Analysis in S_. R package version 2.37-7, URL: <http://CRAN.R-project.org/package=survival>.
- Wickham, H. 2011. The Split-Apply-Combine Strategy for Data Analysis. *Journal of Statistical Software*, 40(1), 1-29. URL <http://www.jstatsoft.org/v40/i01/>.
- Wickham, H. 2014. ggplot2: elegant graphics for data analysis. Springer New York, 2009.

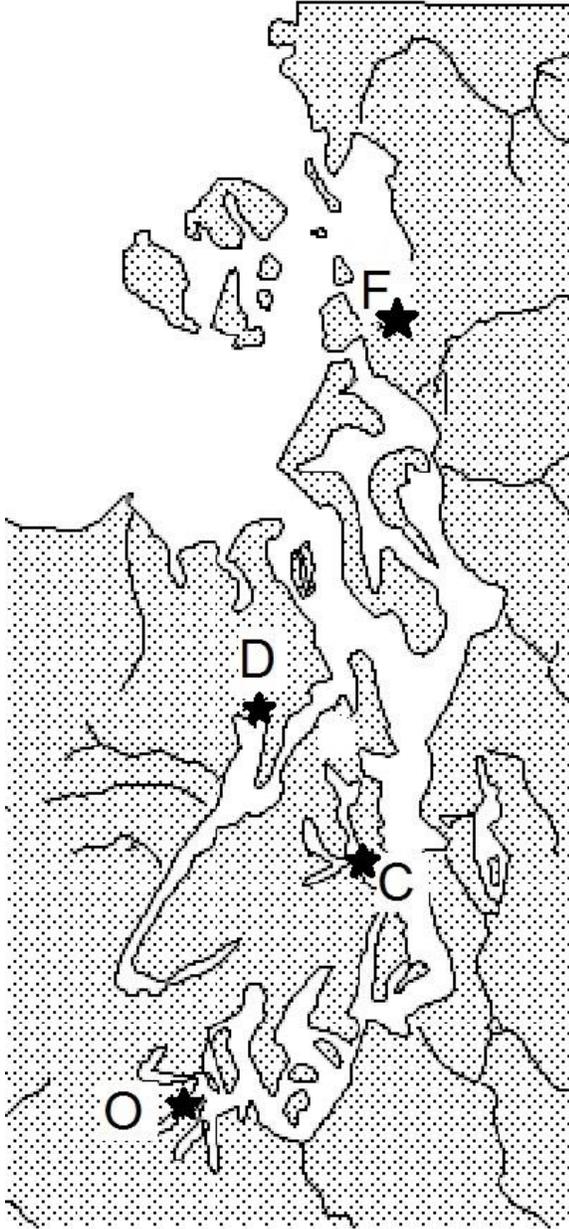
Figures

Figure 1. Map of Puget Sound with *Ostrea lurida* broodstock and outplant sites. Conditioning site was Port Gamble (G). Broodstock collected from Fidalgo Bay (F), Dabob Bay (D), and Oyster Bay (O). Outplanted at Fidalgo Bay also known as the Northern site (F), Dabob Bay also known as the Hood Canal Site (D), Clam Bay also known as the Central site (C), and Oyster Bay also known as the Southern site (O).

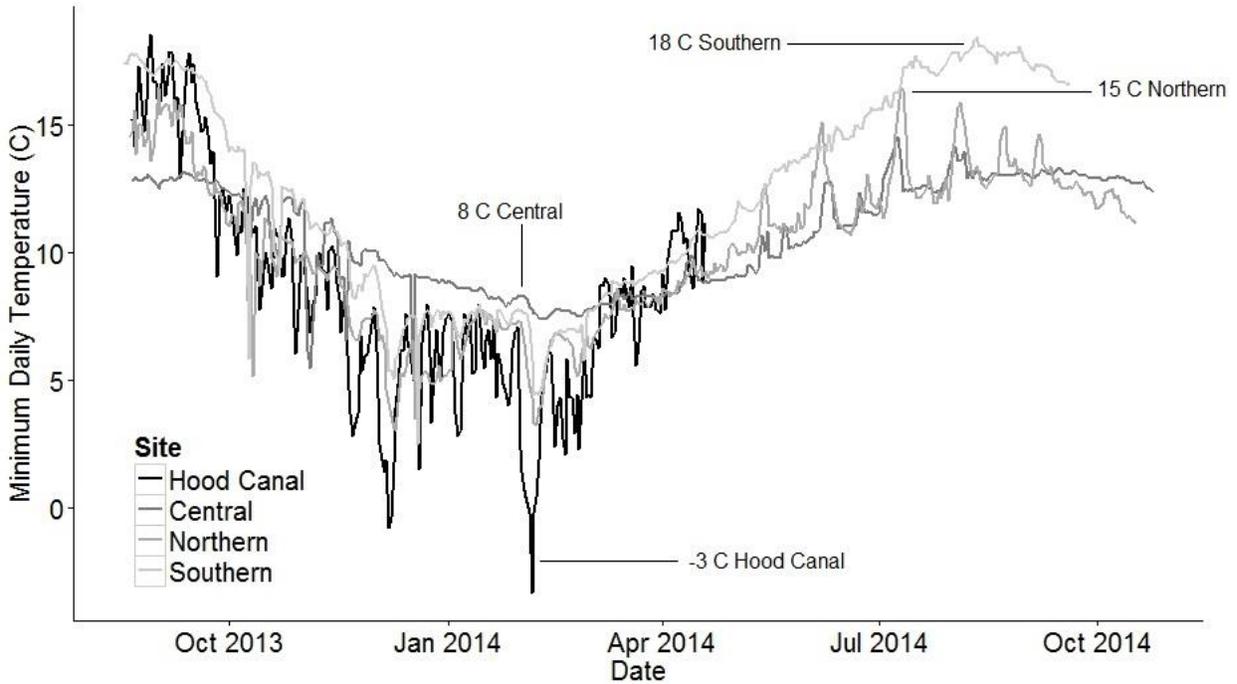


Figure 2. Minimum observed daily temperatures for all sites.

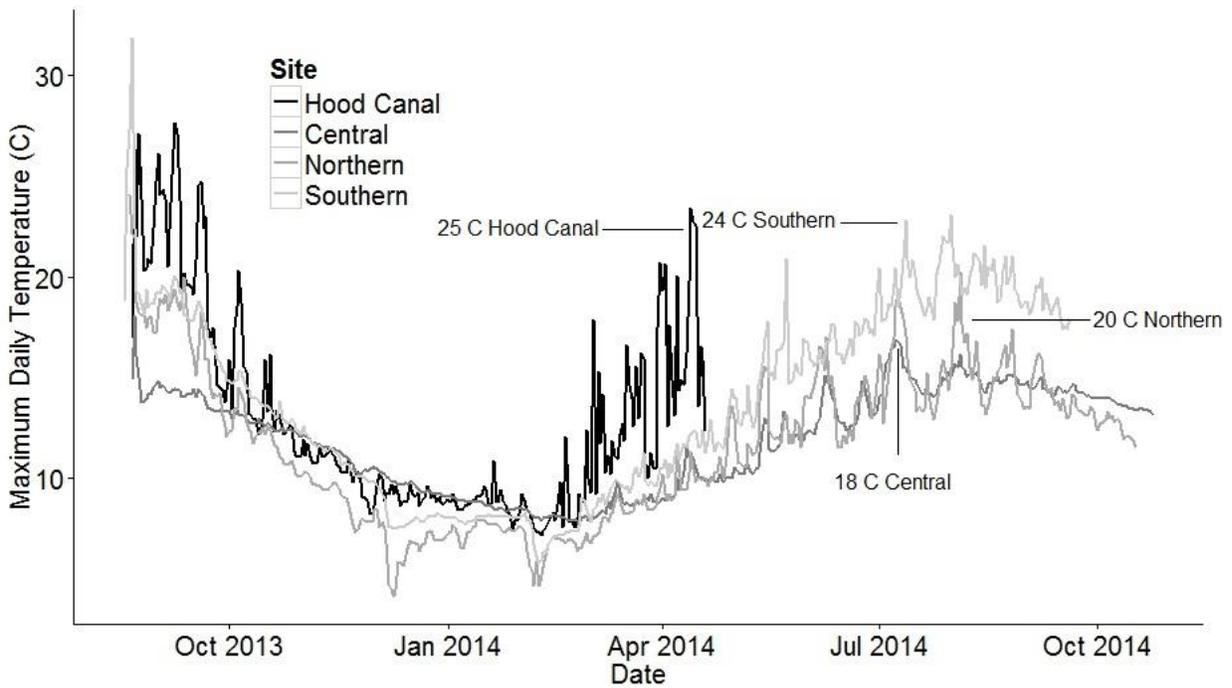


Figure 3. Maximum observed daily temperatures for all sites.

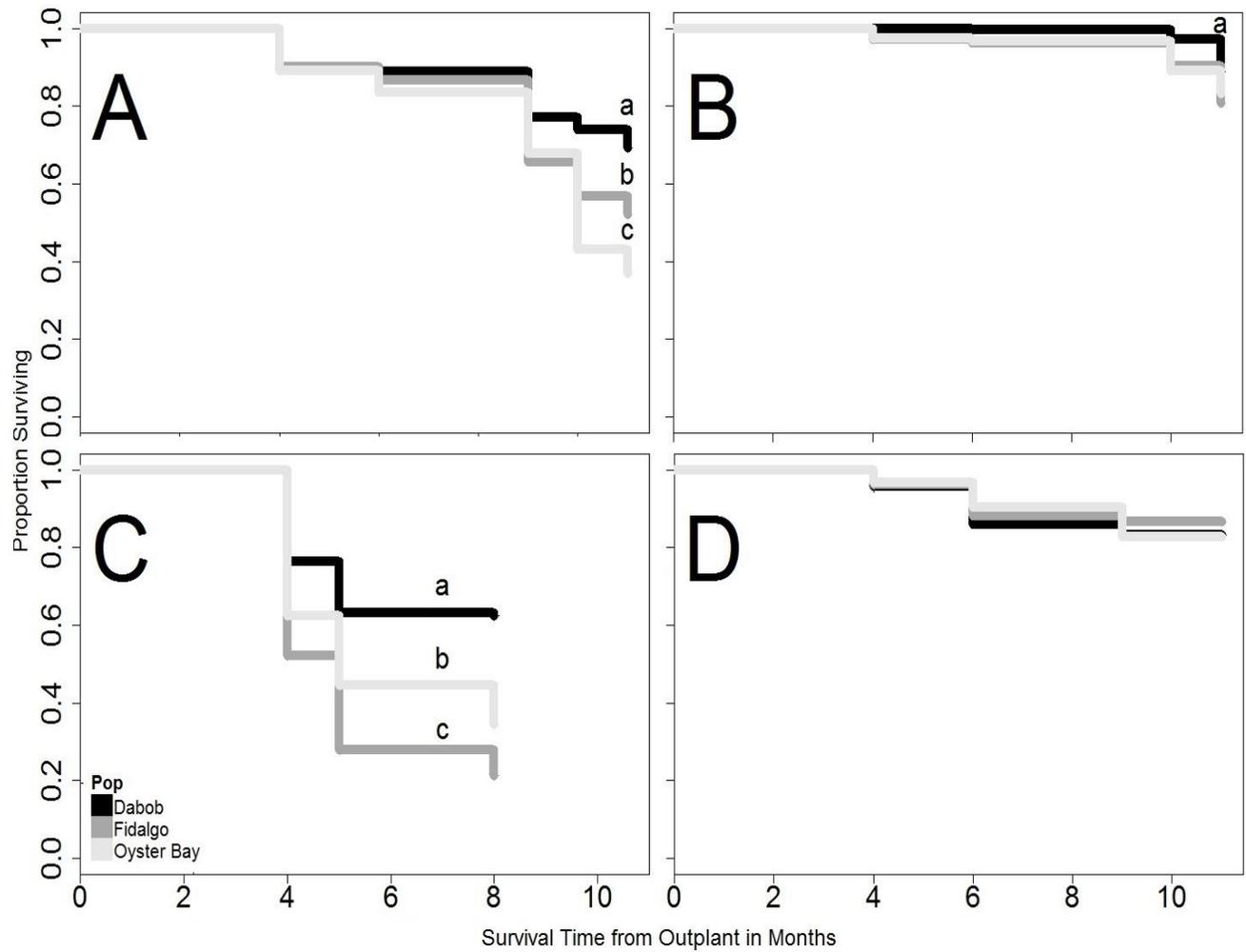


Figure 4. Proportion survival for three *Ostrea lurida* populations at four locations; Southern site (A), Central site (B), Hood Canal site (C), and Northern site (D). Lowercase letters (a, b, c) are significant differences.

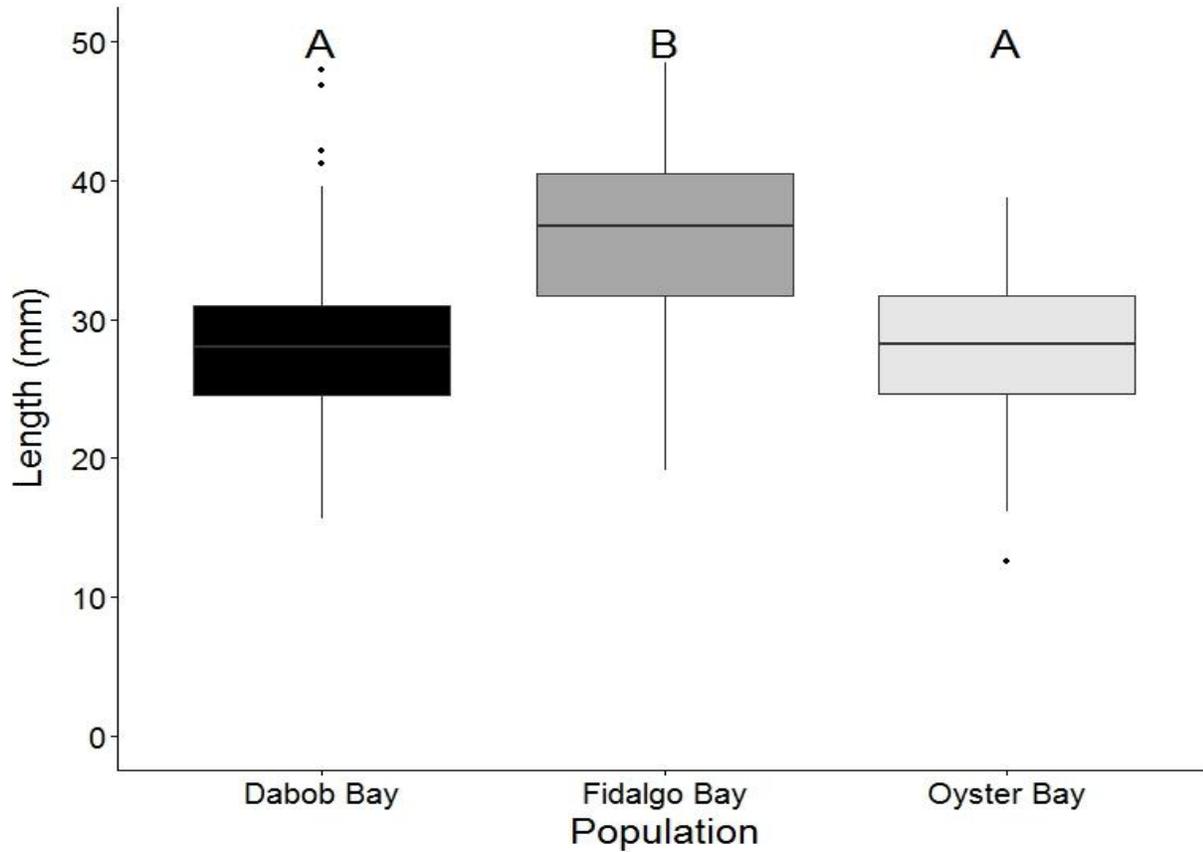


Figure 5. *Ostrea lurida* shell length in September 2014 at Southern site. Boxplots with mean SL as central line and boxes represent second and third quartile. Horizontal lines are 1st and 4th quartile with dots representing outliers from data set. Letters indicate significant differences. Fidalgo Bay oysters were considered different due to Nemenyi Post Hoc test with $P < 0.0001$ (Oyster Bay and Dabob Bay oysters).

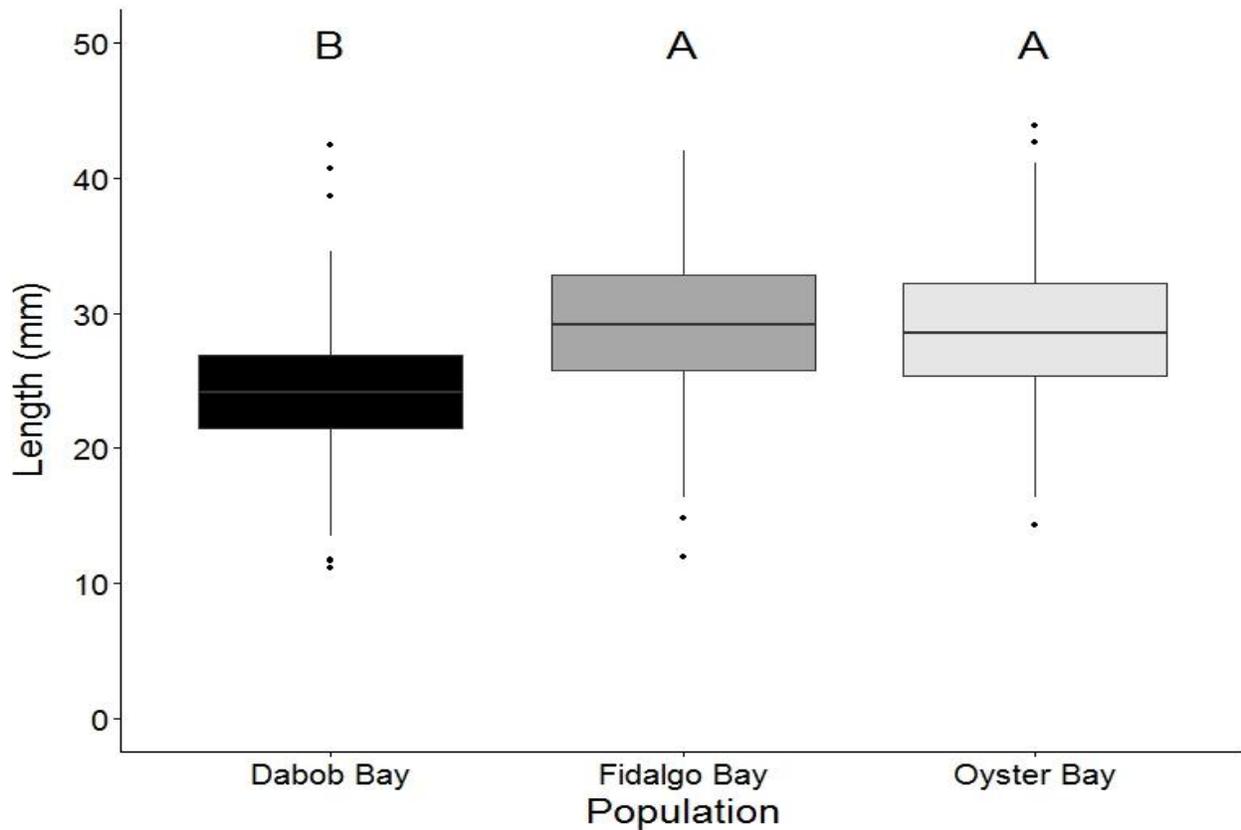


Figure 6. *Ostrea lurida* shell length in October 2014 at Northern site. Boxplots with mean SL as central line and boxes represent second and third quartile. Horizontal lines are 1st and 4th quartile with dots representing outliers from data set. Letters indicate significant differences. Dabob Bay oysters were considered different due to Nemenyi Post Hoc test with $P < 0.0001$ (Fidalgo Bay and Oyster Bay oysters).

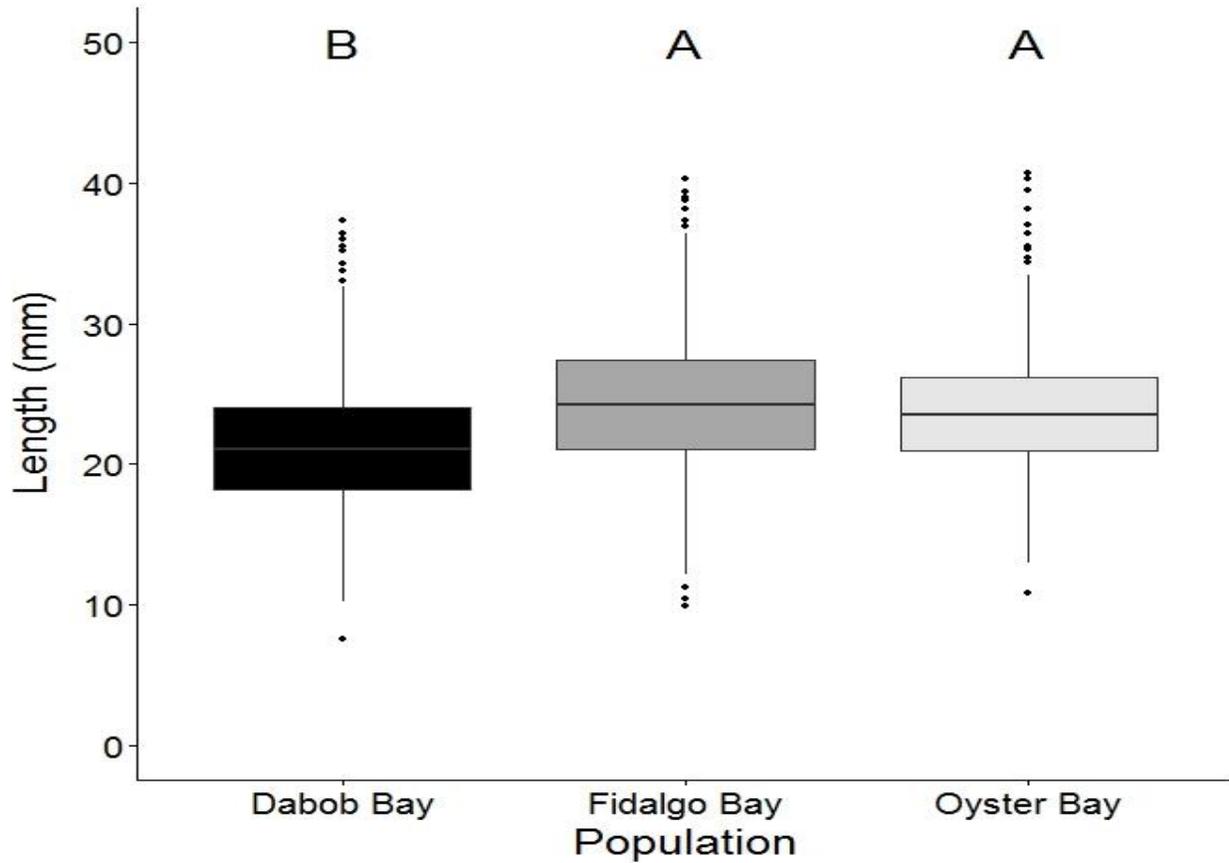


Figure 7. *Ostrea lurida* shell length in October 2014 at Central site. Boxplots with mean SL as central line and boxes represent second and third quartile. Horizontal lines are 1st and 4th quartile with dots representing outliers from data set. Letters indicate significant differences. Dabob Bay oysters were considered different due to Nemenyi Post Hoc test with $P=0.00028$ (Oyster Bay oysters) and $P<0.0001$ (Fidalgo Bay oysters).

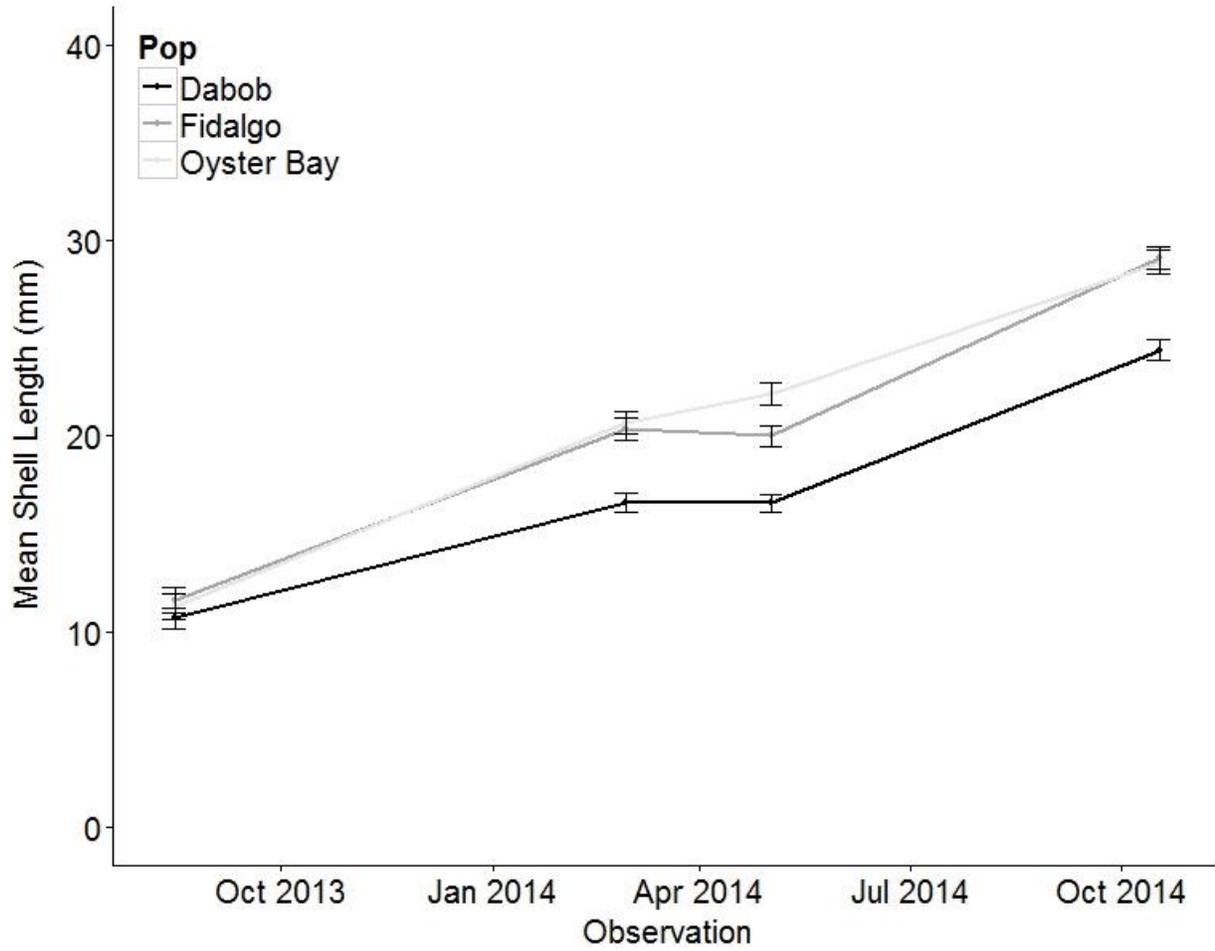


Figure 8. Growth rate of mean shell length in *Ostrea lurida* outplanted at Northern site. Error bars indicate 95% confidence intervals at each time point.

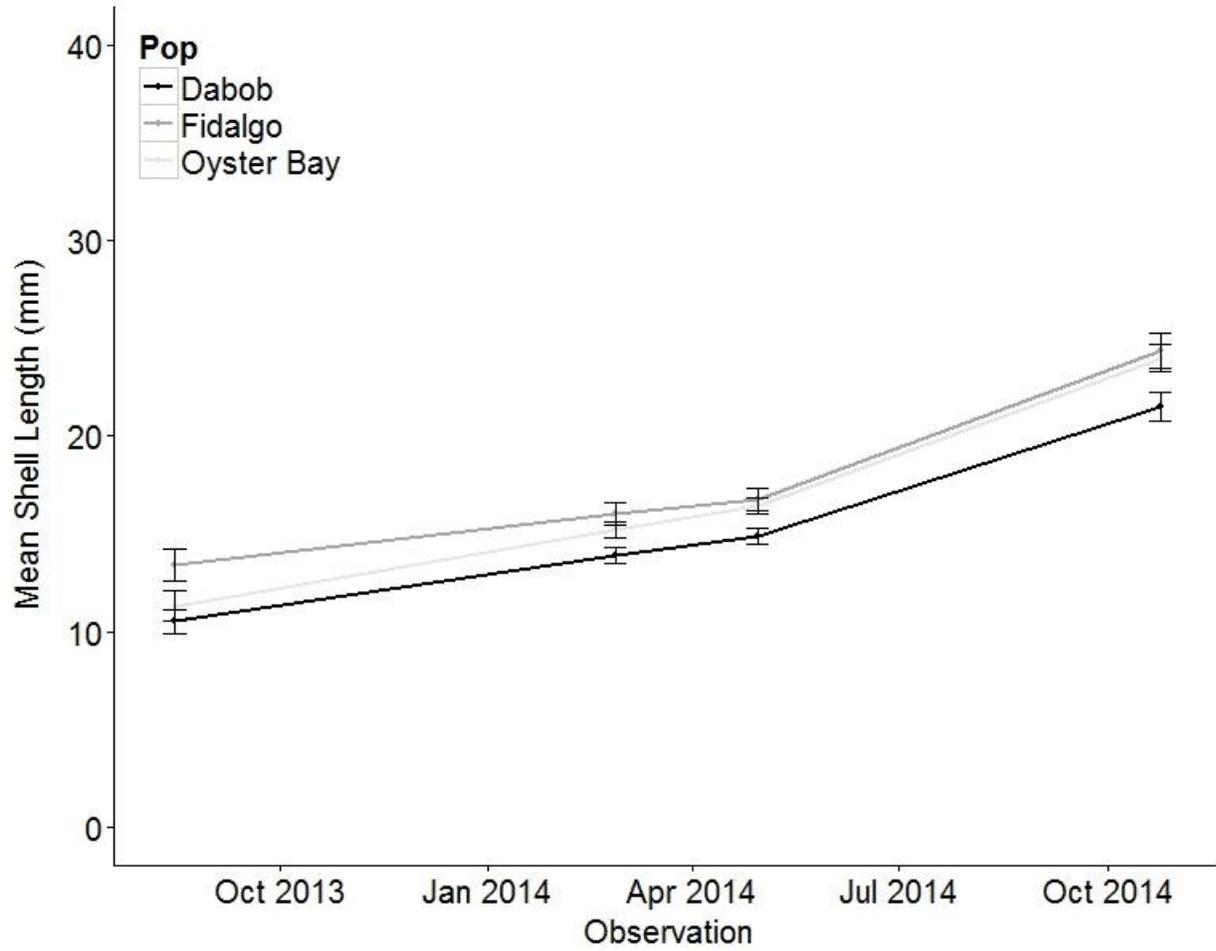


Figure 9. Growth rate of mean shell length in *Ostrea lurida* outplanted at Central site. Error bars indicate 95% confidence intervals at each time point.

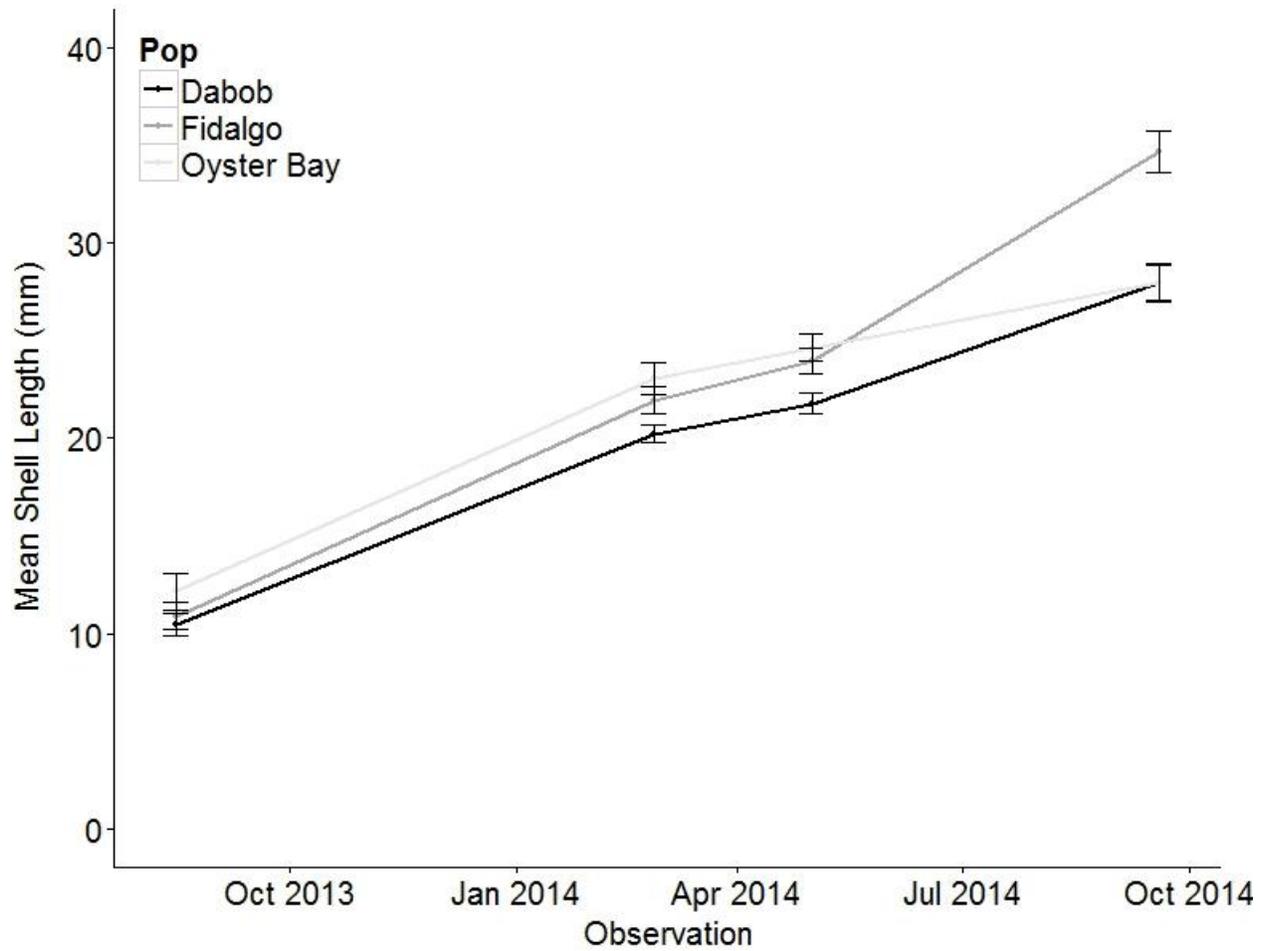


Figure 10. Growth rate of mean shell length in *Ostrea lurida* outplanted at Southern site. Error bars indicate 95% confidence intervals at each time point.

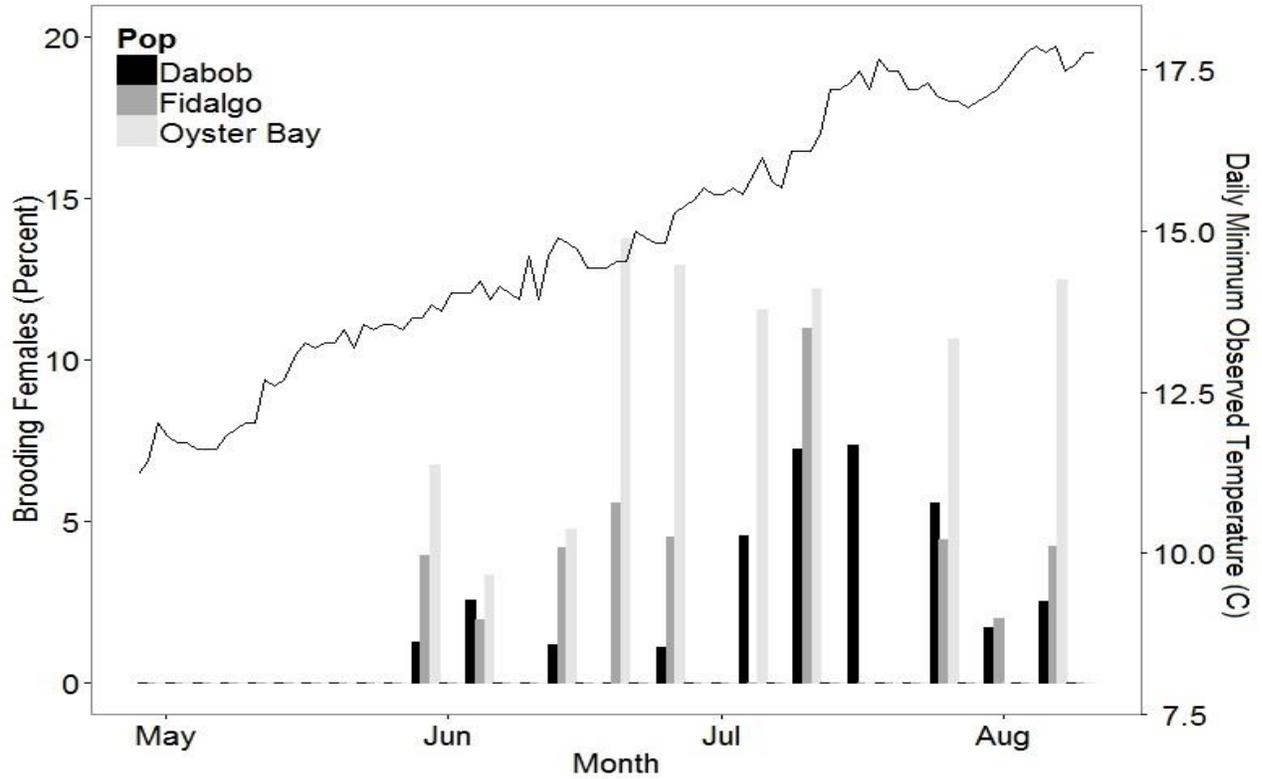


Figure 11. Percent *Ostrea lurida* brooding females from each population at each sample date at Southern site. Percent determined by number of brooding females (Br) divided by number of open oysters (T) or $\% = (\text{Br}/\text{T}) * 100$.

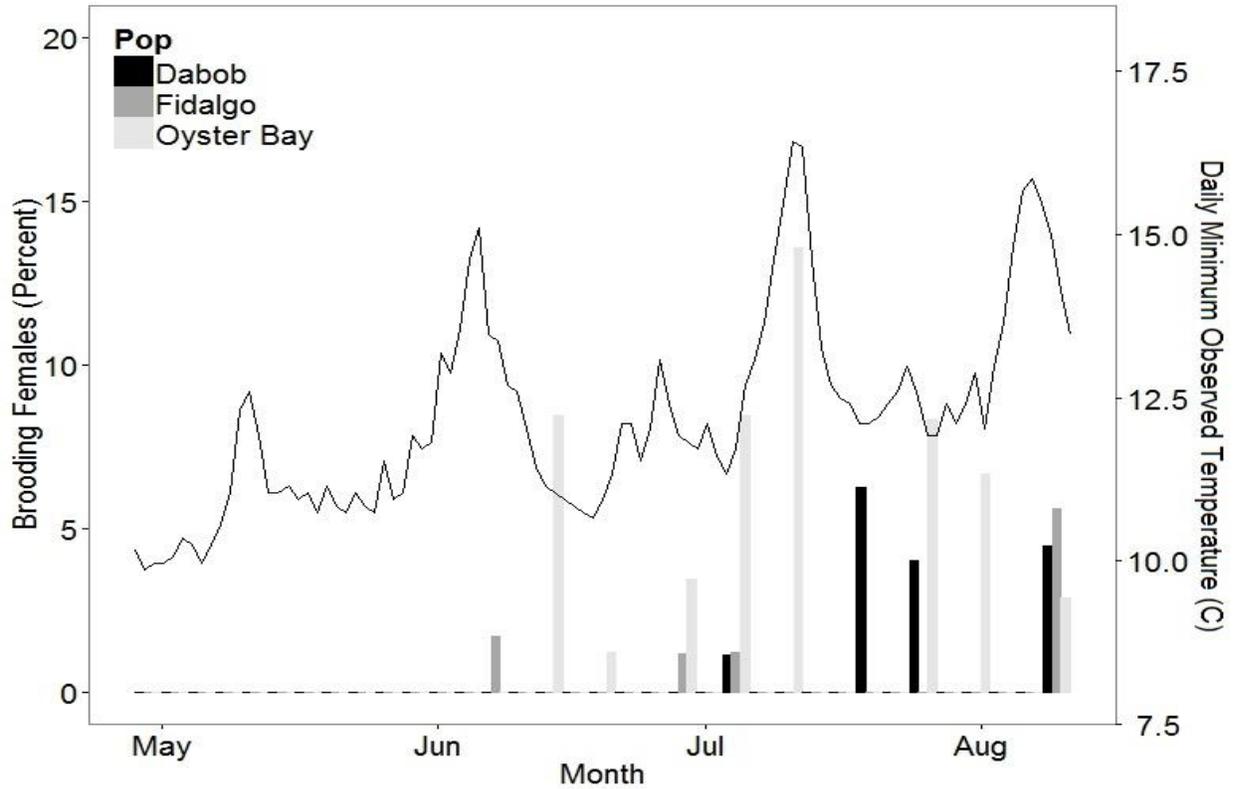


Figure 12. Percent *Ostrea lurida* brooding females from each population at each sample date at Northern site. Percent determined by number of brooding females (Br) divided by number of open oysters (T) or $\% = (\text{Br}/\text{T}) * 100$.

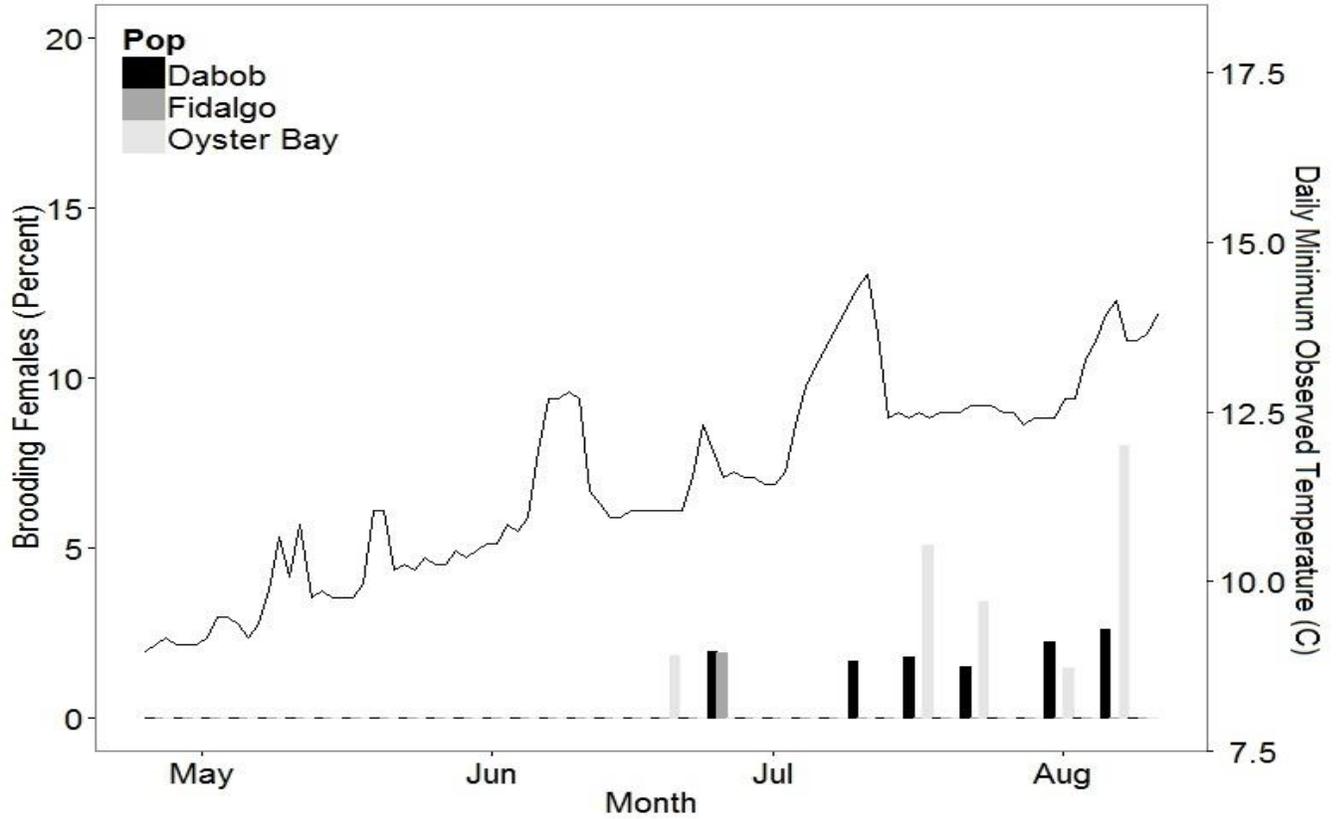


Figure 13. Percent *Ostrea lurida* brooding females from each population at each sample date at Central site. Percent determined by number of brooding females (Br) divided by number of open oysters (T) or $\% = (\text{Br}/\text{T}) * 100$.

Chapter 2: Differential response to stress in *Ostrea lurida* as measured by gene expression.

Abstract

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

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. *O. lurida* is typically smaller than the invasive Pacific oyster, *Crassostrea gigas*, with adults attaining an average size between 40 – 60 mm (Hopkins, 1937; Baker, 1995). As protandric hermaphrodites, Olympia

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

Thermal stress has been widely studied in mollusks, especially bivalves. It is suspected that mass summer mortalities of *C. gigas* may be linked to the effects of heat stress during spawning events (Li et al., 2007). The California mussel, *Mytilus californianus*, has been found to divert resources to physiological defense during thermal stress events (Petes, Menge, and Harris, 2008; Fitzgerald-Dehoog, Browning and Allen, 2012). Expression of homeostasis related genes such as HSP70, Glutamine synthetase, Citrate synthase in *C. gigas* have been shown to fluctuate under prolonged heat stress at 25 °C for 24 days (Meistertzheim et al. 2007). Temperature stress has been shown to induce a variety of up and down regulation of genes to maintain homeostasis (Tomanek, 2010). In oysters, there has been a significant amount of work examining the change

in heat shock protein family gene expression. Specifically seasonal variation of HSPs and HSCs, heat shock cognates, in response to ambient temperatures for *C. gigas* (Hamdoun, Cheney, and Cherr, 2003; Farcy et al., 2009), induction of HSP70 and HSP69 in *Ostrea edulis* at temperatures greater than 38 °C (Piano et al., 2005), and characterization of HSP70 in Antarctic clams (*Laternula elliptica*) (Park, Ahn, and Lee, 2007). Tolerance to heat shock has also been shown to be a heritable trait in oyster species (Lang et al., 2009).

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

Here we set out to examine the response to temperature and mechanical stress in *Ostrea lurida*, while comparing differences in three local populations (Heare et al., 2015). Each of the three

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

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

demonstrate a more effective response to stress that could be evident by greater changes in expression of immune response genes.

Materials and Methods

Experimental Design

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

RNA Isolation

RNA was isolated according to the manufacturer's protocol for total RNA isolation (RNazol RT (Molecular Research Center, Inc.)). Briefly, 400µL of 0.1% DEPC-H₂O was added to the homogenized ctenidia tissue (~1mg), vortexed for 15 seconds, and incubated at room temperature for 15 minutes. The samples were centrifuged for 15 minutes, 16,000g, at room temperature. After centrifugation, 750µL of the supernatant was transferred to a clean tube, added an equal volume of isopropanol (750µL), mixed, and incubated at room temperature for 15 minutes. The samples were centrifuged at 12,000g for 10 minutes at room temperature. The

supernatant was discarded and the pellets were washed with 500 μ L 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 100 μ L 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, 1.5 μ g of total RNA was treated in 0.5mL tubes in a reaction volume of 50 μ L. The samples were incubated with 1 μ L of DNase for 30 minutes at 37C. An additional 1 μ L of DNase was added to each sample and incubated at 37°C for an additional 30 minutes. The DNase was inactivated with 0.2 volumes of the inactivation reagent according to the manufacturer's protocol. Samples were quantified using a NanoDrop1000 (ThermoFisher) and verified to be free of gDNA via quantitative PCR (qPCR).

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

Quantitative PCR

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

Gene

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	TGGTTATCAA CAGCCCCGAC	GTTGTTGACCC CAGGAGGAG
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	ACAAAGATTC CACCCGGCAA	ACACCAACGA CAGGAAGTGG
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	TGCTTTCIGTG TGCCCTTCT	TATCACACCC CGTCACTTGC
comp24065_c0_seq1	O75594	PGRP1_HUMAN	Peptidoglycan recognition protein 1	Assists with recognition of bacteria in an immune response	PGRP	2.00E-42	GAGACTTCAC CTCGCACAA	AACTGGTTTG CCCACATCA
comp44273_c0_seq2	Q8MWP4	Q8MWP4_OSTED	Heat Shock Protein 70kDa	Molecular chaperone and protein preservation in heat response	HSP70	0	TTGTCGCCATT TTCTCGCT	GTTCCGATTG TTCCGTGCC
comp7183_c0_seq1	P12643	BMP2_HUMAN	Bone morphogenetic protein 2	Directs calcification in shell creation	BMP2	2.00E-93	TGAAGGAACG ACCAAAGCCA	TCCGGTTGAA GAACCTCGTG
comp10127_c0_seq1	P62994	GRB2_RAT	Growth factor receptor-bound protein 2	Assists in signal transduction/cell communication	GRB2	1.00E-83	AACTTGTCCA CCCAGACGG	CCAGTTGCAG TCCACTTCT
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	ACAGCGACGG ACGATTTTCT	ATGGCAGACG TTACCCAACA
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	AGCAGGGCAT CAAACCTCTCC	ACAAGTCGCA CTGGCTACAA
comp30443_c0_seq2	Q8TA69	Q8TA69_CRAGI	Actin	Housekeeping gene used for baseline	Actin	0	GACCAGCCAA ATCCAGACGA	CGGTCGTACC ACTGGTATCG

Table 1. Table of qPCR Primers for genes of interest. Full sequences for primer creation are available. (Heare et al., 2015b).

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

Statistical Analysis

To determine original RNA copy number for each gene, cycle threshold (Ct) values were calculated by the BioRad CFX Manager 3.1 (version 3.1.1517.0823, Windows 8.1) and Opticon Manager 3 (Windows 8.1). This was accomplished by subtracting global minimum fluorescence from samples and determining the point in the cycle which amplification reached exponential amplification phase. To standardize the Ct values between runs, default settings were accepted for each program to ensure reproducibility. The BioRad CFX Manager used default settings of single threshold for Cq determination and baseline subtracted curved fit for each run. The Opticon Manager used default settings of subtract baseline via global minimum which estimated the threshold as being between 0.019 and 0.028. Log scale for these programs were checked to determine and correct any issue the default settings may have caused. Ct values for technical replicates (≥ 2) were averaged. Gene expression values were determined as normalized mRNA levels ($2^{-(\text{mean Ct housekeeping genes} - \text{mean Ct target})}$) as described by Pfaffl (2001). The dCt was then log transformed (logdCt) for statistical analysis. Two way ANOVA followed by

Tukey's Honestly Significant Difference post hoc test (*base*, R Core Team, 2014) were performed on logdCt for each target ($p < 0.05$).

Results

Mortality

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

Gene Expression Analysis

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

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

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

Discussion

This work provides the first gene expression study with *Ostrea lurida* focused on multiple stressors and multiple functional gene targets. Some limitations in this study confound findings because we were unable to determine sex or reproductive stage of the animals prior to running this experiment. Both of which may have had an effect on mortality or gene expression.

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

Response to Temperature Stress

The response of *Ostrea lurida* to acute heat stress appears to include an alteration in gene regulatory activity, likely in an effort to conserve energy resources. This is based on an increase of CARM and H2AV gene expression 1 hour following temperature stress. Histone-arginine methyltransferase 1, CARM, is involved in methylation of histones, which regulates binding of DNA in chromatin (Biel, Wascholowski, and Giannis, 2005). This in turn affects the ability of transcription factors to bind and transcription to proceed. Increase in CARM expression could indicate that overall gene regulatory activity is decreased (via transcription factor binding inhibition) to conserve energy resources necessary to effectively respond to temperature stress. These results are similar to those of Wang et al. (2011) where researchers described an increase in expression of Histone-arginine methyltransferase in the sea cucumber, *Apostichus japonicus*, after experiencing 25 °C temperatures for 7 days. They suggested that this was due to an induced dormancy and lower metabolic rate, to provide resources for stress resilience. Histone 2A.v, H2AV, is a variant of the Histone H2A protein. This variant has been shown to act as a transcription promoter agent as well as assist with heterochromatin formation. Truebano et al. (2010) characterized changes in transcription in Antarctic clams, *L. elliptica*, and found that gene modifiers including an H2A variant were significantly upregulated under heat stress conditions (3 °C for 12 hours). As indicated here, they also suggested that changes in gene regulation were important in maintaining cell function during stress conditions.

Heat shock proteins are often found in response to overt stress but this study only found a significant difference of mRNA expression of HSP70 in the Oyster Bay population between mechanical and heat stress. Brown et al. (2004) found the maximum heat shock protein

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

Response to Mechanical Stress

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

Population differences

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

While only speculation at this point, the gene expression pattern differences observed here with oysters from Oyster Bay coupled with corresponding field-based observation that this population has the greatest reproductive activity, could indicate this population has a greater ability to effectively respond to energy demands. Another way to consider this is that the Oyster Bay

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

The other consideration is to what degree does phenotypic plasticity (or the rate of obtaining a new phenotype, in this case) have in restoration. At one level the ability to achieve a number of phenotypes with a given genotype could be advantageous, particularly in a rapidly the changing environment. There is a paradox in the fact that too much plasticity negates the ability of natural

selection to function. Populations with high phenotypic plasticity become deprived of negative selection and thus are often able to survive in rapidly changing environments as long as the changes are consistent and somewhat predictable. However, with this increased adaptive ability, genetic diversity and adaptation become limited within a population that may be unable to properly respond to novel challenges in the future (Crispo, 2008). Alternatively, the Baldwin effect may enhance longterm genetic diversity by allowing species to colonize novel habitats and, with phenotypic plasticity, and eventually genetically diverge from the source population through induced genetic adaptations (Crispo, 2007). For longterm restoration of *O. lurida* populations in Puget Sound, understanding the phenotypic plasticity of individual populations will help determine proper supplementation procedures for existing and historic habitats.

Citations

- Aladaileh, S., Nair, S., & D. Raftos. 2008. Effects of noradrenaline on immunological activity in Sydney rock oysters. *Dev. & Comp. Immunology* 32:627–636.
- Angilletta Jr, M., Wilson, R., Navas, C., & R. James R. 2003. Tradeoffs and the evolution of thermal reaction norms. *Trends in Eco. & Evo.* 18:234–240.
- Arch, R., Gedrich, R., & C. Thompson. 1998. Tumor necrosis factor receptor-associated factors (TRAFs)—a family of adapter proteins that regulates life and death. *Genes & Dev.* 12:2821–2830.
- Baker, P. 1995. Review of Ecology and Fishery of the Olympia Oyster, *Ostrea lurida*, with Annotated Bibliography. *J. of Shellfish Res.* 14:503–518.
- Biel, M., Wascholowski, V., & A. Giannis. 2005. Epigenetics—An Epicenter of Gene Regulation: Histones and Histone-Modifying Enzymes. *Ang. Chem. Intl. Ed.* 44:3186–3216.
- Brown, H. M., A. Briden, T. Stokell, F. J. Griffin, & G. N. Cherr. 2004. Thermotolerance and Hsp70 profiles in adult and embryonic California native oysters, *Ostrea conchaphila* (Carpenter, 1857). *J. of Shellfish Res.* 23:135-141.
- Camara, M. & B. Vadopalas. 2009. Genetic Aspects of Restoring Olympia Oysters and Other Native Bivalves: Balancing the Need for Action, Good Intentions, and the Risks of Making Things Worse. *J. of Shellfish Res.* 28:121–145.

- Clegg, J., Uhlinger, K., Jackson, S., Cherr, G., Rifkin, E., & C. Friedman. 1998. Induced thermotolerance and the heat shock protein-70 family in the Pacific oyster *Crassostrea gigas*. *Mol. Mar. Bio and Biotech.* 7:21-30
- Crispo, E. 2008. Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. *J. of Evo. Bio.* 21:1460–1469.
- Crispo, E. 2007. The Baldwin Effect and Genetic Assimilation: Revisiting Two Mechanisms of Evolutionary Change Mediated by Phenotypic Plasticity. *Evolution* 61:2469–2479.
- Davis, H. 1955. Mortality of Olympia Oysters at Low Temperatures. *Bio. Bulletin* 109:404–406.
- Farcy, E., Voiseux, C., Lebel, J-M., & B. Fievet. 2009. Transcriptional expression levels of cell stress marker genes in the Pacific oyster *Crassostrea gigas* exposed to acute thermal stress. *Cell Str. & Chap.* 14:371–380.
- Fitzgerald-Dehoog, L., Browning, J., & B.J. Allen. 2012. Food and Heat Stress in the California Mussel: Evidence for an Energetic Trade-off Between Survival and Growth. *Bio. Bulletin* 223:205–216.
- Franzellitti S. & E. Fabbri. 2005. Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. *Biochem. & Biophys. Res. Comm.* 336:1157–1163.
- Hamdoun, A., Cheney, D., & G. Cherr. 2003. Phenotypic Plasticity of HSP70 and HSP70 Gene Expression in the Pacific Oyster (*Crassostrea gigas*): Implications for Thermal Limits and Induction of Thermal Tolerance. *Bio. Bulletin* 205:160–169.
- Heare, J., Blake, B., Davis, J., Vadopalas, B., & S.B. Roberts. 2015. Evidence of *Ostrea lurida* (Carpenter 1864) population structure in Puget Sound, WA. PeerJ preprint. <https://peerj.com/preprints/704/>
- Heare, J., & S.B. Roberts. 2015b. <https://github.com/jheare/OluridaGeneExpression/tree/v1.0>
- Hopkins, A. 1936. Ecological Observations on Spawning and Early Larval Development in the Olympia Oyster (*Ostrea lurida*). *Ecology* 17:551–566.
- Kuchel, R., Raftos, D., & S. Nair. 2010. Immunosuppressive effects of environmental stressors on immunological function in *Pinctada imbricata*. *Fish & Shellfish Immunology* 29:930–936.
- Lacoste, A., Malham, S., Cueff, A., Jalabert, F., Gelebart, F., & S. Poulet. 2001a. Evidence for a form of adrenergic response to stress in the mollusc *Crassostrea gigas*. *J. of Exp. Bio.* 204:1247–1255.
- Lacoste, A., Malham, S., Cueff, A., & S. Poulet SA. 2001b. Noradrenaline modulates hemocyte reactive oxygen species production via β -adrenergic receptors in the oyster *Crassostrea gigas*. *Dev. & Comp. Immunology* 25:285–289.
- Lacoste, A., Malham, S., Cueff, A., & S. Poulet. 2001c. Stress-Induced Catecholamine Changes in the Hemolymph of the Oyster *Crassostrea gigas*. *Gen. and Comp. Endo.* 122:181–188.

- Lacoste, A., Jalabert, F., Malham, S., Cueff, A., & S. Poulet. 2001d. Stress and Stress-Induced Neuroendocrine Changes Increase the Susceptibility of Juvenile Oysters (*Crassostrea gigas*) to *Vibrio splendidus*. *App. and Env. Micro.* 67:2304–2309.
- Lang, R., Bayne, C., Camara, M., Cunningham, C., Jenny, M., & C. Langdon. 2009. Transcriptome Profiling of Selectively Bred Pacific Oyster *Crassostrea gigas* Families that Differ in Tolerance of Heat Shock. *Mar. Biotech.* 11:650–668.
- Lathlean, J., & T. Minchinton. 2012. Manipulating thermal stress on rocky shores to predict patterns of recruitment of marine invertebrates under a changing climate. *Fac. of Sci. - Papers (Archive)*:121–136.
- Li, Y., Qin, J., Abbott, C., Li, X., & K. Benkendorff. 2007. Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas*: an explanation for summer mortality in Pacific oysters. *AJP: Reg., Integr. & Comp. Phys.* 293:R2353–R2362.
- Malham, S., Lacoste, A., Gélébart, F., Cueff, A., & S. Poulet. 2003. Evidence for a direct link between stress and immunity in the mollusc *Haliotis tuberculata*. *J. of Exp. Zoo. Part A: Comp. Exp. Bio.* 295A:136–144.
- Meistertzheim, A-L., Tanguy, A., Moraga, D., & M-T. Thébault. 2007. Identification of differentially expressed genes of the Pacific oyster *Crassostrea gigas* exposed to prolonged thermal stress. *FEBS Journal* 274:6392–6402.
- Park, H., Ahn, I-Y., & H. Lee. 2007. Expression of heat shock protein 70 in the thermally stressed Antarctic clam *Laternula elliptica*. *Cell Str. & Chap.* 12:275–282.
- Pereira Mouriès, L., Almeida, M-J., Milet, C., Berland, S., & E. Lopez. 2002. Bioactivity of nacre water-soluble organic matrix from the bivalve mollusk *Pinctada maxima* in three mammalian cell types: fibroblasts, bone marrow stromal cells and osteoblasts. *Comp. Biochem. & Phys. Part B: Biochem. & Mol. Bio.* 132:217–229.
- Petes, L., Menge, B., & A. Harris. 2008. Intertidal mussels exhibit energetic trade-offs between reproduction and stress resistance. *Ecol. Mono.* 78:387–402.
- Pfaffl, M. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* 29:e45–e45.
- Piano, A., Franzellitti, S., Tinti, F., & E. Fabbri. 2005. Sequencing and expression pattern of inducible heat shock gene products in the European flat oyster, *Ostrea edulis*. *Gene* 361:119–126.
- Qu, Y., Li, X., Yu, Y., Vandepuer, M., Babidge, P., Clarke, S., Bott, K., & H. Li. 2009. The effect of different grading equipment on stress levels assessed by catecholamine measurements in Pacific oysters, *Crassostrea gigas* (Thunberg). *Aquacul. Engin.* 40:11–16.
- R: A Language and Environment for Statistical Computing* 2013. Vienna, Austria: R Foundation for Statistical Computing.

- Roberts, S., Sunila, I., & G. Wikfors. 2011. Immune response and mechanical stress susceptibility in diseased oysters, *Crassostrea virginica*. *J. of Comp. Phys. B* 182:41–48.
- Samain, J., Dégremont, L., Soletchnik, P., Haure, J., Bédier, E., Ropert, M., Moal, J., Huvet, A., Bacca, H., Van Wormhoudt, A., Delaporte, M., Costil, K., Pouvreau, S., Lambert, C., Boulo, V., Soudant, P., Nicolas, J., Le Roux, F., Renault, T., Gagnaire, B., Geret, F., Boutet, I., Burgeot, T., & P. Boudry. 2007. Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. *Aquaculture* 268:227–243.
- Sokolova, I., Frederich, M., Bagwe, R., Lannig, G., & A. Sukhotin. 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Mar. Env. Res.* 79:1–15.
- Tomanek, L. 2010. Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. *J. of Exp. Bio.* 213:971–979.
- Truebano, M., Burns, G., Thorne, M., Hillyard, G., Peck, L., Skibinski, D., & M. Clark. 2010. Transcriptional response to heat stress in the Antarctic bivalve *Laternula elliptica*. *J. of Exp. Mar. Bio. and Eco.* 391:65–72.
- Wang, TW., 杨红生, HY., 赵欢, HZ., 陈慕雁, MC., & B. 王兵. 2011. Transcriptional changes in epigenetic modifiers associated with gene silencing in the intestine of the sea cucumber, *Apostichopus japonicus* (Selenka), during aestivation. *Chinese J. of Ocean. and Limn.* 29:1267–1274.
- White, J., Ruesink, J., & A. Trimble. 2009. The nearly forgotten oyster: *Ostrea lurida* Carpenter 1864 (Olympia oyster) history and management in Washington State. *J. of Shellfish Res.* 28:43–49.
- Wickham, H. 2014. *plyr: Tools for splitting, applying and combining data*.
- Wickham, H., & W. Chang. 2014. *ggplot2: An implementation of the Grammar of Graphics*.
- Yao, C-L., & G. Somero. 2012. The impact of acute temperature stress on hemocytes of invasive and native mussels (*Mytilus galloprovincialis* and *Mytilus californianus*): DNA damage, membrane integrity, apoptosis and signaling pathways. *J. of Exp. Bio.* 215:4267–4277.

Figures

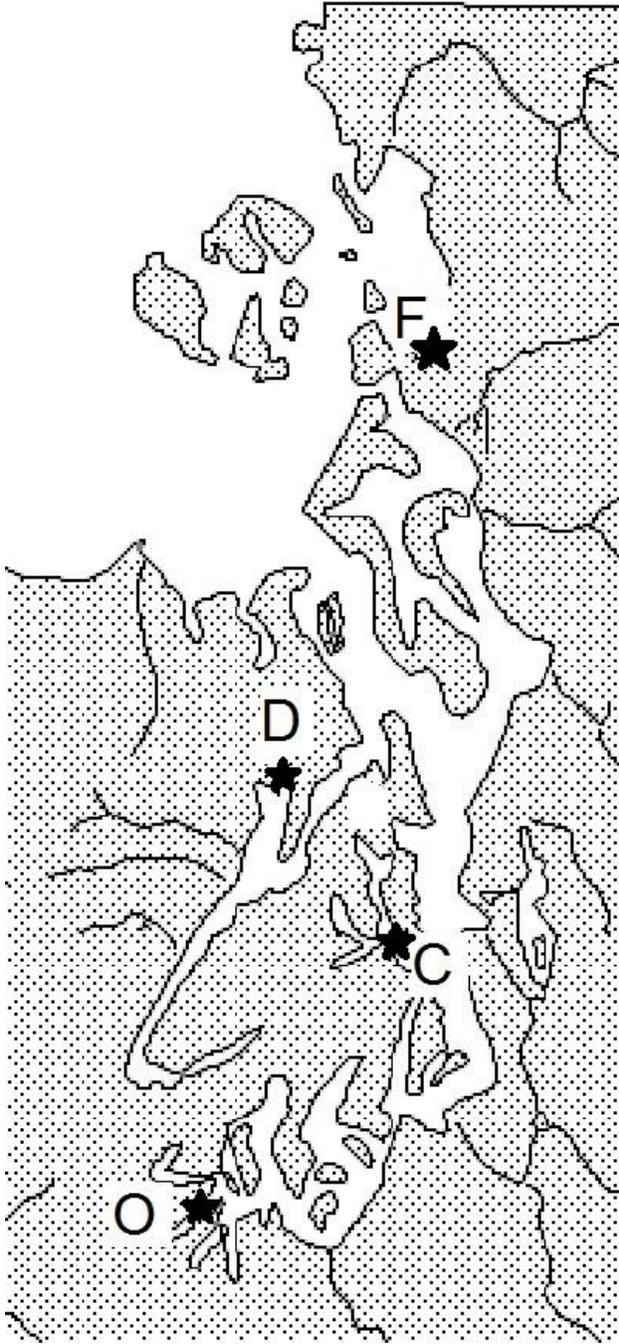


Figure 1. Map of Puget Sound with *Ostrea lurida* broodstock and outplant sites. Conditioning site was Port Gamble (G). Broodstock collected from Fidalgo Bay (F), Dabob Bay (D), and Oyster Bay (O).

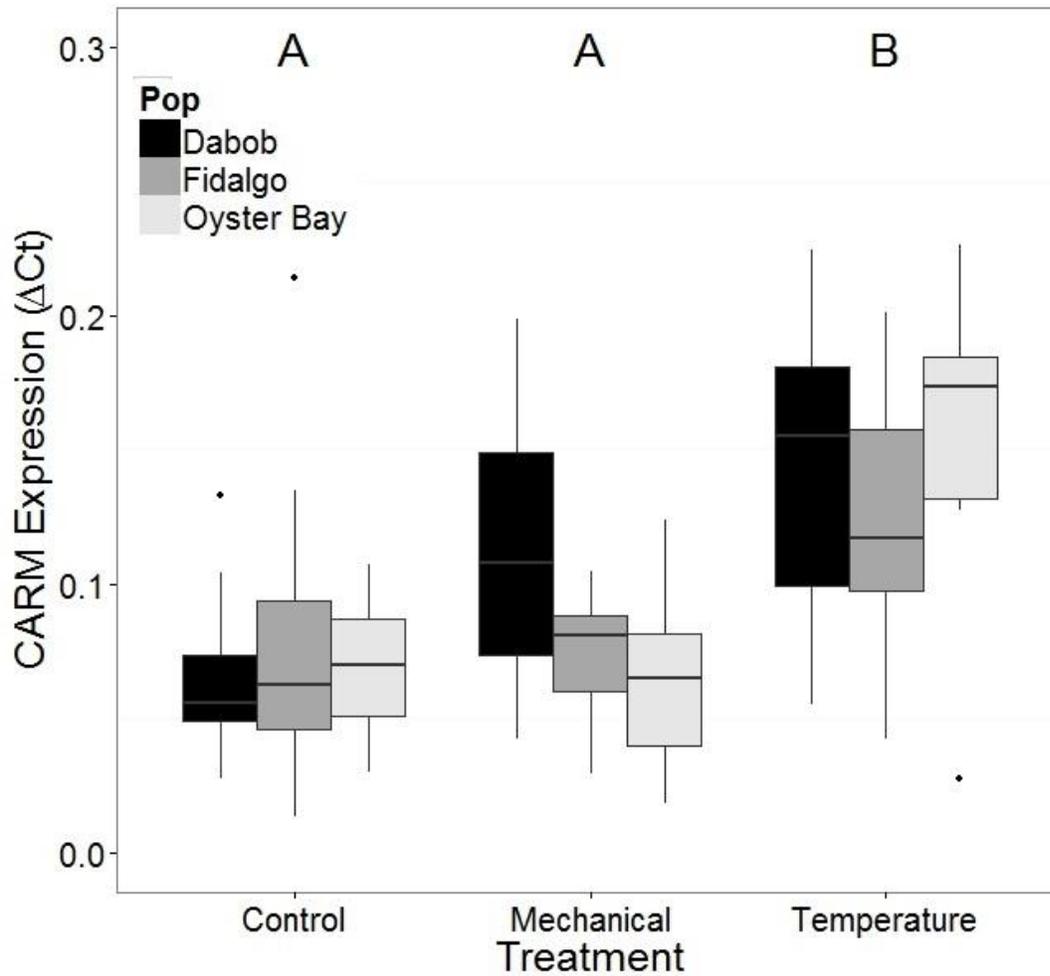


Figure 2. Expression of CARM mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences ($p < 0.05$) between treatments within a population. Capital letters indicate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

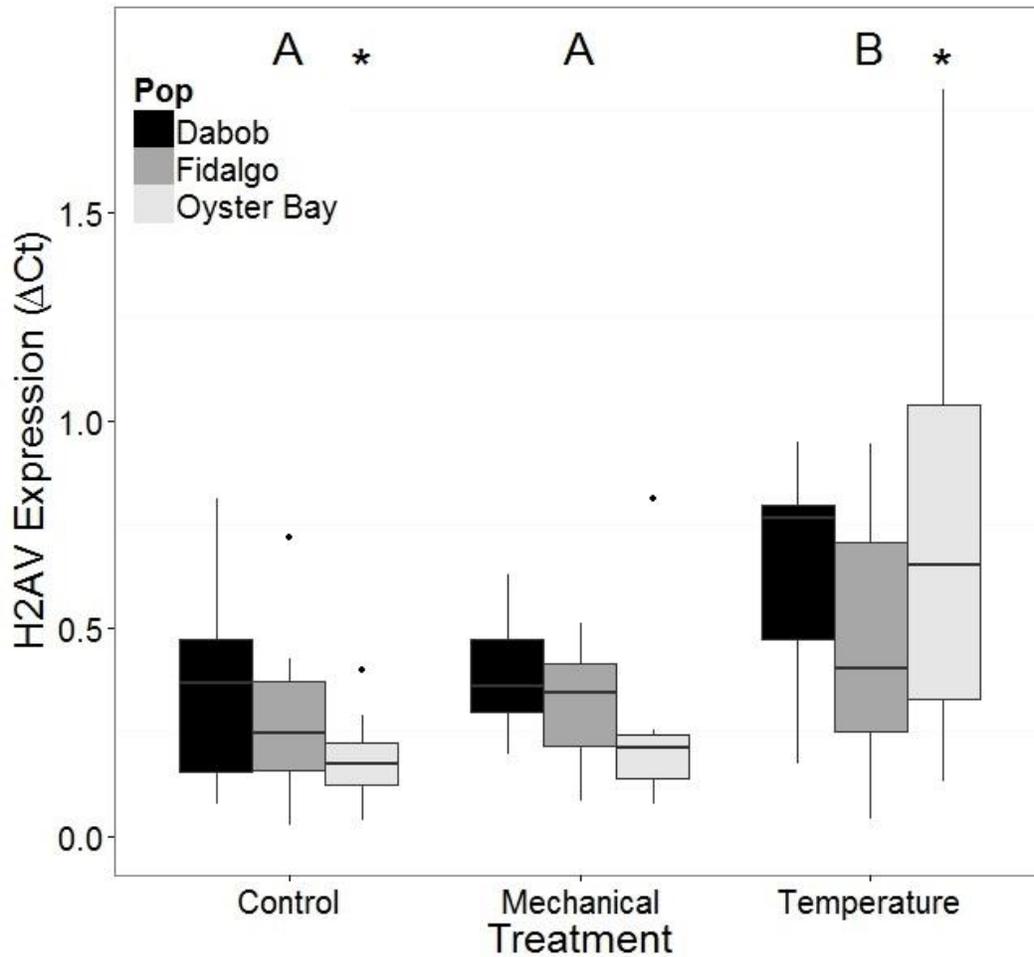


Figure 3. Expression of H2AV mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences ($p < 0.05$) between treatments within a population. Capital letters indicate significant differences ($p < 0.05$) between overall treatment groups.

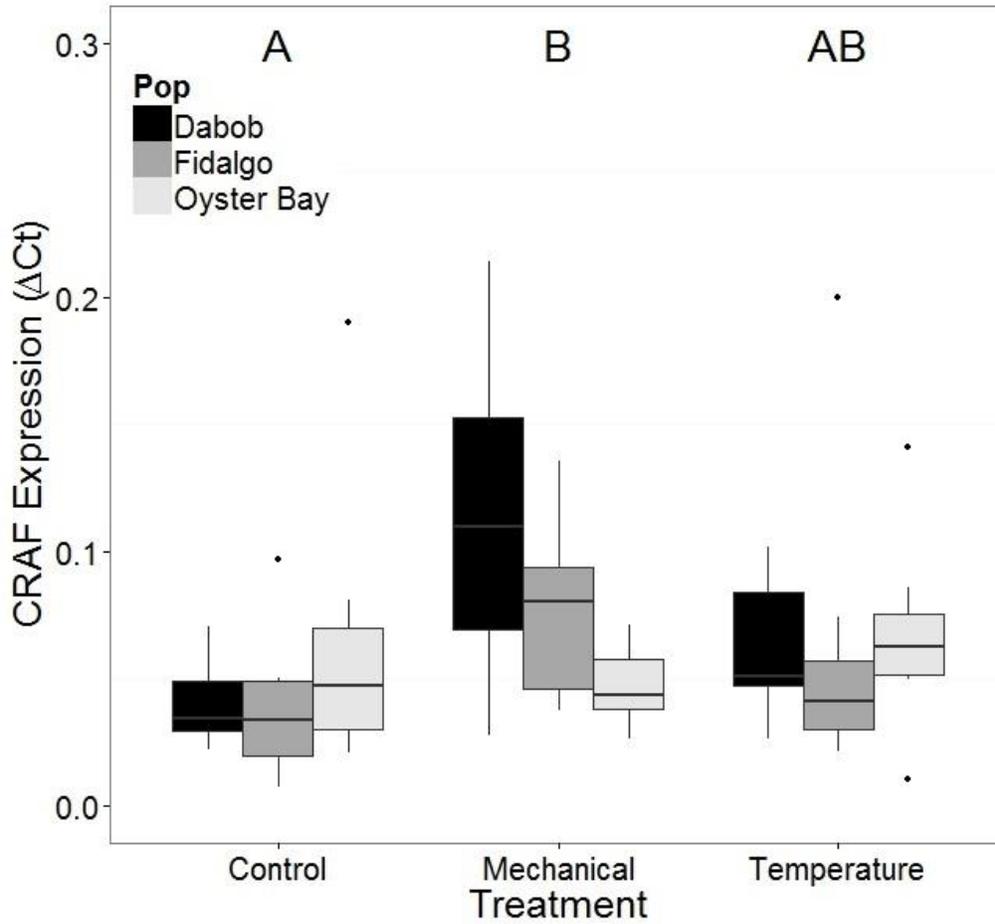


Figure 4. Expression of CRAF mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences ($p < 0.05$) between treatments within a population. Capital letters indicate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

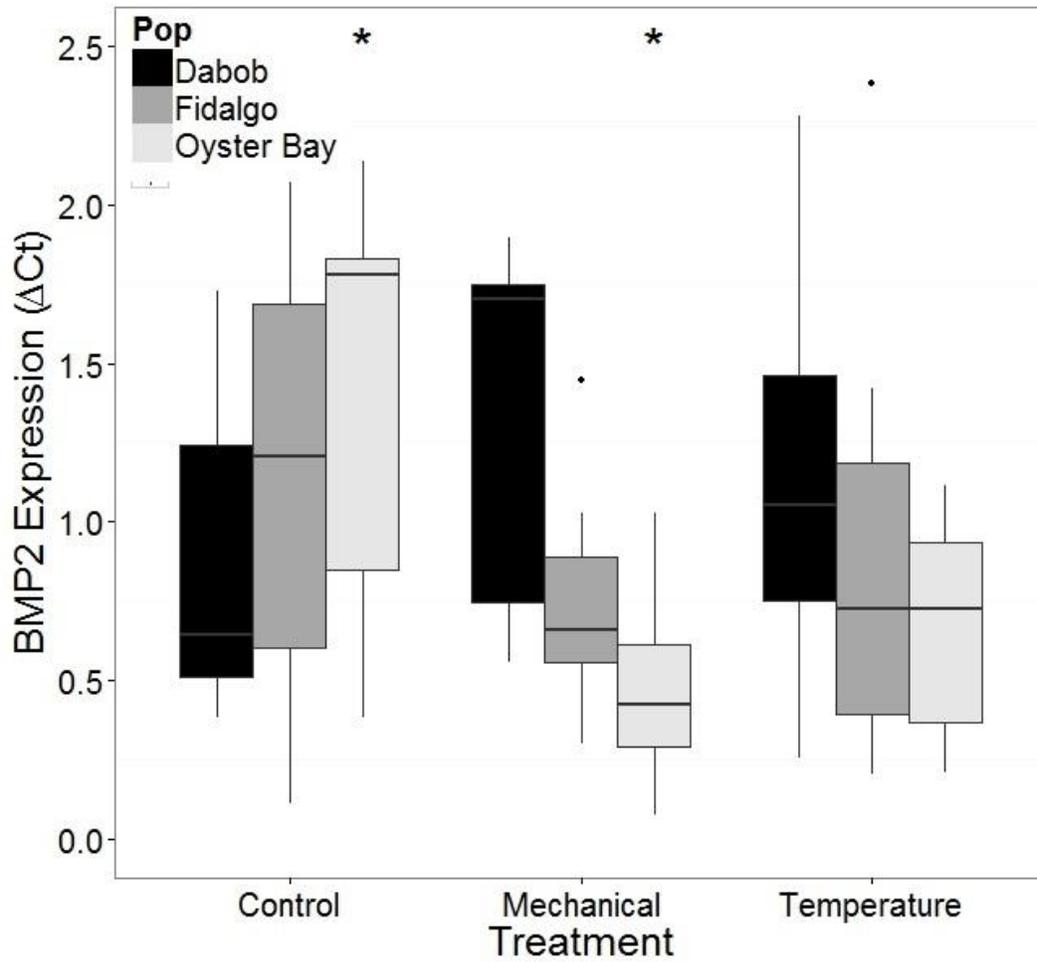


Figure 5. Expression of BMP2 mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences ($p < 0.05$) between treatments within a population. Capital letters indicate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

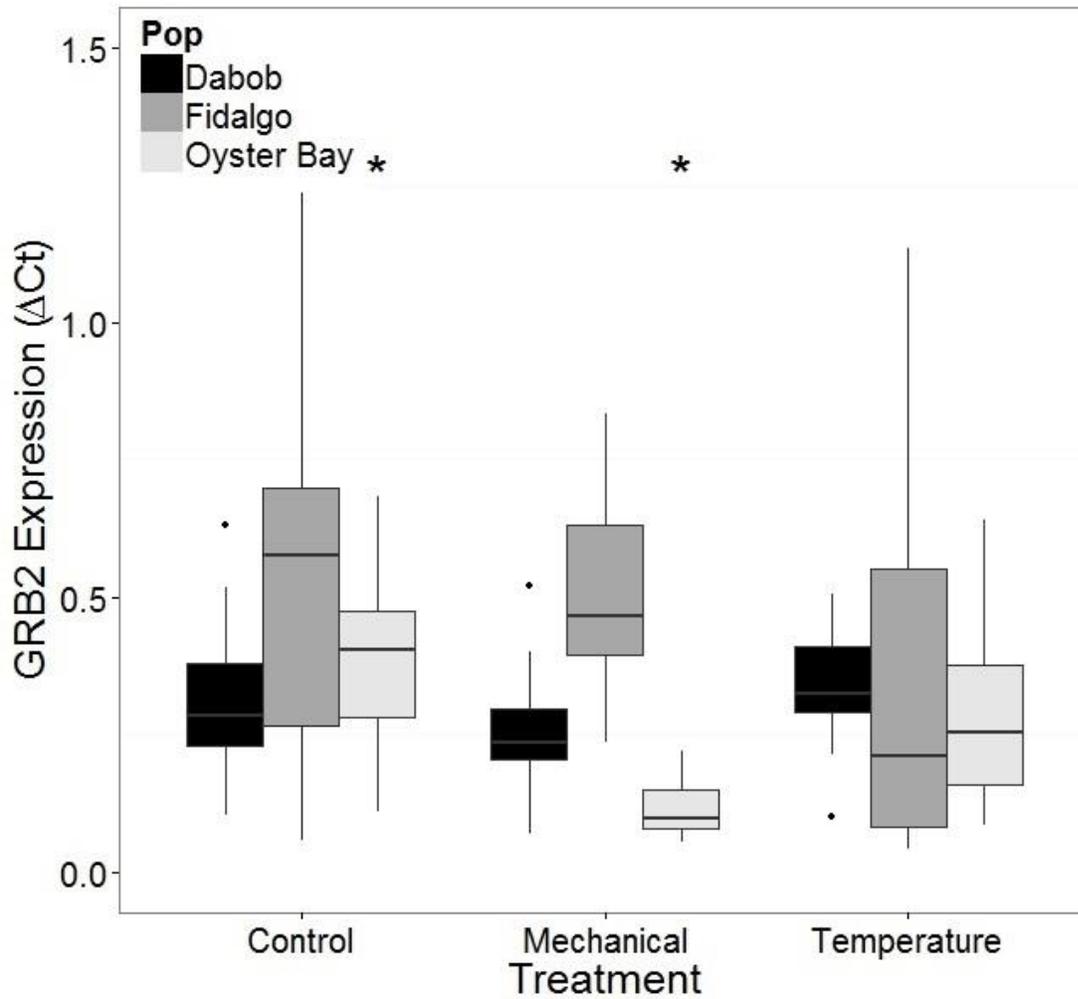


Figure 6. Expression of GRB2 mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences ($p < 0.05$) between treatments within a population. Capital letters indicate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

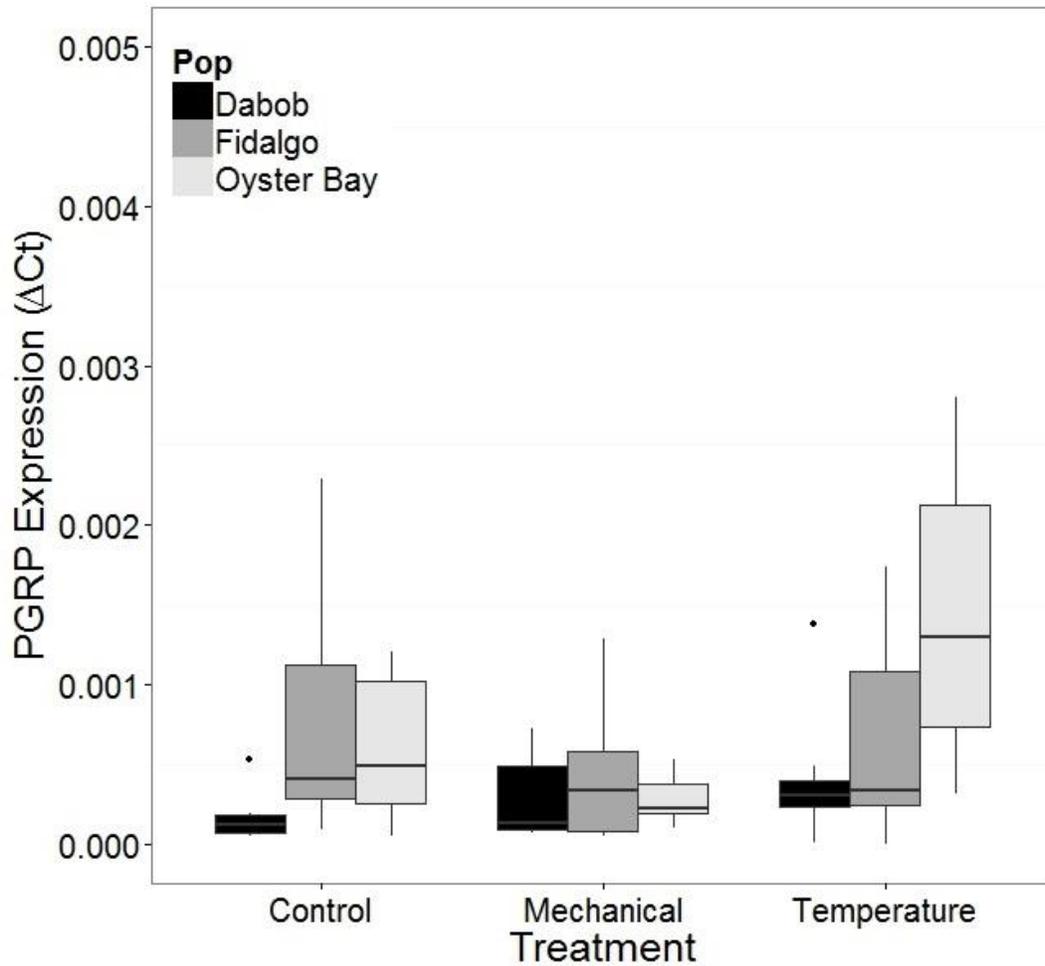


Figure 7. Expression of PGRP mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences ($p < 0.05$) between treatments within a population. Capital letters indicate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

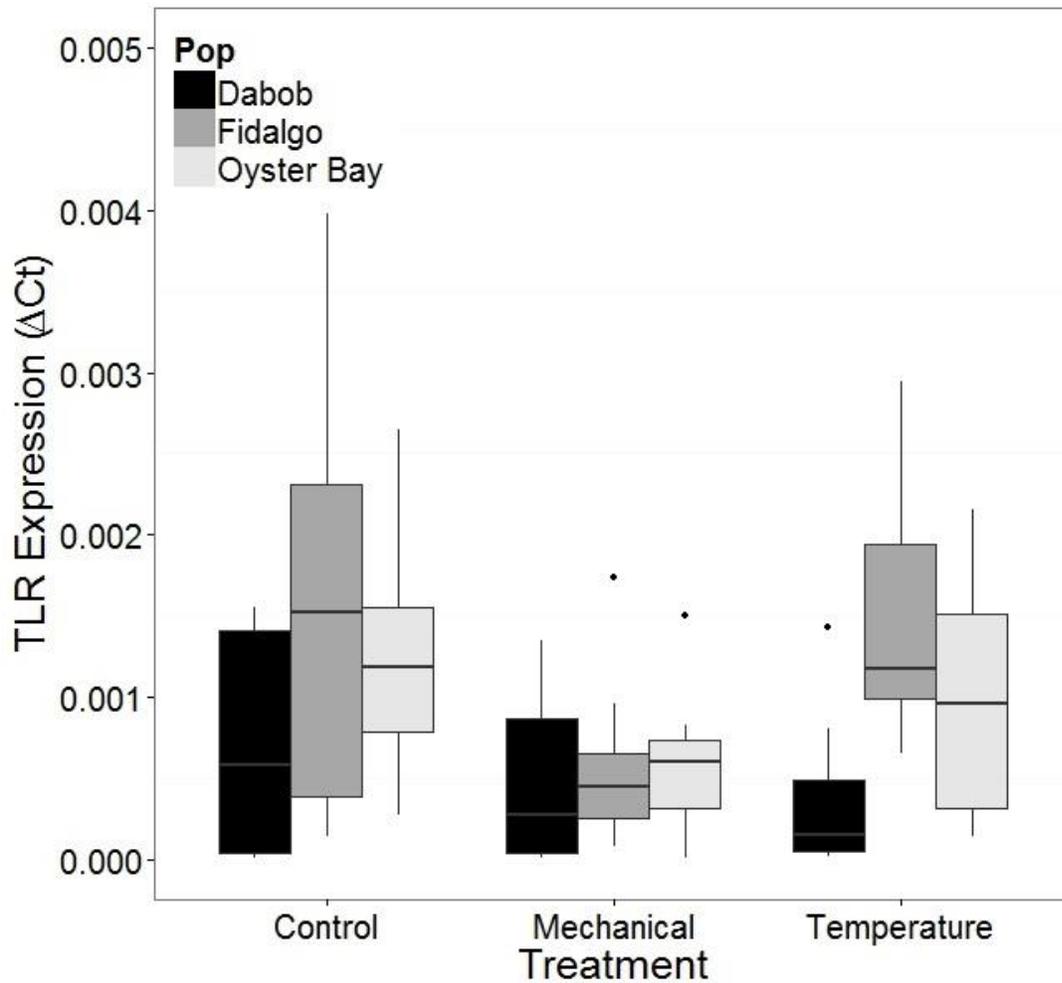


Figure 8. Expression of TLR mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences ($p < 0.05$) between treatments within a population. Capital letters indicate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

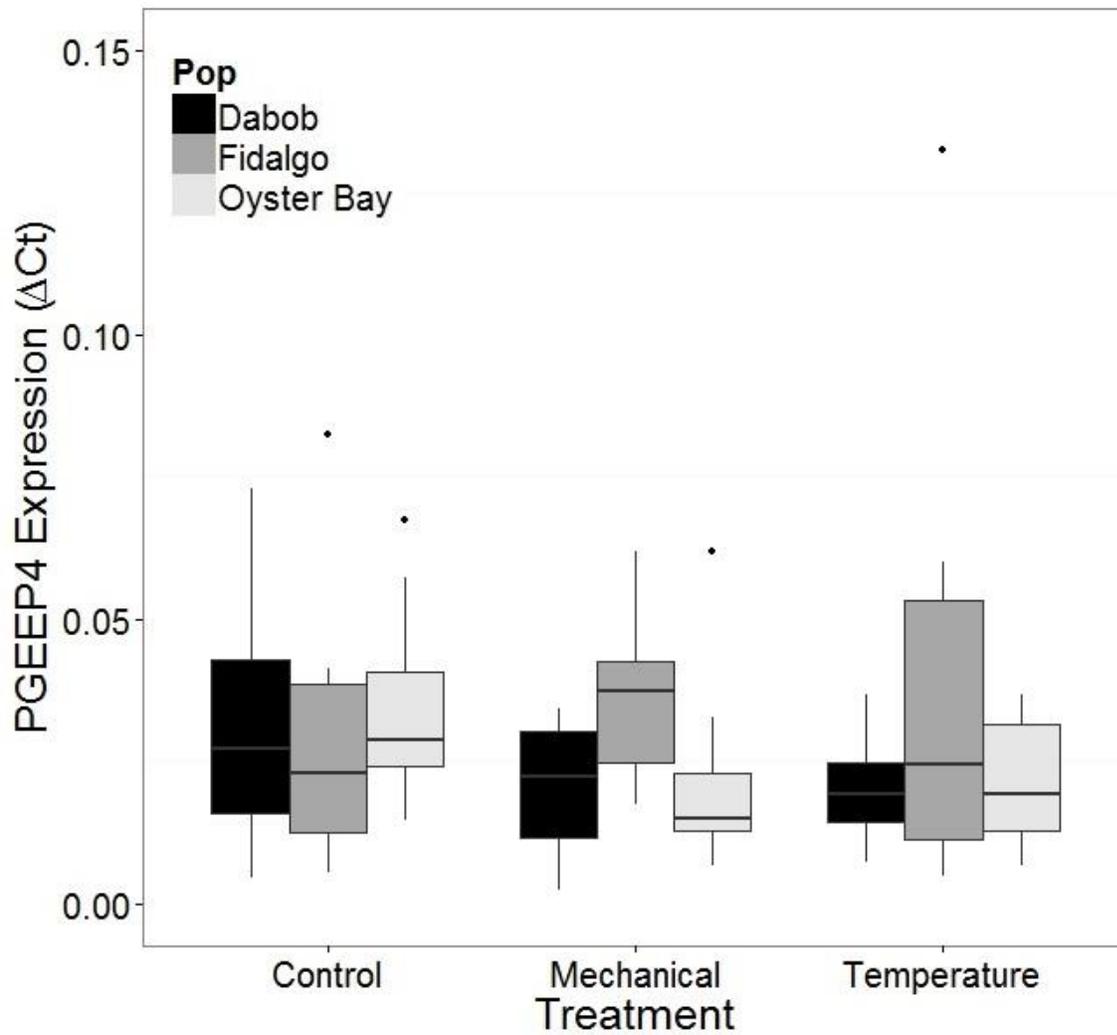


Figure 9. Expression of PGEEP4 mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences (p < 0.05) between treatments within a population. Capital letters indicate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

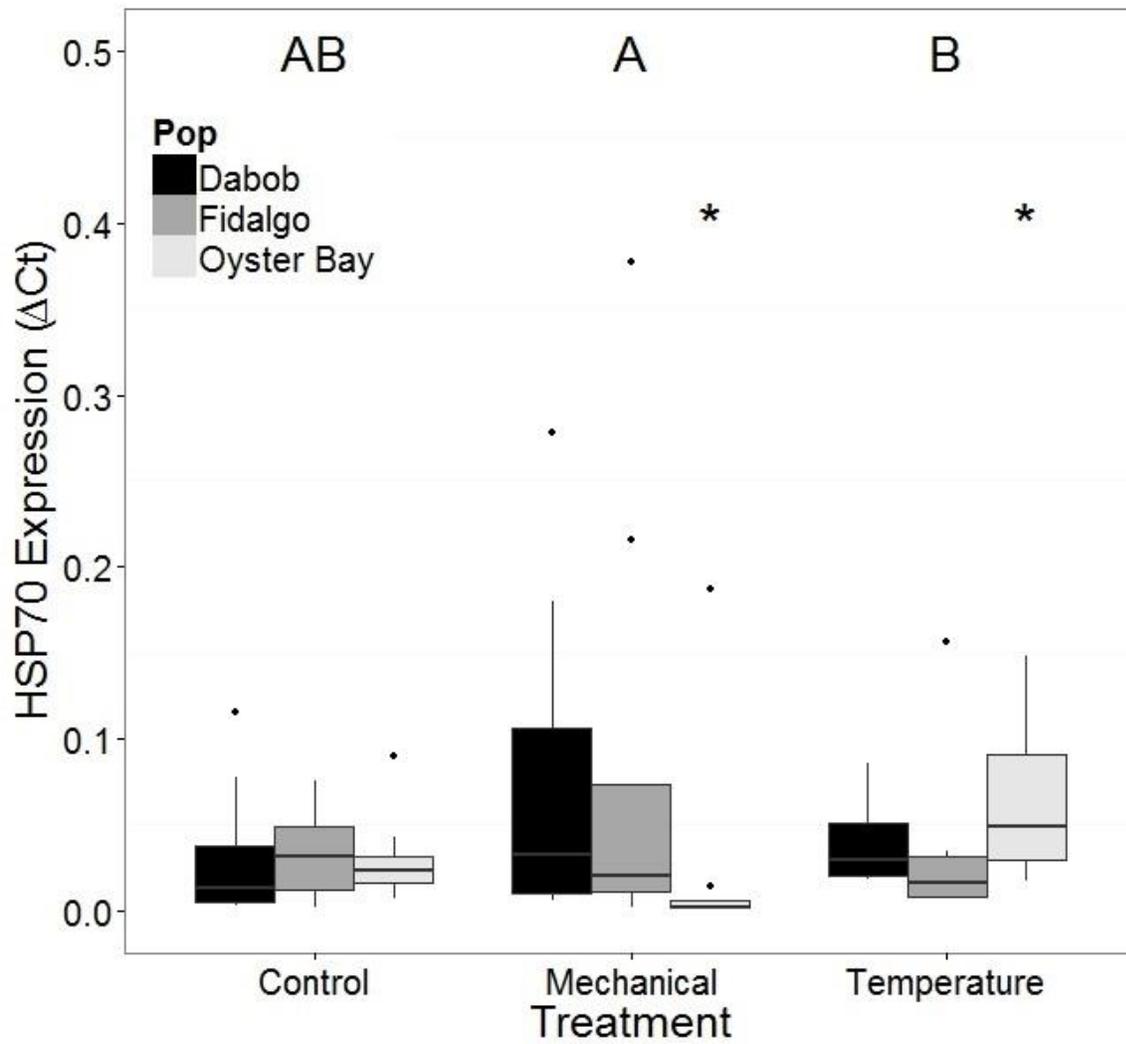


Figure 10. Expression of HSP70 mRNA. Mean delta Ct indicated by line in middle of box plot. Shaded boxes are 2nd and 3rd quartile. Lines are 1st and 4th quartiles. Dots indicate outliers. Asterisks indicate significant differences ($p < 0.05$) between treatments within a population. Capital letters indicate significant differences ($p < 0.05$) between overall treatment groups.

Appendix A. Annotated Bibliography for *Ostrea lurida*

Bolded items were of significant value to this project and considered exceptional resources for *Ostrea lurida* research.

Baker P. 1995. Review of Ecology and Fishery of the Olympia Oyster, *Ostrea lurida*, with Annotated Bibliography. *Journal of Shellfish Research* 14:503–518.

<http://dx.doi.org/10.2983/035.028.0110>

Review of important literature on *O. lurida* distribution, biology, ecology, scientific taxonomy, and commercial use of *O. lurida*; and annotated bibliography of known literature pertaining to *O. lurida* to date of publication.

Blake B., Ermgassen PSEZ. 2015. The History and Decline of *Ostrea lurida* in Willapa Bay, Washington. *Journal of Shellfish Research* 34:273–280. <http://dx.doi.org/10.2983/035.034.0208>

Review of the over exploitation of *O. lurida* in the area with cultivation statistics and reasons for the collapse of the industry. Historically, up to 27% of the floor of Willapa Bay was used for oyster culture.

Brumbaugh RD., Coen LD. 2009. Contemporary Approaches for Small-Scale Oyster Reef Restoration to Address Substrate Versus Recruitment Limitation: A Review and Comments Relevant for the Olympia Oyster, *Ostrea lurida* Carpenter 1864. *Journal of Shellfish Research* 28:147–161. <http://dx.doi.org/10.2983/035.028.0105>

Appropriate use of differing oyster restoration methods are discussed with ecological services vs. fishery outcomes. Recruitment bottlenecks and substrate limitation are identified for small-scale restoration sites.

Camara MD., Vadopalas B. 2009. Genetic Aspects of Restoring Olympia Oysters and Other Native Bivalves: Balancing the Need for Action, Good Intentions, and the Risks of Making Things Worse. *Journal of Shellfish Research* 28:121–145.

<http://dx.doi.org/10.2983/035.028.0104>

Adaptive and quantitative genetic variation with populations is discussed and the obstacles associated with the use of neutral molecular genetic variation. Restoration efforts are reviewed.

Carson HS. 2010. Population connectivity of the Olympia oyster in southern California. *Limnology and Oceanography* 55:134–148. <http://dx.doi.org/10.4319/lo.2010.55.1.0134>

Oyster larvae can spread throughout the aquatic ecosystem, as far as 75 km away from their point of origin. Recruitment pulses possibly account for genetic variation amongst estuaries.

Coe WR. 1932. Development of the Gonads and the Sequence of the Sexual Phases in the California Oyster (*Ostrea lurida*). *Scripps Institution of Oceanography*.

Sexual reproduction methods of *O. lurida* are discussed, including spawning temperatures, peaking spawning times, and sexual phases. Sperm ball and ova production are noted with image plates.

Davis HC. 1955. Mortality of Olympia Oysters at Low Temperatures. *Biological Bulletin* 109:404–406. 10.2307/1539172

Oysters kept at temperatures below 10C die.

Deck AK. 2011. Effects of interspecific competition and coastal oceanography on population dynamics of the Olympia oyster, *Ostrea lurida*, along estuarine gradients. *California Sea Grant College Program*. Permalink:<http://escholarship.org/uc/item/6xq0z4fs>

Master's thesis divided into two chapters. Predation, competition, and tidal influx into estuaries influence recruitment of *O. lurida*. While this study found no competition in the oyster populations, however, total recruitment was affected by the presence of competitors.

Dinnel PA., Peabody B., Peter-Contesse T. 2009. Rebuilding Olympia Oysters, *Ostrea lurida* Carpenter 1864, in Fidalgo Bay, Washington. *Journal of Shellfish Research* 28:79–
85. <http://dx.doi.org/10.2983/035.028.0114>

History of *O. lurida* in Puget Sound is covered along with its over exploitation and environmental pollutants which nearly drove it to extinction. Restoration efforts are discussed along with larval recruitment to new enhanced sites.

Ermgassen PSE zu., Gray MW., Langdon CJ., Spalding MD., Brumbaugh RD. 2013. Quantifying the historic contribution of Olympia oysters to filtration in Pacific Coast (USA) estuaries and the implications for restoration objectives. *Aquatic Ecology* 47:149–
161. <http://dx.doi.org/10.1007/s10452-013-9431-6>

Filtration rates and ecological impact of *O. lurida* was estimated for five Pacific estuaries. Historically, *O. lurida* populations did not filter the full water amount in the estuaries between tides.

Gillespie GE. 2009. Status of the Olympia Oyster, *Ostrea lurida*, in British Columbia, Canada. *Journal of Shellfish Research* 28:59–68. <http://dx.doi.org/10.2983/035.028.0112>

A review of *O. lurida* in British Columbia, Canada, discusses regularly low recruitment levels in estuaries. While population numbers remain low overall, there are no plans for commercialization, and further protection and restoration plans are mentioned.

Grosholz ED., Zabin C. 2010. Investigating the Limits of Native Oyster Recovery and Restoration. *California Sea Grant College Program*. Permalink:<http://escholarship.org/uc/item/8zr5h69f>

Native crab populations protect oysters by preying on native whelks, however, invasive crabs and invasive whelks overwhelm oyster populations. Temperature and salinity gradients do play an important role in oyster growth and recruitment.

Hettinger A., Sanford E., Hill TM., Russell AD., Sato KNS., Hoey J., Forsch M., Page HN., Gaylord B. 2012. Persistent carry-over effects of planktonic exposure to ocean acidification in the Olympia oyster. *Ecology* 93:2758–2768. <http://dx.doi.org/10.1890/12-0567.1>

Adverse environmental conditions experienced by oyster larvae can impede growth and affect later development. Studies or interventions focusing on just one life stage may miss the bigger picture.

Hopkins AE. 1936. Ecological Observations on Spawning and Early Larval Development in the Olympia Oyster (*Ostrea lurida*). *Ecology* 17:551–566. <http://dx.doi.org/10.2307/1932760>

Observational study of *Ostrea lurida* reproduction and early life history in southern Puget Sound. Denotes temperatures for spawning, peak spawn timing, and larval abundance in water column.

McGraw KA. 2009. The Olympia Oyster, *Ostrea lurida* Carpenter 1864 Along the West Coast of North America. *Journal of Shellfish Research* 28:5–10. <http://dx.doi.org/10.2983/035.028.0110>

An overview of the Olympia Oyster. Natural distributions, historic exploitation of the species, and current scientific studies are reviewed.

Meyer GR., Lowe GJ., Kim E., Abbott CL., Johnson SC., Gilmore SR. 2010. Health Status of Olympia Oysters (*Ostrea lurida*) in British Columbia, Canada. *Journal of Shellfish Research* 29:181–185. <http://dx.doi.org/10.2983/035.029.0112>

PCR assays and histological work were used to test for pathogens and disease in declining Olympia Oyster populations. Histological examination discovered five parasite/symbiotic organisms, and PCR assays detected six pathogens. However, none of the pathogen/disease levels were noted to be of detrimental significance.

Polson MP., Hewson WE., Eernisse DJ., Baker PK., Zacherl DC. 2009. You Say Conchaphila, I Say *Lurida*: Molecular Evidence for Restricting the Olympia Oyster (*Ostrea lurida* Carpenter

1864) to Temperate Western North America. *Journal of Shellfish Research* 28:11–

21. <http://dx.doi.org/10.2983/035.028.0102>

Molecular evidence clearly indicates that *O. conchaphila* and *O. lurida* are two separate species.

Polson MP., Zacherl DC. 2009. Geographic Distribution and Intertidal Population Status for the Olympia Oyster, *Ostrea lurida* Carpenter 1864, from Alaska to Baja. *Journal of Shellfish Research* 28:69–77. <http://dx.doi.org/10.2983/035.028.0113>

A geographic survey of *Ostrea lurida* populations along the Pacific coast. This survey identifies southern California sites for possible restoration.

Pritchard C., Shanks A., Rimler R., Oates M., Rumrill S. 2015. The Olympia Oyster *Ostrea lurida*: Recent Advances in Natural History, Ecology, and Restoration. *Journal of Shellfish Research* 34:259–271. <http://dx.doi.org/10.2983/035.034.0207>

A literature review of all aspects relating to *O. lurida* (ecology, biology, reproduction, phylogeny, history, restoration efforts) since Baker's review in 1995.

Seale EM., Zacherl DC. 2009. Seasonal Settlement of Olympia Oyster Larvae, *Ostrea lurida* Carpenter 1864 and Its Relationship to Seawater Temperature in Two Southern California Estuaries. *Journal of Shellfish Research* 28:113–120. <http://dx.doi.org/10.2983/035.028.0103>

No clear initiation or termination water temperature was noted for oyster settlement.

Trimble AC., Ruesink JL., Dumbauld BR. 2009. Factors Preventing the Recovery of a Historically Overexploited Shellfish Species, *Ostrea lurida* Carpenter 1864. *Journal of Shellfish Research* 28:97–106. <http://dx.doi.org/10.2983/035.028.0116>

Olympia Oyster recovery is retarded by native oyster shell removal, competition, and by novel settlement substrate.

White JM., Buhle ER., Ruesink JL., Trimble AC. 2009. Evaluation of Olympia Oyster (*Ostrea lurida* Carpenter 1864) Status and Restoration Techniques in Puget Sound, Washington, United States. *Journal of Shellfish Research* 28:107–112. <http://dx.doi.org/10.2983/035.028.0101>

Restoration efforts should focus on habit addition of more *O. lurida* shells for larvae to settle.

White J., Ruesink JL., Trimble AC. 2009. The nearly forgotten oyster: *Ostrea lurida* Carpenter 1864 (Olympia oyster) history and management in Washington State. *Journal of Shellfish Research* 28:43–49. <http://dx.doi.org/10.2983/035.028.0109>

Historical events have led to *O. lurida*'s poor recovery. Remaining Olympia oyster populations were not protected, instead were setup for further commercial exploitation. Water pollution, nonnative predators, and lost habitat continue to retard restoration efforts.

Xiao S., Wu X., Li L., Yu Z. 2015. Complete mitochondrial genome of the Olympia oyster *Ostrea lurida* (Bivalvia, Ostreidae). *Mitochondrial DNA* 26:471–472. <http://dx.doi.org/10.3109/19401736.2013.834428>

Complete sequencing of the mitochondrial DNA of *Ostrea lurida*

Zacherl DC., Morgan SG., Swearer SE., Warner RR. 2009. A Shell of Its Former Self: Can *Ostrea lurida* Carpenter 1864 Larval Shells Reveal Information About a Recruit's Birth Location? *Journal of Shellfish Research* 28:23–32. <http://dx.doi.org/10.2983/035.028.0107>

Chemical signature of larval shells is a fingerprint of the conditions within the bay that the larvae were brooded in.

Appendix B. Data Analysis R Codes

All R scripts and data can be found in github repositories:

Chapter 1 R scripts: <https://github.com/jheare/OluridaSurvey2014/tree/v3.0>

Chapter 2 R scripts: <https://github.com/jheare/OluridaGeneExpression/tree/v1.0>

Script Index:

Subsection: Chapter 1 R scripts

- Figures 2 and 3. Generate temperature graphs.....80
- Figure 4. Generates Kaplan-Meier graphs.....84
- Figures 5,6,7, & 14. Generates size boxplots and brooder size dotplot.....88
- Figures 8,9,10. Generates growthrate linegraph.....93
- Figure 11. Generates percent brooding graph for Oyster Bay.....96
- Figure 12. Generates percent brooding graph for Fidalgo Bay.....99
- Figure 13. Generates percent brooding graph for Clam Bay.....102
- Drill Data Analysis and Figure. Generates analysis for drill related mortality observations.....105

Subsection: Chapter 2 R Scripts

- Figures 1-9. Generates boxplots for gene expression data.....107

Chapter 1. R Scripts

Figures 2 and 3.

```
#install.packages("plyr")
#install.packages("ggplot2")
require(plyr)
require(ggplot2)
```

```
#set working directory to local copy of repository
setwd<-("**your directory here**")
```

```
daby1edit<-read.csv("./data/Dabob-temp-2014.csv")
#reads in edited CSV with raw data.
```

```
daby1edit$Date<-as.Date(daby1edit$Date,"%m/%d/%Y")
#Tells R that the date column contains dates so it knows how to deal with them
```

```
dabmeantemp<-
ddply(daby1edit,.(Date),summarise,mean_temp=mean(Temp,na.rm=T),min_temp=min(Temp,na
.rm=T),max_temp=max(Temp,na.rm=T))
#finds the mean, minimum, and maximum daily temps from raw data and creates dataframe with
them for Dabob
```

```
many1v3<-read.csv('./data/Manchester-temp-2014.csv')
#reads in edited CSV with raw data.
```

```
many1v3$Date<-as.Date(many1v3$Date,"%m/%d/%Y")
#Tells R that the date column contains dates so it knows how to deal with them
```

```
manmeantemp<-
ddply(many1v3,.(Date),summarise,mean_temp=mean(Temp,na.rm=T),min_temp=min(Temp,na
.rm=T),max_temp=max(Temp,na.rm=T))
#finds the mean, minimum, and maximum daily temps from raw data and creates dataframe with
them for Manchester
```

```
fidy1v3<-read.csv("./data/Fidalgo-temp-2014.csv")
#reads in edited CSV with raw data.
```

```
fidy1v3$Date<-as.Date(fidy1v3$Date,"%m/%d/%Y")
#Tells R that the date column contains dates so it knows how to deal with them
```

```
fidmeantemp<-
ddply(fidylv3,.(Date),summarise,mean_temp=mean(Temp,na.rm=T),min_temp=min(Temp,na.r
m=T),max_temp=max(Temp,na.rm=T))
#finds the mean, minimum, and maximum daily temps from raw data and creates dataframe with
them for Fidalgo
```

```
oysy1edit<-read.csv("./data/OysterBay-temp-2014.csv")
#reads in edited CSV with raw data.
```

```
oysy1edit$Date<-as.Date(oysy1edit$Date, "%m/%d/%Y")
#Tells R that the date column contains dates so it knows how to deal with them
```

```
oysmeantemp<-
ddply(oysy1edit,.(Date),summarise,mean_temp=mean(Temp,na.rm=T),min_temp=min(Temp,na
.rm=T),max_temp=max(Temp,na.rm=T))
#finds the mean, minimum, and maximum daily temps from raw data and creates dataframe with
them for Oyster Bay
```

```
ggplot()+
  geom_line(data=dabmeantemp, aes(x=Date, y=mean_temp,
group=1),col="forestgreen",size=1,guide=T)+
  geom_line(data=manmeantemp, aes(x=Date, y=mean_temp, group=1),col="blue",size=1)+
  geom_line(data=fidmeantemp, aes(x=Date, y=mean_temp, group=1),col="purple",size=1)+
  geom_line(data=oysmeantemp, aes(x=Date, y=mean_temp, group=1),col="orange",size=1)+
  labs(x="Date",y="Average Daily Temperature (C)")+
  theme_bw()+theme(panel.border = element_blank(),
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  axis.line = element_line(colour = "black"))
```

```
#Creates a graph with each line representing average daily temps for each site
```

```
ggplot()+
  geom_line(data=dabmeantemp, aes(x=Date, y=mean_temp, group=1, colour="1"),size=1)+
  geom_line(data=manmeantemp, aes(x=Date, y=mean_temp, group=1, colour="2"),size=1)+
  geom_line(data=fidmeantemp, aes(x=Date, y=mean_temp, group=1, colour="3"),size=1)+
  geom_line(data=oysmeantemp, aes(x=Date, y=mean_temp, group=1, colour="4"),size=1)+
  geom_hline(aes(yintercept=12.5,colour="5"),size=1)+
  scale_colour_manual(values=c("forestgreen","blue","purple","orange","red"),
  name="Site",
```

```

      labels=c("Dabob Bay", "Clam Bay", "Fidalgo Bay", "Oyster Bay", "Spawn
Thresh"))+
labs(x="Date", y="Average Daily Temperature (C)")+
theme_bw()+
theme(legend.position=c(0.13,0.15),
      legend.text=element_text(size=20),
      legend.title=element_text(size=20),
      axis.text.x=element_text(size=20),
      axis.title.x=element_text(size=20),
      axis.text.y=element_text(size=20),
      axis.title.y=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))
#Creates a graph with each line representing average daily temps for each site also adds red line
to indicate Spawning threshold

```

```

ggplot()+
geom_line(data=dabmeantemp, aes(x=Date, y=min_temp, group=1, colour="1"),size=1)+
geom_line(data=manmeantemp, aes(x=Date, y=min_temp, group=1, colour="2"),size=1)+
geom_line(data=fidmeantemp, aes(x=Date, y=min_temp, group=1, colour="3"),size=1)+
geom_line(data=oysmeantemp, aes(x=Date, y=min_temp, group=1, colour="4"),size=1)+
scale_colour_manual(values=c("#333333", "#cccccc", "#999999", "#666666"),
                    name="Site",
                    labels=c("Dabob Bay", "Clam Bay", "Fidalgo Bay", "Oyster Bay"))+
labs(x="Date", y="Minimum Daily Temperature (C)")+
theme_bw()+
theme(legend.position=c(0.13,0.18),
      legend.text=element_text(size=20),
      legend.title=element_text(size=20),
      axis.text.x=element_text(size=20),
      axis.title.x=element_text(size=20),
      axis.text.y=element_text(size=20),
      axis.title.y=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))
#Creates a graph with each line representing observed minimum daily temps for each site

```

```

ggplot()+
geom_line(data=dabmeantemp, aes(x=Date, y=max_temp, group=1, colour="1"),size=1)+
geom_line(data=manmeantemp, aes(x=Date, y=max_temp, group=1, colour="2"),size=1)+

```

```

geom_line(data=fidmeantemp, aes(x=Date, y=max_temp, group=1, colour="3"),size=1)+
geom_line(data=oysmeantemp, aes(x=Date, y=max_temp, group=1, colour="4"),size=1)+
scale_colour_manual(values=c("#333333", "#cccccc", "#999999", "#666666"),
  name="Site",
  labels=c("Dabob Bay", "Clam Bay", "Fidalgo Bay", "Oyster Bay"))+
labs(x="Date", y="Maximum Daily Temperature (C)")+
theme_bw()+
theme(legend.position=c(0.25,0.8),
  legend.text=element_text(size=20),
  legend.title=element_text(size=20),
  axis.text.x=element_text(size=20),
  axis.title.x=element_text(size=20),
  axis.text.y=element_text(size=20),
  axis.title.y=element_text(size=20))+
theme(panel.border = element_blank(),
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  axis.line = element_line(colour = "black"))
#Creates a graph with each line representing observed maximum daily temps for each site

#Dates Min Temp met Spawn Threshold
oyspawntemp<-oysmeantemp[(oysmeantemp$min_temp >= 12.5 &
format(oysmeantemp$Date,"% Y")=="2014"),]
manspawntemp<-manmeantemp[(manmeantemp$min_temp >= 12.5 &
format(manmeantemp$Date,"% Y")=="2014"),]
fidspawntemp<-fidmeantemp[(fidmeantemp$min_temp >= 12.5 &
format(fidmeantemp$Date,"% Y")=="2014"),]

```

Figure 4.

```

=====
#
#UNCOMMENT the lines below if you do have the packages already installed
#
#install.packages("RVAideMemoire")
#install.packages("multcomp")
=====

#loads required packages
require(survival)
require(RVAideMemoire)
require(multcomp)

setwd("**your directory here**")

#reads in Kaplan Meier formatted survival data
kmdab=read.csv("./data/KMdataDabob.csv")

#shows names
names(kmdab)

#Finds the mean and variance for each population at site
with(kmdab, tapply(Death[Status==1],Population[Status==1],mean))
with(kmdab, tapply(Death[Status==1],Population[Status==1],var))

#Generates summary statistics and survival info for Kaplan Meier
fit1=with(kmdab,survfit(Surv(Death,Status)~Population))

#Shows summary statistics for Survival
summary(fit1)

#Plots Kaplan Meier graph
plot(fit1,xlim=c(0,11), col=c("blue","purple","orange"), xlab="Survival Time from Outplant in
Months", ylab="Proportion Surviving", lwd=10)
legend("bottomleft", title="Population", c("Hood Canal","Northern","Southern"),
fill=c("blue","purple","orange"))

#reads in Kaplan Meier formatted survival data
kmman=read.csv("./data/KMdataMan.csv")

#shows names
names(kmman)

#Finds the mean and variance for each population at site

```

```

with(kmman, tapply(Death[Status==1],Population[Status==1],mean))
with(kmman, tapply(Death[Status==1],Population[Status==1],var))

#Generates summary statistics and survival info for Kaplan Meier
fit2=with(kmman, survfit(Surv(Death,Status)~Population))

#Shows summary statistics for Survival
summary(fit2)

#Plots Kaplan Meier graph
plot(fit2, col=c("blue","purple","orange"), xlab="Survival Time from Outplant in Months",
ylab="Proportion Surviving", lwd=2)

#reads in Kaplan Meier formatted survival data
kmfid=read.csv("./data/KMdataFid.csv")

#Finds the mean and variance for each population at site
with(kmfid, tapply(Death[Status==1],Population[Status==1],mean))
with(kmfid, tapply(Death[Status==1],Population[Status==1],var))

#Generates summary statistics and survival info for Kaplan Meier
fit3=with(kmfid, survfit(Surv(Death,Status)~Population))

#Shows summary statistics for Survival
summary(fit3)

#Plots Kaplan Meier graph
plot(fit3, col=c("blue","purple","orange"), xlab="Survival Time from Outplant in Months",
ylab="Proportion Surviving", lwd=2)

#reads in Kaplan Meier formatted survival data
kmoys=read.csv("./data/KMdataOys.csv")

#Finds the mean and variance for each population at site
with(kmoys, tapply(Death[Status==1],Population[Status==1],mean))
with(kmoys, tapply(Death[Status==1],Population[Status==1],var))

#Generates summary statistics and survival info for Kaplan Meier
fit4=with(kmoys, survfit(Surv(Death,Status)~Population))

#Shows summary statistics for Survival
summary(fit4)

#Plots Kaplan Meier graph

```

```
plot(fit4, col=c("blue", "purple", "orange"), xlab="Survival Time from Outplant in Months",
ylab="Proportion Surviving", lwd=10)
```

```
#Summary of Survival Information
mansum<-summary(survfit(Surv(Death,Status)~Population,data=kmman))
print(mansum)
```

```
#calculates p-values for differences in Survival between groups
mansurv<-survdif(Surv(Death,Status)~Population,data=kmman,rho=1)
#prints survdiff statistics to show significant differences between groups
print(mansurv)
```

```
#Summary of Survival Information
dabsum<-summary(survfit(Surv(Death,Status)~Population,data=kmdab))
print(dabsum)
```

```
#calculates p-values for differences in Survival between groups
dabsurv<-survdif(Surv(Death,Status)~Population,data=kmdab)
```

```
#prints survdiff statistics to show significant differences between groups
print(dabsurv)
```

```
#calculates p-values for differences in Survival between groups
fidsurv<-survdif(Surv(Death,Status)~Population,data=kmfid)
```

```
#prints survdiff statistics to show significant differences between groups
print(fidsurv)
```

```
#Summary of Survival Information
fidsum<-summary(survfit(Surv(Death,Status)~Population,data=kmfid))
print(fidsum)
```

```
#calculates p-values for differences in Survival between groups
oyssurv<-survdif(Surv(Death,Status)~Population,data=kmoys)
```

```
#prints survdiff statistics to show significant differences between groups
print(oyssurv)
```

```
#Summary of Survival Information
oyssum<-summary(survfit(Surv(Death,Status)~Population,data=kmoys))
print(oyssum)
```

```
#Exploratory Statistics for all data
kmall=read.csv("../data/KMdataAll.csv")
names(kmall)
```

```
allfit<-coxph(Surv(Death,Status)~Site+Population+Site:Population,data=kmall)
thingy<-cox.zph(allfit)
plot(thingy[5])
allaov<-anova(allfit)
```

```
allaov
summary(allfit)
```

```
allsurv<-survdifff(Surv(Death,Status)~Site+Population,data=kmall)
print(allsurv)
plot(allsurv)
fitall2=(Surv(Death,Status)~as.factor(Site)+as.factor(Population),data=kmall)
anova(fitall2)
TukeyHSD(allfit)
```

#Code for developing publication quality figures.

```
plot(fit1,xlim=c(0,11), col=gray.colors(3,start=0, end=0.9), xlab="Survival Time from Outplant
in Months", ylab="Proportion Surviving",cex.axis=2,cex.lab=1.5,lwd=10)
legend("bottomleft", title="Population", c("Hood Canal","Northern","Southern"),
fill=gray.colors(3,start=0, end=0.9))
text(0.8, 0.8, "C", cex=6)
text(7, 0.7, "a", cex=2)
text(7, 0.52, "b", cex=2)
text(7, 0.33, "c", cex=2)
plot(fit2, col=gray.colors(3,start=0, end=0.9), xlab="Survival Time from Outplant in Months",
ylab="Proportion Surviving",cex.axis=2,cex.lab=1.5,lwd=10)
text(0.8, 0.8, "B", cex=6)
text(11, 1.01, "a", cex=2)
plot(fit3, col=gray.colors(3,start=0, end=0.9), xlab="Survival Time from Outplant in Months",
ylab="Proportion Surviving",cex.axis=2,cex.lab=1.5,lwd=10)
text(0.8, 0.8, "D", cex=6)
plot(fit4, col=gray.colors(3,start=0, end=0.9), xlab="Survival Time from Outplant in Months",
ylab="Proportion Surviving",cex.axis=2,cex.lab=1.5,lwd=10)
text(0.8, 0.8, "A", cex=6)
text(11, 0.8, "a", cex=2)
text(11, 0.63, "b", cex=2)
text(11, 0.48, "c", cex=2)
```

Figures 5, 6, 7, & 14.

```

=====
#
#UNCOMMENT the lines below if you do have the packages already installed
#
#install.packages("ggplot2")
#install.packages("plyr")
#install.packages("splitstackshape")
#install.packages("nparcomp")
#install.packages("PMCMR")
#install.packages("afex")
=====

#loads required packages
require(ggplot2)
require(plyr)
require(splitstackshape)
require(nparcomp)
require(PMCMR)
require(pastecs)
require(afex)

#set working directory
setwd("**your directory here**")

#creates dataframe and reads in the CSV file for sizes
y1size=read.csv('./data/Size-outplant-end-all-2013-14.csv')

#check data
View(y1size)

#make R understand dates
y1size$Date<-as.Date(y1size$Date, "%m/%d/%Y")

#create table of ave size for outplant and year one for each pop at each site
y1meansize<-ddply(y1size,.(Date,Site,Pop),summarise, mean_size=mean(Length.mm,na.rm=T))

#print it out
print(y1meansize)

#create at DF with Average size for Each Site
y1sitemeansize<-ddply(y1size,.(Date,Site),summarise, mean_size=mean(Length.mm,na.rm=T))

#print it out
print(y1sitemeansize)

```

```

#produce some descriptive statistics using stat describe
y1sitestats<-ddply(y1size,.(Date, Site),summarise,
stats=stat.desc(Length.mm)[c(9,10,11,12,13)])

#print it out, stats are in the order (mean, STD Error, 95% Confidence Interval, Variance, STD
Deviation)
print(y1sitestats)

#now we need to create subsets for each site for out plant and end of year 1
outmany1<-ddply(y1size,.(Length.mm,Pop,Tray,Sample,Area),subset,Date=="2013-08-
16"&Site=="Manchester")
outfidyl1<-ddply(y1size,.(Length.mm,Pop,Tray,Sample,Area),subset,Date=="2013-08-
16"&Site=="Fidalgo")
outoosyl1<-ddply(y1size,.(Length.mm,Pop,Tray,Sample,Area),subset,Date=="2013-08-
16"&Site=="Oyster Bay")
endmany1<-ddply(y1size,.(Length.mm,Pop,Tray,Sample,Area),subset,Date=="2014-10-
24"&Site=="Manchester")
endfidyl1<-ddply(y1size,.(Length.mm,Pop,Tray,Sample,Area),subset,Date=="2014-10-
17"&Site=="Fidalgo")
endoosyl1<-ddply(y1size,.(Length.mm,Pop,Tray,Sample,Area),subset,Date=="2014-09-
19"&Site=="Oyster Bay")

#using ggplot2 to create Boxplots

ggplot()+
  geom_boxplot(data=endmany1,aes(x=Pop,y=Length.mm,fill=Pop))+
  scale_colour_grey(start=0, end=0.9,guide=F)+
  scale_fill_grey(start=0, end=0.9, guide=F)+
  ylim(c(0,50))+
  labs(x="Population",y="Length (mm)")+
  scale_x_discrete(labels=c("Dabob Bay","Fidalgo Bay","Oyster Bay"))+
  annotate("text", x=c("4N","4H","4S"),y=50, label=c("A","B","A"),size=10)+
  theme_bw()+
  theme(axis.text.x=element_text(size=20),
        axis.title.x=element_text(size=25, vjust=0.1),
        axis.title.y=element_text(size=25, vjust=2),
        axis.text.y=element_text(size=20))+
  theme(panel.border = element_blank(),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))

ggplot()+
  geom_boxplot(data=endfidyl1,aes(x=Pop,y=Length.mm,fill=Pop))+

```

```

scale_colour_grey(start=0, end=0.9,guide=F)+
scale_fill_grey(start=0, end=0.9, guide=F)+
ylim(c(0,50))+
labs(x="Population",y="Length (mm)")+
scale_x_discrete(labels=c("Dabob Bay","Fidalgo Bay","Oyster Bay"))+
annotate("text", x=c("2N","2H","2S"),y=50, label=c("A","B","A"),size=10)+
theme_bw()+
theme(axis.text.x=element_text(size=20),
      axis.title.x=element_text(size=25, vjust=0.1),
      axis.title.y=element_text(size=25, vjust=2),
      axis.text.y=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))

ggplot()+
geom_boxplot(data=endoysy1,aes(x=Pop,y=Length.mm,fill=Pop))+
scale_colour_grey(start=0, end=0.9,guide=F)+
scale_fill_grey(start=0, end=0.9, guide=F)+
ylim(c(0,50))+
labs(x="Population",y="Length (mm)")+
scale_x_discrete(labels=c("Dabob Bay","Fidalgo Bay","Oyster Bay"))+
annotate("text", x=c("1N","1H","1S"),y=50, label=c("B","A","A"),size=10)+
theme_bw()+
theme(axis.text.x=element_text(size=20),
      axis.title.x=element_text(size=25, vjust=0.1),
      axis.title.y=element_text(size=25, vjust=2),
      axis.text.y=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))

#Check Data for Normality
normality<-
ddply(y1size,.(Date,Site,Pop),summarize,n=length(Length.mm),sw=shapiro.test(as.numeric(Length.mm))[2])
#Data is not normally distributed based on P-Values from Shapiro Wilkes test.

#Create New column with Pop labels for analysis
y1size$Pop2<-y1size$Pop
y1size$Pop2<-
revalue(y1size$Pop2,c("1H"="H","2H"="H","4H"="H","1N"="N","2N"="N","4N"="N","1S"="S","2S"="S","4S"="S"))

```

```

#Here we subset the data set to only include data from the end of year 1
endy1<-ddply(y1size,.(Length.mm,Site,Pop,Tray,Sample,Area,Pop2),subset,Date>="2014-09-
19")

#check for Normality again
normality<-
ddply(endy1,.(Date,Site,Pop),summarize,n=length(Length.mm),sw=shapiro.test(as.numeric(Len
gth.mm))[2])
#Still Not normal, must use nonparametric test.

#using size data from the final sampling for Kruskal Wallis test to compare size versus site
sizekw<-kruskal.test(endy1$Length.mm~endy1$Site,endy1)
print(sizekw)

#using size data from the final sampling for Kruskal Wallis test to compare size versus
Population
sizekwpop<-kruskal.test(endy1$Length.mm~endy1$Pop2,endy1)
print(sizekwpop)

#Using the size data to produce a Post Hoc Nemenyi Test to generate p-values for each
comparison for Size vs Site
sizenemenyi1<-posthoc.kruskal.nemenyi.test(x=endy1$Length.mm,g=endy1$Site,
method="Tukey")
sizenemenyi1

#Using the size data to produce a Post Hoc Nemenyi Test to generate p-values for each
comparison for Size vs Population
sizenemenyi2<-posthoc.kruskal.nemenyi.test(x=endy1$Length.mm,g=endy1$Pop2,
method="Tukey")
sizenemenyi2

#Using the size data to produce a Post Hoc Nemenyi Test to generate p-values for each
comparison for Size vs Site/Population Interaction
sizenemenyi3<-posthoc.kruskal.nemenyi.test(x=endy1$Length.mm,g=endy1$Site:endy1$Pop2,
method="Tukey")
sizenemenyi3

#Brooder Size Data
broodersizes<-read.csv('./data/Broodersizes.csv')
#Let R know what format dates are in
broodersizes$Date<-as.Date(broodersizes$Date,"%m/%d/%Y")

```

```

#Run ANOVA on Brooding female size
brdszaov<-
aov(broodersizes$Size~broodersizes$Site+broodersizes$Population+broodersizes$Site:broodersizes$Population,brodersizes)
print(brdszaov)
summary(brdszaov)
#Run TukeyHSD post hoc on Brooding Female Sizes
tkbrdsz<-TukeyHSD(brdszaov)
print(tkbrdsz)

```

```

#Dotplot for Brooding Female Sizes
ggplot(broodersizes, aes(x=Site, fill=Population, y=Size))+
  geom_dotplot(binwidth=0.5,binaxis='y',stackdir="center",
  position=position_dodge(width=0.5))+
  scale_fill_grey(start=0, end=.9, labels=c("Dabob", "Fidalgo", "Oyster Bay"))+
  theme_bw()+
  labs(x="Site",y="Length (mm)")+
  scale_x_discrete(labels=c("Northern", "Central", "Southern"))+
  theme(axis.text.x=element_text(size=20),
        axis.title.x=element_text(size=25, vjust=0.1),
        axis.title.y=element_text(size=25, vjust=2),
        axis.text.y=element_text(size=20),
        legend.justification=c(0,1),
        legend.position=c(0,1),
        legend.title=element_text(size=15),
        legend.text=element_text(size=15))+
  theme(panel.border = element_blank(),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))

```

```

#mixed effects models with p-value generation
sizelme<-lmer(Length.mm~Pop2*Site+(~Pop2|Tray))
sizelme
sizep<-mixed(Length.mm~Pop2*Site+(Pop2|Tray),data=endy1)
sizep

```

Figures 8,9,10.

```

=====
#
#UNCOMMENT the lines below if you do have the packages already installed
#
#install.packages("ggplot2")
#install.packages("plyr")
#install.packages("splitstackshape")

=====

#loads required packages
require(ggplot2)
require(plyr)
require(splitstackshape)

#set working directory
setwd("***your directory here**")

#reads in growth rate data
grate<-read.csv("./data/ImageJ-size-data-2013-2014.csv",header=T)

#formats data frame to work with plyr and ggplot2
grate1<-cSplit(grate, "Tray", sep=" ", drop=F)
grate1$Pop[1:9215]<-grate1$Tray_1[1:9215]
grate1$Tray2[1:9215]<-grate1$Tray_2[1:9215]
grate1$Site<-gsub("manchester", "Manchester",grate1$Site)
grate1$Tray<-NULL
grate1$Tray_1<-NULL
grate1$Tray_2<-NULL
grate1$Date<-as.Date(grate1$Date, "%m/%d/%Y")

#creates a data table of summary statistics for all samples
gratemean<-ddply(grate1,.(Date,Pop,Site),summarise,mean_length=mean(Length.mm,na.rm=T),
                sd=sd(Length.mm,na.rm=T), N=length(Length.mm),se=sd/sqrt(N))
ciMult<-qt(0.975/2+.5,gratemean$N-1)
gratemean$ci<-gratemean$se*ciMult

#subsets summary statistics table into site related relevant data while removing messy summer
data
grfid<-gratemean[which(gratemean$Site=="Fidalgo" & gratemean$Date<="2014-05-
02"|gratemean$Site=="Fidalgo" & gratemean$Date>="2014-09-19"),]
grman<-gratemean[which(gratemean$Site=="Manchester" & gratemean$Date<="2014-05-
02"|gratemean$Site=="Manchester" & gratemean$Date>="2014-09-19"),]

```

```
groys<-gratemean[which(gratemean$Site=="Oyster Bay"& gratemean$Date<="2014-05-02"|gratemean$Site=="Oyster Bay"&gratemean$Date>="2014-09-19"),]
```

```
#Plots all growth rates from all sizes and populations against each other.
```

```
ggplot(gratemean, aes(x=Date,y=mean_length,color=Pop))+
  geom_point(size=4)+geom_line(size=2)+
  geom_errorbar(aes(x=Date, ymin=mean_length-ci,ymax=mean_length+ci),size=3)+
  scale_colour_grey(start=0, end=.9,labels=c("Dabob", "Fidalgo", "Oyster
Bay", "Dabob", "Fidalgo", "Oyster Bay", "Dabob", "Fidalgo", "Oyster Bay"))+
  scale_fill_grey(start=0, end=.9,labels=c("Dabob", "Fidalgo", "Oyster
Bay", "Dabob", "Fidalgo", "Oyster Bay", "Dabob", "Fidalgo", "Oyster Bay"))+
  theme_bw()+facet_wrap(~Site,ncol=1)
```

```
#Plots growth rates for all populations at Fidalgo Bay
```

```
ggplot(grfid, aes(x=Date,y=mean_length,color=Pop))+
  geom_point(size=2)+geom_line(size=1)+
  geom_errorbar(aes(ymin=mean_length-ci,ymax=mean_length+ci),color="black",width=10)+
  scale_colour_grey(start=0, end=.9,labels=c("Dabob", "Fidalgo", "Oyster Bay"))+
  scale_fill_grey(start=0, end=.9,labels=c("Dabob", "Fidalgo", "Oyster Bay"))+
  theme_bw()+labs(x="Observation",y="Mean Shell Length (mm)")+
  theme(axis.text.x=element_text(size=20),
        axis.title.x=element_text(size=25, vjust=0.1),
        axis.title.y=element_text(size=25, vjust=2),
        axis.text.y=element_text(size=20))+
  theme(panel.border = element_blank(),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))+
  theme(legend.justification=c(0,1),
        legend.position=c(0,1),
        axis.text.x=element_text(size=20),
        axis.text.y=element_text(size=20),
        axis.title.x=element_text(size=20),
        axis.title.y=element_text(size=20),
        legend.title=element_text(size=20),
        legend.text=element_text(size=20))+
  ylim(0,40)
```

```
#Plots growth rates for all populations at Clam Bay
```

```
ggplot(grman, aes(x=Date,y=mean_length,color=Pop))+
  geom_point(size=2)+geom_line(size=1)+
  geom_errorbar(aes(ymin=mean_length-ci,ymax=mean_length+ci),color="black",width=10)+
  scale_colour_grey(start=0, end=.9,labels=c("Dabob", "Fidalgo", "Oyster Bay"))+
  scale_fill_grey(start=0, end=.9,labels=c("Dabob", "Fidalgo", "Oyster Bay"))+
  theme_bw()+labs(x="Observation",y="Mean Shell Length (mm)")+
  theme(axis.text.x=element_text(size=20),
```

```

axis.title.x=element_text(size=25, vjust=0.1),
axis.title.y=element_text(size=25, vjust=2),
axis.text.y=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))+
theme(legend.justification=c(0,1),
      legend.position=c(0,1),
      axis.text.x=element_text(size=20),
      axis.text.y=element_text(size=20),
      axis.title.x=element_text(size=20),
      axis.title.y=element_text(size=20),
      legend.title=element_text(size=20),
      legend.text=element_text(size=20))+
ylim(0,40)

```

```

#Plots growth rates for all populations at Oyster Bay
ggplot(groys, aes(x=Date,y=mean_length,color=Pop))+
  geom_point(size=2)+geom_line(size=1)+
  geom_errorbar(aes(ymin=mean_length-ci,ymax=mean_length+ci),color="black",width=10)+
  scale_colour_grey(start=0, end=.9,labels=c("Dabob","Fidalgo","Oyster Bay"))+
  scale_fill_grey(start=0, end=.9,labels=c("Dabob","Fidalgo","Oyster Bay"))+
  theme_bw()+labs(x="Observation",y="Mean Shell Length (mm)")+
  theme(axis.text.x=element_text(size=20),
        axis.title.x=element_text(size=25, vjust=0.1),
        axis.title.y=element_text(size=25, vjust=2),
        axis.text.y=element_text(size=20))+
  theme(panel.border = element_blank(),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.line = element_line(colour = "black"))+
  theme(legend.justification=c(0,1),
        legend.position=c(0,1),
        axis.text.x=element_text(size=20),
        axis.text.y=element_text(size=20),
        axis.title.x=element_text(size=20),
        axis.title.y=element_text(size=20),
        legend.title=element_text(size=20),
        legend.text=element_text(size=20))+
  ylim(0,40)

```

Figure 11.

```

=====
#
#UNCOMMENT the lines below if you do have the packages already installed
#
#install.packages("plyr")
#install.packages("ggplot2")
#install.packages("scales")
#install.packages("grid")
#install.packages("gtable")
=====

#Load Required Packages
require(plyr)
require(ggplot2)
require(scales)
require(grid)
require(gtable)

#set working directory
setwd("**your directory here**")

#read in brood numbers csv with all brooding data
brood<-read.csv("./data/Brood-numbers-all-2014.csv")

#Make sure dates are understood to be Dates
brood$Date<-as.Date(brood$Date, "%m/%d/%Y")

#subset only the data for Oyster Bay
oysbay<-subset(brood, Site=="Oyster Bay")

#The script below creates an object out of the percent brooding graph which will be stitched to
the
#temperature graph we will create to make a double Y axis graph.

grid.newpage()
p1<-ggplot(data=oysbay, aes(x=Date, weight=Percent, colour=Pop, fill=Pop))+
  geom_bar(binwidth=3, position=position_dodge())+
  ylim(c(0,20))+
  theme(axis.text.x=element_text(angle=90, size=10, vjust=0.5))+

scale_colour_manual(values=c("#cccccc", "#999999", "#666666"), labels=c("Dabob", "Fidalgo",
Oyster Bay"))+

```

```

scale_fill_manual(values=c("#cccccc", "#999999", "#666666"), labels=c("Dabob", "Fidalgo", "Oyster Bay"))+
labs(x="Sample Date", y="Percent Brooding")+
theme_bw()+
theme(legend.justification=c(0,1),
      legend.position=c(0,1),
      axis.text.x=element_text(size=20),
      axis.text.y=element_text(size=20),
      axis.title.x=element_text(size=20),
      axis.title.y=element_text(size=20),
      legend.title=element_text(size=20),
      legend.text=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))

#reads in temperature csv for Oyster Bay
oystemp<-read.csv("./data/OysterBay-temp-2014.csv")

#Make sure Dates are understood as Dates
oystemp$Date<-as.Date(oystemp$Date, "%m/%d/%Y")

#Finds the daily minimum temperatures
oysmintemp<-ddply(oystemp,.(Date),summarise,min_temp=min(Temp,na.rm=T))

#creates a subset of temperatures for only our sampling period
oysmintemprep<-subset(oysmintemp, Date>="2014-05-01"& Date<="2014-08-07")

#creates an object out of our temperature data graph that will be overlaid on the percent brooding graph
p2<-ggplot()+geom_line(data=oysmintemprep,
                        aes(x=Date, y=min_temp), color="black")+
ylim(c(8,18))+
theme_bw() %+replace%
theme(panel.background = element_rect(fill = NA),
      panel.grid.major.x=element_blank(),
      panel.grid.minor.x=element_blank(),
      panel.grid.major.y=element_blank(),
      panel.grid.minor.y=element_blank(),
      axis.text.y=element_text(size=20,color="black"),
      axis.title.y=element_text(size=20))

#this code tells R to overlay the temperature graph on the percent brooding graph
g1<-ggplot_gtable(ggplot_build(p1))

```

```

g2<-ggplot_gtable(ggplot_build(p2))

pp<-c(subset(g1$layout,name=="panel",se=t:r))
g<-gtable_add_grob(g1, g2$grobs[[which(g2$layout$name=="panel")]],pp$t,pp$l,pp$b,pp$l)

ia<-which(g2$layout$name=="axis-l")
ga <- g2$grobs[[ia]]
ax <- ga$children[[2]]
ax$widths <- rev(ax$widths)
ax$grobs <- rev(ax$grobs)
ax$grobs[[1]]$x <- ax$grobs[[1]]$x - unit(1, "npc") + unit(0.15, "cm")
g <- gtable_add_cols(g, g2$widths[g2$layout[ia, ]$l], length(g$widths) - 1)
g <- gtable_add_grob(g, ax, pp$t, length(g$widths) - 1, pp$b)

#generates the final graph
grid.draw(g)

#General statistics on brooding using ANOVA
brood$arcperc<-asin(brood$Percent/100)
BroodANOVA<-aov(brood$arcperc~brood$Site+brood$Pop+brood$Site:brood$Pop,brood)
print(BroodANOVA)
summary(BroodANOVA)

#Tukey Post Hoc test for Brooding
broodTukey<-TukeyHSD(BroodANOVA)
print(broodTukey)

```

Figure 12.

```

=====
#
#UNCOMMENT the lines below if you do have the packages already installed
#
#install.packages("plyr")
#install.packages("ggplot2")
#install.packages("scales")
#install.packages("grid")
#install.packages("gtable")
=====

#Loads required packages
require(plyr)
require(ggplot2)
require(scales)
require(grid)
require(gtable)

#set working directory
setwd("**your directory here**")

#read in brood numbers csv with all brooding data
brood<-read.csv("./data/Brood-numbers-all-2014.csv")

#Make sure dates are understood to be Dates
brood$Date<-as.Date(brood$Date, "%m/%d/%Y")

#subset only the data for Fidalgo
fidrep<-subset(brood, Site=="Fidalgo")

#The script below creates an object out of the percent brooding graph which will be stitched to
the
#temperature graph we will create to make a double Y axis graph.

grid.newpage()
p1<-ggplot(data=fidrep, aes(x=Date, weight=Percent, colour=Pop, fill=Pop))+
  geom_bar(binwidth=3, position=position_dodge())+
  ylim(c(0,20))+
  theme(axis.text.x=element_text(angle=90, size=10, vjust=0.5))+

scale_colour_manual(values=c("#cccccc", "#999999", "#666666"), labels=c("Dabob", "Fidalgo",
Oyster Bay"))+

scale_fill_manual(values=c("#cccccc", "#999999", "#666666"), labels=c("Dabob", "Fidalgo", "Oyst
er Bay"))+

```

```

labs(x="Sample Date", y="Percent Brooding")+
theme_bw()+
theme(legend.justification=c(0,1),
      legend.position=c(0,1),
      axis.text.x=element_text(size=20),
      axis.text.y=element_text(size=20),
      axis.title.x=element_text(size=20),
      axis.title.y=element_text(size=20),
      legend.title=element_text(size=20),
      legend.text=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))

```

```

#reads in temperature CSV for Fidalgo Bay
fidtemp<-read.csv("./data/Fidalgo-temp-2014.csv")

```

```

#Make sure dates are understood as Dates
fidtemp$Date<-as.Date(fidtemp$Date,"%m/%d/%Y")

```

```

#Finds minimum daily temps for Fidalgo
fidmintemp<-ddply(fidtemp,.(Date),summarise,min_temp=min(Temp,na.rm=T))

```

```

#Subsets data to dates for our Sampling period
fidmintemprep<-subset(fidmintemp, Date>="2014-05-02"& Date<="2014-08-08")

```

```

#creates an object out of our temperature data graph that will be overlaid on the percent brooding
graph

```

```

p2<-ggplot()+
  geom_line(data=fidmintemprep,aes(x=Date, y=min_temp), color="black")+
  labs(y="Daily Minimum Water Temperature (C)")+
  ylim(c(8,18))+
  theme_bw() %+replace%
  theme(panel.background = element_rect(fill = NA),
        panel.grid.major.x=element_blank(),
        panel.grid.minor.x=element_blank(),
        panel.grid.major.y=element_blank(),
        panel.grid.minor.y=element_blank(),
        axis.text.y=element_text(size=20,color="black"),
        axis.title.y=element_text(size=20))

```

```

#this code tells R to overlay the temperature graph on the percent brooding graph
g1<-ggplot_gtable(ggplot_build(p1))

```

```
g2<-ggplot_gtable(ggplot_build(p2))

pp<-c(subset(g1$layout,name=="panel",se=t:r))
g<-gtable_add_grob(g1, g2$grobs[[which(g2$layout$name=="panel")]],pp$t,pp$l,pp$b,pp$l)

ia<-which(g2$layout$name=="axis-l")
ga <- g2$grobs[[ia]]
ax <- ga$children[[2]]
ax$widths <- rev(ax$widths)
ax$grobs <- rev(ax$grobs)
ax$grobs[[1]]$x <- ax$grobs[[1]]$x - unit(1, "npc") + unit(0.15, "cm")
g <- gtable_add_cols(g, g2$widths[g2$layout[ia, ]$l], length(g$widths) - 1)
g <- gtable_add_grob(g, ax, pp$t, length(g$widths) - 1, pp$b)

#Generates final graph
grid.draw(g)
```

Figure 13.

```

=====
#
#UNCOMMENT the lines below if you do have the packages already installed
#
#install.packages("plyr")
#install.packages("ggplot2")
#install.packages("scales")
#install.packages("grid")
#install.packages("gtable")
=====

#Loads required packages
require(plyr)
require(ggplot2)
require(scales)
require(grid)
require(gtable)

#set working directory
setwd("**your directory here**")

#read in brood numbers csv with all brooding data
brood<-read.csv("./data/Brood-numbers-all-2014.csv")

#Make sure dates are understood to be Dates
brood$Date<-as.Date(brood$Date, "%m/%d/%Y")

#subset only the data for Manchester
manrep<-subset(brood, Site=="Manchester")

#The script below creates an object out of the percent brooding graph which will be stitched to
the
#temperature graph we will create to make a double Y axis graph.

grid.newpage()
p1<-ggplot(data=manrep, aes(x=Date, weight=Percent, colour=Pop, fill=Pop))+
  geom_bar(binwidth=3, position=position_dodge())+
  ylim(c(0,20))+
  theme(axis.text.x=element_text(angle=90, size=10, vjust=0.5))+

scale_colour_manual(values=c("#cccccc", "#999999", "#666666"), labels=c("Dabob", "Fidalgo",
Oyster Bay"))+

```

```

scale_fill_manual(values=c("#cccccc", "#999999", "#666666"), labels=c("Dabob", "Fidalgo", "Oyster Bay"))+
labs(x="Sample Date", y="Percent Brooding")+
theme_bw()+
theme(legend.justification=c(0,1),
      legend.position=c(0,1),
      axis.text.x=element_text(size=20),
      axis.text.y=element_text(size=20),
      axis.title.x=element_text(size=20),
      axis.title.y=element_text(size=20),
      legend.title=element_text(size=20),
      legend.text=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))

#reads in temperature csv for Manchester
manch<-read.csv('./data/Manchester-temp-2014.csv')

#Makes sure Dates are understood as Dates
manch$Date<-as.Date(manch$Date,"%m/%d/%Y")

#Finds daily minimum temps for Manchester
manmintemp<-ddply(manch,.(Date),summarise,min_temp=min(Temp,na.rm=T))

#Subsets temperature data for our sampling period
manmintemprep<-subset(manmintemp, Date>="2014-04-30"& Date<="2014-08-06")

#creates an object out of our temperature data graph that will be overlaid on the percent brooding
graph
p2<-ggplot()+geom_line(data=manmintemprep,
                        aes(x=Date, y=min_temp), color="black")+
ylim(c(8,18))+
theme_bw() %+replace%
theme(panel.background = element_rect(fill = NA),
      panel.grid.major.x=element_blank(),
      panel.grid.minor.x=element_blank(),
      panel.grid.major.y=element_blank(),
      panel.grid.minor.y=element_blank(),
      axis.text.y=element_text(size=20,color="black"),
      axis.title.y=element_text(size=20))

```

```
#this code tells R to overlay the temperature graph on the percent brooding graph
g1<-ggplot_gtable(ggplot_build(p1))
g2<-ggplot_gtable(ggplot_build(p2))

pp<-c(subset(g1$layout,name=="panel",se=t:r))
g<-gtable_add_grob(g1, g2$grobs[[which(g2$layout$name=="panel")]],pp$t,pp$l,pp$b,pp$l)

ia<-which(g2$layout$name=="axis-l")
ga <- g2$grobs[[ia]]
ax <- ga$children[[2]]
ax$widths <- rev(ax$widths)
ax$grobs <- rev(ax$grobs)
ax$grobs[[1]]$x <- ax$grobs[[1]]$x - unit(1, "npc") + unit(0.15, "cm")
g <- gtable_add_cols(g, g2$widths[g2$layout[ia, ]$l], length(g$widths) - 1)
g <- gtable_add_grob(g, ax, pp$t, length(g$widths) - 1, pp$b)

#generates the final graph
grid.draw(g)
```

Drill Data Analysis and Figure

```

=====
#
#UNCOMMENT the lines below if you do have the packages already installed
#
#install.packages("ggplot2")
#install.packages("plyr")
#install.packages("dispmod")
#install.packages("lme4")

=====

#loads required packages
require(lme4)
require(plyr)
require(ggplot2)
require(dispmod)

#set working directory
setwd("**your directory here**")

#reads in data csv
drill<-read.csv("./data/Drill-mortality-2013-2014.csv",header=T)

#tells R to register date notation as Dates
drill$Date<-as.factor(as.Date(drill$Date, "%m/%d/%Y"))

#creates summary of all drill data
drsum<-ddply(drill,.(Date,Pop),summarise,drill1shell=sum(Drill.1.Shell,na.rm=T),
  drill2shell=sum(Drill.2.shell,na.rm=T),
  nodrill1shell=sum(No.Drill.1,na.rm=T),
  nodrill2shell=sum(No.Drill.2,na.rm=T),
  drills=(drill1shell/2+drill2shell),
  nodrills=(nodrill1shell/2+nodrill2shell),
  N=round(drills+nodrills),
  prop=(drills/N))

#creates bargraph plot to visualize drill data
ggplot(drsum,aes(x=Date,y=prop,colour=Pop, fill=Pop))+
  geom_bar(stat="identity",position=position_dodge())+
  geom_text(aes(label=N), color="black",
    stat="identity",position=position_dodge(width=0.9),
    vjust=-0.25)+
  scale_colour_grey(start=0, end=.9,labels=c("Dabob","Fidalgo","Oyster Bay"))+
  scale_fill_grey(start=0, end=.9,labels=c("Dabob","Fidalgo","Oyster Bay"))+
  theme_bw()+labs(x="Observation Date",y="Oysters with Drill Holes (proportion)")+

```

```
theme(axis.text.x=element_text(size=20),
      axis.title.x=element_text(size=25, vjust=0.1),
      axis.title.y=element_text(size=25, vjust=2),
      axis.text.y=element_text(size=20))+
theme(panel.border = element_blank(),
      panel.grid.major = element_blank(),
      panel.grid.minor = element_blank(),
      axis.line = element_line(colour = "black"))+
theme(legend.justification=c(0,1),
      legend.position=c(0,1),
      axis.text.x=element_text(size=20),
      axis.text.y=element_text(size=20),
      axis.title.x=element_text(size=20),
      axis.title.y=element_text(size=20),
      legend.title=element_text(size=20),
      legend.text=element_text(size=20))
```

```
#runs a general linear model on drill data
```

```
drglm<-glm((cbind(round(drills),round(nodrills)))~Pop,family=binomial(logit),data=drsum)
summary(drglm)
```

```
#corrects for overdispersion in GLM
```

```
drglm.mod<-glm.binomial.disp(drglm)
```

Chapter 2. Data Analysis and Figures R Code

Figures 1-9.

```

=====
#
#UNCOMMENT the lines below if you do have the packages already installed
#
#install.packages("ggplot2")
#install.packages("plyr")
#install.packages("splitstackshape")

=====

#Necessary Packages to manipulate data and plot values.
require(plyr)
require(ggplot2)
require(splitstackshape)

#set working directory
setwd("**your directory here**")

#Read in mean Ct value table
dCt<-read.csv("./data/ct-values-2015.csv", header=T)

#Split SAMPLE_ID column to create columns for population, treatment, and sample number
dCt<-cSplit(dCt,"SAMPLE_ID", sep= "_", drop=F)

#rename columns appropriately
dCt<-
rename(dCt,replace=c("SAMPLE_ID_1"="Pop", "SAMPLE_ID_2"="Treat", "SAMPLE_ID_3"=
"Sample"))

#divide each target of interest by the mean Ct value of the Actin Normalizing gene
dCt$CARM<-2^(dCt$Actinmeanct-dCt$CarmmeanCt)
dCt$TLR<-2^(dCt$Actinmeanct-dCt$TLRaverage)
dCt$CRAF<-2^(dCt$Actinmeanct-dCt$CRAFctaverage)
dCt$H2AV<-2^(dCt$Actinmeanct-dCt$H2AVavgct)
dCt$PGRP<-2^(dCt$Actinmeanct-dCt$PGRPaverage)
dCt$HSP70<-2^(dCt$Actinmeanct-dCt$HSP70averageCt)
dCt$BMP2<-2^(dCt$Actinmeanct-dCt$BMP2average)
dCt$GRB2<-2^(dCt$Actinmeanct-dCt$GRB2average)
dCt$PGEEP4<-2^(dCt$Actinmeanct-dCt$PGEEP4ctav)

#log transform the data to develop normality in data

```

```
dCt$CARMlog<-log(dCt$CARM)
dCt$TLRlog<-log(dCt$TLR)
dCt$H2AVlog<-log(dCt$H2AV)
dCt$PGRPlog<-log(dCt$PGRP)
dCt$HSP70log<-log(dCt$HSP70)
dCt$BMP2log<-log(dCt$BMP2)
dCt$GRB2log<-log(dCt$GRB2)
dCt$PGEEP4log<-log(dCt$PGEEP4)
dCt$CRAFlog<-log(dCt$CRAF)
```

```
#Run ANOVA's on all log transformed data as well as Tukey's Honestly Significant Difference
post hoc test
```

```
CARM<-aov(CARMlog~Pop+Treat+Pop:Treat, data=dCt)
CARM
TukeyHSD(CARM)
```

```
TLR<-aov(TLRlog~Pop+Treat+Pop:Treat, data=dCt)
TLR
TukeyHSD(TLR)
```

```
H2AV<-aov(H2AVlog~Pop+Treat+Pop:Treat, data=dCt)
H2AV
TukeyHSD(H2AV)
```

```
PGRP<-aov(PGRPlog~Pop+Treat+Pop:Treat, data=dCt)
PGRP
TukeyHSD(PGRP)
```

```
HSP70<-aov(HSP70log~Pop+Treat+Pop:Treat, data=dCt)
HSP70
TukeyHSD(HSP70)
```

```
BMP2<-aov(BMP2log~Pop+Treat+Pop:Treat, data=dCt)
BMP2
TukeyHSD(BMP2)
```

```
GRB2<-aov(GRB2log~Pop+Treat+Pop:Treat, data=dCt)
GRB2
TukeyHSD(GRB2)
```

```
PGEEP4<-aov(PGEEP4log~Pop+Treat+Pop:Treat, data=dCt)
PGEEP4
TukeyHSD(PGEEP4)
```

```
CRAF<-aov(CRAFlog~Pop+Treat+Pop:Treat, data=dCt)
CRAF
```

TukeyHSD(CRAF)

#graph all raw mean Ct values to produce boxplots to visualize data

```
ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=CARM,fill=Pop))+theme_bw()+
  scale_fill_grey(start=0, end=.9,
    labels=c("Dabob Bay", "Fidalgo Bay", "Oyster Bay"))+
  guides(fill=guide_legend(title="Population"))+
  theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
    axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
    legend.position=c(.09,.87),panel.grid.major=element_blank(),
    legend.key=element_rect(fill=NA))+
  ylim(c(0,0.3))+scale_x_discrete(labels=c("Control", "Mechanical", "Temperature"))+
  annotate("text",x=c("C", "M", "T"), y=0.3, label=c("A", "A", "B"), size=10)+
  labs(x="Treatment", y=expression(paste("CARM Expression (",Delta,"Ct)")))
```

```
ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=TLR, fill=Pop))+theme_bw()+
  scale_fill_grey(start=0, end=.9,
    labels=c("Dabob Bay", "Fidalgo Bay", "Oyster Bay"))+
  guides(fill=guide_legend(title="Population"))+
  theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
    axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
    legend.position=c(.09,.87),panel.grid.major=element_blank(),
    legend.key=element_rect(fill=NA))+
  ylim(c(0,0.005))+scale_x_discrete(labels=c("Control", "Mechanical", "Temperature"))+
  labs(x="Treatment", y=expression(paste("TLR Expression (",Delta,"Ct)")))
```

```
ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=H2AV,fill=Pop))+theme_bw()+
  scale_fill_grey(start=0, end=.9,
    labels=c("Dabob Bay", "Fidalgo Bay", "Oyster Bay"))+
  guides(fill=guide_legend(title="Population"))+
  theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
    axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
    legend.position=c(.09,.87),panel.grid.major=element_blank(),
    legend.key=element_rect(fill=NA))+
  ylim(c(0,1.9))+scale_x_discrete(labels=c("Control", "Mechanical", "Temperature"))+
  annotate("text",x=c("C", "M", "T"), y=1.9, label=c("A", "A", "B"), size=10)+
  annotate("text",x=c(1.25,3.25), y=1.85, label=c("*", "*"), size=12)+
  labs(x="Treatment", y=expression(paste("H2AV Expression (",Delta,"Ct)")))
```

```
ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=PGRP,fill=Pop))+theme_bw()+
  scale_fill_grey(start=0, end=.9,
    labels=c("Dabob Bay", "Fidalgo Bay", "Oyster Bay"))+
  guides(fill=guide_legend(title="Population"))+
  theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
```

```

axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
legend.position=c(.09,.87),panel.grid.major=element_blank(),
legend.key=element_rect(fill=NA))+
ylim(c(0,0.005))+scale_x_discrete(labels=c("Control","Mechanical","Temperature"))+
labs(x="Treatment", y=expression(paste("PGRP Expression (",Delta,"Ct)"))))

ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=HSP70,fill=Pop))+theme_bw()+
scale_fill_grey(start=0, end=.9,
  labels=c("Dabob Bay","Fidalgo Bay","Oyster Bay"))+
guides(fill=guide_legend(title="Population"))+
theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
  axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
  legend.position=c(.09,.80),panel.grid.major=element_blank(),
  legend.key=element_rect(fill=NA))+
ylim(c(0,.5))+scale_x_discrete(labels=c("Control","Mechanical","Temperature"))+
annotate("text",x=c("C","M","T"), y=.5, label=c("AB", "A", "B"), size=10)+
annotate("text",x=c(2.25,3.25), y=.4, label=c("*","*"), size=12)+
labs(x="Treatment", y=expression(paste("HSP70 Expression (",Delta,"Ct)"))))

ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=BMP2,fill=Pop))+theme_bw()+
scale_fill_grey(start=0, end=.9,
  labels=c("Dabob Bay","Fidalgo Bay","Oyster Bay"))+
guides(fill=guide_legend(title="Population"))+
theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
  axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
  legend.position=c(.09,.87),panel.grid.major=element_blank(),
  legend.key=element_rect(fill=NA))+
ylim(c(0,2.5))+scale_x_discrete(labels=c("Control","Mechanical","Temperature"))+
annotate("text",x=c(1.25,2.25), y=2.5, label=c("*","*"), size=12)+
labs(x="Treatment", y=expression(paste("BMP2 Expression (",Delta,"Ct)"))))

ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=GRB2,fill=Pop))+theme_bw()+
scale_fill_grey(start=0, end=.9,
  labels=c("Dabob Bay","Fidalgo Bay","Oyster Bay"))+
guides(fill=guide_legend(title="Population"))+
theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
  axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
  legend.position=c(.09,.87),panel.grid.major=element_blank(),
  legend.key=element_rect(fill=NA))+
ylim(c(0,1.5))+scale_x_discrete(labels=c("Control","Mechanical","Temperature"))+
annotate("text",x=c(1.25,2.25), y=1.27, label=c("*","*"), size=12)+
labs(x="Treatment", y=expression(paste("GRB2 Expression (",Delta,"Ct)"))))

ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=PGEEP4,fill=Pop))+theme_bw()+
scale_fill_grey(start=0, end=.9,
  labels=c("Dabob Bay","Fidalgo Bay","Oyster Bay"))+

```

```

guides(fill=guide_legend(title="Population"))+
theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
      axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
      legend.position=c(.09,.87),panel.grid.major=element_blank(),
      legend.key=element_rect(fill=NA))+
ylim(c(0,0.15))+scale_x_discrete(labels=c("Control","Mechanical","Temperature"))+
labs(x="Treatment", y=expression(paste("PGEEP4 Expression (",Delta,"Ct)")))

ggplot(data=dCt)+geom_boxplot(aes(x=Treat, y=CRAF,fill=Pop))+theme_bw()+
scale_fill_grey(start=0, end=.9,
                labels=c("Dabob Bay", "Fidalgo Bay", "Oyster Bay"))+
guides(fill=guide_legend(title="Population"))+
theme(axis.text.x=element_text(size=20), axis.text.y=element_text(size=20),
      axis.title.x=element_text(size=25), axis.title.y=element_text(size=25),
      legend.position=c(.09,.87),panel.grid.major=element_blank(),
      legend.key=element_rect(fill=NA))+
ylim(c(0,.3))+scale_x_discrete(labels=c("Control","Mechanical","Temperature"))+
annotate("text",x=c("C","M","T"), y=.3, label=c("A", "B", "AB"), size=10)+
labs(x="Treatment", y=expression(paste("CRAF Expression (",Delta,"Ct)")))

```