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Natural Variation and Evolutionary Responses to Climate Change Stressors in Marine Invertebrates

Joanna Sarah Griffiths
Louisiana State University and Agricultural and Mechanical College

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NATURAL VARIATION AND EVOLUTIONARY RESPONSES TO CLIMATE CHANGE STRESSORS IN MARINE INVERTEBRATES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by
Joanna Sarah Griffiths
B.S. Pacific Lutheran University 2014
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Abstract

Our rapidly changing climate is putting many species at risk of extinction and there is an urgent need to understand how species will respond to these changes. In this dissertation, I evaluate how three species of marine invertebrates (corals, oysters, and copepods) respond to stressful conditions in their current environments and how plasticity and evolutionary adaptation could alter their response to future climate change stressors. I first employed a space for time study to elucidate population differences in the response of cold-water corals, *Balanophyllia elegans*, to future ocean acidification. I found evidence that upwelling history (natural low pH exposure) influences the physiological and transcriptomic response of *B. elegans* to laboratory low pH exposure. I found that populations that naturally experience low pH (due to more frequent upwelling events) may be more tolerant to future ocean acidification. I also used a space for time study on two Louisiana populations of the eastern oyster, *Crassostrea virginica*, and found evidence of local adaptation and plasticity that influences their response to stressful low salinities. The population that naturally experiences high salinity was unable to maintain growth in low salinity conditions and employed a different plastic response to the low salinity population. Finally, I used a combination of quantitative genetics and experimental evolution studies to identify standing genetic variation in populations of *C. virginica*, and the species as a whole, involved in tolerance to current and potentially future stressful environments. I show that the two Louisiana populations of *C. virginica* have ample genetic variation for selection to act on and that they are capable of adapting to low salinity. I also demonstrate the use of hybridization and experimental evolution to increase the fitness of the copepod, *Tigriopus californicus*, to future warming scenarios. In my dissertation, I have contributed to our knowledge of how marine invertebrates respond to stressors in their environments which will inform conservation efforts under future climate change scenarios.
Chapter 1.
Introduction

Our climate is changing at unprecedented rates due to anthropogenic emissions, posing a major threat to biodiversity and the many ecosystem services that we depend upon. As a result of our agricultural practices and the burning of fossil fuels, which emits excess greenhouse gases into our atmosphere, we are experiencing rising temperatures, increases in ocean acidification, and dramatic changes in precipitation (IPCC 2014). Already, we have recorded temperature increases in the atmosphere and ocean of 0.85°C as well as a drop in ocean pH by 0.1 units (IPCC 2014).

We are altering the climate and habitat of entire ecosystems and the result is rapid declines in populations and the extinction of species worldwide. With documented extinction of 1000-10,000 species per year, some scientists classify this as the sixth mass extinction (Chivian and Bernstein, 2008). The key to conserving these ecosystems requires an understanding of how species are responding—will all populations be at equal risk or are some populations more resilient than others? There is evidence that some species may be capable of evading extinction potentially through changing their distributions. A meta-analysis of 1700 species found that range shifts as fast as 6.1 - 16.9 km/decade have been reported (Parmesan and Yohe 2003; Chen et al. 2011).

Current models that aim to predict a species persistence in future climate change scenarios typically estimate the species’ environmental niche from its current range and extrapolate to predict new ranges as the climate changes (Urban 2015). However, these predictions omit important biological factors that may influence the species’ ability to migrate, such as dispersal ability, life-history, physiology, and evolution. Accurate predictions of a species’ response to climate change requires detailed knowledge of that species’ biology and evolutionary potential (Bay et al. 2017). In addition, many species may not be able to shift their distribution(s) as fast as the climate changes due to dispersal limitations, such as habitat fragmentation and reproductive rates (Loarie et al. 2009).

In this dissertation, I aim to further our knowledge of how species will respond to current stressors in their environment and how this may influence their response to future changes in our climate. Using three species of marine invertebrates (corals, oysters, and copepods), I examine how marine invertebrates deal with current stressors such as ocean acidification, salinity, and temperature in order to determine a holistic approach to how they may respond to climate change. To do this, I utilize three different approaches (which I reviewed in Griffiths & Kelly, 2018) for identifying evolutionary responses to climate change, as described below.

1.1. Space for Time Studies

When a species spans a heterogenous landscape, populations often display varying phenotypes in these different environments, either through phenotypic plasticity or through local adaptation. Local adaptation is defined as a process where populations have a higher fitness in their local environment than immigrants. In contrast, if populations are phenotypically plastic, individuals can change their phenotype in response to spatially or temporally changing environments to increase their fitness. A space for time study can elucidate whether local adaptation and/or phenotypic plasticity plays a role in a species’ response to environmental stressors (Griffiths and Kelly 2018). These types of studies substitute space (how a stressor
varies spatially) for time (how a stressor may have varied in the past or with future climate change). Understanding the mechanisms through which populations have adapted to their current environment may help explain how they would adapt to future environments. For example, populations that are currently experiencing extreme conditions may be more likely to persist in future climate change scenarios as those extreme conditions become the norm. These studies take a common-garden approach where different populations are exposed to the same ambient and stressful conditions. If both populations similarly change their response to the stressful conditions, this is evidence for phenotypic plasticity. However, if the population that already experiences the more stressful environment has a higher fitness in the stressful common garden, that population is locally adapted. The interplay of these two mechanisms has a pivotal role in whether that species will be able to persist through future climate change. I explore the role of local adaptation and phenotypic plasticity in the cold-water coral, Balanophyllia elegans, in response to future ocean acidification (Chapter 2) and in the eastern oyster, Crassostrea virginica, in response to stressful low salinities (Chapter 3).

1.2. Quantitative Genetics: Breeding Experiments

A population’s capacity to adapt to future environmental stressors will depend upon the standing genetic variation present within the population for selection to act on. Phenotypic variation in a trait can be partitioned onto its environmental and genetic components. If the trait in question is strongly influenced by the environment and genetic variation is low, heritability is low and there would be little potential for that trait to evolve in response to future climate change. However, if the trait is controlled entirely by the parental genotype and genetic variation is high, then there is enough genetic variation for selection to act on. Heritability can be estimated for a single trait under contrasting environmental conditions using pedigree information and measuring variation in the trait within and among families. For example, Kelly, Padilla-Gamiño, & Hofmann (2013) documented the response of the purple sea urchin, Stronglyocentrotus purpuratus, to future pCO₂ conditions by measuring larvae size from known parental crosses. Although they observed a reduction in body size under stressful pCO₂ conditions, genetic variation and heritability was high, suggesting that body size could evolve. However, not all species contain the capacity to evolve to future environmental conditions. The mussel, Mytilis trossulus, had close to zero genetic variation (and thus a heritability close to zero) for body size under stressful pCO₂ conditions (Sunday et al. 2011). Population persistence models that incorporate estimates of heritability often predict population decline to be much less severe under future climate change scenarios (Kelly, Padilla-Gamiño, et al., 2013).

To assess the capacity of an economically important Gulf of Mexico species to adapt to future environmental stressors, I identify the presence of genetic variation and estimate heritability in the eastern oyster, C. virginica, in response to stressful low salinities (Chapter 3).

1.3. Experimental Evolution

Experimental evolution is another method to assess the capacity of a species to adapt to future climate change stressors (Griffiths and Kelly 2018). This method subjects populations to strong selection for multiple generations and then assesses the fitness of the population. Natural selection removes unfit individuals from the population and only the survivors to reproduce the next generation. This method also assesses standing genetic variation present within a
population; if a population is unable to maintain or increase its fitness after multiple generations of selection—or if the population goes extinct—then that population lacks the genetic variation required to adapt to the stressor in question. However, if a phenotypic change is observed in association with changes in allele frequencies, then the population has the potential to adapt. Using experimental evolution, I explore the evolutionary potential in the eastern oyster, *C. virginica*, in response to stressful low salinities (Chapter 4) and in the copepod, *Tigriopus californicus*, in response to warming temperatures (Chapter 5). In Chapter 4, I show that populations of *C. virginica* possess the genetic variation for low salinity tolerance after a single generation of selection. In contrast, populations of *T. californicus* have previously been shown to be unable to increase their heat tolerance after 15 generations of heat selection. However, variation among populations of this species would suggest there is ample genetic variation for heat tolerance across their broad geographic range extending from Southeast Alaska to Baja California. In Chapter 5, I demonstrate the potential for evolutionary adaptation to future warming through hybridization of populations with contrasting heat tolerances.
Chapter 2.

Differential Responses to Ocean Acidification Between Populations of *Balanophyllia elegans* Corals from High and Low Upwelling Environments*

2.1. Introduction

An important consequence of anthropogenic greenhouse gas emissions is ocean acidification, the global decrease in surface water pH, which is rapidly altering marine ecosystems (Raven et al. 2005; Doney et al. 2012; Guinotte and Fabry 2008). Sensitivity to acidification varies among and even within species, confounding the ability to predict the response of marine ecosystems to ocean acidification (Kroeker et al. 2013) and warranting the need for finer scale predictions, especially at the population and individual level (Valladares et al. 2014). Two important sources of intraspecific variation in sensitivity are local adaptation and phenotypic plasticity to environmental gradients (Calosi et al. 2017; Lardies et al. 2014; Bednarsek et al. 2017; Kelly, Sanford, and Grosberg 2012). In the California Current Large Marine Ecosystem (CCLME), seasonal environmental gradients are created by regional differences in the strength of upwelling; strong winds push the surface water offshore which is then replaced by deep water. This deeper water is more acidic from the accumulation of CO$_2$ from organic respiration (Fassbender et al., 2011). These seasonal occurrences of low pH events can present physiological challenges, in part because they lead to water conditions undersaturated in aragonite, a soluble form of calcium carbonate, which many organisms use to build their skeleton (Albright et al. 2018).

The CCLME is a hot-spot for upwelling activity, where differences in pH regimes do not follow a latitudinal gradient, but instead form a spatial mosaic; coastal sites in Oregon, Northern California, and Baja California, Mexico, spend more time exposed to a pH < 7.8 than Washington and Southern California (Fig. 2.1.; Hofmann et al., 2014; Kelly, Padilla-Gamiño, & Hofmann, 2013; Kroeker et al., 2016). Geographical differences in the strength of upwelling-associated low pH events occur from year to year (Chan et al., 2017) and even over millennia (van Geen et al. 1992), which has the potential to drive divergent selection among populations. As a result populations may have evolved phenotypic differences that provide a fitness advantage in their local environment (Sanford and Kelly 2011). Indeed, many marine organisms show population variation that is correlated with local pH conditions in the CCLME (G. E. Hofmann et al. 2014), such as red abalone (de Wit & Palumbi, 2013), coralline algae (Padilla-Gamiño et al. 2016), and purple sea urchins (Evans et al. 2013; Kelly, Padilla-Gamiño, and Hofmann 2013). For example, when the purple sea urchin, *Strongylocentrotus purpuratus*, is acclimated to upwelling conditions (e.g., ecologically relevant high pCO$_2$ conditions), it has larger offspring than parents acclimated to non-upwelling conditions (Wong, Johnson, Kelly, & Hofmann, 2018).

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Despite documented effects of acidification on cold-water calcifiers, and a substantial body of work on its effects on tropical corals (Chan & Connolly, 2013; Foster, Gilmour, Chua, Falter, & McCulloch, 2015; Hoegh-Guldberg et al., 2007; Shaw, Carpenter, Lantz, & Edmunds, 2016), few studies have focused on cold-water corals’ response to future ocean acidification (Form and Riebesell 2012; Movilla et al. 2016). In tropical corals, some studies have observed reductions in growth, calcification, and ultimately decreased survival under low pH exposure (Erez et al. 2011; Kroecker et al. 2013), while other studies have shown some coral species to be resistant to low pH (McCulloch, Falter, Trotter, & Montagna, 2012; Schoepf et al., 2013). However, cold-water corals may be more susceptible to future ocean acidification because they are found at higher latitudes, depth, and upwelling sites where aragonite saturation is naturally low (Raven et al. 2005). While corals in the CCLME have been exposed to upwelling over millennia, ocean acidification will exacerbate the effects of upwelling and expose organisms to pH and aragonite saturation conditions below what they currently experience (Chan et al., 2017; Feely, Sabine, Hernandez-Ayon, Ianson, & Hales, 2008). The resulting decreased availability of calcium and carbonate ions that must be pumped into the calcifying fluid, and the hydrogen ions that must be pumped out of the calcifying fluid against the increased seawater pH gradient, makes calcification energetically unfavorable (McCulloch et al., 2012; McCulloch, D’Olivo, Falter, Holcomb, & Trotter, 2017).

This study focused on the effects of acidification on Balanophyllia elegans, a solitary cold-water coral that lives in the subtidal down to ~300m in depth, and has a wide geographic range extending from Baja California to Southeast Alaska (Gerrodette 1981). The upwelling mosaic of the CCLME combined with B. elegans’ non-planktonic larval stage, which restricts gene flow, creates the potential for population variation through local adaptation or phenotypic plasticity when exposed to low pH (Sanford and Kelly 2011). Previous research has shown B. elegans to be sensitive to low pH, with juvenile mortality 10-20% higher under pH 7.6 compared to ambient conditions (pH 8.0) (Crook et al. 2013). However, only a single population from Monterey Bay, California, was investigated, where exposure to pH < 7.8 is rare (Kroeker et al. 2016; Evans et al. 2017). Other locations in California are exposed to pH < 7.8 for a greater amount of time due to greater upwelling intensity (Kroeker et al. 2016; Kelly, Padilla-Gamiño, and Hofmann 2013), and these populations of B. elegans corals may be more resilient to future ocean acidification.

I compared the physiological and transcriptomic responses of two populations of B. elegans from different upwelling environments (Fig. 2.1) when exposed to experimental seawater acidification. While ocean acidification is a slow, global process that also includes daily pH variations, what I measured is the corals’ response to acute stable pH stress. Nevertheless, it is a measure of the current population’s genetic variability and plasticity for responding to future low pH events caused by ocean acidification. The transcriptomic response can elucidate the mechanism by which populations differ in their response to pH change (Kenkel, Meyer, & Matz, 2013) and larger responses have been observed in intolerant populations (Schoville et al. 2012; Barshis et al. 2013; Gleason and Burton 2015). However, transcriptomic data alone are unable to confirm if these changes translate into differences in the whole-organism phenotype (DeBiasse and Kelly 2015). Therefore, I measured respiration rates and total lipid and protein content. I hypothesized that the response of Balanophyllia elegans corals to future ocean acidification is population-specific and is influenced by its upwelling history. I predicted that corals from the low upwelling environment will have higher gene expression and physiological changes between low and high pH exposure than corals from the high upwelling environment. Across all response
variables, I observed differing physiological and transcriptomic responses to low pH, suggesting these populations may not be equally tolerant to future ocean acidification.

2.2. Methods

2.2.1. Coral Collection

In June 2016, 27 and 30 individual *B. elegans* corals were collected from two locations near Point Arena Cove, CA (PAC, 38.914000°N, 123.711441°W) and Goleta, CA (GOL, 34.41775°N, 119.90153°W), respectively, and transported to Friday Harbor Laboratories (FHL) in San Juan, WA. These two sites are located near deployed sensors that collected continuous pH and $pCO_2$ measurements during the upwelling season of May to September of 2011 (Fig. 2.1.; Chan et al., 2017). Corals from the PAC population experience lower mean pH levels due to higher upwelling activity compared to the GOL population. Corals from PAC will be referred to as the HU (high upwelling) population and corals from GOL will be referred to as the LU (low upwelling) population. Corals were acclimated for two weeks at FHL at ambient conditions (pH 7.8 and 13°C) and fed *Artemia* nauplii.

Figure 2.1. (a) Histogram of hourly pH measurements at Van Damme State Park (VD) and Lompoc Landing (LOL) collected from May to September 2011 (Chan et al., 2017 and adapted from Kelly et al., 2013). pH was measured hourly with a modified version of the Honeywell DuraFET® (Honeywell, Fort Washington, PA, USA), an ion-sensitive field-effect transistor (ISFET), with an integrated data logger and power supply (modified from Martz, Connery, & Johnson, 2010). (b) Squares denote sensor locations (VD, 39.28°N, 123.80°W; LOL, 34.72°N, 120.61°W) and circles denote Balanophyllia elegans collection locations at Point Arena Cove (PAC, 38.914000°N, 123.711441°W) and Goleta (GOL, 34.41775°N, 119.90153°W). Corals collected from PAC will be referred to as the HU (high upwelling) population and corals collected from from GOL will be referred to as the LU (low upwelling) population.

2.2.2. Experimental Treatments

Corals from each population were exposed to $pCO_2$ levels of 785 µatm (ambient incoming seawater) and 2367 µatm (pH of 7.8 and 7.4 respectively; Table A.1.) in one gallon
jars for 29 days. Four to five corals were placed in each jar to achieve three replicates per population per pH exposure for a total of 12 jars. The $p$CO$_2$ conditions were achieved using the system at the Ocean Acidification Environmental Laboratory (OAEL) at FHL. At this facility, flow-through coolers are set up with 1µm filtered UV sterilized seawater that is scrubbed of excess CO$_2$ before CO$_2$ is replaced at the desired concentration through a venturi injector. The desired $p$CO$_2$ conditions are maintained with continuous pH measurements using a Honeywell Durafet pH electrode and a feedback loop system. The Durafets were calibrated weekly using the spectrophotometer pH method with the indicator dye $m$-cresol purple following standard operating procedures (SOP) 6b (Dickson et al., 2007).

Corals were kept in a climate-controlled room set at 13°C. Experimental water with a pH of 7.9 and 7.4 was collected from the flow-through coolers and used to fill the one gallon jars. Oxygen levels were replenished by circulating the water in the jar with the header space using a 70gph pump. The low pH was maintained by filling the overhead space in the jars with mixed air (1900ppm $p$CO$_2$ and 20.9% O$_2$). High pH jars were partially sealed to allow air circulation and equilibration with atmospheric $p$CO$_2$ levels. Water changes occurred every three days and corals were fed live Artemia nauplii in excess concentrations (Crook et al. 2013) three hours before water changes, allowing ample time for corals to consume the Artemia. Artemia were removed with water changes to reduce the possibility of additional pH decreases from Artemia respiration.

Physical parameters of experimental seawater were verified by measuring temperature, salinity, pH, and total alkalinity (Table A.1.). Temperature and total pH in the jars were measured daily using a conductivity meter (YSI 60) that was calibrated daily with the OAEL Durafet probes. Total pH was measured in the OAEL coolers and jars once a week following SOP 6b (Dickson et al., 2007) using the spectrophotometer pH method with the indicator dye $m$-cresol purple. TA was measured once a week following SOP 3b (Dickson et al., 2007) using a Mettler Toledo DL15 Titrator (Schwerzenbach, Switzerland). Certified reference materials (Dickson Laboratory) were first titrated prior to experimental samples to ensure precision and accuracy of TA measurements. Salinity was measured with a conductivity probe at time of pH and TA measurements. These physical parameters were entered into CO$_2$CALC (Robbins et al. 2010) with carbonic acid dissociation constants from (Mehrbach et al. 1973) to estimate $p$CO$_2$, $\Omega_{\text{ars}}$, and $\Omega_{\text{cal}}$ (Table A.1.).

2.2.3. Physiologic Measurements

Respiration measurements were performed on day 9 and 29 of the exposure. Individual corals (nine per treatment per population) were measured by placing them into sealed vials filled with ~10ml of 0.45µm filtered seawater from their corresponding pH treatments. Each vial contained a stir bar and sat over a stir plate to prevent oxygen layer formation. Total oxygen consumption was calculated by measuring percent oxygen levels in the vial every hour for three hours using a NeoFox Phase Fluorometer and oxygen sensor probes with red eye patches placed inside the vials (Dunedin, FL, USA). The oxygen sensor was calibrated using 100% O$_2$ saturated seawater and 0% O$_2$ standard solution (NaSO$_3$ saturated DI water). Coral respiration rates were calculated by subtracting the background respiration (negligible in all cases but one) from the slope of oxygen consumed per hour for each coral individual. Respiration rates were normalized by coral weight and log transformed, then fit with a linear mixed-effect model with corals nested within jars as random factors using the R package lme4 (Bates et al. 2014). All data were normally distributed (Shapiro-Wilk test $p > 0.05$), except HU corals on day 29 at pH 7.8, but this
group was not involved in any significant effects. A three-way ANOVA was performed using the car package (J. Fox and Weisberg 2011) and a Tukey’s post-hoc test was performed using the package emmeans (Lenth 2018) in R v. 3.4.4 (R core team, 2018).

After nine days in the experimental treatments, one coral from each jar (three corals from each population per pH treatment) were dabbed dry and cut in half with one half preserved in RNAlater and the other half frozen immediately at -80°C for lipid and protein analysis. At the end of the experiment all corals were similarly sacrificed 24 hours after respiration measurements to avoid confounding short-term oxygen stress with long-term pH acclimation.

Lipid and protein analyses were done at the University of Southern California following the methods described in Pan et al. (2018). Prior to biochemical content analyses, samples were placed in a 65°C drying oven overnight and weighed to the nearest 0.1 mg. These samples were then homogenized using Tenbroeck tissue grinders sonicated using a microprobe, and centrifuged to remove the insoluble fraction. For protein quantification, total protein in tissue homogenates was precipitated in 5% trichloroacetic acid, resolubilized in NaOH, and quantified using the Bradford dye-binding assay modified for marine invertebrates. For lipid quantification, major lipid classes (phospholipid, triacylglycerol, sterol, free fatty acid, and wax ester) were extracted in a water-methanol-chloroform mixture. Extracted samples were spotted onto Chromarods (Iatron Laboratories Inc.), and lipid classes were separated using a solvent system containing hexane and ethyl ether. Chromarods were analyzed using an Iatroscan Flame Ionization Detector system (Iatroscan MK-5, Iatron Laboratories Inc.). Total lipid content in each coral sample was determined as the sum of the major lipid classes. Total protein, total lipid, and each lipid-class contents were normalized by coral dry weight, square-root transformed, then fit with a linear mixed-effect model with jars as random factors using the R package lme4 (Bates et al. 2014). A two-way ANOVA was performed using the car package (J. Fox and Weisberg 2011) in R v. 3.4.4 (R core team, 2018).

2.2.4. Transcriptomic Analysis

One coral from each jar (three per treatment per population) was collected at each of two time points (day 9 and 29) for a total of 24 samples for RNA sequencing analyses to compare changes in gene expression. Total RNA was extracted using a RNeasy® Plus Mini Kit (Qiagen Inc., Valencia, CA, USA), following manufacturer’s instructions, and yield and quantity was assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). I constructed 24 cDNA libraries from mRNA using the NEB Illumina RNASeq prep kit (version 2; Illumina, San Diego, CA, USA), following manufacturer’s instructions. Library size was assessed using a Bioanalyzer (Agilent, Santa Clara, CA, USA) and concentration was quantified with qPCR (Kapa Biosystems kit; Illumina, Wilmington, MA). Each library received an individual barcode adapter and 12 libraries were haphazardly pooled for a total of two pools. Paired-end (2x100bp) sequencing was performed at Iowa State University’s DNA Facility (Ames, IA, USA) on two lanes of an Illumina HiSeq 3000.

I first assembled a transcriptome with Trinity-v2.3.2 (Grabherr et al. 2011) using all of the reads (both HU and LU combined), but this yielded an assembly with 127,739 contigs. This large number of contigs suggests that high sequence divergence between populations was causing population-specific alleles to be assembled as separate contigs in the hybrid assembly (Schoville et al. 2012; Debiasse, Kawji, and Kelly 2018). This becomes problematic when comparing differential gene expression between populations because reads preferentially map to
contigs from their own population, potentially creating a signal of population-specific gene expression, even where none exists. By assembling population-specific transcriptomes and identifying orthologous contigs between populations we were able to reduce the total number of contigs to 37,631. I describe this procedure below.

Low quality bases (Q < 20) and adapters were removed using TrimGalore-v.0.4.4, which uses FastQC v0.11.2 (Andrews 2010) and Cutadapt v1.7 (Martin 2011), before being assembled using the default parameters in Trinity-v2.3.2 (Grabherr et al. 2011). Transcriptomes were assembled using all 12 individuals from each population. After assembly, contigs were collapsed using a 98% similarity threshold using CD-Hit (Fu et al. 2012) and contigs less than 500 bp in length were removed to reduce the number of fragmented contigs. Transcriptome completeness was assessed using BUSCO-v3 (Simão et al. 2015). The two transcriptomes were combined into one consensus transcriptome by identifying orthologs within and between both populations’ transcriptomes using the program OrthoFinder-v.1.1.4. (Emms and Kelly 2015). OrthoFinder performs a reciprocal blast search within and between transcriptomes with an e-value cut off of 0.001 to group contigs into OrthoGroups. An OrthoGroup consisted of multiple contigs from the HU population-specific transcriptome and the LU population-specific transcriptome. All OrthoGroups with a single contig from one population, but not the other population, which also did not get further sorted into other OrthoGroups via an independent blast search, were removed from the final consensus transcriptome.

Transcripts were mapped as single-end to their population-specific transcriptome using RSEM-v.1.3.0 to achieve maximum mapping success (Li and Dewey 2014). Using a custom python script, an OrthoGroup consensus count matrix was created by first matching total contig counts (from the RSEM population-specific count matrix) to their corresponding OrthoGroup. The total read count for each OrthoGroup was calculated by summing all contig read counts within that OrthoGroup (https://github.com/JoannaGriffiths/Coral-population-responses-to-acidification). Differential gene expression analysis was performed using the DESeq2 package in R (v.3.3.1) using an adjusted p-value cut off of 0.05 (Love, Huber, and Anders 2014). I performed multiple comparisons (one-way, two-way, and three-way interactions) to test for interactions among various combinations of time points, populations, and treatments (See Table A.4.). OrthoGroups were assigned gene ontology (GO) terms using InterProScan-v.5.24-63.0 (Jones et al. 2014). Functionally enriched GO terms were identified using a rank-based gene ontology analysis with adaptive clustering (Wright et al., 2015) which uses a two-sided Mann-Whitney U-test and a false discovery rate correction.

To describe global gene expression patterns, we performed a Principal Coordinate Analysis (PCoA) on both the complete transcriptomic dataset and each sampling time-point using the R package vegan (Oksanen 2015). PCoA analyses were completed using manhattan distances computed from the r-log transformed expression data. The Adonis function (as implemented in the R package vegan) was subsequently used to identify significant effects of time point, population, and pH treatment on global gene expression levels.

2.2.5. WGCNA and Functional enrichment

To identify molecular pathways associated with the observed physiological responses to low pH exposure, I performed a Weighted Gene Co-expression Network Analysis (WGCNA) (Langfelder and Horvath 2008). This method identifies gene networks, or modules, that are co-regulated across samples (in this case, pre-defined groups of population and pH treatment)
assignments). The resulting eigengene expressions were then tested for correlations post-hoc with the observed physiological characteristics. The genes associated with each module were also tested for functional enrichment of GO terms using a Mann-Whitney U-test.

2.2.6. Discriminant Analysis of Principal Components

To further examine gene expression associations between populations, pH treatments, and the physiological data, I performed a discriminant analysis of principal components (DAPC) on day 29 gene expression data. This analysis converts variation between pre-identified groups into a single axis that can be displayed on a principal components biplot. I performed DAPC by first maximizing differences between populations and then pH treatments using the R package adegenet (Jombart, Devillard, and Balloux 2010). These discriminant analyses were then plotted as a vector (scaled by correlation coefficient) onto a PCA of physiological data. Correlation with PCA axes was computed using the R function envfit (R package vegan).

2.3. Results

2.3.1. Respiration Rates

Overall respiration rates were similar among populations and treatments on day 9, but a three-way ANOVA found a significant population by day interaction (F\(_{1,32}=7.14\) p = 0.01; Fig. 2.2.A & Table A.2.). Further investigation with a Tukey’s post hoc test revealed respiration rates within the LU population at low pH decreased with time (p = 0.011, Table A.3.). HU corals showed no changes in respiration during the experiment, whereas LU corals suppressed their respiration by 67.7% over the course of the experimental low pH exposure.

2.3.2. Protein and Lipid Content

There was a nearly significant pH treatment by location interaction for total protein content (F\(_{1,9.3}=4.68\) p = 0.058, Fig. 2.2.B & Table A.2.), but no significant interaction effect for total lipid content (F\(_{1,9.3}=2.89\) p = 0.122, Fig. 2.2.C & Table A.2.). Broken down further into the different lipid-classes, there was a significant pH treatment by location interaction for fatty acids (F\(_{1,9.3}=5.79\) p = 0.04, Fig. 2.2.D & Table A.2.), with lower fatty acid content in the LU population at low pH exposure relative to the HU population. Phospholipid content (F\(_{1,9.3}=3.34\) p = 0.09, Fig. 2.2.F & Table A.2.) and sterol content (F\(_{1,10.5}=2.71\) p = 0.13, Fig. 2.2.E & Table A.2.) did not differ between populations or pH treatment. The LU population had significantly lower amounts of triacylglycerol (F\(_{1,10.5}=21.12\) p < 0.001, Fig. 2.2.G & Table A.2) and wax esters (F\(_{1,10.5}=15.09\) p < 0.001, Fig. 2.2.H & Table A.2.) than the HU population regardless of pH treatment.

2.3.3. Transcriptome Assembly

The HU and LU coral transcriptomes were constructed from 415 million and 349 million pairs of 100 bp reads, respectively. The collapsed HU population transcriptome contained 80,876 contigs, with a N50 of 1,231 and GC content of 40.14%. The collapsed LU population transcriptome contained 80,181 contigs, with a N50 of 1,215 and GC content of 41.9%. The final
combined OrthoGroup transcriptome contained 37,631 contigs. BUSCO analyses demonstrated that the HU and LU transcriptomes were 98.1% and 97.7% complete, respectively, after removal of contigs that did not pair with the other population (i.e. OrthoGroups with a single contig from one population). The OrthoGroup transcriptome was 99.3% complete. HU and LU coral reads that mapped to the OrthoGroup transcriptome had an average mapping success of 54.7% (range of 43.7-71.3%) and 53.1% (range of 41.6-63.2%), respectively.

Figure 2.2. (a) Respiration rates on day 9 and day 29 of HU and LU corals at a pH of 7.8 and 7.4. (b) Protein content on day 29. (c) Lipid content on day 29. (d–h) Lipid–class content (fatty acid, sterol, phospholipid, triacylglycerol, and wax ester) on day 29. Means per total dry weight and standard error bars are presented. Statistical tests from ANOVA (Supporting Information Table A.2.) are presented in the top right corner of the graph. Significant codes: *p < 0.05

2.3.4. Gene Expression

The PCoA of whole transcriptome expression data revealed significant clustering by day (Adonis p<sub>day</sub> < 0.001), suggesting a similar change in gene expression by both populations over time (Fig 2.3.A). The number of differentially expressed genes (DEGs) between time points was
large, ranging from ~8000 to 11,500 genes (Table A.4.), for each population in each pH treatment. Additionally, the low pH treatment for both populations had a larger change in gene expression over the course of the experiment, with ~3000 DEGs more than the high pH treatment.

To observe potential pH treatment and population effects on gene expression, I performed a PCoA for each time point. On day 9, the gene expression response was similar between populations and pH treatments (Adonis $p_{\text{population}} = 0.16$, $p_{\text{pH}} = 0.67$; Fig. 2.3.B). However, there was a different gene expression response between the two populations after 29 days (Adonis $p_{\text{population}} = 0.03$, $p_{\text{pH}} = 0.24$; Fig. 2.3.C). This pattern is also evident in the number of DEGs. In response to the high pH treatment, there was a greater number of DEGs between populations on day 29 (490) than on day 9 (117) (Table A.4.), with 18 shared DEGs between the two time points (Fig. A.1.A). In response to the low pH treatment, the change in the number of DEGs between populations was not as great from day 9 (211 DEGs) to day 29 (244 DEGs) (Table A.4.), with 18 shared DEGs shared between the two time points (Fig. A.1.A). The small number of shared DEGs between the two time points suggests that the populations are acclimating over time using a different set of genes, with the greatest divergence between populations at the high pH treatment on day 29.

To understand each population’s response to pH treatment at each time point, I compared DEGs between the two pH treatments (Fig. 2.3.D). Both populations increased the total number of DEGs from day 9 to 29, but the LU population had more total DEGs than the HU population at both time points (Fig. 2.3.D). The populations are also expressing different sets of genes, with little (one at day 29) to no (day 9) overlap in the number of DEGs (Fig. A.1.B). This is further supported by a test for a population by pH by time point interaction that identified 15 DEGs. In summary, each population had a distinct response to pH treatment over time, but this response was more pronounced in the LU population. Finally, a test of the population by pH interaction identified 17 DEGs on day 29 and 4 DEGs on day 9 (Table A.4.), further supporting transcriptomic divergence between populations in response to low pH as the length of exposure increases.

To see if gene expression was related to changes in physiology, I used density plots of discriminant function values for day 29 gene expression differences between populations (Fig. 2.4.A) and pH treatments (Fig. 2.4.B) and plotted the vectors onto a physiological PCA. The PCA identified axis 1 representing physiological fitness, whereby all physiology data had a significant fit with PC1 ($p < 0.05$) while respiration rates had a significant fit with PC2 ($p < 0.05$) (Fig. 2.4.C). Fit of gene expression differences between populations on day 29 (GE_pop) is statistically significant ($p = 0.011$), while gene expression differences between pH treatments on day 29 (GE_pH) is not ($p = 0.839$; Fig. 2.4.C). Fit of gene expression differences between populations points in a similar direction to triacylglycerol and wax ester, both of which showed population-specific differences—higher concentrations in the HU population (Fig. 2.2.G,H). Fit of gene expression differences between pH treatments, although not significant, pointed in the same direction as fatty acids and proteins (which showed significant or nearly significant population by pH treatment interaction, with LU corals having lower fatty acid and protein content under low pH).
2.3.5. Functional Enrichment and WGCNA

Rank-based gene ontology analysis was performed using all genes to determine the molecular functions and biological processes involved in the low pH response. For the biological processes GO category, the short term (day 9) initial response to low pH was dominated by an upregulation of RNA metabolic and biosynthetic processing genes in both populations (Fig. 2.5.A,C). By day 29, after continued exposure to low pH, the direction of regulation switches and both populations downregulated these same genes (Fig. 2.5.B,D). One population difference was the upregulation of DNA metabolic processes in LU corals and downregulation in HU corals on day 29. Other genes only enriched in the HU population included ion and transmembrane transport genes that were downregulated on day 9 (Fig. 2.5.A), but upregulated on day 29 (Fig. 2.5.B).

![Figure 2.3](image_url)

Figure 2.3. Manhattan distance based principal coordinate analysis (PCoA) of r-log transformed expression data testing pH treatment by population by day effects (a), pH treatment by population for day 9 (b), and pH treatment by population for day 29 (c). Differentially expressed genes between the high pH (7.8) and low pH (7.4) treatment (d). The negative and positive values refer to the number of genes that were downregulated or upregulated, respectively, in response to low pH as compared to the high pH treatment.
Genes with molecular functions that changed expression levels between pH treatments also showed differences between populations and time points. Only HU corals were significantly enriched for calcium-ion binding, voltage-gated calcium channel, endopeptidase, and serine-type peptidase in the upregulated gene set on day 29 (Fig. A.2.A,B). But only LU corals were significantly enriched for lipid, phospholipid, and phosphatidylinositol binding genes in the upregulated gene set at day 29 (Fig. A.2.C,D).

Figure 2.4. (a–b) Density plots of discriminant function values for day 29 gene expression by population (a) and pH treatment (b). (c) Principal coordinate biplot of physiological data on day 29. All physiology data had a significant fit with PC1 (p < 0.05) while respiration rates had a significant fit with PC2 (p < 0.05). Fit of gene expression by population on day 29 (GE_pop) is statistically significant, while gene expression of pH treatment on day 29 (GE_pH) is not.

WGCNA analysis assigned day 29 transcriptomic data to 28 modules that were significantly correlated with coral traits (Fig. A.3.), seven of which showed significant correlations with population and some other physiological characteristic. For example, module blue2 and brown4 showed significant correlations with population, wax ester, and triacylglycerol content—although these modules had no functionally enriched gene sets (Fig. A.3.). There were only two modules which showed significant correlations with pH treatment and some other
physiological characteristic—royalblue and mediumpurple (Fig. 2.6.A & Fig. A.3.). While these modules were both correlated with pH treatment and protein content, only royalblue had any functionally enriched gene sets, including regulation of cytoskeleton organization, supramolecular fiber organization, and regulation of cellular component organization (Fig. 2.6.A). Other modules significantly correlated with pH treatment included, lightblue2, where LU corals downregulated genes related to ribosomal biogenesis, such as ribosomal subunit, intracellular ribonucleoprotein complex, and structural constituent of ribosomes, in response to low pH and upregulated these same gene sets in response to high pH (Fig. 2.6.B). In contrast, HU corals showed no change in expression of these genes in response to pH exposure (Fig. 2.6.B).

Figure 2.5. Biological processes GO categories for genes significantly enriched at low pH either upregulated (red) or downregulated (blue). (a) HU day 9. (b) HU day 29. (c) LU day 9. (d) LU day 29. The dendrogram on the side is a hierarchical clustering of the GO categories based on shared genes. The fractions correspond to the number of genes that exceeded the unadjusted p < 0.05 over the total number of genes in that GO category.
2.4. Discussion

Populations of marine organisms from high intensity upwelling environments are currently experiencing pH values that are not predicted in the open ocean until the end of the century (Hofmann et al., 2011). As a result, these populations must acclimate or adapt to low pH or risk extirpation. In this study, I found evidence for population divergence in the physiological and transcriptomic responses of corals from different upwelling environments. While I was unable to measure growth rates in this study, I measured several other traits that correlate with fitness, such as respiration and protein and lipid content. These results imply that populations are not always responding equally to low pH and highlights the importance of future research focusing on population-specific responses to ocean acidification.
2.4.1. Population-Specific Responses in Respiration Rates

Although there were no differences in respiration rates at low pH between LU and HU corals, LU corals reduced their respiration rates in response to low pH over the length of the exposure (from day 9 to day 29), whereas the HU corals maintained the same respiration rate throughout (Fig. 2.2.A). Reductions in respiration rates in response to low pH have been observed in tropical (Albright and Langdon 2011; Edmunds 2012; Nakamura et al. 2011) and cold-water corals (Hennige et al. 2014), although some cold-water corals exhibit no change (Maier et al. 2013). Reductions in respiration rates can initially be an adaptive response to short term low pH, achieved through a decrease in energetically expensive protein synthesis (Langenbuch et al. 2006). For example, LU corals in this study downregulated ribosomal protein genes (Fig. 2.6.B). However, continued reductions in respiration rates under long term exposure comes at a cost to growth and reproduction (Guppy and Withers 1999; Fabry et al. 2008).

2.4.2. Population-Specific Responses in Lipid and Protein Content

Lipid and protein reserves are important indicators of a coral’s energy balance and overall health (Anthony et al. 2009; Rodrigues, Grottoli, and Pease 2008). While total lipid, sterols, and phospholipids were unaffected by pH treatment and population, fatty acids were significantly reduced in the LU population under low pH (Fig. 2.2.). In addition, triacylglycerol and wax esters were more abundant in the HU population regardless of pH treatment (Fig. 2.2.G,H). Triacylglycerol and wax esters (composed largely of fatty acids) are important storage lipids while sterols and phospholipids are structural lipids (Harland et al. 1993; Imbs et al. 2010). The lack of change in structural lipids suggests that the structural integrity of the cells have not been compromised due to low pH. In contrast, the reduction in storage lipids could be detrimental in the long run (Anthony et al. 2009) and may be tied to reduced respiration in the LU population after corals deplete their energy reserves. Other studies examining tropical and cold-water coral responses found no effect of low pH on lipid and protein reserves (Movilla et al. 2016; 2014; Bramanti et al. 2013). However, the low pH treatment in these studies ranged from 7.7 to 7.8, which may not have been stressful enough to elicit a change to lipid and protein content. Other studies have observed species-specific responses in lipid content at low pH (Schoepf et al. 2013) and lipid content is known to vary among populations after heat stress (Li et al. 2018). However, I demonstrate an additional population response in corals to pH stress.

In coral larvae, triacylglycerols can be quickly hydrolyzed for immediate energy needs whereas wax esters are for more long-term storage, but both can be depleted in just 24 hours under combined high temperatures and low pH stress (Rivest and Hofmann 2015). However, the higher abundance of triacylglycerol and wax esters in the HU population could indicate a potential for genetic variation for the two main storage lipid classes due to different environmental factors, such as upwelling. Alternatively, the HU and LU corals may have been collected under different environmental conditions as a result of higher food availability during upwelling conditions which influenced their initial nutritional status at the start of this experiment. Analyzing lipid content from B. elegans populations collected at the start of the experiment, or in the field, may further elucidate causes for the observed differences between populations.
2.4.3. Greater Differential Gene Expression in the LU Population

The number of DEGs varied between populations, pH treatments, and time points. The overall transcriptomic response showed a clear separation between the two sampled time points (Fig 2.3.A). This response is further depicted by an increase in the total number of DEGs from day 9 to day 29 (Fig. 2.3.D, Table A.4.) for each population, which suggests there is a larger transcriptomic response as the length of low pH exposure increases. The two populations also differed in the total number of genes differentially expressed; at both time points, LU corals had more DEGs than HU corals (Fig. 2.3.). This pattern has been observed in copepods (Schoville et al. 2012), corals (Barshis et al. 2013), and snails (Gleason and Burton 2015) exposed to stressful temperatures, where greater numbers of DEGs are observed in the thermally intolerant population. Taken together, the difference in the physiological response, coupled with a greater overall transcriptomic response and more DEGs between treatments in the LU population, illustrates divergent responses to low pH between these two populations.

2.4.4. Functional Enrichment

Contrasting pH treatments for each population and time point (Fig. 2.3.D) revealed little to no overlap in shared DEGs and a three-way interaction test for population, pH treatment, and time point revealed only 15 genes that were differentially expressed (Table A.4.). However, functional enrichment analyses show that populations share GO terms, suggesting that populations may be utilizing similar processes at low pH, but using a different set of genes. The initial upregulation of RNA metabolic processing genes on day 9 and then downregulation on day 29, suggests that both populations are halting transcription processes (mRNA and tRNA processing and RNA splicing). However, DNA metabolic processes, with functions such as DNA replication and initiation, were downregulated in the HU population and upregulated in the LU population on day 29, suggesting that the HU population arrested cell proliferation. On day 29 at low pH, LU corals downregulated genes related to ribosomal biogenesis, such as ribosomal subunit, intracellular ribonucleoprotein complex, and structural constituent of ribosomes, while HU corals upregulated many of these same genes. Downregulation of ribosomal biogenesis is a common response to environmental stress (López-Maury, Marguerat, and Bähler 2008). The results suggest that LU corals may be less able to cope with low pH exposure compared to HU corals. This stress response has also been documented in corals when transplanted to new thermal environments and between coral populations living in different water qualities (Kenkel & Matz, 2016; Rocker, Kenkel, Francis, Willis, & Bay, 2019).

On day 29, LU corals upregulated genes with functions related to lipid and phospholipid binding (Fig. A.1.), phosphorylation, protein localization, and carbohydrate biosynthetic processes (Fig. 2.5.D) in response to low pH. Consequently, LU corals may be mobilizing energy reserves, which is also observed in the reduced protein and lipid-class content at low pH. They may also be experiencing post-translational protein modifications, such as phosphorylation which regulates protein activity in the cell (Mumby and Brekken 2005). Changes to phosphorylation have been shown to be important mechanisms of acclimation to pH changes (Wong et al., 2011). Protein localization can be a rapid and efficient response to environmental change that moves pre-existing proteins between cells, thereby regulating their functions and reducing the energy used to make new transcripts (Bauer et al., 2015). HU corals also show post-
translational modifications through the upregulation of endopeptidase and serine-type peptidase genes. Both of these enzymes are involved in proteolysis, a key process that degrades deformed proteins following stressful exposures.

2.4.5. Physiological and Gene Expression Correlations

The biplot of physiological and gene expression data (Fig. 2.4.C) showed gene expression differences between populations was significantly correlated with all lipid and protein content (the first PC axis), but most strongly with triacylglycerol and wax ester content. This suggests that the greatest gene expression differences among populations was also correlated with the two lipid-classes that showed population-wide differentiation. These results were also evident in WGCNA analyses, such as the two modules that were correlated with population, triacylglycerol, and wax ester content (Fig. A.3.). The fit of gene expression differences by pH treatment was not significantly correlated with all lipid and protein content, but this might be the result of population-specific responses to low pH as observed by a lack of overlap in LU corals’ gene expression between the two pH treatments in the DAPC (Fig. 2.4.B).

Although there were 28 significant modules that were correlated with coral traits, only a few were significantly correlated with either population or pH treatment and some other physiological trait. The more coral traits being tested, the higher the likelihood for more gene co-regulations to be observed and testing these gene sets for functional enrichment is a tool for further analyzing the biological meaning (if any) behind these gene modules. One of the modules with functional enrichment was the royalblue module which included genes related to cytoskeletal and fiber organization. Both populations downregulated these genes at low pH, suggesting that there is an overall response for cellular re-organization.

Future work should measure growth and mortality to clarify tolerant and intolerant populations. This would allow correlations to be drawn between the transcriptomic and physiological response in relation to mortality. Gene expression patterns have been used to successfully predict coral colony survival under stressful changes in temperature (Bay and Palumbi 2017) and many of the expression patterns observed in this study have been replicated in other species and stressors, as discussed in this paper (see Table 2.1. summary), suggesting that there is a ubiquitous response to stressful conditions.

2.4.6. Ion Transport and Calcium Ion Binding: Key to Low pH Tolerance?

On day 29, HU corals were upregulating genes that have previously demonstrated expression changes during the initiation of calcification (De Wit, Durland, Ventura, & Langdon, 2018), such as ion transport, transmembrane transport (Fig. 2.4.), and calcium ion binding (Fig. A.2.). For calcification to occur in corals, they must increase the internal cellular pH relative to seawater (McCulloch et al., 2012; McCulloch et al., 2017). This is achieved by pumping calcium ions into the calcifying fluid and pumping hydrogen ions out. An upregulation of ion transporters may be required for corals to maintain pH homeostasis and calcification under low pH conditions (Vidal-Dupiol et al. 2013).

Calcifying and non-calcifying species adapted to variable or low pH environments have been shown to have an increased physical capacity for ion transport (Pan, Applebaum, & Manahan, 2015; Seibel, 2003) as well as an upregulation of genes involved in ion transport (Evans and Watson-Wynn 2014; Kaniewska et al. 2012; Vidal-Dupiol et al. 2013). Organisms
actively remove cellular hydrogen ions through Na+/H+ exchangers and pumps, similar to removal of metabolically produced CO$_2$, (Fabry et al., 2008; Seibel & Walsh, 2001). Only HU corals upregulated sodium ion transporters, sodium ion channels (Fig. A.1.), and sodium ion transport (Fig. 2.4.) under low pH, suggesting that both an increase in the total number of transporters and the capacity of those transporters is needed to maintain pH homeostasis under low pH conditions. The movement of ions across membranes is energy intensive, especially under acidification (Pan et al., 2015), but HU corals were able to maintain high respiration rates under low pH, potentially allowing them to put this energy towards pH homeostasis and calcification.

2.4.7. Conclusions and Future Implications

Multiple lines of evidence suggest that there are divergent responses between populations of *Balanophyllia elegans* corals to experimental seawater acidification. HU corals in the low pH treatment maintained their respiration rate throughout the exposure, had higher protein and lipid content, and upregulated genes involved in critical processes, such as pH homoeostasis, when exposed to future low pH. The additional downregulation of stress response genes in the LU population lends support to LU corals being under greater levels of stress than the HU population. While I was unable to distinguish between local adaptation and developmental plasticity, HU corals may have unique traits that allow them to be more resilient to future ocean acidification.

My ambient pH treatment (pH 7.8) was lower than intended and may in fact better reflect the corals’ response to upwelling conditions. Both populations experience a pH of 7.8 for less than 5% of the time, but this pH would be more of a shock to the LU populations (Fig. 2.1.). This hypothesis may be supported by lower triacylglycerols and wax esters in the LU population compared to the HU population. Additionally, this might explain the lack of differences among overall gene expression patterns due to pH and the overall large differences among populations (Fig. 2.3.C). However, there are some differences among pH treatments when observing DEGs, WGCNA modules, and functionally enriched genes sets.

This study has important implications for making conservation decisions based on the sensitivity of a species to ocean acidification. I caution against future research that attempts to extrapolate the population response to the species level without first taking into consideration the selective forces imposed by environmental variation. By studying multiple populations, I have a more reliable prediction about the future of the entire species. Finally, resolving variation in tolerance among populations will provide insight into the mechanisms of persistence under future ocean acidification.
Table 2.1. Over-represented GO terms that were up or down regulated for each specific treatment. Studies cited in this study that also find these sets of GO terms over-represented are displayed displayed with the corresponding stressor and species. The p-values are based on the functional enrichment test (same as Fig. 2.5. and Fig. A.2.).

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Chapter 3.
Transgenerational Plasticity and Parental Genotype Alter the Response to Low Salinity in the Eastern Oyster, *Crassostrea virginica*

3.1. Introduction

Our rapidly changing climate will expose populations to novel and potentially stressful environments. Many studies have demonstrated that organisms can adapt over ecological timescales that may keep pace with climate change (Phillip Gienapp et al. 2013; Sanford and Kelly 2011; R. G. Shaw and Etterson 2012). For this to occur, there must be heritable genetic variation present for traits that improve fitness in the novel environment (Lynch and Walsh 1998). Quantitative genetic studies test for the presence of genetic variation in a particular trait under selection and estimate the population’s capacity to adapt to a new environment. If there is a lack of genetic variation within the population, this would suggest that there is limited potential to adapt to a changing climate. However, some studies have successfully identified variation among genotypes in their response to future warming and acidification in a variety of taxa, including sea urchins, corals, and fish (Kenkel, Setta, and Matz 2015; Malvezzi et al. 2015; Foo and Byrne 2016; Kelly, Padilla-Gamiño, and Hofmann 2013; Sunday et al. 2011).

Phenotypic plasticity is another mechanism which may promote survival in stressful and novel environments. Adaptive plasticity moves individuals closer to the optimum for the new environment, so that individuals that are more plastic have a higher fitness. However, plasticity can also be maladaptive, especially if it causes individuals to have a lower fitness in the novel environment (Ghalambor et al. 2007). Plasticity can also be inherited across generations, termed transgenerational plasticity, whereby a plastic change induced in the parental population is inherited and expressed in the offspring generation.

Studies that attempt to identify mechanisms of transgenerational plasticity have traditionally focused on maternal investment, such as through mRNA, lipids, or proteins passed on in the egg. (Mousseau and Fox 1998). However, paternal effects have also been observed to affect offspring fitness, suggesting that sires contribute more than just genetic material, such as through epigenetic processes (Crean and Bonduriansky 2014). Consequently, both epigenetic (e.g. DNA methylation, histone modifications) and maternal effects are mechanisms through which the parental environment influences offspring fitness. In cases where the parental environment accurately predicts and matches the offspring environment, and the plastic response is adaptive, offspring inherit the parents’ plastic trait change, increasing fitness (Burgess and Marshall 2014). For example, when offspring of the marine tubeworm, *Galeolaria caespitosa*, are exposed to ecologically relevant temperatures, they have higher survival when the rearing temperature matches that of the parental environment (Chirgwin et al. 2018). Parental exposure to ecologically relevant stressful environments can also promote increased fitness in novel environments predicted under future climate change scenarios. Hoshijima & Hofmann (2019) observed increased resistance to future low pH conditions in *Strongylocentrotus purpuratus* sea urchins when their parents were conditioned in naturally low pH environments. However, these beneficial effects may not always be prominent (Uller, Nakagawa, and English 2013) or stressful parental conditioning could be non-adaptive (Ghalambor et al. 2007; Dustin J. Marshall and Uller 2007; Guillaume, Monro, and Marshall 2016).

The interplay of intra-generational and transgenerational plasticity and evolution needs further investigation to accurately predict species responses to future climate change. These
mechanisms may work in tandem, whereby plasticity prevents a population from going extinct by extending the time over which selection has the opportunity to act (Chevin 2010, Kopp & Matuszewski, 2014, Munday 2013). The plastic response may even influence the evolutionary trajectory of the population by either speeding up or impeding the effects of selection (Räsänen and Kruuk 2007). However, transgenerational plasticity may not be enough to buffer populations from continually changing conditions (Gienapp, Teplitsky, Alho, Mills, & Merilä, 2008), therefore, the presence of additive genetic variation (or the capacity to evolve) will play an important role in determining a population’s response to future climate change.

I explore the influence of genetic variation and transgenerational plasticity on larval growth rates in the eastern oyster, *Crassostrea virginica*, an ecologically and economically important species in the Gulf of Mexico. *C. virginica* is a widespread euryhaline species but is sensitive to low salinities, with documented reductions in growth and overall production when salinity is below 5 psu (La Peyre et al. 2016). While oysters are capable of withstanding low salinity for short periods of time, their tolerance rapidly declines in conjunction with high temperatures. Under future climate change scenarios, precipitation is expected to increase during peak temperatures in the summer and fall (Ning and Abdollahi 2003; Swenson 2003), drastically reducing the salinity in estuaries that oysters inhabit. In addition to climate change related stressors, oysters will be more immediately affected by human-driven alterations to the Mississippi river. Government planned Mississippi river diversions are predicted to drastically alter the estuarine landscape in Louisiana by increasing freshwater flow into productive oyster habitats. Under large-scale predicted diversions, oyster growth and production will be negatively impacted (Wang et al. 2017).

To investigate factors contributing to variation in *C. virginica* larval tolerance of low salinity, I measured larval size in the F2 generation when oysters were reared at a stressful low or ambient high salinity. Larval size is an important determinant of fitness because larger larvae typically have more energy available giving them a competitive advantage and the ability to escape predation (Dustin J. Marshall, Pettersen, and Cameron 2018). I investigated the influence the environment, genotype, and transgenerational plasticity on low salinity tolerance in *C. virginica* larvae. To do this, the progeny (F1 generation) of the Calcasieu Lake population were acclimated to a low and high salinity environment (Fig. 3.1.A) to determine the influence of the environment and transgenerational plasticity when their offspring (F2 generation) were reared at low or high salinity in the lab. Finally, I measured the capacity of the *C. virginica* to evolve increased tolerance of low salinity at the larval stage using a quantitative breeding design to evaluate the influence of additive genetic variation (contributed by sires) on larval body size.

### 3.2. Methods

#### 3.2.1. Population Origin and F1 Acclimation

Oysters were collected from a high salinity site in October 2016 from Calcasieu Lake, Louisiana (29°50'58.02"N, 93°17'1.32"W; Fig 3.1.A). The hatchery staff at the Louisiana Sea Grant's Grand Isle oyster research lab performed crosses. There were a total of 7 males and 17 females contribute to the cross. Spawning was induced using warm temperatures (28°C) and the addition of sterilized sperm. Oysters were raised in the hatchery in an upwelling system for 4 months before being deployed at the low and high salinity acclimation sites during February 2017. A total of 240 oysters were deployed at each acclimation site where they acclimated for
two years; a low salinity site at Louisiana Universities Marine Consortium (LUMCON; 29°15'12.5"N 90°39'46.0"W) and a high salinity site at Grand Isle (29°14'19.3"N 90°00'11.1"W; Fig. 3.1.A).

Figure 3.1. (A) Map of population origin sites (LC: Lake Calcasieu; VB: Vermilion Bay) and F1 acclimation sites (LUM: LUMCON; GI: Grand Isle) with mean monthly salinity from 2009-2018 in psu with standard error in parentheses. (B) F1 modified North Carolina II breeding design. Each male was crossed with two females of the same acclimation site and two females of the other acclimation site. F2 offspring were reared at either high or low salinity treatments. Blue colors represent low salinities and orange represents high salinity.

3.2.2. Crosses and Larval Culturing

In May 2019, I collected oysters that had been acclimated at the two salinity sites (LUMCON and Grand Isle). These oysters were brought to the Grand Isle oyster research lab to perform crosses. Oysters were strip spawned and their sex and gamete quality were assessed under a microscope. Crosses were made following a modified North Carolina II breeding design with a total of 16 blocks (Lynch & Walsh, 1998; Fig. 3.1.B). Each block consisted of one male from each acclimation site and two females from each acclimation site. Each male was crossed with four females, two from his own acclimation site and two from the other acclimation site. Each female was crossed with two males, one from her own acclimation site and one from the other. Due to uneven sex ratios, some females were used in multiple blocks and were subsequently crossed with a total of three to four males. A total of 16 males were used from each acclimation site. A total of 19 females were used from LUMCON and 21 females were used from Grand Isle.

Sperm was passed through a 13 µm filter and eggs were passed through 35 µm filter to remove debris. Fertilization occurred at 15 psu and 28°C at an egg concentration of 10,000 eggs per mL in 100 mL. Larvae from each family were split into a low (8 psu) and high (15 psu)
rearing salinity. To minimize experimental treatment shock for the low salinity treatment, embryos were given 24 hours to acclimatize to half of their experimental salinity treatment. Following 48 hours post fertilization, larvae were transferred to their final salinity conditions. Larvae were reared in 40 mL Falcon tubes with 35 µm mesh sides and bottoms that were floating in 10-gallon aerated tanks at their final salinity conditions. Larvae were stocked at 15 embryos per mL.

Algae are grown at the Grand Isle oyster facility and larvae were fed in equal ratios of 5 species: Tisochrysis lutea, Isochrysis galbana, Pavlova lutheri, Chaetoceros muelleri, and Chaetoceros calcitrans. Larvae were fed twice a day at 20-25,000 cells/mL on day 2 and 3 and 30-40,000 cells/mL on day 4 and 5 (Wallace et al., 2008). Five days post-fertilization, larvae were collected and stained with neutral red dye for 30 min. Finally, larvae were filtered down to 2 mL centrifuge tubes and preserved with buffered formaldehyde added at 5% concentration. Due to the difficulty in visually determining egg and sperm quality, many crosses experienced 100% mortality. Thus, the following analyses were based on 18 sires and 18 dams for a total of 40 families.

3.2.3. Egg Quality Assessment

Eggs samples were first lyophilized in a freeze dryer and subsequently sonicated using a microprobe in 2 ml Tris-HCl buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Multiple aliquots were removed from egg homogenate to determine DNA content, total protein content, and lipid content. For double-stranded DNA measurements, 100 µl homogenates were removed from each sample and treated with 1 µl RNase A (Qiagen) for 2 min at room temperature. The amount of DNA was measured using PicoGreen assays following the manufacturer’s protocol (Invitrogen). Protein content was determined from 10 µl aliquots using Bradford assays modified for developmental stages of marine bivalves (Pan et al. 2018). For lipid content measurements, 300 µl egg homogenates were removed from each sample and treated with chloroform and methanol to extract lipids (Pan et al. 2018). Total lipids were separated into different lipid classes using thin-layer chromatography, and the amount of each lipid class was determined using Iatroscan Flame Ionization Detector system (Iatroscan MK-5, Iatron Laboratories Inc.). For each sample, amounts of DNA, protein, and lipid were assayed in triplicates.

The biochemical content was standardized per egg and the total number of eggs in each sample was estimated by the amount of double-stranded DNA present. A set of measurements was undertaken to measure the average DNA content per egg in the current experiment. Samples containing a known number of eggs from a subset of female oysters were analyzed following the method described above for DNA content measurements. The result shows that an egg contained 1.16 pg DNA (± 0.11 SD, n = 7). This number is in close agreement with previously published C-value for this species (Hinegardner 1974) and similar to the DNA content in eggs of the Pacific oyster (Crassostrea gigas), a species with a known genome size.

Each egg quality measurement (protein, hydrocarbons, triacylglycerols, free fatty acids, sterols, and phospholipids) was normalized by a log transformation and then fit with a linear mixed-effect model in R. To determine if crosses with 100% mortality were dependent on egg quality, I ran a one-way ANOVA using the nlme package in R. A two-way ANOVA was performed to test the influence of dam acclimation conditions on egg quality. Finally, the
influence of egg quality on larval body size was assessed by running a one-way ANOVA with Dam ID and Sire ID as random effects using the lme4 package in R.

### 3.2.4. Larval Survival and Morphometrics

I estimated survival and measured body size on photographs of 5-day old preserved larvae (Nikon Digital Sight DS-Fi2). Survival was estimated by counting the ratio of empty shells to neutral red stained larvae. Using ImageJ under 13.5 x compound magnification, I measured body size (maximum anterior-posterior length) for 1248 and 1379 larvae (~50 larvae per cross per treatment) for larval rearing conditions at low and high salinity, respectively. Using a three-way ANOVA, I tested the effects of larval rearing salinity, dam acclimation salinity, and sire acclimation salinity on larval body size with Dam ID and Sire ID as random factors in the car package (Fox & Weisberg, 2011). I further tested these effects using a Tukey's 'Honest Significant Difference’ method in the package emmeans (Lenth 2018) in r v. 3.4.4 (R Core Team, 2018).

### 3.2.5. Parental Genotype and Heritability

To determine whether larval size under low or high salinity has the capacity to evolve, I estimated variance components under both low and high larval rearing salinity. To do this, I used an animal model which is a form of a mixed model that can estimate the genetic and environmental components of phenotypic variation using pedigree data (Kruuk 2004). Specifically, I used a generalized linear-mixed model (GLMM) using Markov Chain Monte Carlo (MCMC) in the R package MCMCglm (Hadfield 2010). Following tutorials in Wilson et al. (2010), I estimated variance components and narrow-sense heritability ($h^2$) for larval size at each larval rearing salinity using the model:

\[ y = \mu + Z_1 \text{Animal} + Z_2 \text{DamID} + \beta_1 \text{DamAcclim} + \beta_2 \text{SireAcclim} + \varepsilon \]

where $\mu$ is the population mean, animal is a random effect of the additive genetic effects of each individual, and dam ID is a random effect due to non-genetic components (i.e. maternal effects) that also contribute to phenotypic variance. Finally, dam and sire acclimation site are fixed effects and $\varepsilon$ is random residual error. I calculated narrow-sense heritability as the ratio of additive genetic variance and total phenotypic variance. I also calculated maternal effects, which was the ratio of variance contributed by each dam ID to the total phenotypic variance.

The package MCMCglm uses a Bayesian framework to estimate the variance contributed by each effect in the model. Thus, I set the priors to equally partition variation among all random effects (animal, Dam ID, and residual error). I confirmed that other priors did not significantly change the outcome of the model by comparing posterior results. The MCMC chains were run for 1,300,000 generations with a burn-in of 300,000, which I then visually checked for convergence as recommended in the Wilson et al. (2010) tutorial. Our autocorrelation values for the parameters were near zero, confirming that convergence had occurred and there were no trends in the parameters over successive generations of the model.
3.3. Results

3.3.1. Environmental Effects

To test the influence of environmental effects on larval survival and size, I reared F2 offspring at either low or high salinity for five days. By measuring larval traits in the F2 generation, I was able to omit the influence of maternal effects from the source population (Sanford and Kelly 2011; Kawecki and Ebert 2004). I reared a total of 40 families created from crosses where parents were acclimated at either a low or high salinity site. For crosses that survived to 5 dpf, I observed no differences in mortality between F2 low or high larval rearing salinities ($F_{1,28.3} = 0.16$, $p=0.70$; Table B.1.), with mean mortalities around 27%. For larval size, I found that larvae reared at high salinity were 4.5 % ($\pm$ 0.46 % SE) larger ($F_{1,2607} = 39.5$, $p < 0.001$; Table B.2.; Fig. 3.2.). This suggests that the environment does have an effect on larval size, with slower growth at low salinity conditions.

![Figure 3.2. Mean anterior-posterior length of F2 larvae reared at either high (15 psu) or low salinity (8 psu).](image)

3.3.2. Transgenerational Plasticity

To test for effects of transgenerational plasticity (i.e. parental acclimation) on larval size, I crossed the F1 generation within and between low and high salinity acclimation sites. For both populations, I observed no differences in larval mortality between dam acclimation site ($F_{1,37} = 0.02$, $p=0.89$; Table B.1.), nor sire acclimation site ($F_{1,36.8} = 0.64$, $p=0.43$; Table B.1.).

Transgenerational plastic effects contributed by dams significantly contributed to larval body size (Fig. 3.3.A). When dams were acclimated at the low salinity site, I observed a significant difference in larval size between low and high larval rearing salinities, $F_{1,2613} = 43.7$, $p<0.001$; Table B.2.; Fig. 3.3.A). Larvae reared in high salinity conditions were 9.1 % ($\pm$ 0.64 % SE) larger compared to low salinity conditions when their dams were acclimated to low salinity (Tukey, $p<0.05$; Fig. 3.3.A; Table B.3.). There was no difference in larval size reared at...
low and high salinities when their dams were acclimated at the high salinity site (Tukey, p > 0.05; Fig. 3.3.A; Table B.3.).

Transgenerational plastic effects contributed by sires had a nearly significant effect on larval body size ($F_{1,2611} = 3.7$, $p = 0.055$; Table B.2.; Fig. 3.3.B). When sires were acclimated to high salinity, larvae were 5.8% ($\pm 0.67$ % SE) larger when reared at high salinity compared to low salinity (Tukey, $p < 0.05$; Fig. 3.3.B; Table B.3.). When sires were acclimated to low salinity, larvae were 2.5% ($\pm 0.63$ % SE) larger when reared at high salinity compared to low salinity (Tukey, $p < 0.05$; Fig. 3.3.B; Table B.3.).

![Figure 3.3.](image)

Figure 3.3. Mean anterior-posterior length of F2 larvae reared at either high (15 psu) or low salinity (8 psu). Background colors represent larvae with a dam or site that was acclimated to the high salinity site (orange) or the low salinity site (blue). (A) Comparison dam acclimation effect on larvae length originating. (B) Comparison of sire acclimation effect on larvae length. Tukey post-hoc results are represented as an asterisk over significant comparisons.

3.3.3. Egg Quality

To further tease apart the mechanisms of transgenerational plasticity in dams, I measured the egg quality of females in the crosses. To test whether maternal acclimation site affected egg quality, I measured protein, hydrocarbons, and lipid content (triacylglycerol, free fatty acids, sterols, and phospholipids) in the eggs of dams from the crosses. However, I observed no differences in egg content between dam acclimation sites ($p > 0.05$; Table B.4.). In addition, egg quality had no influence on whether crosses experienced 100% mortality ($p > 0.05$; Table B.5.). We also tested whether egg quality was a good predictor of larval size, however, I observed no correlations among any egg quality measurements and larval size ($p > 0.05$; Table B.6.).

3.3.4. Capacity to Evolve

Finally, I was able to estimate the capacity of this population to evolve to either low or high salinity in F2 generation larvae. Using half-siblings, I was able to partition the effects of size due to additive genetic variation in sires and maternal effects in dams. Narrow sense
heritability was estimated to have a similar influence on larval size at high rearing salinity, 0.66, and low rearing salinity, 0.68 (Fig. 3.4.). I also measured the influence of maternal effects, which are contributions that are above and beyond what is contributed by the genotype (i.e. transgenerational plasticity or epigenetic effects). Maternal effects had a significantly lower influence on larval size at both high (0.16) and low (0.11) larval rearing salinity (Fig. 3.4.). In all cases, narrow sense heritability was high, suggesting that there is ample genetic variation present and oysters have the potential to adapt to either low or high salinity conditions.

![Graph showing narrow sense heritability and maternal effects estimated for larvae reared at either low or high salinity](image)

Figure 3.4. Narrow sense heritability and maternal effects (with 95% confidence intervals) estimated for larvae reared at either low or high salinity.

3.4. Discussion

Salinity conditions in the Gulf of Mexico are predicted to undergo drastic changes due to climate change and government planned Mississippi river diversions (Ning and Abdollahi 2003; Swenson 2003), which will negatively affect oyster productivity (Wang et al. 2017). Low salinity has been shown to negatively affect oyster growth and is especially stressful in conjunction with other factors, such as temperature (Jones, Johnson, & Kelly, 2019; La Peyre et al., 2016). I measured larval size to investigate mechanisms of resilience to stressful low salinity conditions. My results show that genetic variation within the population, as well as transgenerational plasticity, contribute to variation in low salinity tolerance in *C. virginica*.

3.4.1. Influence of Environmental Effects

I first demonstrated that the environment (or larval rearing salinity) significantly affects larval size. I observed reduced larval growth at low salinity conditions. Similar patterns were observed in the adult F1 generation acclimated at the two salinity sites. Oysters at the low salinity site (LUMCON) were smaller than oysters acclimated at the low salinity site (Grand Isle). These results are in agreement with other studies that have found a similar effect on oyster size at multiple life stages in *C. virginica* (Davis & Calabrese, 1964; La Peyre et al., 2016;
A reduction in growth may affect their ability to be competitive when they get older. However, these observations are based on modern populations in future low salinity conditions and transgenerational plasticity and evolutionary adaptation may modulate these responses, as I describe below.

3.4.2. Influence of Transgenerational Plasticity

Transgenerational plasticity is predicted to be adaptive when the parental environment matches that of the offspring environment (Chirgwin et al. 2018). However, we did not observe increased growth in larvae reared in low salinity conditions when their dams were also acclimated to low salinity conditions. This suggests that transgenerational plasticity contributed by dams may not be a mechanism through which they can increase offspring fitness in future low salinity environments. Not all species seem to benefit from stressful parental conditions, as observed by poor-quality offspring when reared in the same stressful conditions (Guillaume, Monro, and Marshall 2016; Shama and Wegner 2014).

Nevertheless, I observed that transgenerational plasticity contributed by dams was an important contributor to larval size when reared in high salinity conditions. When offspring are reared in high salinity conditions, they were larger when their dams were acclimated to low salinity, suggesting an adaptive effect. Contrary to predictions, my results suggest that a mismatch between the parent and offspring environment can promote offspring growth. I propose that maternal transgenerational plasticity is adaptive when stressful maternal conditioning promotes higher fitness in offspring reared in a high salinity or beneficial environment (Marshall and Uller 2007). This effect may be the result of mothers producing larger, but possibly fewer eggs (resulting in larger offspring) when they are in a low quality or low food availability environment (Fox, Thakar, & Mousseau, 1997; Plaistow, Lapsley, & Benton, 2006). However, there was no difference in egg quality between the two dam acclimation sites (Table B.3.). In addition, previous studies have observed that higher egg quality is not always correlated with faster larval growth (Gallager and Mann 1986; Powell et al. 2002). These results suggest that other mechanisms of maternal effects, besides egg quality, are important for larval survival, such as epigenetics.

We also observed higher growth in larvae reared in high salinity conditions when sires were acclimated at the low salinity site. This suggests that transgenerational plasticity contributed by sires also plays an important role in body size. Sires are only considered to contribute genetic material to the next generation, thus the transgenerational effects I observed may be explained by epigenetic effects.

3.4.3. High Capacity to Evolve

Typically, highly plastic traits demonstrate low genetic variation or heritability (Hallsson and Björklund 2012; Lynch and Walsh 1998), since the trait is largely controlled by the environment. However, despite the high plasticity seen in body size, I also observed high narrow-sense heritability, suggesting that there is ample genetic variation present within the population to adapt to both low and high salinity conditions. Other studies have found reduced genetic variation under stressful conditions (Sunday et al. 2011; Charmantier and Garant 2005) or sometimes increase genetic variation (Mcguigan et al. 2010), which is termed cryptic variation revealed due to stress. However, I find similar levels of genetic variation in larvae reared in high
and low salinity conditions. Our low salinity conditions may not have been stressful enough to reveal either reduced genetic variation or cryptic genetic variation.

Maternal effects (i.e. transgenerational plasticity and epigenetic effects) also significantly contributed to larval size; however, additive genetic variation had a larger influence on larval size than did maternal effects (Fig. 3.4.). This suggests that evolution will be the primary mechanism through which populations of *C. virginica* may persist in future low salinity conditions.

### 3.4.4. Conclusions

I investigated the role of the environment and genotype in determining larval size and how transgenerational plasticity may modulate these two effects. I observed significant differences in larval size in their response to rearing salinity. However, these responses to salinity are also modulated through transgenerational plasticity contributed by dams and sires, whereby parental acclimation to stressful salinities is adaptive and increases offspring growth. Since egg quality had no influence on larval growth and transgenerational plasticity contributed by sires was influential on larval growth, this suggests that these plastic responses may be regulated through epigenetic mechanisms. However, transgenerational plasticity may not play a role in persisting to future low salinity conditions since I observed no differences in larval size reared at low salinity when their parents were acclimated to either low or high salinity conditions. Variance in larval size was mostly controlled by parental genotype, suggesting that this trait can instead evolve under low salinity conditions. Overall, when choosing parental stocks for increasing oyster resilience to low salinity, it is important to take the individual genotype and acclimation history into consideration.
Chapter 4.
Evolutionary Change in the Eastern Oyster, *Crassostrea virginica*, Following an Experimental Low Salinity Event

4.1. Introduction

For many marine organisms, current rates of dispersal and plasticity will not be sufficient to keep pace with predicted rates of ocean change, such as increased temperatures, acidification, and precipitation (Sunday et al. 2015). Persistence will therefore depend on the species’ capacity for evolutionary adaptation (Hoffmann and Sgrò 2011). Adaptation to current climate change is unlikely to occur from new mutations due to the fast rate of environmental change (Orr and Unckless 2008), but pre-existing genetic variation among populations may provide the raw material necessary for evolution to occur (Pespeni and Palumbi 2013; Barrett and Schluter 2008). Genetic variation among populations can arise and be maintained by strong selective pressures imposed by environmental gradients, such as temperature and salinity, resulting in locally adapted populations (Kelly, Padilla-Gamiño, and Hofmann 2013; Kawecki and Ebert 2004). Alternatively, variation may be maintained through balanced polymorphisms within populations, usually when there are high levels of gene flow combined with balancing selection imposed by fine grained environmental heterogeneity or negative frequency dependent selection (Silliman 2019; Bay and Palumbi 2014; Schmidt, Bertness, and Rand 2000). Species with broad geographic ranges and species which inhabit heterogeneous environments may harbor the genetic diversity for natural selection to act on and promote the adaptation of species to future climate change (Alberto et al. 2013).

Evidence for rapid adaptation to climate change stressors has been documented in many species. For example, shifts in allele frequencies in response to seasonal fluctuations in temperature, extreme heat waves, and gradual warming have been observed in *Drosophila* (Umina 2005; Bergland et al. 2014; Rodríguez-Trelles, Tarrío, and Santos 2013). Other studies have identified shifts in standing genetic variation in marine organisms, such as sea urchins and mussels, that would allow them to potentially adapt to future ocean acidification (Brennan et al. 2019; Bitter et al. 2019). By assessing the presence of genetic variation and shifts in response to climate change stressors, we can more accurately determine population resiliency.

While the effects of rapid changes in precipitation have been studied in terrestrial environments (Weltzin et al. 2003), less well-known are the effects of precipitation-associated salinity changes on coastal ocean habitats. In the Gulf of Mexico, the increasing frequency and intensity of storm events can result in extreme salinity changes (Goldenberg et al. 2001; Walker 2001). A species of particular concern, is the eastern oyster, *Crassostrea virginica*; a widespread euryhaline species that is a major fishery in Louisiana, but also provides many other services, such as stabilizing coastlines in the Gulf of Mexico (Meyer, Townsend, and Thayer 1997) and providing habitat for other fishery species (Plunket and La Peyre 2005). While oysters can withstand low salinity for short periods of time, growth and competitive ability are ultimately compromised, especially in the early life stages (La Peyre et al. 2013). However, salinity conditions are expected to change in the Gulf of Mexico. Projected diversions to the Mississippi river to combat subsidence issues in Louisiana will expose oyster habitats to surges of fresh water, which are predicted to negatively impact oyster production (Wang et al. 2017). However, the broad geographic range of *C. virginica* exposes them to salinity regimes ranging from as low as 4 psu to full strength seawater at 35 psu (Fig. 4.1.). While the species seems to be highly
plastic, there is also some evidence that they might be locally adapted to different salinity conditions (Chapter 3). Genetic variation for low salinity tolerance is present within and among populations in Louisiana, as evidenced by population-specific larval growth patterns under stressful low salinities. In Chapter 3, I reported significantheritabilities for larval growth, suggesting that parental genotype (and thus standing genetic variation) is an important determinant of a species’ response to changing salinity conditions.

In this study, I quantified the presence of standing genetic variation in multiple populations of *C. virginica* by imposing a strong low salinity selection event on oyster larvae. I hypothesize that genetic variation for survival at low salinities is present within each population and that selection will result in changes to allele frequencies for genes associated with salinity tolerance. The substantial body of work on salinity tolerance in oysters, and the publicly available *C. virginica* genome, sets the foundation for exome capture of genes that have been shown to be involved in low salinity tolerance in oysters from four studies (Jones, Johnson, and Kelly 2019; She et al. 2018; Meng et al. 2013; Johnson and Kelly 2019). I expect to observe changes in allele frequencies in ecologically relevant genes, consistent with standing genetic variation for low salinity tolerance present in each population.

![Map of population origin sites with mean (+- SE) salinity at each site (collected from USGS data).](image)

### 4.2. Methods

#### 4.2.1. Oyster Collection and Parental Crosses

Oysters were collected from three locations in the Gulf of Mexico that experience three distinct salinity regimes that I classify as high salinity (HS; Aransas Bay, Texas), mid-salinity (MS; Sister Lake, Louisiana), and a low salinity site (LS; Vermilion Bay, Louisiana; Fig. 4.1.). Crosses were performed in two different years of 2018 and 2019. For crosses performed during 2018 (high salinity and low salinity sites), the oysters were brought back to the Texas A&M
University Corpus Christi campus in February 2018 and acclimated them to 30 psu for three months prior to breeding. For crosses performed during 2019 (mid and low salinity sites), oysters were spawned in 2017 and their offspring were outplanted at the Grand Isle oyster hatchery site for two years prior to breeding. For all crosses, oysters were naturally spawned (induced by warming water to 30°C), or strip spawned if they did not naturally spawn, and sex and gamete quality were assessed under a microscope. Within populations crosses were performed using a single male and a single female. There were four HS crosses and two LS crosses performed in 2018 at Texas A&M. There was one LS cross and four MS crosses performed in 2019 at the Grand Isle oyster hatchery. Sperm was passed through a 13μm filter and eggs were passed through 35μm filter to remove debris. Fertilization occurred at 30 psu (2018 crosses) or 15 psu (2019 crosses) and 28°C.

4.2.2. Selection Experiment

Following 12-hours post-fertilization (hpf), I subjected larvae from each cross to a low salinity selection experiment. Prior to selection I took a sample of approximately 600,000 larvae that were immediately flash-frozen for pre-selection genetic analyses. I subjected the remaining larvae (approximately 1 million) to a low salinity of either 7, 4, or 3 psu. I chose the salinity for each selection experiment based on preliminary tests of what salinity would provide at least 50% larval knock-down in each set of crosses. All crosses performed in 2018 (four high salinity crosses and 2 low salinity crosses) were exposed to 7 psu (Table 4.1.). I chose slightly lower salinities for crosses performed in 2019 to increase the strength of selection while also trying to prevent 100% mortality. The one LS cross performed in 2019 was exposed to 3 psu and the four MS crosses in 2019 were exposed to 4 psu (Table 4.1.). Low salinity exposure occurred in a 2L beaker at a concentration of ~250 larvae/mL. Following 12 hours of low salinity exposure, I carefully poured off the top 90% the water to ensure larvae that had died and settled to the bottom of the beaker were not disturbed. I filtered down the larvae that were swimming in the top part of the beaker which were then immediately flash frozen for post-selection genetic analyses. To demonstrate that we had imposed a selection event, I sampled the non-swimming portion of the beaker and larvae were stained with neutral red dye for 30min. Any larvae that are still alive will consume the red dye and will be stained a bright red color. Larvae were then preserved with buffered formaldehyde added at 5% concentration. Using these preserved samples, I estimated the strength of the selection experiment by calculating the percent mortality.

For two of the crosses (MS cross 3 and 4), I also performed a control experiment, whereby larvae were kept at their ambient spawning salinity for 12 hours. After the 12 hours of exposure, I sampled larvae as described above and estimated allele frequency shifts to the same pre-selection samples for cross 3 and 4. In this control experiment, no loci should be found to be under selection.
Table 4.1. For each cross, the low salinity (psu) exposure is displayed along with metrics for the strength of selection (percent swimming and total number of larvae used for post-selection genetic analyses). The number of genes under selection in each cross are displayed as well as the total for each population.

<table>
<thead>
<tr>
<th>Pop Cross</th>
<th>Salinity (psu)</th>
<th>Swimming (%)</th>
<th>Swimming (# larvae)</th>
<th># genes under selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS 1</td>
<td>7</td>
<td>1.27</td>
<td>7,654</td>
<td>0</td>
</tr>
<tr>
<td>LS 2</td>
<td>7</td>
<td>0.56</td>
<td>3,383</td>
<td>0</td>
</tr>
<tr>
<td>LS 3</td>
<td>3</td>
<td>1</td>
<td>5500</td>
<td>49</td>
</tr>
<tr>
<td>MS 1</td>
<td>4</td>
<td>2</td>
<td>12,000</td>
<td>2</td>
</tr>
<tr>
<td>MS 2</td>
<td>4</td>
<td>2</td>
<td>15,000</td>
<td>2</td>
</tr>
<tr>
<td>MS 3</td>
<td>4</td>
<td>4</td>
<td>20,000</td>
<td>0</td>
</tr>
<tr>
<td>MS 4</td>
<td>4</td>
<td>15</td>
<td>103,845</td>
<td>0</td>
</tr>
<tr>
<td>HS 1</td>
<td>7</td>
<td>0.38</td>
<td>2,337</td>
<td>11</td>
</tr>
<tr>
<td>HS 2</td>
<td>7</td>
<td>20</td>
<td>123,600</td>
<td>0</td>
</tr>
<tr>
<td>HS 3</td>
<td>7</td>
<td>33</td>
<td>199,800</td>
<td>4</td>
</tr>
<tr>
<td>HS 4</td>
<td>7</td>
<td>67</td>
<td>406,008</td>
<td>8</td>
</tr>
<tr>
<td>LS average</td>
<td>7 &amp; 3</td>
<td>0.91</td>
<td>5,518</td>
<td>49 total</td>
</tr>
<tr>
<td>MS average</td>
<td>4</td>
<td>5.75</td>
<td>25,973</td>
<td>4 total</td>
</tr>
<tr>
<td>HS average</td>
<td>7</td>
<td>30</td>
<td>108,579</td>
<td>20 total</td>
</tr>
</tbody>
</table>

4.2.3. Exome Capture Design

A full genome assembly available for *C. virginica* provided a unique opportunity to use exome capture to sequence genes that previous studies have associated with low salinity tolerance. I created probes for 152 genes that had been previously correlated with salinity responses in oysters in four different studies. I selected 47 genes that show high levels differential gene expression in Louisiana populations of *C. virginica* when exposed to low and high salinity (Jones et al. 2019). I selected 26 genes that show high $F_{ST}$ between low and high salinity sites in Louisiana populations of *C. virginica* (Johnson and Kelly 2019). I also selected genes that have previously been shown to be involved in low salinity tolerance in a closely related species, *Crassostrea gigas*. This included 33 genes with high levels polymorphism associated with salinity adaptation between populations of *C. gigas* (She et al. 2018). Another 30 genes were selected from *C. gigas* that show changes in free amino acid metabolism pathways (such as taurine, glycine, proline, alanine, etc.) (Meng et al. 2013). For genes identified in *C. gigas*, I used BLAST to identify orthologs in the *C. virginica* genome. There were 27 genes that fell into 2 or more source categories (Fig. C.1.). I also haphazardly selected 42 genes across the genome to ensure that no large blocks of each chromosome were omitted from sequencing. The 152 genes were grouped into 12 functional categories: chemical defense, immune response, ion and water balance, elongation factors, free amino acid metabolism, fatty acid hydrolysis, ubiquitin-related, proteolysis, ROS-related, RNA polymerase, free amino acid rich proteins, or uncharacterized. Probes were designed to start 2000 bp upstream of the gene and extend for 8000 bp (which often resulted in spanning the entire gene and downstream of the gene).
4.2.4. Exome Capture and Sequencing

One sequencing library was made for each parent and each pooled larval sample pre and post low salinity exposure for each cross (a total of 46 libraries for 11 crosses). DNA was extracted using an OMEGA E.Z.N.A. Tissue DNA extraction kit following manufacturer’s instructions. DNA quantity was assessed using a NanoDrop spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA). Each extraction was sonicated for a median fragment size of 500 bp. I constructed 46 whole genome libraries from sonicated DNA using the NEBNext Ultra DNA Library Prep Kit for Illumina (Illumina, San Diego, CA, USA), following manufacturer’s instructions. Concentration and fragment size distributions for each library were assessed using a Bioanalyzer (Agilent, Santa Clara, CA, USA). Each library received an individual barcode adapter using the Multiplex Oligos for Illumina. I pooled 4 to 5 libraries prior to exome capture based on similar fragment size distributions. Probes were hybridized overnight using the myBaits® Target capture kits (Arbor Biosciences, Ann Arbor, MI, USA). Samples collected in 2018 were sequenced on half a lane of an Illumina HiSeq 3000 with a goal coverage of 100x (assuming a 40% capture efficiency). Single-end (1 x 150 bp) sequencing was performed at Iowa State University’s DNA Facility (Ames, IA, USA). Samples collected in 2019 were sequenced on half a lane of an Illumina HiSeq X with a goal coverage of 100x. Paired-end (2 x 150 bp) sequencing was performed at NovoGen (Beijing, China).

4.2.5. Data Analysis

Low quality bases and adapters were removed using FastQC v0.11.5 (Andrews 2010). Reads were mapped to the C. virginica genome (GCF_002022765.2) using bowtie2 (Langmead and Slazberg 2013). I then followed the pipeline outlined by Popoolation2 (Kofler, Pandey, and Schlötterer 2011) to analyze pool-seq data. Ambiguously mapped reads were removed using samtoolsview and sort (Li et al. 2009). A synchronized file containing SNP frequencies for each sample was created using the samtools mpileup option and the perl script mpileup2sync.pl through Popoolation2. I performed a Fisher’s exact test to identify strong changes in allele frequencies pre- and post-low salinity exposure for each cross. I then used a false discovery rate correction for individual SNP p-values. Since I performed single parent crosses, minor alleles are expected to have a frequency of 25% in the offspring. Therefore, I filtered SNPs with a significant change in allele frequencies, by requiring a starting frequency (pre-selection) between 15 - 35%. This conservative window omits SNPs that are of low frequency and may be due to sequencing error. I also required SNPs to increase in frequency post-selection which removes SNPs from the analyses that were present in the sample, but which I failed to observe due to sequencing error. To account for large linkage blocks of selection, I required at least three significant SNPs within a gene to categorize a gene as under selection.

To elucidate genetic relatedness among samples, I performed a principal coordinate analysis (PCoA) for each population and a principal component analysis (PCA) for all samples in R v.3.4.4 (R core team, 2018). Euclidean distances were computed from allele frequencies in each sample (each parent and each pool of larvae pre- and post-selection from population PCoAs).
4.2.6. Annotation of Genes Under Selection

To determine if SNPs were more likely to be upstream of the gene (and therefore potentially involved in regulatory changes), I first identified the location of genetic variation in the pre-selection larval samples (SNPs with a starting frequency between 15 - 35%). I then performed a Chi-Square test in R to determine whether SNPs post-selection were more likely to be found upstream, within, or downstream of the genes based on available SNPs pre-selection. I also determined if genes under selection were enriched in a particular functional or source category (e.g., high gene expression, high FST, etc.) by performing a Chi-Square test in R.

4.3. Results

4.3.1. Validation of Selection Experiment

I confirmed that I had imposed a lethal selection event by estimating the percent survival of larvae in the water column of the beaker. I observed survival rates between ~1 % and 67 % in each cross, confirming that I successfully imposed a strong selection event (Table 4.1.). The HS population had the highest survival with a mean of 67%, the MS population had a mean survival rate of 6%, and the LS population had the lowest survival at 1%. High survival rates in the HS population may be explained by the comparatively weak selection event that was imposed on this population; the HS population was exposed to a salinity of 7 psu which was not as extreme as the low salinity exposure experienced by the MS and LS populations. However, the LS population was exposed to a low salinity of 7 and 3 psu but had similar survival rates across both salinities.

4.3.2. Allele Frequency Shifts Pre- and Post-Selection

For each cross, I estimated the probability an allele frequency shift occurred due to selection rather than genetic drift. To do this, I filtered SNPs with a pre-selection frequency of ~25% and increased in frequency after the low salinity exposure (post-selection). In addition, to consider an entire gene as under selection, I required at least three SNPs to pass the significance threshold. For the HS population, I observed significant changes in allele frequencies in three out of the four crosses (crosses 1, 3, and 4; Fig. 4.2.A-C), but I saw no variation in low salinity tolerance segregating in cross 2 (Fig. C.2.A). This suggests that not all individuals in the population harbored variation for low salinity tolerance that can be acted upon by selection. In the crosses that did have significant changes in allele frequencies, there were some genes that were under selection in multiple crosses (Table 4.2.). However, the majority of genes were cross-dependent, suggesting that low salinity tolerance is not only polygenic, but also possibly redundant as each cross had multiple unique genes under selection. This suggests that not all alleles that increase low salinity tolerance are necessary, but the presence of at least a few of these alleles is enough for survival at low salinities.

For the MS population, I observed considerably lower variation for low salinity tolerance. Only two crosses had significant changes in allele frequencies (Fig. 4.2.D,E), of which there were only two genes under selection in each, neither of which overlapped between the two crosses. There was no variation for low salinity tolerance segregating in cross 3 or 4 (Fig. C.2.B,C). For crosses 3 and 4, I also performed a control experiment, whereby larvae were kept...
at their ambient spawning salinity for 12 hours. As expected, I observed no significant changes in allele frequencies for these crosses (Fig. C.3.A,B).

For the LS population, only one out of the three crosses showed variation for low salinity tolerance (Fig. 4.2.F). However, this cross had 49 genes that were under selection, suggesting that there still may be ample variation for low salinity tolerance in this population. There was no variation for low salinity tolerance segregating in cross 1 or 2 (Fig. C.2.D,E).

Figure 4.2. Manhattan plot showing SNPs that had a significant change allele frequencies with a starting frequency on ~25% pre-selection and then increased in frequency post-selection. P-values are displayed as −log from CMH test. The blue horizontal line represents the significance threshold after a false discovery rate correction. SNPs colored green pass the threshold for considering the gene to be under selection (each arrow points to a single gene that was under selection). Crosses displayed had significant genes under selection for the high salinity population (A-C), mid-salinity population (D-E), and the low salinity population (F). The low salinity exposure for each cross is displayed next to cross info.

PCoAs for each population also demonstrated shifts in allele frequencies pre- and post-selection (Fig. 4.3.A-C). Overall distances between larvae pre-and post-selection were not larger if that cross had genes under selection ($r = -1.22, p = 0.24$; Table C.1.). However, allele frequencies used in PCoAs are not filtered by SNP starting frequency or positive increase post-selection. Interestingly, the PCA for all populations grouped the HS and LS as most similar even though these two populations are geographically the most isolated (Fig. 4.1.).
Table 4.2. Genes that were under selection in one or more cross along with their function and source category.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Gene</th>
<th>Cross</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>035782</td>
<td>XM_022464577.1</td>
<td>HS4, LS3</td>
<td>Uncharacterized</td>
<td>C. virginica High F_ST &amp; gene expression</td>
</tr>
<tr>
<td>035782</td>
<td>XM_022469890.1</td>
<td>HS3, LS3</td>
<td>Uncharacterized</td>
<td>C. virginica High F_ST &amp; gene expression</td>
</tr>
<tr>
<td>035784</td>
<td>XM_022430406.1</td>
<td>HS1, HS4, LS3</td>
<td>Immune system</td>
<td>C. virginica High F_ST</td>
</tr>
<tr>
<td>035784</td>
<td>XM_022482159.1</td>
<td>HS1, HS3</td>
<td>Ion-water balance</td>
<td>C. gigas metabolic pathways</td>
</tr>
<tr>
<td>035786</td>
<td>XM_022436855.1</td>
<td>HS1, HS4, LS3</td>
<td>Ubiquitin</td>
<td>Filler</td>
</tr>
<tr>
<td>035787</td>
<td>XM_022442587.1</td>
<td>HS4, LS3</td>
<td>Ion/water balance</td>
<td>Filler</td>
</tr>
<tr>
<td>035787</td>
<td>XM_022447067.1</td>
<td>MS1, LS3</td>
<td>Chemical defense</td>
<td>C. gigas high polymorphism</td>
</tr>
<tr>
<td>035788</td>
<td>XM_022452045.1</td>
<td>HS4, LS3</td>
<td>Immune system</td>
<td>C. gigas high polymorphism</td>
</tr>
</tbody>
</table>

Figure 4.3. PCoA of allele frequencies for each parental and larval sample for the high (A), Mid (B), and low (C) salinity populations. Each family consists of a dam, sire, larvae pre- and post-selection, and larvae with no selection—if applicable for that cross—which are represented by different shapes. Each family is a different color. Arrows point in direction of allele frequency change pre- and post-selection. Cross names are displayed next to arrows with the number of significant genes displayed below (see Fig. 4.2.). For (D), PCA of all samples are displayed and populations are grouped by color.
4.3.3. Annotation of Genes Under Selection

To determine if genes under selection were enriched in a particular functional or source category, I performed a Chi-Square test. I found that genes under selection were not more likely to be enriched in a particular functional or source category (p > 0.05 for all crosses; Table C.2.). The overall lack of functional and source category enrichment suggests that the linkage blocks are large. This is expected as I performed a single generation selection experiment with single parental crosses.

Since I also sequenced ~2000 bp upstream and slightly downstream of each gene body, I was able to determine whether variation for low salinity tolerance was more likely to be upstream rather than within the gene body. An excess of variation upstream of the gene body would suggest that regulatory changes may be more important than structural changes to the protein for low salinity tolerance. In the LS population post-selection, there was an increase in SNPs found upstream (p < 0.001; Table C.2.). However, for the MS and HS populations, SNPs were not more likely to be upstream than within the gene body (p > 0.05 for all crosses; Table C.2.).

4.4. Discussion

Rather than gradual changes in salinity, extreme weather events that are increasing in frequency and intensity (Goldenberg et al. 2001) are expected to pose a major threat to oyster growth and survival in the Gulf of Mexico. High intensity storms are more likely to occur during the summer months when oysters are spawning, ultimately forcing selection to occur in the early life stages of oysters. I evaluated the capacity of populations of *C. virginica* to respond to low salinity selection events using experimental evolution on a single generation. I found that standing genetic variation exists within all populations and could facilitate rapid adaptation to low salinity conditions.

4.4.1. Presence of Standing Genetic Variation

I observed strong changes in allele frequencies in at least one cross in each of the populations from a high, mid, and low salinity site. In addition, multiple chromosomes had genes under selection in each cross, suggesting that low salinity tolerance is polygenic, as has been demonstrated in other species adapting to environmental stressors (Bergland et al. 2014; Rodríguez-Trelles, Tarrió, and Santos 2013; Bitter et al. 2019; Brennan et al. 2019). However, not all crosses had genetic variation for low salinity tolerance that segregated within the cross, suggesting that some individuals within each population lacked heterozygosity for any loci involved in low salinity tolerance. Alternatively, the lack of observed variation within these crosses could be because traits that are polygenic are typically controlled by many alleles of small effect, and these alleles are harder to detect since there may not be strong changes in allele frequencies at each locus (Yeaman 2015).

There was no correlation between the number of genes detected as being under selection and percent survival (Table 4.1.). For example, I might expect the LS population to have higher survival under low salinity with lower genetic variation if they were locally adapted to low salinity conditions. This would follow expected patterns of selective sweeps where genetic diversity is reduced after multiple generations of selection (Przeworski 2002; Savolainen,
Lascoux, and Merilä 2013). In contrast, I might expect the HS population to have low survival with high genetic variation—genetic diversity has not yet been removed by selection. While I do see evidence for more crosses in the HS population having genes under selection (demonstrating high genetic variation within the population), these crosses had the highest survival compared to all of the populations. This may be because I did not expose the HS population to a salinity as low as the other populations. Nevertheless, the low salinity exposure is outside of the range normally experienced by the HS and MS populations (Fig. 4.1.). Thus, we might expect prior selection against low salinity tolerance in the HS and MS populations and alleles that promote low salinity tolerance to be rare in these populations (Tobler et al. 2014). While the experimental design required SNPs to begin at a low frequency for consideration as under selection, other experimental evolution studies with larvae have similarly demonstrated the importance of rare variants in response to temperature and pH stressors (Brennan et al. 2019; Barghi et al. 2019; Tobler et al. 2014). I show that variation that exists at low frequencies is also important for low salinity tolerance.

The LS population was exposed to a salinity of 7 and 3 psu, of which only 3 psu is expected to impose a challenge. Thus, we might expect prior selection for low salinity conditions to cause low salinity tolerance alleles to be common in the population. Under these expectations, we might not expect many crosses from the LS population to have any genes under selection because the alleles are already close to fixation. Following these expected patterns, only one cross from the LS population had alleles segregate under low salinity, but this occurred under the most stressful salinity exposure of 3 psu which may still pose a challenge to this population.

4.4.2. Mechanisms of Response to Low Salinity

The lack of enrichment in a particular source or functional category suggests that the linkage blocks in the experiment are large. While this prevents us from identifying the causative genes under selection, I have identified potential loci that contribute to genetic variation for low salinity tolerance. In the LS population cross 3, I demonstrate that loci that contain more variation upstream of the gene are under selection, suggesting that regulatory changes are important for low salinity tolerance. This cross had the greatest number of genes under selection, which may have provided enough power to detect this pattern.

Some loci identified as under selection were also identified in other crosses (Table 4.2.), suggesting that some regions of the genome may be universally important for low salinity tolerance. However, most loci were unique to each cross and each population, suggesting that low salinity tolerance is redundant as well as polygenic. In addition, I don’t expect variation for low salinity tolerance to be present within all individuals of a population. Other studies have similarly found that polygenic traits are also redundant in nature (Barghi et al. 2019).

4.4.3. Local Adaptation and Population Structure

My results suggest that selection during the larval phase could act as a filter on genotypes found within populations. However, the extent to which this influences the population structure I observe among populations is unclear. Patterns of local adaptation have been observed in C. virginica (Chapter 3), however, high levels of gene flow are expected to swamp out locally adapted alleles (Haldane 1930). Local adaptation in the face of high gene flow can be maintained through differential survival of oyster larvae at each site. For example, I would expect LS alleles
to have a higher fitness in low salinity conditions and HS populations to have low fitness under low salinity conditions. Fewer crosses responded to selection (only 1 cross had alleles segregating for low salinity tolerance) in the LS population, suggesting that alleles for low salinity tolerance may be at a higher frequency in the population. A genotype-by-environment mismatch may explain patterns of population structure (D. J. Marshall et al. 2010; Pespeni and Palumbi 2013); however, my results are unable to fully elucidate this possible mechanism. I was only able to perform a few crosses per population—the parental genotypes originated from a maximum of 8 individuals from each population, which is not representative of an entire population’s genetic variation. Additional crosses for each population would allow us to further quantify the extent of low salinity tolerance present within each population.

Nevertheless, the fact that I did see one cross in the LS population that had alleles segregating for low salinity tolerance suggests that this population has potentially not experienced a decrease in genetic variation for low salinity—as might be expected under strong selection because alleles for low salinity tolerance would be swept to high frequencies. This variation may be maintained by a few possible mechanisms. High gene flow among populations may promote balanced polymorphism for salinity tolerance. In this scenario, the migration-selection balance in the wild is not strong enough to remove unfit alleles from the environment (Savolainen, Lascoux, and Merilä 2013) or temporally fluctuating stressors may maintain all genotypes in a population (Pedersen, Hunt, and Scheibling 2000). Alternatively, low salinity tolerance may have negative consequences and trade-offs might exist (Mitchell-Olds, Willis, and Goldstein 2007). Thus, high salinity genotypes in the population may still have a fitness advantage.

4.4.4. Conclusion

I observed standing genetic variation for low salinity tolerance, suggesting that each population has the potential to respond to future salinity changes in the wild. While I was unable to identify the causative genes under selection, I show that low salinity tolerance is polygenic, as well as redundant. My exome capture technique allowed us to increase sequencing depth by focusing on only 152 genes that are spread across all chromosomes. This list of genes may be of value to other researchers hoping to elucidate mechanisms of low salinity tolerance in a variety of oyster species.
Chapter 5.
An Experimental Test of Adaptive Introgression in Locally Adapted Populations of a Tidepool Copepod, *Tigriopus californicus*

5.1. Introduction

As the climate warms, populations of animals and plants that have adapted to historical climates will experience strong selection to respond to new temperature regimes (Jump and Penuelas 2005; Atkins and Travis 2010; Kuparinen, Savolainen, and Schurr 2010; Hoffmann and Sgrò 2011; Aitken et al. 2008; Alberto et al. 2013; Shaw and Etterson 2012). Rapid adaptation to changing climates has already been observed in several species (Bradshaw and Holzapfel 2001; Franks, Sim, and Weis 2007), but current rates of climate change may also outpace adaptive evolution *in situ*, leading to population declines (Møller, Rubolini, and Lehikoinen 2008; Smale et al. 2019). However, in populations that are locally adapted to current gradients in temperature, evolutionary rescue may be facilitated by gene flow, with warming temperatures favoring alleles from equatorward or lower elevation populations (Davis and Shaw 2001). As a result, ‘rear edge’ populations are thought to be an important reservoir of genetic variation necessary for adaptation to warming (Hampe and Petit 2005).

Yet, even when genetic variation for adaptive responses to warming exists with a species, adaptive introgression may be hampered by several important factors. First, warm adapted alleles may arrive in the new population in a genetic background that is not otherwise well-adapted to the new location (Schiffrers et al. 2012). For example, if local adaptation has occurred along multiple environmental axes, equatorward genotypes may contain warm adapted alleles that are favored by climate change, but also other alleles that are maladaptive in the new population. Maladaptation of foreign genotypes may also stem from intralocus selection, if negative epistatic interactions cause alleles from equatorward population to be disfavored in the predominant genetic background of the new population. Second, the power of poleward gene flow to promote evolutionary rescue will be weakened when the trait under selection is highly polygenic. Introggression of a single beneficial allele can happen quickly (Oziolor et al. 2019; Miao, Wang, and Li 2016), but recombination will break up unlinked loci underlying highly polygenic traits and stymie their coordinated introgression (Yeaman and Whitlock 2011; Sachdeva and Barton 2018). Thus, the probability of evolutionary rescue through gene flow will depend on the degree of maladaptation of the genetic background in which the warm adapted alleles are found, the rate of recombination (which will determine the capacity of the warm adapted alleles to escape the maladaptive genetic background), and the genomic architecture of the trait under selection. While all of these factors have been discussed in the theoretical literature (Sachdeva and Barton 2018; Sachdeva and Barton 2018), few studies have empirically examined the dynamics of introgression for complex traits that are likely to be under selection in the context of climate change.

Here, I examine adaptive introgression using the model organism, *Tigriopus californicus*, a tidepool copepod that has a reference genome and has been studied intensively for its adaptation to thermal stress. *T. californicus* populations are highly differentiated with respect to heat tolerance across a geographic range that spans > 27 degrees of latitude, suggesting that, at the species level, there is ample genetic variation to adapt to warming temperatures. However, *T. californicus* populations are also highly subdivided, with individual populations containing about 1% of the quantitative genetic variation for heat tolerance found at the species level (Kelly,
As a result, laboratory selection experiments indicate that individual populations can evolve only about ~0.5°C additional heat tolerance before exhausting the standing genetic variation available within populations. A species like *T. californicus*, with local adaptation to temperature and limited variation within populations, would seem to be an excellent candidate for evolutionary rescue through gene flow from warm-adapted populations. However, decades of research have also demonstrated that this species exhibits strong outbreeding depression, driven in part by mito-nuclear incompatibilities in inter-population crosses (Edmands 1999; Ellison and Burton 2010; Willett 2012).

By describing the genomic architecture of thermal tolerance in *T. californicus*, I can evaluate the likelihood of evolutionary rescue via gene flow from more tolerant equatorward populations in the face of mito-nuclear incompatibilities. To do this, I developed a technique which I term Hybridize Evolve and Re-sequence (HER, modified from ‘Evolve and Re-sequence’; Turner et al. 2011), which I use to assess the genomic architecture of heat tolerance variation among populations of *T. californicus*. Evolve and re-sequence methods combine experimental evolution (Kawecki et al. 2012) with sequencing to identify the targets of selection. This approach has been used to map the genetic basis of a variety of traits in *Drosophila*, including body size (Turner et al. 2011) and courtship song (Turner and Miller 2012), but I am aware of no study, to date, that has applied this approach to a marine metazoan.

By hybridizing two populations with divergent heat tolerance limits and subjecting subsequent generations to strong heat selection, I can test the ability of selection to fix alleles responsible for heat tolerance via whole genome re-sequencing. After 15 generations of heat selection, hybrids were able to increase their thermal tolerance in comparison to hybrids under control conditions. This corresponded with repeatable changes in allele frequencies at multiple loci across all independent selected lines. All 12 chromosomes had regions of strong changes in allele frequencies, providing evidence that heat tolerance is a highly polygenic trait. Taken together, the results suggest that adaptive introgression of a polygenic trait can occur even with a maladaptive genetic background, provided that there is strong selection for the introgressed alleles.

### 5.2. Methods

#### 5.2.1. Field Collection, Hybrid Crosses, and Experimental Selection

Laboratory cultures were established from two populations of *T. californicus* collected during 2014 from Bodega Marine Reserve in northern California, USA (38°04’ N, 123°19’ W) and San Diego in southern California, USA (32°49’ N, 117°16’ W) (Fig. 5.1.). Cultures were maintained at ambient temperature (20°C) and salinity (35 ppt) under 12- h light/12-h dark conditions and fed ground spirulina fish food *ad libitum* for two generations (~ 8 weeks) before initiating crosses.

These two populations have different thermal tolerances, with median lethal (LT$_{50}$) temperatures at 34.8 and 36.5°C for Bodega and San Diego, respectively (Kelly, Sanford, and Grosberg 2012). To create hybrid laboratory populations, 20 females from Bodega and 20 males from San Diego were crossed. Copepods form mate-guarding pairs, in which a male copepod guards the virgin female by attaching to her with specialized antennae. These pairs from each population can be teased apart with a fine probe and then paired with a partner from the opposite population. Newly formed pairs were held in a 24-well tissue culture plate until the female produced her first brood. Most genetic variation for thermal tolerance is partitioned between
populations (Kelly, Sanford, and Grosberg 2012), therefore a starting population size of 20 individuals from each population was sufficient to sample the genetic variation within and between populations. To ensure the direction of the cross did not affect the LT$_{50}$ temperature for the F1 generation, I also performed a similar cross with males and females from the opposite population, i.e. 20 males from Bodega and 20 females from San Diego. I then compared the LT$_{50}$ temperatures following the methods described under ‘Heat tolerance measurements’.

Once the F1 generation (for maternal Bodega and paternal San Diego crosses) reached adulthood and began forming mate-guarding pairs, I established five replicate lines with 30 mate-guarding pairs each. Parental and F1 generations were maintained at ambient conditions as described above. Starting with the F2 generation, from each of the five replicate lines, 40 mate-guarding pairs were haphazardly chosen for the ‘selection’ line and 40 pairs for the ‘control’ line. Experimental exposure for the selected lines is described below. In the field, temperatures in shallow pools can increase by 20°C over the course of 3 hours. To select for increased heat tolerance, I exposed the selected lines to the temperature that produced 50–90% mortality for 1 h (36.0°C in the first generation of selection, increasing by ~0.1°C per generation of selection). This initial temperature was determined by calculating the LT$_{50}$ temperature for the F1 generation hybrid crosses, as described below. The next generation was then established with 40 of the surviving mate-guarding pairs. The control lines were propagated for 21 generations, while the selected lines were propagated for a total of 15-16 generations, due to the increased amount of time per generation to achieve adequate numbers of individuals for the selection treatment.

5.2.2. Heat Tolerance Measurements

I determined LT$_{50}$ temperatures for each parental population line (Bodega and San Diego), the F1 generation for both hybrid crosses (Bodega-maternal and San Diego-paternal; Bodega-paternal and San Diego-maternal), and after 15-21 generations for each selected and control line derived from Bodega-females and San Diego-males hybrid crosses. For each of these groups, individual mate-guarding pairs were collected and placed into PCR tubes with seawater. For each of the heat-selected lines and controls, I exposed sets of nine mate-guarding pairs to a target temperature in a thermocycler for 1 h, allowed 48 h for recovery, and then assessed survival. I did this for a series of 5–10 temperatures at 0.2°C intervals, spanning from the temperature that produced 90–100% survival to the temperature that produced 90–100% mortality, and then used the mortality at each temperature to estimate LT$_{50}$ for each line via logistic regression and the R package MASS (Venables and Ripley 2002). Females and males were scored separately for survival since females typically have slightly higher heat tolerances than males (Kelly, Sanford, and Grosberg 2012). A two-way ANOVA was performed to test the effects of selection and sex on LT$_{50}$ temperatures with each line treated as a random factor using the R package nlme (Pinheiro et al. 2019) in R v. 3.4.4 (R Core Team, 2018).

5.2.3. DNA Extraction and Library Preparation for Whole Genome Resequencing, Pool-Seq

One sequencing library was made for each of the 5 selected lines and 5 control lines plus one sequencing library for each of the parental pure populations (12 libraries total). For each library, 100 copepods were pooled and concentrated to remove as much water as possible. DNA was extracted using a DNA isolation kit from Sigma Aldrich kit following manufacturer’s instructions. DNA quantity was assessed using a NanoDrop spectrophotometer.
(NanoDrop Technologies, Wilmington, DE, USA). Each extraction was sonicated for a median fragment size of 500 bp. I constructed 12 whole genome libraries from sonicated DNA pools using the NEBNext Ultra DNA Library Prep Kit for Illumina (Illumina, San Diego, CA, USA), following manufacturer’s instructions. Concentration and fragment size distributions for each library were assessed using a Bioanalyzer (Agilent, Santa Clara, CA, USA). Each library received an individual barcode adapter using the Multiplex Oligos for Illumina. Libraries were pooled in two sets composed of 5 and 7 libraries and then sequenced on two lanes of an Illumina HiSeq 3000 with a goal coverage of 30x. Paired-end (2x150 bp) sequencing was performed at Iowa State University’s DNA Facility (Ames, IA, USA).

5.2.4. Identifying Windows Under Selection

Low quality bases and adapters were removed using FastQC v0.11.5 (Andrews 2010). Reads were mapped to the *Tigriopus californicus* genome version 2.1 (Barreto et al. 2018) using bowtie2 (Langmead and Slazberg 2013). I then followed the pipeline outlined by Popoolation2 (Kofler, Pandey, and Schlötterer 2011) for estimation of \( F_{ST} \) between selected lines and the pure Bodega population. Ambiguously mapped reads were removed using samtools view and sort (Li et al. 2009). A synchronized file containing SNP frequencies for each sample was created using the samtools mpileup option and the perl script mpileup2sync.pl through Popoolation2. \( F_{ST} \) values were calculated using the sliding window approach with the fst-sliding.pl perl script (Kofler, Pandey, and Schlötterer 2011). A window size and step-size of 10,000 bp was used with a minimum coverage of 20x. \( F_{ST} \) comparisons were computed for each selected and control line against the pure Bodega population. Regions with high \( F_{ST} \) for selected vs. Bodega, but not control vs. Bodega, are expected to be loci under selection for heat tolerance, since heat tolerance selection should mostly favor alleles from the heat tolerant (San Diego) population. Since all copepods have a Bodega maternal line, mito-nuclear incompatibilities should favor nuclear Bodega alleles compatible with the Bodega mitochondrial background. Regions with low \( F_{ST} \) relative to Bodega in both the control and selected lines are expected to be loci with high mito-nuclear incompatibilities or loci where the Bodega allele had higher fitness under laboratory conditions.

To estimate consistent allele frequency differences across selected lines relative to controls, I first pooled all control line reads into a single fastq file. In the F1 generation, all lines begin with a 50/50 ratio of San Diego and Bodega alleles. In the absence of selection, these allele frequencies are expected to depart from their initial frequencies due to drift only. However, in *T. californicus*, mito-nuclear incompatibilities will select for nuclear Bodega alleles that are compatible with the Bodega mitochondrial alleles, causing allele frequencies to depart from expectations under drift. Therefore, reads from control lines were pooled so I could individually compare each selected line to all control lines to identify changes in allele frequencies only due to heat tolerance selection relative to allele frequencies generated by drift + intralocus selection in controls. I then followed the suggested pipeline outlined by Popoolation2 and performed a Cochran–Mantel–Haenszel (CMH) test obtain a single p-value for consistent changes in allele frequencies across all replicate lines in comparison to the pooled control line (minimum coverage of 50x). The CMH calculates p-values for SNPs which are assumed to be non-independent, which greatly inflated the significance. To correct for the large linkage blocks in the experiment, I calculated the geometric mean of p-values within a 10,000 bp window. Windows were identified to be under heat tolerance selection after passing the Bonferroni correction threshold for multiple comparisons (total number of windows identified: 18,491).
5.2.5. Annotation of Windows Under Selection

Windows that passed the Bonferroni correction for allele frequency differences were consequently annotated. For windows that overlap with gene regions, we extracted BLAST annotations from the Genome GFF file. I also extracted GO terms from the GFF file and used a Fisher’s exact test for presence-absence of functional categories with a false discovery rate correction (Wright et al. 2015). I also performed a Fisher’s exact test to determine if significant windows were enriched in genes that were differentially expressed in selected lines and in response to heat shock in an earlier experiment (Kelly, Pankey, et al. 2016).

5.2.6. Gene Expression

To test whether windows under selection in this experiment also contain genes that are differentially expressed, I re-analyzed transcriptomic data from (Kelly, Pankey, et al. 2016) and re-mapped RNA-seq reads to the newly available reference genome (Barreto et al. 2018). In this previous experiment, hybrid individuals were created with the same methods described here and selected and control lines were propagated for 4 generations following the same experimental selection procedure described here. Raw reads were cleaned using TrimGalore-v.0.4.4, which removed low quality bases (Q < 20) and adapters with FastQC v0.11.7 (Andrews 2010) and Cutadapt v1.18 (Martin 2011). Cleaned reads were mapped as single-end to the reference genome v2.1 (Barreto et al. 2018) using RSEM-v.1.3.0 (Li and Dewey 2014). I performed differential gene expression analysis using the DESeq2 package (v1.18.1) in R (v.3.4.4) using an adjusted p-value cut off of 0.05 (Love, Anders, and Huber 2014). I tested the gene expression response to heat shock for both selected and control lines. I also tested for differences in expression between selected and control lines under ambient conditions. I then compared DEGs to genes that were found to be under selection from whole-genome re-sequencing to determine if there was any overlap. A Fisher’s exact test was run in R to determine if genes within identified significant windows were also enriched in DEGs.

5.2.7. Sequence Divergence

To test whether windows under selection also contained genes with high sequence divergence between populations, I compared sequencing reads from the Bodega population to the reference genome, which was assembled with individuals from the San Diego population. Following the GATK pipeline, cleaned reads were mapped to the reference genome using bwa (v0.7.17) (Li and Durbin 2009). Duplicate reads were removed and then remaining reads were indexed using Picard tools. Finally, SNPs and indels were identified using HaplotypeCaller with GATK v4.0.2.1. The resulting VCF file was annotated using snpEff, a tool to annotate and predict the effects of SNPs (Cingolani et al. 2012). I then used a python script to identify Ka/Ks ratios for each gene using the annotated VCF file (Cadzow et al. 2014). The results of a Student’s t-test for the observed p-value was compared to the distribution of 10,000 permutations to determine if genes within identified significant windows also had a divergent Ka/Ks ratio from the genome-wide mean Ka/Ks ratio.
5.2.8. Estimates of Linkage Disequilibrium

Evidence for positive selection can be inferred through identification of elevated linkage disequilibrium around the locus, concurrent with patterns of a selective sweep (Kim and Neilsen 2004; Przeworski 2002). I estimated linkage disequilibrium between pairs of SNPs within a single read using LDx (Feder 2012), a program that estimates linkage disequilibrium (LD) using pool-sequencing data when multiple timepoints do not exist within a dataset. While this program is unable to give estimates of the size of linkage blocks under selection, I can infer the rate of LD decay across small distances of SNPs that are under selection. Following the pipeline in Brennan et al. (2019), I estimate LD decay by regressing the log of the distance between SNPs and their maximum likelihood estimate of LD score with replicate lines as a random effect using the R package nlme (Pinheiro et al. 2019). I compared the LD decay of SNPs that are within the windows identified as significant from the CMH results (SNPs under “selection”) to SNPs within windows identified as nonsignificant (“neutral” SNPs) within the same replicate line. A slower LD decay rate in the selected SNPs compared to neutral SNPs would follow my expectations that the most strongly selected SNPs have higher LD, following patterns of a selective sweep. I also compared the LD decay rates of the same selected SNPs from the CMH results across selected and control lines. Slopes and intercepts of LD decay of the selected SNPs and selected lines were compared to 500 permuted distributions of randomly subsampled data in R.

5.3. Results

5.3.1. Increased Heat Tolerance in Selected Lines

The two parental population LT50 temperatures agreed with previous findings (Kelly, Sanford, and Grosberg 2012), with San Diego copepods having a ~2°C higher heat tolerance than Bodega copepods (Fig. 5.1.). The F1 generation of both reciprocal crosses had an LT50 value in between that of the two parental populations, with the direction of the cross having no influence on the LT50 value (Fig. 5.1.). For the selection experiment, the females of the selected lines had a significantly higher LT50 temperature than control lines (F1,16 = 92.8 p < 0.001; Fig. 5.1.) with selected lines exhibiting a ~1.2°C higher tolerance. Male hybrids showed a similar response to females and there was no difference in LT50 temperatures between sexes (F1,16 = 3.15 p = 0.09; Fig. D.1.). The difference in LT50 temperatures between the selected and control lines demonstrate that I successfully selected for increased heat tolerance in hybrid copepods. Additionally, both males and females from the selected lines either exceeded or were within ~ 0.5 °C of the San Diego population LT50 temperature.

5.3.2. Pool-Seq

Mean mapping success of all libraries was 64.2% (range of 59.9-74.8%). The San Diego population had the highest mean mapping rate (74.80%) as expected since the genome was assembled from the same population, while the Bodega population had a mean mapping rate of 60.98%. The selected and control lines had a mean mapping rate of 65.95% (range of 62.31-69.03%) and 60.90% (range of 59.90-61.55%), respectively.
5.3.3. Consistent Allele Frequency Changes Among Replicate Selected Lines

1,860,985 SNPs met the coverage requirements across all selected lines and the pooled control lines. I calculated the geometric mean of the p-value from the CMH test of all SNPs within a 10,000 bp window since these SNPs are non-independent due to large linkage blocks. After Bonferroni correction (p < 0.05 / 18,491 windows), there were 589 windows that had a significant change in allele frequency across all selected lines compared to the pooled control line (Fig. 5.2.A). These windows are strong candidates for loci that are under selection for heat tolerance. These loci spanned all 12 of the chromosomes and on some chromosomes there were multiple loci under selection.

![Figure 5.1. Median lethal temperatures (LT₅₀) for females from hybrid lines selected for heat tolerance (Selected 1-5) and controls lines 1-5. Parental copepods from the pure Bodega and San Diego population are displayed as well as the F1 hybrid generation crossed as either Bodega-San Diego or San Diego-Bodega (maternal-paternal respectively).](image)

5.3.4. Southern Alleles Under Selection for Heat Tolerance

I compared the mean F_{ST} for selected and control lines to the Bodega pure population so that loci of high F_{ST} corresponded to a higher frequency of alleles from the heat tolerant San Diego population. The overall genome-wide mean F_{ST} for selected lines vs. the pure Bodega population was higher (0.367) than control lines vs. Bodega (0.159). This matches my expectations that the selected lines will have a higher frequency of alleles from the heat tolerant San Diego population and control lines will have a higher frequency of alleles from the Bodega population that would be compatible with the Bodega mitochondrial genes. Windows identified to be under heat tolerance selection from Fig. 5.2.A also had a high frequency of San Diego alleles when comparing the mean F_{ST} for each of the five selected lines (0.395, 0.391, 0.267,
0.245, and 0.539 for selected lines 1-5, respectively; Fig. 5.3.A-E, represented as green dots). Thus, windows that had high allele frequency change between selected and control lines (Fig. 2.A) also had high F_{ST} values when comparing the selected lines to the Bodega pure population. As expected, windows under selection had low F_{ST} values in each control line compared to the pure Bodega population (0.159, 0.134, 0.120, 0.179, and 0.219 for control lines 1-5, respectively; Fig. D.2.).

Figure 5.2. (A) Manhattan plot showing 10,000bp windows that had consistent changes in allele frequencies across all selected lines compared to pooled control lines. P-values are displayed as –log from CMH test. The blue horizontal line represents the significance threshold after Bonferroni correction. (B) FST average of 10,000bp window of all selected lines vs the pure Bodega population. (C) FST average of 10,000 bp window of all control lines vs the pure Bodega population. Windows colored green on FST plots are loci that were above the Bonferroni correction from A.
Figure 5.3. FST for each selected line 1-5 (A-E, respectively) compared to the pure BR population. Windows colored green on FST plots are loci that were above the Bonferroni correction from figure 5.2.A.
The Manhattan plots of both the selected and control lines also allow us to make an interesting investigation of universally beneficial alleles. For example, there were loci with high F\textsubscript{ST} values relative to the Bodega population on chromosome 1, 2, 4, 7, and 9 in both the selected and control lines. These regions of universal high F\textsubscript{ST} (i.e. higher frequency of San Diego alleles) may provide a fitness advantage under laboratory conditions.

5.3.5. Characterization of Windows Under Selection

There were 374 genes that overlapped with the windows that were identified as under selection from the CMH test. These genes had BLAST annotations with functions involved in ATP creation in the electron transport chain, phosphorylation, apoptosis, and sodium and potassium channels. However, these windows were not functionally enriched in any category.

Of the 27 annotated Heat Shock Proteins (HSP) in the T. californicus genome, three were found within the windows under selection. These HSPs were annotated as two paralogs of HSP67 and one HSP26. A Fisher’s exact test revealed that windows under heat tolerance selection were enriched in HSPs (p = 0.033). In addition, the gene encoding HSP67 has previously been demonstrated to be differentially expressed in control lines in response to one hour of heat shock (Kelly, Pankey, et al. 2016).

I re-analyzed transcriptomic data from Kelly et al. (2016) using the newly available genome to determine the number of differentially expressed genes after 4 generations of selection for increased heat tolerance. The total number of DEG corresponded closely with results from Kelly et al. (2016) when testing the response of selected and control lines between heat-shocked and ambient temperature treatments (Table D.2.). I found a total of 2317 unique genes that were differentially expressed in response to heat shock and/or between selected vs. control lines. I tested to see if windows under selection for heat tolerance were also enriched in genes that are differentially expressed. Out of the 374 windows under heat tolerance selection that overlapped with a gene region, 62 of these windows contained a gene that was differentially expressed in one of the comparisons. However, a Fisher’s exact test revealed windows under selection were not enriched in DEG that showed a genotype-by-environment interaction (line by short-term heat shock; p = 0.46; Table D.2.), nor in genes differentially expressed only in the selected lines in response to short-term heat shock (p = 0.21; Table D.2.). However, windows under heat tolerance selection were enriched in DEG in both the selected and control lines in response to a short-term heat shock (591 DEG; p = 0.003; Table D.2.). This suggests that loci under selection, while responsive to heat shock, do not differ in their responsiveness to heat shock between populations or in the more heat tolerant lines. Thus, the feature of these loci that actually generates the observed differences in heat tolerance may be structural changes to proteins rather than regulatory changes to the protein’s abundance.

To test if windows under selection for heat tolerance contained genes with high Ka/Ks values between populations (regions of the genome where there is high coding sequence divergence between the Bodega and San Diego population), I obtained an estimate of Ka/Ks for all genes in the genome. The mean Ka/Ks value for the genes under selection was (0.479) which was lower than the genome-wide average Ka/Ks of 0.667. A Student’s t-test suggested that this difference was statistically significant (t\textsubscript{563} = 7.84, p = 2.25e-14). A permutation test confirmed this significant difference in Ka/Ks values as the mean Ka/Ks value for genes under selection (0.479) fell below the range of the 95% permutation distribution (0.576, 0.797). This suggests
that genes under heat tolerance selection are also under purifying selection between the two populations and that these genes may be highly conserved in the genome of both populations.

5.3.6. LD of Selected SNPs in Selected and Control Lines

A permutation test showed no significant difference in the LD decay rate between SNPs within the selected windows (slope: -0.113) compared to the neutral SNPs within the same replicate selected line (slope: -0.120) (Fig. 5.4.). Similarly, for the intercept, or zero distance, the selected SNPs’ LD (intercept: 0.743) and neutral SNPs (intercept: 0.741) were within the permuted 95% distributions (0.705, 0.845; Fig. 5.4.).

A permutation test also showed no significant difference between the LD decay rates of SNPs under selection in the CMH test in the selected lines (slope: -0.113) versus the control lines (slope: -0.133) (Fig. 5.4.). Similarly, for the intercept the selected lines LD (intercept: 0.743) and control lines LD (intercept: 0.784) were within the permuted 95% distributions (0.705, 0.845; Fig. 5.4.).

![Figure 5.4. LD decay of selected SNPs in the selected lines (solid red) compared to neutral SNPs in the selected lines (solid blue) and selected SNPs in the control lines (dashed blue). Models incorporated either all selected or all control lines with replicate as a random effect. Grey shading is the 95% confidence interval of 500 permutations of randomly sampled SNPs across the genome (based on CMH results from the pooled selected lines).](image-url)
5.4. Discussion

The possibility of evolutionary rescue through gene flow has been discussed in the theoretical literature (Bell 2013; Sachdeva and Barton 2018; Sachdeva and Barton 2018), but few studies have empirically tested this idea. I demonstrate the capacity of warm adapted alleles to increase in frequency in hybrid copepods under heat selection in the face of mitonculear incompatibilities. Using an approach I call Hybridization Evolve and Re-Sequencing (HER), I observed consistent shifts in allele frequencies across replicate lines at multiple loci, suggesting that heat tolerance is a highly polygenic trait. These results contribute to the growing body of evidence that introgression of complex adaptive traits can occur relatively quickly, even in an otherwise maladaptive genetic background (Kovach et al. 2016; Bay, Taylor, and Schluter 2019; Matute et al. 2019; Oziolor et al. 2019).

5.4.1. Polygenic Basis for Heat Tolerance

Selection can drive large-effect loci to fixation rather quickly and are easier to identify than small effect loci (Yeaman 2015), which explains the bias in the literature describing short term evolutionary changes controlled by one or a few loci. However, most traits are polygenic, often with many alleles of small-effect (Pritchard and Di Rienzo 2010; Pritchard, Pickrell, and Coop 2010; Yeaman 2015). I identified loci across all 12 chromosomes that reached high frequencies of heat tolerant alleles originating from the warm adapted Southern population after just 15 generations of selection.

Heat tolerance has been shown to be a highly polygenic trait in a few other species, such as *Drosophila* (Michalak et al. 2019; Tobler et al. 2014). For example, Barghi et al. (2019) identified many loci under heat selection in *D. simulans*, but only a subset of these were under selection in each of the 10 different selected lines, suggesting that this trait is not only polygenic but also genetically redundant.

Possibly due to its polygenic nature, the genomic architecture and genetic basis for intraspecific variation in heat tolerance is poorly understood for most species. Nevertheless, this is an important area of research as temperature plays a major role in all levels of biological organization, from setting species’ distributions (Bozinovic, Calosi, and Spicer 2011) to affecting both the physical states of all biological structures and the rates of biochemical reactions. I urge the continuation of studies that describe the genomic architecture of population variation in thermal tolerance for species that may be at risk in the warming climate.

5.4.2. Linkage Disequilibrium

Since I sequenced pools of individuals at a single time point, I was unable to estimate the size of LD blocks. To accurately estimate LD block size, haplotype information is required *a priori* in order to observe correlated changes in allele frequencies at multiple loci in the population over time (Feder, Petrov, and Bergland 2012). Future experiments should not omit this step, because the power to detect causative alleles depends on the size of the LD block (Baldwin-Brown, Long, and Thornton 2014). The smaller the linkage block that selection is acting on the greater the power to accurately identify causative SNPs for the observed phenotypic changes. However, the ability to identify the causative region or loci under selection is also influenced by the strength of selection, population size, and the number of replicates.
I estimate the size of LD blocks in the hybrids to be ~120 kb based on the total number of predicted recombination events (½ each generation due to sex dependent recombination). Thus, I believe the chosen window size of 10 kb accurately captures allele frequency changes without assuming the location of each block and avoiding an overly conservative estimate of non-independent SNPs. While the large size of the LD blocks, small population size, and modest number of replicates may prevent us from identifying causative SNPs under selection for increased heat tolerance (according to Baldwin 2014 standards), I subjected hybrids to strong selection (50% mortality each generation), making it possible for us to identify causative loci or regions that may be under selection.

Loci under selection are assumed to have greater LD as a selective sweep occurs (Kim and Neilsen 2004; Przeworski 2002). However, I observed no difference in the decay rate between selected and neutral SNPs within the same line (Fig. 5.4.). Additionally, I did not observe a difference between the decay rate of SNPs under selection in the selected lines and the selected SNPs in the control lines. Consequently, SNPs under selection for heat tolerance have similar LD decay rates to the rest of the genome in both the selected and control lines. This suggests that most of the genome is affected by selection by virtue of being linked to SNPs related to heat tolerance or mito-nuclear incompatibilities (Kern and Hahn 2018).

5.4.3. Genes Under Selection

Sixty-four % of windows under selection spanned a gene region—however, these genes were not functionally enriched in a particular category. This may be due in part to the large size of the LD blocks which may be causing neutral SNPs contained within or nearby genes to increase in frequency, thus reducing my ability to identify genes contributing to heat tolerance.

Windows under selection were enriched, however, for genes that were differentially expressed in both the selected and control lines in response to a 1-hr heat shock. This suggests that genes that are important for short-term heat shock are also important for long-term adaptation to heat stress. However, windows under selection were not enriched in DEG with a genotype-by-environment interaction (line by short-term heat shock interaction) nor in response to short-term heat shock in selected lines only. This suggests that alleles under selection are those that are differentially expressed in response to heat shock, but not alleles whose expression response changes in response to selection.

Because the selection experiments subjected copepods to acute heat stress, the experimental design may be selecting for proteins that resist denaturation under heat stress, rather than selecting for alleles with a more plastic response to heat stress. Prior exposure to heat stress can extend the upper lethal limit of T. californicus by 0.5-1.0°C. This is a form of phenotypic plasticity known as 'thermal acclimation' or 'heat hardening’ (Phillips et al. 2016; Telonis-Scott et al. 2014) that can occur through transcriptomic changes of genes involved in heat tolerance (Schoville et al. 2012; Kenkel et al. 2013; Barshis et al. 2013). We might expect a different set of loci to be under selection—perhaps more loci enriched in DEG with genotype-by-environment interactions—if I were to modify the experiment by imposing a sublethal heat shock first (to induce a plastic response) followed by a lethal heat shock event to impose selection.

Similar to results from heat shock selection in Drosophila (Barghi et al. 2019), I found HSPs to be under selection, as well as differentially regulated (Kelly, Pankey, et al. 2016). However, it was surprising that the more commonly studied HSP70 was not under selection even
though it was also differentially regulated (Kelly, Pankey, et al. 2016). Other HSPs besides HSP70 may also play an important role in heat tolerance that should be further investigated.

I found that genes under selection for heat tolerance have lower Ka/Ks values than the genome-wide average. This surprising result complements my hypothesis that structural changes to proteins that make them more resistant to denaturation may be more important than regulatory changes to the proteins abundance. Indeed, proteins involved in thermal tolerance in other species are highly conserved (Fields 2001), and a single amino acid change was sufficient to alter the thermal stability of an otherwise highly conserved protein. (Johns and Somero 2004; Fields, Rudomin, and Somero 2006; Dong and Somero 2009). These results highlight the utility of the Hybridization Evolve and Re-sequence technique. Standard population comparison techniques identify regions under selection that are the most differentiated among populations (e.g. high Ka/Ks or high $F_{ST}$), however, I find the opposite response. Loci under selection in my study contained genes that are under high purifying selection between the two populations (low Ka/Ks). These loci would not have been identified as being under divergent selection in typical population comparison analyses.

5.4.4. Conclusion

My results suggest that evolutionary rescue through introgression from more stress tolerant populations can be a possible avenue to prevent population extirpation from climate change. Individual populations subjected to generations of heat tolerance selection were unable to increase their heat tolerance more than half a degree (Kelly, Sanford, and Grosberg 2012), but I show that hybrid copepods are able to increase their heat tolerance to levels seen in lower latitude populations. Heat tolerant alleles can reach high frequencies in hybrid copepods despite the strong mito-nuclear incompatibilities developed over years of separation between these two populations. However, I did not test possible fitness tradeoffs in the hybrid individuals in this study even though it has been previously demonstrated that hybrids have reduced fecundity and plasticity (Kelly, Grosberg, and Sanford 2013; Kelly, DeBiasse, et al. 2016). In addition, evolutionary rescue is unlikely to occur naturally in this species due to their limited dispersal. Assisted evolution through breeding populations with tolerant alleles may hold promise for preventing species extinction, but the ethics of doing this still need to be further explored.
Chapter 6.
Conclusions

In this dissertation, I aimed to evaluate evolutionary responses to climate change stressors in three marine invertebrates, corals, oysters, and copepods. There is an emerging need to understand how species might respond to the rapidly changing climate. Evolution is one mechanism that could promote species persistence. However, for a species to evolve, genetic variation must be present within or among populations. I attempted to identify the presence of genetic variation in marine invertebrate populations in each chapter.

In chapter 2, I identified divergent reaction norms between populations with contrasting upwelling histories. I compared two Californian populations of the coral, *Balanophyllia elegans*, from distinct upwelling regimes and tested their physiological and transcriptomic responses to experimental seawater acidification (Griffiths, Pan, & Kelly, 2019). I measured respiration rates, protein and lipid content, and gene expression in corals from both populations exposed to pH levels of 7.8 and 7.4 for 29 days. Corals from the low upwelling site (high pH) had reduced respiration rates, protein content and lipid–class content at low pH exposure, suggesting they have depleted their energy reserves. We found that corals from the low upwelling site downregulated stress response genes at low pH exposure, a common response when organisms are under stress. Overall, the low upwelling population exhibited evidence of exhibiting a stress response under future ocean acidification. Overall these population-specific responses highlight the importance of multi-population studies for predicting a species’ response to future OA.

In chapter 2, I demonstrated how a species with a broad geographic range may have contrasting responses to future climate change stressors as a result of their environmental histories. As a result, some populations may be more tolerant to future stressors. A follow-up to these results would be to determine if these responses to stress are heritable and whether the population will be able to evolve. However, I am limited to answering these questions with *B. elegans* with their long reproduction time and limited number of offspring produced. Instead, I focused on the eastern oyster, *Crassostrea virginica*, which has a shorter generation time and produces thousands to millions of larvae within a single reproductive event.

In chapter 3, I tested for the presence genetic variation within and among populations to assess their capacity to evolve. I found that the low salinity population was locally adapted to low salinity conditions, whereas the high salinity population was unable to maintain its body size under low salinity. My quantitative breeding design also allowed us to estimate the influence of transgenerational plasticity as a mechanism of tolerant to low salinity. I found that maternal transgenerational plasticity significantly contributed to larval body size in both populations. However, this effect was adaptive in the high salinity population and maladaptive in the low salinity population. Finally, I found genetic variation within both populations for offspring reared at low and high salinity, suggesting that body size is heritable, and oysters have the capacity to evolve at both salinities.

In chapter 3, I was able to identify the presence of genetic variation and that this variation is heritable. A further step to elucidate the mechanisms of tolerance to climate change stressors is identifying which genes or loci are under selection. Experimental evolution studies can be used to identify allele frequency changes pre- and post-selection. In chapter 4, I exposed larvae to a low salinity selection event across three populations that experience contrasting salinity regimes. Using exome capture on 152 genes, I measured changes in allele frequency pre- and post-selection to assess whether genetic variation for survival exists at low salinities within
populations. I observed 90-95% mortality in all crosses and saw changes in allele frequencies in at least one cross per population. I concluded that variation for low salinity tolerance is present in all populations, but not all individuals (as evidenced by some crosses having no alleles segregating for at low salinity). In addition, I can conclude that low salinity tolerance polygenic and potentially redundant.

*C. virginica* is an example of genetic variation for tolerance to a particular stressor that exists within populations. However, species that have limited dispersal and become isolated have lost variation within populations. For example, previous work suggests that individual populations of *Tigriopus californicus* are unable to increase their heat tolerance to keep pace with the rapidly warming climate (Kelly et al., 2012). However, there is ample genetic variation for heat tolerance at the species level of the copepods’ broad geographic range extending down the west coast from Washington to Baja California. In chapter 5, I hybridized two populations of *T. californicus* with divergent heat tolerances and subjected subsequent generations to strong heat selection. I successfully increased the heat tolerance in selected lines and observed repeatable changes in allele frequencies towards higher frequencies of alleles from the southern (heat tolerant) population. All 12 chromosomes contained loci with strong changes in allele frequencies across all independent selected lines, providing evidence that heat tolerance is a highly polygenic trait.

Overall, I demonstrate the adaptive capacity to evolve in multiple species of marine invertebrates in response to multiple climate change stressors. While further research will be needed to determine the extent of evolution that can occur in the wild, an important first step is identifying which populations exhibit increased tolerance to future stressors. Populations that harbor genetic variation for tolerance to climate change stressors should be prioritized for conservation efforts.
Appendix A.
Supplementary Information for Chapter 2

Figure A.1. Venn diagrams of the number of shared DEG from Table A.2. (A) Population comparisons of shared DEG across pH treatment by day interactions. (B) pH treatment comparisons of shared DEG across population by day interactions.
Figure A.2. Molecular function GO categories for genes significantly enriched at low pH either upregulated (red) or downregulated (blue). (A) HU day 9. (B) HU day 29. (C) LU day 9. (D) LU day 29. The dendrogram on the side is a hierarchical clustering of the GO categories based on shared genes. The fractions correspond to the number of genes that exceeded the unadjusted $p < 0.05$ over the total number of genes in that GO category.
Figure A.3. WGCNA module correlations among coral traits and eigengene expression. The two numbers reported in each box are the Pearson’s correlation coefficient and p-value of the correlation test. Red indicates a positive correlation and blue a negative correlation.
Table A.1. Physical parameters of experimental seawater were verified by measuring temperature, salinity, pH, and total alkalinity (TA). Temperature and total pH was checked in the jars daily using a conductivity meter (YSI 60) and calibrated daily with the OAEL Durafet probes. Total pH was measured in the OAEL coolers and jars with corals once a week following standard operating procedure (SOP) 6b (Dickson et al., 2007) using the spectrophotometer pH method with the indicator dye m-cresol purple. TA was measured once a week following SOP 3b (Dickson et al., 2007) using a Metler Titrator. Certified reference materials (Dickson Laboratory) were first titrated prior to experimental samples to ensure the precision and accuracy of TA measurements. Salinity was measured with a conductivity probe at time of pH and TA measurements. These physical parameters were entered into CO₂CALC (Robbins et al., 2010) with carbonic acid dissociation constants from (Mehrbach et al., 1973) to estimate pCO₂, Ω_{aragonite}, and Ω_{calcite}. LU: Low Upwelling population; HU: High Upwelling population

<table>
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<tr>
<th>Parameters</th>
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<th></th>
<th><strong>High pH</strong></th>
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<td>HU</td>
<td>Ave</td>
<td>LU</td>
<td>HU</td>
<td>Ave</td>
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<td>13.0</td>
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<td>2074.7</td>
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<td>2072.4</td>
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<td>pCO₂(μatm)</td>
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Table A.2. Statistical results from a Tukey’s post-hoc test for respiration rates across treatments (pH 7.4 or 7.8), location (upwelling environment), and days (day 9 and day 29). Significant codes: \( p < 0.1 \sim, \ p < 0.05 \ *, \ p < 0.01 \ **. \)

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<th>P value</th>
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Table A.3. The number of differentially expressed genes (p<0.05) by three-way, two-way, and one-way interactions. The number of these significant DEG that had GO terms are also presented. Since DESeq2 is primarily a pair-wise comparison test, we performed multiple comparisons to test for interactions among various combinations of time points, populations, and treatments.

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<th># genes with GO terms (p&lt;0.05)</th>
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<td>3</td>
</tr>
<tr>
<td>All LU</td>
<td>Treatment*tp</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>All HU</td>
<td>Treatment*tp</td>
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<tr>
<td>All tp1</td>
<td>Pop*tp</td>
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<td>1</td>
</tr>
<tr>
<td>All tp2</td>
<td>Pop*tp</td>
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</tr>
<tr>
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<tr>
<td>All high pH</td>
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<td>0</td>
</tr>
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<td>Treatment</td>
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<tr>
<td>Tp 2 LU</td>
<td>Treatment</td>
<td>183</td>
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<tr>
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<td>Treatment</td>
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<tr>
<td>Tp 2 HU</td>
<td>Treatment</td>
<td>99</td>
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<tr>
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<td>Population</td>
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<tr>
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<td>Population</td>
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Appendix B.
Supplementary Information for Chapter 3

Table B.1. Statistical results from a three-way ANOVA using all data to compare the influence of dam and sire acclimation salinity and F2 rearing salinity on larval survival. Significant codes: p <0.1 ~, p < 0.05 *, p < 0.01 **.

<table>
<thead>
<tr>
<th>Effect</th>
<th>F</th>
<th>DF</th>
<th>DF.Res</th>
<th>p</th>
</tr>
</thead>
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<td>SireAcclim</td>
<td>0.637</td>
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<tr>
<td>DamAcclim</td>
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<tr>
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<td>SireAcclim*DamAcclim</td>
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<td>29.298</td>
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Table B.2. Statistical results from a three-way ANOVA using all data to compare the influence of dam and sire acclimation salinity and F2 rearing salinity on larval size. Significant codes: p <0.1 ~, p < 0.05 *, p < 0.01 **.

<table>
<thead>
<tr>
<th>Effect</th>
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<th>DF</th>
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<th>p</th>
</tr>
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Table B.3. Tukey test results comparing larval rearing salinity interactions with dam and sire acclimation conditions on larval body size. Significant codes: p <0.1 ~, p < 0.05 *, p < 0.01 **.

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<td>0.01279579</td>
<td>0.04963017</td>
<td>17.79</td>
<td>0.258</td>
<td>0.9938</td>
</tr>
<tr>
<td>L,H - H,L</td>
<td>-0.0789236</td>
<td>0.04956335</td>
<td>17.67</td>
<td>-1.592</td>
<td>0.4078</td>
</tr>
<tr>
<td>L,H - L,L</td>
<td>0.00816091</td>
<td>0.04963017</td>
<td>17.87</td>
<td>0.164</td>
<td>0.9984</td>
</tr>
<tr>
<td>H,L - L,L</td>
<td>0.08708451</td>
<td>0.0098239</td>
<td>2615.76</td>
<td>8.865</td>
<td>&lt;.0001 **</td>
</tr>
</tbody>
</table>

Table B.4. Statistical results from a two-way ANOVA to compare the influence of population origin and Dam acclimation conditions on egg content (protein, hydrocarbons, triacylglycerols, free fatty acids, sterols, and phospholipids). Significant codes: p <0.1 ~, p < 0.05 *, p < 0.01 **.

<table>
<thead>
<tr>
<th>Egg Content</th>
<th>F</th>
<th>DF</th>
<th>Sum Sq</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DamAcclim</td>
<td>0.1</td>
<td>1</td>
<td>0.03</td>
<td>0.75</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DamAcclim</td>
<td>0.34</td>
<td>1</td>
<td>0.13</td>
<td>0.57</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DamAcclim</td>
<td>0.34</td>
<td>1</td>
<td>0.23</td>
<td>0.56</td>
</tr>
<tr>
<td>Fatty Acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DamAcclim</td>
<td>2.33</td>
<td>1</td>
<td>1.01</td>
<td>0.14</td>
</tr>
<tr>
<td>Sterols</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DamAcclim</td>
<td>0.54</td>
<td>1</td>
<td>0.33</td>
<td>0.47</td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DamAcclim</td>
<td>0.04</td>
<td>1</td>
<td>0.01</td>
<td>0.85</td>
</tr>
</tbody>
</table>
Table B.5. Statistical results from a one-way ANOVA to determine whether crosses that experienced 100% mortality were dependent on egg quality content (protein, hydrocarbons, triacylglycerols, free fatty acids, sterols, and phospholipids).

<table>
<thead>
<tr>
<th>Egg Content</th>
<th>F</th>
<th>DF</th>
<th>Sum Sq</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.16</td>
<td>2</td>
<td>0.09</td>
<td>0.85</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>0.34</td>
<td>2</td>
<td>0.20</td>
<td>0.79</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>0.62</td>
<td>2</td>
<td>1.21</td>
<td>0.56</td>
</tr>
<tr>
<td>Fatty Acids</td>
<td>0.07</td>
<td>2</td>
<td>0.08</td>
<td>0.93</td>
</tr>
<tr>
<td>Sterols</td>
<td>0.65</td>
<td>2</td>
<td>0.79</td>
<td>0.53</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.54</td>
<td>2</td>
<td>0.74</td>
<td>0.59</td>
</tr>
</tbody>
</table>

Table B.6. Statistical results from a one-way ANOVA to determine whether larval size was correlated with egg quality content (protein, hydrocarbons, triacylglycerols, free fatty acids, sterols, and phospholipids).

<table>
<thead>
<tr>
<th>Egg Content</th>
<th>F</th>
<th>DF</th>
<th>DF.res</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>0.28</td>
<td>1</td>
<td>11.6</td>
<td>0.61</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>0.16</td>
<td>1</td>
<td>13.6</td>
<td>0.70</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>0.05</td>
<td>1</td>
<td>13.8</td>
<td>0.83</td>
</tr>
<tr>
<td>Fatty Acids</td>
<td>0.15</td>
<td>1</td>
<td>13.5</td>
<td>0.70</td>
</tr>
<tr>
<td>Sterols</td>
<td>0.005</td>
<td>1</td>
<td>14.6</td>
<td>0.94</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.002</td>
<td>1</td>
<td>12.6</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Table B.7. All variance components (in μm) for larval size estimated from four different animal models. In parentheses are 95% confidence intervals.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>V_A</th>
<th>V_DamAcclim</th>
<th>V_SireAcclim</th>
<th>V_DamOffs</th>
<th>V_SireOffs</th>
<th>V_P</th>
<th>Mean length</th>
<th>Heritability</th>
<th>Maternal effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2 Offspring</td>
<td>1248</td>
<td>140.7</td>
<td>0.9</td>
<td>1.5</td>
<td>19.3</td>
<td>188.6</td>
<td>67.51</td>
<td>73.51</td>
<td>0.68</td>
<td>0.11</td>
</tr>
<tr>
<td>low salinity</td>
<td></td>
<td>(68.6 – 176.0)</td>
<td>(-7.8 – 8.3)</td>
<td>(-3.5 – 8.9)</td>
<td>(4.65 – 82.3)</td>
<td>(145.6 – 245.3)</td>
<td>(0.39 – 0.89)</td>
<td>(0.03 – 0.36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2 Offspring</td>
<td>1379</td>
<td>122.3</td>
<td>5.9</td>
<td>0.76</td>
<td>25.4</td>
<td>187.5</td>
<td>77.63</td>
<td>77.63</td>
<td>0.66</td>
<td>0.16</td>
</tr>
<tr>
<td>high salinity</td>
<td></td>
<td>(65.1 – 180.1)</td>
<td>(-1.1 – 13.9)</td>
<td>(-5.6 – 6.1)</td>
<td>(5.8 – 83.0)</td>
<td>(155.8 – 252.8)</td>
<td>(0.36 – 0.86)</td>
<td>(0.04 – 0.35)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix C.
Supplementary Information for Chapter 4

Figure C.1. Venn diagram displaying the number of genes selected for exome capture design that were highly expressed in *C. virginica* (Jones et al. 2019), displayed high FST among populations of *C. virginica* (Johnson et al. 2019), displayed high polymorphisms among populations of *C. gigas* (She et al. 2018), and finally genes that changed their metabolic and expression pathways in *C. gigas* in response to low salinity (Meng et al. 2013). Some genes appeared in multiple categories.
Figure C.2. Manhattan plot showing crosses that had no significant allele frequency shifts. SNPs were required to have a starting frequency on ~25% pre-selection and then increased in frequency post-selection. P-values are displayed as $-\log$ from CMH test. The blue horizontal line represents our significance threshold after FDR correction. Crosses displayed had no significant genes under selection for the high salinity population (A), mid-salinity population (B-C), and the low salinity population (D-E). The low salinity exposure for each cross is displayed next to cross info.
Figure C.3. Manhattan plot for MS crosses 3 and 4 that were not exposed to low salinity. SNPs were required to have a starting frequency of ~25% pre-selection and then increased in frequency post-selection. P-values are displayed as −log from CMH test. The blue horizontal line represents our significance threshold after FDR correction. Crosses displayed had no significant genes under selection for the MS population cross 3 (A) and 3(B).

Table C.1. Euclidean distances for larval samples pre- and post-selection (or no selection: NS). Distances are derived from each population PcoA (Fig. 3.A-C).

<table>
<thead>
<tr>
<th>Cross</th>
<th>PCoA Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS1</td>
<td>1.0e-04</td>
</tr>
<tr>
<td>LS2</td>
<td>9.5e-05</td>
</tr>
<tr>
<td>LS3</td>
<td>4.8e-04</td>
</tr>
<tr>
<td>MS1</td>
<td>8.4e-05</td>
</tr>
<tr>
<td>MS2</td>
<td>3.9e-05</td>
</tr>
<tr>
<td>MS3</td>
<td>5.8e-05</td>
</tr>
<tr>
<td>MS3-NS</td>
<td>4.6e-05</td>
</tr>
<tr>
<td>MS4</td>
<td>1.4e-04</td>
</tr>
<tr>
<td>MS4-NS</td>
<td>6.6e-05</td>
</tr>
<tr>
<td>HS1</td>
<td>6.0e-05</td>
</tr>
<tr>
<td>HS2</td>
<td>1.1e-04</td>
</tr>
<tr>
<td>HS3</td>
<td>6.8e-05</td>
</tr>
<tr>
<td>HS4</td>
<td>3.0e-04</td>
</tr>
</tbody>
</table>
Table C.2. For crosses that had genes under selection, we performed a Chi-Square test (p-value displayed) to determine if the genes were enriched in a source or functional category.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Source category (p-value)</th>
<th>Functional category (p-value)</th>
<th>SNP location (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS3</td>
<td>0.33</td>
<td>0.96</td>
<td>2.2e-05 *</td>
</tr>
<tr>
<td>MS1</td>
<td>0.64</td>
<td>0.72</td>
<td>0.40</td>
</tr>
<tr>
<td>MS2</td>
<td>0.65</td>
<td>0.93</td>
<td>0.08</td>
</tr>
<tr>
<td>HS1</td>
<td>0.55</td>
<td>0.30</td>
<td>0.33</td>
</tr>
<tr>
<td>HS3</td>
<td>0.50</td>
<td>0.84</td>
<td>0.32</td>
</tr>
<tr>
<td>HS4</td>
<td>0.67</td>
<td>0.38</td>
<td>0.56</td>
</tr>
</tbody>
</table>
Appendix D.
Supplementary Information for Chapter 5

Figure D.1. Median lethal temperatures (LT50) for males from hybrid lines selected for heat tolerance (Selected 1-5) and control lines 1-5. Parental copepods from the pure Bodega and San Diego population are displayed as well as the F1 hybrid generation crossed as either Bodega-San Diego or San Diego-Bodega (maternal-paternal respectively).
Figure D.2. FST for each control line 1-5 (A-E, respectively) compared to the pure Bodega population. Windows colored green on FST plots are loci that were above our Bonferroni correction from Fig. 5.2.A.
Table D.1. We re-analyzed transcriptomic data from Kelly et al. (27) using the newly available genome to determine the number of differentially expressed genes after 4 generations of heat selection. Displayed is the number of DEGs from mapping to the newly available genome (56) using the DESeq2 package compared to the number of DEGs from de novo mapping using the Limma package in the Kelly et al. (27) methods. Unique DEGs from all the comparisons were used to determine overlap with genes within windows under heat tolerance selection. The p-value for the Fisher's exact test (FET) is displayed in parentheses to test for enrichment for each DEG category.

<table>
<thead>
<tr>
<th>Input</th>
<th>Formula</th>
<th>Genome mapping &amp; DeSeq2: DEG</th>
<th>Kelly et al. (pop mapping &amp; Limma): DEG</th>
<th># windows under selection that overlap with DEG (FET p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>Line*Heat shock</td>
<td>333</td>
<td>-</td>
<td>5 (p=0.46)</td>
</tr>
<tr>
<td>All</td>
<td>Heat shock</td>
<td>591</td>
<td>506</td>
<td>27 (p=0.003) *</td>
</tr>
<tr>
<td>All</td>
<td>Line</td>
<td>30</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Heat shock</td>
<td>Line</td>
<td>184</td>
<td>0</td>
<td>4 (p=1)</td>
</tr>
<tr>
<td>No heat shock</td>
<td>Line</td>
<td>148</td>
<td>-</td>
<td>4 (p=0.78)</td>
</tr>
<tr>
<td>Selected line</td>
<td>Heat shock</td>
<td>117</td>
<td>93</td>
<td>5 (p=0.21)</td>
</tr>
<tr>
<td>Control line</td>
<td>Heat shock</td>
<td>1981</td>
<td>462</td>
<td>50 (p=0.70)</td>
</tr>
</tbody>
</table>
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Vita

Joanna Sarah Griffiths was born in Croydon, England (UK) to Steve and Julie Griffiths. She moved back and forth between the UK and the US for much of her childhood before settling down in Portland, Oregon during Middle School. Her love for the outdoors began in the Pacific Northwest in the mountains and at the beach. Her interest in biology stemmed from numerous visits to the Oregon Zoo, which holds a special place in her heart. She volunteered at the Zoo all throughout high school and received the opportunity to work with the veterinarians behind the scenes working with the polar bears. It was this encounter that brought her attention to climate change issues and their affects not only on polar bears but other marine organisms as well. Joanna received her Bachelor’s in Science from Pacific Lutheran University with a major in Biology and minor in Geosciences. She received a research opportunity during her undergraduate work at LUMCON to work on the effects of the BP oil spill on blue crabs and Gulf fish. This opportunity confirmed her interest in continuing her education and she began her doctoral work with Dr. Morgan Kelly at Louisiana State university in August 2014. She anticipates graduating from LSU in May 2020 and beginning postgraduate work at UC Davis.