AN ABSTRACT OF THE DISSERTATION OF

Evan R. Durland for the degree of Doctor of Philosophy in Fisheries Science presented on May 14, 2019.

Title: The Genetics of Larval Fitness in the Pacific Oyster: Responses to Acidified Seawater and Temporally Dynamic Selection Processes.

Abstract approved:

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The Pacific Oyster (*Crassostrea gigas*) is one of the most economically and ecologically significant shellfish species worldwide. In the Pacific Northwest United States (PNW), the sustainability oyster stocks is increasingly threatened by ocean acidification (OA), which has had significant negative effects on the aquaculture industry in this region over the last decade. Currently, little is known with regards to stock-based differences in larval fitness for PNW populations of *C. gigas* in ambient or high pCO_2 conditions. Furthermore, no studies have been performed that evaluate the genetic consequences of larval rearing in acidified seawater for Pacific oysters. Here I examined genetic components of larval fitness traits, both in ambient seawater as well as simulated OA conditions. In Chapter 2 my co-authors and I conducted two experiments to compare the relative fitness of larvae from selectively bred aquaculture stocks to those spawned from a naturalized source of broodstock in Willapa Bay, WA. We reared genetically diverse pools of larvae from each group in ambient (~400 μatm) and high (~1600 μatm) pCO_2 seawater for 22-24 days from

fertilization through settlement to juvenile stage. Overall, we found that the impacts of high pCO_2 seawater on larval phenotypes were heterogeneous across larval developmental stages and variable between the two experiments. Nevertheless, larvae from selectively bred lines had consistently higher survival and greater developmental success through settlement than those from naturalized stocks across both conditions and experiments. In Chapter 3 we analyzed the overall changes in genetic composition of each larval pool created in the first experiment. We found an abundance of loci with significantly distorted allele frequencies across larval development, nearly all of which were specific to each broodstock type. There were additional genetic changes owing to high pCO_2 culture that, also, were largely unique to each parental group. Overall, larvae from selectively bred stocks had significantly less genetic change than those from naturalized populations, both for general larval development as well as survival in acidified seawater. We performed functional enrichment analyses on genes associated with distorted loci and found that those with altered allele frequencies in acidified seawater were significantly over-represented by gene-ontology categories concerning membrane structure and function. This finding is consistent with previous studies which also highlighted this aspect of larval physiology as a critical component for survival in acidified conditions. The reduced genetic change in larvae from selected lines suggests that multiple generations of hatchery culture has had a domesticating effect on the genotypes of selectively bred oyster stocks. These results also support the phenotypic results from Chapter 2 which suggested that general improvements to larval fitness have crossover benefits for environmental stressors like OA. In Chapter 4 we further investigated the dynamic

genetic changes taking place in larval populations during development, absent any overt environmental stress. By analyzing temporal patterns of changes in allele frequencies across development, we observed that genetic changes were highly dynamic, with more than a quarter that displayed significant shifts in allele frequencies changing in direction across developmental transitions. Importantly, this resulted in the detection of substantially more genetic changes taking place than were indicated from overall analyses from 'start' and 'end' points in larval culture examined in Chapter 3. These findings represent a novel and unparalleled analysis of the genetic impacts of genotype-dependent mortality in oysters. The patterns of viability selection that we describe likely have extensive consequences on the genetic diversity of this species, and its long term capacity for adaptation.

The Genetics of Larval Fitness in the Pacific Oyster: Responses to Acidified Seawater and Temporally Dynamic Selection Processes.

by
Evan R. Durland

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Evan R. Durland, Author

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CONTRIBUTION OF AUTHORS

Dr. George Waldbusser, Dr. Chris Langdon and Dr. Eli Meyer conceived the project under which I conducted the research described in Chapters 2 and 3. Dr. Waldbusser contributed to the analysis and interpretation of results from Chapter 2. Dr. Pierre De Wit provided me with hands-on training for DNA extraction and library preparation for the samples used in Chapters 3 and 4. Dr. Meyer and Dr. De Wit provided assistance adapting bioinformatic pipelines to analyze pooled DNA samples and helped design a statistical framework to analyze results used in Chapters 3 and 4. Dr. Brett Dumbauld contributed valuable context for Willapa bay oyster populations and provided editorial feedback on the dissertation. Dr. Michael Banks was an invaluable sounding board for ideas and concepts of the 'bigger picture' for some of the themes of my dissertational research and helped me to expand the scope of my discussions beyond the narrow three weeks of oyster development. Dr. Chris Langdon was my primary advisor during all stages of this research. He assisted in the development, execution, analysis, interpretation and editing of this dissertation as a whole.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

DEDICATION

In honor of my grandfather Newton Durland. He taught our family a love of the water and boats, and instilled in me an endless curiosity for all things aquatic.

CHAPTER 1 GENERAL INTRODUCTION

1. Background

The Pacific Oyster (*Crassostrea gigas*) is the most abundantly farmed shellfish species in the Pacific Northwest (PNW) aquaculture industry, with harvests contributing >$86 million to local economies (USDA 2013). This species is native to Japan but was imported to the PNW at the beginning of the 20th century to support the oyster industry that had struggled from the loss of the native Olympia oyster (*Ostrea lurida*) after decades of over-harvesting and habitat mismanagement (White et al. 2009). After a prolonged period of importation of juvenile oysters from Japan (termed 'spat' or 'seed'), stocks of *C. gigas* established naturalized populations in isolated bays and estuaries of Washington State in the 1920s to 1930s (Steele 1964). Naturally recruited spat, from both domestic and Japanese sources, continued to supply the oyster industry in the PNW with juveniles for farming operations up until the 1970s when hatchery operations were developed to supplement growers with a more stable supply of seed (Clark & Langmo 1979).

Hatchery production of oyster seed has greatly increased the productivity of oyster aquaculture on the West coast by offering a consistent supply of seed that can be induced to settle on substrate (termed 'set') and grown at farm sites very distant from natural spawning grounds or the hatchery that produced them (Henderson 1983). A secondary benefit of hatchery technologies is the so-called 'closing of the life cycle', which allows for control of all stages of production of oysters across generations, permitting the use of breeding to enhance performance traits. In 1996, the USDA funded Molluscan Broodstock Program (MBP) was initiated at Oregon

State University in order to conduct a selective breeding program for Pacific oysters farmed on the West Coast of the United States (De Melo et al. 2016). For more than 20 years this program has worked collaboratively with the shellfish industry to enhance the growth and survival of juveniles and adults at farm sites across the West Coast. At the outset, MBP's breeding program was founded from numerous naturalized stocks in the PNW from Washington State and British Columbia. From these founder populations, MBP has executed a family-based breeding design which selects top-performing full-sibling families for future generations while simultaneously maintaining genetic diversity through the use of a pedigree and constraining inbreeding accumulation. MBP is currently in its 7^{th} generation of selection and coordinates a large scale transfer of improved germplasm to industry partners who use them extensively in commercial production.

While the shellfish industry has expanded production over the past 20 years, recent shifts in seawater chemistry have presented a new set of challenges for oysters and oyster farmers alike. Rising atmospheric CO_2 levels, spurred by anthropogenic activities, are tempered by oceanic uptake, which accounts for around a third of the carbon added to the atmosphere through fossil fuel consumption and deforestation (Sabine et al. 2004). The addition of atmospheric carbon into the oceans reduces the pH of seawater and alters the carbonate chemistry, a process known as ocean acidification (OA). The negative effects of OA on the growth and survival of marine bivalve larvae has been well studied in the past decade (e.g. Kurihara et al. 2007, Waldbusser et al. 2013, Gray et al. 2017). Low pH and reduced saturation state of aragonite (Ω_{arag}) that are associated with OA, present a thermodynamic barrier to

shell synthesis (Waldbusser et al. 2015a) and impair larval development (Timmins-Schiffman et al. 2012, Waldbusser et al. 2015b).

OA presents an imminent threat to many marine taxa across much of the globe but, in the PNW, it is already significantly altering coastal ecosystems (Feely et al. 2016). In this region, seasonal upwelling events (May-October) bring deep water onto the continental shelf which is naturally low in pH, owing to microbial respiration, but which has become more strongly acidified in recent years due to OA (Feely et al. 2004). These increasingly frequent and severe periods of upwelling have negatively impacted oyster populations in the region over the last decade (Barton et al. 2012, Hales et al. 2017). In response to these changes, commercial oyster hatcheries in the PNW now routinely buffer incoming seawater to increase pH and Ω_{arag} to sustain larval growth and survival. What remains less well understood is how OA affects naturalized oyster populations in the PNW. It is difficult to establish a correlation between the stochastic spawning and settlement trends of Pacific oysters in these habitats and the dynamic effects of upwelling and OA (Weisberg et al. 2016, Ruesink et al. 2017). Overall, recruitment for these stocks has been highly variable but relatively low for the past 15 years (Dumbauld et al. 2011) and acidified seawater environments are likely to be one of the factors contributing to this trend (Hales et al. 2017).

Extensive research effort has been devoted to investigating the physiological impacts of acidified seawater on oyster larvae, but relatively few studies have evaluated the nature of the variability in fitness responses. Sensitivity to acidified seawater appears to be a trait in marine calcifying larvae that is genetically variable

(Sunday et al. 2011, Frieder et al. 2016), trans-generationally plastic (Parker et al. 2015, Thor & Dupont 2015) and is not constant across populations of the same species (Parker et al. 2010). The long term adaptive consequences of ocean acidification in marine communities is still somewhat uncertain (Kelly & Hofmann 2013, Reusch 2014, Sunday et al. 2014), but variation for resilience phenotypes to OA which exists in current populations suggests that evolutionary forces may potentially drive shifts in fitness optima in the future for numerous taxa (Gaylord et al. 2015).

Whether and how OA has affected the genetic composition of domesticated and naturalized oyster populations in the PNW is unknown, but recent anecdotal evidence from commercial hatcheries suggest that larvae from MBP perform better than larvae spawned from naturalized stocks during periods of heavy upwelling. This is surprising for two reasons, first, over the past two decades MBP has bred exclusively for adult traits in field environments (growth, survival) and no intentional selection pressure was applied to larval performance traits. Secondly, from the standpoint of adaptation, the greater genetic diversity in wild stocks and heightened exposure to acidified seawater in natural settings should theoretically advantage them to accumulate genes conferring fitness benefits in OA environments faster than in MBP. Observed larval fitness advantages in MBP lines, anecdotal though they may be, raise interesting questions regarding the relative performance of larvae from selectively bred stocks and their recently related counterparts in naturalized populations in both ambient and acidified seawater. We evaluated these fitness differences across larval development in two replicated experiments in Chapter 2. In

order to investigate the genetic components of survival in high pCO_2 seawater, we also analyzed changes in allele frequencies for ~1200 genome-wide molecular markers in MBP and wild larvae at the beginning and end of larval culture. We compared overall patterns of genetic change in these groups as well as changes specific to acidified seawater (Chapter 3).

In Chapter 4, we more comprehensively analyzed the pronounced genetic changes taking place during larval oyster development. Prior research has indicated that a high mutation rate in oysters renders a vast majority of all larvae spawned 'genetically inviable' (Plough et al. 2016) and largely accounts for the low survivorship to spat stage, independent of environmental stress. Very few studies have evaluated the stage-specific genetic changes taking place in larval populations and all prior examples suffer from a paucity of markers that were able to be assessed in temporal analyses. The results from Chapter 4 represent a novel analysis, which evaluates the allele frequencies of n = 847 markers at numerous time points during development and indicates that temporally contrasting selection pressures may have a balancing effect on allele frequency for more than ~25% of markers analyzed. This finding carries broad implications for the adaptive capacity of oysters and how high rates of mutation are seemingly persistent in large outbred populations.

2. Purpose of Chapter 2

Chapter 2 is entitled: "Comparison of larval development in domesticated and naturalized stocks of the Pacific oyster *Crassostrea gigas* exposed to high pCO_2

conditions." The purposes of this chapter were to 1) evaluate larvae from MBP lines and 'wild' stocks from Willapa bay, WA reared in 'control' (ambient) conditions as well as simulated 'OA' (high pCO_2) conditions, and 2) to evaluate the phenotypic impacts of acidified seawater across all stages of larval development, from embryogenesis to settlement.

3. Purpose of Chapter 3

Chapter 3 is entitled: "Comparing the genetic effects of larval development in acidified seawater for wild and selectively bred stocks of the Pacific oyster on the West coast United States." In this chapter we analyzed the genetic changes in larval groups used for the first experiment in chapter 2. By comparing genetic composition in larvae at the start of development (day 2) to the same cultures after settlement (day 22) we evaluated changes specific to broodstock type (MBP or wild) as well as changes attributable to acidified seawater. Furthermore, we conducted functional enrichment analyses on the genes associated with significantly shifted loci to putatively identify physiological functions that are being selected for in acidified seawater.

4. Purpose of Chapter 4

Chapter 4 is entitled: "Patterns of genetic change and temporally balanced polymorphisms during larval development of the Pacific oyster (*Crassostrea gigas*) inferred from pooled DNA analyses." In this chapter we more comprehensively

evaluate the dynamic patterns of genetic change taking place during larval oyster development. We compare the complexity of changes we observed through a set of pooled DNA samples collected at separate time points to previous studies which evaluated overall, or 'final', results of larval development and discuss the potential inferences these results have for adaptation and population genetics of oysters.

5. Purpose of Chapter 5

In chapter 5 "General Discussion" I summarize the major themes that the research for my dissertation has addressed including 1) The stage-specific and variable effects of OA on fitness traits in larval oysters, 2) The disparate genetic effects of larval development in MBP and wild oyster stocks, 3) The genetic components of larval survival in high pCO_2 seawater, and, 4) The highly dynamic patterns of genetic selection during larval oyster development.

6. Summary

The overall aim of my dissertation is to investigate the genetic components of larval fitness in oysters. Oysters are an important species in many marine environments and are innately sensitive to changes to their environment. The long term impacts of Ocean acidification on marine bivalves is uncertain, but both natural and farmed oyster populations in the PNW will be impacted from increasingly acidified seawater. Oysters, however, are a very plastic and adaptive species and they have been able to successfully inhabit a range of volatile environments. If we can

better understand how selective pressures shape the potential of oysters to adapt to myriad effects of climate change, it will help us to better forecast the expected future of the imperiled oyster industry and, potentially, offer some genetic tools to help offset some of the negative impacts of ocean acidification.

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COMPARISON OF LARVAL DEVELOPMENT IN DOMESTICATED AND NATURALIZED STOCKS OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS* EXPOSED TO HIGH pCO_2 CONDITIONS.

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Abstract:

Ocean acidification (OA) has had significant negative effects on oyster populations on the West Coast of North America over the past decade. Many studies have focused on the physiological challenges experienced by young oyster larvae in high pCO_2/low pH seawater with reduced aragonite saturation state (Ω_{arag}) which is characteristic of OA. Relatively few, by contrast, have evaluated these impacts upon fitness traits across multiple larval stages and between discrete oyster populations. In this study we conducted two replicated experiments, in 2015 and 2016, using larvae from naturalized 'wild' and selectively bred stocks of the Pacific oyster (*Crassostrea gigas*) from the Pacific Northwest USA and reared them in ambient (~400 μatm) or high (~1600 μatm) pCO_2 seawater from fertilization through final metamorphosis to juvenile 'spat'. In each year, high pCO_2 seawater inhibited early larval development and affected the timing, but not the magnitude, of mortality during this stage. The effects of acidified seawater on metamorphosis of pediveligers to spat was variable between years, with no effect of seawater pCO_2 in one experiment but a ~42%

reduction in spat in the second. Despite this variability, larvae from selectively bred oysters produced, on average, more (+ 55% and 37%) and larger (+ 5% and 23%) spat in ambient and high pCO_2 seawater, respectively. These findings highlight the variable and stage-specific sensitivity of larval oysters to acidified seawater and the influence that genetic factors have in determining the larval performance of *C. gigas* exposed to high pCO_2 seawater.

KEY WORDS: Ocean acidification, Pacific oyster, *Crassostrea gigas*, Larval development, Genetics, Domestication

1. Introduction

1.1. Effects of ocean acidification on bivalve larval fitness

The effects of ocean acidification (OA) upon marine organisms have been widely studied by biologists (Kroeker et al. 2013), ecologists (Miller et al. 2009, Gaylord et al. 2015) and aquaculturists (Barton et al. 2012, 2015) over the past decade. While OA conditions have diverse impacts on marine ecosystems, marine bivalves are particularly vulnerable to shifts in seawater carbonate chemistry, especially during embryogenesis when the initial larval shell is being formed (prodissoconch I or PDI stage) and the sites of calcification are exposed to ambient seawater conditions (Waldbusser et al. 2013). Reduced seawater aragonite saturation (Ω_{arag}), which is a typical consequence of OA conditions, leads to an increase in morphological abnormalities of PDI or "D-hinge" veliger larvae at ~24-48 hours post fertilization (hpf) as well as smaller normally developed larvae (Kurihara et al. 2007, Waldbusser et al. 2015a). In addition to impeding calcification, the multi-stressor environment of OA conditions (low pH/ high pCO_2/ reduced Ω_{arag}) has diverse effects upon larval physiology and metabolism (Dineshram et al. 2012, Waldbusser et al. 2015b), many of which may be difficult to assess (Frieder et al. 2016). Oyster larval development is also complex; it encompasses two distinct metamorphic transitions, is impacted by numerous water quality parameters (Portner 2010, Przeslawski et al. 2015), and fitness traits are prone to variation based on maternal/egg quality (Gallager et al. 1986, Myrina et al. 2015) and nutritional aspects of the larval culture (Kheder et al. 2010, Marshall et al. 2010). While much attention has been paid to early development and shell formation in bivalve larvae, especially with regards to

bioenergetics, calcification and egg lipids (Waldbusser et al. 2013, Frieder et al. 2016), the impacts of OA conditions across the entire larval period are important to consider in order to expand the scope of inference from discrete physiological impacts to overall population survival and fitness (Gobler & Talmage 2013, Brunner et al. 2016).

1.2. Impacts of OA on West coast oyster stocks

In the Pacific Northwest of the United States (PNW), Pacific oysters (*Crassostrea gigas*) were established from numerous importations of Japanese founder stocks in the early 20th century. They have successfully established self-recruiting naturalized populations in several bays of Washington State that are frequently exposed to upwelled seawater during summer months (May-September). These upwelling events bring deep water onto the continental shelf which is naturally low in pH, owing to microbial respiration, and which has become more strongly acidified in recent years due to OA (Feely et al. 2004) and affects seawater chemistry of coastal bays in the PNW (Feely et al. 2008, Feely et al. 2016). It is difficult to establish a correlation between the inherently stochastic spawning and settlement trends of Pacific oysters in these habitats and the dynamic effects of upwelling and OA (Weisberg et al. 2016, Ruesink et al. 2017) but overall recruitment for these stocks has been in decline for the past 30 years (Dumbauld et al. 2011) and increasingly acidified seawater environments are likely to be one of the factors contributing to this trend (Hales et al. 2017).

Notably, the same upwelling events that affect naturalized Pacific oyster populations have also had severe impacts on shellfish hatcheries operating in this region which have experienced significantly reduced rates of larval growth and survival during upwelling events in past decade (Barton et al. 2012). In response to these challenges, commercial hatcheries in this region now measure seawater carbonate chemistry, strategically time larval production cycles, chemically buffer incoming seawater in order to maintain optimal carbonate chemistry conditions and are implementing breeding programs to produce shellfish stocks that may have greater resistance to OA effects (Barton et al. 2015). The potential for breeding to improve OA specific larval fitness traits remains uncertain but growth and survival of larvae in high pCO_2 seawater appear to be fitness traits which are partially genotype dependent, displaying significant variation within bivalve populations (Sunday et al. 2011, Frieder et al. 2016) and between them (Parker et al. 2010, Wright et al. 2014). The variable response of bivalve larvae to acidified conditions is also consistent with broader phenotypic plasticity and adaptive capacity to OA observed in other marine invertebrates (Kelly & Hofmann 2013, Sunday et al. 2014, Thor & Dupont 2015).

1.3. Oyster breeding for improved fitness

Since 1996, the Molluscan Broodstock Program (MBP) at Oregon State University has conducted a selective breeding program for Pacific oysters farmed on the West Coast of the United States (De Melo et al. 2016). The commercial shellfish industry has collaborated with MBP to enhance commercially valuable field traits (growth and survival of juveniles and adults at farm sites) while maintaining genetic

diversity and limiting inbreeding accumulation in the stocks through family-based structured mating designs using bi-parental crosses. To date, there has been no selection directed upon any larval performance traits, but six generations of rearing larval cohorts (defined here as a group of separate or mixed families reared simultaneously) in hatchery environments may have resulted in unintentional selection that improves mean larval growth and survival under hatchery conditions. Additionally, larvae from most of these MBP cohorts were cultured during summer periods with likely, but unmeasured, upwelling events and may have acquired additional traits which benefitted their survival in high pCO_2 seawater. Interestingly, over the past ~10 years, commercial shellfish hatcheries that partner with MBP have reported that during periods of intense upwelling, larvae spawned from MBP broodstock survive and grow better when compared to larvae spawned from naturalized populations (Sue Cudd; personal communication). Little is known about stock-based sensitivity of Pacific oysters in the PNW to the effects of OA and anecdotal evidence that selected lines exhibit increased larval fitness in acidified conditions is surprising given that any such gain in MBP stocks would be incidental. Furthermore, naturalized oysters, which possess substantially larger populations and greater genetic diversity (Camara 2011, Sun & Hedgecock 2017) and are exposed to upwelled, low Ω_{arag} conditions more frequently (Hales et al. 2017), should be theoretically favored to more rapidly acquire beneficial adaptations to these stressors.

More directly, any realized differences in larval fitness between selectively bred, or ‘domesticated’, lines of oysters and naturalized stocks has important implications for the sustainability of the shellfish industry in the PNW along with

potential genetic consequences for naturalized stocks of *C. gigas* in the region. Improved resilience of domesticated oyster larvae to acidified conditions would not only be a commercially valuable trait but could also potentially alter population dynamics between domesticated oysters and naturally occurring populations which are located close to aquaculture operations and frequently interbreed (Sun & Hedgecock 2017). Differences in larval fitness between the stocks, especially for performance in high pCO_2 conditions, could possibly augment the rate of gene flow from hatchery reared, domesticated lines to established natural stocks and have unknown long term implications for these populations.

In this study we aimed to compare the resilience of larvae from selected MBP broodstock with that of oysters collected from Willapa Bay (the most abundant local source of naturalized *C. gigas*) when cultured in both ambient and high pCO_2 seawater. The structured pedigree of MBP oyster families (De Melo et al. 2016) allows for the creation of genetically diverse pools of larvae for experimentation that are not compromised by inbreeding and are similar to those used by commercial hatcheries. Additionally, access to pedigreed oyster families allows the use of similar genetic pools across multiple experiments, enabling a level of comparison unavailable to other studies that use broodstock of unknown genetic composition. Utilizing this resource, we conducted two long term (22-24 day) experiments across two years in which we compared the performance of larvae from pools of mixed MBP families to those of larvae from Willapa stocks (of unknown genetic structure) to determine stage-specific sensitivities in ambient (~400 μatm) and high (~1600 μatm) pCO_2 conditions.

2. Materials and Methods

2.1. Broodstock selection and conditioning

For the first experiment, conducted in 2015, full sibling oyster families from MBP's fifth generation cohorts were ranked based on farm yields (De Melo et al. 2016) and the top 20 performing families were selected for spawning. In April 2014, naturalized oysters were obtained from the Naselle region of Willapa bay, WA (46°25'15.2" N, 123°51'47.6" W) and transported to the Hatfield Marine Science Center (HMSC), OR where all MBP breeding stocks are maintained in the Yaquina Bay (44°37'27.6" N, 124°02'35.2" W). In late April 2015, n~20 broodstock from each MBP family, (~400 total) and Willapa broodstock (~60 total) were transferred from the broodstock repository to conditioning tanks (approx. 50 liters, n = 12) to facilitate gonadal development. Oysters in conditioning tanks were provided with seawater (~2 liters $minute^{-1}$ flow-through, from a common head tank) and fed on a microalgal diet of 50/50 (by cell concentration) of *Isochrysis galbana* (C-iso) and *Chaetoceros gracilis* at approximately 20-30,000 cells ml^{-1}. Seawater was filtered to ~10 μm and buffered with sodium carbonate to maintain a pH ~8.0 for the duration of the conditioning period. After a three day acclimation period in ambient conditions (11 ^{o}C), water temperature was increased by one degree Celsius per day until it reached 20 ^{o}C, where it was maintained for 25 days until broodstock were removed for spawning (see below). For the second experiment, in 2016, no Willapa oysters remained from the previous year's supply; therefore, new adult oysters were collected from the intertidal rocks at Stony Point in Willapa bay (46°40'21.3" N, 123°55'31.4" W). Recent analyses by Sun and Hedgecock (2017) suggest that there is little genetic

differentiation between naturalized *C. gigas* stocks across a broad range of PNW environments so we do not expect that these broodstock were substantially genetically distinct from those used in 2015. In April 2016, n ~60 adult oysters were collected from this site and acclimated in seawater holding troughs at HMSC for four weeks. In May of 2016, MBP oysters from each of the same families used previously (n ~20 each, ~400 total) were obtained from the broodstock repository and were transferred to conditioning tanks along with acclimated Willapa broodstock. After this point, conditioning protocols for the 2016 experiment were identical to those of the prior year.

2.2. Cross design and spawning:

For each broodstock group, in each year, 95 single pair matings (1♀ x 1♂) were created from available parents. For MBP, families were crossed in a semi-factorial fashion: one male and one female oyster from each family were individually paired with 4-6 individuals (of opposite gender) from other MBP families with a low coefficient of co-ancestry (<10%) with no reciprocation. Crosses were conducted in this way with 19 and 16 MBP families in 2015 and 2016, respectively. Oysters from Willapa bay in both years exhibited a heavily skewed sex ratio (~10:1 female to male) and thus 95 crosses were made from 19 females and 5 males in each year with a fully crossed mating design (every male paired with every female).

Crosses were performed by stripping ripe gametes from each male and female, and suspending them in beakers filled with seawater. Eggs from each female were enumerated (see 'larval sampling and measurement' below) and divided into five

replicate beakers for fertilization. Each replicate beaker was independently fertilized with appropriate aliquots of sperm suspension from different males. After one hour, each cross was checked for fertilization by sampling eggs and visually confirming polar bodies or cell division. Eggs were then rinsed of excess sperm on a 25 μm screen and proportionally combined to form two composite larval pools (one each for MBP and Willapa) that contained approximately equal quantities of fertilized eggs from each of the 95 crosses. Pooling larvae in this way - with approximately equal input from each of 95 crosses and ~19 females - spread the effects of individual parental contributions to larval fitness (maternal, genetic or both) to an aggregate group. This method should provide a better representation of 'mean' fitness of these two populations than could be practically obtained by rearing a much more limited number of families separately.

2.3. Seawater manipulations and sampling:

Ambient seawater (~400 μatm CO_2, pH = 7.9-8.1 , Ω_{arag} = 2.3-2.7) was created by filling a 200 liter tank with standard hatchery seawater (25 ^{o}C, 32ppt , 10 μm-filtered) and equilibrating it overnight via vigorous aeration with outside air. High pCO_2 treatment water (~1600 μatm CO_2, pH ~ 7.5-7.6, Ω_{arag} = 0.9-1.2) was created by filling an identical tank with hatchery seawater and vigorously aerating it for several hours with a gas mixture of CO_2-stripped air and pure CO_2 to result in a final pCO_2 concentration of ~1600 μatm. Gas mixing was controlled by paired mass flow controllers (Alicat, Tuscon, AZ): one each for air and CO_2. Culture units consisted of 10-liter polycarbonate chambers (BearVault, San Diego, CA) fitted with a sealing lid,

and rubber ring seal (McMaster-Carr, Santa Fe Springs, CA). No supplemental aeration was supplied to the larval rearing units throughout the experimental period.

Seawater conditions of individual culture chambers were monitored by daily measurements of pH (Orion Star A11; Thermo-Fisher USA) calibrated with NIST buffers (calibrated to a seawater standard: Batch 22, A.G. Dickson, Scripps Institution of Oceanography, USA) and dissolved oxygen (YSI 85; YSI, Yellow Springs, OH, USA). Seawater samples for carbonate analysis were also obtained from seawater used to fill the chambers after 48 hours of culture (before each water change) to account for changes in chemistry arising from off-gassing or respiration. These samples were stored in sealed, gas-tight 350 ml amber glass bottles and poisoned with 30 µl of saturated mercuric chloride ($HgCl_2$) solution for later analysis. These water samples were analyzed at the lab of Dr. Burke Hales at Oregon State University, following the procedure outlined by Hales et al. (2005) and Bandstra et al. (2006) to obtain values for sample total dissolved carbon dioxide (TCO_2), pCO_2, and seawater pH, from which Ω_{arag} and Ω_{calc} values were calculated. This method has been shown to be highly accurate, providing TCO_2 and pCO_2 estimates with <0.2% and <5% uncertainty, respectively (Waldbusser et al. 2013). A summary of seawater carbonate chemistries is found in Table 2.1 and changes in pH and Ω_{arag} values throughout each experiment are represented in Figure 2.1.

2.4. Larval culture

The embryo pools of each group (MBP and Willapa) were counted and aliquots of ~200,000 embryos were distributed among culture units approximately five hours post fertilization for an effective stocking rate of 20 larvae ml^{-1}. Culture units were filled with either ambient (~ 400 µatm) or high (~1600 µatm) pCO_2 seawater. In 2015, each treatment level (broodstock x water treatment) was replicated five times, resulting in a total of 20 culture units. In 2016, each level was replicated six times, to improve statistical power, resulting in 24 culture units. Larval culture protocols were similar to those of Langdon et al. (2003), briefly: every two days, culture water was exchanged with fresh seawater, equilibrated to treatment pCO_2 levels. The contents of culture units were poured onto a sieve (see screen sizes below) to retain larvae then washed, re-filled, resupplied with algae and re-stocked with larvae. Antibiotics were added prophylactically in order to reduce bacterial respiration in culture units that would unduly affect seawater carbonate chemistry (Waldbusser et al. 2015b). Antibiotics were alternated at each water change between a chloramphenicol/ampicillin mixture (2 ppm and 10 ppm, respectively) and 20 ppm streptomycin to reduce the risks of development of resistant bacterial strains.

Microalgal diets were supplied once daily, starting 2 dpf, beginning with C-iso (*Isochrysis galbana*) at 20,000 cells ml^{-1}. Rations were increased by 5,000 cells ml^{-1} per day and the diatom species *Chaetoceros gracilis* was gradually incorporated into the diet starting at 5% (based on cell concentrations) on day 4, until it accounted for 50% of the algal diet by day 11, where it was maintained for the duration of the

experiments. Fertilized eggs were stocked at a rate of 20 ml^{-1} and larval densities were reduced to: 10 ml^{-1} on day 2, 5 ml^{-1} on day 6 and 1 ml^{-1} at the pediveliger stage - day 14 in 2015 or day 12 in 2016. Larval density was maintained in this fashion to provide equal and optimal environments for growth and survival and limit respiratory contribution to seawater pCO_2 levels. One ambient pCO_2 culture unit in 2015 suffered complete larval mortality on day 10 but all prior data points were retained for analysis.

Throughout the experiments, there was no selection for larval size and larvae were screened on conservative screen sizes to retain slow-growing individuals: 25 μm to day 4, 37 μm on day 6, 45 μm on day 8, 64 μm on days 10 and 12 and 80 μm for the remainder of the experiments. Reductions in larval density (as above) were carried out in the same way for all larval populations with no selection on size. After the appearance of eyed larvae on day 16, larvae were additionally screened on a 240 μm sieve to retain pediveliger larvae which were subsequently induced to settle by exposure to $1.8E10^{-4}$ M epinephrine for two hours (Coon et al. 1986). After this time, all larvae and newly settled spat were rinsed in seawater and returned to the culture vessel with the remainder of the larval group. Additional experiments were conducted that confirmed that neither seawater pCO_2 nor the antibiotics we used had interactive effects with epinephrine in determining settlement success (Figure A2.1).

2.5. Larval sampling and measurement:

Larval survival was estimated from counts of larvae in each culture unit on days 2, 6, 10, 14, 16 and 22 (day 24 in 2016). Counts were obtained by concentrating larvae from each 10-liter culture unit into a 250 ml beaker with 100-150 ml of seawater and removing five subsamples (~30-50 µl each) for counts, with additional re-sampling when the coefficient of variation among samples exceeded 10%. Survival estimates accounted for animals removed for sampling and for adjustments in densities by multiplying survivorship between sampling/counting events to obtain a cumulative survival estimate across the entire experimental period. Samples for larval size analysis were preserved by collecting ~200 larvae from each replicate, adding them to 10 ml seawater and fixing them with 200 µl of 10% buffered (pH = 8.1 – 8.3) formalin. Mean larval size was measured as the maximal anterior-posterior shell width parallel to the hinge for 30 to 50 larvae from each sample. Developmental stages were assessed as the proportion of 'normal' D-hinge larvae on day 2, pediveliger or 'eyed' larvae after day 16 and spat on day 22 in 2015 and day 24 in 2016. Day 2, 'normal' larvae were characterized by a straight hinge, smooth shell along the perimeter of the valve and tissue contained within the translucent shell (ASTM International 2012). Images were analyzed using ImageJ (NIH, USA).

2.6. Lipid analysis:

Directly after fertilization, eggs were sub-sampled from each fertilized egg pool, filtered on a pre-ashed glass fiber filter (Whatman GF/A), stored under

chloroform and nitrogen gas at -4^{o}C for later analysis. In 2015, ~20,000 eggs were sampled, in triplicate, from each pool. In 2016, females yielded fewer eggs overall and only ~5,000 - 6,000 eggs were available for sampling, in triplicate. An internal standard of the fatty acid 23:0 was added prior to extraction at an amount that was approximately 10% of expected total fatty acid content in order correct for losses during extraction and analysis. Lipid extraction, derivatization and analysis of fatty acid methyl esters (FAMEs) followed the methods detailed in Copeman et al. (2016). Given the disparity in sample size between experiments additional egg samples were later collected (in 2018) with sample sizes of: 5,000, 10,000, 20,000, 50,000 and 100,000, in duplicate, and processed in the same fashion to assess the efficiency of the extraction method. No relationship was observed between sample size (# of eggs) and estimated total fatty acid egg^{-1} in this range. Analysis of the fatty acid content and profile of Willapa eggs obtained in 2016 (n = 3) indicated these samples were contaminated during filtration and these were removed from analysis. Lipid composition of each egg pool is summarized in Table A2.1.

2.7. Data analysis:

We categorized larval oyster development into three distinct stages: 1) early larval development (from fertilized egg to D-hinge veliger larvae), 2) mid-veliger stages (from days 6 to 16), and 3) settlement and metamorphosis (from pediveliger stage at day 16 to ‘spat’ at day 22/24). Each of these developmental stages have distinct patterns of feeding, growth, and survival (Figure 2.2). Consequentially, in order to appropriately characterize treatment and broodstock effects upon larval

performance metrics, the data collected in these trials was first analyzed as a whole and subsequently partitioned by developmental stage for analysis. The whole and stage-separated datasets for larval performance metrics were analyzed using generalized linear models with the binary fixed effects: seawater treatment (low or high pCO_2), broodstock type (MBP or Willapa) and year/cohort (2015 or 2016). Each model was subjected to backwards and forward stepwise selection (Venables & Ripley 2002) to resolve a final model with fixed effects and interactions that minimized residuals. Models for spat size data included survival rate as a covariate to test for the influence of density-dependent growth during this stage. All data satisfied normality and homogeneity of variance assumptions as evaluated with Bartlett's and Levene's test and significance was determined at $p<0.05$ on the Type III sums of squares. All statistical analyses were conducted in R version 3.4.1(R core R Core team 2015). All models, summary tables and associated p-values can be found in the appendices.

3. Results

3.1. Carbonate chemistry:

Both ambient (~400 pCO_2, pH ~8.0, Ω ~2.7) and high pCO_2 (~1600 pCO_2, pH ~7.6, Ω ~1.0) culture replicates showed fluctuations in carbonate chemistry between water changes. For ambient cultures, pH averaged 7.89 ± 0.13 (mean ± standard deviation) and 7.79 ± 0.13 in 2015 and 2016 with an average pCO_2 level of 645 ± 210 and 840 ± 305 μatm, respectively. High pCO_2 cultures had an average pH of 7.48 ± 0.09 and 7.43 ± 0.19 in 2015 and 2016 with average pCO_2 levels of 1790 ± 345 and

2222 ± 788, respectively (Table 2.1, Figure 2.1A). The metabolically produced CO_2 in these static systems contributed to these fluctuations but Ω_{arag} levels (which are arguably the most biologically relevant parameter for early larvae; Waldbusser et al. (2015a)) remained distinct during these experiments: averaging 2.3 ± 0.5 and 1.95 ± 0.46 in ambient conditions and 1.00 ± 0.16 and 0.995 ± 0.58 in high pCO_2 replicates in 2015 and 2016, respectively ($p << 0.001$; Table 2.1, Figure 2.1B). There were no significant differences in treatment conditions between the broodstock groups in a given year, but, overall, the 2016 trial had lower Ω_{arag} for both ambient and high pCO_2 treatments than the 2015 trial ($p < 0.01$). Despite this, Ω_{arag} treatments for both experiments were maintained above and below the Ω_{arag} 'threshold' (~1.5-1.6) for oyster larvae that has been recently suggested as minimum conditions for early development and commercial production of *C. gigas* (Barton et al. 2012, Gimenez et al. 2018).

3.2. Overall survival and growth:

When analyzed over the total period of larval development, larval survival from fertilization (day 0) through metamorphosis and settlement (day 22 in 2015 and day 24 in 2016) was similar between experiments, treatments and broodstock groups. Both experiments had low initial survival for young larvae (~30% surviving at day 6) followed by stable populations of mid-veliger larvae and additional mortality occurring during the settlement period (Table 2.2, Figure 2.2A). Cumulative survival of all larvae and spat at the termination of the experiments averaged ~11.2 ± 5.0 % in

2015, compared to ~7.0 ± 3.5 % surviving in 2016. Group-specific survival and size estimates at each stage are found in Table 2.2. Growth rates of larvae were similarly uniform across groups and experiments: ~10.4 ± 0.5 μm day^{-1} during veliger larval stages and ~28.7 ± 10.4 μm day^{-1} for post-metamorphic spat (Figure 2.2B). The overall effects of broodstock type, seawater treatment and experiment iteration upon larval growth and survival during the entire 22 (24) day culture period were not statistically different (supplemental tables A2.2-A2.4). Stage-specific larval performance metrics, however, provide greater insight into how treatment conditions impacted developing oyster larvae.

3.3. Early larval development:

Survival from fertilization to shelled D-hinge larvae at 48 hours was higher in 2015 than 2016 with an average of 60.0 ± 5.3 % and 69.6 ± 12.2 % of MBP and Willapa larvae surviving in 2015, compared to 52.0 ± 8.0% and 38.2 ± 5.6 %, respectively, in 2016 (Table 2.2). The difference in survival to 48 hpf between the years, however, was significant only for Willapa larvae in 2016 ($p << 0.001$; Figure 2.3A, Table A2.5). Survival was slightly greater in high pCO_2 treatments overall (+ ~4.6%), although this effect was statistically marginal ($p = 0.063$; Table A2.5). The difference in larval survival to 48 hpf between the years did not appear to be a function of lipid content of the eggs: the mean total fatty acid content of eggs from MBP and Willapa groups in 2015 was 3.38 ± 0.31 and 3.66 ± 0.39 ng egg^{-1},

respectively, compared with 4.33 ± 0.35 ng egg^{-1} for the 2016 MBP cohort ($p = 0.0134$, Figure A2.2, Table 2.1).

Among the surviving larvae at 48 hours, an average of 75% were fully shelled 'normal' D-hinge larvae in ambient pCO_2 conditions; ~13% more, on average, than their high pCO_2 counterparts ($p << 0.001$; Fig. 2.3B, Table A2.6). Normal larvae in ambient replicates were also ~1.3 ± 0.5 μm larger, on average, than those in high pCO_2 conditions ($p = 0.011$, Fig. 2.3D, Table A2.7). When the total number of D-larvae (*# of all larvae* x *% normal D-hinge*) is estimated, however, there is no significant difference between seawater types in either year ($p = 0.37$; Table A2.8, Figure 2.3C) and by day 6 all groups in both experiments averaged 30.8 ± 1.2% cumulative survival of all larvae with no significant differences between broodstock types, seawater treatment or years (Figure 2.2, Table A2.2). Additionally, the proportion of normal larvae at 48 hpf was correlated with neither subsequent survival (from day 2 to 6) nor mean larval size at day 6 (Fig. 2.4 A&C). Total larval survival (normal and abnormal) at day 2, however, displayed a significant negative correlation with survival from day 2 to 6 ($p = 0.027$, Fig. 2.4B, Table A2.9) and weak but positive correlation with larval size at day 6 ($p = 0.08$, Fig. 2.4D, Table A2.10). Among all the early larval development performance metrics, broodstock effects were marginal and inconsistent between years suggesting little overall differences between MBP and Willapa larvae at this age (Fig 2.3).

3.4. Veliger stages:

During veliger development, from day 6 to 16, survival was high in all cultures, with an average mortality rate of 1.37 ± 0.09 % day^{-1} with no significant effect of broodstock or water treatment (Fig. 2.2, Table A2.11). At the last sampling point prior to metamorphosis, cultures had an average cumulative survival (from fertilization) of 18.3 ± 4.1%. Similarly, growth rates were relatively uniform during this period, averaging a daily 10.4 ± 0.5 μm increase in shell length across all treatments in both experiments (Table A2.12). Prior to induction of settlement, pediveliger larvae were, on average, 213.2 ± 46.4 μm at day 16 in 2015 and 172.7 ± 10.4 μm at day 12 in 2016. Although pediveliger larvae (day 16) were not measured in 2016, the similarity in growth rates between treatments and years over this period resulted in a linear model of best fit with only age (dpf) as an explanatory variable, with a model-predicted mean larval size of 213.5 ± 40.7 μm at day 16 for all treatments.

3.5. Settlement and Metamorphosis:

Survival rates for all larvae and spat from pediveliger stage through the settlement phase (day 16 through 22) averaged 59.2 ± 12.8% in 2015, with no significant effect of broodstock or seawater pCO_2 ($p > 0.1$, Fig. 2.5A, Table A2.13). In 2016, survival rates during this stage were similar for ambient conditions: ~66% and ~53% in MBP and Willapa groups, respectively, but high pCO_2 seawater significantly reduced survivorship of larvae: only ~18% in MBP and ~30% in Willapa were surviving at day 24 in acidified cultures in this year ($p < 0.001$, Fig.

2.4A). The final larval/spat samples collected at the conclusion of the trial were size separated on a 240 μm screen (see methods above). In all replicates (for both experiments), <1% of larvae/spat in the >240 μm size fraction were dead or moribund, indicating that mortality during this phase was almost entirely due to the loss of slow growing larvae (<240 μm) and not due to spat mortality.

The disparate effects of high pCO_2 culture between the years were also reflected in the settlement rates of these groups. In 2015 there was no apparent effect of acidified seawater on settlement with ~35% of MBP and ~19% of Willapa pediveligers successfully metamorphosing to spat by day 22, unaffected by seawater treatment ($p = 0.56$). In 2016, by contrast, ~51% vs 9% of pediveligers in MBP groups and ~36% vs 6% in Willapa groups settled out in ambient and high pCO_2 conditions, respectively. This represents an average ~42% reduction in settlement success in acidified seawater ($p < 0.001$; Fig. 2.5B, Table A2.14). Overall, broodstock effects upon settlement in both trials were significant (Table A2.15). On average, MBP pediveliger larvae resulted in 55% and 37 % more spat in ambient and acidified conditions overall, respectively, when compared to Willapa groups ($p = 0.036$, Fig. 2.5B, Table A2.14).

Larvae from MBP stocks also resulted in larger spat, on average, at the conclusion of both experiments; averaging a ~28.0 μm (5.1 %) increase in ambient and ~123.1 μm (22.5 %) increase in high pCO_2 conditions across both experiments ($p = 0.042$ and $p = 0.027$, respectively, Fig. 2.5C; Table A2.16). The mean size of spat was, on average, significantly greater in all groups in 2016 (+ ~255.5 μm), likely owing to the additional two days of growth in this trial (24 vs 22 dpf). Survival rates

of all larvae during settlement (from day 16) were modeled as a covariate in the analysis of spat size to account for possible density-dependent growth. In 2016, where survival differences were more pronounced, survival was significantly negatively correlated with spat size: an estimated ~2.2 μm reduction in mean spat size for every percent increase in total survival ($p = 0.005$, Table A2.16). This effect was not significant, however, in the 2015 experiment ($p = 0.296$) where survival rates were relatively uniform. A summary of the most significant overall effects of broodstock type, seawater treatment and their interaction is depicted in Figure 2.6.

4. Discussion

These experiments contribute to a relatively sparse body of work concerning the long term effects of low Ω_{arag} seawater upon Pacific oyster larvae and build on this information by comparing the relative sensitivity of domesticated and naturalized stocks to these conditions. The stage-specific analysis of the response of oyster larvae to acidified seawater provides greater insight into the physiological effects of low Ω_{arag} seawater than analyses based on overall impacts or those upon single larval stage alone. These experiments also uniquely re-create both the larval rearing environment and genetic composition of MBP larval pools in two experiments, allowing us to examine inherent differences between experiments that arise from factors not incorporated into typical designs of ocean acidification experiments (e.g. Gimenez et al. 2018).

4.1. Early larval development

Shell morphology is a useful and sensitive metric to assess the fitness of 'D-larvae' at 48 hpf and their sensitivity to low Ω_{arag} seawater during formation of the PDI and early larval development (Kurihara et al. 2007, Parker et al. 2010, Waldbusser et al. 2015a). In these experiments, we observed a similar reduction in the proportion (%) and size (μm) of normal larvae in both MBP and Willapa larval groups reared in high pCO_2 seawater (Fig. 3B&D). In addition to % normal D-larvae (which is the toxological standard for larval fitness at this age; ASTM 2012), here we also report the total percent survival of all larvae at 48 hpf (abnormal and normal alike) relative to the stocking density of eggs following fertilization (20 ml^{-1}; ~200,000 $replicate^{-1}$; Fig. 2.3A). Although this distinction may seem to be a minor detail, there was an overall, if subtle, higher total survival rate of all larvae in high pCO_2 seawater treatments compared with ambient treatments ($\bar{x}$ = +4.6%, p = 0.063). Interestingly, the average difference in total normal larvae (*% survival x % normal*) in these experiments is statistically indistinguishable between low (~1) and high (>2) Ω_{arag} seawater conditions within each experiment (p=0.37; Table A2.8, Fig. 2.3C). This difference of effect between proportion-normal and total-normal is due to the greater abundance of abnormal larvae surviving to this age (48 hpf) in low Ω_{arag} replicates. While low Ω_{arag} is likely to impede precipitation of the PDI, it is unlikely that the physio-chemical barrier to calcification presented by these conditions (Lannig et al. 2010, Waldbusser et al. 2013, 2015a) should also lead to increased total survival of abnormal larvae to this age. This finding may, instead, result from an overlap of

two other coinciding biologic processes: developmental delays incurred by low Ω_{arag} (De Wit et al. 2018), and stage-specific genetic inviability (Plough et al. 2016).

4.2. Genetic load and larval mortality

The broadly adapted and highly polymorphic genome of the Pacific oyster (Zhang et al. 2012) contains an abundance of negative or deleterious alleles, referred to as a high "genetic load" (Launey & Hedgecock 2001). Many of these alleles appear to be involved with developmental transitions (Plough 2011, 2018), resulting in stage-specific mortality patterns that render a large majority of *C. gigas* larvae genetically inviable (Plough et al. 2016). Larval mortality owning to these genetic impediments are manifest as a function of developmental progression rather than absolute time (Plough 2018). In the context of our experiments, under ambient seawater conditions early larval development and associated genetic mortality proceed at a 'normal' rate and samples taken at 48 hpf cumulatively represent both processes. In low Ω_{arag} seawater conditions, in contrast, developmental processes are delayed and protracted across a longer time frame (De Wit et al. 2018), which not only results in smaller fully-formed surviving larvae (Fig. 2.3D; also observed in Waldbusser et al. 2014) but also an increased total abundance of 'abnormal' larvae, many of which may have genetic impediments to proper development (Fig. 2.3 A&B). The hypothesis that low Ω_{arag} seawater delays both embryogenesis and the timing of genetically mediated mortality during this stage, is supported by the comparison of larval performance metrics at 48 hpf with that of subsequent larval growth and survival to 6 dpf. Total survival rate at day 2 has a surprisingly robust

negative correlation with subsequent survival between day 2 and 6 (Fig. 2.4B), indicating that seawater pCO_2 (and correlated carbonate parameters) appears to have had an effect on the timing, but not the magnitude, of early larval mortality to day 6 in our experiments. These data also suggest that total survival and proportional normality at 48 hours in these experiments are ephemeral performance metrics - by day 6 cumulative larval survival is similar among all groups (~30%) and we find no correlation between proportion normal at 48 hpf and subsequent survival or larval size at day 6 (Fig. 2.4 A&C). A general (albeit ambiguous) positive correlation between survival at day 2 and mean larval size at day 6 (Fig. 2.4D) suggests that culture conditions in both seawater pCO_2 treatments were adequate to maintain healthy growing larvae, and survival to 6 dpf was not negatively affected by larval density or food availability.

4.3. Veliger growth and survival

During mid-veliger stages, from six to 16 dpf, we observed no discernable differences in survival or growth rate of veliger larvae in either water treatment, broodstock group or experiment. Shelled veliger larvae are somewhat more resilient to elevated seawater pCO_2 and low Ω_{arag} (Ramesh et al. 2017) but these conditions have, nevertheless, been shown to exhibit distinct effects on the physiology of larval oysters. Timmins-Schiffman et al. (2012) and Frieder et al. (2016) indicated that acidified seawater reduces net calcification rates of early larvae and Pan et al. (2015) and Frieder et al. (2018) have suggested that acidification stress alters the allocation of metabolic energy within larvae. Dineshram et al. (2012, 2013, 2016) demonstrated

similar impacts on global proteomic expression. Despite the acknowledged significance of bioenergetics in larval physiology under acidification stress (Waldbusser et al. 2013, 2015a, Frieder et al. 2018), many studies which report reduced larval growth rates in high pCO_2 conditions frequently overlook the quality of microalgal diets and research is undertaken with larvae fed on a mono-specific diet of *Isochrysis galbana* (e.g. Miller et al. 2009, Talmage & Gobler 2010, Frieder et al. 2016) . This species of microalgae is widely used in bivalve culture but is nutritionally sub-optimal for larval oysters, leading to slower growth, poorer survival and reduced settlement when compared to mixed-species diets that include diatoms, such as those in the *Chaetoceros* genus (Langdon & Robinson 1996, Rico-Villa et al. 2006, Marshall et al. 2010). Sub-optimal algal diets may result in nutritional stress of larvae, affecting their response to the additional stress of acidified seawater. The more complete diets used in this study may have improved mid-veliger performance in high pCO_2 seawater but null effects of acidified conditions on the growth of veliger larvae are not without precedent; for example, Miller et al. (2009) saw no reduced growth of *Crassostrea ariakensis* larvae when reared in seawater with Ω_{arag} as low as 0.6, and others (Thiyagarajan & Ginger 2012, Hettinger et al. 2013, Ko et al. 2014) have suggested that the negative effects of acidified seawater on growth and survival may be negated by increased culture temperature or food availability. The lack of measurable effects of acidified seawater we report during mid-veliger stages does not suggest that oyster larvae were not impacted by the high pCO_2 experimental conditions but, rather, in our culture environment their effects were not discernable

during this stage from gross performance metrics such as size and survival, as suggested by Brunner et al. (2016).

4.4. Settlement and Metamorphosis

In contrast to similar growth and survival rates between pCO_2 treatments observed during the veliger stages, the metamorphic period - from 16 to 22 dpf (2015) or to 24 dpf (2016) - displayed distinct yet variable effects of high pCO_2 seawater. During metamorphosis, pediveliger larvae settled with relatively similar success in ambient conditions in 2015 and 2016, resulting in ~43% and ~28% settlement success, on average, in MBP and Willapa groups, respectively (Fig 2.5B). Larvae undergoing metamorphosis in high pCO_2/low Ω_{arag} replicates in the first experiment (2015) appeared unaffected by acidification but in the second experiment (2016) high pCO_2/low Ω_{arag} conditions resulted in ~40% greater mortality during metamorphosis and ~42% fewer spat, on average, when compared to ambient conditions(Fig 2.5A&B). In both of these experiments, however, ~99% of the observed mortality occurred with small, under-developed, larvae that passed through a 240 μm screen and we observed almost no mortality of size-competent pediveliger larvae (>240 μm; Coon et al. 1990) or settled spat. This distinction is a useful detail because it indicates that the increased mortality observed during settlement in high pCO_2 replicates in 2016 was a function of development to pediveliger competency and not due to an effect of acidified seawater on the metamorphic transition from pediveliger to spat. It appears that the chronic stress of high pCO_2 seawater in the 2016 experiment was masked in growth and survival measurements through much of

the veliger stage (6-16 dpf). The cumulative physiological impacts of this stress, nevertheless, inhibited development to pediveliger competency for a large portion of the larval population in these cultures, resulting in death. In 2015, larval oysters likely experienced similar physiological challenges in high pCO_2 cultures, but it appears that some unknown additional set of factors (discussed in section 4.6. below) helped ameliorate these effects enough to permit comparable rates of survival and settlement in ambient and high pCO_2 treatments.

It is worth acknowledging that the environmental and behavioral factors contributing to the stochastic nature of oyster settlement (Fitt et al. 1990, Tamburri et al. 1992, Turner et al. 1994) were circumvented in these experiments by the use of epinephrine to chemically induce metamorphosis (Coon et al. 1986, Bonar et al. 1990). We adopted this technique in order to standardize the culture period between replicates and treatments and facilitate counting of spat. Haws et al. (1993) demonstrated that this method increases the rate but not the magnitude of larval settlement, relative to 'natural' controls, and we conducted additional experiments that confirmed that neither seawater pCO_2 nor the antibiotics we used had interactive effects with epinephrine in determining settlement success (Figure A2.1). Nevertheless, this methodology is an artificial one, and the results should be interpreted with caution in other contexts where environmental cues, such as seawater pCO_2, may have behavioral effects upon pediveliger larvae and influence settlement timing and success (Pechenik 1990, Pechenik et al. 1990).

4.5. Comparison with previous work

Several studies have documented the physiological effects of acidified seawater environments on larval bivalves over multiple developmental stages (e.g. Miller et al. 2009, Talmage & Gobler 2011, Frieder et al. 2016). Among these, however, many initiated experimental exposure likely after PDI development (~16 hpf; Miller et al. 2009, Talmage & Gobler, 2009, Thiyagarajan & Ginger, 2012, Ko et al. 2013, Gobler & Talmage, 2014, Clark & Gobler, 2016), provided potentially inadequate microalgal diets (mentioned previously), and base estimates of size and developmental progression on a limited number of remaining individuals (Talmage & Gobler 2009, 2012, Gobler & Talmage 2014), all of which can skew results and weaken conclusions with regards to treatment effects. Additionally, few studies have examined larval performance over multiple cohorts in the same analysis. The reported variability between experiments evaluating the long term response of oyster larvae to OA conditions are large; for example, researchers (Talmage & Gobler 2009, 2012, Gobler & Talmage 2014, Clark & Gobler 2016) reported average larval survival to the pediveliger stage of *Crassostrea virginica* under ambient conditions (~400 pCO_2) ranged from ~15 to 50% (from initial stocking at ~6-24 hpf), and the final percentage of larvae that settled to produce spat ranged from ~25-45% (for those experiments which included metamorphosis). Low Ω_{arag} seawater in these studies (0.39-1.52) had generally negative impacts on survival and metamorphosis of *C. virginica* but also produced variable results, relative to the control. In two of the four studies, moderate Ω_{arag} levels (1.83-1.91) resulted in positive effects on larval performance, compared to high Ω_{arag} controls (2.91-3.68; (Talmage & Gobler 2009,

Gobler & Talmage 2014). This substantial variation in overall and relative larval performance among these studies (and between the two experiments reported here) demonstrate the inherent variability between larval oyster cohorts and the importance of replicated experiments and extensive sampling regimes to better understand larval responses to OA conditions.

In this case, the differential effect of seawater pCO_2 upon spat production between the 2015 and 2016 experiments similarly highlights the sensitivity of larval fitness to a multitude of factors. High pCO_2 cultures in 2016 had lower mean Ω_{arag} values overall (0.995 ± 0.58 compared to 1.00 ± 0.16 in 2015) but this slight difference is unlikely to be the sole explanatory variable for explaining the substantially disparate effects of acidified seawater on larval fitness between experiments. Underlying variation in water quality parameters that traditionally go unmeasured, such as algal blooms and marine toxins (such as those observed earlier in 2015; McCabe et al. 2016), may also potentially affect larval fitness and relative sensitivity to stressors like high pCO_2 (e.g. Gimenez et al. 2018). In our experiments we were unable to discern any substantial difference in larval performance in control (ambient) conditions that suggest the presence of any such confounding factors, but it remains a possibility that we cannot entirely reject. Differences in the quality of broodstock available for each of the experiments is another plausible explanation for the observed difference in larval sensitivity to high pCO_2 seawater. Larval growth and survival is strongly influenced by broodstock egg quality (Gallager et al. 1986, Kennedy 1996, Bochenek et al. 2001) and although broodstock in both years were conditioned similarly for ~4 weeks prior to spawning, it has been reported that

gametes are formed from reserves that are accumulated ~6 months prior to conditioning (Berthelin et al. 2000). A strong El Niño event during the 2015-2016 winter (NOAA 2018) produced both warmer seawater temperatures and increased precipitation at the broodstock holding site (HMSC; Fig. A2.3), potentially disrupting natural patterns of gonad re-absorption and gametogenesis (Dutertre et al. 2009) and reducing feeding rates (Gray & Langdon 2018). Sub-optimal overwintering conditions likely contributed to the observed poor condition of broodstock in 2016 and significantly fewer eggs obtained per female (Fig. A2.4) despite having significantly greater estimates of total lipid per egg (Fig. A2.2). Differences in overwintering location for 2016 broodstock (Yaquina vs Willapa bay) could have also impacted egg quality, resulting in early larval survival differences between the broodstock types (Fig 2.3) but uniform performance over the veliger period and consistent broodstock-level effects on settlement metrics suggest that season, more than overwintering location, was a stronger source of variation. Gametes physically removed from underdeveloped broodstock (i.e. strip spawning) may remove 'un-ripe' gametes that are compromised in other ways that are difficult to assess (Pauletto et al. 2017) and, as noted by Myrina et al. (2015), total lipid content may not be a reliable predictor of oocyte quality. Natural spawning is likely a preferred method for producing robust larvae for experimentation, but the extensive cross design adopted in these experiments made this approach unfeasible.

4.6. Effect of broodstock type

Despite substantial inter-experimental variation in the effect of seawater treatment upon settlement results, pediveliger larvae from selected MBP broodstock, when evaluated across both experiments and seawater types, resulted in significantly more and larger spat, on average, when compared to their naturalized counterparts (Table 2.2, Fig. 2.5B&C, Tables A2.14 & A2.16). The increased settlement rate of MBP groups in ambient seawater appeared to be somewhat muted by high pCO_2 conditions (+55% vs +37%, respectively, overall) but differences in mean spat size were magnified by acidified environments: MBP spat were, on average, ~5 % and ~23% larger in ambient and high pCO_2 conditions, respectively, than those of Willapa spat. (Fig. 2.5C & Table A2.16). The overall trends for increased size of MBP spat are somewhat obscured by inter-annual variation and interactive effects: in the 2015 experiment, MBP spat were larger overall (+76 μm, on average, calculated from model predicted estimates) with little effect of treatment and in 2016 there was a stronger interactive effect between seawater treatment and broodstock type which resulted in larger spat for MBP groups specifically in high pCO_2 treatments (+130 um, on average, calculated as above), but less significant size differences in ambient cultures (Table A2.16, Table A2.17). This multi-level variation in relative performance may be due to variability in culture conditions between the experiments (discussed previously) or minor differences in genetic composition of Willapa larval pools of unknown background (discussed below). While it is tempting to interpret size differences as evidence for an improved growth rate of spat, the sampling design in these experiments makes that conclusion tenuous. Metamorphosis and settlement

are dynamic and sporadic processes in larval oyster populations and with a single sampling point we cannot distinguish from these data whether differences in spat size between broodstock groups or treatments are due to altered timing of settlement or differences in spat growth rates. Further studies are warranted to investigate the specific effects of acidified seawater on bivalve settlement behavior and physiology to better parse these two distinct potential effects.

MBP Pacific oyster families have been selectively bred over five generations for improved yields on farms (De Melo et al. 2016) but improved larval performance has not been intentionally selected, so differences of this magnitude in the performance of larvae and juveniles of MBP and Willapa stocks are surprising. Larval performance traits have been found to be heritable (Ernande et al. 2003) and five generations of larval rearing in hatchery systems is likely to have resulted in unintentional selection for faster growth and higher survival in these conditions (stable 25 ^{o}C seawater together with abundant, high quality algal diets). Although the genetic background of Willapa broodstock potentially varied between the two sites from which they were collected for 2015 and 2016, the difference is expected to be small (Sun and Hedgecock 2017). A reduced sire input from Willapa groups in each year (5♂, compared to 19♂ for MBP) likely reduces the overall genetic diversity for Willapa larval pools to a degree but the large number of females used in each year (19♀ each) still results in 95 individual crosses that represent a broad combination of genotypes for experimentation. Moreover, the general comparisons between MBP larval pools (which had a high degree of genetic similarity) and Willapa counterparts were highly consistent between years, with only minor significant differences

observed in early larval stages (Fig 2.3). Epinephrine-induced settlement could also be a selective pressure that favors MBP groups in these experiments (resulting in increased settlement) but we did not see a higher proportion of 'unresponsive' pediveliger larvae at the final sampling point in Willapa groups to support this hypothesis (Fig. A2.5). In 2016, high pCO_2 seawater negatively impacted both MBP and Willapa larvae, but MBP pediveligers still produced ~50% more spat than Willapa groups in these adverse conditions (Tables 2.2 & A2.15). It is possible that one or more previous generations in the MBP breeding program were spawned during a period of coastal upwelling, thus inadvertently exerting a selection pressure for genotypes that are resilient to high pCO_2 conditions. More broadly, improvements in general larval performance traits such as metabolic efficiency, growth and survival could also possibly reduce the negative effects of acidified seawater upon larval physiology. This latter explanation is, perhaps, more plausible as we see no apparent performance advantage in MBP stocks for initial shell formation (assessed at 48 hpf), a stage when the impacts of acidified seawater are most pronounced and consistent. Increased spat production from MBP groups in all treatment combinations, however, suggests that incidental selection for larval performance has unintentionally improved early life fitness traits in stable hatchery conditions for these selected lines.

4.7. Implications for C. gigas *populations in the PNW*

The effect of larval fitness differences between domesticated and naturalized oyster stocks on population dynamics in natural environments in the PNW is difficult to predict. In theory, improved larval fitness represents a competitive reproductive

advantage for domesticated oysters but it is uncertain whether these advantages are maintained in the highly variable and dynamic environments that naturalized Pacific oyster larvae experience (Hales et al. 2017). Furthermore, reproductive success of *C. gigas* in the PNW is highly stochastic, temporally variable and vulnerable to the effects of 'sweepstakes recruitment' which severely limits the effective population size (N_e) and increases the rate of inbreeding accumulation (Hedgecock et al. 1992). All of these factors increase the relative strength of genetic drift over directive selection as a mechanism of genetic change and may reduce or overwhelm any more subtle effects that the introduction of domesticated genotypes have on current populations of naturalized stocks. The continuing effects of climate change on atmospheric and oceanic processes, however, make for an uncertain future for *C. gigas* populations in the PNW (Barton et al. 2015, Lemasson et al. 2018). If the Pacific oyster aquaculture industry in the region becomes increasingly reliant on hatchery reared, selectively bred oysters for continued production, the genetic impacts of domesticated stocks on naturalized 'wild' populations may also increase accordingly.

5. Conclusions

We demonstrate that Pacific oyster larvae display sensitivities to high pCO_2 culture conditions that differed across larval developmental periods; furthermore, we found that larval settlement and survival in simulated OA conditions is highly variable between spawning cohorts. The negative effects of low Ω_{arag} seawater upon shell formation and initial larval development were consistent with the reported

literature (e.g. Waldbusser et al. 2015a). These impacts, however, did not result in a significant decrease of the total number of 'normal' D-stage larvae at 48 hours post fertilization nor did they display any persistently negative effects on later veliger larval stages. High pCO_2 seawater had variable effects on total metamorphic success in these experiments but, importantly, did not appear to directly impede the physiological process of metamorphosis from pediveliger larvae to juvenile spat. The negative overall impacts of high pCO_2 seawater we observed in the 2016 experiment, but the lack of effect of similar conditions in the experiment conducted in 2015, suggest that stress responses due to acidification are influenced by other biotic and abiotic parameters e.g. broodstock quality, food availability and culture conditions, as suggested by Hettinger et al. (2013), Thomsen et al. (2013), and Cole et al. (2016). The consistently improved settlement results of MBP stocks relative to that of larvae from Willapa oysters, in both ambient and high pCO_2 seawater (Fig. 2.6), supports previous findings which indicate a genetic effect on larval resilience to OA (Parker et al. 2010, Sunday et al. 2011, Goncalves et al. 2018) and a potential for Pacific oysters to adapt to acidified environments.

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Figures and Tables

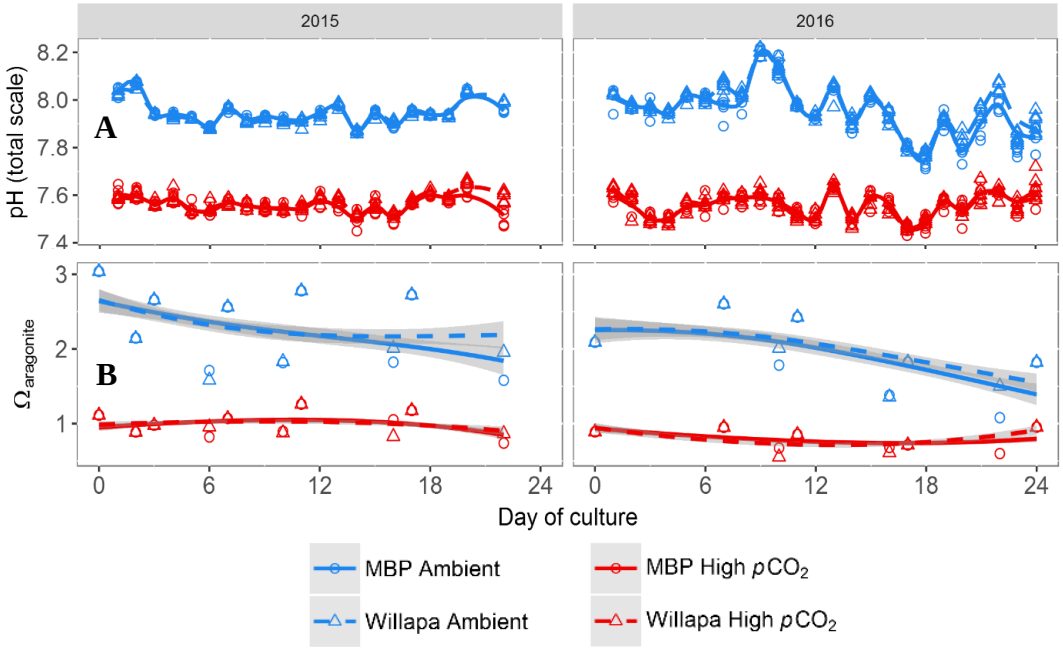

Figure 2.1 Seawater carbonate chemistry.
(A) Daily pH measurements of MBP and Willapa larvae static cultures reared at ambient and high pCO_2 levels for 0 to 22 (24) days post fertilization in 2015 and 2016 trials. Seawater (prepared at ~400 and ~1600 µatm for ambient and high pCO_2, respectively) was replaced in each culture every 48 hours, fluctuations in pH values reflect changes due to respiration between water changes.
(B) Aragonite saturation state (Ω_{arag}) in ambient (~400 µatm) and high (~1600 µatm) pCO_2 cultures across the culture period. Individual points represent estimates from discrete samples, alternating between those obtained from newly prepared seawater (equilibrated to ~400 and ~1600 pCO_2) and in situ samples from vessels after 48 hours of static culture. Lines represent local regression (LOESS) estimates of mean values.

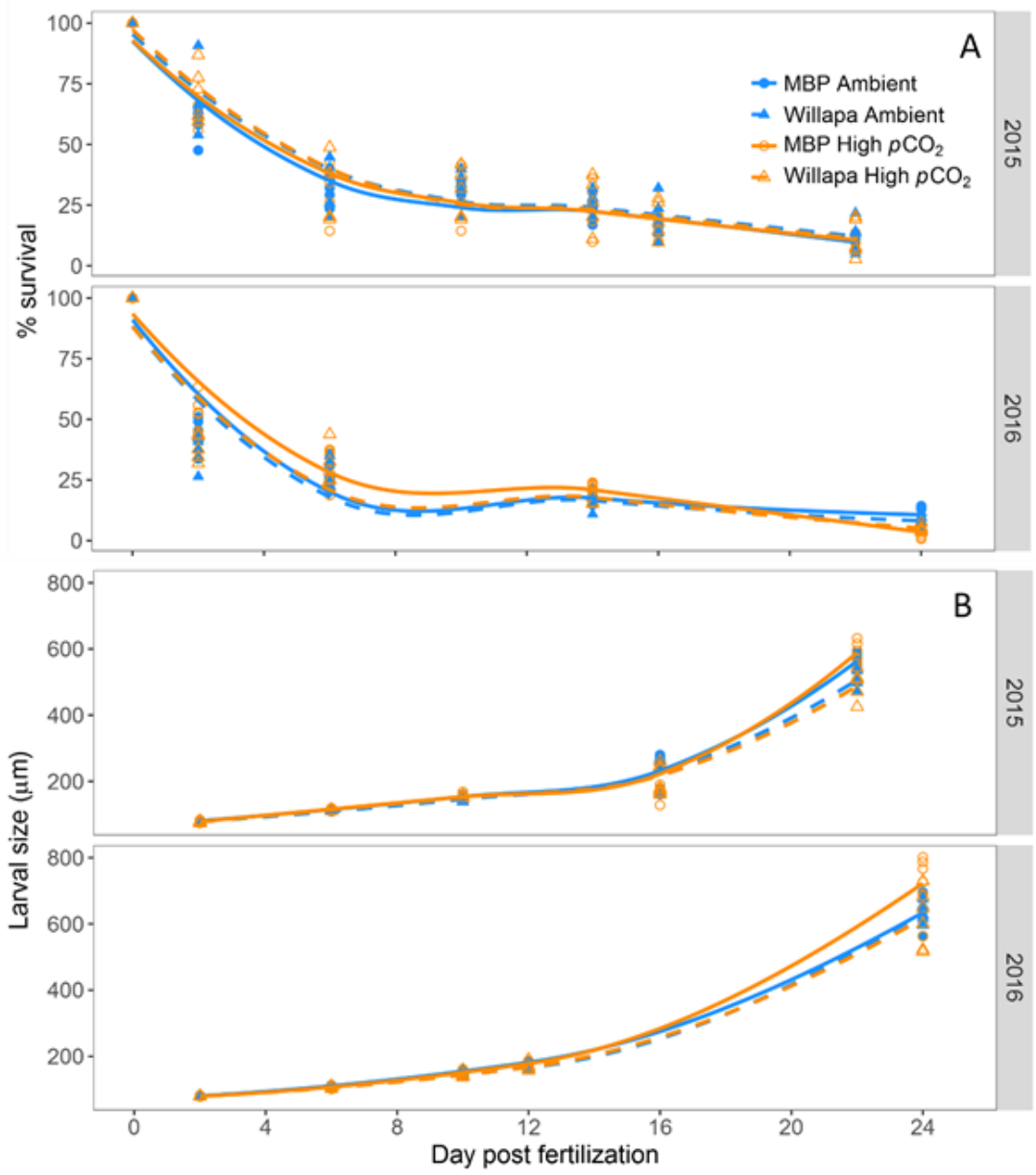

Figure 2.2 Cumulative survival (A) and larval size (B) from 0 to 22 (24) days postfertilization for MBP and Willapa larvae reared at ambient and high pCO_2 conditions in replicated experiments in 2015 and 2016. * Final sizes in (B) at day 22 and 24 in 2015 and 2016, respectively, are representative of metamorphosed spat only. Pre-metamorphic veliger larvae were measured but not represented in this figure.

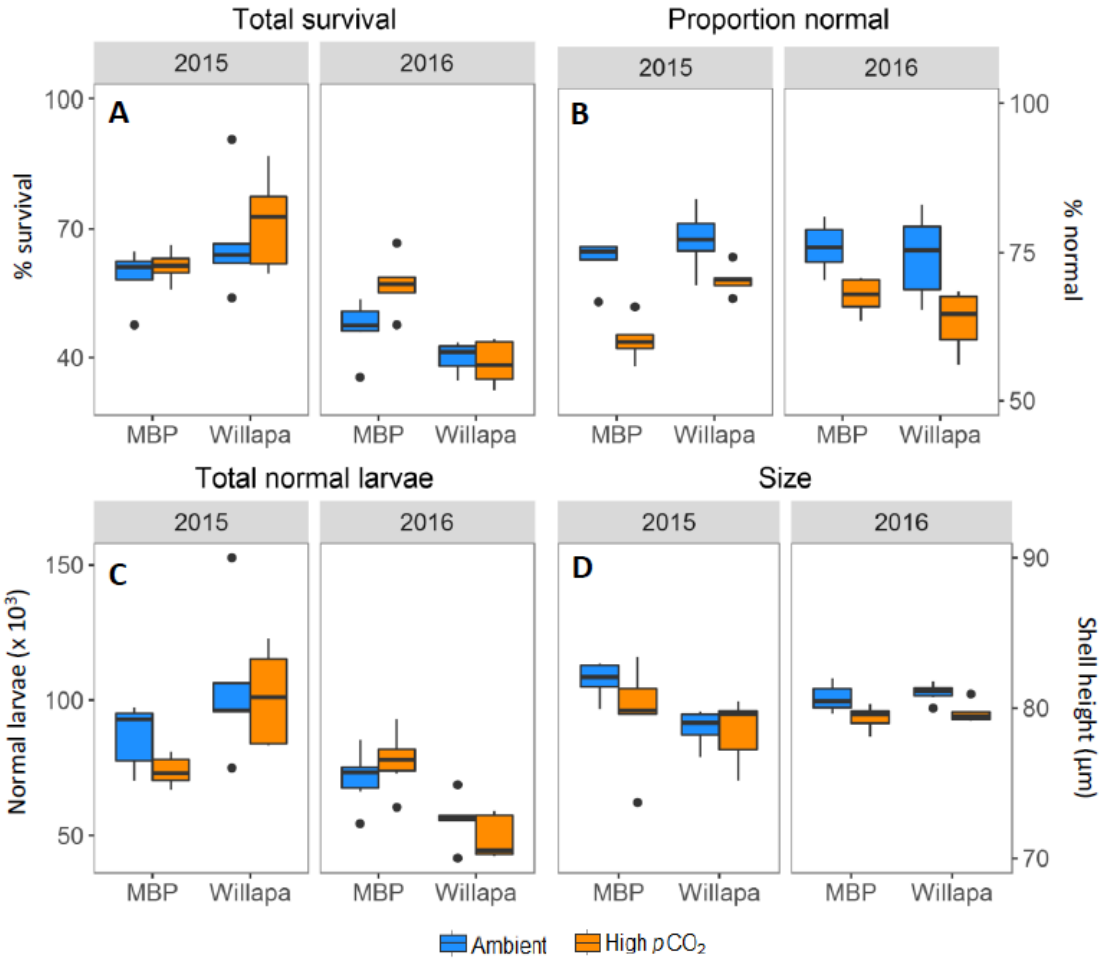

Figure 2.3 Larval fitness. Total larval survival (A) proportion normal (B) total number of normal larvae present (C) and mean size (shell height; µm) (D) for D-hinge veliger larvae at 48 hours post fertilization in MBP and Willapa larval groups reared in ambient and high pCO_2 seawater conditions in 2015 and 2016. The horizontal band in each box represents the median, the bottom and top of the boxes represent the inter-quartile range (IQR; at 25th and 75th percentiles, respectively). The error bars define range of the data, up to 1.5 * IQR (data points falling outside these percentiles are marked by dots)

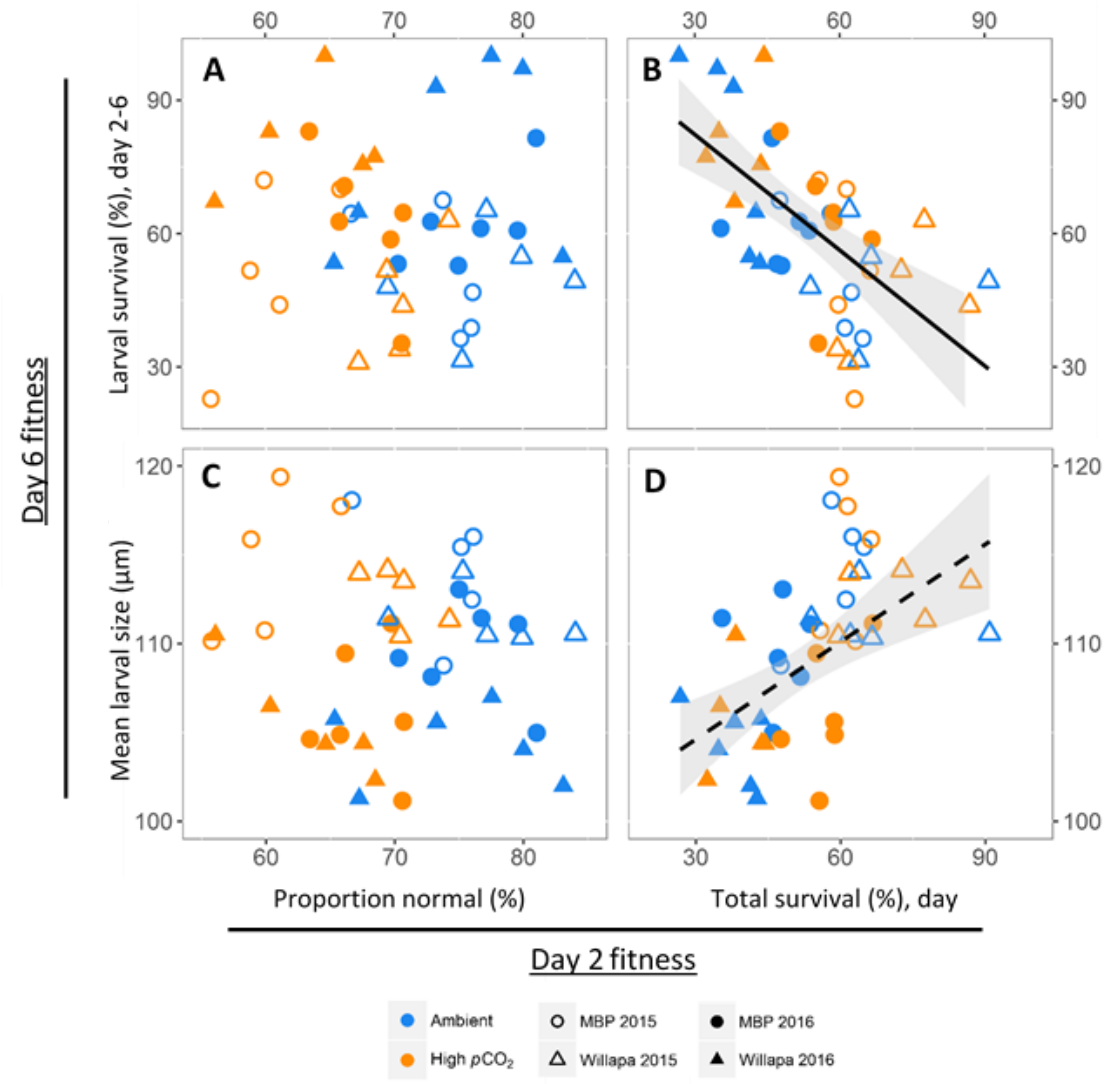

Figure 2.4 Correlation between day 2 and day 6 fitness metrics. Correlation between proportion 'normal' D-hinge veliger larvae and total larval survival at 2 days post fertilization to subsequent larval survival between days 2 and 6 (A&B) and mean larval size at day 6 (C&D). Each point represents one culture replicate: orange and blue points correspond to ambient and high pCO_2 seawater conditions, respectively. Open and filled points refer to experiments conducted in 2015 and 2016, respectively. Lines in plots B and D represent significant first order correlations between main factors, solid and dashed lines represent relationships significant at $p < 0.05$ and $p < 0.1$ levels, respectively and grey 'ribbons' are 95% confidence intervals.

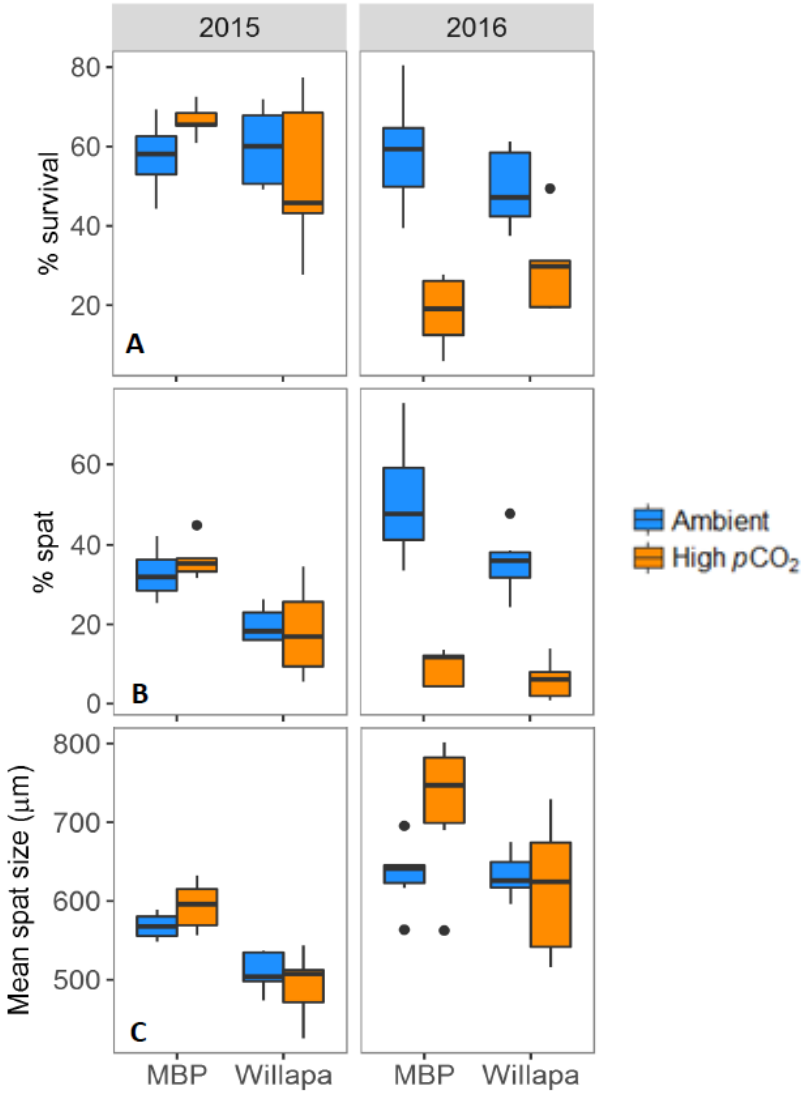

Figure 2.5 Settlement performance. Percent survival (A) and settlement success (% spat; B), and mean shell length (µm; C) at 22 and 24 days post fertilization in MBP and Willapa larval groups in 2015 and 2016. Percentage survival and settlement are calculated from pediveliger stage, not initial stocking density. The horizontal band in each box represents the median, the bottom and top of the boxes represent the inter-quartile range (IQR; at 25th and 75th percentiles, respectively). The error bars define range of the data, up to 1.5 * IQR (data points falling outside these percentiles are marked by dots).

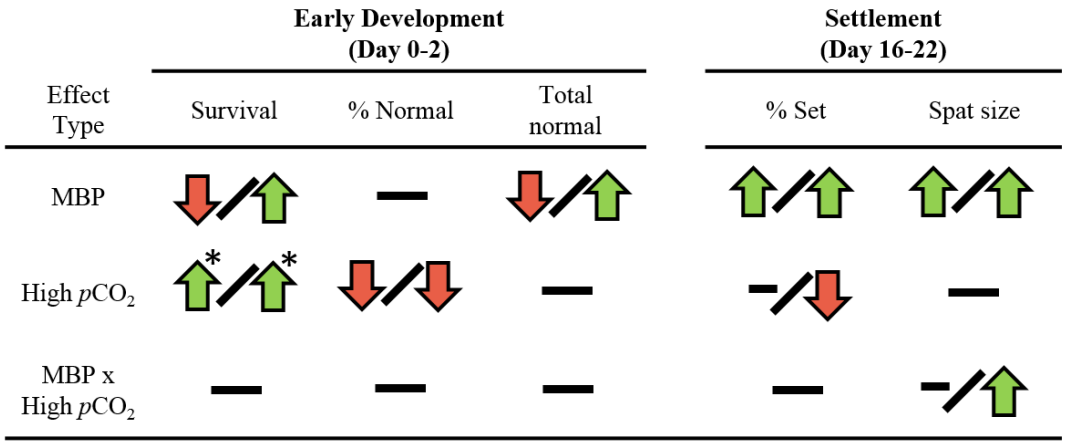

Figure 2.6 Summary of significant net effects of broodstock type (MBP), seawater treatment (High pCO_2), and their interaction (MBP x High pCO_2) on larval performance. Early larval development metrics (0-2 dpf) include: survival, proportion normal (%) and total normal D-larvae. Metrics for performance over the settlement stage from pediveliger to spat (16-22 days) include: percent metamorphosed (% set) and size of spat (spat size). Green and red arrows indicate a positive or negative effect, respectively, with year specific effects separated by a "/" (e.g. '2015/2016').

* *The positive effect of High pCO_2 culture on larval survival was marginally significant ($p=0.063$).*

Table 2.1 Water quality measurements. Mean (±SD) temperature, salinity, total alkalinity (μeq kg^{-1}), total CO_2 (TCO_2), partial pressure CO_2 (pCO_2), bicarbonate (μmol kg^{-1}), carbonate (μmol kg^{-1}), pH (pH_T = pH on the total scale) and saturation state of calcite (Ω_{calc}) and aragonite (Ω_{arag}) for ambient and high pCO_2 seawater treatments in MBP and Willapa larval culture replicates across the 22-24 day culture period.

			Parameter									
			Temp. (°C)	Salinity	Alkalinity (μeq kg^{-1})	TCO_2 (μmol kg^{-1})	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	pH_T	Ω_{calc}	Ω_{arag}
2015	MBP	Ambient	24.5	29.8	2249	2069	651	1911	139.3	7.89	3.43	2.28
			± 0.86	± 0.27	± 34	± 50	± 215.1	± 72	± 32.1	± 0.13	± 0.79	± 0.53
		High pCO_2	24.4	30.5	2294	2260	1791	2146	61.0	7.48	1.50	1.00
			± 1.02	± 0.26	± 31	± 18	± 364.3	± 17	± 10.3	± 0.09	± 0.25	± 0.16
	Willapa	Ambient	24.5	29.8	2267	2083	640	1922	142.0	7.89	3.50	2.33
			± 0.90	± 2.27	± 30	± 56	± 203.1	± 77	± 29.7	± 0.12	± 0.73	± 0.48
		High pCO_2	24.3	30.5	2304	2270	1790	2156	61.1	7.48	1.50	1.00
			± 1.00	± 0.36	± 23	± 24	± 326.8	± 23	± 9.2	± 0.08	± 0.22	± 0.15
2016	MBP	Ambient	24.6	30.9	2246	2104	862	1963	115.7	7.78	2.82	1.88
			± 0.5	± 0.2	± 58	± 80	± 321.8	± 96	± 31.0	± 0.14	± 0.76	± 0.50
		High pCO_2	24.6	30.8	2302	2296	2318	2177	51.4	7.38	1.25	0.83
			± 0.9	± 0.4	± 50	± 74	± 610.7	± 69	± 13.9	± 0.12	± 0.34	± 0.23
	Willapa	Ambient	24.8	30.8	2262	2112	817	1968	120.5	7.8	2.93	1.95
			± 0.7	± 0.3	± 59	± 91	± 289.6	± 105	± 26.3	± 0.12	± 0.64	± 0.42
		High pCO_2	24.6	30.8	2340	2302	2126	2168	71.3	7.46	1.74	1.16
			± 0.9	± 0.4	± 115	± 72	± 969.2	± 67	± 56.8	± 0.26	± 1.4	± 0.93

Table 2.2 Mean cumulative percent survival (%), shell length (µm) and (± standard deviation) at D-hinge, pediveliger and spat stages for MBP and Willapa larvae reared at ambient and high pCO_2 conditions in replicated experiments in 2015 and 2016.
[a] Pediveliger larvae were sampled at day 14 and 12 in 2015 and 2016 experiments, respectively.
[b] Spat stage metrics have been separated into 1) all larvae and spat (Total), 2) pre-settlement larvae (Pre-set) and 3) settled spat (Spat).

				Larval Stage				
						Spat[b]		
				D-hinge	Pediveliger[a]	Total	Pre-set	Spat
2015	MBP	Ambient	%	58.8(6.7)	17.3(3)	10.1(3.1)	4.3(1.9)	5.8(1.9)
			µm	81.8(1.2)	220(56.0)	-	304.6(25.9)	568.2(18.4)
		High pCO_2	%	61.3(3.9)	16.6(5)	11.1(3.4)	5.0(1.6)	6.1(1.6)
			µm	79.6(3.6)	209.2(47.3)	-	302.4(31.8)	593.9(31.5)
	Willapa	Ambient	%	67.4(13.9)	19.6(8.7)	12.3(6.7)	8.2(2.6)	4.1(2.6)
			µm	78.7(1.2)	216.7(49.2)	-	295.4(28.2)	509.4(26.7)
		High pCO_2	%	71.7(11.3)	19.2(7.7)	11.3(7.9)	7.3(3.9)	4.0(3.9)
			µm	78.5(2.2)	208.1(40.6)	-	299.0(32.8)	492.1(45.3)
2016	MBP	Ambient	%	44.6(6.0)	18(2.6)	10.7(3.4)	1.4(3.4)	9.3(3.4)
			µm	80.7(0.9)	181.2(5.8)	-	248.7(13.7)	634.2(43.1)
		High pCO_2	%	54.2(5.9)	21.6(2.3)	3.5(2.4)	1.5(1.0)	2.0(1.0)
			µm	79.4(0.8)	177.4(8.0)	-	240.6(21.2)	722.6(88.4)
	Willapa	Ambient	%	37.3(6.2)	16.7(3.2)	8.2(2.2)	2.3(2.2)	5.9(2.2)
			µm	81.0(0.6)	164.5(5.2)	-	280.1(15.4)	632.4(28.7)
		High pCO_2	%	38.2(5.2)	17.8(2.7)	5.1(1.5)	4.0(0.8)	1.0(0.8)
			µm	79.7(0.7)	167.5(12.2)	-	273.7(14.5)	616.6(86.5)

CHAPTER 3 COMPARING THE GENETIC EFFECTS OF LARVAL DEVELOPMENT IN ACIDIFIED SEAWATER FOR WILD AND SELECTIVELY BRED STOCKS OF THE PACIFIC OYSTER ON THE WEST COAST UNITED STATES

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Abstract:

Many previous works have evaluated the negative effects of acidified seawater on the physiology of larval bivalves but, to date, very few studies have investigated the genetic impacts of larval development in high pCO_2 environments. Here, we created two pools of Pacific oyster (*Crassostrea gigas*) larvae from naturalized 'wild' and selectively bred stocks in the Pacific Northwest USA and reared them in ambient (~400 μatm) or high (~1600 μatm) pCO_2 seawater from fertilization through settlement to juvenile 'spat'. Using n= 1288 genomic Single Nucleotide Polymorphisms (SNPs) from samples of the larval pools at two and 22 days post fertilization we evaluated the change in allele frequencies across larval development and between ambient and high pCO_2 seawater environments. Larvae from wild stocks had ~26% more loci changing across developmental stages and >2x more loci affected by acidified culture conditions, compared to larvae from selectively bred stocks. Interestingly, the affected loci were largely exclusive to the parental stock, suggesting that disparate larval physiological processes are targets of

selection within each group or that substantial genetic differentiation has occurred between these two recently related populations. Despite these differences, functional analyses of the genes associated with loci affected by acidification stress revealed that the structure and function of cellular membranes were traits that were disproportionately affected by high pCO_2 culture in both groups. Collectively, these results provide evidence of stock-specific genetic responses to larval development and an adaptive capacity in Pacific oysters for resiliency to acidified seawater.

Keywords: Ocean acidification, Pacific oyster, *Crassostrea gigas*, Larval development, Genetics, Domestication, Functional enrichment, Poolseq

1. Introduction

The negative effects of ocean acidification (OA) on the growth and survival of larvae of the Pacific oyster (*Crassostrea gigas*) has been well researched and documented in the past decade (e.g. Kurihara et al. 2007, Waldbusser et al. 2013, Gray et al. 2017). Low pH and reduced saturation state of aragonite (Ω_{arag}) that are associated with OA, present a thermodynamic barrier to shell formation and synthesis (Waldbusser et al. 2015a) and impair larval development (Timmins-Schiffman et al. 2012, Waldbusser et al. 2015b). These impacts have often been reported to result in increased mortality by the end of larval development at metamorphosis to juvenile 'spat' (Talmage & Gobler 2010, Gimenez et al. 2018, Durland et al. *in press*). Sensitivity to acidified seawater, however, appears to be a trait in marine calcifying larvae that is genetically variable (Sunday et al. 2011, Frieder et al. 2016), trans-generationally mutable (Parker et al. 2015, Thor & Dupont 2015) and is not fixed across populations (Parker et al. 2010). The long term adaptive consequences of ocean acidification in marine communities is uncertain (Kelly & Hofmann 2013, Reusch 2014, Sunday et al. 2014) but existing variation for resilience phenotypes to OA suggests that evolutionary forces may drive future shifts in fitness optima in numerous taxa (Gaylord et al. 2015).

While OA presents an imminent threat to a diverse list of marine taxa across much of the globe, in the Pacific Northwest of the United States (PNW), it is already exhibiting significant effects on coastal ecosystems (Feely et al. 2016). In this region, seasonal upwelling brings deep water onto the continental shelf during summer months (May-October) which is naturally low in pH, owing to microbial

respiration, but which has become more strongly acidified in recent years due to OA (Feely et al. 2004). This has substantially affected the water chemistry of coastal bays (Feely et al. 2008) and negatively impacted oyster populations in the region over the last decade (Barton et al. 2012, Hales et al. 2017). In response to these changes, commercial oyster hatcheries in the PNW now routinely buffer incoming seawater to increase pH and Ω_{arag} to sustain larval growth and survival. Seawater buffering, along with additional adaptive management practices, has been effective in reducing much of the increased larval mortality that is associated with periods of high pCO_2/low Ω_{arag} in coastal seawater but it is not a complete solution for the increasingly significant effects of OA (Barton et al. 2015).

The scientific community is developing a better understanding of many aspects of the specific and acute effects of high pCO_2 seawater on larval oyster fitness traits but still very little is known with regards to the genetic impacts of OA on oyster larvae developing in acidified seawater. Pespeni et al. (2013) reported rapid and dramatic shifts in allele frequency of 30 genes in larval populations of purple sea urchins (*Strongylocentrotus purpuratus*) reared at elevated pCO_2, but a comparable analysis for oysters has yet to be conducted. For Pacific oysters, Plough and Hedgecock (2011), and Plough et al. (2016) found that overall larval survival is largely dictated by a high load of negative alleles in both inbred and wild oyster populations. Environmental stress may also magnify the effects of genetic load during larval oyster development (Plough 2012) but it is unclear how viability selection from negative alleles interacts with the adaptive selection pressures that

exposure to low Ω_{arag} seawater is likely to exert on larval oyster phenotypes and genotypes.

Pacific oysters have established several naturalized 'wild' populations in bays across Washington State and British Columbia with varying exposure to upwelling and OA conditions. This oyster species is also extensively farmed across the region but the sensitivity of different stocks of *C. gigas* to acidification stress is largely unknown. Since 1996, the Molluscan Broodstock Program (MBP) at Oregon State University has been breeding Pacific oysters for improved adult traits (growth and survival) in aquaculture field sites across the PNW (De Melo et al. 2016, De Melo et al. 2018) and has been supplying commercial hatcheries with 'domesticated' lines of broodstock for production over the past decade. It appears over 5-6 generations of breeding, that MBP has carried out passive selection for larval performance in hatchery conditions (~25^{o}C seawater, abundant food) that has unintentionally improved the growth and survival of larvae from MBP lines relative to wild controls (Durland et al. 2019 *in press*). Interestingly, this fitness advantage also appears to offer carry-over benefits in high pCO_2 culture conditions (~1600 μatm), where MBP larvae produced more and larger spat than comparable larval groups spawned from wild stocks. What is uncertain, however, is whether these differences in phenotypic performance are also reflected by differential genetic changes in these groups across larval development and whether or how high pCO_2 seawater influences overall changes in allele frequencies in larvae of wild and selectively bred oysters.

In order to investigate these questions, we conducted an experiment to analyze genetic changes in two diverse pools of larvae, spawned from either wild stocks from

Willapa Bay, WA or MBP selected broodstock. These two populations represent the most significant source of wild and selectively bred oysters, respectively, for the shellfish industry in the PNW. Larvae from these groups were reared in ambient (~400 μatm CO_2) or high pCO_2 (~1600 μatm) seawater from fertilization through to settlement. By comparing the genetic composition of larvae at 'D-hinge' veliger stage (48 hours post fertilization) with that of juvenile spat and settlement competent larvae at 22 days post fertilization, we can gain insight into the overall nature and scale of genetic changes during larval development between selectively bred stocks (MBP) and their wild counterparts. By comparing these changes between ambient and high pCO_2 seawater at both stages, we can also evaluate how acidified seawater conditions impact the genetic shifts during larval oyster development and the relative genetic sensitivity of each of these stocks to simulated OA conditions.

2. Materials and Methods

2.1 Broodstock selection and conditioning:

This experiment was conducted in 2015 as part of a broader comparison of the phenotypic effects of high pCO_2 seawater on MBP and wild oyster stocks across two spawns (years) - detailed methods are described by Durland et al. (2019 *in press*). Briefly, in the spring of 2015, n~400 oysters from the 20 top performing families from MBP (ranked based on farm yields; De Melo et al. (2016)) and n~60 wild oysters from Willapa bay, WA (collected in 2014) were transferred from the broodstock repository at the Hatfield Marine Science Center (HMSC) on Yaquina bay, Oregon, to broodstock conditioning tanks. Broodstock were provided with flow-

through seawater (~2 liters min^{-1}) and a mixed algal diet of 50/50 (by cell concentration) *Isochrysis galbana* (C-iso) and *Chaetoceros gracilis* at approximately 20-30,000 cells ml^{-1}. Water temperature was increased from ambient (11ºC) to 20ºC over the course of two weeks and maintained at this temperature for 25 additional days to facilitate gonad development prior to spawning.

2.2 Cross design and spawning:

For both MBP and wild broodstock groups, 95 single-pair matings (1♀ x 1♂) were created from available parents. MBP families were crossed in a semi-factorial fashion: one male and one female oyster from each family were individually paired with 4-6 individuals (of opposite gender) from other MBP families with a low coefficient of co-ancestry (<10%) with no reciprocation. Crossing 19 MBP families in this way yielded 95 crosses. Wild broodstock had a heavily skewed sex ratio (~10:1 female to male) and thus 95 crosses were made from 19 females and 5 males in a fully factorial mating design (every male paired with every female). For each cross, eggs and sperm were manually removed from ripe parents (strip spawned) and suspended in seawater. The total number of eggs from each female were counted and divided equally into 4-6 replicate beakers and fertilized with sperm suspensions from different males. After one hour, fertilization was confirmed visually and eggs were washed of excess sperm on a 25 μm screen. Fertilized eggs were proportionally combined to form two composite embryo pools (one each for MBP and wild) that contained approximately equal proportions of each of the 95 crosses.

2.3 Seawater manipulation and sampling:

Ambient (~400 μatm CO_2, pH = 7.9-8.1 , Ω_{arag} = 2.3-2.7) and high pCO_2 (~1600 μatm CO_2, pH ~ 7.5-7.6, Ω_{arag} = 0.9-1.2) treatment conditions were created by filling two identical 200 liter tanks with standard hatchery seawater (25 ^{o}C, 32ppt , 10 μm-filtered) and equilibrating them overnight via vigorous aeration with outside air. High pCO_2 treatment water was then vigorously aerated for several hours with a gas mixture of CO_2-stripped air and pure CO_2 to result in a final pCO_2 concentration of ~1600 μatm. Gas mixing was controlled by paired mass flow controllers (Alicat, Tuscon, AZ): one each for air and CO_2. Culture units consisted of 10-liter polycarbonate chambers (BearVault, San Diego, CA) fitted with a sealing lid, and rubber ring seal (McMaster-Carr, Santa Fe Springs, CA). No supplemental aeration was supplied to the larval rearing units throughout the experimental period.

The pH, temperature and dissolved oxygen of individual culture chambers were monitored daily: nominal pH and temperature values were measured with an Orion Star A11 pH meter (Thermo-Fisher USA) with a Ross Ultra pH/ATC triode, calibrated with NIST buffers and adjusted with a seawater standard (Batch 22, A.G. Dickson, Scripps Institution of Oceanography, USA). Dissolved oxygen was measured with a YSI 85 meter (YSI, Yellow Springs, OH, USA). Seawater samples for carbonate analysis were also obtained from seawater used to fill the chambers and after 48 hours of culture (before each water change) to account for changes in chemistry arising from off-gassing or respiration. These samples were stored in gas-tight 350 ml amber glass bottles, poisoned with 30 μl of saturated mercuric chloride ($HgCl_2$) solution and sealed with a metal crimp cap for later analysis. Samples were

analyzed at the lab of Dr. Burke Hales at Oregon State University, following the procedure outlined by Hales et al. (2005) and Bandstra et al. (2006) to obtain values for sample total dissolved carbon dioxide (TCO_2), pCO_2, and seawater pH, from which Ω_{arag} and Ω_{calc} values were calculated. A summary of seawater carbonate chemistry of the experimental treatments is found in Figure A3.1.

2.4 Larval culture:

Approximately five hours after fertilization ~200,000 embryos from MBP or wild pools were distributed to each culture replicate resulting in an effective stocking density of 20 larvae ml^{-1}. Culture units were filled with either ambient (~400 μatm) or high (~1600 μatm) pCO_2 seawater. Each treatment level (broodstock x water type) was replicated five times, for a total of 20 culture units. Water was changed every 48 hours by sieving oyster larvae on a mesh screen, filling the culture units with fresh seawater equilibrated to treatment pCO_2 levels, and re-stocking the larvae. Antibiotics were added prophylactically in order to reduce bacterial respiration in culture units that would unduly affect seawater carbonate chemistry (Waldbusser et al. 2015a). Antibiotics were alternated at each water change between a mixture of chloramphenicol/ampicillin (2 ppm and 10 ppm, respectively) and 20 ppm streptomycin to reduce the risks of development of resistant bacterial strains.

Microalgal diets were supplied once daily, starting 2 days post fertilization (dpf), beginning with C-iso (*Isochrysis galbana*) at 20,000 cells ml^{-1} and increasing the ration by 5,000 cells ml^{-1} day^{-1}. The diatom species *Chaetoceros gracilis* was gradually incorporated into the diet on day 4, starting at 5% (based on cell

concentrations) until it accounted for 50% of the algal diet by day 11, where it was maintained for the duration of the experiment. Larval densities were reduced to 10 ml^{-1} on day 2, 5 ml^{-1} on day 6 and 1 ml^{-1} at the pediveliger stage at day 14. Larval density was reduced randomly, with no selection on size, and maintained in this fashion to provide equal and optimal environments for growth and survival and limit respiratory contribution to seawater pCO_2 levels. One ambient pCO_2 culture unit suffered complete larval mortality on day 10 but all prior data points and samples were retained for analysis. After the appearance of eyed larvae (indicating a readiness to metamorphose), larvae were screened on a 240 μm sieve to retain pediveliger larvae which were subsequently induced to metamorphose by exposure to $1.8E10^{-4}$ M epinephrine for two hours (Coon et al. 1986). After this time, all larvae and newly metamorphosed juvenile 'spat' were rinsed in seawater and returned to the culture vessel with the remainder of the larval group. Metamorphosis was induced in this fashion on days 16, 18 and 20. The experiment was terminated on day 22 after the majority of potentially competent "eyed" larvae had metamorphosed.

2.5 Larval Sampling

Larval survival was estimated from counts of larvae in each culture unit on days 2, 6, 10, 14, 16 and 22. Counts were conducted by concentrating the larvae from each culture unit in a 250ml beaker with ~150 ml of seawater and obtaining five subsamples (~30-50 μl each) which were enumerated, averaged, and volumetrically adjusted to estimate total number of larvae in each culture unit. Survival estimates accounted for larvae removed for sampling and for adjustments in densities by

multiplying survivorship between sampling/counting events to obtain a cumulative survival estimate across the entire experimental period. Larvae sampled (n~200) for size and developmental stage were taken from each replicate when densities were reduced (days 2,6,10,14) and at the conclusion of the experiment. Samples were transferred to vials with 10 ml seawater and preserved by adding 200 µl of 10% buffered formalin (pH= 8.1-8.3). Developmental stages were assessed as the proportion of 'normal' D-hinge larvae on day 2, pediveliger or 'eyed' larvae after day 16 and spat on day 22. Day 2, 'normal' larvae were characterized by a straight hinge, smooth shell along the perimeter of the valve and tissue contained within the translucent shell (ASTM International 2012).

2.6 Sampling and extraction of DNA

Larval samples for DNA extraction contained ~3000 and ~200 individual oysters per culture unit for 2 and 22 dpf, respectively (Figure 3.1). The samples from day 22 were a composite of metamorphosed juvenile spat as well as eyed larvae that were retained on a 240 µm screen. From each sample, genomic DNA was extracted using a CTAB extraction method with RNAse treatment detailed by (Panova et al. 2016). DNA concentration and purity were assessed using a Qubit fluorometer (Thermo Scientific). 2bRAD libraries were prepared following a modified protocol based on Wang et al. (2012), using the BcgI restriction enzyme. Protocols for library preparation can be found in the repository at: https://github.com/E-Durland/oyster-poolseq. All individual samples (n=39) were given unique barcodes and pooled (with samples from a larger study) in sets of ~32 samples per sequencing lane (n=5).

Single-read, 50 base-pair (bp) target length sequencing was conducted on an Illumina HiSeq2500 platform in the SNP&SEQ Technology Platform at Uppsala University, Sweden.

2.7 Bioinformatics

The bioinformatic analysis of DNA sequences are described in detail at the github repository (see section 2.6). First, de-duplexed raw reads were truncated to 36 bp in length and quality filtered for reads that had <10 bp with phred quality scores < 20. The remaining reads were further filtered for adaptor sequence contaminants using BBDuk (part of the BBtools package; Bushnell, 2014) and a kmer size of 12. Cleaned, high quality reads were mapped to the reference genome (Zhang et al. 2012) using the SHRiMP software package (Rumble et al. 2009) with default mapping parameters but allowing for a maximum of three genomic alignments per read and retaining the single best alignment. Alignments were then filtered to retain only those with ≥ 30 bp matching the reference sequence. At the end of these steps, the files contained an average of 1.02×10^6 reads across ~2.6×10^5 loci for an average coverage depth of ~4x across all loci. Quality filtering and mapping statistics can be found in Table A3.1. Sequence alignment map (SAM) files were converted to tab-delimited files with read counts of each nucleotide at each locus and merged. A custom Python script was used to then remove reads with a sequencing depth of < 50 or > 1000 (Schlötterer et al. 2014) and loci with >2 alleles observed. Lastly, polymorphic loci were filtered to one SNP per 36 bp tag to limit complications arising from potential ambiguities in mapping of the reads which may erroneously result in the detection of

multiple polymorphisms in close proximity to one another. All bioinformatic analyses were conducted on computing infrastructure at the Center for Genomic Research and Biocomputing (CGRB) Core Laboratories at Oregon State University.

2.8 Detecting change in allele frequency:

A total of 8514 SNPs were retained from the filtering steps above for statistical analysis. Loci in this dataset were filtered for missing data and rare polymorphisms; keeping only loci for which three or more replicates per treatment level had reads present and starting (day 2) minor allele frequency (MAF) in at least one group was > 1%. This left 1288 SNPs for statistical modeling. The read count data for each allele at each remaining locus was analyzed with a binomial generalized linear model (GLM) with the formula: $(A_1 : A_2) \sim \beta_{Stage} + \beta_{Trt} + (\beta_{Stage}\ x\ \beta_{Trt})$ where A_1 and A_2 refer to the read counts of alternative alleles at each locus and β_{Stage} and β_{Trt} represent estimates for developmental stage (2 or 22 dpf) and treatment (seawater pCO_2), respectively. Initially, these data were modeled with broodstock type (MBP vs Wild) as a third fixed effect (and corresponding interactions) but this resulted in an overabundance of two and three-way interactions that: 1) complicated the interpretation of results and 2) effectively over-fit variation on many loci that were data poor. Analyzing the effects of development and seawater treatment on genetic changes in larval pools of each broodstock group individually allowed for a more robust detection of variation arising from these independent variables and for comparison of these changes among stocks. For each locus, in both the MBP and Wild populations, p-values were obtained from the type III sum of squares of the

GLM and mean allele frequencies of each treatment level (Broodstock(2) x Stage(2) x Trt(2); n=8) were calculated. p-values were adjusted with a Benjamini-Hochberg false discovery rate correction and determined significant at $p < 0.05$.

2.9 Mapping markers to linkage groups:

For analyses of genetic change relative to the architecture of the genome, loci were assigned to linkage groups (LGs) by comparing genomic mapping locations to markers on the published linkage map (Hedgecock et al. 2015). To accomplish this, loci in our dataset which were found on a scaffold that was also represented in the linkage map were assigned the same position as the reported mapped marker. If multiple SNPs on one scaffold were found in our dataset but only a single locus from that scaffold was represented in the linkage map, all the corresponding SNPs from our dataset were assigned the (same) genomic position to that of the mapped marker. When multiple SNP markers from the same scaffold were found on the linkage map, SNPs in our dataset were assigned the linkage position of the marker that was nearest in the scaffold. Loci existing on genomic scaffolds which were not found on the linkage map or scaffolds which appeared on multiple linkage groups were omitted from this step of the analysis. In total, n=334 markers (~25%) were mapped to linkage groups in this way. For the remainder of this paper we will refer to these as 'mapped loci'.

2.10 Functional analyses:

Lastly, for functional analyses, genomic regions within 5kb up and down-stream of each mapped locus were searched for gene annotations in the reference genome (Zhang et al. 2012), yielding 331 genes in total. This list of genes was then entered into the UniProtKB database (www.uniprot.org) which provided putative functions and gene ontology (GO) terms for 204 of the genes predicted in the annotation. Many genes are assigned with multiple, partially overlapping, GO terms resulting in 483 unique GO IDs for this gene set. The entire gene list, and their associated GO terms, were then analyzed with a gene score resampling procedure (GSR) in ErmineJ (Gillis et al. 2010), which compares GO terms associated with genes near markers that were significantly changed by developmental stage or seawater treatment in each group to GO terms stemming from genes in proximity to markers that were unchanged. This technique does not strictly discriminate on p-value thresholds (e.g. $p<0.05$) but, instead, incorporates p-values as a continuous measure of significance and evaluates functional over-representation of specific GO terms from the entire dataset. We conducted GSR analyses for p-values from each of the main effects ('Stage' and 'Trt'; Table 3.1) and include the results from these analyses here. We also performed the test on the p-values for interaction effects ('Stage*Trt') but, in this case, no functional categories were over-represented and so results were not included. We additionally analyzed the GO term list with REViGO (Supek et al. 2011) to visualize the contrasts in functional differences of putative genes associated with only those loci which were determined by the linear model

(above) to be significantly affected by development and seawater treatment (main effects) within MBP and wild broodstock groups.

3. Results

3.1 Survival and development

At 48 hours post fertilization, larval survival was not significantly different between MBP or wild broodstock groups or between ambient and high pCO_2 seawater (ANOVA, $p > 0.05$). The proportion of fully formed 'normal' D-hinge larvae at day 2, however, was ~13 % lower in high pCO_2 seawater (ANOVA $p < 0.05$; Fig. A3.2). Larval growth and survival through the remainder of veliger stages (to day 16), however, was statistically uniform between all cultures. By day 22, after settlement and metamorphosis, MBP groups had significantly more spat than wild groups (ANOVA, $p < 0.05$), but overall survival of all larvae and spat was not different between MBP and wild. High pCO_2 seawater did not have any significant effect on growth, survival or metamorphic success in either group (ANOVA, $p > 0.05$; Fig. A3.2). For detailed reporting on the phenotypic effects of high pCO_2 seawater across all developmental stages we direct readers to Durland et al. (2019 *in press*).

3.2 Genetic effects of larval development and seawater treatment

Among the 1288 SNPs available for analysis, 66% (n=855) exhibited no significant change in allele frequency owing to larval development or seawater treatment in either MBP or wild larval groups. The remaining ~34% of SNPs were

found to be significantly different between larval developmental period (Stage), seawater pCO_2 treatment (Trt), both (Stage + Trt), or an interaction between these factors (Stage * Trt) in one or both groups. Overall, the assignment of SNPs by category of change into each group (e.g. 'none', 'Stage' etc.) was found to be statistically significant $X^2(df = 1, n = 1288) = 9.53, p < 2.2x10^{-16}$ and wild larval pools had ~1.7x more SNPs with significantly altered allele frequencies (n=330 or ~25.6% of the total) than MBP larvae (n=190 or ~14.8% of the total; Table 3.1, Figure 3.2).

For both broodstock types, larval development had the greatest impact in total number of SNPs that changed in frequency with 107 and 145 SNPs in MBP and wild groups, respectively, displaying treatment-independent changes between 2 and 22 dpf which represents a ~26% increase in the number of loci affected by development in wild larval groups. The effects of high pCO_2 seawater treatment on genetic changes in larval groups, however, were even more distinctly different between the two broodstock groups. Wild larval pools had n=185 SNPs with distorted allele frequencies owing to high pCO_2 seawater compared to n=83 found in MBP groups, representing a ~2.3 fold increase. Seawater treatment effects also additively overlapped with 'Stage' effects (Stage + Trt) more so in Wild (n=65) than MBP (n=7) groups but interactive effects between development and treatment (Stage * Trt) were approximately equally abundant in MBP (n=53) and Wild larvae (n=56). Interestingly, among all the significantly affected loci, there were very few that were shared between the groups (Table 3.1). Changes in allele frequency are visualized in Figure 3.3, comparing the average allele frequency (among replicates) of each SNP at

2 and 22 dpf in ambient conditions (Fig. 3.3A&B), as well as genetic differences between day 22 populations in each broodstock group reared in ambient and high pCO_2 conditions (Fig. 3.3C&D).

The assignment of loci to linkage groups via scaffold association resulted in a significant reduction of the size of the dataset but the remaining 334 SNPs, for which genomic position could be approximated, provide moderate coverage on each of the 10 linkage groups. Manhattan plots were used to assess the scale of genetic change of each of these factors across the genome in both MBP and wild larval groups (Figure 3.4). These plots highlight the increased abundance and significance of genetic changes in wild groups, relative to the domesticated MBP controls, over larval development in general (Fig. 3.4A&B) and when cultured in high pCO_2 seawater (Fig. 3.4C&D). Additionally, significantly affected loci occur across the genome in a relatively homogenous fashion, without any clear clustering in specific regions. It is worth noting that the way in which these markers were assigned positions on LGs results in several cases where numerous markers occupy the same approximate genomic position, based on scaffold association on the reference genome (see methods). Therefore these instances, such as the tail-end of LG 7, may seem to represent an outlier genomic region under selection, but should be interpreted with caution.

3.3 Functional enrichment

Functional enrichment analyses are somewhat under-powered when using datasets of this scale and nature but, nevertheless, these procedures successfully identified several GO terms and processes which were overrepresented in the genes near mapped loci which were determined to be significantly affected by developmental stage or seawater pCO_2 treatment (Table 3.2). In MBP larvae, significant effects of larval development were associated with GO terms for protein modification (GO:0036211), helicase activity (GO:0004386), anion binding (GO:0043618), lipid binding (GO:0008289) and organo-nitrogen compound metabolic processes (GO: 0016817). In wild groups, larval development was over-represented by only two discrete functions: cell adhesion (GO:0016021) and 'integral component of membrane' (GO:0016021), both of which were only identified by these analyses with marginal significance ($p > 0.05$).

In contrast with larval development, seawater treatment effects seemed to have more consistent functional effects in MBP and wild larvae. In both groups, the overrepresented functions were associated with membranes: phospholipid binding (GO:0005543) in MBP larvae, two different types of membrane components in wild larvae (GO:0044425 and GO:0016020) and integral component of membrane (GO:0016021) was shared by both MBP and wild groups as a significant function. It is worth noting that the over-represented functional categories putatively affected by larval development were generally broad, with high multi-functionality (MF) scores (0.217-0.901) and relatively few genes assigning to each group (n=2-11). This is contrasted by the results analyzing the same set of genes and GO terms with p-values

for change owing to seawater pCO_2, which yielded categories associated with membrane function that had higher specificity (lower MF scores; 0.007-0.209) and more gene assignments to each (n=2-52; Table 3.2). This is an indication of the relative strength of the results from these analyses for each component of detected genetic effect.

Lastly, GO terms associated with genes found in association with significantly altered markers (as opposed to the entire GO dataset) were compared between MBP and wild groups by visualizing functional similarity with REViGO (Supek et al. 2011). These figures represent the 'biological processes' category of the associated GO terms for these genes, and suggest the breadth of change potentially incurred by general larval development (e.g. 'multicellular organism development; Fig. 3.5A) and the somewhat more specific functions impacted by seawater pCO_2 (e.g. ATP coupled proton transport; Fig 3.5B). As noted, these functional analyses are designed for gene expression data of much greater size and with tighter correlation to biological processes so they should be interpreted with caution in the context they are applied here: they provide interesting visual contrasts but cannot robustly link the changes in allele frequency observed in these larval populations to specific physiological processes.

4. Discussion

These results contribute to a growing body of literature examining the genetic changes that oysters undergo during larval development (Plough 2011, Plough 2018) and the genetic consequences of ocean acidification in marine invertebrate

populations (Hoffmann & Sgrò 2011). The results we present here highlight the complexity of the interaction between genetics, environmental stress and larval development that for oysters, as in many marine invertebrates, encompasses several distinct life stages and morphologies. Our results indicate that the relative genetic impacts of larval development, as well as the changes in genetic composition of larval pools in response to acidified seawater, are significantly reduced in selected lines of oysters when compared to their wild counterparts reared under the same hatchery conditions. The contrast in the nature of genetic changes in these groups also provides an interesting view into the potentially broad network of physiological functions which are crucial to successful larval development overall as well as the potentially more specific set of functions that are affected in high pCO_2/low Ω_{arag} environments.

4.1 Genetic effects intrinsic to larval development

Larval development was a strong driver of changes to the genetic composition of larval pools in this study, uniquely accounting for ~56% and ~43% of all the markers significantly changed in MBP and wild larvae, respectively ('Stage' effects in Table 3.1). Interestingly, larvae from wild stocks had ~26% more markers significantly affected by larval development alone when compared to those of domesticated groups; furthermore, the markers affected by developmental 'Stage' in each group were not substantially shared between them (only n=4 in total; Table 3.1). The incongruity between the SNPs affected by development in MBP and wild groups, however, suggests that the genetic impacts of larval development in MBP and wild

groups are largely dissimilar - a surprising finding which, to our knowledge, has not been reported elsewhere for oysters or any other species.

4.2 Genetic load and viability selection

The observation of substantial genetic changes occurring during larval development in oysters is not novel; previous studies have reported the presence of a high genetic load in Pacific oysters which is associated with high mortality rates in early life stages (~99%; Plough 2016). It is difficult, however, to directly compare the results from this study to those of Plough (2011, 2018), and Plough et al. (2016). These previous researchers measured the effects of viability selection based on several quantitative trait loci exhibiting viability selection (termed 'vQTL') through distortions in Mendelian segregation ratios in individually genotyped progeny from a relatively smaller group of full-sibling oyster families (n=2-4). In this study, we utilize many more oyster families (n= 95 x 2) and a larger set of genetic markers but the use of pooled DNA samples obscures both parentage and individual genotypes which limits our ability to infer mechanisms of genetic causes of larval mortality.

We can expect that genetic load and viability selection is influencing changes in the genetic composition of the larval pools in this study but, in pooled populations, the net change in allele frequency at a specific QTL is a function both of strength of selection and abundance in the population as a whole. For loci that exhibit significant changes in allele frequency, it is difficult to distinguish between the effects of strong viability selection for vQTLs that vary between discrete oyster families (as observed in Plough et al. 2016) and less intense directional selection for alleles which are

common to many families in the pool. When comparing changes between MBP and wild larvae, group-level differences in minor allele frequencies (MAF) for individual SNPs may also affect the detection of change when comparing pooled DNA samples, and possibly account for a portion of the lack of shared 'Stage' SNPs between groups in this dataset. Generally, however, there is not a large enough contrast in MAF between MBP and wild groups (Figure A3.3) that can substantially explain the observed disparity in genetic changes over larval development between MBP and wild larval groups.

4.3 Changes in the context of genomic architecture

Mapping individual SNPs into linkage groups allows for an evaluation of the scale of genetic changes relative to the architecture of the genome (Figure 3.3 A&B). This visualization not only reinforces the interpretation that the genetic impacts of larval development are substantially greater in wild larvae but also suggests that outlier loci in each group (determined by genome wide thresholds) appear to be dispersed haphazardly across each of the 10 linkage groups. There are no genomic regions that appear to possess an overabundance of outlier loci that are common to both MBP and wild groups that would indicate similar genetic changes taking place. As stated previously, with this dataset we cannot distinguish the nature of genetic change for individual markers or determine whether the overall changes are indicative of viability selection or other selection pressures (e.g. balancing, directional etc.). The fact that ~95% of the 'Stage' SNPs in this dataset are group-specific, however, suggests that either vQTLs for MBP and wild groups are largely distinct or that

physiological processes driving changes in allele frequency differ between the groups.

Larval survival has been demonstrated to be a significantly heritable trait in Pacific oysters (Ernande et al. 2003) so it is possible that the routine and repeated passive selection for improved larval performance in the hatchery (warm 25^{o}C seawater, abundant food) may have resulted in MBP genotypes which are more adapted to these conditions (i.e. domestication; Taris et al. 2006, Taris et al. 2007). Wild populations, on the other hand, possess a greater diversity of genotypes (Camara 2011) which are perhaps advantageous to more variable larval conditions experienced in natural settings (Grangeré et al. 2009, Hales et al. 2017). These putative domestication effects are a plausible explanation to account for the reduced and disparate genetic impact of larval development in MBP larvae. It is also possible, however, that the structured breeding design of MBP, which strictly limits inbreeding accumulation across generations, has reduced the overall mortality attributable to high genetic load or even purged some deleterious alleles.

Traditional theories of genetic load in natural populations predict that purging of negative mutations is favored by inbreeding (Crnokrak & Barrett 2002, Agrawal & Whitlock 2012) but these models are largely predicated on the assumption that mutations are rare and recessive and that breeding is random within the population. For Pacific oysters, by contrast, mutations are frequent, many deleterious alleles are additive or dominant in nature (Plough et al. 2016) and wild populations have been characterized as having a chronically reduced effective population size and ‘sweepstakes’ reproductive success (Hedgecock & Pudovkin 2011). These genetic

factors and population dynamics have been proposed as the primary mechanisms by which high genetic load is maintained in this species (Plough 2016). While overall rates of mutation in oysters are not likely to have been affected by selective breeding, the reduction of inbreeding in MBP stocks may have distributed the load more evenly among families which may reduce the selection strength of individual vQTLs and, perhaps, may allow for some degree of purifying selection in these stocks by reducing the effects of genetic drift. These two hypotheses - domestication and reduced genetic load - are not mutually exclusive and both are reasonable ways to explain the consistently improved phenotypic performance of MBP larvae (Durland et al. 2019 *in press*) as well as the reduced genetic change over larval development found in MBP groups. Further research investigating the genetic load of oysters in wild populations and structured breeding programs would provide useful insights to clarify the relative influence of mutation rate and population dynamics in the maintenance of negative adaptations in these species. Additionally, the broadly disparate genetic changes over larval development between these recently related stocks warrants an evaluation of the efficacy and stability of QTLs in oyster breeding to improve traits by marker assisted selection (e.g. Wang et al. 2016).

4.4 Genetic changes in oyster larvae in response to acidified seawater

In this experiment high pCO_2 seawater did not have significant effects upon total larval survival, either at day 2 or after settlement to spat stage at day 22 post fertilization (Fig. A3.2). Numerous other studies have reported negative effects of high pCO_2/low Ω_{arag} conditions on larval growth and survival during early stages (e.g.

Kurihara et al. 2007, Waldbusser et al. 2015a) and we observed an increased proportion of abnormal larvae and smaller average size of shelled larvae at 48 hours post fertilization in low Ω_{arag} treatments, which is consistent with these previous findings (Fig. A3.2). Relatively few studies, however, have evaluated the long-term impacts of acidified seawater on larval survival, growth and development through to juvenile spat stage. In an associated study, we found that the chronic effects of low Ω_{arag} on net larval survival and metamorphosis by day 22-24 post fertilization was highly variable and likely influenced by additional parameters that are difficult to predict or control (see discussion in Durland et al. 2019 *in press*). In the larval populations for this experiment (2015), although high pCO_2 seawater did not negatively affect the net average performance metrics of larvae from either broodstock group as a whole, the genetic changes in both groups suggest that high pCO_2 culture did result in significant, but different, changes to the genetic composition of both MBP and wild larval groups.

Similar to the genetic effects of larval development, high pCO_2 seawater also had a markedly greater impact on larval pools from wild than MBP broodstock yet few of these markers were shared between them (Table 3.1, Figure 3.1). The SNPs which were significantly distorted between seawater treatments in each group are also more or less evenly distributed across the genome, with no clear consistency between MBP and wild larval groups nor specific outlier regions of the genome (Figure 3.4 C&D). The effects of seawater treatment on larval survival also interacted extensively with larval developmental processes, in both compounding and contrasting ways. In wild larvae, ~1/3 of all SNPs affected by high pCO_2 seawater

were also additively impacted by developmental stage (Stage + Trt; Table 3.1, Fig. 3.2) meaning that both effects altered allele frequency significantly and in a uniform direction. This type of interaction is consistent with the findings of Plough (2012), who reported that, during larval oyster development, environmental stress (diet quality in that study) doubled the number of detected vQTLs and increased both strength of selection and dominance of the negative alleles. In MBP groups, a very small number of SNPs were affected by these processes in tandem (n=7), which also suggests both a reduced genetic load and adaptation to hatchery conditions in these selected lines.

Interestingly, in addition to the additive effects of pCO_2 stress and larval development, there was also an abundance of loci which exhibited interactive effects between these two processes (Stage * Trt; Table 3.1, Fig. 3.2). Positive interactions indicate a consistent and magnified effect of pCO_2 stress on allele frequency distortion across larval development but among the loci which showed significant interactive effects, ~89% and ~75% in MBP and wild groups, respectively, had negative interactions. For these loci, the differences in mean allele frequency between ambient and high pCO_2 environments at day two were reversed by day 22 (Fig. A3.4). This is a curious finding which implies that there is a disconnect between some of the genetic components of larval survival early in development and those contributing to later larval fitness under acidified conditions. Just as we found that larval oysters express phenotypic impacts to acidified seawater that are stage specific (Durland et al. 2019 *in press*), it also appears that genetic effects of these conditions are not homogeneous across larval development or life stages.

Conceptually, this is similar to the findings of Ernande et al. (2003) who demonstrated that larval growth and survival are substantially heritable traits but that they are genetically independent from those in juvenile stages. In this case, these negative interactions indicate that, under acidification stress, there may also be some antagonistic selection pressures between developmental stages that raise interesting questions regarding the dynamic relationship between genetics and fitness traits in larval oysters. More broadly, these results suggest caution is warranted when evaluating the phenotypic or genetic impacts of environmental stressors (such as OA) on larvae at a discrete time point or developmental stage and extending the scope of inference to overall performance of organisms, species or populations (e.g. Wittmann & Pörtner 2013).

4.5 Functional analyses of loci under selection during development

Although we found that the majority of loci that were significantly changed in either MBP or wild larvae were group-specific, it is possible that specific changes at discrete loci nevertheless relate to selective pressures on a similar physiological function. In order to investigate this, we used functional enrichment analyses to examine if genes associated with mapped loci were over-represented by one or more functional categories. In general, the genes we found to be associated with mapped loci that were affected by larval development were physiologically broad in scope. For MBP larvae, stage effects spanned multiple functional categories: protein modification, helicase activity, metabolic processes and anion and lipid binding (Table 3.2). The breadth of these functions, coupled with their high MF scores and

low number of genes for each category, suggests that selective pressures during larval development were applied to numerous, loosely associated aspects of larval physiology. The lack of strong over-representation of GO terms in wild larvae supports this concept: despite the markedly greater and more significant overall genetic impact in these groups (Table 3.1, Fig. 3.4), no functional categories were found to be disproportionately more impacted than any other (at $p < 0.05$). This is reflected in Figure 3.5A - far more functional groups appear to be affected in wild larvae (warm colors) than in MBP larvae (cool colors) and they do not exhibit clear clustering to common functions. This finding is similar to those of Pespeni et al. (2013) who reported similar temporal genetic changes in the gene expression of sea urchin larvae yet these authors were unable to significantly identify discrete functional categories to which they could be attributed. Larval development is a complex process which involves thousands of genes in bivalves (Huan et al. 2012, De Wit et al. 2018), so it is perhaps not surprising that it is difficult for functional analyses to narrowly define a specific subset of GO categories in this context.

4.6 Functional analyses of loci under selection in acidified conditions

In contrast to the broad effects of larval development, high pCO_2 seawater appeared to have much more specific and consistent effects on both MBP and wild groups. In this case, aspects of membrane function and structure appear to be strongly over-represented in both larval populations: phospholipid binding, and membrane structure and function were strongly identified in these analyses with much lower MF scores, many more genes assigned to each category, and, in the case

of wild larvae, much more significant p-values (Table 3.2). This finding is surprisingly uniform considering the observed disparity in the changes of specific loci between MBP and wild larvae. Although membranes are a diverse component of the structure of cells that interact with myriad physiological processes, they are predicted to play an especially important role for bivalve larvae reared in acidified environments by maintaining ion homeostasis within cells (Pan et al. 2015, Frieder et al. 2018) as well as modifying Ω_{arag} in the extracellular space at the site of calcification (Ramesh et al. 2017, De Wit et al. 2018). These results suggest that genes contributing to membrane structure in MBP and wild larvae in this study may have both been subjected to selection pressures in high pCO_2 seawater, but that the response to selection was stronger in wild larvae (Fig. 3.3B). Acidified environments have diverse effects on larval physiology in marine calcifiers (O'Donnell et al. 2010, Dineshram et al. 2012, De Wit et al. 2018) so it is surprising that these data identified a plausible and discrete set of functions that were affected by high pCO_2 seawater conditions. These findings are also strikingly similar to those of Pespeni et al. (2013) who determined that genes related to ion regulation and membrane structure are targets of selection in urchin larvae reared in high pCO_2 conditions. As stated previously, GO analyses of this type are under-powered for data of this nature and, consequentially, these results should be interpreted as tentative and further work investigating the genetic and transcriptomic changes in oyster larvae during development and under acidification stress should be undertaken to more confidently identify causative mechanisms for survival or mortality at different life stages and between populations.

5. Conclusions

We have found that the genetic changes in larval oyster populations across developmental transitions are profound, abundant genome-wide, and likely associated with numerous aspects of larval physiology. This result is broadly consistent with the 'genetic inviability' hypothesis previously proposed by Plough et al. (2016) but in this work we additionally demonstrate that selectively bred stocks (MBP) showed significantly less genetic change than wild counterparts. This is evidence to suggest that MBP lines have been either domesticated to hatchery conditions or that these stocks have a reduced load of negative alleles in the breeding population. Additionally, the changes in allele frequencies taking place in each group were largely dissimilar, indicating either substantial differentiation between these recently related populations or significantly distinct targets of selection pressures during larval stages.

Despite no significant impact of high pCO_2 seawater on total larval survival or settlement success in this experiment, culture in acidified seawater did, nevertheless, have significant impact on the genetic makeup of larval groups by the time of settlement and metamorphosis. Overall, acidified seawater resulted in >2x more changes to genetic markers in wild than MBP larvae, potentially indicating that selected lines are more genetically resilient to the net stresses of acidified seawater in hatchery settings. The predicted genes in association with markers affected by high pCO_2 seawater were disproportionately attributable to functional categories concerning membrane structure and function. This finding reinforces the hypothesis that the regulation of ionic gradients across cellular membranes is a critical

physiological function necessary for the survival of larval oysters in acidified seawater. Larval oyster development in high pCO_2 seawater results in significant changes to the genetic composition of larval groups, relative to control conditions, but specific changes are likely to be genotype-dependent and variable based on genetic background of parental stocks. The complex interaction between the genetic changes during larval oyster development and those that result from acidification stress also complicate predictions of the heritability of these traits in selectively bred stocks and the adaptive capacity of wild oysters in natural settings. Taken together, these findings suggest that genetic marker-based approaches (QTLs) for predicting breeding values for traits, such as fitness in high pCO_2 seawater, may be ineffective and have limited applicability among different populations. The genetic changes in larval oysters during development appear to be complex and dynamic and further work is necessary to resolve how these changes are affected by the myriad abiotic stressors which impact larval fitness.

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Figures and tables

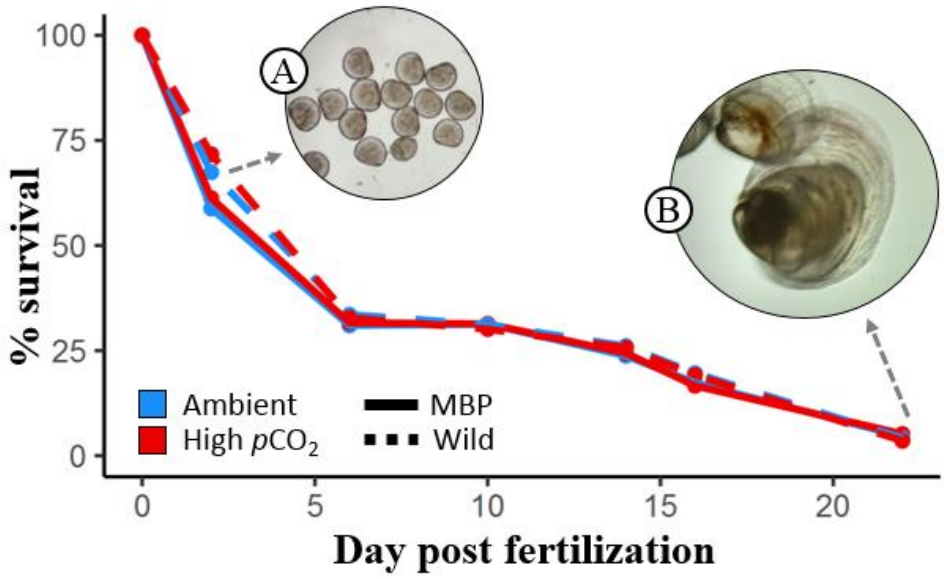

Figure 3.1 Survival and sampling points. Total survival for larvae from MBP (solid lines) and Wild (dashed lines) groups reared in ambient (blue) and high (red) pCO_2 seawater. Samples of D-larvae at 2 dpf (A) and spat and eyed pediveliger larvae at 22 dpf (B) were obtained for DNA extraction and sequencing.

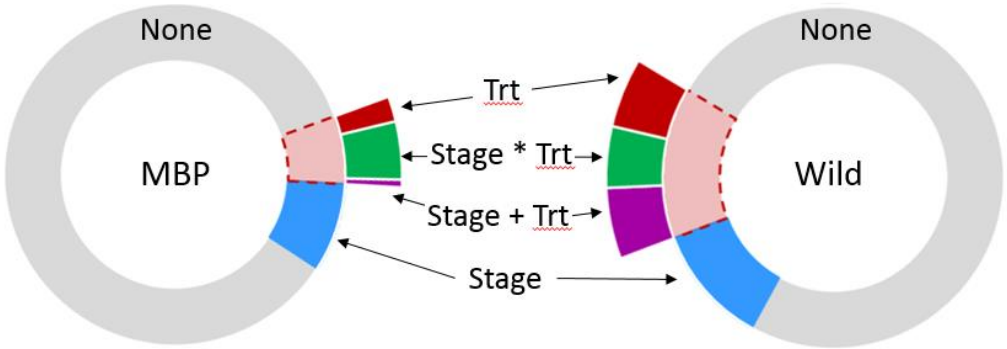

Figure 3.2 Graphical representation of SNPs by category of change for MBP and wild larval groups presented in Table 3.1.

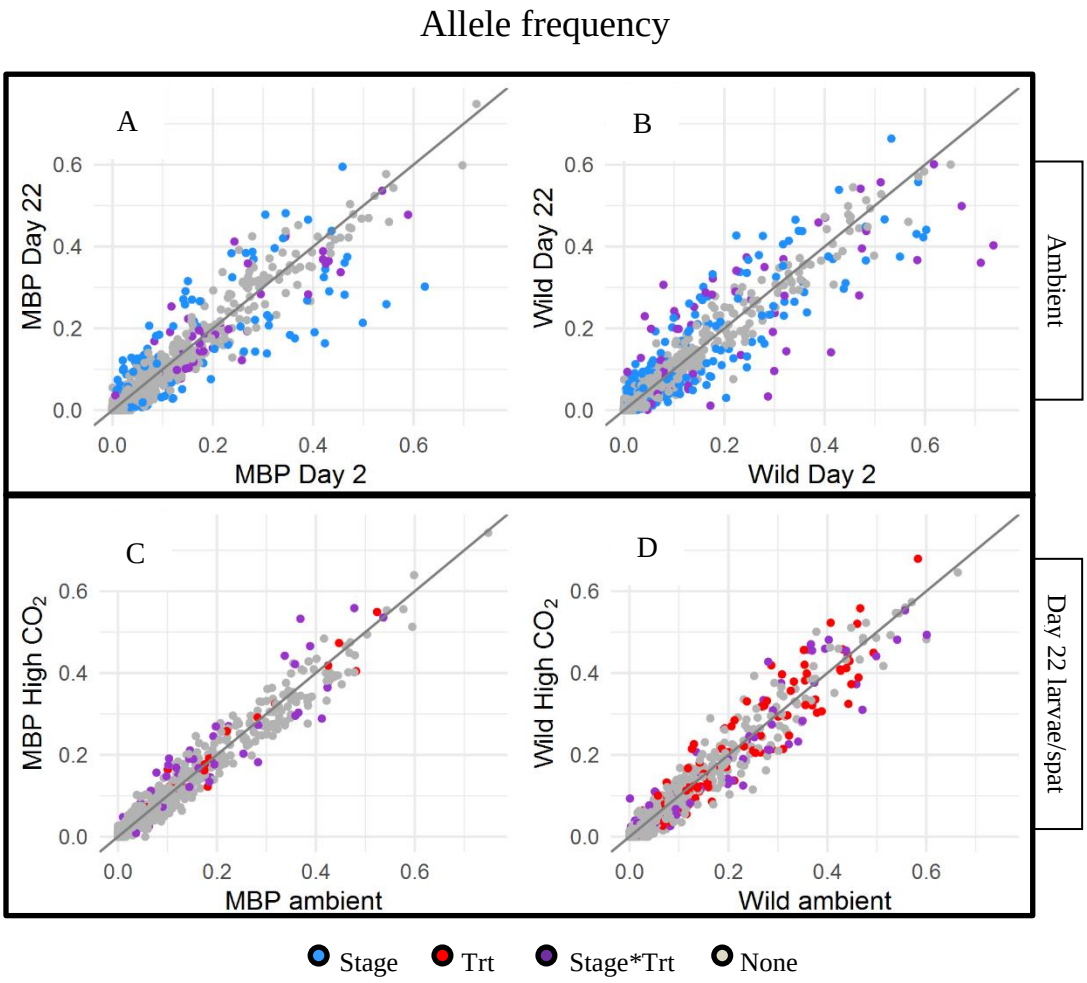

Figure 3.3 Changes in allele frequency owing to development and seawater pCO_2. A comparison of minor allele frequencies in MBP (A) and wild (B) larval groups between day 2 and 22 in ambient conditions and between spat/larval populations (at day 22) for MBP (C) and wild (D) reared in ambient and high pCO_2 treatments. Point color corresponds to significant changes ($p < 0.05$) based on effect: blue for 'Stage', red for seawater treatment ('Trt') and purple for an interaction of the two (Stage*Trt). Grey points represent SNPs for which no significant change was detected.

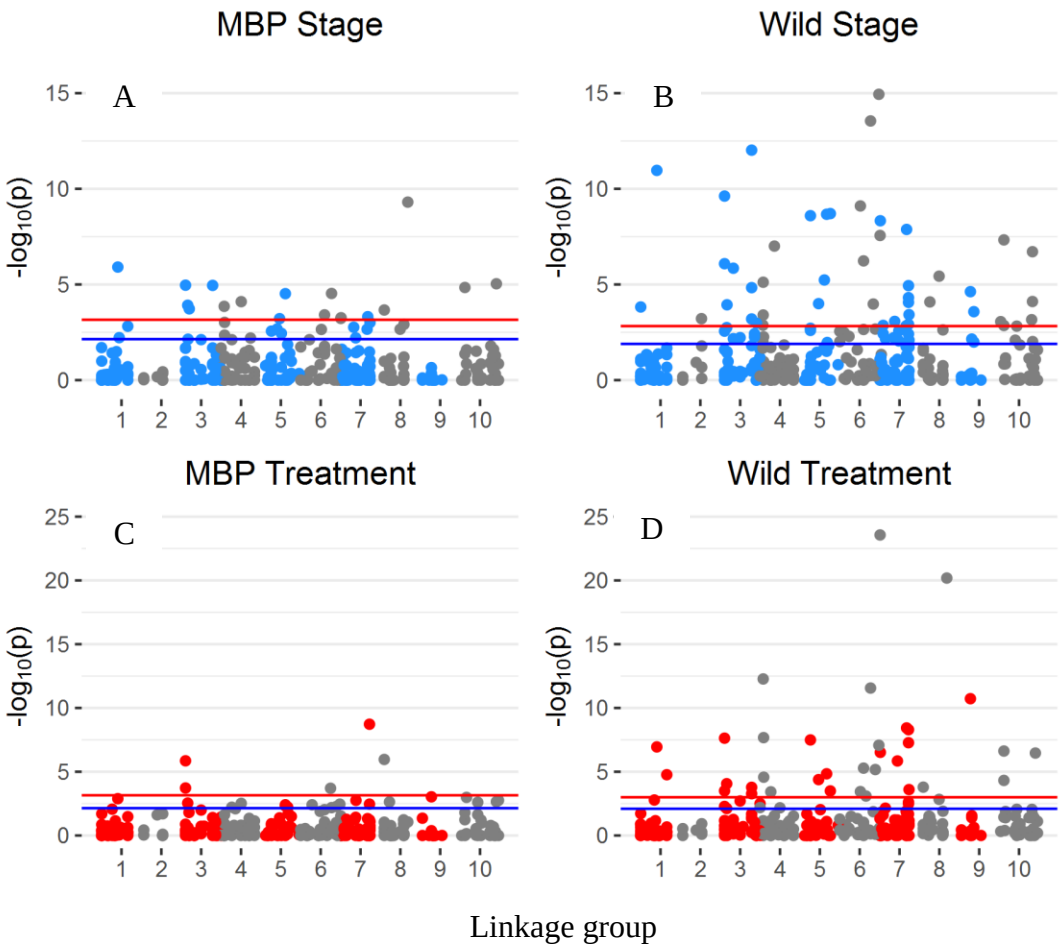

Figure 3.4 Manhattan plots. Mapped SNPs (n=334) and the –log10 (p-value) of significance for change in allele frequency owing to developmental 'Stage' (blue; A&B) and seawater treatment (red; C&D). Red and blue horizontal lines on each plot reflect genome-wide significance thresholds of p=0.05 and p=0.01, respectively, after a Benjamini-Hochberg correction to the raw p-values.

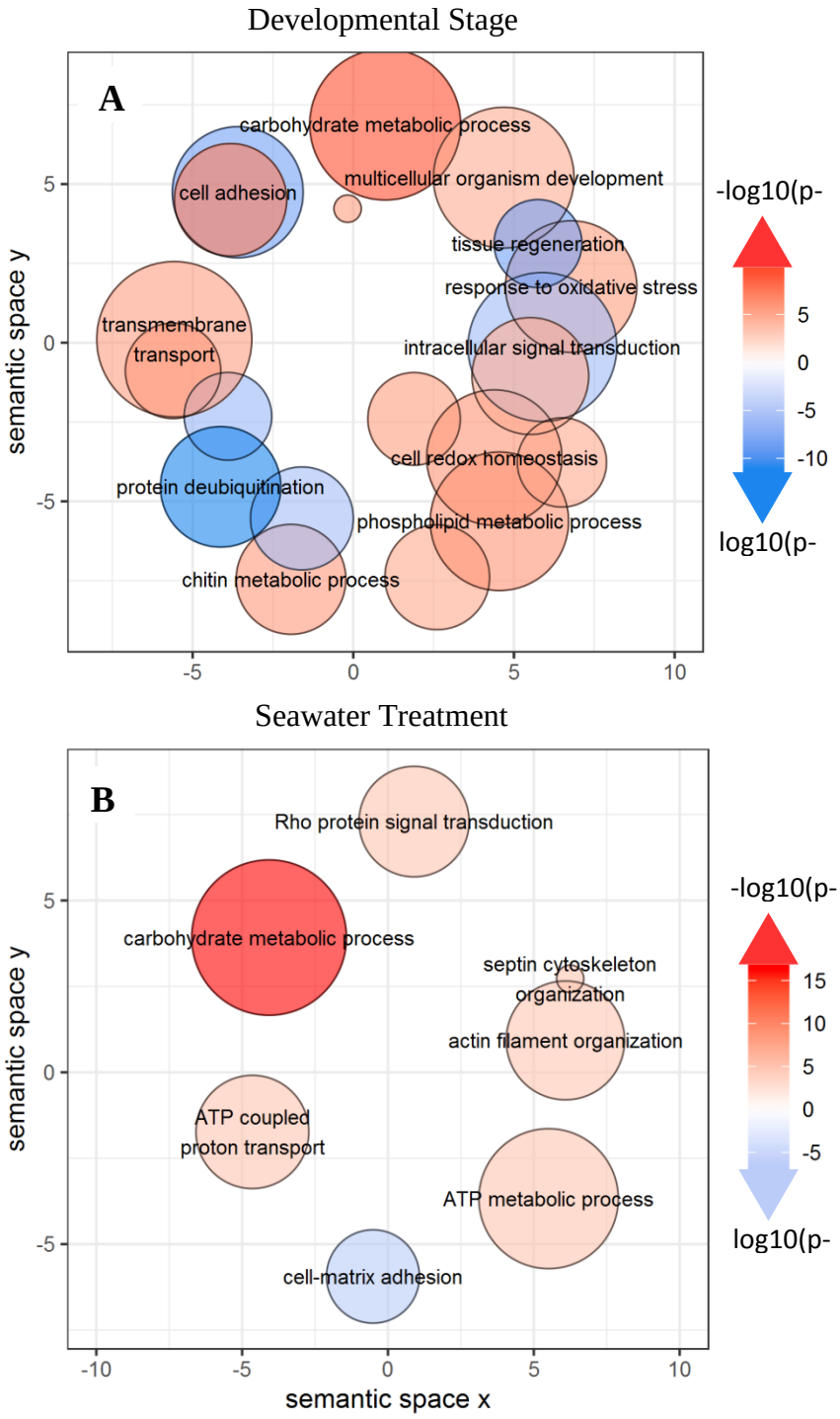

Figure 3.5 REViGO Scatterplot comparing the enriched GO clusters from genes associated with loci altered by developmental stage (A) and seawater treatment (B) in MBP and Wild larval groups. P-values were log(10) transformed for MBP groups, yielding negative integers and –log(10) transformed for Wild groups, resulting in positive values. Scaling the numeric range to a blue to red color scale results in 'warmer' colors assigned to clusters arising from Wild groups and 'cooler' colors stemming from MBP.

		Wild					
		None	Stage	Trt	Stage + Trt	Stage * Trt	Total
MBP	None	855	131	46	40	26	**1098**
	Stage	60	4	9	15	19	**107**
	Trt	13	4	3	3	0	**23**
	Stage + Trt	4	0	0	1	2	**7**
	Stage * Trt	26	6	6	6	9	**53**
	Total	**958**	**145**	**64**	**65**	**56**	**1288**

Table 3.1 Cross-tabulation of the number of SNPs found significantly different ($p < 0.05$) by effect type in MBP and wild larval oyster groups. 'Stage' refers to changes detected between developmental states of 'D-larvae' at day 2 and eyed larvae and spat at day 22. Treatment (Trt) refers to changes detected between ambient and high pCO2 seawater environments.

Table 3.2 Functional enrichment. Over-represented functional groups identified by Gene Score Resampling (GSR) in ermineJ (Gillis et al. 2010) with a p-value cutoff of < 0.05. Number (#) of genes indicates the total amount of genes which were assigned to this group. Multi-functionality (MF) is a measure, from 0-1, of estimated functional specificity, with higher values being more broad and smaller values more specific. 'MF p-value' is a scaling metric taking into account the adjusted p-value in conjunction with multi-functionality. Gene ontology ID's reported are the primary, but not exclusive, ID associated with this function. A comprehensive list of GO ID terms and genes assigned to each is found in the supplementary information. **These p-values are only marginally significant (p>0.05)*

Type	Group	Name	Gene Ontology ID	# Genes	p-value	Multi-functionality (MF)	MF p-value
Stage	MBP	protein modification process	GO:0036211	2	0.031	0.434	0.022
		helicase activity	GO:0004386	2	0.032	0.217	0.064
		anion binding	GO:0043168	11	0.039	0.849	0.083
		lipid binding	GO:0008289	2	0.044	0.243	0.034
		organo-nitrogen compound metabolic process	GO:0016817	7	0.049	0.901	0.055
	Wild	cell adhesion	GO:0007155	2	0.051*	0.143	0.048
		Integral component of membrane	GO:0016021	43	0.087*	0.016	0.287
Treatment	MBP	phospholipid binding	GO:0005543	2	0.042	0.209	0.040
		Integral component of membrane	GO:0016021	52	0.049	0.011	0.313
	Wild	membrane part	GO:0044425	45	4.10E-03	0.027	0.294
		integral component of membrane	GO:0016021	44	6.00E-03	0.014	0.284
		Membrane	GO:0016020	48	9.50E-03	0.007	0.343

CHAPTER 4 PATTERNS OF GENETIC CHANGE AND TEMPORALLY BALANCED POLYMORPHISMS DURING LARVAL DEVELOPMENT OF THE PACIFIC OYSTER (*CRASSOSTREA GIGAS*) INFERRED FROM POOLED DNA ANALYSES

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Abstract

For marine shellfish species, high fecundity is frequently accompanied with low rates of survival through larval stages to recruitment. For oysters, much of this early mortality has recently been attributed to genetic factors that appear to render a large proportion of larvae genetically incapable of surviving through metamorphosis to juvenile stage. Previous studies of viability selection in oysters have largely focused on the 'outcome' of lethal alleles, resulting in distorted genotypes of juveniles relative to Mendelian expectations but temporal patterns of genotype-dependent mortality during larval development remain largely unresolved. In this study we reared a genotypically diverse pool of larvae from fertilization to settlement in replicated laboratory conditions and examined consensus changes in marker allele frequency at six points across larval development. By sequencing n=847 genome wide polymorphisms from pooled larval DNA samples we observed patterns of genetic change that were much more dynamic than previously indicated. Approximately 60% of all loci exhibited significant distortions in allele frequency

across development and ~25% exhibited significant shifts in allele frequencies that altered in direction between developmental stages. Collectively, ~62% of all loci changing during larval development had shifts in mean allele frequency which were temporally significant but statistically indistinct comparing starting and ending points alone. These results imply that temporally balanced patterns of viability selection target more than one lethal allele at a locus and genotype-specific fitness is temporally heterogeneous. This evidence for balancing selection may help to explain how a high load of lethal mutations are maintained in oyster populations.

Keywords: Pacific oyster, *Crassostrea gigas*, Larval development, Genetics, Poolseq, Balancing selection.

1. Introduction

Oysters are among the most highly fecund animals in the world but also typically experience very high levels of mortality during the larval phase, early in their life cycle (Llorda 2002). There are many exogenous sources of mortality in natural settings, both biotic and abiotic, that could affect larval survival and recruitment success (Baker & Mann 1992, Barnes et al. 2010). Laboratory and hatchery settings remove many of these threats and are optimized for larval performance but, nevertheless, oysters raised there routinely experience very low rates of larval survival from fertilization through settlement and metamorphosis to juveniles, termed 'spat' (e.g. Ernande et al. 2003, Durland et al. *in press*). Numerous studies have indicated that larval survival in oysters is strongly influenced by a high genetic load, or abundance of negative mutations in the genome, resulting in significant genotype distortions in post-larval oysters, relative to Mendelian expectations (e.g. Hu et al. 1993, Bierne et al. 1998, Launey & Hedgecock 2001, Plough & Hedgecock 2011).

Initially, evidence for genotype-dependent mortality was interpreted through the lens of inbreeding depression, as oyster families used for experimentation were frequently the product of small populations or inbred crosses. More recently, however, Plough et al. (2016) conducted similar analyses with randomly bred 'wild-type' Pacific oysters (*Crassostrea gigas*), collected from naturalized stocks in Dabob Bay, WA and found that there was no substantial reduction in genotype-dependent mortality in these outbred populations. Additionally, these results demonstrated that

loci exhibiting effects of viability selection vary substantially across oyster families and negative alleles are potentially abundant in naturalized populations. It was estimated that, for naturalized stocks of *C. gigas*, an average of 11-19 deleterious alleles per oyster 'family' (full-sibling cross) render >95% of all larvae 'genetically inviable'. In traditional examples, lethal or negative alleles are subject to purifying selection which purges these allelic variants from the population (Crnokrak & Barrett 2002). For oysters, how such an apparently high level of lethal mutations are maintained in natural populations remains somewhat uncertain but the reported high mutation rate in this species (Plough et al. 2016), coupled with population dynamics which exacerbate the effects of inbreeding and genetic drift (e.g. sweepstakes reproduction; Hedgecock et al. 1992), are two putative explanatory factors (see Plough 2016 for a thorough review of genetic load in marine animals).

It has been shown that genetic load and viability selection result in high rates of mortality in larval oysters, but what is less well resolved is the overall patterns of genetic change in larval oysters over the developmental stages ending with metamorphosis and settlement. Plough and Hedgecock (2011) examined the timing or 'expression' for lethal alleles (i.e. mortality, evaluated by distortions in segregating microsatellite markers) in two F_2 hybrid families during larval development and they reported that around half of all the lethal alleles they detected were significantly altered around the time of metamorphosis from pediveliger larvae to spat. Similarly, Bierne et al. (1998) evaluated segregation ratios of genetic markers during larval development in *Ostrea edulis* and reported an excess of heterozygotes after metamorphosis. Both of these temporal studies provide good evidence for the 'final'

outcome of segregation distortion of alleles after completion of larval development. The small number of markers that were available in these previous studies for analysis across larval stages, however, makes it difficult to robustly resolve 'when and how' viability selection affects putative negative alleles during larval development. Additionally, the limited number of families evaluated and a lack of replicated cultures makes it impossible to determine whether these changes were family- or culture-specific.

In order to attempt to better resolve temporal patterns of genetic changes during development of Pacific oyster larvae, we created a genetically diverse pool of larvae from naturalized broodstock from Willapa Bay, WA and reared them in replicated hatchery conditions for 22 days, from fertilization to settlement. We monitored mortality over developmental stages and sampled larvae from each culture unit at six time points over the culture period. By evaluating changes in allele frequencies for genome-wide single nucleotide polymorphisms (SNPs) from pooled DNA samples, we could not only detect significant changes in genetic composition related to mortality events across replicates, but also observe overall patterns of change during larval development with greater detail than ever before. These analyses help to further our understanding of the timing of genotype-dependent mortality in larval oysters and the mechanisms through which genetic load is potentially preserved in oyster populations.

2. Methods

2.1 Broodstock conditioning and spawning

In spring of 2014, n~60 naturally recruited oysters from the Washington State oyster reserves in the Naselle region of Willapa Bay, WA (46°25'15.2"N 123°51'47.6"W) were transferred to the broodstock holding site at the Hatfield Marine Science Center (HMSC) located at the mouth of the Yaquina estuary, Oregon. In spring 2015, these animals were transferred to conditioning tanks to prepare them for spawning. Broodstock were provided with flow-through seawater (~2 liters min^{-1}) and a mixed algal diet of 50/50 (by cell concentration) *Isochrysis galbana* (C-iso) and *Chaetoceros gracilis* at approximately 20-30,000 cells ml^{-1}. Water temperature was increased from ambient (≈11ºC) to 20ºC over the course of two weeks and maintained at this temperature for 25 additional days to facilitate gonad development prior to spawning. The naturalized broodstock used for this study had a heavily skewed sex ratio (~10:1 female to male), therefore, we used 19 females and 5 males, crossed in a fully factorial mating design (every male paired with every female), to create 95 full sibling (1♀x 1♂) crosses. For each cross, eggs and sperm were manually removed from ripe parents (strip spawned) and suspended in seawater. The numbers of eggs from each female were counted and divided equally into 4-6 replicate beakers and fertilized with sperm suspensions from different males. After one hour, fertilization was confirmed visually and eggs were washed of excess sperm on a 25 μm screen. Fertilized eggs were proportionally combined to form a composite embryo pool that contained approximately equal proportions of each of the 95 crosses.

2.2 Culture units and water quality

Larval culture units consisted of 10-liter polycarbonate chambers (BearVault, San Diego, CA) fitted with a sealing lid, and rubber ring seal (McMaster-Carr, Santa Fe Springs, CA). Units were filled with hatchery seawater (25^{o} C, 32ppt , 10 μm-filtered) that had been vigorously aerated overnight to equilibrate dissolved pCO_2 to ambient levels (~400 μatm CO_2, pH = 7.9-8.1 , Ω_{arag} = 2.3-2.7) No supplemental aeration was supplied to the larval rearing units throughout the experimental period because this experiment was part of a larger study on the effects of acidified seawater on larval development and aeration would have altered dissolved CO_2 levels in the culture environments. The pH, temperature and dissolved oxygen of individual culture chambers were monitored daily: nominal pH and temperature values were measured with an Orion Star A11 pH meter (Thermo-Fisher USA) with a Ross Ultra pH/ATC triode, calibrated with NIST buffers and adjusted with a seawater standard (Batch 22, A.G. Dickson, Scripps Institution of Oceanography, USA) and dissolved oxygen was measured with a YSI 85 meter (YSI, Yellow Springs, OH, USA). Dissolved oxygen and pH did not deviate from optimal levels and further details on carbonate chemistry parameters can be found in the methods of Durland et al. (*in press*).

2.3 Larval culture

Approximately five hours after fertilization ~200,000 embryos from the pool were distributed to each of five culture replicates resulting in an effective stocking

density of 20 larvae ml^{-1}. Water was changed every 48 hours by sieving oyster larvae on a mesh screen (see sizes on Table 4.1.), filling the culture units with fresh seawater, and re-stocking the larvae. Antibiotics were added prophylactically in order to reduce bacterial respiration in culture units that could unduly affect seawater carbonate chemistry (Waldbusser et al. 2015). Antibiotics were alternated at each water change between a mixture of chloramphenicol/ampicillin (2 ppm and 10 ppm, respectively) and 20 ppm streptomycin to reduce the risks of development of resistant bacterial strains. Microalgal diets were supplied once daily, starting 2 days post fertilization (dpf), beginning with C-iso (*Isochrysis galbana*) at 20,000 cells ml^{-1} and increasing the ration by 5,000 cells ml^{-1} day^{-1}. The diatom species *Chaetoceros gracilis* was gradually incorporated into the diet on day 4, starting at 5% (based on cell concentrations) until it accounted for 50% of the algal diet by day 11, where it was maintained for the duration of the experiment. Larval densities were reduced to 10 ml^{-1} on day 2, 5 ml^{-1} on day 6 and 1 ml^{-1} at the pediveliger stage on day 14. Larval density was reduced randomly, with no selection for size, and maintained in this fashion to optimize potential growth and survival while simultaneously accommodating the sampling regime for DNA which required thousands of larvae to be removed from each culture unit. After the appearance of eyed larvae (indicating a readiness to metamorphose), larvae were screened on a 240 μm sieve to retain pediveliger larvae which were subsequently induced to metamorphose by exposure to $1.8E10^{-4}$ M epinephrine for two hours (Coon et al. 1986). After this time, all larvae and newly metamorphosed spat were rinsed in seawater and returned to the appropriate culture vessel with the remainder of the non-metamorphosed larvae.

Metamorphosis was induced in this fashion on days 16, 18 and 20. The experiment was terminated on day 22 after the majority of pediveligers had metamorphosed.

2.4 Larval Sampling

Larval survival was estimated from counts of larvae in each culture unit on days 2, 6, 10, 14, 16 and 22. Counts were conducted by concentrating the larvae from each culture unit in a 250ml beaker with ~150 ml of seawater and obtaining five subsamples (~30-50 µl each) which were enumerated, averaged, and volumetrically adjusted to estimate the total numbers of larvae in each culture unit. Survival estimates accounted for larvae removed for sampling and for adjustments in densities by multiplying survivorship between sampling/counting events to obtain a cumulative survival estimate across the entire experimental period. Cumulative survival for all five replicate cultures is represented in Figure 4.1. Egg and larval samples for DNA extraction were obtained on days 0, 2, 6, 10, 16 and 22, (n=1 egg and n = 25 larval samples in total) containing ~200-3000 individual oysters (depending on age) per culture unit (Table 4.1). The samples from day 22 were a composite of metamorphosed juvenile spat as well as eyed larvae that were retained on a 240 µm screen. From all samples, genomic DNA was extracted using a CTAB extraction method with RNAse treatment detailed by Panova et al. (2016) and DNA concentrations were assessed using a Qubit fluorometer (Thermo Scientific). 2bRAD libraries were prepared following a modified protocol based on Wang et al. (2012), using the BcgI restriction enzyme. Protocols for library preparation can be found in a github repository at: https://github.com/E-Durland/oyster-poolseq. All individual samples were given unique barcodes and pooled (with samples from a larger study) in

sets of ~32 samples per sequencing lane (n=5). Single-read, 50 base-pair (bp) target length sequencing was conducted on an Illumina HiSeq2500 platform in the SNP&SEQ Technology Platform at Uppsala University, Sweden.

2.5 Bioinformatics:

The bioinformatic analyses of DNA sequences are also detailed at the github page (above). First, de-duplexed raw reads were truncated to 36 bp in length and quality filtered for reads that had <10 bp with phred quality scores < 20. The remaining reads were further filtered for adaptor sequence contaminants using BBDuk (part of the BBtools package; Bushnell (2014)) and a kmer size of 12. Cleaned, high quality reads were mapped to the reference genome (Zhang et al. 2012) using the SHRiMP software package (Rumble et al. 2009) with default mapping parameters but allowing for a maximum of three genomic alignments per read and retaining the single best alignment. Alignments were then filtered to retain only those with ≥ 30 bp matching the reference sequence. At the end of these steps, the files contained an average of 1.02×10^6 reads across ~2.6×10^5 loci for an average coverage depth of ~4x across all loci. Quality filtering and mapping statistics are found in Table A4.1. Sequence alignment map (SAM) files were converted to tab-delimited files with read counts of each nucleotide at each locus and merged. A custom Python script was used to then remove reads with a sequencing depth of < 50 or > 1000 (Schlötterer et al. 2014) and loci with >2 alleles observed. 'Minor' alleles were determined as the less abundant of the two alleles for each SNP, averaged across all samples and, therefore, single samples or time points may have 'minor' allele

frequencies above 50%. Lastly, polymorphic loci were filtered to one SNP per 36 bp tag (with the highest minor allele frequency) to avoid analyzing multiple closely linked markers or those that may arise from potential ambiguities in mapping of the reads which may erroneously result in the detection of multiple polymorphisms in close proximity to one another. All bioinformatic analyses were conducted on computing infrastructure at the Center for Genomic Research and Biocomputing (CGRB) Core Laboratories at Oregon State University.

2.6 Data analysis

2.6.1 Detecting changes in allele frequencies

A total of 5373 SNPs were retained from the filtering steps above for statistical analysis. Loci in this dataset were filtered for missing data and rare polymorphisms by keeping only loci for which three or more replicates per time point had reads present and the minor allele frequency (MAF) of the fertilized egg pool was > 1%. This left 847 SNPs for statistical modeling. The read count data for each allele at each remaining locus was analyzed with a binomial generalized linear model (GLM) with the formula: $(A_1 : A_2) \sim \beta_{Age}$ where A_1 and A_2 refer to the read counts of alternative alleles at each locus and β_{Age} is the parameter estimate for age of the sample (0 to 22 dpf). Age was incorporated into the model as a multi-level factor rather than a continuous variable because the predominant nature of genetic change we observed was variable between ages and non-linear. For each locus, the p-value for 'age' effects (Type I sum of squares) were adjusted with a Benjamini-Hochberg

false discovery rate and determined significant at $p < 0.05$. All statistical procedures were conducted in R (R Core team 2015).

2.6.2 Evaluating patterns of change

In order to investigate the dynamic patterns of change in allele frequency between time points for loci with significant 'age' effects, we used three tools: pair-wise multiple comparison tests (Tukey's) for changes in mean allele frequencies on sequential time points, k-means clustering for overall patterns of change in allele frequency (ΔAF) and linear discriminant analyses to validate cluster assignments. Pair-wise Tukey's tests for differences in minor allele frequencies conservatively detect significant and abrupt changes in allele frequency between two time points while controlling for false-discovery inflation across multiple comparisons within the same locus (Miller 2012). We rationalized significant changes (or lack thereof) sequentially between sampling points to resolve an acute pattern of change and categorized them as: 1) 'gradual', 2) 'uni-directional' or 3) 'bi-directional'. 'Gradual' changes were those that had significant overall effects of 'age' in the linear model but sequential pair-wise tests did not indicate any single transition as significantly different. 'Uni-directional' changes were assigned to loci for which sequential pair-wise tests (SPT) on mean allele frequency indicated one or more sampling intervals during which a minor allele frequency significantly changed and all changes were in a single or common direction (i.e. negative or positive). Loci which had two or more significant changes in allele frequency (determined by SPT) that were not in the same direction were categorized as 'bi-directional'. Example trajectories in mean allele

frequency for each category are seen in Figure 4.2A and the derived change in allele frequency (ΔAF) between each sampling interval (e.g. $\Delta AF(D0-2) = AF(D2) - AF(D0)$ for MAF at day 2 and day 0, respectively) is represented in Figure 4.2B.

In these analyses, SPT can qualitatively assign statistical significance to a sequence of changes in allele frequencies but they are incapable of quantifying the magnitude of changes and overall patterns of change across all loci which had significantly different mean allele frequencies at one or more ages. To accomplish this, we adopted unsupervised machine learning algorithms similar to those used for time-series gene expression data (McDowell et al. 2018). We applied a k-means clustering algorithm to sort the patterns of ΔAF between each sampling point for each locus into similar clusters. We first used a bootstrapping cluster method to determine the appropriate number of clusters (k=10, Jacard index= 0.75; 'fpc' package in R; Hennig (2018)) and then executed the clustering algorithm ('kmeans' in R) to group each locus to a cluster for further interpretation. Lastly, in order to cross-validate cluster assignments, we used linear discriminant analyses ('lda' in the MASS package for R) to determine the stability of each of the 10 clusters and to evaluate the relative influence of each time interval on their assignment. By integrating results from all three approaches we can more robustly and efficiently evaluate both the specific and overall changes in allele frequency taking place during larval development in these populations.

2.6.3 Simulating starting genotype frequencies

The primary limitation of pooled DNA samples is that they obscure individual genotypes and limit the ability to connect changes in overall minor allele frequencies to specific shifts in the relative abundance of homozygous and heterozygous genotypes. With a factorial mating design and bi-allelic SNPs (A/B), however, initial genotypic proportions of the larval pool for each marker can be estimated with reasonable accuracy through simulation. To accomplish this, we simulated random parental gene pools based on the observed minor allele frequency (MAF) of the fertilized eggs and conducted a set of *in silico* crosses to generate the estimated genotype composition and MAF of the final pool. For example: if a locus had an observed MAF=25% in the egg pool, we generated a simulated set of 48 alleles (two for each of 24 broodstock) with 36 'A' alleles and 12 'B' alleles (12/48= 25%). We then randomly 'drew' from this set of alleles, without replacement, and assigned them to five paternal and 19 maternal genotypes. Using Mendelian segregation ratios, we then 'crossed' each male to each female to generate 4 possible genotype per cross (i.e. AB x AB = AA/AB/AB/BB) and 95 total crosses for n= 380 simulated genotypes. From this simulated egg pool the total MAF was calculated, along with relative abundance of each genotype. Simulations were repeated n=20 times for each 1% increment from 1-50 % MAF for a total of 1000 iterations. Although the unbalanced cross design tends to produce a slightly higher than expected abundance of heterozygotes, the genotypic proportion of the pool generally follows expectations under Hardy-Weinberg equilibrium. The relationship between starting MAF and estimated genotype proportions are found in figure A4.1.

3. Results

3.1 Sequential pair-wise tests

Among the 847 SNPs which passed filtering steps, 516 (~61%) exhibited significant 'age' effects, with distorted allele frequencies at one or more time points during larval development. Comparing changes between specific time points with SPT indicates that 166 loci (~32% of those that changed) were 'gradual', 219 (~42%) were 'uni-directional' (UD) and 131 (~25%) had alternating directions of change and classified as 'bi-directional' (BD) loci. When we account for changes for both UD and BD loci relative to the developmental progression of larvae (Figure 4.3), we observe that during later stages (days 10-22) there are substantially more genetic changes taking place (n= 230 loci, ~45% of those that changed) than during initial development (days 0-10; n=78 loci; ~15% of those that changed). Additionally, there are very few UD loci which appear to have significantly changed in frequency in the same direction at more than one time point ('recurring uni-directional' in Figure 4.3). In general, loci with increasing magnitudes of change (gradual < UD < BD) had higher minor allele frequencies both at the beginning and end of the larval culture period (Figure 4.4) but the detection of all three categories of change at low starting MAF (<10%) indicates that parametric tests were sufficiently sensitive to discern small changes in allele frequencies for rare variants (i.e. alleles with low MAF change so little they may escape detection as significantly different).

3.2 Structural analyses and genomic coverage

In order to rationalize these changes in the context of genomic architecture, we placed SNPs on the linkage map (Hedgecock et al. 2015) through scaffold level association (Fig. 4.5; see methods in Chapter 3). Loci undergoing each type of change (gradual, UD, BD) are spread more-or-less evenly across linkage groups and no regions of the genome appear as clear outliers that would indicate structural association between loci with similar patterns of change. By assigning SNPs to the linkage map, we retain n=225 (~27%) of the markers and the average distance between them is ~6.3 cM (excluding LG 2, for which few markers have been mapped; Hedgecock et al. 2015). If we assume the distribution of the remaining ~73% of markers to be similar to those that were able to be mapped, this brings the estimated average marker distance to ~1.6 cM. Collectively, these 847 markers appear to reasonably cover a large portion of the genome.

3.3 k-means clustering and linear discriminant analysis

With unsupervised machine learning algorithms we are able to broaden the scope from the specific and significant changes detected by the previous SPT method to more comprehensively characterize patterns of allele frequency change in these larval populations over development. Using k=10 clusters (determined from bootstrapping, see methods), each locus was assigned to a group with other loci that shared their overall pattern of change in mean allele frequencies between time points (Figure 4.6). We further cross-validated the strength of cluster assignments with linear discriminant analyses which indicated that, overall, cluster assignment was

relatively robust ($\bar{x}$= 78.6% overlap between methods; Table 4.2), especially for loci in clusters characterized by highly dynamic patterns of change (clusters 7-10). Group means for each cluster and time interval, along with details regarding the linear discriminant analysis are found in Table A4.2. Results from the machine learning algorithms also agree well with the SPT approach - a greater proportion of 'gradual' loci are abundant in clusters with reduced change (clusters 1-4) and the proportion of BD loci in each cluster increases with overall dynamic nature of change trajectory (clusters 7-10; Fig. 4.5, Table 4.2). The correlation between increasingly dynamic patterns of change and higher starting MAF seen in SPT categories is also mirrored in k-means cluster groups where progressively higher numbered clusters (more dynamic trajectories) had significantly higher starting MAF in the egg pool (ANOVA $p < 0.05$, Figure 4.7A).

3.4 Comparing temporal to overall changes

Comparing the changes in allele frequency observed during larval development to overall changes between day two (the earliest time point for which we have replicated samples) and day 22 indicates that less than half of all loci (n=194; ~38%) had significantly altered 'final' allele frequencies at the end of the larval culture period (Figure 4.7B). For the remaining ~62% of loci, substantial changes in allele frequency at one stage during larval development were essentially offset by additional changes in the opposite direction, resulting in little overall change in average MAF across the breadth of larval development. Despite apparently strong selection pressures against potentially lethal alleles associated with many loci, no rare

allelic variants were actually purged (i.e. driven to fixation for the alternate allele) from the larval population in this study (Figure 4.8). For a majority of the loci analyzed in these larval populations, there appear to be significant selective pressures occurring during larval development that have some level of a balancing effect on allele frequency and are essentially obscured by analyses of initial and final allele frequencies alone.

4. Discussion

4.1 Mortality and genetic changes across development

The pattern of larval mortality we observed in this study (Fig. 4.1) is common for oysters (e.g. Lannan 1980, Ernande et al. 2003, Durland et al. *in press*) and the low survivorship routinely observed through early life stages has been largely attributed to genetic load in previous studies (e.g. Plough & Hedgecock 2011, Plough et al. 2016). The temporal patterns of viability selection at quantitative trait loci (vQTL) during larval development that were reported by Plough and Hedgecock (2011) are broadly similar to our findings. In both cases, few loci exhibited distortions in allele frequency in early life stages and mortality during metamorphosis accounted for around half of all distorted loci (Fig. 4.3). In this study we are able to more directly correlate temporal distortions in allele frequencies to measured rates of mortality, which previously had only been approximated. In this context, we see that a substantial amount of mortality occurs early in larval development (~70% before 6 dpf; Fig. 4.1) that is not matched by an abundance of distortions in allele frequency at this age (~15% of all loci; Fig. 4.3). With pooled data, we are unable to accurately

calculate strength of selection for individual loci at discrete time points but Plough and Hedgecock (2011) similarly identified distortions at only two vQTL prior to 2 dpf and acknowledged that there was insufficient evidence for viability selection to account for the high level of mortality (~50-80 %) typically observed at this age in cultures of larval oysters.

Low fertilization rates would explain a portion of early larval mortality but fertilization was closely monitored in this study as well as by Plough and Hedgecock (2011) and no substantial fertilization failure was observed in either case. Egg quality is an additional parameter that is likely to influence early larval development and survival (Corporeau et al. 2012, Myrina et al. 2015) but gamete quality of the broodstock used to make the crosses for this experiment was generally very good (see discussion in Durland et al. *in press*). Manual extraction of gametes (stripping), which is a common practice both for experimental and commercial spawns, also likely removes a portion of oocytes from female broodstock oysters that are not fully mature, and may compromise early larval development. More broadly, high rates of early larval mortality for bivalves have been documented for decades (e.g. Kraeuter et al. 1982, Hedgecock et al. 1995, Ernande et al. 2003) and the more recent investigations into molecular genetic changes of larval populations at this age, including this study, have been curiously unable to substantially attribute this phenomenon to heritable components. The hypothesis that *de novo* germ-line mutations contribute to this early mortality (as proposed by Plough and Hedgecock, 2011) is appealing but, as yet, unproven. It remains unclear what suite of factors contribute to this rather uniform, yet unexplained, period of high mortality for larval

oysters, but this uncertainty is an important point to consider when estimating the scale of genotype-dependent mortality and genetic load in larval oysters (i.e. Plough et al. 2016).

4.2 Genotype inferences from pooled samples

With pooled DNA samples, inferring genotype-specific mortality based on changes in the overall MAF is inherently imprecise, but not entirely impossible. Through simulations of possible starting initial genotype proportions at a given level of MAF in the egg pool, we can estimate with some certainty that the genotypes begin near those predicted by Hardy-Weinberg equilibria (Fig. A4.2). In an idealized scenario, where each parental oyster is heterozygous for two alleles at a locus (AB), there would be a 1:2:1 ratio of AA : AB : BB genotypes in the larval pool and the minor allele frequency of the population would be 50%. Any change in larval allele frequency would necessitate the loss of homozygotes (AA or BB) because an elimination of a heterozygote (AB) would not change the group-wide mean allele frequency. Alternatively, a locus with low initial MAF (<20 %) is expected to have very few homozygous genotypes for the minor allele (BB ; $q^2 = 0.2^2 = 0.04$ or 4%) and mortality of the 'BB' genotype would shift the overall minor allele frequency relatively little. Lethal alleles which express dominance effects would also remove heterozygotes (AB) from the population and, if we assume 'B' to be the negative allele in this latter example, it would also result in a downward shift of the MAF. Notably, this type of distortion in MAF requires >2x greater mortality of heterozygotes than a comparable loss of homozygotes to achieve a similar net effect

on mean allele frequency (removing one minor and one major allele vs two alleles of the same type). In these pooled genotype data we predict that loci with higher initial MAF have more balanced genotypic proportions of the larval pool and are, therefore, more likely to be sensitive to distortions of the mean allele frequency owing to genotype-dependent mortality. This hypothesis is supported by finding that the mean starting MAF in egg pools are significantly greater in increasing categories of dynamic change (SPT = gradual < UD < BD and k-means = 1<2…<10; Fig. 4.7A).

4.3 Evaluating patterns of change

The abrupt shifts in MAF we observe for UD and BD loci represent mortality events in the larval population which rapidly change the overall genetic composition of the group over the course of a few short days. It is likely that these significant changes in allele frequency are similar to the reported effects of viability selection for negative alleles in Pacific oysters previously identified by Plough and Hedgecock (2011) and Plough et al. (2016). In this study, however, we additionally describe a much greater degree of dynamic genetic change across larval development than has previously been reported. Among the n=350 loci which exhibit temporally specific changes in allele frequency (UD and BD loci), more than a third (~37%) do so in a bi-directional pattern, with opposing shifts in MAF at different stages of development. For instance, one SNP in cluster 10 (Figure 4.6) started at a MAF= 49% in eggs, dropped to a mean of 19% by day six, increased to 62% at day 16 and, after metamorphosis, ended at 30%.

It is possible that some number of these highly dynamic patterns of change are an artifact of experimental or bioinformatic error but these results represent the mean changes in allele frequencies across a minimum of three culture replicates and after two levels of p-value correction (Benjimini-Hochberg on the locus level, Tukey's on the temporal level). Collectively, type I error, or 'false discovery' is an implausible way to explain these observations. Instead, for these highly dynamic loci, dramatic shifts in allele frequency seem to be a biological reality and imply strong selection pressures that target genes associated with both allelic variants of these markers at different stages of development. Although we cannot measure changes in genotypic frequencies for each locus across development, for loci with strong bi-directional trajectories of MAF, sequential mortality primarily targeting homozygous states (AA and BB) is the most parsimonious way to explain the scale of change (e.g. Clusters 9&10 in Fig. 4.6) relative to the estimated rate of mortality in the larval population. This is not to say that the mortality events driving these changes were exclusive to these genotypes but the marginal loss of heterozygotes (AB) would have a diminished effect on shifts in mean allele frequencies (as described previously) and are unlikely to completely account for the observed MAF trajectories.

4.4 Comparison to previous temporal studies

Conceptually, the 'bi-directional' loci which we report in this study are similar to the sequential patterns of homozygote deficiency observed in the bi-allelic markers evaluated by Plough and Hedgecock (2011). In that study, 7/10 of the two-allele markers (ABxAB) became homozygote deficient for each genotype (AA and BB) at

different times in larval development and an additional two markers lost both homozygote genotypes around the time of metamorphosis. Plough (2018) also reported a heterozygote advantage for a marker involved with viability selection during metamorphosis. In that case, the marker appeared to be linked to two different genes in repulsion phase (each with different selection effects; i.e. pseudo-overdominance). The author observed that one homozygous state (AA) was associated with swift mortality of pediveliger larvae early in settlement and the other (BB) appeared to inhibit metamorphosis to juvenile spat. In these previous examples, temporally contrasting patterns of genotype-dependent mortality were detected, but rarely. In our study, we estimate that this type of viability selection is not exceptional and may account for one quarter (or more) of all loci affected by larval development.

Ultimately, we cannot distinguish whether these patterns of change are due to linkage of markers to multiple genes in repulsion phase or whether a single linked gene has differential fitness consequences at discrete life stages - it is possible that both cases may be true. In pooled analyses, bi-allelic SNPs also oversimplify the representation of genotypes by anonymizing haplotype phasing and assuming each locus is unlinked to others in the dataset. The highly fragmented reference genome (Zhang et al. 2012) limits our ability to robustly evaluate significantly affected loci relative to the architecture of the genome but we were able to assign ~27% of SNPs to the linkage groups reported by Hedgecock et al. (2015) through scaffold level association (see methods in Chapter 3). The markers used in this study assign to all 10 linkage groups with reasonable genomic coverage (average map distance ~6.3 cM) and there is no compelling evidence to suggest that specific genomic regions are

responsible for a disproportionate amount of distorted loci (Fig. 4.5). Regardless of the mechanism, these results demonstrate that genotype-dependent mortality in larval oysters is likely more complex than previously estimated and many loci do not fit traditional paradigms of simple dominant or additive effects. Over-dominance or heterosis, which have been suggested as genetic mechanisms behind many oyster traits (reviewed by Launey and Hedgecock 2001 and Hedgecock and Davis 2007) would seem to appropriately characterize the end result for the bi-directional loci in this analysis but both terms over-simplify the contrasting pattern of selection at these loci across developmental stages. 'Interrupted heterotic resilience' is, perhaps, a suitable phrase to describe the net effects we observe for these bi-directional SNPs- where a genetic 'hedging of bets' with one allele of each type results in increased, but not guaranteed, chances of survival across the morphological transitions during larval development.

4.5 Temporally balanced polymorphisms

Over 22 days of larval development, from fertilization to settlement, we find that many loci are undergoing significant changes in allele frequency. Approximately 60% of all loci evaluated exhibit detectable shifts in allele frequencies at one or more points in larval development. This estimate is more than two fold greater than what would be concluded from pair wise comparison tests between two and 22 days post fertilization alone (Fig. 4.7B). This disparity is explained by the fact that, for many loci, the dynamic changes in allele frequency during larval development have a balancing effect on shifts in overall MAF. Through SPT tests we detect some of

these changes as significantly 'bi-directional' in nature, but many that take place over broader time frames are not found significant (at $p < 0.05$) in tests examining sequential changes in isolation. Overall, only 91 of the 291 UD loci (~31%) and 51 of the 131 BD loci (~39%) have significantly altered minor allele frequencies comparing samples from 2 and 22 dpf (Fig. 4.7B).

Collectively, these data provide interesting evidence to support a hypothesis for balancing selection on the majority of loci we evaluated during larval development. Balancing selection is a long debated mechanism for maintaining genetic diversity in a population, the prevalence and significance of which has yet to be resolved (Nielsen 2005, Fijarczyk & Babik 2015). In natural populations, evidence for balancing selection is rare and generally limited to genes and traits involved in co-evolutionary adaption, such as immune responses (Ferrer-Admetlla et al. 2008, Pespeni et al. 2012). In these cases it is proposed that, for heterozygotes, any reduction in base fitness from the inferior allele is compensated for by a more diverse set of genes with which to overcome variable exogenous stressors such as pathogens. For a thorough review of the molecular evidence for balancing selection we refer the reader to Fijarczyk and Babik (2015).

Obviously, pooled DNA samples are not the best equipped tool to demonstrate conclusively that balancing selection is favoring heterozygotes and causing the observed genetic changes in these larval populations. The overall trends we observe in this study, however, are consistent with expectations under theories of over-dominance or heterosis, but specifically dis-advantaging homozygous genotypes at different points during larval development. Furthermore, the increased allele

frequency for loci undergoing significant change, especially bi-directional change (Fig. 4.4 & 4.7A), is consistent with the theoretical expectations under balancing selection (Box 4; Fijarczyk & Babik 2015). If true, this hypothesis of 'temporally balanced polymorphism' would also help to explain how oysters, which are burdened by high rates of genetic load, seem to maintain lethal alleles in natural populations (Plough 2016). For oysters, mutation rates are estimated to be >90 times that for drosophila (Plough et al. 2016) and this increases the accumulation of negative alleles across generations. Oyster populations in natural settings also have been shown to have lower effective population sizes (N_e) than expected and are likely subject to sweepstakes recruitment success (Hedgecock et al. 1992). These population dynamics may enhance the effects of inbreeding and drift in these communities and may be antagonistic to the pressures of purifying selection. In this scenario, where mutation load is potentially high and persistent, 'heterotic resilience' may represent a genetic refuge for individuals if allelic variants have contrasting and lethal consequences on larval fitness across developmental time points. The consequence, however, is that heterozygotes also perpetuate negative alleles to future generations, further limiting the strength of purifying selection in these populations. For instance, in this larval group, despite a cumulative ~96% mortality and substantial genetic distortions, not one locus became fixed for a preferable allele, completely removing a rare (and potentially lethal) variant by the end of larval culture (Fig. 4.8). This demonstrates the difficulty of purging highly lethal alleles from populations when heterozygosity is even marginally favorable.

For oysters, substantial genetic load and low rates of larval survival, however, may be acceptable trade-offs for high fecundity and phenotypic plasticity (Llorda 2002). Balancing selection increases standing genetic variation of a population, enhancing the adaptive capacity, which possibly helps explain how the Pacific oyster has been able to so effectively colonize volatile environments (Ruesink et al. 2017) in coastal regions across the globe (Schmidt et al. 2008, Dolmer et al. 2014). Understanding the evolutionary implications of balancing selection in oysters will necessitate a better grasp of the genetic mechanisms and physiological processes behind these dynamic selective pressures which have yet to be resolved. Future efforts to characterize temporal changes in genotype frequency across larval development will require a more robust method to infer genotypic composition of the larval pool and currently it is not feasible to create high quality genomic libraries from individual larvae for high-throughput sequencing platforms. For pooled sequencing approaches, emerging techniques to reconstruct haplotypes from pooled DNA samples (Franssen et al. 2017) and an improved reference genome, like that of the recently available chromosomal assembly for *Crassostrea virginica* (Gomez-Chiarri et al. 2015) are likely to be valuable resources in the future.

5. Conclusions

In summary, by analyzing temporal changes in the genetic structure of Pacific oysters over larval development, we can begin to better understand the complex nature of genotype-dependent mortality taking place in these populations during larval development and metamorphosis. This study builds on previous findings of

substantial genotype-dependent mortality in oysters (Plough et al. 2016) and these results suggest that not only do negative alleles cause shifts in genetic composition of surviving larvae, but that patterns of selection are not uniformly binary 'for or against' specific alleles and genotypes. Our results suggest that at least one quarter of all loci affected by development are subject to selection pressures against multiple negative alleles, and these loci exhibit stage-specific mortality patterns that eliminate genotypes possessing both allelic variants, but at different time points. The patterns of genetic change during larval development vary among loci, and the trajectory of mean allele frequency in these groups is often dynamic across developmental transitions. Results from this study provide interesting evidence to support a hypothesis for balancing selection as a parsimonious way to describe the net effects of 'interrupted heterotic resilience' we observe for loci with patterns of change that appear to select against homozygous genotypes. If this theory is supported, it would add to the relatively few cases where this mode of selection has been robustly demonstrated to influence long-term genetic variation and aid our understanding of the mechanisms by which genetic diversity and high levels of genetic load are maintained in highly fecund marine invertebrates.

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Figures and Tables

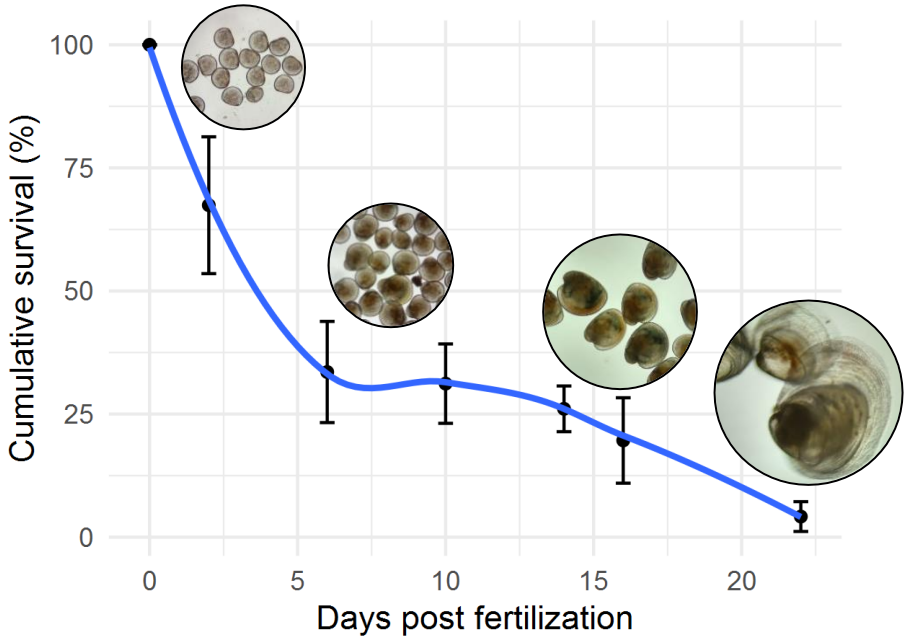

Figure 4.1 Cumulative larval survival from fertilization (day 0) through metamorphosis and settlement (day 22). Images represent larval samples taken at each respective age.

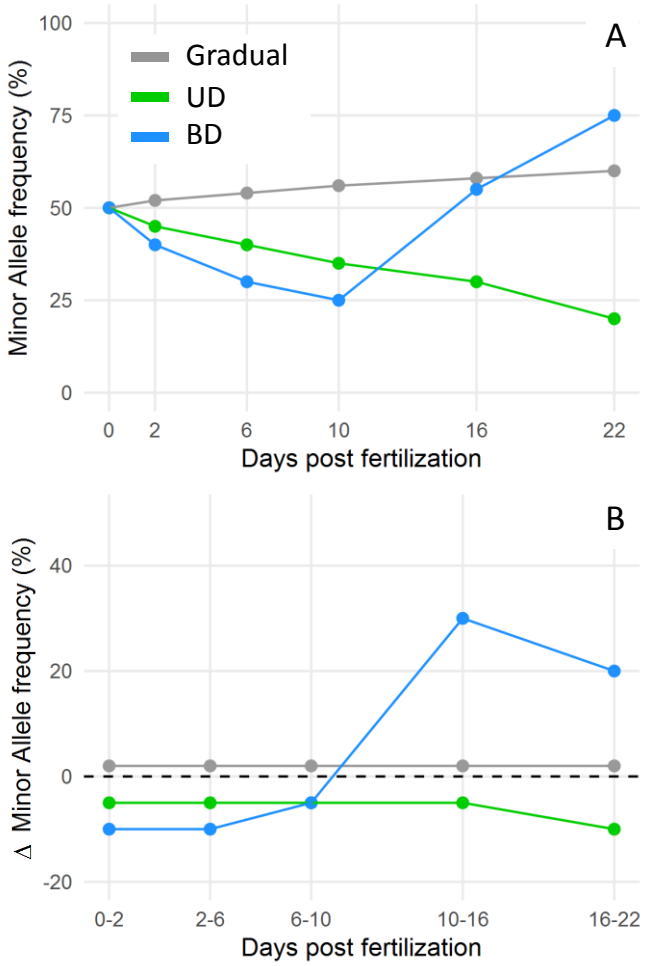

Figure 4.2 Examples of three trajectories of mean allele frequency in larval pools across development from fertilization through metamorphosis and settlement (day 22). Overall minor allele frequency (A) and relative change (Δ) in allele frequency (B) for 'gradual', 'uni-directional' (UD) and 'bi-directional' (BD) changes are represented by grey, green and blue lines, respectively.

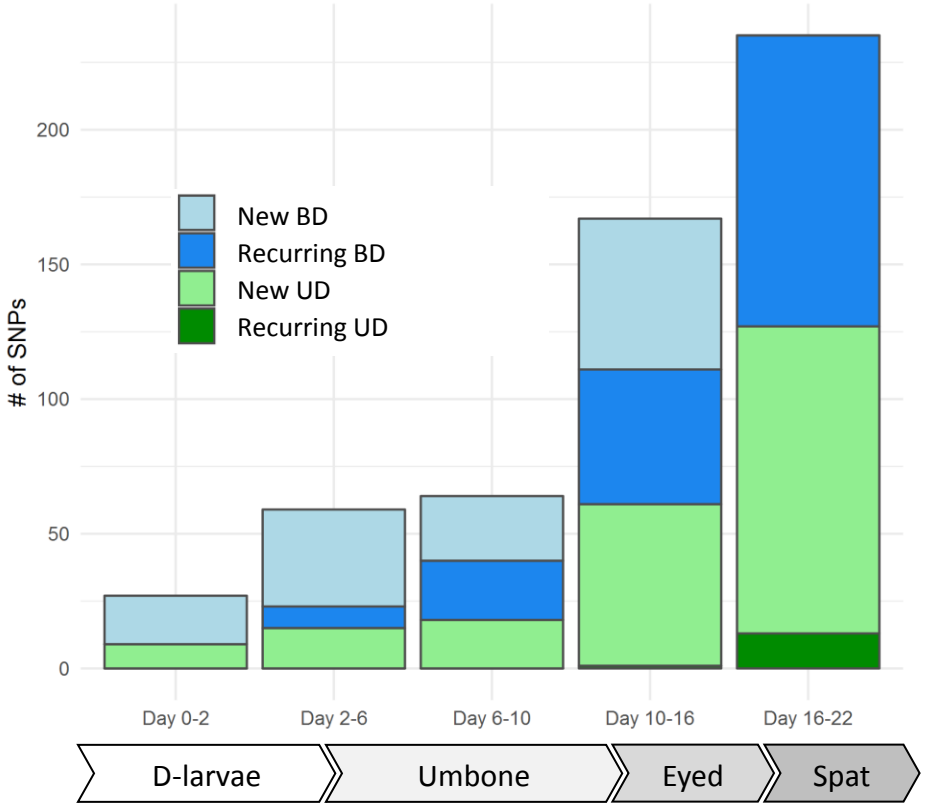

Figure 4.3 Significant changes in mean allele frequency across larval development. Bars represent new and recurring 'uni-directional' (UD) and bi-directional' (BD) changes at each time interval. Larval developmental stages are represented in block arrows below the plot.

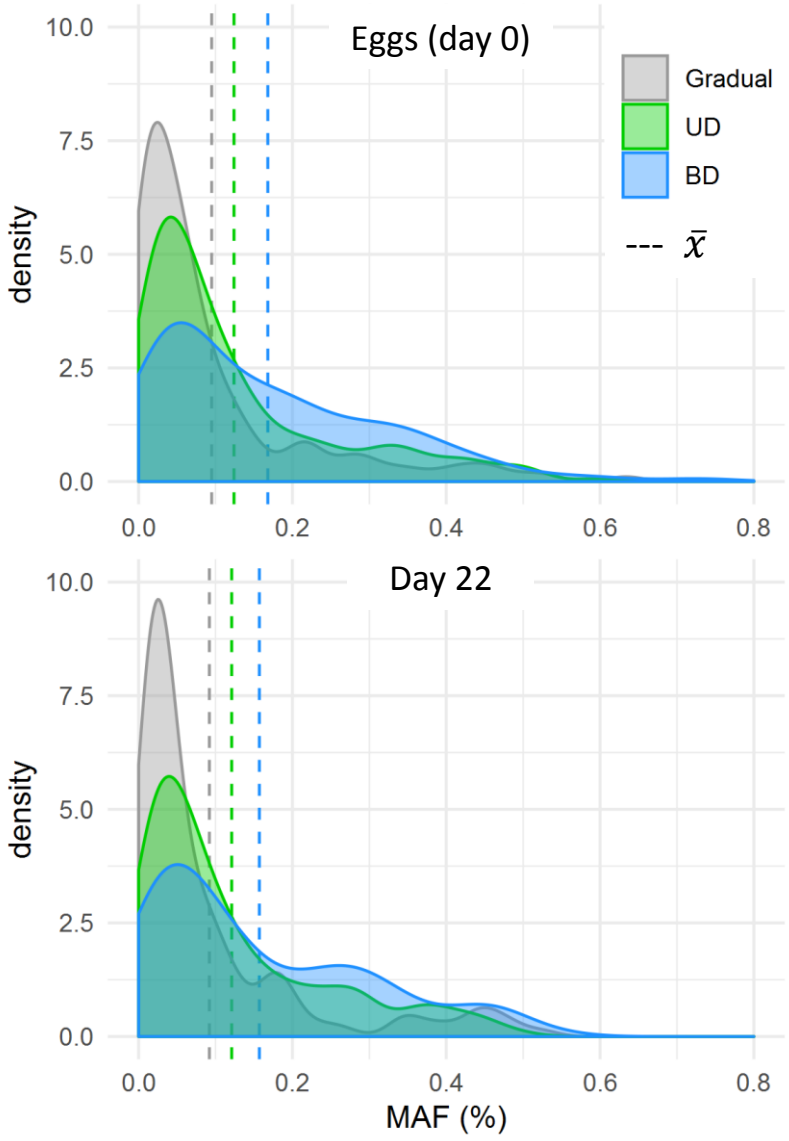

Figure 4.4 Allele frequency distribution of n=516 SNPs which have significantly changed minor allele frequencies (MAF) over larval development. Minor allele frequency for these markers in fertilized eggs (top) and spat/larvae at day 22 (bottom). Grey, green and blue colors refer to 'gradual' , 'uni-directional' (UD) and 'bi-directional' (BD) categories of change, determined by sequential pair-wise tests (SPT) of mean allele frequency. Dashed lines indicate means of each distribution. MAF means for gradual, UD and BD loci were 0.095, 0.124 and .0168 at day 0 and 0.092, 0.121 and 0.157 at day 22, respectively. The distributions for each category within each time point are significantly different (K-S test; $p < 0.0001$).

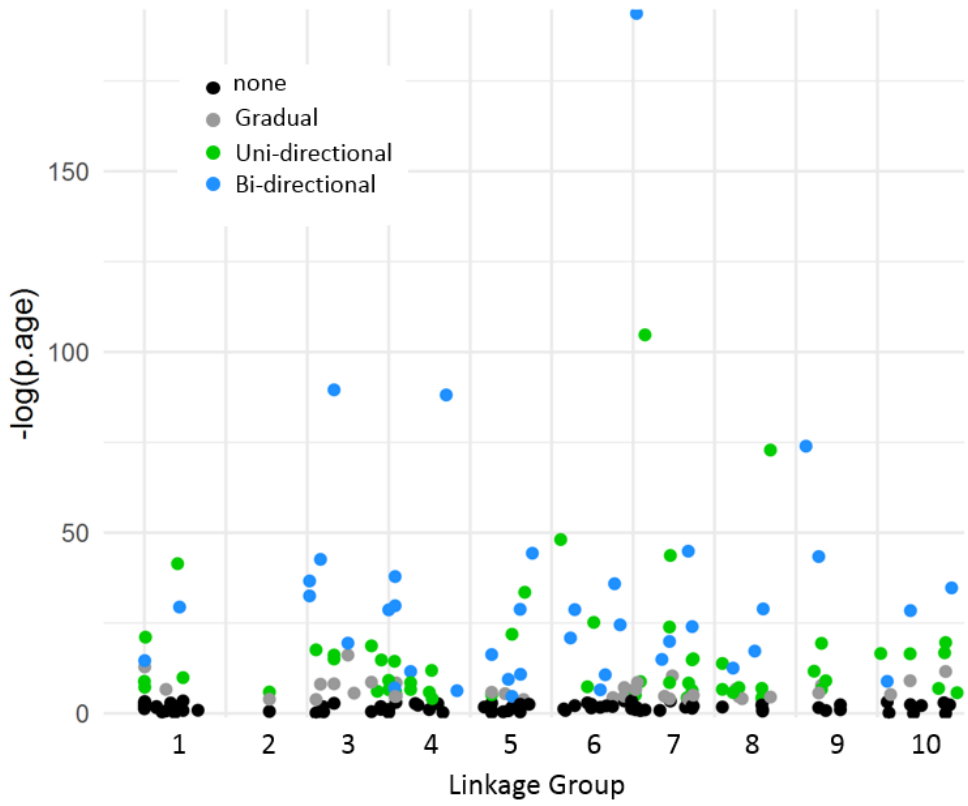

Figure 4.5 Manhattan plot of mapped loci. A total of n=225 loci mapped to linkage groups (from Hedgecock et al. 2015) relative to significance of change (-log(p.age)). Grey, green and blue points correspond to gradual, uni-directional and bi-directional categories of change determined by SPT. Black points are mapped loci that did not significantly change over larval development.

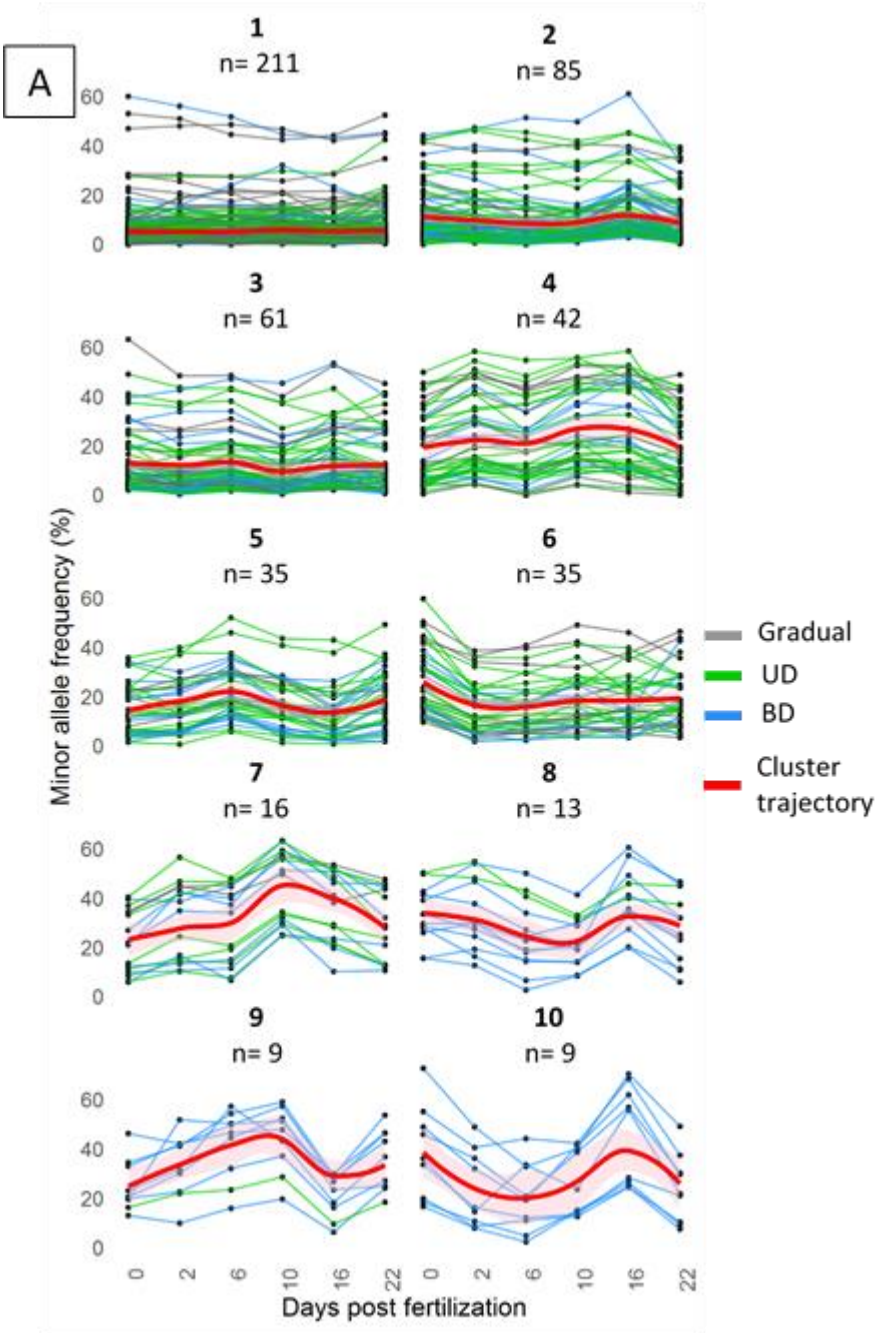
A
1
n= 211
2
n= 85
3
n= 61
4
n= 42
5
n= 35
6
n= 35
7
n= 16
8
n= 13
9
n= 9
10
n= 9
Minor allele frequency (%)
0
20
40
60
0
2
6
10
16
22
Days post fertilization
Gradual
UD
BD
Cluster trajectory

Figure 4.6

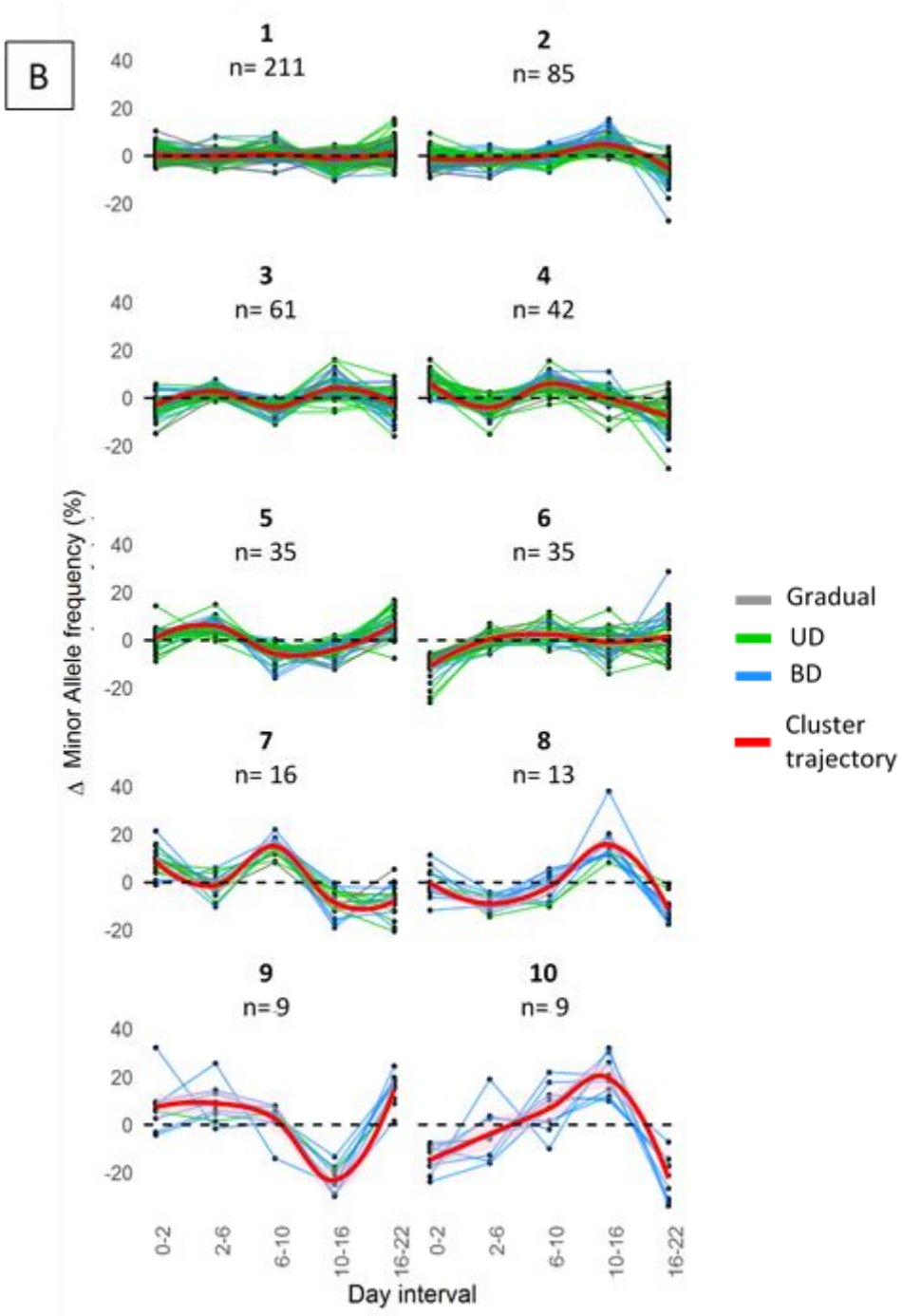

Figure 4.6 (continued) Clustered trajectories of minor allele frequency (A) and change (Δ) in allele frequency (B) for n= 516 SNPs found significantly changed in one or more ages by the linear model. Clusters 1-10 were generated by a k-means clustering algorithm evaluating change in allele frequency between sampling points. Line colors correspond to patterns of change identified by sequential pair-wise Tukey's tests. Bold red lines are localized estimate (loess) trajectories for all SNPs within a cluster (pink ribbons ± SD).

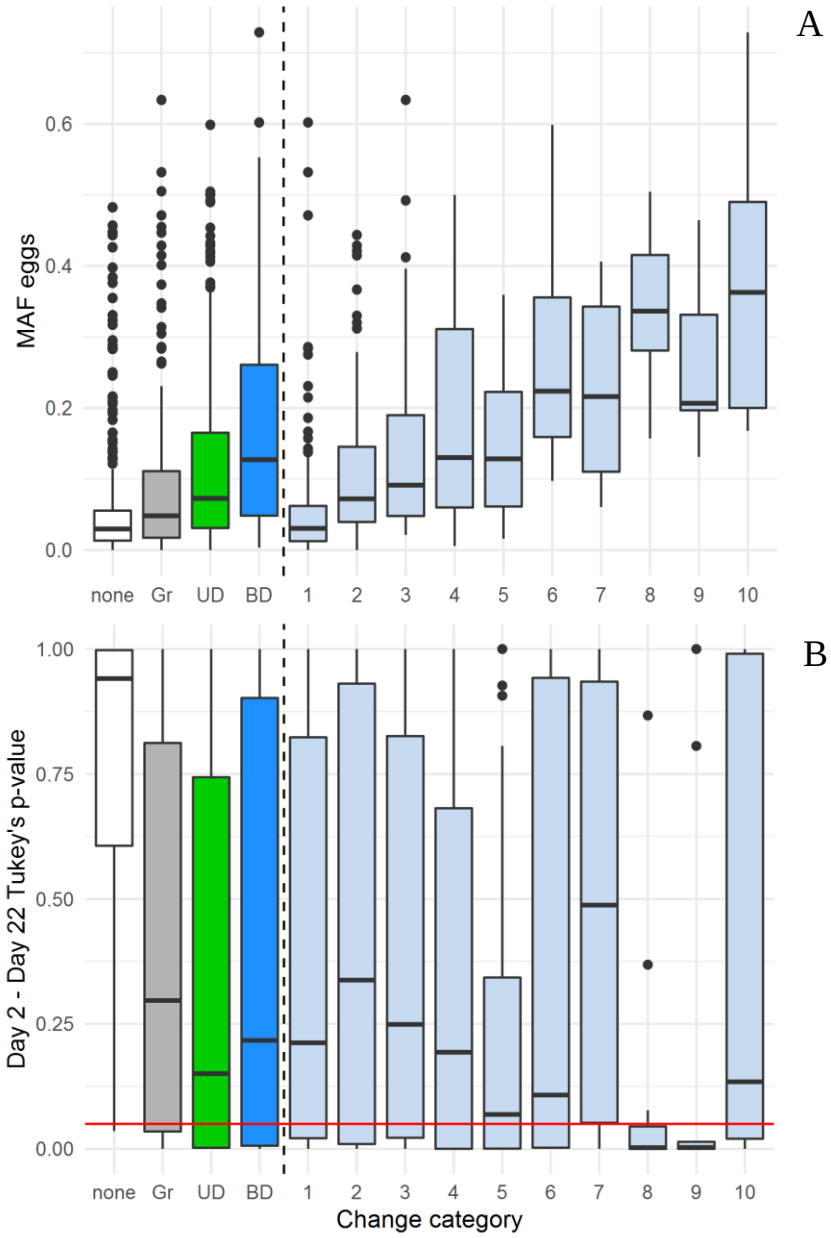

Figure 4.7 Initial minor allele frequency (MAF) and significance of overall distortion for loci changing over larval development. (A) Starting MAF in egg pool and (B) p-value for pair-wise test (Tukey's) of difference in MAF between day two and 22. Each box represents a 'category of change' for loci which had significantly distorted allele frequencies over larval development. To the left of the dashed line are the categories of change determined by sequential pair-wise Tukeys tests (SPT): 'none' (white), 'gradual' (Gr; grey), 'uni-directional' (UD; green) and 'bi-directional' (BD; blue). The boxes to the right of the dashed line are groups (1-10) determined by cluster analysis. Red line in (B) represents p =0.05, only n=194 of 516 (~38%) loci that changed over larval development resulted in significant shifts in MAF between day two and 22.

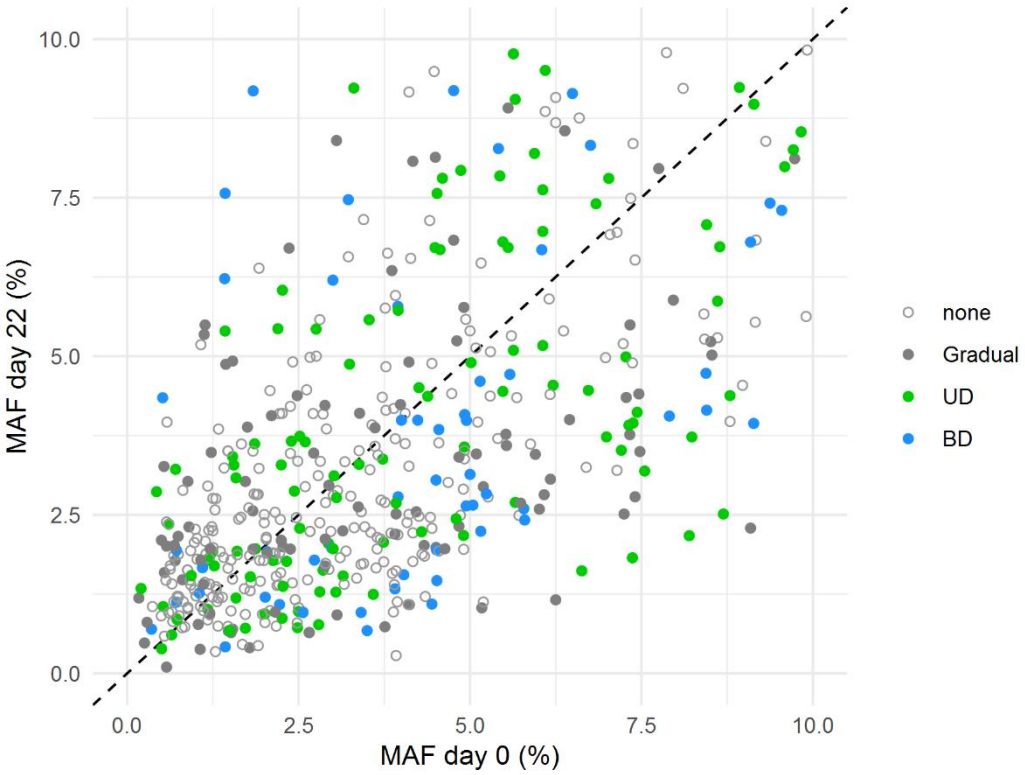

Figure 4.8 Relationship between starting and final minor allele frequency (MAF) for loci with <10 % minor alleles. After 22 days of larval culture and ~96% mortality, no loci had alleles which were removed from the population entirely (y=0).

Day	# larvae sampled	Screen size (μm)
0	10,000	25
2	3000	25
6	1000	37
10	800	64
16	1000	80/240
22	200	240

Table 4.1 Average size of larval samples and screen size at different time points during larval culture. From day 16 onward, larvae were screened on two screens: 240 μm on top of 80 μm to separate pediveligers out for settlement with epinephrine (see methods)

		k-means clustering										
	Cluster #	1	2	3	4	5	6	7	8	9	10	
LDA	1	**209**	31	25	11	4	6	0	0	0	0	
	2	0	**54**	4	1	0	3	0	1	0	1	
	3	2	0	**32**	0	2	0	0	0	0	0	
	4	0	0	0	**30**	0	0	1	0	0	1	
	5	0	0	0	0	**29**	0	0	0	0	0	
	6	0	0	0	0	0	**25**	0	0	0	0	
	7	0	0	0	0	0	0	**15**	0	0	0	
	8	0	0	0	0	0	0	0	**12**	0	2	
	9	0	0	0	0	0	0	0	0	**9**	0	
	10	0	0	0	0	0	1	0	0	0	**5**	
	% agreement	99	64	53	71	83	71	93	92	100	56	
SPT	BD	21	29	17	8	13	8	7	11	8	9	**n= 131**
	UD	77	41	31	24	18	18	7	2	1	0	**n= 219**
	G	113	15	13	10	4	9	2	0	0	0	**n= 166**

Table 4.2 Comparison between techniques to classify patterns of genetic change. K-means clusters (1-10) are across the top horizontal bar, categorization by linear discriminant analyses (LDA) is in the vertical column on the left. The cluster-specific agreement between the two methods (% agreement) is represented below the main matrix. Cluster-wise membership of categories determined by sequential pair-wise tests (SPT) for 'gradual' (G), uni-directional (UD) and bi-directional (BD) changes are in grey, green and blue colors, respectively.

CHAPTER 5 GENERAL CONCLUSIONS

1. Overview of general conclusions

The following chapter summarizes the main themes addressed in my dissertational research. Specific themes include: 1) the stage specific and variable sensitivity of oyster larvae to acidified seawater, 2) the difference in relative fitness of larvae from selectively bred (MBP) stocks compared to those from naturalized populations, 3) genetic changes occurring during larval development in MBP groups versus those of naturalized stocks, 4) genetic changes occurring across larval development in MBP and naturalized groups in simulated OA conditions, and, 5) the dynamic pattern of genetic selection over larval development and inferences for balancing selection, adaptation, and genetic diversity.

2. Stage-specific effects of acidified seawater on oyster larvae

The study described in Chapter 2 constituted the core of the experimental work for all of the research that I conducted for this dissertation. At the outset the goal of this project was to examine the relative differences in larval performance between parental oysters from naturalized stocks of *Crassostrea gigas* in the PNW and domesticated lines from MBP in ambient and high pCO_2 conditions. What we found over the course of two experiments, however, became a much more complex set of results that provided unique insights into some of the aspects of acidification stress on oysters that are underreported in the scientific literature.

Collectively, we found that initial larval development was distinctly and consistently impaired by high pCO_2/low Ω_{arag} conditions, resulting in an increased

proportion (+ ~13%) of malformed, or 'abnormal' larvae after two days of culture. This observation is altogether consistent with numerous previous studies which reported similar effects of low Ω_{arag} on embryogenesis and shell formation in marine bivalves (e.g. Waldbusser et al. 2015). What we were also able to additionally demonstrate, however, was that larval performance metrics at 2 days post fertilization did not exhibit significant correlations to any subsequent performance metrics such as growth or survival of larvae through veliger stages (to day 16). This finding is not evidence to suggest that the negative effects of OA dissipate after initial shell formation in larval oysters, but simply that early impediments to larval development may not always have fatal outcomes and the ongoing stresses of acidified seawater may not be detected by measurements of external phenotypes such as size, survival and morphology.

Over the metamorphic transition from pediveliger larvae (~day 16) to juvenile spat, high pCO_2 seawater conditions had variable effects on total settlement success of larval populations. In the first experiment simulated OA conditions did not exhibit any significantly adverse impacts on settlement - the total number and size of spat for each group was unaffected by 22 days of culture in high pCO_2 seawater. In the second experiment, by contrast, similarly acidified conditions ultimately impaired larval development to settlement 'competency' resulting in increased mortality of smaller larvae (< 240μm) and ~42% fewer spat for both groups at the end of the experiment (Fig. 2.5B). Given the high degree of replication in these experiments (culture conditions, genetic stocks etc.) and relatively uniform performance of larvae prior to metamorphosis, it is surprising to observe such a high degree of variability in the

response of larvae to seawater pCO_2. For much of the larval period, however, acidification stress is not a parameter which influences overall larval fitness independently. Several other studies have reported significant interactive effects between additional environmental parameters and seawater pCO_2 (Hettinger et al. 2013, Thomsen et al. 2013, Cole et al. 2016) and the results from Chapter 2 demonstrate that experimental variables which are difficult to measure or control (such as gamete quality) may strongly influence larval fitness and overall sensitivity to environmental stressors.

Future studies that seek to evaluate the responses of small marine organisms, such as larval oysters, to environmental stressors would be advised to consider evaluating these organisms across multiple life stages and in replicated experiments. It is clear from our study that if the scope of experimentation was constrained to initial larval development (as is common for OA research; e.g. Kurihara et al. 2007) or was limited to one of the two trials (also common; e.g. Talmage & Gobler 2010), completely different conclusions would be reached which obscure the complexity of larval sensitivity to high pCO_2 culture conditions.

3. Relative fitness of larvae from MBP and Willapa stocks

Amidst all of the variability in larval response to high pCO_2 from Chapter 2, one trend which was consistent across experiments was the performance of MBP larvae, relative to counterparts from naturalized stocks in Willapa bay. On average, MBP larval groups produced ~55% and ~37% more spat that were ~5% and ~23% larger in ambient and high pCO_2 conditions, respectively. These results were a

surprise, considering that MBP has focused entirely on genetic improvements for adult performance traits, not larval fitness (de Melo et al. 2016). It appears, however, that five generations of passive selection in hatchery environments has had a domesticating effect within MBP lines and unintentionally improved larval traits (similar to Taris et al. 2006, and Taris et al. 2007). These general fitness improvements also appear to have cross-over benefits for larvae exposed to OA conditions but the specific aspects of larval physiology which account for increased spat production in MBP groups have yet to be determined. Larvae from selected lines did not have improved rates of growth and survival during veliger stages (to day 16) so perhaps more subtle advantages, such as increased metabolic efficiencies and greater energy reserves, improved settlement rates and spat growth. Regardless of mechanism, these results broadly suggest that genetics play a significant role in larval fitness, and that heritable genetic variation can alter fitness optima for oyster larvae.

4. Overall genetic changes over larval development in MBP and Willapa groups.

For oysters, the high mortality rate which is frequently observed during larval stages is reported to be accompanied by significant changes in genetic composition of the population over this period (Launey & Hedgecock 2001, Plough et al. 2016). In Chapter 3, we expanded on these previous studies by evaluating changes in a greater number of genome wide markers and comparing the relative changes in domesticated (MBP) and naturally breeding (Willapa) oyster populations. Similar to prior estimates, we found abundant genetic changes in larval oyster populations across developmental transitions but MBP stocks showed ~26% less genetic change than

their Willapa counterparts. Interestingly, nearly all changes attributable to development were unique to either MBP or Willapa groups with no significant changes to allele frequencies of the same loci in the other population.

In Chapter 3 we discussed some of the mechanisms which may have contributed to the reduced and disparate genetic changes taking place in MBP lines but the important outcomes of this research are that: 1) the increased survival of MBP larvae in hatchery conditions (Chapter 2) is matched with a reduced genetic change of the larval population and 2) The loci which undergo changes during development are associated with numerous and diverse physiological processes and 3) The disparate genetic changes between stocks implies different physiological vulnerabilities of larvae and/or substantial and rapid genetic differentiation of these populations. From the standpoint of oyster breeding, these results support with molecular evidence what we observed with phenotypic results: that traditional selection methods, although unintentional, have improved larval fitness phenotypes in MBP lines. The disparity of changes between these stocks, however, suggests that molecular methods, such as marker assisted selection (e.g. Hollenbeck & Johnston 2018), may be difficult to employ in oysters and have a limited scope of application.

5. Genetic changes of larvae in response to OA exposure

The phenotypic impacts of acidified seawater on the fitness of larval marine calcifying organisms has been widely studied (Kroeker et al. 2010). What remains less well investigated is the genetic components underpinning both vulnerability and resilience to acidification stress. In the first experiment for Chapter 2, we did not find

any significant impacts of high pCO_2 seawater on total larval survival or settlement success. By comparing the genetic changes in larval groups in ambient seawater pCO_2 to those taking place in simulated OA conditions, however, we found that acidified seawater significantly altered the genetic makeup of pediveliger larvae and spat by the end of the experiment. This result is similar to the findings of Pespeni et al. (2013) who reported no net phenotypic effect of acidification stress on urchin larvae yet substantial genetic changes taking place in populations exposed to high pCO_2 seawater. Overall, in our study, acidified seawater had more than a two-fold greater impact on the number of genetic markers changed in Willapa larval groups, compared with MBP. This potentially indicates that domesticated stocks, that have less genetic change over larval development in general, are also more genetically resilient to the net stresses of acidified seawater in hatchery settings.

While genes associated with general developmental changes were functionally diverse, those associated with loci changing in high pCO_2 conditions for both groups were significantly over-represented by functional categories involved with membrane structure and function in both groups (Table 3.2). This finding is consistent with previous physiological studies (Pan et al. 2015, Frieder et al. 2018) that suggested the regulation of ionic gradients across cellular membranes is a critical physiological function for the survival of larval oysters in acidified seawater. It is important to recognize that the majority of genetic changes we found in response to high pCO_2 seawater exposure were deeply intertwined with overarching genetic changes taking place during larval development. Many of these changes were in a consistent direction, i.e. development and pCO_2 stress shifted allele frequency in a common

way, but many also had significantly negative interactions, i.e. selection during development opposed selective pressures of acidified seawater. The genetic aspects of larval development of oysters in acidified seawater are complex and temporally dynamic. Further work will be necessary to determine how viability selection during development itself interacts with the stresses of high pCO_2 seawater in order to better understand the potential long term impacts of OA on adaptation in oyster populations.

6. Patterns of genetic change over larval development

The results from Chapters 2 and 3 demonstrate that larval development in oysters is a highly dynamic process and the genetic components of survival are extensive and complex. Previous studies of genetic load in oysters, and its consequences on larval survival, have focused almost entirely on the 'final' outcome of viability selection in juvenile or adult oysters (e.g. Launey & Hedgecock 2001, Plough et al. 2016). In Chapter 4, we utilized temporal pooled DNA samples from larval populations to evaluate the nature and scale of genetic changes during larval development with greater resolution than previously achieved.

Our results suggest that, similar to previous findings, numerous negative alleles result in shifts of the genetic composition of surviving larvae. The temporal patterns of selection, however, do not exclusively favor one allele or genotype at each locus. Around a quarter of all loci affected by development appear to be impacted by selection pressures against multiple negative alleles, and these loci exhibit stage-specific mortality patterns that purge genotypes possessing both allelic variants. These so-called 'bi-directional' changes in allele frequency that we found provide

evidence to support a hypothesis for temporally balanced polymorphisms. For these loci, negative or lethal alleles may persist in the population as a result of 'interrupted heterotic resilience' whereby homozygous genotypes are potentially selected against at sequential points in developmental time. With pooled DNA samples it is difficult to conclusively demonstrate changes in genotypic proportions for a given shift in allele frequency at a locus but if our interpretation is correct, this example would contribute to the relatively few cases where balancing selection has been demonstrated to influence long-term genetic variation. This evidence also furthers our understanding of the mechanisms by which genetic diversity and high levels of genetic load are maintained in oysters and, potentially, other highly fecund marine species (Plough 2016).

Through the course of this research, the aim of this dissertation evolved from a comparative analysis between naturalized and selectively bred Pacific oysters in the PNW to a detailed investigation into the genetic aspects of larval fitness and survival of oysters. The OA and breeding aspects of these studies have important implications for the future of the global shellfish industry as it faces changing ocean conditions and new environmental threats. The techniques for genetic analyses of pools of larval oysters we developed not only shed some light on the cryptic network of genetic-based fitness traits for larval marine invertebrates but also demonstrate some new tools for future studies to efficiently advance research in this field.

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APPENDICES

APPENDIX 1 SUPPLEMENTAL TABLES

	2015		2016	
	MBP	Willapa	MBP	Willapa
# eggs sampled	20000	20213	6489	omitted due to contamination
sum SFA	37.88	41.67	40.73	
Sum MUFA	29.51	29.99	26.91	
sum PUFA	31.04	26.78	30.55	
PUFA/SFA	0.82	0.64	0.75	
Sum n-3	18.46	14.82	19.36	
DHA/EPA	0.71	0.72	0.67	
Total per sample	67.65	73.92	28.10	
Mean lipid egg^{-1}	**3.38**	**3.66**	**4.33***	

Table A2.1 Summary lipid composition of fertilized egg pools in 2015 and 2016 from MBP and Willapa groups. Mean lipid egg^{-1} was significantly greater for MBP eggs in 2016 (*). Lipid composition, however was highly similar. Willapa egg samples from 2016 were removed from analysis due to contamination during collection (see methods).

				Day post fertilization						
				2	6	10	12	16	22	24
2015	MBP	Ambient	%	58.8(6.7)	29.2(5.9)	31.3(2.0)	-	17.3(3.0)	10.1(3.1)	-
			µm	81.8(1.2)	114.2(3.6)	154.8(5.9)	-	220(56.0)	568.2(18.4)	-
		High pCO_2	%	61.3(3.9)	31.6(11.6)	31.4(11.2)	-	16.6(5.0)	11.1(3.4)	-
			µm	79.6(3.6)	114.8(4.1)	154.6(9.8)	-	209.2(47.3)	593.9(31.5)	-
	Willapa	Ambient	%	67.4(13.9)	33.5(10.3)	31.2(8.1)	-	19.6(8.7)	12.3(6.7)	-
			µm	78.7(1.2)	111.4(1.6)	144.8(6.0)	-	216.7(49.2)	509.4(26.7)	-
		High pCO_2	%	71.7(11.3)	32.8(12.8)	30.1(10.2)	-	19.2(7.7)	11.3(7.9)	-
			µm	78.5(2.2)	112.7(1.7)	152.4(3.5)	-	208.1(40.6)	492.1(45.3)	-
2016	MBP	Ambient	%	44.6(6.0)	27.7(5.7)	-	18(2.6)	-	-	10.7(3.4)
			µm	80.7(0.9)	109.7(2.9)	156.3(4.9)	181.2(5.8)	-	-	634.2(43.1)
		High pCO_2	%	54.2(5.9)	33.6(7.3)	-	21.6(2.3)	-	-	3.5(2.4)
			µm	79.4(0.8)	106.1(3.6)	153.2(5.4)	177.4(8.0)	-	-	722.6(88.4)
	Willapa	Ambient	%	37.3(6.2)	27.9(5.2)	-	16.7(3.2)	-	-	8.2(2.2)
			µm	81.0(0.6)	104.3(2.3)	150.6(4.3)	164.5(5.2)	-	-	632.4(28.7)
		High pCO_2	%	38.2(5.2)	31(7.8)	-	17.8(2.7)	-	-	5.1(1.5)
			µm	79.7(0.7)	105.1(3.1)	143.6(8.2)	167.5(12.2)	-	-	616.6(86.5)

Table A2.2 Detailed larval performance. Mean cumulative percent survival (%) and size (µm) of MBP and Willapa larvae reared in ambient and high pCO_2 seawater conditions in replicate experiments in 2015 and 2016. Means are averaged from n=5 and n=6 experimental replicates in 2015 and 2016, respectively.

$$Cumulative\ survival \sim \beta_{Type}\ x\ \beta_{Treatment}\ x\ \beta_{Year}\ x\ \beta_{Day}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	7.30E-01	5.34E-02	13.663	**<2e-16**
Type = Willapa	3.12E-02	7.51E-02	0.415	0.678
Day	-3.45E-02	4.51E-03	-7.64	**5.48E-13**
Treatment = High pCO_2	6.76E-03	7.51E-02	0.09	0.928
Year = 2016	-6.42E-02	7.26E-02	-0.884	0.377
Willapa x Day	-1.94E-04	6.20E-03	-0.031	0.975
Willapa x High pCO_2	6.79E-03	1.06E-01	0.064	0.949
Day x High pCO_2	2.87E-04	6.20E-03	0.046	0.963
Willapa x 2016	-5.54E-02	1.02E-01	-0.541	0.589
Day x 2016	5.15E-03	6.01E-03	0.857	0.392
High pCO_2 x 2016	5.90E-02	1.02E-01	0.577	0.565
Willapa x Day x High pCO_2	-1.56E-03	8.64E-03	-0.18	0.857
Willapa x Day x 2016	4.45E-04	8.36E-03	0.053	0.958
Willapa x High pCO_2 x 2016	-5.51E-02	1.46E-01	-0.378	0.706
Day x High pCO_2 x 2016	-4.94E-03	8.36E-03	-0.591	0.555
Willapa x Day x High pCO_2 x 2016	4.75E-03	1.19E-02	0.401	0.689

Residual Std. Error = 0.186 on 235 degrees of freedom

Adjusted R^2 = 0.651

$F_{15,235}$ = 32.08 , p-value: 2.2×10^{-16}

Table A2.3 Summary table of linear model of cumulative survival of larvae from day 0 to 22 in 2015 and day 0 to 24 in 2016. Type III sum of squared residuals are reported for the fixed effects: broodstock type, seawater treatment, day post fertilization, year of experiment and all interactions between them. The random effect of culture replicate (Replicate) is used to account for multiple measurements. Reference level (intercept) is: Day=0, Type=MBP, Treatment=Ambient and Year=2015. Day is found as the only significant parameter in this model and when this model is submitted to bi-directional stepwise AIC selection, the final model of best fit is *Survival ~ Day*.

$$log(mean\ size) \sim \beta_{Type}\ x\ \beta_{Treatment}\ x\ \beta_{Year}\ x\ \beta_{Day}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	4.25E+00	7.12E-02	59.667	**<2e-16**
Type = Willapa	-2.67E-02	9.99E-02	-0.267	0.789
Day	7.42E-02	4.83E-03	15.37	**<2e-16**
Trt = High pCO_2	-2.53E-02	9.99E-02	-0.253	0.801
Year = 2016	4.42E-02	1.03E-01	0.429	0.668
Willapa x Age	-6.42E-04	6.65E-03	-0.096	0.923
Willapa x High pCO_2	3.11E-02	1.41E-01	0.221	0.825
Day x High pCO_2	8.64E-04	6.65E-03	0.130	0.897
Willapa x Year 2016	-3.94E-02	1.45E-01	-0.271	0.786
Day x Year 2016	-4.47E-03	6.82E-03	-0.655	0.513
High pCO_2 x Year 2016	-1.91E-02	1.45E-01	-0.131	0.896
Willapa x Age x High pCO_2	-1.61E-03	9.28E-03	-0.173	0.863
Willapa x Age x 2016	5.51E-03	9.52E-03	0.579	0.563
Willapa x High pCO_2 x 2016	6.45E-03	2.05E-01	0.031	0.975
Day x High pCO_2 x 2016	2.54E-03	9.52E-03	0.266	0.790
Willapa x Age x High pCO_2 x 2016	-2.78E-03	1.34E-02	-0.208	0.836

Residual Std. Error = 0.242 on 302 degrees of freedom

Adjusted $R^2 = 0.858$

$F_{15,302} = 128.8$, p-value: 2.2e-16

Table A2.4 Summary table of linear model of mean size of larvae (log transformed for normality) from day 0 to 22 in 2015 and day 0 to 24 in 2016. Type III sum of squared residuals are reported for the fixed effects: broodstock type, seawater treatment, day post fertilization, year of experiment and all interactions between them. Reference level (intercept) is: Age=0, Type=MBP, Treatment=Ambient and Year=2015. Day is found as the only significant parameter in this model and when this model is submitted to bi-directional stepwise AIC selection, the final model of best fit is *log(size) ~ Day*.

$$Survival \sim \beta_{Type} + \beta_{Treatment} + \beta_{Year} + \beta_{Type\ x\ Year}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	0.5772	0.02777	20.788	**2.00E-16**
Type = Willapa	0.09546	0.03536	2.699	**0.0103**
Treatment = High pCO_2	0.04616	0.02414	1.912	0.0634
Year = 2016	-0.08027	0.03386	-2.371	**0.0229**
Willapa x 2016	-0.2311	0.04839	-4.776	**2.66E-05**

Residual std. error: 0.079 on 38 degrees of freedom

Adjusted R-squared: 0.6786

$F_{4,38}$ = 23.17, p-value: 9.319e-10

Table A2.5 Summary table of linear model (lm) estimating percent survival of 48 hour D-larvae. Type III sum of squared residuals are reported for the fixed effects: broodstock type (Type), seawater treatment (Trt), year of experiment (Year) and an interaction of Type*Year (Type:Year). Reference level (Intercept) is MBP, Ambient and 2015 for Type, Trt and Year, respectively. A highly significant interaction of Willapa and Year ($p=2.66 \times 10^{-5}$) suggest this effect overshadows the independent effects of each of the contributing parameters.

$$\%\, normal \sim \beta_{Type}\, x\, \beta_{Treatment}\, x\, \beta_{Year}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	0.73546	0.02067	35.587	< **2e-16**
Type = Willapa	0.03635	0.02923	1.244	0.222
Treatment = High pCO_2	-0.1326	0.02923	-4.537	**6.44E-05**
Year = 2016	0.02376	0.02798	0.849	0.402
Willapa x High pCO_2	0.06491	0.04133	1.570	0.125
Willapa x 2016	-0.05143	0.03957	-1.300	0.202
High pCO_2 x 2016	0.05063	0.03957	1.279	0.209
Willapa x High pCO_2 x 2016	-0.09293	0.0566	-1.642	0.11

Residual std. error: 0.046 on 35 degrees of freedom

Adjusted R-squared: 0.5737

$F_{7,35}$ = 9.075 , p-value: 2.392e-06

Table A2.6 Summary table of linear model (lm) estimating percent normal larvae among 48 hour D-larvae. Type III sum of squared residuals are reported for the model with fixed effects: broodstock type, seawater treatment, year of experiment and all 2-way and 3-way interactions between them. Reference level (Intercept) is MBP, Ambient and 2015 for Type, Trt and Year, respectively. While the full model was selected as best fit for the data via stepAIC, (Venables and Ripley, 2002), only seawater treatment (high pCO_2) is a significant effect, without interactions.

$$Average\ size \sim \beta_{Type} + \beta_{Treatment} + \beta_{Year} + \beta_{Type\ x\ Year}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	81.3443	0.5622	144.678	**< 2e-16**
Treatment = High pCO_2	-1.2881	0.4839	-2.662	**0.01123**
Type = Willapa	-2.1416	0.7177	-2.984	**0.00489**
Year = 2016	-0.6901	0.6872	-1.004	0.32144
Willapa x 2016	2.4682	0.9718	2.54	**0.01519**

Residual std. error: 1.605 on 39 degrees of freedom

Adjusted R-squared: 0.2388

$F_{4,39}$ = 4.373 , p-value: 0.005125

Table A2.7: Summary table of linear model estimating average shell height (μm) of larvae at 48 hours. Type III sum of squared residuals are reported for the model with fixed effects: seawater treatment, broodstock type, year of experiment and an interaction of Type and Year. High pCO_2 had significant negative effects across both broodstock types in both years. Willapa is a significant as a primary coefficient (est = -2.14 μm) as well as in an interaction with Year (+2.47). This indicates that Willapa larvae at 48 hours were significantly smaller in 2015 but had similar average size in 2016.

A

$$Total\ normal\ larvae \sim \beta_{Type} + \beta_{Year} + \beta_{Type\ x\ Year}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	0.40101	0.0224	17.9210	**< 2e-16**
Type = Willapa	0.11495	0.0317	3.6330	**0.000807**
Year = 2016	-0.02912	0.0303	-0.9610	0.342379
Willapa x 2016	-0.22263	0.0433	-5.1430	**7.97E-06**

Residual std. error: 0.071 on 39 degrees of freedom

Adjusted R-squared: 0.6046

$F_{3,39}$ = 22.41 , p-value: 1.338e-08

B

Analysis of Variance Table

Model 1: $Total\ normal\ larvae \sim \beta_{Type} + \beta_{Year} + \beta_{Treatment} + \beta_{Type\ x\ Year}$

Model 2: $Total\ normal\ larvae \sim \beta_{Type} + \beta_{Year} + \beta_{Type\ x\ Year}$

Model	Residual DF	Residual Sum of Squares	DF	Sum of Squares	F	P-value
1	38	0.1912				
2	39	0.19528	-1	-0.00408	0.8103	0.3737

Table A2.8 Total normal larvae

A) Summary table of linear model estimating total percent normal larvae at 48 hours, from total stocked at fertilization. Type III sum of squared residuals are reported for the model with fixed effects: broodstock type, year of experiment and an interaction of type and year. Reference level (Intercept) is MBP and 2015 for Type and Year, respectively. The highly significant (and negative) interaction between Willapa and 2016 fixed effects ($p=7.97 \times 10^{-6}$) suggests the main (and positive) effect of Willapa in 2015 is, while significant, relatively minor.

B) Analysis of variance table comparing a model including $\beta_{Treatment}$ and the model in A), above, chosen by AIC stepwise selection. The large p-value (0.3737) indicates that including '*treatment'* effects in this model is not justified .

$$Survival\ Day\ 2 - Day\ 6 \sim \beta_{Type} + \beta_{Treatment} + \beta_{Year} + \beta_{Survival\ Day\ 0-2}$$

Coefficients	Estimate	SE	t value	P-value
(Intercept)	0.81816	0.16238	5.039	**1.18E-05**
Type = Willapa	0.05261	0.04639	1.134	0.2638
Treatment = High pCO_2	0.0263	0.0474	0.555	0.5823
Year = 2016	0.10071	0.06586	1.529	0.1345
Survival Day0-Day2	-0.56197	0.24377	-2.305	**0.0267**

Residual std. error: 0.1503 on 38 degrees of freedom

Adjusted R-squared: 0.3637

$F_{4,38}$ = 7.002 , p-value: 0.0002515

Table A2.9 Summary table of the linear model estimating the survival of larvae from day 2 to 6 post fertilization. Type III sum of squared residuals are reported for the model with fixed effects: broodstock type, seawater treatment, year of experiment and survival from fertilization from Day 0 to Day 2 of the same culture unit. Initial survival to day 2 was significantly negatively correlated to subsequent survival from day 2 to day 6 (p=0.267).

$$Average\ Day\ 6\ size\ (\mu m) \sim \beta_{Type} + \beta_{Treatment} + \beta_{Year} + \beta_{Survival\ Day\ 0-2} + \beta_{Type\ x\ Treatment} + \beta_{Type\ x\ Year} + \beta_{Treatment\ x\ Year} + \beta_{Type\ x\ Survival\ Day\ 0-2} + \beta_{Type\ x\ Treatment\ x\ Year}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	102.1317	6.9517	14.692	**4.91E-16**
Type = Willapa	11.0641	8.6345	1.281	0.209
Treatment = High pCO_2	0.1181	1.8773	0.063	0.9502
Year = 2016	-2.0811	2.2468	-0.926	0.361
Survival Day0-Day2	20.4497	11.6098	1.761	**0.0874**
Willapa x High pCO_2	1.2945	2.6581	0.487	0.6295
Willapa x 2016	-5.815	3.5961	-1.617	0.1154
High pCO_2 x 2016	-5.6919	2.6645	-2.136	**0.0402**
Willapa x Survival Day0-Day2	-23.1521	13.7357	-1.686	0.1013
Willapa x High pCO_2 x 2016	5.6417	3.7095	1.521	0.1378

Residual std. error: 2.934 on 33 degrees of freedom

Adjusted R-squared: 0.6128

$F_{9,33}$ = 8.384 , p-value: 2.231e-06

Table A2.10 Summary table of the linear model estimating the average size (μm) of larvae at day 6 post fertilization. Type III sum of squared residuals are reported for the model with fixed effects: broodstock type, seawater treatment, year of experiment and survival from fertilization from Day 0 to Day 2 of the same culture unit. Multiple interactions between these parameters are also retained in the optimal fit of this model (by forward/backward AIC selection). Larvae reared in High pCO_2 groups in 2016 were ~5% smaller than other groups (est -5.7 μm, p=0.04) but survival from day 0 to 2 was weakly (p=0.087) but positively correlated to size at day 6 (est +20.5 μm).

$$Cumulative\ Survival \sim \beta_{Day} + \beta_{Year}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	41.9495	2.0770	20.197	**<2e-16**
Day	-1.3447	0.1668	-8.063	**6.32e-13**
Year = 2016	-4.2441	1.3708	-3.096	**0.00244**

Residual std. error: 7.244 on 120 degrees of freedom

Adjusted R-squared: 0.351

$F_{2,120}$ = 33.99 , p-value: 2.016e-12

Table A2.11. Summary table of linear model estimating percent cumulative survival of larvae from day 6 to 16. Type III sum of squared residuals are reported for the fixed effects: day post fertilization (Day) and year of experiment. Reference level (intercept) is Day 0 in 2015, outside the range of data used to create the model (Day 6-16). The significant effect of 'Year = 2016' in this model suggests these data are best fit by a parallel lines model, with 2016 having a lower intercept but not a reduced survival rate. * Note: the estimate for intercept (Day 0, 2015) is ~42%; much lower than the actual 100%. This is due to the heterogeneous survival pattern seen in larvae during these experiments: mortality rate in day 0-6 is much greater than subsequent stages. Survival rates over this timeframe are sufficiently linear for this model, however prediction intervals outside of this data range are unreliable.

$$Average\ size\ (\mu m) \sim \beta_{Day}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	47.305	5.9488	7.952	**4.48E-13**
Day	10.384	0.5259	19.748	**< 2e-16**

Residual std. error: 24.1 on 147 degrees of freedom

Adjusted R-squared: 0.7244

$F_{1,147}$ = 390 , p-value: < 2.2e-16

Table A2.12. Summary of linear model estimating the effect of age (days post fertilization) on larval size (μm) to pediveliger stage (day 16). After forwards/backwards stepwise AIC selection, all other parameters (seawater pCO_2, broodstock type and year of experiment) were eliminated as insignificant.

$$Total\ survival\ over\ settlement \sim \beta_{Type}\ x\ \beta_{Treatment}\ x\ \beta_{Year}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	57.47	0.061	9.404	**7.38E-11**
Treatment = High pCO_2	9.07	0.082	1.106	0.277
Type = Willapa	2.44	0.082	0.298	0.768
Year = 2016	1.16	0.079	0.146	0.884
High pCO_2 x Willapa	-16.44	0.113	-1.459	0.154
High pCO_2 x 2016	-49.43	0.110	-4.475	**8.59E-05**
Willapa x 2016	-11.72	0.108	-1.083	0.287
High pCO_2 x Willapa x 2016	37.29	0.154	2.425	**0.021**

Residual std. error: 12.22 on 33 degrees of freedom

Adjusted R-squared: 0.5886

$F_{7,33}$ = 9.176 , p-value: 2.947e-06

Table A2.13: Summary table of linear model estimating percent survival of larvae from pediveliger stage to day 22 in 2015 and 24 in 2016. Type III sum of squared residuals are reported for the model with fixed effects: broodstock type (Type), seawater treatment (Trt), year of experiment (Year) and all 2-way and 3-way interactions between them. Reference level (Intercept) is MBP, Ambient and 2015 for Type, Trt and Year, respectively. High CO_2 * 2016 had a strong interactive effect ($p=8.59 \times 10^{-5}$) and the three way interaction 'High CO_2 x Willapa x 2016' was significant (p=0.021) although the net estimated effect (+ 37.3%) is nearly offset by the independent and 2 way interactions beneath it: High CO_2 (+9.1%) + Willapa (+2.4) + 2016 (1.2%) + High CO_2*Willapa (-49.4%) = -36.7%

$$\% \, Settlement \sim \beta_{Type} \, x \, \beta_{Treatment} \, x \, \beta_{Year}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	32.88	4.40	7.466	**1.60E-08**
Trt = High pCO_2	3.51	5.91	0.594	0.55681
Type = Willapa	-12.99	5.91	-2.199	**0.03607**
Year = 2016	18.24	5.69	3.209	**0.00313**
High pCO_2 x Willapa	-4.99	8.12	-0.615	0.54281
High pCO_2 x 2016	-45.40	7.96	-5.704	**2.57E-06**
Willapa x 2016	-2.53	7.80	-0.324	0.74938
High pCO_2 x Willapa x 2016	19.13	11.08	1.726	0.12782

Residual std. error: 8.863 on 33 degrees of freedom

Adjusted R-squared: 0.7264

$F_{7,33}$ = 16.17 , p-value: 5.079e-09

Table A2.14 Percent settlement. Summary table of linear model (lm) estimating percent metamorphosis and settlement (% Settlement) of pediveliger larvae to juvenile spat on day 22 in 2015 and day 24 in 2016. Type III sum of squared residuals are reported for the model with fixed effects: broodstock type, seawater treatment, year of experiment and all 2-way and 3-way interactions between them. Reference level (Intercept) is MBP, Ambient and 2015 for Type, Treatment and Year, respectively.

A) Year= 2015

$$\%\ Settlement \sim \beta_{Type}\ x\ \beta_{Treatment}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	32.88	3.89	8.44	**4.4E-07**
Trt = High pCO_2	3.51	5.22	0.67	0.5122
Type = Willapa	-12.99	5.22	-2.49	**0.0251**
High pCO_2 x Willapa	-4.99	7.18	-0.70	0.4974

Residual std. error: 7.78 on 15 degrees of freedom
Adjusted R-squared: 0.4822
$F_{3,15}$ = 6.587 , p-value: 0.0047

B) Year= 2016

$$\%\ Settlement \sim \beta_{Type}\ x\ \beta_{Treatment}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	51.12	3.95	12.95	**1.47E-10**
Trt = High pCO_2	-41.89	5.85	-7.16	**1.16E-06**
Type = Willapa	-15.52	5.58	-2.78	**0.0124**
High pCO_2 x Willapa	12.43	8.28	1.50	0.1507

Residual std. error: 9.669 on 18 degrees of freedom
Adjusted R-squared: 0.7905
$F_{3,18}$ = 27.42 , p-value: 6.256e-07

Table A2.15 Summary table of linear models estimating % settlement for experiments in 2015 (A) and 2016 (B) separately (in comparison with the combined analysis in Table A2.14). Type III sum of squared residuals are reported for the models with fixed effects: broodstock type, seawater treatment, and a 2-way interaction between Treatment x Type. Reference level (Intercept) is: Type = MBP, Treatment = ambient. In both years, MBP pediveliger larvae yielded more spat, on average, than Willapa counterparts and in 2016, high pCO2 treatment effects significantly reduced settlement rate in both groups.

A) Model Summary

$$Spat\ size\ (\mu m) \sim \beta_{Type} + \beta_{Treatment} + \beta_{Year} + \beta_{Survival} + \beta_{Treatment\ x\ Type} + \beta_{Year\ x\ Survival}$$

Coefficients	Estimate	SE	t value	P-value
(Intercept)	513.16	49.74	10.317	**5.22E-12**
Treatment = High pCO_2	25.1	23.32	1.076	0.28934
Type = Willapa	-39.41	18.68	-2.11	**0.04227**
Year = 2016	255.49	56.37	4.533	**6.88E-05**
Survival	0.859	80.94	1.061	0.296
High pCO_2 x Willapa	-64.72	27.99	-2.312	**0.02697**
2016 x Survival	-3.08	1.04	-2.967	**0.00547**

Residual std. error: 41.82 on 34 degrees of freedom

Adjusted R-squared: 0.7748

$F_{6,34}$ = 23.94 , p-value: 7.119e-11

B) Predicted estimates (μm)

Type	Treatment	Year	Fitted est.	Lower	Upper
MBP	Ambient	2015	562.53	470.87	645.20
MBP	High CO_2	2015	595.42	504.28	686.57
Willapa	Ambient	2015	525.22	435.00	615.44
Willapa	High CO_2	2015	497.27	388.77	569.76
MBP	Ambient	2016	637.96	547.69	728.23
MBP	High CO_2	2016	753.03	661.88	844.17
Willapa	Ambient	2016	619.22	529.44	709.00
Willapa	High CO_2	2016	623.10	532.60	713.59

Table A2.16: Spat size. A) Summary table of linear model estimating mean shell length of spat (μm) at day 22 in 2015 and 24 in 2016. Type III sum of squared residuals are reported for the model with fixed effects: broodstock type, seawater treatment, year of experiment, survival from day 16-22(24), two 2-way interactions: Treatment x Type and Year x Survival. Reference level (Intercept) is: Type = MBP, Treatment = ambient, Year = 2015, Survival = 100%. B) Estimated values from model in A) above. Estimates incorporate parameter coefficients from treatment combination as well as mean survival of the group. Spat were much larger in 2016, owing to an extended culture period but Willapa broodstock produced significantly smaller larvae than MBP in both ambient and high pCO2 conditions in both years. Survival rate of the culture replicate was negatively correlated to the mean size of spat therein, with an estimated correlation of -3.08 μm (% survival-1).

A) Year= 2015

$$Spat\ size\ (\mu m) \sim \beta_{Type} + \beta_{Treatment} + \beta_{Survival} + \beta_{Treatment\ x\ Type}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	505.15	38.79	13.02	**3.25E-09**
Type = Willapa	-61.53	20.58	-2.99	**0.0097**
Trt = High pCO_2	15.71	21.27	0.74	0.4720
Survival	1.10	0.62	1.77	0.0986*
High pCO_2 x Willapa	-24.89	29.99	-0.83	0.4210

Residual std. error: 30.59 on 14 degrees of freedom

Adjusted R-squared: 0.6624

$F_{4,14}$ = 9.828 , p-value: 0.00053

B) Year= 2016

$$Spat\ size\ (\mu m) \sim \beta_{Type} + \beta_{Treatment} + \beta_{Survival} + \beta_{Treatment\ x\ Type}$$

Coefficients	Estimate	SE	t-value	P-value
(Intercept)	783.66	61.57	12.73	**4.07E-10**
Type = Willapa	-25.39	30.07	-0.84	0.4102
Trt = High pCO_2	17.49	50.04	0.35	0.7310
Survival	-2.55	0.99	-2.57	**0.0198**
High pCO_2 x Willapa	-89.40	47.22	-1.89	0.0755*

Residual std. error: 49.58 on 18 degrees of freedom

Adjusted R-squared: 0.5812

$F_{4,17}$ = 8.286 , p-value: 0.0006724

**marginally significant (0.05 < p < 0.1)*

Table A2.17: Summary table of linear models estimating mean shell length of spat (μm) for experiments in 2015 (A) and 2016 (B) separately (in comparison with the combined analysis in Table A2.16**)**. Type III sum of squared residuals are reported for the models with fixed effects: broodstock type, seawater treatment, survival and a 2-way interaction between Treatment x Type. Reference level (Intercept) is: Type = MBP, Treatment = ambient, Survival = 100%. In 2015, MBP spat were ~61 μm larger, on average, than Willapa spat (p=0.0097) with no significant effect of seawater treatment (p= 0.472). In 2016, the overall difference in mean spat size between MBP and Willapa groups was reduced in ambient conditions (~25.4 μm larger in MBP, p=0.410) but was increased in high pCO_2 replicates (~89.4 μm larger in MBP, p=0.0755). Survival also played a more significant role in determining spat size in 2016, with an average reduction of ~2.55 μm in average spat size for every 1% increase in survival over the settlement period (p=0.0198).

Table A3.1 Bioinformatic statistics for DNA samples.

Sample	Type	Age	Trt	Raw reads	Quality reads	All mappings	Quality mappings	Unique reads remaining	Number mapped loci
MA1-D2	MBP	2	A	13946577	13609336	8454632	3703048	2822873	340824
MA2-D2	MBP	2	A	1878491	1661569	373398	158630	117693	89955
MA3-D2	MBP	2	A	5030559	4956775	3258779	1404331	1050563	333172
MA4-D2	MBP	2	A	5145627	5078867	3311382	1349460	988082	245130
MA5-D2	MBP	2	A	3551893	3497740	1860992	744245	558421	161773
MA1-D22	MBP	22	A	6372958	5864260	3417879	1519760	1160113	261163
MA3-D22	MBP	22	A	4322480	4194316	2538489	1157826	885551	299614
MA4-D22	MBP	22	A	5226179	5090249	3062730	1374266	1055124	285365
MA5-D22	MBP	22	A	5068494	4975223	3161363	1366917	1052107	296861
MO1-D2	MBP	2	OA	5348346	5265760	3262127	1347399	997339	231149
MO2-D2	MBP	2	OA	3630852	3567163	2314608	929514	702745	210175
MO3-D2	MBP	2	OA	3393679	3244250	1772359	767890	590477	213617
MO4-D2	MBP	2	OA	8565784	8437935	5518882	2238158	1659401	312304
MO5-D2	MBP	2	OA	5606621	5500992	3177689	1252132	953462	186084
MO1-D22	MBP	22	OA	2929883	2861900	1737413	798114	610453	287675
MO2-D22	MBP	22	OA	6158540	6042958	3761322	1663138	1266772	303578
MO3-D22	MBP	22	OA	5106231	4931591	3081673	1397872	1076024	278556
MO4-D22	MBP	22	OA	5474384	5369225	3318855	1554615	1181114	369039
MO5-D22	MBP	22	OA	5453414	5312945	3236995	1434309	1104277	295178
WA1-D2	Wild	2	A	12125947	11950469	7952953	3221936	2408811	343709
WA2-D2	Wild	2	A	2229377	2158559	1295826	533812	401305	162203
WA3-D2	Wild	2	A	4396436	4332553	2536505	1066913	799664	214750
WA4-D2	Wild	2	A	2357303	2322815	1421060	581084	441223	193189
WA5-D2	Wild	2	A	3912891	3774395	1323729	532327	399884	200158
WA1-D22	Wild	22	A	8761300	8576215	5074431	2144308	1655043	274894
WA2-D22	Wild	22	A	4693999	4588280	2763362	1233426	950859	284362
WA3-D22	Wild	22	A	8970549	8835842	5614330	2465819	1884638	396450
WA4-D22	Wild	22	A	5238631	5097166	2864427	1254374	967790	241909
WA5-D22	Wild	22	A	5369862	5222245	3278707	1438718	1101121	337526
WO1-D2	Wild	2	OA	4873652	4781479	3095205	1361693	1038373	354941
WO2-D2	Wild	2	OA	6753058	6633904	4180111	1827768	1401833	282888
WO3-D2	Wild	2	OA	4961648	4827942	2196793	835724	641487	146499
WO4-D2	Wild	2	OA	4476514	4390193	2803751	1121140	843131	222740
WO5-D2	Wild	2	OA	10911586	10713430	6573287	2803862	2129212	265857
WO1-D22	Wild	22	OA	5668343	5390948	2989686	1203517	926895	179327
WO2-D22	Wild	22	OA	7069057	6889332	4152001	1754670	1359443	252823
WO3-D22	Wild	22	OA	2176120	1949669	1128344	441215	336634	216265
WO4-D22	Wild	22	OA	2757234	2692144	1455484	624514	483767	217103
WO5-D22	Wild	22	OA	4509721	4398410	2680780	1215334	927933	319052

Table A4.1 Bioinformatic statistics for temporal DNA samples

Culture replicate	Day	Raw reads	Quality reads	All mappings	Quality mappings	Unique reads remaining
Eggs	0	4937567	4740502	2998430	1281478	966417
1	2	12125947	11950469	7952953	3221936	2408811
2	2	2229377	2158559	1295826	533812	401305
3	2	4396436	4332553	2536505	1066913	799664
4	2	2357303	2322815	1421060	581084	441223
5	2	3912891	3774395	1323729	532327	399884
1	6	1823375	881824	85172	38127	22922
3	6	3447370	3387980	2048810	873660	670446
4	6	3772550	3359812	2042372	842698	631238
5	6	2234758	2196227	1441303	608330	443906
1	10	7255330	7029740	4508597	1842833	1365617
2	10	7316634	7001121	4618729	1883362	1409273
3	10	5763903	5409838	3778068	1272128	967368
4	10	5137535	5016963	3244697	1333484	1003318
5	10	4549583	4454315	2997133	1221735	917561
1	16	5749373	5681726	3715734	1579167	1195793
2	16	6314380	6208816	3723647	1319456	1025771
3	16	1486092	1440380	795590	235284	184371
5	16	8082566	7983074	5345891	2314287	1703473
1	22	8761300	8576215	5074431	2144308	1655043
2	22	4693999	4588280	2763362	1233426	950859
3	22	8970549	8835842	5614330	2465819	1884638
4	22	5238631	5097166	2864427	1254374	967790
5	22	5369862	5222245	3278707	1438718	1101121

(A)

k-means cluster	ΔAF				
	Day 0-2	Day 2-6	Day 6-10	Day 10-16	Day 16-22
1	-0.029	0.089	-0.193	-0.091	0.299
2	0.603	-0.605	0.208	0.034	-0.312
3	-0.966	-0.376	0.219	0.631	-0.470
4	0.338	0.415	1.401	-1.228	-0.280
5	-1.890	0.418	-0.344	-0.358	1.341
6	0.326	1.440	-1.307	-0.633	1.076
7	1.725	-1.207	2.223	-0.383	-1.200
8	-0.004	0.657	-1.246	1.338	-0.877
9	1.368	2.156	0.341	-3.506	2.206
10	-1.280	-2.288	0.870	2.699	-2.262

(B)

	Coefficients of linear discriminants:				
	LD1	LD2	LD3	LD4	LD5
Day 0-2	0.239	-0.918	1.206	0.409	0.480
Day 2-6	0.879	-0.031	-0.472	1.153	0.714
Day 6-10	-0.347	-1.476	-1.026	0.299	1.135
Day 10-16	-0.957	0.233	-0.013	0.433	1.754
Day 16-22	0.772	0.054	-0.055	-0.489	1.372

(C)

Proportion of trace:				
LD1	LD2	LD3	LD4	LD5
0.451	0.412	0.096	0.039	0.002

Table A4.2 Summary output from machine learning algorithms. (A) Cluster group means in change of allele frequency (ΔAF) at each time interval for k-means clustering. (B) Coefficients of linear discriminants (LD) for each interval, and (C) 'proportion of trace' or proportion of variance explained by each discriminant vector. LD1 and LD2 collectively account for ~86% of all the variance.

APPENDIX 2 SUPPLEMENTAL FIGURES

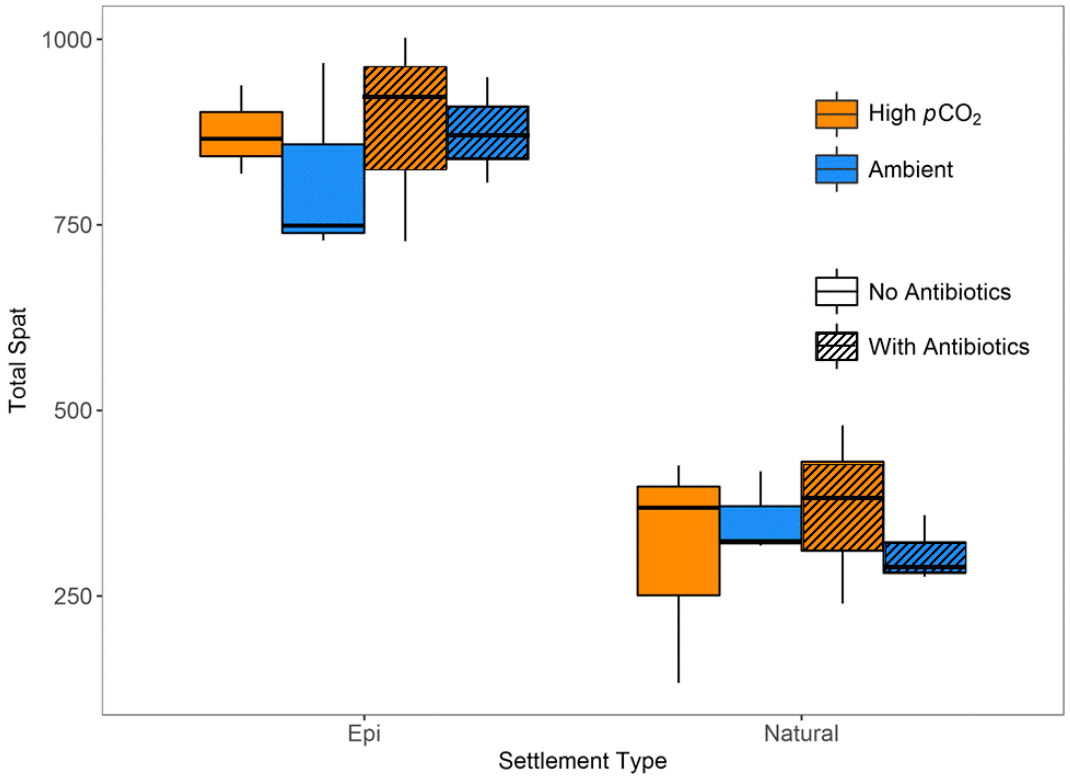

Figure A2.1 Comparing epinephrine (Epi) induced versus natural settlement in low (~400) and high (~1600) pCO_2 seawater with and without antibiotics.

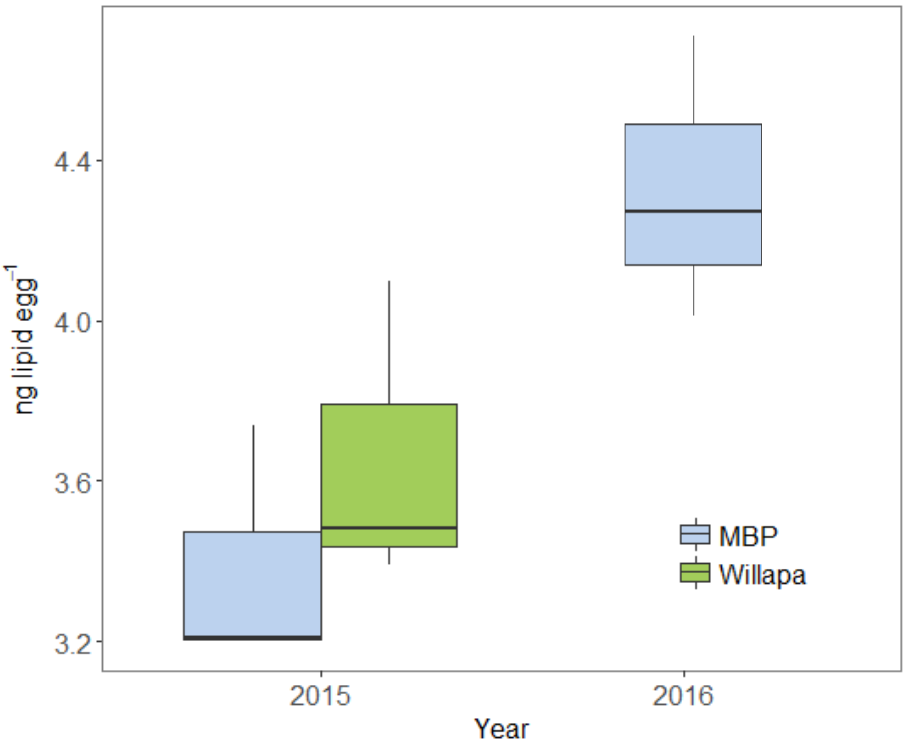

Figure A2.2. Mean lipid content (ng egg^{-1}) of eggs for MBP and Willapa pools in 2015 and MBP in 2016.

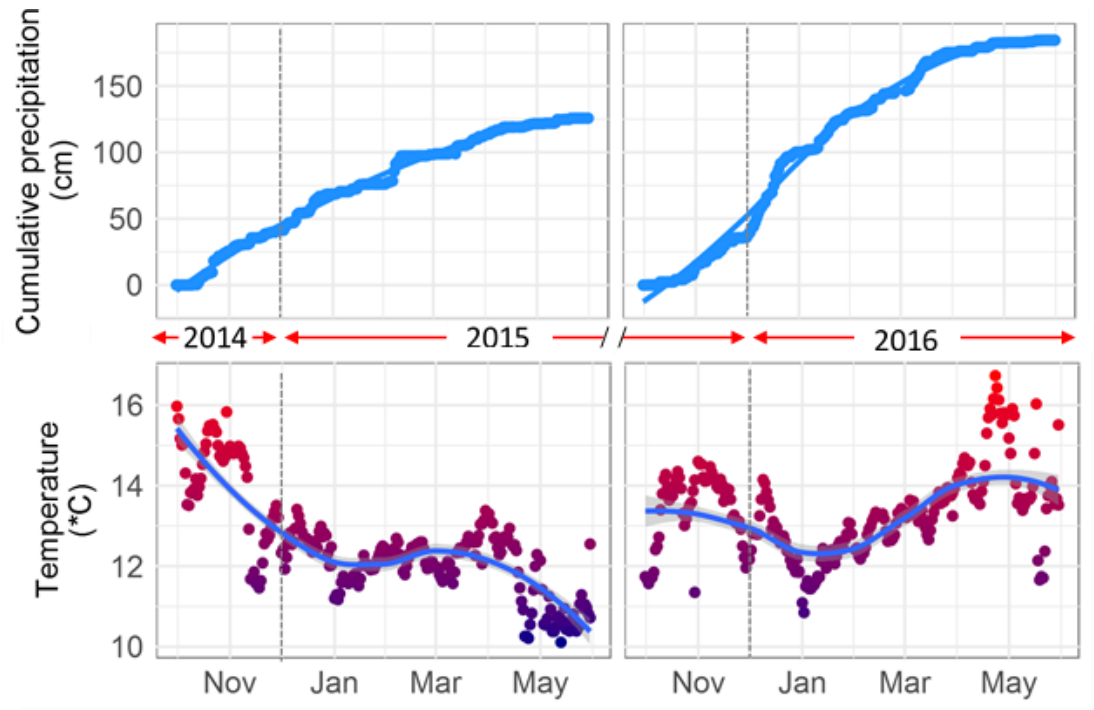

Figure A2.3. Cumulative precipitation and seawater temperatures for Yaquina Bay, Oregon during the 2015 and 2016 oyster conditioning seasons. 'Conditioning season' is defined here as the winter period during which oysters reabsorb old gonad tissue and begin gametogenesis anew, denoted by red arrows and dashed vertical lines to delineate seasons. Warmer seawater temperatures and higher cumulative precipitation in 2016 were potentially causative factors contributing to poorer overall gonad quality observed in broodstock for this year's spawn.

Precipitation data was obtained from NOAA weather stations at:
https://www.ncdc.noaa.gov/cdo-web/
station ID: ZIP:97365.

Seawater temperature data was obtained from NOAA's national data buoy center at:
http://www.ndbc.noaa.gov/
station ID: SBE03-9435380

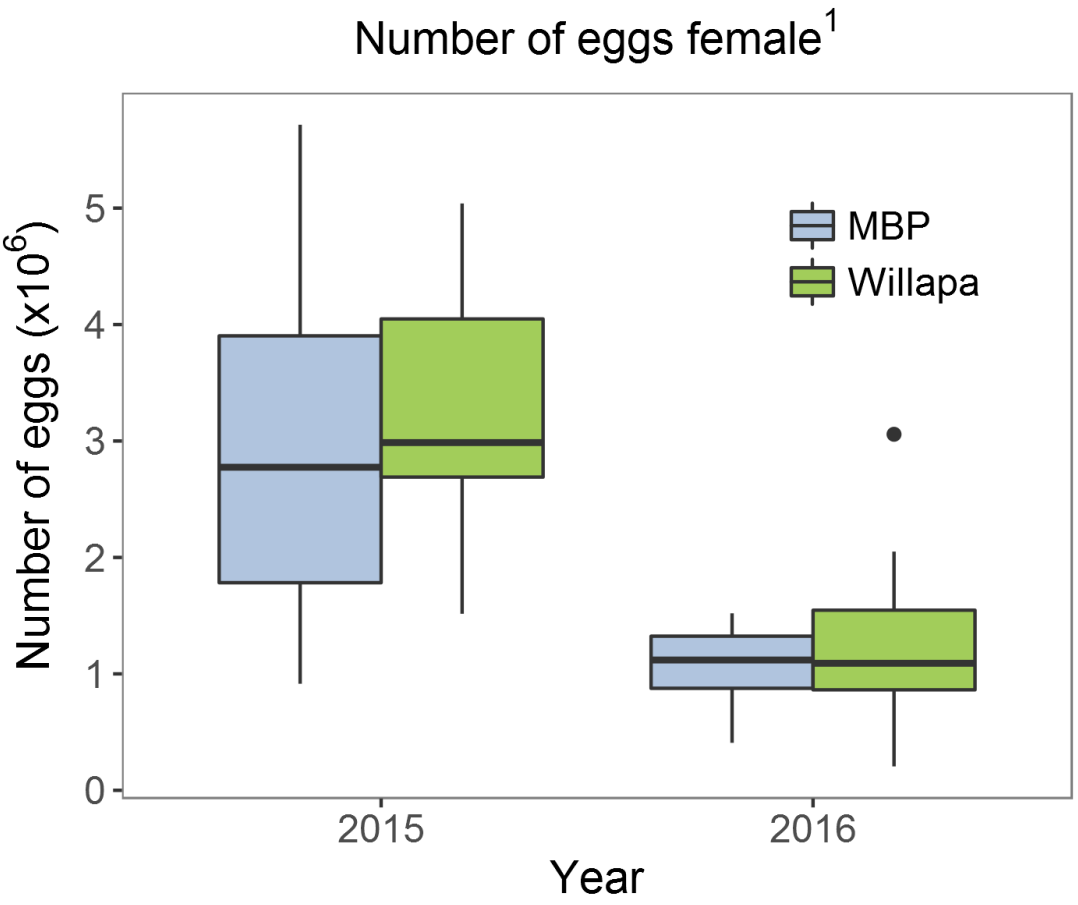

Figure A2.4 Average number of eggs female^{-1} in MBP and Willapa broodstock used in 2015 and 2016

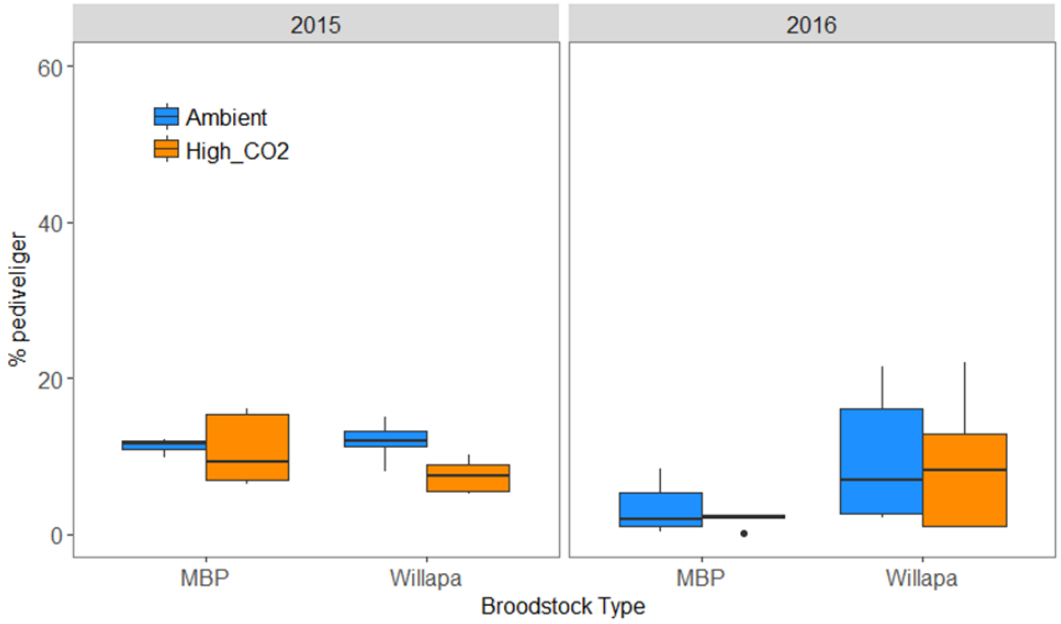

Figure A2.5. Percentage of pediveliger 'eyed' larvae at the conclusion of the experiments: day 22 in 2015, day 24 in 2016. Calculated as: *% pediveliger larvae / total larvae at day 16*

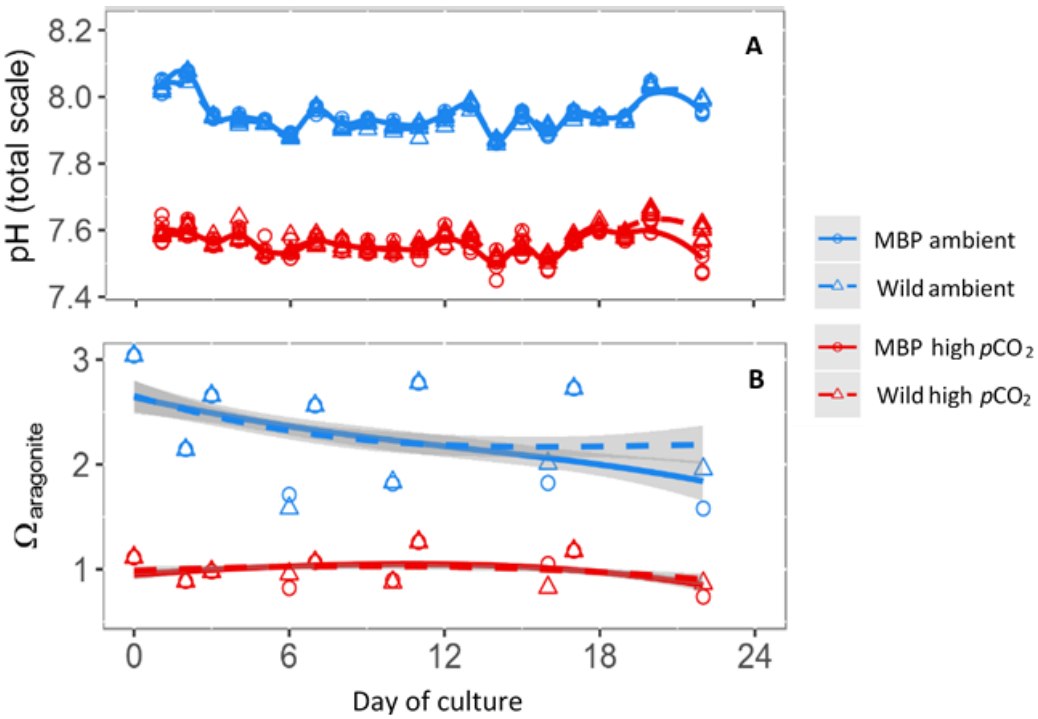

Figure A3.1: Summary of water chemistry. A) pH and B) saturation state of aragonite ($\Omega_{aragonite}$) values across the 22 day experimental period for MBP (solid lines) and Wild (dashed lines) larvae reared at ambient (blue) and high (red) pCO2 levels

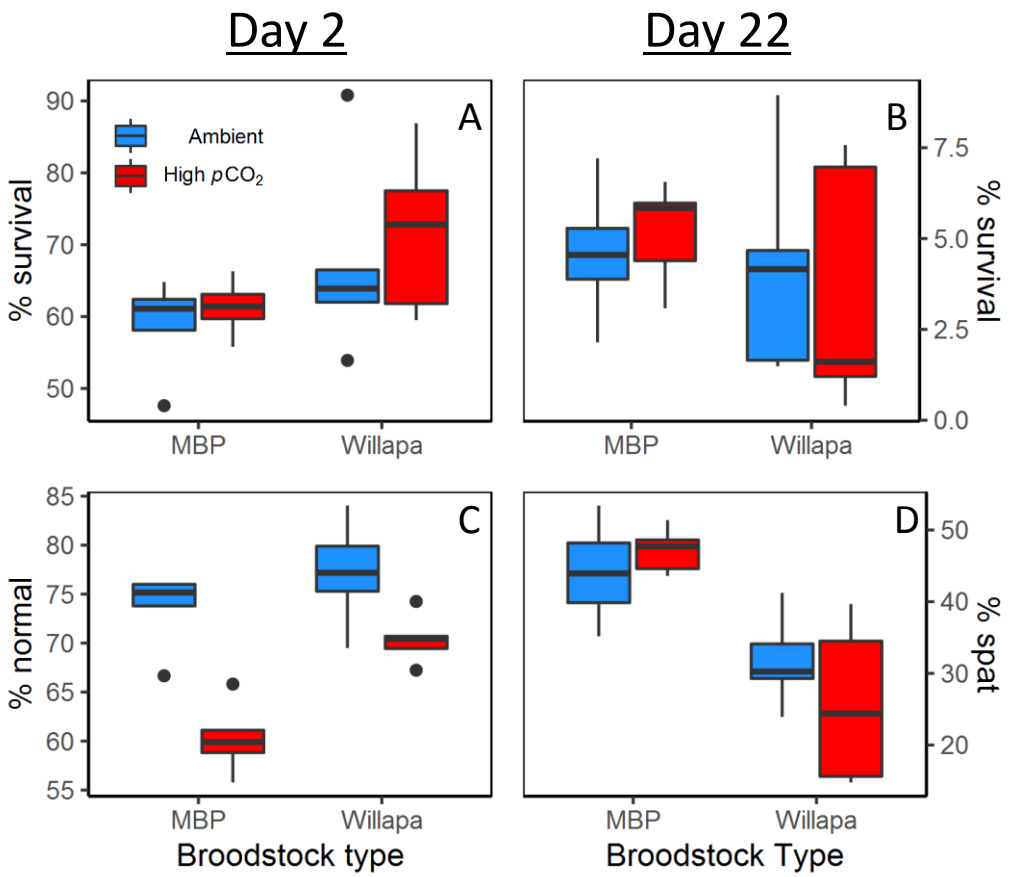

Figure A3.2 Survival of all larvae at day 2 (A) and 22 (B) post fertilization and percent 'normal' D-hinge larvae at day 2 (C) and percent spat at day 22 (D) for MBP and wild groups reared in ambient and high pCO_2 conditions.

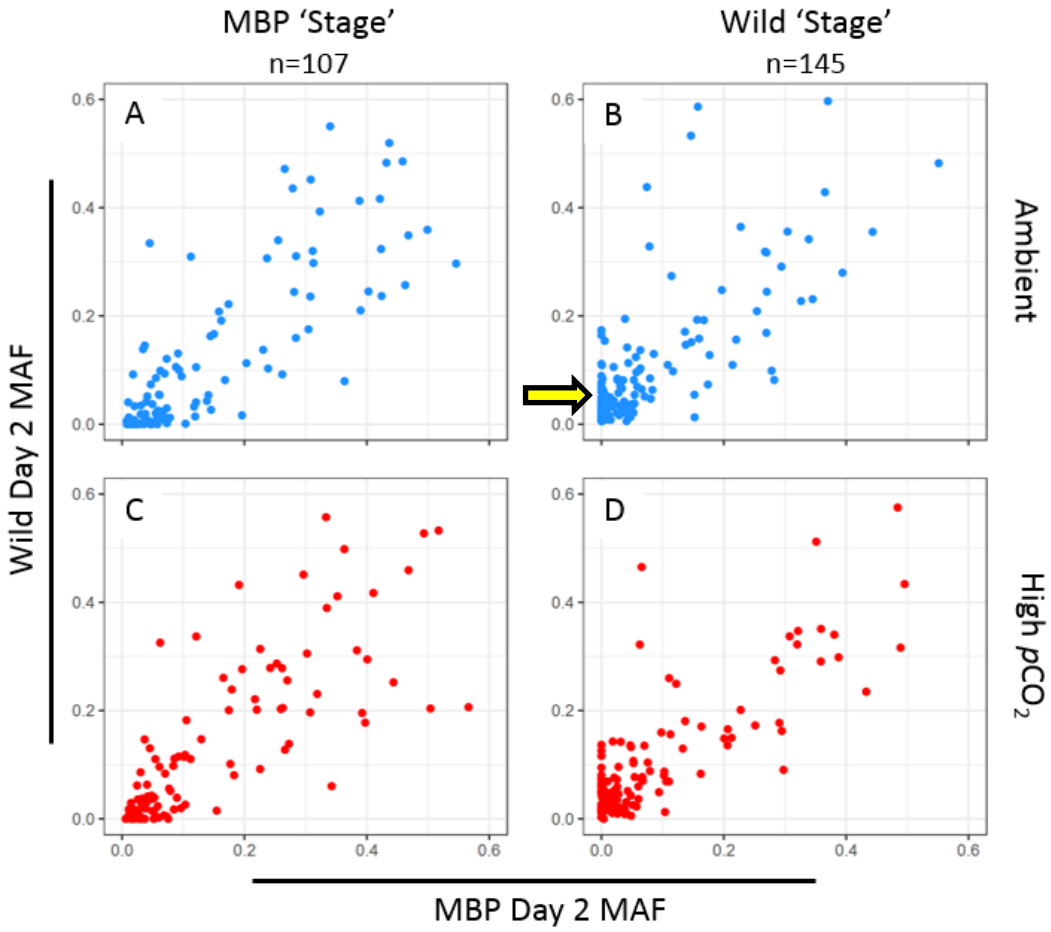

Figure A3.3 'Starting' minor allele frequency comparisons at Day 2 for MBP and Wild larvae in Ambient (blue; A&B) and High pCO_2 (red; C&D) conditions. Figures (A) and (C) represent n=107 SNPs which were found significantly changed by larval development ('Stage') in MBP groups. Figures (B) and (D) represent n=145 SNPs 'Stage' SNPs for Wild larval groups. There are only n=4 SNPs significantly changed by 'Stage' in both groups. The yellow arrow in (B) indicates SNPs for which a low minor allele frequency in one group (MBP) may obscure the detectable change in frequency which, in the other (Wild) had a more moderate starting MAF.

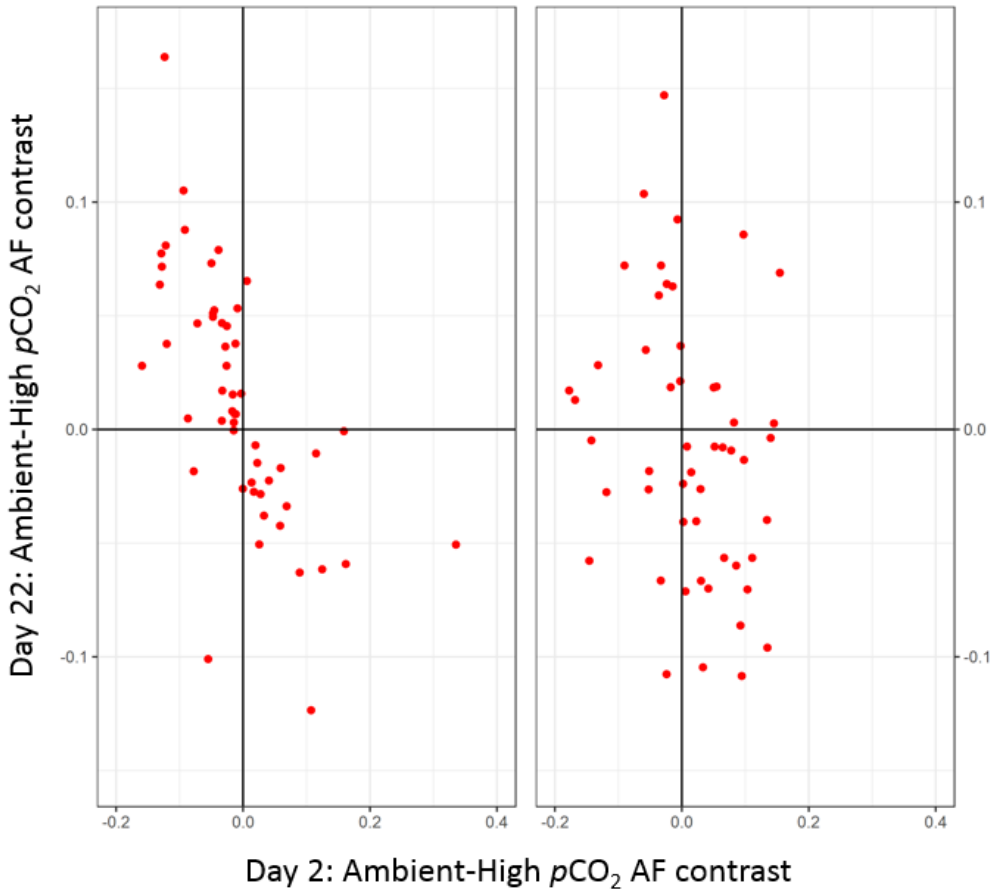

Figure A3.4 Interactive effects between larval development and seawater treatment. Contrasts in mean allele frequency (AF) for significant 'Stage * Trt' loci between ambient and high pCO_2 seawater at day 2 (x-axis) and day 22 (y-axis) post fertilization. Per-locus contrasts in AF are calculated as the mean allele frequency of acidified cultures minus that of ambient replicates. Approximately 89% of MBP and 75% of wild loci (points) have negative interactive effects, represented by falling in the upper left and lower right quadrants of the plots.

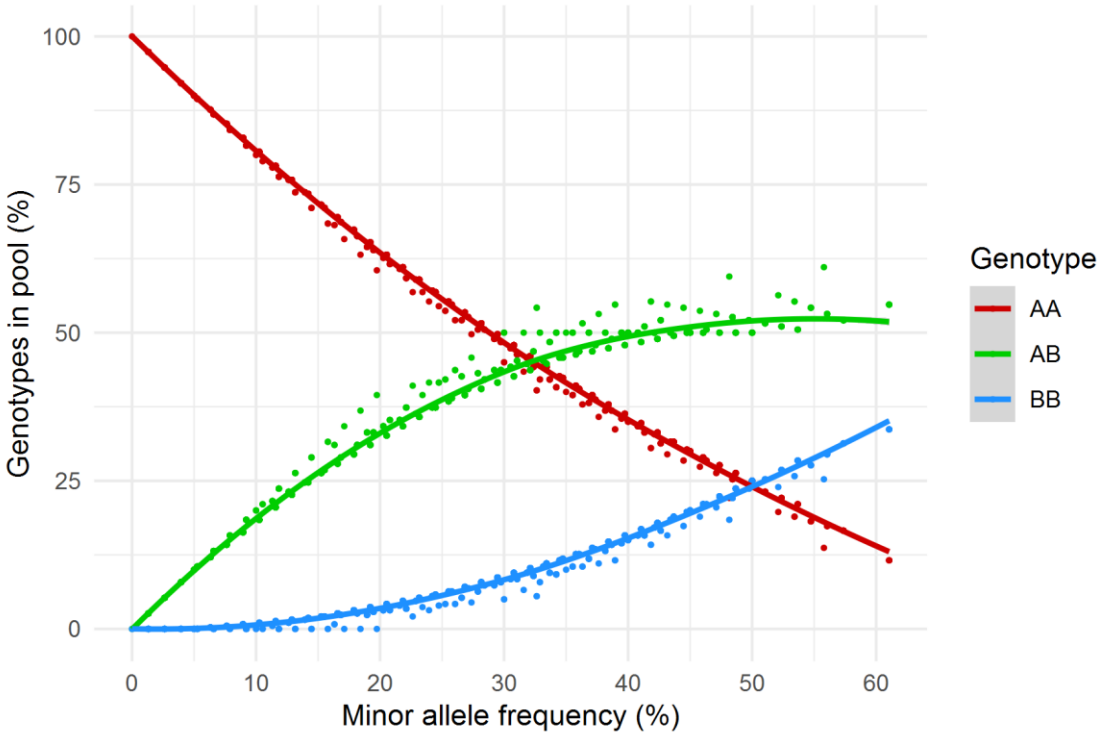

Figure A4.1. Estimated genotypic proportions in the larval pool based on initial minor allele frequency (MAF).