Physiological response of shellfish native to the North American Pacific Coast to ocean acidification and warming

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A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington
2021

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Program Authorized to Offer Degree:
School of Aquatic and Fishery Sciences

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Abstract

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Following observations of shifting ocean conditions an enormous scientific effort has explored the response of marine species to ocean acidification and warming. Empirical data has established that many species are vulnerable to ocean conditions projected for this century, particularly calcifying invertebrates, affecting a range of physiological processes over the lifetime of an organism. However, these studies also indicate that biological responses are quite variable, related to an organism’s genetic and environmental ancestries. Some species are more tolerant to the effects of acidification than others, as are some populations within species. There is also evidence that transgenerational carryover effects may alleviate some negative effects by buffering future generations against challenging conditions. The future of marine ecosystems and food systems hinges in part upon our ability to identify, conserve, and invest in individuals that

can tolerate shifting ocean conditions, and to understand the role of transgenerational carryover effects in shaping future populations.

The aim of this dissertation work is to examine the physiological and molecular responses of two invertebrate species native to the North American Pacific Coast, the Olympia oyster (*Ostrea lurida*) and Pacific geoduck (*Panopea generosa*), to ocean acidification and warming. Both species inhabit dynamic, heterogeneous estuarine environments that are influenced by coastal upwelling, and through adaptation and/or carryover effects may be relatively tolerant of ocean change. By testing multiple species, populations, life stages, and generations I provide evidence that these Pacific Coast natives are uniquely equipped for the effects of ocean acidification, and that warming will be a more impactful, but not necessarily negative, driver of physiological changes.

Chapter 1 characterizes the proteomes of Pacific geoduck in varying natural environments and habitat-specific pH conditions. Juvenile geoduck were deployed in eelgrass and adjacent unvegetated habitats for 30 days while pH, temperature, dissolved oxygen, and salinity were monitored. Across the four deployment locations pH was lower in unvegetated habitats compared to eelgrass habitats. While geoduck growth and proteomes were not affected by pH, they were sensitive to temperature and dissolved oxygen, but neither affected survival rates. Chapter 1 demonstrates that geoduck may be resilient to acidification in a natural setting and temperature may have a greater influence on geoduck physiology.

Chapter 2 examines the intra- and inter-generational carryover effects of ocean warming in the Olympia oyster. In many species reproductive and metabolic processes are tightly linked to the seasonal change from winter to spring, yet we know little about how these processes will shift as winters become milder. Therefore, in Chapter 2 I exposed adult Olympia oysters to elevated winter temperature and monitored effects to reproduction and offspring viability in spring. Parental exposure to warming did not affect overall larval production or survival, however it did increase the size and development of gametes, and the size of larval offspring. In the wild more developed gametes and larger larvae following milder winters could greatly impact recruitment patterns, possibly benefitting *O. lurida* populations. The results of Chapter 2 suggest that *O. lurida* is at minimum resilient to winter warming, and at best could benefit from it due to improved larval viability.

Chapter 3 continues exploring carryover effects in the Olympia oyster by examining the effects of combined ocean warming and acidification across three distinct *O. lurida* populations. Larval production was higher and began sooner following winter warming, was reduced by acidification, but was unaffected by combined stressors. Offspring of parents exposed to acidification, which were reared in common conditions for one year, had higher survival rates when tested in the field. Results of Chapter 3 indicate that altered recruitment patterns may follow warmer winters due to a prolonged reproductive season and/or increased production, but these effects may be masked by coincidental high pCO_2. Furthermore, Olympia oysters may be more resilient in certain environments when progenitors are pre-conditioned in stressful conditions. This carryover effect demonstrates that parental conditions can have substantial ecologically relevant impacts that should be considered when predicting impacts of environmental change.

Chapter 4 further describes three *O. lurida* populations' responses to acidification by examining growth, reproductive development, gene expression, and signals in offspring. Responses reveal energetic trade-offs that range from a robust transcriptional response in one population (Dabob Bay) without impacts to growth or reproduction, to no detectable

transcriptional response but negative impacts to growth and reproduction in another (Oyster Bay). While exposure to acidification did not affect gene expression in the next generation’s larval stage, it did increase larval size in the Oyster Bay, which could partially alleviate negative effects of acidification in the wild in that population. Given the distinct transcriptional response of the Dabob Bay population to acidification and its high survival rates in previous studies, we identified genes unique to that population, which provide insight into the mechanisms behind a stress-tolerant oyster population. Chapter 4 provides the first description of molecular processes responsive to acidification in an *Ostrea* spp, and demonstrates that species inhabiting heterogeneous environments, even on small geographic scales, offer natural reservoirs of biodiversity.

This dissertation work reveals the resilience of bivalves native to the Northeast Pacific Ocean to ocean change, and suggests that that Olympia oyster and Pacific geoduck are good candidates for aquaculture investment and conservation efforts. Furthermore, population-specific responses and carryover effects observed in Olympia oyster suggests that both fine-scale genetic structure and parental priming can influence how an organism responds to ocean change, and should be considered by conservationists and managers, and in future studies.

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ACKNOWLEDGEMENTS

Thank you to my advisor Dr. Steven Roberts for your intellectual guidance, encouragement, and support. You went beyond the role of a mentor, and became my advocate, collaborator, hatchery technician, and even my IT specialist. Among your many skills is your incredible ability to find the story in your science, which I will attempt to emulate in my research career.

To members of the Roberts Lab – past and present – your insight and bioinformatics prowess were invaluable. Of note was the near-instantaneous help you provided with code/computing issues via GitHub, and the comprehensive (and terrifying) feedback you gave during practice talks. Thank you also for being part of my COVID-19 support team.

Thank you to my committee members – Rick Goetz, Jackie Padilla-Gamiño, and Jennifer Ruesink. I could not have asked for a more supportive group of experts to advise me through my dissertation.

Many thanks to the shellfish research and restoration community, particularly those of you on the Pacific Coast and especially the Puget Sound Restoration Fund. Your knowledge, work-ethic, mission, and disposition make you the ideal colleagues and collaborators.

To the “seis buddies” – your companionship, particularly during the initial COVID quarantine, helped me maintain sanity. Thank you also for keeping Ian company when I wasn’t able to.

To my parents, sibling and their families, cousins, aunts and uncles, and in-laws, thank you for being genuinely excited by oysters. Your enthusiasm helped me stay focused on why I study shellfish – which to eat them always and forever.

Thank you to my family. Ian – my decision to leave a cushy (but uninspiring) downtown Seattle job for a low-paying summer internship with PSRF, which is what launched my career in a field that I love, was in large part thanks to you. You never settle for “safe”; thank you for emphatically encouraging me to do the same. Thank you also for sticking with me through the many stressful periods of graduate school, and for making me have some damn fun once in a while. Calvin - thank for being such a good baby.

This dissertation work would not have been possible without the National Science Foundation, who funded my research, coursework, and internships with their many programs: the Graduate Research Fellowship Program (GRFP), the Graduate Research Internship Program (GRIP), the Graduate Opportunities Worldwide Program (GROW), and the Non-Academic Research Internship for Graduate Students (INTERN).

Chapter 1. PACIFIC GEODUCK (*PANOPEA GENEROSA*) RESILIENCE TO NATURAL PH VARIATION

A version of this chapter was published as: Spencer, L. H., Horwith, M., Lowe, A. T., Venkataraman, Y. R., Timmins-Schiffman, E., Nunn, B. L., & Roberts, S. B. (2019). Pacific geoduck (*Panopea generosa*) resilience to natural pH variation. *Comparative Biochemistry and Physiology. Part D, Genomics & Proteomics*, *30*, 91–101. https://doi.org/10.1016/j.cbd.2019.01.010

1.1 ABSTRACT

Pacific geoduck aquaculture is a growing industry, however, little is known about how geoduck respond to varying environmental conditions, or how the industry will fare under projected climate conditions. To understand how geoduck production may be impacted by low pH associated with ocean acidification, multi-faceted environmental heterogeneity needs to be included to understand species and community responses. In this study, eelgrass habitats and environmental heterogeneity across four estuarine bays were leveraged to examine low pH effects on geoduck under different natural regimes, using targeted proteomics to assess physiology. Juvenile geoduck were deployed in eelgrass and adjacent unvegetated habitats for 30 days while pH, temperature, dissolved oxygen, and salinity were monitored. Across the four bays, pH was lower in unvegetated habitats compared to eelgrass habitats. However this did not impact geoduck growth, survival, or proteomic abundance patterns in gill tissue. Temperature and dissolved oxygen differences across all locations corresponded to differences in growth and targeted protein abundance patterns. Specifically, three protein abundance levels (trifunctional-enzyme β-subunit, puromycin-sensitive aminopeptidase, and heat shock protein 90-α) and shell growth positively correlated with dissolved oxygen variability and inversely correlated with mean temperature. These results demonstrate that geoduck may be resilient to low pH in a natural setting, but other

abiotic factors (i.e. temperature, dissolved oxygen variability) may have a greater influence on geoduck physiology. In addition this study contributes to the understanding of how eelgrass patches influences water chemistry.

1.2 INTRODUCTION

The Pacific geoduck, *Panopea generosa,* is native to the North American Pacific Coast and is a burgeoning aquaculture species with strong overseas demand as a luxury commodity (Coan et al. 2000; Shamshak and King 2015; Vadopalas et al. 2010). As the largest burrowing clam in the world, cultured geoduck reach upwards of 180mm and are harvested after growing approximately 6-7 years in sub- or intertidal sediment (Vadopalas et al. 2015; Washington DNR website 2017; Washington Sea Grant 2013). The long grow-out period and high per-animal value highlights the importance of site selection for farmers to maximize investment; however, there remains a paucity of data on the optimal environmental conditions for geoduck aquaculture.

As marine calcifiers, geoduck may be vulnerable to ocean acidification due to their reliance on calcite and aragonite (forms of calcium carbonate) for shell secretion (Orr et al. 2005; Weiss et al. 2002), both of which become less biologically available as seawater pH declines with pCO_2 enrichment (Feely et al. 2008). While there are no ocean acidification studies on *Panopea* clams to date, a growing body of research on marine calcifiers generally indicates that projected low pH will shift organisms' physiology to the detriment of species-wide abundances and distributions (Pörtner 2008; Pörtner and Farrell 2008). However, broad generalizations of how ocean acidification affects calcifiers are few due to varying pH sensitivity between taxa (Gazeau et al. 2007; Ries et al. 2009) and life stage (Kurihara 2008; Kroeker et al. 2010). For example, in the deeply studied oyster genus *Crassostrea*, Miller et al. (2009) found that larvae of two species varied in their response to elevated pCO_2, as calcification rates were significantly reduced in the

Eastern oyster (*C. virginica*), but the Suminoe oyster (*C. ariakesnsis*) showed no negative response. Thus, lessons learned from other bivalve species cannot directly be applied to geoduck.

The effect of low pH on cultured geoduck needs to be explored to help the aquaculture industry make informed site selection, selective breeding, and investment decisions. For practical application, geoduck ocean acidification studies should best replicate the natural environment in which they are grown. Ninety percent of global production occurs in the Puget Sound estuary of Washington State, where environmental drivers vary between subbasin, season, and diurnal cycle (Moore et al. 2008; Shamshak and King 2015). This habitat heterogeneity exposes geoduck to a variety of secondary stressors when outplanted. Similarly, there is substantial evidence that low pH is not occurring in isolation, but rather in conjunction with changes in other environmental drivers such as temperature (meta-analyses: Byrne and Przeslawski 2013; Harvey et al. 2013; Kroeker et al. 2013), dissolved oxygen (Gobler et al. 2014), and salinity (Przeslawski et al. 2015). Thus, single-stressor studies are limited in their predictive capacity of response to broad scale environmental change. For example, an additive, negative effect of elevated pCO_2 and temperature was observed in juvenile giant fluted clam survival (*Tridacna squamosa*) (Watson et al. 2012). Another consideration is the incorporation of naturally-occurring diurnal pH variability into ocean acidification studies, as variable pH can have differing effects on marine calcifiers compared to persistent low pH (Review, Boyd et al. 2016). Porcelain crabs, for example, exposed to diurnally variable pH and temperature conditions demonstrated significantly slower metabolism than when crabs were exposed to less variability, or to temperature or pH variability alone (Paganini et al. 2014).

To best predict the effect of ocean acidification on geoduck aquaculture, this project deployed geoduck in variable environmental conditions and leveraged the natural pH differences between eelgrass and unvegetated habitats in Washington State estuaries. Ocean acidification studies are increasingly exploiting naturally low pH systems to monitor the environmental heterogeneity alongside test organisms, in hydrothermal vents (Tunnicliffe et al. 2009; Kerrison et al. 2011), shallow CO_2 seeps (Duquette et al. 2017), coastal upwelling regions and eutrophic estuaries (Howarth et al. 2011; Thomsen et al. 2013). Compared to controlled laboratory studies, these deployment studies can uniquely incorporate natural ranges and daily cycles in air exposure, temperature, pH, dissolved oxygen, salinity, and food availability. For instance, Ringwood and Keppler (2002) deployed hard clams (*Mercenaria mercenaria*) in the Charleston Harbor estuary in South Carolina while collecting physical-chemical parameters. They observed that while salinity was the primary determinant of growth, pH was also important particularly when salinity was low, and when pH dropped below 7.5, a nuanced finding that is more likely to be captured in a natural environment.

Estuaries along the United States Pacific Coast are ideal, natural mesocosms for examining the effect of ocean acidification on commercially vital calcifiers, as they contain dense macroalgae beds (Bulthuis 1995), environmental conditions that vary considerably between subbasins (Banas et al. 2004; Moore et al. 2008), and have rich communities of native and cultured shellfish (Dethier et al. 2006; Miller et al. 2009; Washington Sea Grant 2015). Furthermore, coastal estuaries have already shifted towards lower pH and warmer temperature averages, and are projected to continue along this trend (Abatzoglou et al. 2013; Busch et al. 2013; Doney et al. 2007; Feely et al. 2012, 2010, 2008; Mote and Salathé 2010). The buffering capacity of macroalgae (seagrass meadows, kelp forests) allows for block-designed experiments to examine the effect of pH, while controlling for varying background environments and maintaining diurnal fluctuations (Middelboe and Hansen 2007; Palacios and Zimmerman 2007; Wahl et al. 2018).

In order to better inform geoduck aquaculture practices, we set out to examine how low pH and other natural variation in environmental conditions influence geoduck growth and physiology, using native eelgrass (*Zostera marina*) as a primary determinant of water chemistry. Physiology was evaluated with a unique two-phase proteomics approach using Selected Reaction Monitoring, with targets identified using Data Independent Acquisition, and selected based on prior environmental stress response studies.

Ocean acidification contributes to an elevation of reactive oxygen species in marine invertebrates (Tomanek 2015). Reactive oxygen species (ROS), or free-radicals, result in oxidative stress and in addition to low pH, higher ROS levels are associated with other environmental stressors including temperature, oxygen variability, salinity, and heavy metals (Review, Lushchak, 2011). Upregulation of anti-oxidants such as catalase, peroxiredoxins, and superoxide dismutase (among others) have consistently been observed in bivalves under low pH and heat stress (Tomanek et al. 2011; Matozzo et al. 2013; Hu et al. 2015), and under heavy metal exposure (Giarratano et al. 2014). In addition to ROS response, ocean acidification is thought to elicit a broader and generic molecular stress response in marine bivalves. Notably, the inducible heat shock proteins are associated with response to hypercapnia, in addition to acute heat, inflammation, and heavy metals, as they act as chaperones to recognize and bind to unfolded or improperly folded proteins (Bozaykut et al. 2014). Induction of HSP90, for example, has been universally observed thus far in bivalve species (Fabbri et al. 2008). Metabolic function is also altered under low pH, hypoxia, and salinity stress, generally shifting to anaerobic metabolism to minimize the mitochondrial ROS production associated with aerobic metabolism (Tomanek 2014).

In addition to antioxidant, metabolic, and general stress-response proteins, this study targeted proteins involved in mitotic growth, detoxification, acid-base balance, and ion regulation, all quantified simultaneously to characterize the physiological response in the Pacific geoduck under variable pH environments. This novel application demonstrates the advances in proteomic research and the potential it has to improve aquaculture production.

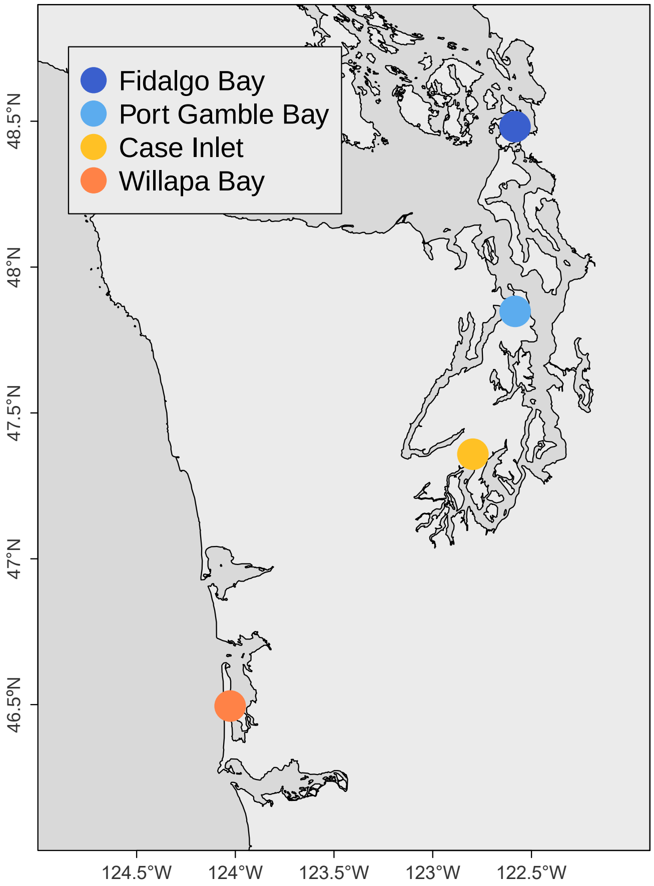

Figure 1.1. Geoduck juveniles were deployed for 30 days in 2 habitats (eelgrass beds, unvegetated) within 4 bays in Western Washington State.

1.3 METHODS

1.3.1 *Experimental Design*

Panopea generosa juveniles (14.0 ± 0.85 mm) were used in this experiment. Animals were of the same cohort, hatched from broodstock harvested from Puget Sound in Washington State, and reared in a commercial facility in Dabob Bay, WA in controlled conditions (18°C, salinity of 30ppt and pH 8.2). Geoduck were out-planted in four bays throughout Western Washington State from June 21 to July 21, 2016: Fidalgo Bay (FB), Port Gamble Bay (PG), and Case Inlet (CI) in Puget Sound, and Willapa Bay (WB) located off the southwest Pacific Coast of Washington (Table 1.1, Figure 1.1). All locations were selected based on the criteria that both *Z. marina* eelgrass beds ("eelgrass"), and unvegetated sediment ("unvegetated") habitats were present. Clams were placed in 10 cm diameter polymerizing vinyl chloride (PVC) pipes buried in sediment with 5 cm exposed; this method replicates aquaculture techniques. Five clams were placed in each of the 3 tubes in both the eelgrass and unvegetated habitat, with a total of 30 clams across 6 tubes per bay. Pipes were covered with a protective mesh exclosure to limit predation. The replicate structures surrounded and were equidistant to a suite of water quality sensors capturing pH (Honeywell Durafet II Electrode, in custom-built housing), salinity (via conductivity, Dataflow Systems Ltd. Odyssey Conductivity and Temperature Logger), dissolved oxygen (Precision Measurement Engineering MiniDOT Logger), and temperature (via dissolved oxygen probes). Sensors were modified for submersible, autonomous data collection, and logged at 10-minute intervals for the duration of the 30-day outplant.

Table 1.1. Coordinates for geoduck placement.

Bay	Eelgrass Habitat	Unvegetated Habitat
Fidalgo Bay	48° 28' 52.8312" N 122° 35' 0.7044" W	48° 28' 54.0876" N 122° 35' 0.708" W
Port Gamble Bay	47° 50' 52.7388" N 122° 34' 58.5084" W	47° 50' 33.6336" N 122° 35' 1.7952" W
Case Inlet	47° 21' 30.3808" N 122° 47' 47.2182" W	47° 21' 28.5721" N 122° 47' 44.7457" W
Willapa Bay	46° 29' 42.288" N 124° 1' 35.472" W	46° 29' 40.124" N 124° 1' 34.0882" W

1.3.2 *Collection and Sampling*

Animals were collected during low tide and transferred on wet ice to shore where mortality and size were recorded. Live animals were dissected, and ctenidia tissue was isolated and flash-frozen in an ethanol-dry ice bath. Ctenidia was selected for proteomic analysis due to its direct interaction with the environment, importance in gas and ion regulation, and its implication in environmental stress response (Timmins-Schiffman et al., 2014, Matozzo et al. 2013, Zhang et al. 2015, Thompson et al. 2015). During sampling all instruments were sterilized between samples with bleach then ethanol, and rinsed with nanopure water. Samples were held on dry ice while transported back to the lab and stored at -80ºC.

1.3.3 *Environmental and Growth Data*

Temperature, pH, salinity and dissolved oxygen data were compared between outplant locations. Outliers for all environmental parameters were removed, as determined using Tukey Interquartile Range (IQR) method (Tukey 1977), excluding data outside the inner fence (1.5*IQR). Tidal charts from WWW Tide/Current Predictor and salinity data (<20ppt) were also used to remove DO and pH data corresponding to periods of tidal exposure. Four probe failures occurred during deployment and these data were not included in the analysis (pH at Port Gamble-eelgrass, salinity at Port Gamble-unvegetated & Case Inlet-eelgrass, DO in Fidalgo Bay-eelgrass). Salinity data from two additional locations was not reliably measured (Willapa Bay-unvegetated, Fidalgo Bay-unvegetated), so habitat comparisons were not performed for salinity data. For each parameter at each location, daily mean and daily standard deviation time series were calculated. Relative growth for each animal was determined as (L_f-L_i), where L_f= final geoduck shell length, L_i = average initial geoduck shell length within each exclosure (n=5). Differences in growth and environmental parameters between habitat were compared using 2-way analysis of variance (ANOVA) applied to regression models (value ~ habitat*bay). Bays and ad-hoc regions (north vs. south bays) were tested using 1-way ANOVA. Pairwise comparisons were tested with the t-statistic. Significance for all tests was defined as $P < 0.05$, corrected for multiple comparisons using the Bonferroni correction.

1.3.4 *Protein Analysis*

1.3.4.1 Protein Preparation

Relative protein abundance was ultimately assessed in a two-phase proteomics approach using Selected Reaction Monitoring (SRM), with targets identified using Data Independent Acquisition (DIA). Tissues were prepared separately for DIA and SRM, both following the protocol in Timmins-Schiffman et al. (2014) with a few exceptions. For DIA, 8 ctenidia tissue samples were analyzed, one sample from each location and habitat: FB-eelgrass (G048), FB-unvegetated (G058), PGB-eelgrass (G077), PGB-unvegetated (G068), CI-eelgrass (G010), CI-unvegetated (G018), WB-eelgrass (G131), WB-unvegetated (G119). For SRM, new ctenidia samples were examined, 12 individuals per bay (Fidalgo Bay, Port Gamble Bay, Case Inlet, Willapa Bay), with 6 from each habitat (eelgrass, unvegetated) for 48 samples total. Tissue was homogenized with sterile plastic pestle in 100 µl lysis buffer (50 mM NH_4HCO_3, 6M urea solution) and sonicated with Sonic Dismembrator (Fisher Scientific, Model 120) at 50% amplitude for ten seconds, three times. Protein concentration was quantified via Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA USA).

1.3.4.2 Mini-Trypsin Digestion

Aliquots of protein (30 µg for DIA, 100 µg for SRM) were suspended in Lysis Buffer (50 mM NH_4HCO_3 + 6 M urea solution) to a total volume of 100 µl followed by: 1) a 1 hour incubation at 37°C with 200 mM Tris(2-carboxyethyl)phosphine (2.5µl) and 1.5 M Tris at pH 8.8 (6.6 µl); 2) 1 hour at room temperature in dark with 200 mM iodoacetamide (20 µl); 3) 1 hour at room temperature with 200 mM diothiothreitol (20 µl); 4) 1 hour at room temperature with 2 ug/µl Lysyl Endopeptidase (Lys-C, Wako Chemicals) (3.3 µg); 5) overnight at room temperature in 25 mM NH_4HCO_3 (800 µl) + high pressure liquid chromatography grade methanol (200 µl) + Pierce Trypsin Protease, MS Grade (1 ug/µl, Thermo Scientific) at 1:30 enzyme:protein ratio (3.3 µg). Samples were evaporated to near dryness at 4°C using a CentriVap Benchtop Vacuum Concentrator.

1.3.4.3 Desalting

Samples were desalted to isolate peptides using MacroSpin Columns (Nest Group, 50-450 µl, Peptide Protein C18). Peptides were reconstituted in 5% acetonitrile + 0.1% trifluoroacetic acid (TFA) (100 µl), then 10% formic acid (70 µl) was added to achieve pH ≤ 2. Columns were washed with 60% acetonitrile + 0.1% TFA (Solvent A, 200 µl) four times, then equilibrated with 5% acetonitrile + 0.1% TFA (Solvent B, 200 µl) three times. Peptides were bound to the column by running the digest through the column twice, followed by peptide elution with two additions each of Solvent A (100 ul). Columns were spun for 3 minutes at 3000 rpm on VWR Galaxy 16DH digital microcentrifuge at each stage. Samples were evaporated to near dryness at 4°C, then reconstituted in the Final Solvent (3% acetonitrile + 0.1% formic acid) (60 µl for 0.5 µg/µl final concentration of protein, and 50 µl for 2 µg/µl final concentration for DIA & SRM, respectively).

1.3.4.4 Peptide sample preparation and internal standard

Final mixtures for mass spectrometry included 3.13 fmol/µl Peptide Retention Time Calibration mixture (PRTC), 0.33 µg/µl and 0.5 µg/µl peptides for DIA and SRM, respectively, in Final Solvent for 15 µl total volume. To confirm that peptides were quantified correctly in SRM, 10 µg from 5 randomly selected geoduck peptide samples were pooled, and 8 dilutions were prepared by combining with oyster peptides at known percentages of total protein content (10%, 13.3%, 20%, 40%, 60%, 80%, 87.7%, 90%) and analyzed alongside other samples.

1.3.5 *Data Independent Acquisition*

1.3.5.1 Data acquisition

Data Independent Acquisition (DIA) was performed to assess global protein abundance patterns and to identify consistently detectable peptides for SRM targets. Eight samples, one per deployment location, were analyzed in technical duplicates via liquid chromatography tandem mass spectrometry (LC-MS/MS) with the Thermo Scientific™ Orbitrap Fusion Lumos™ Tribrid™. Prior to sample analysis, the 30 cm analytical column and 3 cm trap were packed in-house with with C18 beads (Dr. Maisch HPLC, Germany, 0.3 µm). For each sample, 3 µl of geoduck peptides (1.0 µg) + PRTC peptides was injected and analyzed in MS1 over 400-900 *m/z* range, in 5 *m/z* overlapping isolation windows from 450-850 *m/z* with 15K resolution in MS2. Final Solvent blanks were run between each geoduck peptide injection to ensure against peptide carry-over. Lumos MS/MS method and sequence files are available in the project repository (Spencer et al. 2019), and data are available via ProteomeXchange with identifier PXD012266.

1.3.5.2 Protein identification and analysis

Proteins were inferred using an assembled, translated, and annotated *P. generosa* gonad transcriptome (combined male and female) (Timmins-Schiffman et al. 2017; DOI: 10.17605/OSF.IO/3XF6M). Transcriptome peptides were queried against those detected by the Lumos MS/MS using PEptide-Centric Analysis (PECAN) (Ting 2017) to create a peptide spectral library (.blib type file). DIA raw files were first demultiplexed using MSConvert (ProteoWizard version 3.0.10738, 2017-04-12) (Chambers et al. 2012) with filters set to vendor centroiding for msLevels [2,3] (--"peakPicking true 1-2"), and optimizing overlapping spectra ("demultiplex optimization-overlap only"). The transcriptome fasta file was tryptic digested *in silico* in Protein Digestion Simulator (version 2.2.6471.25262), set to Fully Tryptic from 400-6000 fragment mass range, 5 minimum residues allowed, 3 maximum missed cleavages and peak matching thresholds set to 5 ppm mass tolerance, and 0.05 ppm NET tolerance. Skyline version 3.7.0.11317 (MacLean et al. 2010) automatically selected transition peaks and quantified peptide abundances using peak area integration. All PRTC peptide peak selections were manually verified and corrected. Skyline peak selection error rate was calculated by manually checking chromatograms from 100 proteins across all DIA samples. Auto-selected peaks were assigned correct or incorrect selection based on transition retention time alignment across replicates, using PRTC peptides as a reference. Transition peak area, which is assumed to correlate to peptide transition abundance, was exported from Skyline for analysis in R version 2.4-3 (R Core Team 2016). Abundance was normalized by the total ion current (TIC) for each injection. Technical replicate, bay and habitat differences were assessed to inform SRM analysis via non-metric multidimensional scaling (NMDS) analysis using `metaNMDS` in the vegan package (Oksanen et al. 2016) on log(x+1) transformed abundances using a Bray-Curtis dissimilarity matrix. Technical replicate spectral abundances clustered together on NMDS plots, thus were averaged across each sample. Bay and habitat differences in global abundance were visually but not quantitatively analyzed.

Table 1.2. SRM proteins targets selected based on biological function and detectability across DIA samples

SRM Protein Targets	**Top Blast Hit Uniprot SpID (E-value)**	**Peptide sequences**
heat shock protein 90-alpha	P30946 (0)	GVVDSEDLPLNISR EVVQSSAFVER DSSTMGYMAAK
heat shock protein 70	Q91233 (0)	TTPSYVAFNDTER NAVVTVPAYFNDAQR IINEPTAAALAYGLDK
superoxide dismutase	P28757 ($1e^{-57}$)	THGAPTDEER ISLTGPHSIIGR TIVVHADVDDLGK
catalase	P00432 (0)	AGELGGSDPDYAMR LYSYSDTHR LTANIAGHLIGAQEFIQK
peroxiredoxin-1	Q6B4U9 ($2e^{-95}$)	ALFIIDDK QITMNDLPVGR LVQAFQFTDK
puromycin-sensitive aminopeptidase	Q11011 (0)	LNSGSVGVYR SLTENFVTEEQAK SIQQSVENIR
protein disulfide-isomerase	P07237 (0)	NNKPSDYQGGR DNVVVIGFFK MDSTANEVEDVK
ras-related protein rab-11B	O35509 ($1e^{-85}$)	VVLVGDSGVGK STIGVEFATR AQLWDTAGQER
sodium/potassium-transporting ATPase subunit alpha	Q13733 ($9e^{-62}$)	TVIEPMAGDGLR MVTGDNVNTAR LLDQVWPDLR
glycogen phosphorylase (muscle form)	Q9WUB3 (0)	APNSFNLR VLYPNDNFFEGK TSFDAFPDK
trifunctional enzyme β-Subunit (mitochondrial)	O46629 (0)	AAQDNGLLTDVLAYK ALELGLKPK FNLWGGSLSLGHPFGATGVR
cytochrome P450	P00185 ($7e^{-39}$)	IITRPFNVNGLLAYDSR WLDESGVFLPEEHPSR QSLLPFGATGPR
arachidonate 5-lipoxygenase	P09917 ($2e^{-94}$)	APGLPAQIK MDVEGTLPEDLK GLGLGGVPGQNGK

1.3.6 *Selected Reaction Monitoring*

1.3.6.1 Target selection

Thirteen proteins were selected for SRM targets (Table 1.2). First, candidate proteins (~200) were selected based on biological function listed in the Universal Protein Knowledgebase (Apweiler et al. 2004) and evidence of stress response in bivalves from the scientific literature. Candidate proteins were screened for detectability using DIA results. Selected proteins were required to have ≥3 high quality peptides, each with ≥3 transitions, present in all DIA biological and technical replicate data. Quality peptides had uniform peak morphology and retention time in Skyline across replicates. A total of 49 peptides were selected for SRM: 39 to quantify 13 target proteins (116 transitions), and 10 for internal standard (30 transitions). A full list of transition targets are published on https://panoramaweb.org/e0TsuK.url and available in the project repository (Spencer et al. 2019).

1.3.6.2 Data acquisition

SRM samples were analyzed on a Vantage Triple-Stage Quadrupole Mass Spectrometer (Thermo Scientific, San Jose, CA, USA), and injected by a nanoACQUITY UPLC® system (Waters, Milford, MA, USA) at random in two technical replicates. For each sample, 2 µl of peptides + PRTC solution containing 1.0 µg of geoduck peptides was injected, trapped on a 3 cm pre-column and separated on a 30 cm analytical column using a chromatography gradient of 2-60% acetonitrile over 60 minutes. Columns were prepared as in DIA (above). Samples were injected in randomized groups of 5, followed by a Peptide Retention Time Calibration (PRTC) plus bovine serum albumin peptides (BSA) standard, then Final Solvent blank. Vantage MS sequence and method files are available in the project repository (Spencer et al. 2019).

1.3.6.3 Protein identification and quality assurance

Peptides were identified and quantified via Skyline-daily version 3.7.1.11357 (MacLean et al. 2010). Raw SRM files were imported into a Skyline-daily project along with the target protein transitions, and the spectral library (.blib file) created previously in the DIA Protein Identification step. SRM peptides were verified by regressing PRTC peptide retention time (RT) in SRM against retention time in DIA. A fitted model from PRTC peptides predicted RT of protein target peptides. Where necessary, peak selection and boundaries were manually adjusted for SRM peptide chromatograms, and actual RT were regressed against predict RT to confirm correct selection (F(1,38): 5768, p-value: $< 2.2e^{-16}$, Adjusted R-squared: 0.9933). Transition peak area, defined henceforth as abundance, was exported from Skyline for further analysis in R (R Core Team 2016). Abundance results from the separate serial dilution samples were used to remove peptides that did not adhere to the dilution curve. Briefly, dilution abundances (exported from Skyline) for each transition were normalized by the most dilute sample abundance, then regressed against predicted ratios. Peptides with slope coefficient $0.2<x<1.5$ and adjusted $R^2 >0.7$ were included in analysis. Ten of the 39 peptides were discarded from the dataset based on dilution standards results. To determine and remove disparate technical replicates, NMDS analysis was performed as described above. Technical replicates with ordination distance >0.2 were removed, and only samples with two technical replicates were preserved for analysis. Thirteen technical replicates from different samples and all replicates from three sample were discarded, for 84% technical replicate and 94% biological replicate retention. Within samples, transitions with coefficients of variation (CV) > 40% between technical replicates were also discarded (2% of all transitions across 21 samples). In final dataset for differential analysis, 10 proteins, 26 peptides, and 77 transitions were retained. Mean transition abundance was calculated for replicates, with zero in the place of n/a values, which

Skyline generates for replicates without peaks. Transition abundances within each peptide were summed for a total peptide abundance before analyzing for differential abundance.

1.3.7 *Differential protein analysis*

After data quality screening, peptide abundance was analyzed for differences between locations and habitats. NMDS plots visualized patterns in peptide abundances by bay and habitat as described above. Global peptide abundance was compared between bay and habitats using two-way ANOVA on log-transformed abundances. For protein-specific comparisons, peptide abundances were grouped by protein, box-cox transformed (Box and Cox 1964) and normality confirmed via qqplot (Wickham 2017). Two-way ANOVA tested abundances for each protein between eelgrass and unvegetated habitats within and between bays. Pairwise comparisons for differentially abundant proteins were tested with the t-statistic. Peptides within proteins were regressed against each other to confirm stable abundance patterns. For all statistical analyses, significance was defined as alpha ≤ 0.05, corrected for multiple comparisons using the Bonferroni correction.

1.3.8 *Correlative analysis*

To understand how environmental and biometric parameters covaried, Pearson's product-moment correlation and scatter plots were assessed between protein abundances, growth, and environmental summary statistics (mean and variance). Each protein was assessed independently. Due to salinity probe malfunction, salinity data were not included in correlation tests.

All analyses were performed in RStudio version 1.1.383 (R Core Team 2016). R scripts and notebooks (Spencer et al. 2019), raw data (ProteomeXchange PXD012266), and Skyline project files (https://panoramaweb.org/e0TsuK.url) are publicly available.

1.4 RESULTS

1.4.1 *Environmental & Growth Data*

Mean pH differed significantly between habitats across all bays (F(1,206)=180.0, $p=1.1e^{-28}$) (Figure 1.2). During the deployment, pH was recorded from 6.71 to 8.34, with mean pH 7.86±0.15 in eelgrass, and 7.51±0.25 in unvegetated habitats (means are for all locations). Variability in pH was significantly different among bays (F(3,206=43.8, $p=1.0e^{-20}$). Variability did not differ between habitats across all bays, but differences were detected between habitats within Case Inlet and within Willapa Bay (less variable in eelgrass). The locations with the highest and lowest daily mean pH were Fidalgo Bay-eelgrass (7.90±0.19) and Port Gamble Bay-unvegetated, respectively (7.32±0.25). On average across all locations, pH fluctuated daily by 0.46±0.23 pH units. Considerable heterogeneity among bays was observed in the other environmental parameters. Mean temperature was significantly different among all bays (F(3,236) =129.4, $p=2.2e^{-48}$), and temperature decreased with latitude (coldest in northernmost Fidalgo Bay, warmest in southernmost Willapa bay). Temperature did not differ between habitats within bays. Dissolved oxygen (DO) varied among bays in both daily standard deviation (F(3,210)=132.8, $p=4.6e^{-47}$) and mean (F(3,210)=56.7, $p=1.1e^{-25}$). DO variability was substantially higher in the two northern bays (SD was 5.6 and 3.9 mg/L in FB, PGB), as compared to the southern bays (2.5 and 1.4mg/L in CI, WB). Across all bays, DO variability did not differ between habitats, but did differ within Case Inlet and Fidalgo Bay. Mean salinity differed by bay (F(3,136)=254.3, $p=2.3e^{-54}$), with the largest differences between Fidalgo Bay (mean 29.9 ppt) and the other three bays (mean 23.4-27.0 ppt).

Growth significantly differed between northern and southern bays (F(1,97)=54.8, P=4.9-11), but not between habitats either within or across all bays. Geoduck in Fidalgo Bay and Port Gamble Bay grew larger compared to Willapa Bay, and Case Inlet (Figure 1.3). Survival did not differ among locations.

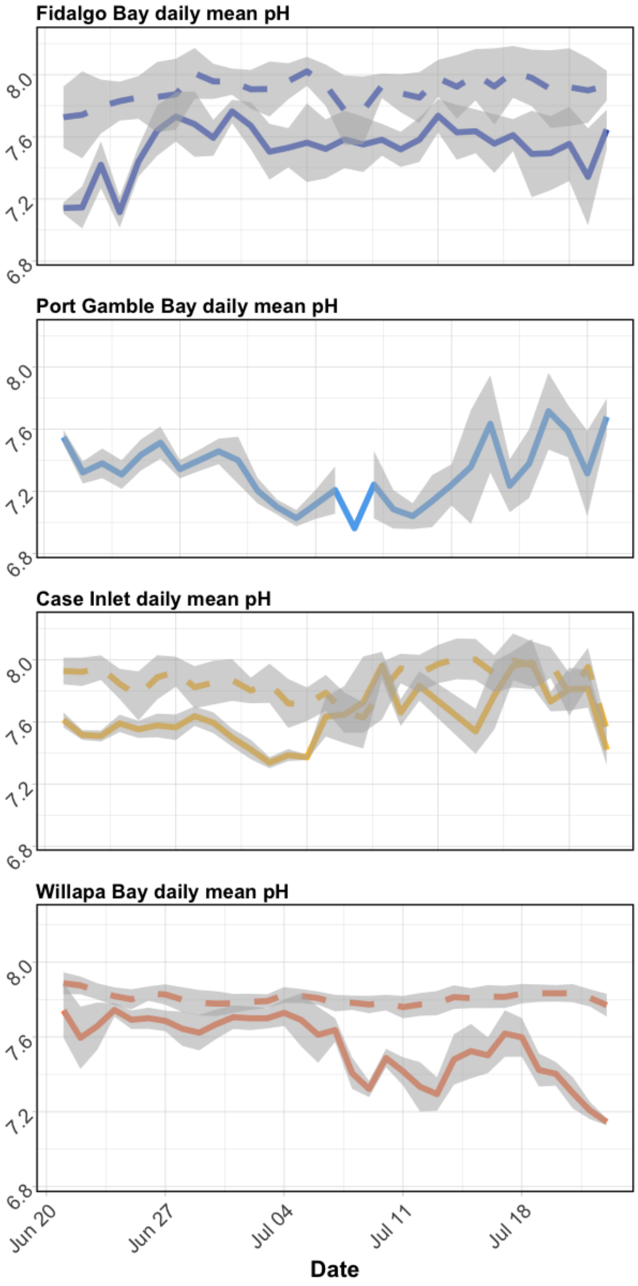

Figure 1.2. Daily mean pH in eelgrass (dashed lines) and unvegetated (solid lines) across bays during geoduck deployment. Gray ribbons denote daily standard deviation. Data from Port Gamble Bay eelgrass are not included due to probe failure.

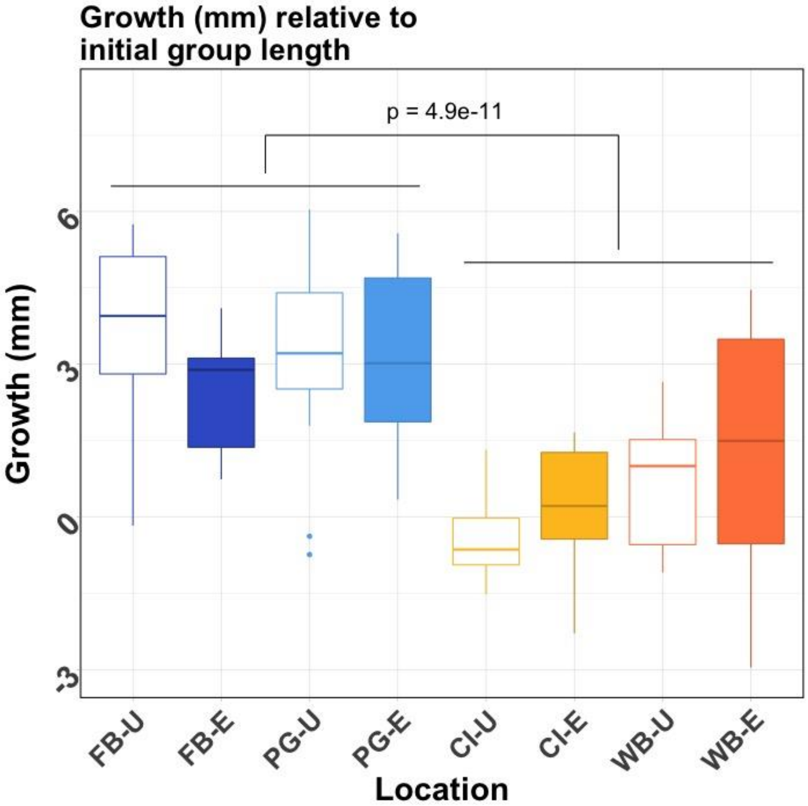

Figure 1.3. Geoduck shell growth after 30 days across Willapa Bay (WB), Case Inlet (CI), Port Gamble Bay (PG), and Fidalgo Bay (FB), where -U and -E represent unvegetated (empty boxes) and eelgrass habitats (filled boxes), respectively. Growth is relative to the mean initial shell length within deployment groups (n=5 per group, 3 groups per location). Boxes contain all biological replicates lying within the interquartile range (IQR), with median growth indicated by line in middle of boxes. Whiskers extend to the largest value no greater than 1.5*IQR, and dots indicate outliers beyond 1.5*IQR. Geoduck that did not survive deployment are not included. Growth differed significantly between southern bays (WB, CI) and northern bays (PG, FB) ($p=4.9e^{-11}$) but not between habitats within bays.

1.4.2 *Protein Detection and Variance*

In DIA, a total of 298,345 peptide transitions were detected from 30,659 distinct peptides across the 8 samples (one sample per habitat from each bay). These peptides were associated with 8,077 proteins, and more than half of the proteins (4,252) were annotated using Universal Protein Resource database (UniProt). Automated peak selection (Skyline) success rate was 71%.

In SRM, the final dataset after screening included 10 proteins, 26 peptides, and 77 transitions. The 3 proteins fully removed from the dataset were heat shock protein 70, peroxiredoxin-1, and ras-related rab. The SRM mean coefficients of variation (CV) of technical replicate abundances across all transitions decreased from 18.2% to 9.6% after screening. Transition abundance CV within bays ranged from 24.9% to 83.2% with mean 50.1%, and within deployment locations CV ranged from 11.6% to 93.0% with mean 48.9%. Within proteins, regression analysis indicated that peptide abundances from the same protein differed slightly, however the relative abundances across samples was consistent. This indicates a small degree of background variability in peptide detection, digestion, or stability within proteins that applied to all samples (Pep1xPep2: R^2_A=0.985, coefficient=0.682; Pep1xPep3: R^2_A=0.990, coefficient=0.954; Pep2xPep3: R^2_A=0.990, coefficient=0.954).

1.4.3 *Protein Abundance Differences*

None of the 10 targeted proteins were differentially abundant between habitats within or across bays (Figure 1.4). NMDS plots of all transitions in DIA and those targeted in SRM revealed clustering of overall proteomic response by bay. In SRM, Fidalgo Bay and Port Gamble Bay samples clustered together (henceforth “northern bays”), and some overlap between Case Inlet and Willapa Bay (“southern bays”) indicated similar protein abundances within these ad-hoc regions. This was verified from the ANOVA results, which detected significant abundance differences between northern and southern bays for three proteins: HSP90-α (HSP90) (F(1,133)=20.5, p-adj=$1.8e^{-4}$), trifunctional-enzyme subunit β-subunit (TEβ) (F(1,88)=11.1, p-adj=0.018), and puromycin-sensitive aminopeptidase (PSA) (F(1,130)=9.11, p-adj=0.043). HSP90 and TEβ abundances were also significantly different between bays (respectively: F(3,131)= 7.80, p-adj=0.0011; F(3,345)= 5.19, p-adj=0.034), but these differences were driven by regional differences, as post-hoc tests detected no differences between Case Inlet and Willapa Bay (southern), or beteen Fidalgo Bay and Port Gamble Bay (northern). For the three differentially abundant proteins, abundances were lowest in Case Inlet (southernmost in Puget Sound) followed by WIllapa Bay (southernmost overall), then Port Gamble Bay, and highest in Fidalgo Bay (northernmost).

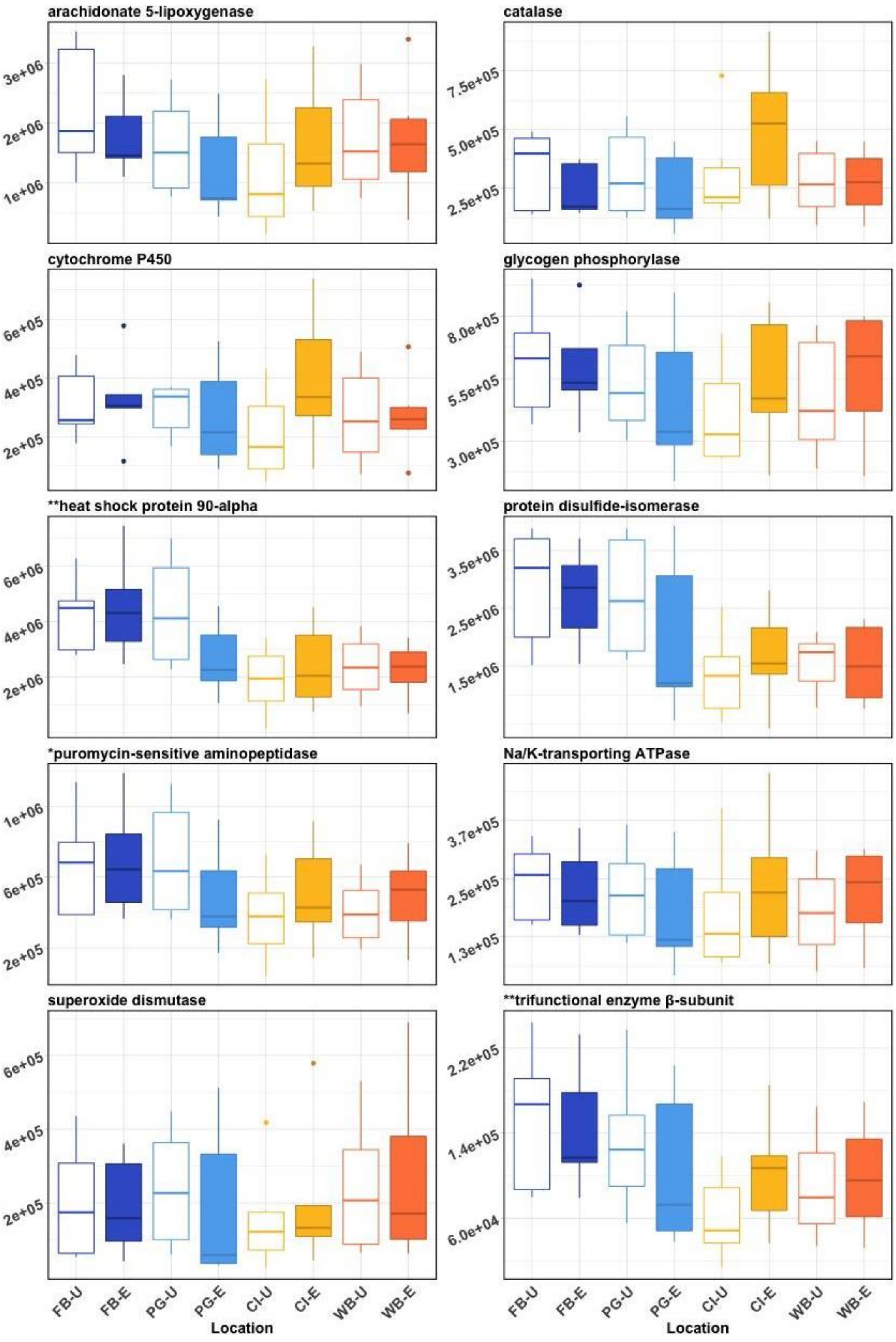

Figure 1.4. Boxplots of protein mean spectral abundances (mean of 2 or 3 peptides targeted for each protein) for Fidalgo Bay (FB), Port Gamble Bay (PG), Case Inlet (CI), and Willapa Bay

(WB), where -U and -E represent unvegetated (white boxes) and eelgrass (filled boxes) habitats, respectively. For each location, n=5 or 6 geoduck. Boxes contain all biological replicates lying within the interquartile range (IQR), with median abundances indicated by line in middle of boxes. Whiskers extend to the largest value no greater than 1.5*IQR, and dots indicate outliers beyond 1.5*IQR. Protein abundance ranges (y-axes) vary between proteins. Differentially abundant proteins between region and bay are indicated by (**), and region only by (*). No protein abundances were significantly different between habitats.

Table 1.3. Correlation analysis results between growth, environmental parameters, and peptide abundance (z-transformed). Correlation coefficient r shown with p-values adjusted via Bonferroni correction (number of comparisons). Correlations deemed significant are in bold.

	Growth	heat shock protein 90	puromycin sensitive aminopeptidase	trifunctional enzyme β-subunit
Growth	---	**r=0.53** **p=5.54e^{-11}**	**r=0.46** **p=1.42e^{-7}**	**r=0.43** **p=9.70e^{-7}**
T$_{mean}$	**r= -0.39** **p=8.11e^{-21}**	r= -0.36 p=0.060	r= -0.24 p=0.48	r= -0.21 p=0.70
T$_{sd}$	**r= 0.39** **p=2.10e^{-20}**	r= 0.24 p=0.42	r= 0.15 p=1	r= 0.11 p= 1
DO$_{mean}$	**r= 0.45** **p=2.12e^{-28}**	r= 0.26 p=0.34	r=0.18 p=0.91	r=0.21 p=0.66
DO$_{sd}$	**r= 0.48** **p=2.64e^{-32}**	**r=0.41** **p=0.021**	r=0.31 p=0.16	r=0.31 p=0.15
pH$_{mean}$	**r= -0.32** **p=1.08e^{-12}**	r= -0.09 p=1	r=-0.02 p=1	r=-0.03 p=1
pH$_{sd}$	**r= 0.30** **p=8.72e^{-11}**	r= 0.22 p=0.73	r=0.06 p=1	r=0.04 p=1

1.4.4 *Correlation between Environment, Abundance, and Growth*

Growth positively correlated with peptide abundance in all but 2 of the 10 targeted proteins (no correlation with catalase and superoxide dismutase), including the three proteins that were differentially abundant between bays (Table 1.3). Growth also correlated with most environmental parameters (excluding salinity SD). Heat Shock Protein 90 correlated positively with dissolved oxygen SD. Mean and SD pH did not correlate with any peptide abundance patterns or growth. None of the environmental parameters, nor growth, correlated significantly with peptide abundances pooled across all proteins.

1.5 DISCUSSION

This study tested the effects of varying pH on geoduck, a valuable aquaculture species in a natural setting, and confirmed that *Zostera marina* eelgrass can effectively alter local pH during warm summer months (June and July). We have shown that ocean acidification research on cultured shellfish can augment findings from controlled laboratory studies with field deployments to incorporate natural variability and relevant environmental drivers associated with an organism's habitat. Targeted proteomics was assessed alongside growth and environmental data for an integrated view of how geoduck respond to varying environmental conditions. Proteomics is a powerful approach suitable for comparative physiological studies of non-model, marine organisms (Tomanek, 2014). Using a two step method, this study detected substantially more proteins (8,077) compared to the previous geoduck study using Data Dependent Acquisition (3,651) (Timmins-Schiffman et al. 2017). This produced a valuable protein catalogue for future projects, as researchers can now skip directly to the targeted SRM phase to greatly reduce the cost and time associated with a discovery analysis.

Geoduck exhibited no phenotypic differences between pH conditions, counter to our predictions. We predicted that pH would be higher within eelgrass habitats, creating a refuge against the less alkaline surrounding waters and reducing oxidative stress. Concordantly, proteins involved in the oxidative stress response would be less abundant inside eelgrass habitats (such as superoxide dismutase, peroxiredoxin-1, catalase, and HSP), possibly translating to differential growth as less energy would be used to counter the pH stress. While pH in eelgrass habitats was found to be consistently higher in this study, no differences in abundance of selected peptides, growth or survival were found between habitats across all four bays. This suggests that juvenile geoduck may tolerate a wide pH range in the context of the natural environment in which they are cultured.

Earlier studies on other clam species point to some degree of pH tolerance, but also describe complex responses to low pH that vary between metrics, species, and when secondary stressors are applied (Ries et al. 2009; Ringwood and Keppler 2002; G. G. Waldbusser et al. 2010). For example, juvenile carpet shell clams (*Ruditapes decussatus*) under ambient (pH 8.2) and reduced pH (7.8, 7.5) for 75 days displayed no difference in size, weight, or calcification rate (Range et al. 2011), but other physiological parameters (clearance, ingestion, respiration, ammonia excretion) differed at day 87 (Fernández-Reiriz et al. 2011). In the hard clam *Mercenaria mercenaria,* protein oxidation, biomineralization, and standard metabolic rate (SMR, measured as resting oxygen consumption) in adults were largely unaffected by hypercapnia alone, but when combined with elevated temperature SMR increased and shell strength decreased (Ivanina et al. 2013; Matoo et al. 2013). Interestingly, the baltic clam (*Macoma balthica*) grew significantly larger in low pH (7.35 vs. 7.85 for 29 days), and were largest when combined with low dissolved oxygen (3.0 mg/L vs. 8.5 mg/L) (Jansson et al. 2015). Geoduck metrics examined in this study were not affected by varying pH, but other physiological parameters (metabolic rate, biomineralization, reproductive development, cytoskeleton), and other tissues such as mantle or hepatopancreas, may be affected and should be examined in future studies.

The complex, mixed responses exhibited in clam species may, in part, be a function of local adaptation to varying environmental drivers. Pacific geoduck are native to the Puget Sound, a region that experiences regular episodes of low pH in certain areas and has significant diel and monthly pH variability (Busch et al. 2013; Feely et al. 2008, 2010). Thus, the species may have evolved under selective pressure to withstand periods of low pH. The native Northeast Pacific Coast oyster, *Ostrea lurida,* also shows signs of pH tolerance as veliger larvae compared to the non-native Pacific oyster (*Crassostrea gigas*) (Waldbusser et al. 2016), a stage primarily found to be vulnerable in other calcifying species (for reviews see Byrne and Przeslawski 2013; Kurihara 2008). Geoduck are also infaunal organisms, extending their long siphons into the water column for feeding and retreating to deep burrows during low tide or when disturbed (Goodwin and Pease 1987). Sediment and burrow chemistry, while influenced by the overlying water column, can have lower pH due to aerobic microbial activity, another potential source of selective pressure shaping this giant clam’s pH tolerance (Gattuso and Hansson 2011; Widdicombe and Spicer 2008). An important future step is to assess the relative influence of sediment pH and overlying water column pH on burrowing calcifiers. This is particularly applicable when comparing habitats that likely have varying bacterial communities and activity.

While pH was not a universal predictor of geoduck phenotype in this study, mean temperature and dissolved oxygen variability correlated significantly with biometric parameters and separated into two groups: northern bays (Fidalgo and Port Gamble Bays), and southern bays (Case Inlet, and Willapa Bay). Geoduck grew less (or not at all, in Case Inlet) and had lower levels of targeted proteins in the southern bays, which were warmer with less variable dissolved oxygen content.

Temperatures in the southern bays (16-18°C) during the deployment dates may have exceeded optimal conditions for juvenile geoduck, resulting in elevated metabolism and less energy available for growth (Newell and Branch 1980). Similar temperature-dependent growth was observed in *M. mercenaria*, where shell calcification rate was highest between 12.8-15.2°C, above which growth negatively correlated with temperature (except for a secondary peak at 23.9°C) (Storr et al. 1982). In *P. generosa*, Goodwin (1973) reported that temperature for normal larval development is between 6-16°C. In adults, the optimal hatchery temperature for reproduction is relatively low (appr. 11°C), and at the highest experimental temperature (19°C) gonad did not regenerate after an initial spawning event (Marshall et al. 2012). Arney et al. (2015) found that in early juveniles (<3.5mm), growth increased with temperature within 7-19°C when fed ad libitum. However, organic weight accumulation (total body ash-free dry weight) was highest between 11-15°C, indicating that the optimal juvenile temperature may be approximately 15°C. In the present study, geoduck grew fastest in cooler, northern bays (15°C), but stress protein abundances (e.g. HSP90) did not suggest an acute thermal stress in the warmer, southern bays (abundances were inversely related to temperature). Southern bays may have exceeded the geoduck upper pejus temperature but remained below acute-stress, which could explain the reduced growth in those locations without a proteomic signal. Conversely, as tissues were collected at day 30, a heat stress signal could have been captured with earlier or more frequent samples. A thermal performance curve for *P. generosa* under natural feeding levels would be valuable for aquaculture siting, but these data suggest that cooler summer temperatures are more suitable for culturing geoduck.

Dissolved oxygen (DO) variability may be an indirect indicator of geoduck performance as it is often correlated to phytoplankton biomass (Khangaonkar et al. 2012). Less DO fluctuation in the southern bays could be an indicator of less phytoplankton biomass, translating to lower food availability (Anderson and Taylor 2001; Bergondo et al. 2005; Winter et al. 1975). While we were unable to monitor chlorophyll during the outplant, both southern bays, Willapa Bay and Case Inlet, may have phytoplankton populations that are controlled by shellfish grazers due to long residence times and aquaculture activity (Banas et al. 2007; Washington Sea Grant 2015). It is possible that food availability was different between northern and southern locations during the outplant period (June-July), and could be the underlying cause of higher growth and abundances of selected proteins in the northern locations (Carmichael, Shriver, and Valiela 2004; Liu et al. 2016; Loosanoff and Davis 1963), although this warrants additional data collection.

1.6 CONCLUSION

This is the first study to investigate geoduck performance alongside varying pH conditions, and contributes a geoduck ctenidia peptide database useful for quantifying multiple proteins simultaneously. The primary finding is that geoduck aquaculture may be less impacted by ocean acidification compared to other environmental stressors, for example ocean warming. Geoduck ocean acidification research is in its infancy, and these results are a snapshot into geoduck physiology at one developmental stage, using one tissue type (ctenidia), with individuals from one genetic pool, and with present-day pH levels in Washington State. To best inform current and future geoduck aquaculture, further foundational studies are needed to elucidate the variability in the species' pH limits in conjunction with more acute environmental stressors, and expanded to include other key tissues and functions (e.g. mantle for shell secretion), and whole-animal physiological studies (e.g. metabolic rate, reproductive development).

This study also demonstrates applied use of systems such as eelgrass beds in estuaries to test pH effects in a natural system. There is growing interest in using macroalgae as an ocean acidification bioremediation tool, also known as phytoremediation (Greiner et al. 2013; Hendriks et al. 2014; Washington State Blue Ribbon Panel on Ocean Acidification 2012; Groner et al. 2018). Incorporating seagrass into shellfish-pH interaction studies can help evaluate the potential for merging mariculture with shellfish aquaculture to improve growing conditions for vulnerable cultured species.

1.7 ACKNOWLEDGEMENTS

Our gratitude to the following people who assisted with this project: Grace Crandall, Kaitlyn Mitchell and Jose Guzman assisted with protein extraction. Jarrett Egertson contributed to DIA design. Austin Keller adapted MSConvert to correctly demultiplex and convert DIA files. Han-Yin Yang, Brian Searle and Sean Bennett assisted with running PECAN. Brittany Taylor and Taylor Shellfish Farms provided geoducks. Thank you to anonymous reviewers for the helpful feedback.

This work was supported in part by the National Science Foundation Graduate Research Fellowship Program, the NOAA Ocean Acidification Program, the University of Washington's Proteomics Resource (UWPR95794), and the Washington Department of Natural Resources.

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Chapter 2. LATENT EFFECTS OF WINTER WARMING ON OLYMPIA OYSTER REPRODUCTION AND LARVAL VIABILITY

A version of this chapter was published as: Spencer, L. H., Horkan, E., Crim, R., & Roberts, S. B. (2021). Latent effects of winter warming on Olympia oyster reproduction and larval viability. *Journal of Experimental Marine Biology and Ecology*, *542-543*, 151604. https://doi.org/10.1016/j.jembe.2021.151604

2.1 ABSTRACT

For ectothermic marine invertebrates living in temperate regions, impacts of ocean warming will vary considerably by season. In many species, reproductive and metabolic processes are tightly linked to the seasonal change from winter to spring, yet we know little about how these processes will shift as winters become milder. This study examined latent effects of winter warming on spring reproduction in the Olympia oyster, *Ostrea lurida*. Adults were collected in autumn from central Puget Sound, WA, USA, and exposed to two winter temperatures (7°C, 10°C) in the presence of food limited (5k algal cells/mL) and food abundant (50k algal cells/mL) environments. Following treatments, adults exposed to elevated winter temperature contained larger oocytes regardless of feeding regime, and those also fed abundant food contained more developed sperm. Adults then spawned in common conditions, and larvae were reared through settlement to assess carryover effects of winter treatments on larval viability. Adults previously exposed to elevated winter temperature (10°C) produced larger larvae, particularly if they were also fed high food levels. More developed gametes and larger larvae suggest that gametogenesis occurred at low levels throughout the winter, possibly resulting in increased maternal provisioning to influence larval size. Interestingly, winter temperature did not impact larval survival, or the

timing or magnitude of larval production. In the wild, more developed gametes and larger larvae following milder winters could greatly impact recruitment patterns, possibly benefitting *O. lurida* populations. In the hatchery setting, larval production and survival is not contingent upon winter conditions, and larval survival does not correlate with oocyte and larval size. Our results suggest that *O. lurida* reproduction is resilient to winter warming. Furthermore, as global temperature continues to rise, winter conditions should not be overlooked when examining reproduction in *O. lurida* and other temperate marine invertebrates with similar reproductive cycles.

2.2 INTRODUCTION

Temperature regulates many reproductive processes in marine invertebrates (Newell & Branch 1980; Hoegh-Guldberg & Pearse 1995). For species that live in temperate regions, reproductive cycles are tightly linked to seasonal temperature changes (Orton 1920). Gametogenesis onset, gamete growth rate, time to ripening, and the act of spawning are all believed to be a function of temperature, along with other environmental drivers such as nutrient availability and photoperiod (Olive 1995; Bates 2005). The precise timing, duration, and frequency of reproductive processes varies among species, but many follow a general seasonal pattern: rapid gametogenesis occurs in the spring; spawning occurs in the late spring and summer; recovery and resorption of residual gametes occurs in autumn, with some early differentiation of next season's gametes; and reproductive activity slows or ceases in the winter when temperatures drop below a minimum for breeding (Orton 1920; Olive 1995).

As global temperatures rise due to anthropogenic inputs, milder winters are anticipated due to increased sea surface temperature and more frequent marine heat waves (Gentemann et al. 2017; IPCC 2019; Holbrook et al. 2020). Ocean warming will invariably alter marine invertebrate reproductive processes (Edwards & Richardson 2004; Lawrence & Soame 2004). For instance, we know that moderately elevated temperature during gametogenesis and spawning (occurring primarily in spring) can accelerate gamete development (Parker et al. 2018), alter sex ratios (Eagling et al. 2018; Zapata-Restrepo et al. 2019), and increase fertilization rates (Rogers-Bennett et al. 2010; Byrne 2011), and could therefore augment recruitment rates. High summer temperatures can result in adult mortality when spawning coincides with acute heat stress (Mori 1979; Sastry 1966; Samain et al. 2007), reducing the number of breeding individuals or surviving offspring. We know little about how changes to winter conditions will impact reproduction. This oversight could be attributed to the relatively little reproductive activity that occurs in the winter compared to other seasons. There is, however, evidence that winter conditions can carry over to greatly impact physiological processes in other seasons. A 19-year dataset of oyster mortality (*Crassostrea gigas*) and climate variability revealed a lagged response, where high summer mortality occurred following warm winters (Thomas et al. 2018). It is therefore important that we explore more comprehensively how warming during winter months could impact reproduction in temperate ectotherms.

Many temperate species are thought to enter reproductive diapause in the winter, until temperatures exceed the physiological minimums for breeding in the spring (Orton 1920; Giese 1959; Pearse 1968; Bayne 1976). Winter warming could therefore result in uninterrupted gametogenesis, precocious spawning, or asynchronous sperm release and ovulation, if spermatogenesis and oogenesis respond differently to winter warming (Philippart et al. 2003; Chevillot et al. 2017). Winter warming could impact offspring phenotype by direct impacts to gametes, or indirectly by impacting the physiological processes of progenitors. Egg size, which is associated with nutritional content, typically correlates negatively with maternal environmental temperature (Atkinson et al. 2001; Moran & McAlister 2009; Gosselin et al. 2019). Maternal

RNAs and lipid composition, which are utilized during embryogenesis and larval development, could differ if their production or composition are sensitive to winter temperature (Krisher 2013; Leroy et al. 2018). It is also possible that elevated metabolic demand during warmer winters could drain maternal glycogen reserves, resulting in smaller or poor-quality oocytes in the spring (Mathieu & Lubet 1993), particularly if food availability remains limited during winter months (Eppley 1972; Winder & Cloern 2010, but see Testa et al. 2018). Elevated winter temperature therefore has capacity to alter wild populations and cultured stocks through wide-scale shifts in reproductive timing and capacity, and offspring viability.

Understanding the impacts of warming on marine species that are of ecological, economic, and cultural importance is particularly pressing. Here, we explore the effects of winter warming in *Ostrea lurida* Carpenter 1864, the Olympia oyster. Once abundant in estuaries along the Northeast Pacific Ocean, overharvest and pollution devastated populations in the early 1900s, and today 2–5% of historic beds remain (Polson & Zacherl 2009; Blake & Bradbury 2012). As the dominant native oyster along the Pacific Coast of North America, *O. lurida* is of cultural and economic significance to tribes and shellfish growers (Peter-Contesse & Peabody 2005; White et al. 2009), is an ecosystem engineer in estuarine habitats (Newell 2004; Coen et al. 2007; Pritchard et al. 2015), and shows some signs of resilience to ocean acidification (Waldbusser et al. 2016; Lawlor & Arellano 2020; Spencer et al. 2020). Restoration efforts are afoot, but *O. lurida* populations are still struggling, and may be further challenged by ocean warming.

O. lurida reproduce seasonally, with one or two larval settlement peaks occurring in spring or summer, depending on the location and conditions (Oates 2013; Pritchard et al. 2015). They are hermaphroditic spermcasters, and eggs are internally fertilized then brooded for approximately 10-12 days before being released as D-stage veliger larvae (Coe 1931; Hopkins 1937). Much of the foundational reproductive knowledge for the species was collected in the early 20th century, and the thermal threshold for gametogenesis was then established as ~12.5°C for *O. lurida* in Washington State (the location of our focal population) (Hopkins 1936, 1937). However, recent observations of low-temperature brooding (10.5°C, Barber et al. 2016) and spermatogenesis (~10°C, Spencer et al. 2020) question the concept that Washington State populations cease reproducing below 12.5°C. Winter temperatures in the region typically reach approximately 6.5-8°C (Moore et al. 2008), so even moderately elevated winter temperature could profoundly alter *O. lurida* reproductive phenology by interrupting winter quiescence or altering spawn timing. Sea surface temperature is projected to increase 1.2°C by the 2040's in the Pacific Northwest (as compared to 1970-1999) under the medium greenhouse gas scenario (Mote & Salathé 2010). However, the region is already experiencing periods of unprecedented warming, such as during the 2013-2015 marine heat wave (coined "The Blob"), which resulted in temperature anomalies exceeding +5°C (Di Lorenzo & Mantua 2016; Gentemann et al. 2017). There were anecdotal reports of poor larval production in an *O. lurida* hatchery in spring 2015 (Ryan Crim, *pers. comm.*), and warm winter temperatures were posited to have interfered with reproduction.

Previously, we explored the combined effects of winter warming and acidification on *O. lurida*, and found that reproduction may begin earlier in the spring following warmer winters, resulting in increased larval production, but when combined with acidification the effects are neutralized (Spencer et al. 2020). Here, we expand the warming-aspect of that study. Adult oysters were exposed to two winter temperatures (7°C, 10°C) in the presence of two feeding regimes (low algal density=5k cells/mL, high algal density= 50k cells/mL). Because winter warming could impact spring reproduction by depleting endogenous energy reserves due to increased metabolic demand (Sokolova et al. 2012), the two feeding regimes were included to assess effects of temperature under an energy limited environment (low algal density) and energy abundant environment (high algal density). The two overwintering temperatures were selected to represent historic (7°C) and elevated (10°C) winter temperatures, with elevated set below the minimum temperature at which *O. lurida* have been found to brood larvae in Washington State (10.5°C in a northern Puget Sound population, Barber et al 2016). Furthermore, the adult oysters were collected from the wild, as opposed to hatchery-reared oysters used in Spencer et al. 2020. Adult growth, survival, and gamete development were monitored during exposure to winter treatments and while spawning in the spring, as well as larval production, size, and survival.

2.3 METHODS

2.3.1 *Adult winter treatments*

Adult *Ostrea lurida* (3.80±0.50 cm) were collected from Mud Bay in Dyes Inlet in Bremerton, WA on November 6, 2017, acclimated to hatchery conditions in filtered (5 um), flow-through seawater supplemented with live algae. On December 8th, the adults were divided among eight flow-through tanks (50-L), each with two bags of 50 animals for a total of 100 oysters per tank. Adults were treated in a factorial design to two temperatures (Ambient: 7°C, Warm: 10°C) and two live algae feeding levels (High: 50,000 cells/mL, and Low: 5,000 cells/mL), with two replicate tanks per treatment (200 oysters per treatment in total), for a total of four treatments (7°C+low-food, 10°C+low-food, 7°C+high-food, 10°C+high-food, Figure 2.1).

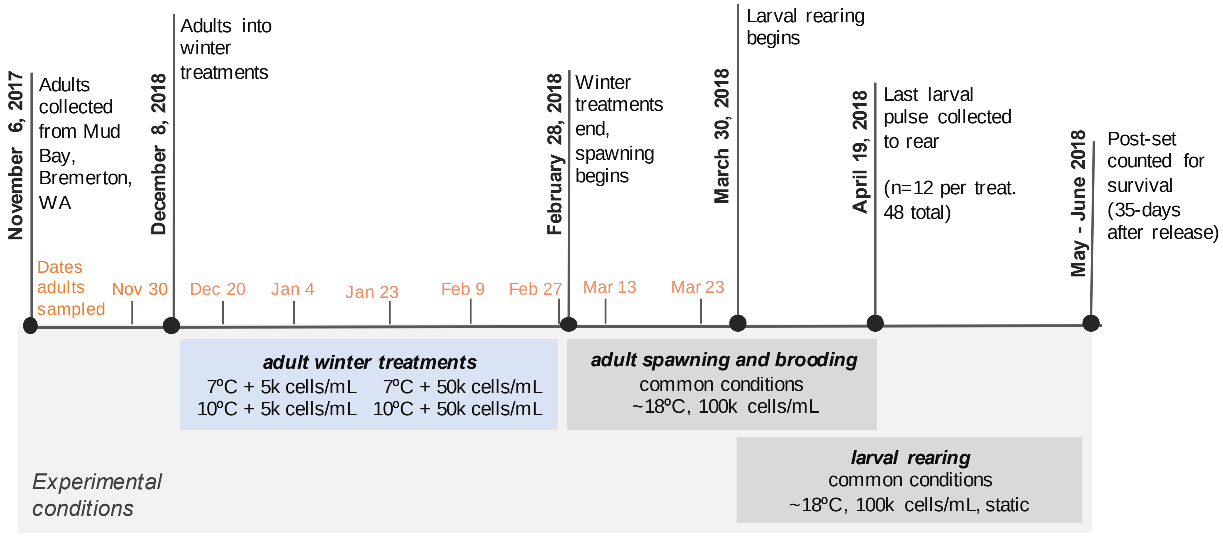

Figure 2.1. Experimental timeline. Cells/mL indicate the concentrations of live algae given to oysters at various stages.

To establish experimental conditions, temperatures were reduced by 0.5°C/day over one and two weeks for the 10°C and 7°C groups, respectively, then maintained for 12 weeks. Temperatures were maintained by recirculating seawater from a reservoir (50-L) through an aquarium chiller (Teco TK-2000 Tank Chiller, 1/3 HP, with built-in heater), before seawater was distributed to experimental tanks at a flow rate of 8L/hr. Feeding rates were maintained using Iwaki metering pumps from a common algae mixture (cocktail of *Tisochrysis lutea*, *Tetraselmis suecica*, *Chaetoceros* sp.). Algae cocktail concentration was estimated daily by manual cell counts and adjusting metering pump rates to achieve 50,000 and 5,000 cells/mL for high and low feeding levels, respectively. Temperature was monitored continuously with Avtech temperature probes and recorded with HOBO Pendant Temperature Data Loggers (UA-002-64). Oysters were cleaned and rotated among experimental tanks and aquarium chillers twice weekly and monitored for mortality. On February 28th the treatment conditions were terminated, and oysters were gradually returned to common conditions for reproductive conditioning and spawning (see section 2.5 for details). Tissue sampling occurred on a regular basis (Figure 2.1).

2.3.2 *Adult tissue sampling and measurements*

Approximately twice monthly during adult treatments (November 30 - February 27, Figure 2.1), 10 oysters per treatment were sacrificed to assess growth and gonad development, sampled evenly across treatment replicates and bags within replicates. Adults were also sampled twice during reproductive conditioning (n=12 per treatment, March 13 and 23). Upon sampling, shell height was measured as the distance from hinge to margin, perpendicular to the hinge, and tissue wet weight was estimated by subtracting shell weight from whole wet weight. Gonad tissue was excised by opening the oyster at the umbo, discarding gill tissue, then preserving the whole visceral mass in the PAXgene Tissue FIX system (PreAnalytiX, Hombrechtikon, Switzerland). Fixed tissues were processed for gonad analysis by Histology Consulting Services (Everson, WA).

Effects of winter treatments on changes to adult size and condition during treatments (November 30 - February 27, Figure 2.1) were estimated using Three-Way Analysis of Covariance. Shell height and estimated wet tissue weight were each modeled as a function of temperature and food level as categorical predictors with time as a continuous covariate using lm(), then main effects of predictor variables and their interactions were assessed using anova() (R Core Team 2021). Estimated wet tissue weight was cube-root transformed prior to analysis to meet normality assumption.

2.3.3 *Gamete development*

2.3.3.1 Gamete stage and sex

The stage and sex of sampled adult oysters were determined from preserved gonad histology sections, using designations adapted from da Silva, Fuentes, and Villalba (2009). As per da Silva, sex was assigned as indeterminate (I), male (M), hermaphroditic primarily-male (HPM), hermaphroditic (H), hermaphroditic primarily-female (HPF), and female (F). Due to the high frequency of hermaphroditism (41.1% of the 316 sampled oysters), male and female gametes within the same oyster were assigned separate developmental stages, then a dominant gonad stage was assigned for each oyster based on the predominant sex. The da Silva designations were applied for stages 1-3 (1: early gametogenesis; 2: advanced gametogenesis; 3: ripe). Departures from da Silva's stage 0 (inactive or resting), stage 4 (partially spawned), and stage 5 (fully spawned/resorbing) are as follows: stage 0 in this study represents empty follicles, or no presence of male or female gonad tissue. Stage 4 represents both spawned and resorbing gonad. This method does not include a separate stage 5, due to the very high frequency of residual gametes, and no distinct partially spawned oysters.

Impacts of treatments on gonad development were assessed using Chi-Square or Fisher Exact tests on contingency tables (depending on sample size), which were constructed from counts of gonad sex, male gamete stage, and female gamete stage. Prior to statistical testing, the six sex categories (I, M, HPM, H, HPF, F) were collapsed into four categories by combining HPF and F into one female designation (F), and HPM and M into one male designation (M). Tests were performed for all treatment weeks combined (December 20 - February 27), termination of winter treatments (February 27), and both reproductive conditioning weeks separately and combined (March 13 & 23). Due to the parsing of gonad data across the four sexes and five developmental stages p-values were computed using a Monte Carlo simulation test with 1,000 iterations (Hope 1968). To account for multiple comparisons, significance was designated as α=0.01. To determine pairwise differences Fisher Exact post-hoc tests were run using the pairwiseNominalIndependence function from the rcompanion package v2.3.7 (Mangiafico 2020).

2.3.3.2 Ripe oocyte size

To estimate impacts of winter treatments on maternal provisioning ripe oocytes were measured upon terminating treatments and during spawning (February 27, March 13 & March 23). The maximum oocyte length was measured for 24 of the largest oocytes in oysters containing stage 3 (ripe) oocytes using a Nikon eclipse Ni microscope and the NIS-Elements BR imaging and measuring software (version 4.60). The maximum length was assessed due to elongated oocyte shape, and the varying orientation of oocytes in mounted histology sections. The number of stage 3 females varied among treatments, and was 6, 14, 7, and 10 for 7°C+low-food, 10°C+low-food, 7°C+high-food, and 10°C+high-food, respectively. Mean oocyte size was calculated for each oyster, square-transformed to meet the assumption of normality, then compared among treatments using Two-Way Analysis of Variance with Type II Sums of Squares using the Anova() function from the car v3.0.10 package for R (Fox & Weisberg 2019).

2.3.4 *Larval production*

After terminating treatments adults were spawned to assess impacts of winter treatments on larval production. Following hatchery procedures, continuous, volitional spawning was induced by holding adults in elevated temperature and nutrition in flow-through tanks (20-L at 26-L/hr). Adults from each winter treatment tank were split into two spawning tanks, for a total of four replicate spawning tanks per winter treatment, each with ~25 oysters. Beginning on February 28, temperature was increased 0.5°C/day for 7°C treatments to 10°C (6 days), then all groups increased 1°C/day to 18°C and fed live algae cocktail at approximately 100,000 cells/mL. Tanks were checked daily for veliger larvae, which are released from the maternal brood chamber approximately 10-14 days after fertilization. Once larval release began, larvae were collected daily for four weeks, and counts were estimated by hand-counting larvae in triplicate subsamples. Twice weekly tanks were cleaned, adults were inspected for mortality, and then rotated among the tank arrangement.

Larval production timing and magnitude were compared among adult winter temperature and food treatments. Release timing was assessed by comparing the date of onset and date of maximum release. Release magnitude was assessed by comparing the total number of larvae released over the 4-week collection period, and the average number of larvae released each day, which estimates the average brood size. Treatment effects were examined using Two-Way Analysis of Variance using anova() and lm() from base R (R Core Team 2021) for all but the date of larval release onset, which did not meet ANOVA assumptions. Larval release onset was therefore first examined with Two-way ANOVA with Robust Estimation using pbad2way() from the WRS2 package for R (est=”mom” and nboot=5000, Mair & Wilcox 2020), then, as there was no significant interaction (simulated p-value < 0.05), main effects were further examined with Kruskal Wallis rank sum tests. All metrics were assessed for homogeneity of variance using Levene’s Tests.

2.3.5 *Larval viability*

2.3.5.1 Larval size

To assess impacts of adult winter treatment on larval size, larvae were measured upon release from the maternal brood chamber. After released larvae were counted, excess larvae were preserved directly in -80°C, then measured using a Nikon eclipse Ni microscope and the NIS-Elements BR imaging and measuring software (version 4.60). Mean shell width (longest distance parallel to hinge) was estimated from at minimum 40 larvae per collection from each tank. The number of larval batches that were measured varied among treatments, and was 30, 38, 23, and 29 for 7°C+low-food, 10°C+low-food, 7°C+high-food, and 10°C+high-food, respectively. Mean width was compared among parental treatments using lme() from the nlme package (Pinheiro et al. 2021) to construct the linear mixed-effect models, and Anova() from the car v3.0.10 (Fox & Weisberg 2019) to construct Analysis of Variance tables with Type II Sums of Squares and to test for significance using Wald chi-square. Larval preservation method changed part-way through sampling: the first 50 larval samples were sacrificed using ethanol, whereas only fresh water was used to collect later samples. Ethanol use impacted the integrity of preserved larval tissue, resulting in larvae that were on average 5.6 µm smaller compared to those that were preserved only with freshwater. We therefore included the use of ethanol as a random variable when testing for effects of parental treatments on larval size.

2.3.5.2 Larval survival

To assess impacts of adult winter treatments on larval survival, a subset of collected larvae were reared through settlement. In total, 48 pulses of larvae were reared, 12 per adult treatment, which were collected over a 19-day period. As multiple females can release larvae on the same day, some larval pulses may represent more than one male x female mating pair, and thus each pulse is henceforth referred to as a “group” (as opposed to “family”). To ensure genetically diverse larval groups within treatments, three groups were collected from each of the four replicate spawning tanks. Upon collecting a larval group, larvae were cleaned of debris using nylon mesh (224 µm) and soaked in fresh water (18°C) for 1 minute. For each larval group, approximately 2,400 larvae were reared in triplicate (800 larvae per tank). Larval stocking error rate (3.1%, mean 824±54 SD larvae) was determined for 12 of the 48 groups by hand counting triplicate samples, taken simultaneous to tank preparation. Larval tanks were constructed from thin-walled polyvinyl chloride pipe (7.6 cm) and nylon mesh (100 µm) placed in individual containers with static water (800±50 mL). Water was changed daily from a common mixture of filtered seawater (<1 µm) and live algae, which consisted of a 1:1 mix by volume of *Chaetoceros muelleri* and *Pavlova pinguis*, for a combined concentration of 100,000 cells/mL. Fourteen days after collecting each larval group, oyster shell fragments (0.5-mL of 224 µm) were sprinkled into larval tanks to serve as settlement substrate. After thirty-five days, survival rate was estimated for each larval tank by hand-counting the number of surviving, metamorphosed larvae, and average post-set survival was calculated from the three replicate tanks for each of the 48 larval groups. Factors influencing larval survival were assessed using quasibinomial generalized linear models (GLM) and Pearson's Chi-squared tests, which were generated with Analysis of Deviance tables using Anova() from car v3.0.10 (Fox & Weisberg 2019). Factors tested included adult temperature treatment, adult feeding level, larval size upon release (shell width), the number of larvae released in a group (i.e., approximate brood size), and the date larvae were collected.

2.4 RESULTS

2.4.1 *Adult survival and size*

During winter treatments there was minimal adult mortality, and no differences in mortality among winter treatments. However, during reproductive conditioning (which began February 26), high mortality occurred in both 7°C groups. Cumulative survival at the end of the experiment was 50% and 70% in the 7°C+high-food and 7°C+low-food groups, and 81% in both 10°C+high-food and 10°C+low-food groups (Figure 2.2).

Adult shell height and estimated wet tissue weight were not affected by winter temperature (height: : F(1,211)=0.053, p=0.82; weight: F(1,211)=0.022, p=0.88) or feeding level (height: F(2,211)=0.33, p=0.72; weight: F(2,211)=0.77, p=0.46), and there was no significant change over time during the treatments (height: F(1,211)=0.14, p=0.70; weight: F(1,211)=3.56, p=0.061).

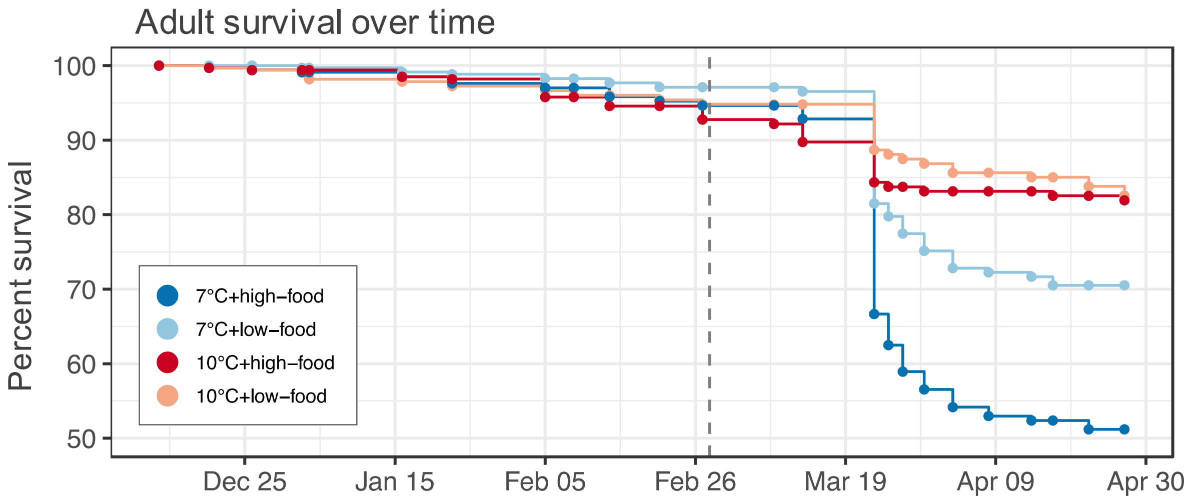

Figure 2.2. Adult survival over time by winter treatment. There was higher mortality in adults that had previously been exposed to 7°C, particularly those also held in the high food environment, but mortality differences were only observed after they were removed from winter treatments (dashed line) and had entered common spawning conditions.

2.4.2 *Gamete development*

Winter warming resulted in more developed sperm, but only in the presence of high food (Figure 2.3, Table 1). There were no significant effects of winter treatments on oocyte stage or gonad sex ratio. However, winter warming resulted in larger ripe oocytes (Figure 2.4). Here we provide more details on sperm development and egg size.

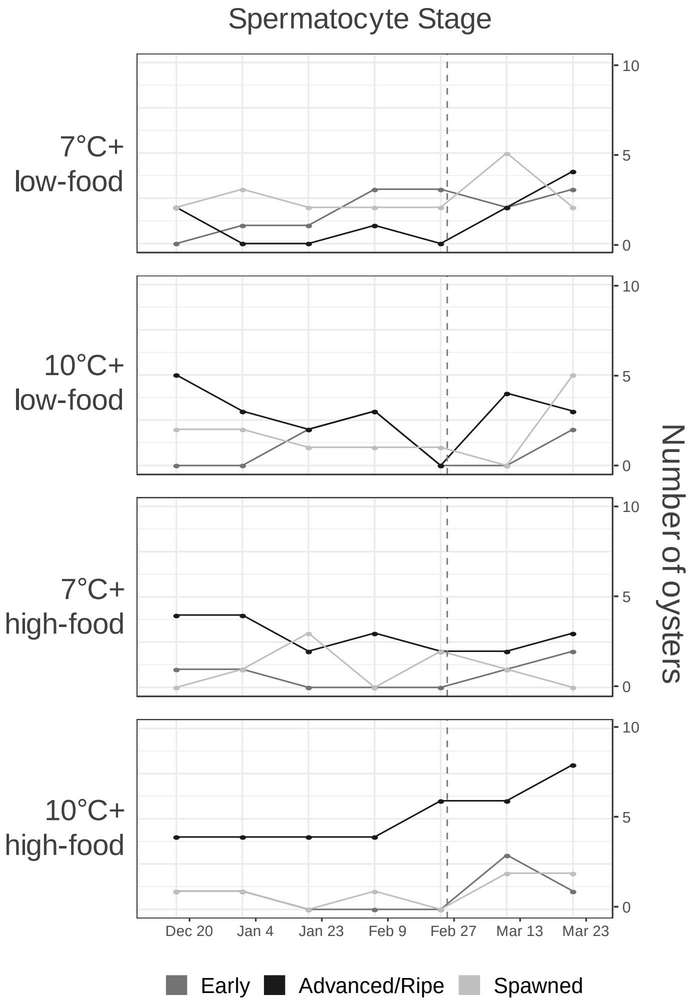

Figure 2.3. Spermatocyte stage for each treatment throughout winter and during spawning. Adults exposed to winter warming and high food had more developed spermatocytes (gray bars) at treatment termination (February 28, dotted line) and while spawning in common conditions (March 23). Oysters were induced to spawn by increasing temperature 1°C/day and feeding with live algae cocktail at approximately 100,000 cells/mL. Frequency of Stage 0 spermatocytes, indicating none present, are omitted.

2.4.2.1 Sperm development

On the final day of adult winter treatments (February 27), sperm developmental stage differed significantly among experimental treatments (Table 2.1). More of the 10°C+high-food oysters contained late-stage spermatocytes (60% of male tissue was advanced or ripe) than the 10°C+low-food (0%) and 7°C+low-food oysters (0%) (Figure 2.3). Sperm stage also differed during the spawning phase (March 13 & 23, Table 1). The 10°C+high-food group contained more late-stage spermatocytes (58% of male tissue was advanced or ripe) and only 2 oysters fully lacked sperm (8%, Figure 2.3), compared the 7°C+high-food treatment, which had fewer oysters with late-stage sperm (21%) but a higher proportion without sperm (63%).

Table 2.1. Impacts of winter treatments on spermatocyte development. Spermatocyte stage was compared among winter treatments using all oysters sampled during exposures (Dec 20 - Feb 27), oysters sampled upon termination of exposures (on Feb 27), and those sampled during spawning in common conditions (Mar 13 & 23) by Pearson's Chi-squared test with simulated p-value (based on 10000 replicates). The four treatments included 7°C+low-food, 10°C+low-food, 7°C+high-food, and 10°C+high-food. Where treatment significantly affected gonad development (*), we include pairwise treatment differences according to Fisher Exact tests (due to multiple comparisons, significance was set as α=0.01).

Sample date(s)	χ^2	**p-simulated**	**Pairwise treatment differences**
All adults sampled during treatments (Dec 20 - Feb 27, N=200)	25.6	0.010	*NA*
End of winter treatments (Feb 27, N=40)	*25.8	0.0065	*10°C+high-food vs. 7°C+low-food* *10°C+high-food vs. 10°C+low-food*
2 weeks in common spawning conditions (Mar 13, N=48)	23.4	0.019	*NA*
3.5 weeks in common spawning conditions (Mar 23, N=48)	*25.2	0.0096	*No sign. pairwise differences*
Both spawning dates combined (Mar 13 & 23, N=96)	*32.2	0.0010	*10°C+high-food vs. 7°C+high-food*

2.4.2.2 Ripe oocyte size

Adults exposed to elevated winter temperature had larger ripe oocytes (stage 3) upon termination of winter treatments and during spawning (Feb 27, Mar 13 & 23 combined) than adults exposed to ambient winter temperature (F(1,33)=6.71, p=0.014, Figure 2.4). Feeding level had no effect on ripe oocyte size (F(1,33)=1.12, p=0.30), nor was there an interaction between temperature and food treatments (F(1,33)=0.17, p=0.69). Mean oocyte length (maximum length) of stage 3 oocytes was 91±14 µm and 83±13 µm for 10°C and 7°C oysters, respectively.

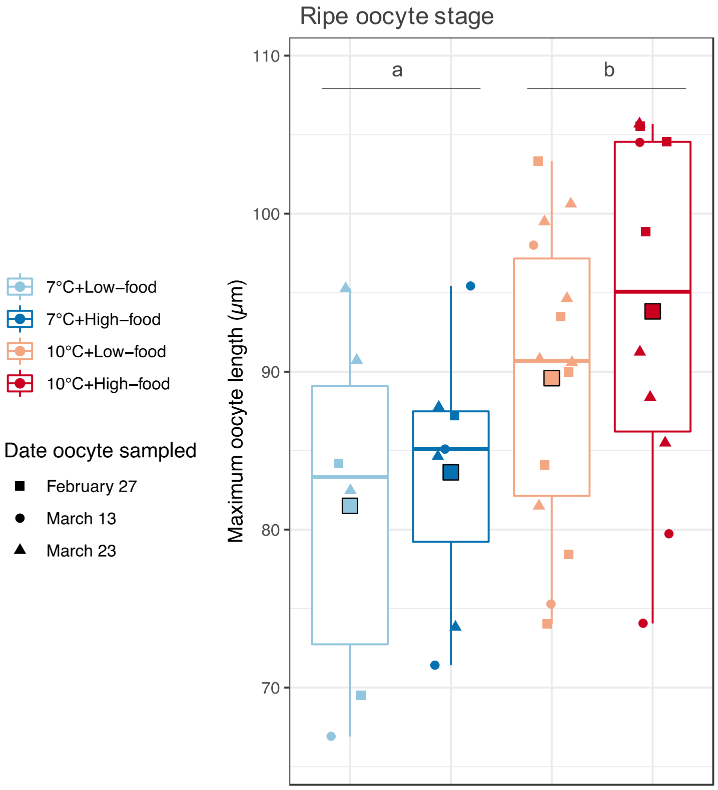

Figure 2.4. Ripe oocyte size by adult winter treatment. Adults exposed to winter warming (10°C) contained larger ripe oocytes than those held at ambient temperature (7°C), regardless of the feeding regime. Each point represents an oyster's average oocyte length, which we report for all oysters containing ripe oocytes (stage 3) at the termination of treatments (February 27) and during volitional spawning (March 13 & 23). Boxes contain values lying within the interquartile range (IQR), with medians indicated by lines in the middle of boxes. Whiskers extend to the largest value no >1.5∗IQR, and points outside the boxes indicate outliers beyond 1.5∗IQR. Square points outlined in black indicate mean oocyte length by adult winter treatment.

2.4.3 *Larval production*

Winter temperature treatment did not influence larval release timing (onset: χ^2 (1, N=16) = 1.33 p=0.25; peak release: F(1,12)=0.57 p=0.46) or magnitude (per-day average: F(1,12)=0.068 p=0.80; total released: F(1,12)=1.15 p=0.30) as a sole factor, nor did it interact with food level (onset: p-sim=0.10; peak release: F(1,12)=4.78 p=0.050; per-day average: F(1,12)=0.005, p=0.98;

total released: F(1,12)=0.58 p=0.46). Food level influenced larval release onset (χ^2(1, N=16) = 10.87, p=9.4e^{-4}). Adults exposed to low food during the winter released larvae earlier than those exposed to high food. The total number of larvae released during the 30-day period did not differ among treatments, nor did the average number of larvae collected per day (which estimates brood size).

Across all treatments, released larvae were first observed 31 days after entering spawning conditions. Brooded embryos were observed on March 23 during adult oyster sampling, 24 days after beginning the spawning phase. Most brooded larvae were collected from oysters exposed to elevated winter temperature. Of the 48 oysters sampled (12 per treatment), six broods were observed from adults exposed to 10°C+high-food (50% brooding), five from 10°C+low-food (42% brooding), one from 7°C+low-food (8% brooding), and zero in 7°C+high-food.

2.4.4 *Larval viability*

2.4.4.1 Larval size at liberation

Adults exposed to elevated temperature produced larger larvae, with larger mean shell width (χ^2=4.69, p=0.030, Figure 2.5). Adult temperature treatment also interacted with adult food level to affect larval shell width, resulting in significantly larger larvae from adults exposed to 10°C+high-food than those exposed to 7°C+high-food (χ^2=4.28, p=0.039). Adult food treatment alone did not affect larval size (χ^2=2.53, p=0.11).

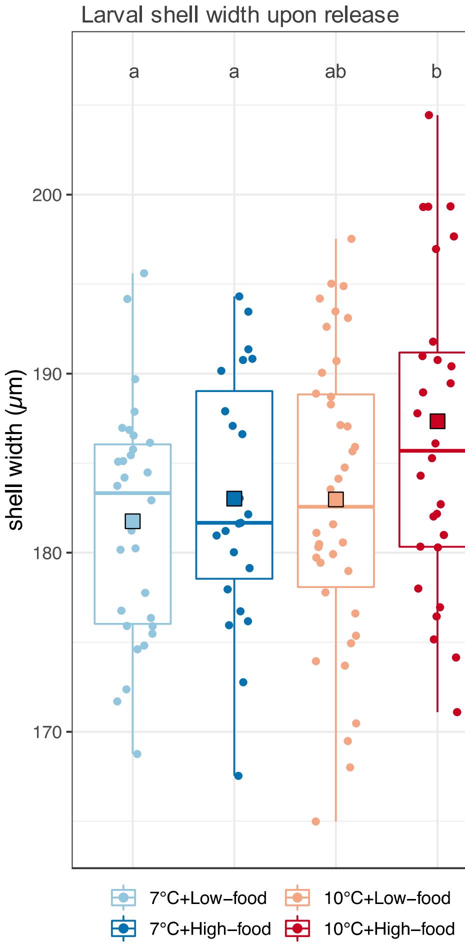

Figure 2.5. Larval size by parental winter treatment. Larvae were larger from adults that had previously been exposed to elevated winter temperature, particularly if they were also fed high food levels. Each point represents the average shell width of a group of veliger larvae released from the maternal brood chamber (N=120). Boxes contain values lying within the interquartile range (IQR), with medians indicated by lines in the middle of boxes. Whiskers extend to the largest value no >1.5∗IQR, and points outside the boxes indicate outliers beyond 1.5∗IQR. Square points outlined in black indicate mean larval shell width by parental winter treatment.

2.4.4.2 Larval survival

Larval survival through metamorphosis (assessed 5 weeks after maternal liberation) was not significantly influenced by adult winter temperature (χ^2=0.13, p=0.72) or food treatment (χ^2=0, p=1), nor was there a significant interaction between the two treatments (χ^2=0.84, p=0.36, Figure 2.6). Larval survival was not associated with larval shell width (χ^2=0.33, p=0.57) upon liberation.

All data and code associated with this project are publicly available (Spencer et al. 2021).

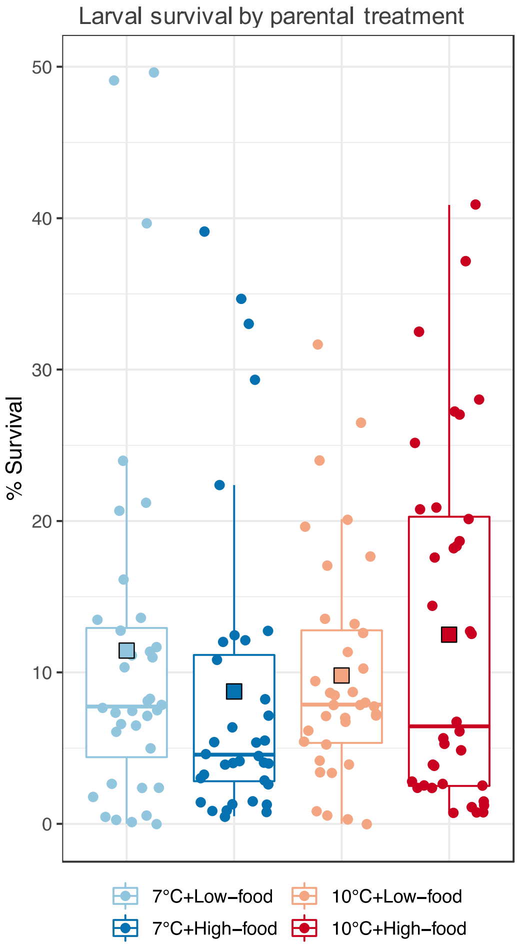

Figure 2.6. Larval survival (%) by parental winter treatment, which were not associated. For each of the 12 spawning tanks (4 per treatment), three larval groups were reared in triplicate from larval pulses released on different days, for a total of 144 larval tanks (each point = average survival in one larval tank). Boxes contain values lying within the interquartile range (IQR), with medians indicated by lines in the middle of boxes. Whiskers extend to the largest value no >1.5∗IQR, and points outside the boxes indicate outliers beyond 1.5∗IQR. Square points outlined in black indicate mean larval survival by parental winter treatment.

2.5 Discussion

This study tested the effects of winter warming on *O. lurida* spring reproduction and larval quality. Adults were exposed to two winter temperatures (7°C, 10°C) and two feeding regimes to examine effects of warming under an energy limited environment (low algal density) and energy abundant environment (high algal density). Winter warming as a sole exposure augmented egg size and larval size and influenced sperm development in the presence of high-food, but warming did not affect fecundity or larval survival under any feeding regime.

For oysters and many other marine invertebrates living in temperate regions, reproduction is tightly regulated by temperature and seasonality (Korringa 1952; Newell & Branch 1980). Ocean warming in winter could greatly affect these populations via latent effects to gametogenesis and maternal provisioning (among other possible effects) (Ottersen et al. 2001; Lawrence & Soame 2004; Sunday et al. 2010). In theory, elevated winter temperature increases an ectothermic organism’s metabolic rate (Schulte et al. 2011; Schulte 2015), and, in the absence of increased food, depletes glycogen and lipid reserves, resulting in less energy available for reproduction (i.e. gamete development) and growth (i.e. glycogen/lipid storage, calcification, Sokolova et al. 2012). Therefore, we predicted that elevated winter temperature (+3°C) would result in poor *O. lurida* larval production and/or low-quality larvae (i.e., smaller, poor survival), unless they were provided ample exogenous energy (high food). Contrary to expectations, winter warming augmented oocyte and larval size, and had no effect on larval production or survival. We expand on these findings below and discuss implications for *O. lurida* populations in future ocean conditions.

2.5.1 *Gamete development*

Winter temperature impacted egg and sperm development. Following exposure to elevated temperature (+3°C), adults contained significantly larger mature oocytes regardless of feeding regime (Figure 2.4), and more developed sperm when they were also exposed to high food (Figure 2.3, Table 1). That winter temperature impacted egg size is not unexpected - in ectotherms, egg size often correlates with environmental temperature, both between and within species (Thorson 1950; Atkinson et al. 2001; Fischer et al. 2003; Moran & McAlister 2009). However, the relationship is typically negative, such that elevated temperature results in smaller eggs. We observed the opposite - larger eggs following elevated temperature. We therefore posit that larger oocytes and more developed sperm are both explained by elevated winter temperature triggering or increasing the rate of gametogenesis. While *O. lurida* reproduction was previously thought to cease below 12.5°C for populations along the species' northern range, recent evidence has indicated that brooding can occur as low as 10.5°C (Barber et al. 2016), and spermatogenesis as low as 10°C (Spencer et al. 2020). Under experimental conditions the observed gamete differences had no bearing on larval production. In a natural setting, however, slight changes in gamete development could greatly influence population dynamics by lengthening the reproductive season (e.g., precocious spawning), and/or increasing spawning rates.

Another possible explanation for the gamete developmental differences is that adults exposed to winter warming experienced less thermal stress when temperature was increased rapidly to induce spawning. Indeed, less mortality was observed in adults that had been exposed to high winter temperature (Figure 2.2), but only when adults from all treatments were in common conditions and undergoing a 1°C/day increase to induce spawning. While large temperature swings are common for populations of Puget Sound *O. lurida* (Becker et al. 2020), the added 3°C increase for the ambient temperature exposed oysters may have been thermally stressful as applied (persistent, unabated temperature increase) which could have increased their susceptibility to bacterial infection. This could have resulted in an energetic shift away from vitellogenesis and towards the stress and immune responses in the ambient-temperature group, resulting in high mortality and less developed sperm and smaller eggs (Bayne et al. 1978; Delaporte et al. 2006; Wendling & Wegner 2013; Lokmer & Wegner 2015). While thermal stress might explain the observed gamete differences during spawning, it does not account for developmental differences observed upon termination of winter treatments, prior to spawning (on February 27th, Figures Figure 2.3 & Figure 2.4, Table 3.1). Therefore, while thermal stress could have contributed to the observed gamete differences between temperature treatments, it is not likely the sole explanation.

Interestingly, we observed a sex-specific interaction between elevated winter temperature and food level that impacted gamete development. Winter warming increased oocyte size regardless of feeding regime, whereas more developed sperm were only observed following combined warming and high food. From previous studies it is clear that temperature and food availability both likely play significant roles in *O. lurida* reproductive cycles (Coe 1931; Loosanoff & Davis 1952; Bulseco 1982; Joyce et al. 2013). However, few studies have deciphered which environmental factors need to be present to trigger oogenesis and spermatogenesis in *O. lurida*. Our data suggest that temperature may be the dominant factor influencing vitellogenesis, whereas temperature and phytoplankton may both be necessary to trigger spermatogenesis. Foundational studies are still needed to determine the minimum temperature required for vitellogenesis and spermatogenesis in *O. lurida*, and whether supplemental algae is a necessary cue for spermatogenesis, or if it simply accelerates it when combined with elevated temperature.

2.5.2 *Larval viability*

Adults exposed to winter warming produced larger larvae. Because we also observed larger eggs in those adults (Figure 2.4), it is very likely that larvae were larger in part because they hatched from larger eggs (Chambers & Leggett 1996). Larvae were measured upon liberation from the maternal brood chamber, which occurs ~10-12 days after fertilization (Coe 1931; Hopkins 1937), therefore size differences may also reflect varying larval growth rates (Helm et al. 1973). Again, larger eggs were observed following elevated winter temperature, which probably reflects increased maternally-provisioned nutrients, and thus more endogenous energy to fuel embryogenesis and pre-feeding larval growth. However, we also observed an interaction between winter temperature and food, such that the largest larvae were produced by adults that were exposed to both elevated winter temperature and high food. Egg size was not similarly affected by food level; therefore, egg size alone may not explain the observed larval size differences. The quality or type of biochemical constituents in eggs may have been influenced by winter food treatment, such as fatty acid composition, which correlates closely with larval growth in *Ostrea edulis* (Jonsson et al. 1999) and can be influenced by adult diet (Helm et al. 1991). Adult exposure to winter warming could also have resulted in changes to epigenetic controls of gene expression in larvae, such as changes to DNA methylation, that influenced overall larval physiology and growth rates. DNA methylation patterns can shift upon heat exposure in the Pacific oyster (*Crassostrea gigas*) (Wang et al. 2020), but whether those changes persist and are heritable is not known. To understand the mechanisms by which parental exposure to winter warming influences larval physiology and size, future studies should assay egg biochemical constituents and other plastic, heritable factors (e.g., DNA methylation) alongside egg size and larval performance metrics.

That adults exposed to elevated winter temperature produced larger larvae and eggs is another signal that *O. lurida* may benefit from winter warming. Larger larvae are considered more viable, since they are more capable feeders and swimmers, may be less susceptible to predation (Bailey 1984), and can have improved metamorphic success and growth (Marshall & Keough 2007; Marshall et al. 2008). In *Ostrea*, egg size and larval size upon liberation have been positively linked to larval performance. Chilean flat oyster (*Ostrea chilensis*) pediveliger size at release positively correlates with larval growth and spat survival, in addition to egg size and biochemical properties (Wilson et al. 1996), and *O. edulis* larval growth and metamorphic competency positively relates to size and lipid content upon release (Helm et al. 1973; Gonzalez Araya et al. 2012). In other molluscs, larval size is linked to larval metamorphic competency and egg size, such as in the Eastern oyster *Crassostrea virginica*, hard clam *Mercenaria mercenaria*, bay scallop *Argopecten irradians* (Kraeuter et al. 1981; Gallager & Mann 1986), and the Japanese abalone *Haliotis discus hannai* (Fukazawa et al. 2005).

Given the influence of adult winter temperature exposure on egg and larval size in the present study, one would expect to see consistent impacts to larval survival. Interestingly, larval survival was not influenced by parental exposure to elevated winter temperature, nor did survival correlate with larval or oocyte size. This indicates that although winter warming may augment provisioning of gametes and larvae, the effect does not necessarily persist to influence larval competency, at least not under this study's experimental conditions. While these results contrast previous studies showing an association between size and survival (Kraeuter et al. 1981; Gallager & Mann 1986; Millican & Helm 1994; Wilson et al. 1996; Fukazawa et al. 2005), they align with the related study, Spencer et al. (2020), which also did not detect temperature effects on larval survival. It is probable that the optimal larval rearing conditions in both studies (*e.g.*, ample food, regular cleaning) negated the benefits that larger oocytes and larvae can have on larval physiology, and buoyed any larvae with energy deficits. Nevertheless, this and the related study (Spencer et al. 2020) indicate that winter warming does not compromise *O. lurida* larval quality when reared in the hatchery, and larval size when released from the brood chamber is not predictive of survival through settlement. In the wild, where there is higher predation risk, conditions are more stressful, and phytoplankton abundance is less consistent, winter warming may benefit Puget Sound *O. lurida* populations by increasing larval recruitment due to increased size and/or growth rate (Swezey et al. 2020).

2.5.3 *Larval production*

Larval production during the 30-day collection period was unaffected by winter temperature, regardless of algal density. The results are in contrast with a complementary study, Spencer et al. (2020), in which elevated temperature exposure prior to spawning resulted in more larvae. The present study specifically expands Spencer et al. (2020), to use a new *O. lurida* population collected from the wild rather than oysters that were bred in captivity. The response of *O. lurida* reproduction to elevated winter temperature may be conditional upon gonad stage prior to treatment, and population-specific reproductive traits (Barber et al. 1991; Silliman et al. 2018). For instance, in this study the percentage of adults that were female was unusually high upon collection (63%), and many already contained late-stage oocytes (55% of females). In comparison, only 28% of all oysters sampled in Spencer et al. (2020) were female, 33% of which contained late-stage oocytes. Oysters that enter the winter with late-stage oocytes may be less influenced by winter conditions, and may require less time and energy for maturation in the spring. The developmental status of Olympia oyster oocytes entering the winter season may therefore influence how winter temperature affects spring reproduction. It must be noted that when we first observed brooded larvae in sampled adults (March 23rd), we found that more of the high-temperature exposed oysters were brooding (42%-50%, vs. only 0%-8% of the ambient-temperature adults). Differing brooding frequencies could be a signal that winter warming did indeed result in precocious spawning by functional females, however this limited data does not allow us to draw any concrete conclusions. Ultimately, that larval production was unaffected by overwintering treatments suggests that *O. lurida* are capable of withstanding a range of winter conditions without compromising spring larval production. Furthermore, this and Spencer et al.

2020 studies find no evidence that the poor larval production in the *O. lurida* hatchery (Ryan Crim, *pers. comm.*) was attributed to the 2013-2015 marine heat wave (Gentemann et al. 2017).

2.5.4 *No carryover effects of winter feeding regime*

Parental feeding regime was not the focal treatment in this study, therefore we will not expound its effects in detail. However, it is interesting that adult winter food level did not influence gametes or larvae as a sole factor. Prior work in *Ostrea* spp. has indicated that parental nutrition at various phases can greatly influence the size, growth rate, and survival of offspring (Lannan et al. 1980; Millican & Helm 1994; Wilson et al. 1996; Berntsson et al. 1997; Gonzalez Araya et al. 2012; Marshall & Keough 2007). Our results indicate that for *O. lurida* to successfully reproduce in the spring, nutritional needs during the winter are flexible. In the wild, warming-induced changes to winter phytoplankton availability or composition (Cavole et al. 2016; McCabe et al. 2016; Peterson et al. 2017) may not have pronounced effects on spring reproduction. This could reflect low clearance rates at temperatures ~10°C and below (Gray & Langdon 2018), resulting in weak responses to varying food levels and reliance on endogenous energy sources to sustain metabolic and reproductive functions through the winter. In locations with higher winter temperature regimes the *O. lurida* feeding response, and thus the impact of winter dietary differences, could be a larger factor influencing reproduction in spring. Of course, our experimental conditions must also be considered, such as the high-quality larval diet, which could have offset any nutritive differences that adult winter diet imparted to offspring. Additionally, adult diet was only limited during treatment, not during spawning, which could have obfuscated effects of winter malnourishment. Starving adults of various condition index through the spawning and brooding phases would expose whether *O. lurida* rely solely on endogenous glycogen to provision gametes (“capital breeder”), or exploit and require exogenous energy for reproduction (“income breeder”)(Bayne 2017).

2.5.5 *Adult mortality*

Poor survival was observed in adults that had been exposed to ambient temperature (7°C), particularly in the presence of high food (Figure 2.2). The majority of mortality occurred when adults from all treatments were in common conditions and undergoing a 1°C/day increase to induce spawning. The added 3°C increase for the ambient temperature exposed oysters may have been thermally stressful, or increased their susceptibility to bacterial infection, resulting in the high mortality (Delaporte et al. 2006; Wendling & Wegner 2013; Lokmer & Wegner 2015). In addition to thermal stress, mortality in the 7°C+high-food treatment could also be due to oxidative stress caused by high reproductive effort. Just prior to spawning, estimated wet tissue weight increased in the 7°C+high-food treatment only. Condition index (a reflection of tissue weight) is positively associated with post-spawning mortality in other oyster species, explained in part by high glycogen reserves fueling rapid gamete proliferation, which elevates metabolism and causes oxidative stress (Alonso-Alvarez et al. 2004; Chávez-Villalba et al. 2007; Huvet et al. 2010). High mortality in the 7°C+high-food adults may therefore be associated with synergistic effects of thermal stress and oxidative stress. We do not report these findings to predict impacts of winter warming on wild populations given the rapid temperature increase during spawning, but rather to highlight potential causes of reproduction-related mortality in *O. lurida*.

2.6 CONCLUSION

There is growing evidence that *O. lurida* is more equipped than other bivalves to handle shifting ocean conditions (Waldbusser et al. 2016; Gray et al. 2019; Lawlor & Arellano 2020; Spencer et al. 2020). While we recognize the limitations of our hatchery-based experimental design in predicting how wild *O. lurida* will respond to ocean warming, our findings do suggest that *O. lurida* reproduction and larval viability are, at the least, not highly sensitive to winter warming, and at best may benefit from it. Our results also provide more evidence that *O. lurida* reproduction is not "on pause" throughout the winter (Barber et al. 2016; Spencer et al. 2020), but rather gametogenesis is likely occurring at temperatures lower than the previously established threshold for reproduction in Washington State populations (12.5°C, Coe 1931, Hopkins 1936). Winter conditions should therefore not be overlooked when examining reproductive cycles in *O. lurida* and other temperate oysters, particularly as the oceans continue to warm and marine heat waves occur more frequently. Additionally, based on histological samples collected throughout the winter, we think that many residual gametes remained viable throughout the winter in our adult oysters. Therefore, winter warming could have influenced larval size by altering factors other than size or stage in gametes, such as macromolecules or RNAs deposited in eggs, or epigenetic modifications to sperm or eggs (e.g., DNA methylation changes). Examining biochemical composition, gene expression, and/or epigenetic factors in gametes after warm and cool winters could reveal the mechanisms responsible for the carryover effects observed here. There are, however, many gaps in our understanding of *Ostrea* spp. reproductive systems, which constrain interpretation of complex molecular data collected from *O. lurida*. It is critical that we untangle how precisely environmental drivers (e.g. temperature, food, tidal cycle, allosperm), and internal drivers (e.g. age, source population), control reproduction across seasons, and in the context of ocean warming. Given the potential resilience of *O. lurida* to ocean change, its reproductive processes may become more pertinent to those culturing, harvesting, conserving, and restoring marine invertebrates.

2.7 ACKNOWLEDGEMENTS

Our gratitude to the following people who assisted with this project: the NOAA Manchester Research Center and Puget Sound Restoration Fund (PSRF) provided facilities and materials, and PSRF staff members Stuart Ryan, Morgan Adkisson, and Josh Bouma assisted with daily feeding and maintenance. Duncan Greeley and Anne Spencer helped with the larval husbandry; Kaitlyn Mitchell assisted with larval measurements; committee members Jackie Padilla-Gamñio, Rick Goetz, and Jennifer Ruesink advised and supported this project.

This work was supported in part by the National Science Foundation Graduate Research Fellowship Program. This publication is partially funded by the Joint Institute for the Study of the Atmosphere and Ocean (JISAO) under NOAA Cooperative Agreement NA15OAR4320063, Contribution No. 2020-1078.

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Chapter 3. CARRYOVER EFFECTS OF TEMPERATURE AND PCO$_2$ ACROSS MULTIPLE OLYMPIA OYSTER POPULATIONS

A version of this chapter was published as: Spencer, L. H., Venkataraman, Y. R., Crim, R., Ryan, S., Horwith, M. J., & Roberts, S. B. (2020). Carryover effects of temperature and pCO2 across multiple Olympia oyster populations. *Ecological Applications: A Publication of the Ecological Society of America*, *30*(3), e02060. https://doi.org/10.1002/eap.2060. Copyright 2020 by John Wiley and Sons. Adapted with permission under license # 5132180138881.

3.1 ABSTRACT

Predicting how populations will respond to ocean change across generations is critical to effective conservation of marine species. One emerging factor is the influence of parental exposures on offspring phenotype, known as intergenerational carryover effects. Parental exposure may deliver beneficial or detrimental characteristics to offspring that can influence larval recruitment patterns, thus shaping how populations and community structure respond to ocean change. Impacts of adult exposure to elevated winter temperature and pCO$_2$ on reproduction and offspring viability were examined in the Olympia oyster (*Ostrea lurida*) using three populations of adult, hatchery-reared *O. lurida,* plus an additional cohort spawned from one of the populations. Oysters were sequentially exposed to elevated temperature (+4°C, at 10°C), followed by elevated pCO$_2$ (+2204 µatm, at 3045 µatm) during winter months. Male gametes were more developed after elevated temperature exposure and less developed after high pCO$_2$ exposure, but there was no impact on female gametes or sex ratios. Oysters previously exposed to elevated winter temperature released larvae earlier, regardless of pCO$_2$ exposure. Those exposed to elevated winter temperature as a sole treatment released more larvae on a daily basis, but when also exposed to high pCO$_2$ there was no effect. These combined results indicate that elevated winter temperature accelerates *O.*

lurida spermatogenesis, resulting in earlier larval release and increased production, with elevated pCO_2 exposure negating effects of elevated temperature. Altered recruitment patterns may therefore follow warmer winters due to precocious spawning, but these effects may be masked by coincidental high pCO_2. Offspring were reared in common conditions for one year, then deployed for three months in four estuarine bays with distinct environmental conditions. Offspring of parents exposed to elevated pCO_2 had higher survival rates in two of the four bays. This carryover effect demonstrates that parental conditions can have substantial ecologically relevant impacts that should be considered when predicting impacts of environmental change. Furthermore, Olympia oysters may be more resilient in certain environments when progenitors are pre-conditioned in stressful conditions. Combined with other recent studies, our work suggests that the Olympia may be more equipped than other oysters for the challenge of a changing ocean.

3.2 INTRODUCTION

The repercussions of ocean warming and acidification on marine invertebrate physiology are complex, but significant recent advances indicate that larval stages of marine taxa are particularly vulnerable (Byrne & Przeslawski, 2013; Kurihara, 2008; Przeslawski, Byrne, & Mellin, 2015). Understanding how shifting conditions will influence larval recruitment patterns is critical to predicting changing population dynamics, and thus community structure. One emerging consideration is whether larval stages benefit from ancestral exposures, based on evidence that memory of environmental stressors can be transferred between generations through non-genetic inheritance (reviewed in Perez & Lehner, 2019; Donelson *et al.* 2018; Eirin-Lopez & Putnam, 2019; Ross, Parker, & Byrne, 2016). Beneficial, or positive, carryover effects may be important acclimatory mechanisms for marine organisms facing rapid change, particularly those that evolved in dynamic environments like estuaries and the intertidal (Donelson, Salinas, Munday, & Shama, 2018; Gavery & Roberts, 2014). These carryover effects are defined as transgenerational when they persist in generations that were never directly exposed. Intergenerational, or parental, effects may be due to direct exposure as germ cells (Perez & Lehner, 2019). Trans- and intergenerational carryover effects are increasingly reported across marine phyla, including Cnidaria (*e.g.* Putnam & Gates, 2015), Echinodermata (*e.g.* Clark *et al.*, 2019), Mollusca (*e.g.* Parker *et al.* 2015), Arthropoda (*e.g.* Thor & Dupont, 2015), and Chordata (Review: Munday 2014).

A foundational series of studies on the Sydney rock oyster (*Saccostrea glomerata*) provide strong evidence for intergenerational carryover effects in bivalves, an ecologically and economically important group of taxa (Dumbauld, Ruesink & Rumrill, 2009). Adult *S. glomerata* exposed to high pCO_2 produced larger larvae that were less sensitive to high pCO_2, and the effect persisted in the successive generation (Parker *et al.*, 2012, 2015). In the presence of secondary stressors, however, parental high pCO_2 exposure rendered larvae more sensitive (Parker *et al.*, 2017). Intergenerational carryover effects are increasingly documented in larvae across other bivalve species, and are beneficial in the mussels *Mytilus chilensis* (Diaz *et al*., 2018) and *Mytilus edulis* (but not juveniles) (Kong *et al*., 2019; Thomsen *et al*., 2017), and detrimental in the clam *Mercenaria mercenaria*, the scallop *Argopecten irradians* (Griffith & Gobler, 2017), and the oyster *Crassostrea gigas* (Venkataraman, Spencer, & Roberts, 2019).

These preliminary studies provide strong evidence for intergenerational carryover effects in bivalves, but the body of work is still narrow in scope. Nearly all studies have exposed parents to stressors during denovo gamete formation (gametogenesis). For many temperate bivalve species, this occurs seasonally in the spring (Bayne, 1976). Yet, challenging periods of acidification and warming can occur during other times of the year (Evans, Hales, & Strutton, 2013; Joesoef, Huang, Gao, & Cai, 2015; McGrath, McGovern, Gregory, & Cave, 2019). The most corrosive carbonate environment in the Puget Sound estuary in Washington State, for example, commonly occurs in the winter when many species are reproductively inactive, while favorable conditions are in the spring when gametogenesis coincides with phytoplankton blooms (Pelletier, Roberts, Keyzers, & Alin, 2018). Thus, adult exposure to severely corrosive conditions during gametogenesis may not represent the natural estuarine system. To our knowledge, only one study has assessed carryover effects of exposure to acidification before reproductive conditioning in a bivalve, the oyster *C. gigas*, and found negative maternal carryover effects on larval survival (Venkataraman, Spencer, & Roberts, 2019), indicating that pre-gametogenic exposure also matters. No studies have yet attempted to examine intergenerational carryover effects of combined winter warming and acidification in bivalves.

To best predict whether intergenerational carryover effects will be beneficial or detrimental, it is also crucial to understand how warming and acidification will impact fertility and reproductive phenology. Temperature is a major driver of bivalve reproduction, and modulates gametogenesis (Joyce, Holthuis, Charrier, & Lindegarth, 2013; Maneiro, Pérez-Parallé, Pazos, Silva, & Sánchez, 2016; Oates, 2013), influences sex determination (Santerre *et al.*, 2013) and, in many species, triggers spawning (Fabioux, Huvet, Le Souchu, Le Pennec, & Pouvreau, 2005) (alongside other factors such as photoperiod, nutrition, lunar/tidal phases). Year-round warming may result in unexpected impacts to larval competency resulting from changes to reproduction. For instance, some temperate bivalve species have a thermal threshold for gametogenesis and enter a period of reproductive inactivity, or “quiescence”, which is believed necessary for successive spawning (Giese, 1959; Hopkins, 1937; Loosanoff, 1942). Warmer winters brought on by global climate change (IPCC, 2013, 2019) may therefore shift species’ reproductive cycles to begin earlier, or eliminate seasonality altogether, resulting in poorly provisioned or ill-timed larvae (Chevillot *et al.*, 2017). Such impacts were clearly demonstrated using a long-term dataset (1973-2001) of estuarine clam *Macoma balthica* reproduction and temperature. Mild winters and earlier springs resulted in low fecundity, earlier spawning, and poor recruitment, which was largely explained by a phenological mismatch between spawning and peak phytoplankton blooms (Philippart *et al.*, 2003). The impacts of winter acidification on estuarine bivalve reproduction are less predictable. The few studies to date show that high pCO_2 delays gametogenesis in the oysters *Crassostrea virginica* and *S. glomerata* (Boulais *et al*., 2017; Parker *et al.*, 2018), but both studies exposed oysters during gametogenesis. Acidification during the winter months could increase energetic requirements (Sokolova, Frederich, Bagwe, Lannig, & Sukhotin, 2012), and deplete glycogen reserves that are later utilized for gametogenesis in the spring (Mathieu & Lubet, 1993), but this hypothesis has yet to be tested.

The purpose of this study was to assess whether warmer, less alkaline winters will affect fecundity and offspring viability in the Olympia oyster, *Ostrea lurida*. The Olympia is native to the Pacific coast of North America (McGraw, 2009). Overharvest and pollution devastated populations in the early 1900s, and today 2-5% of historic beds remain (Blake & Bradbury, 2012; Polson & Zacherl, 2009). Restoration efforts are afoot, but *O. lurida* populations continue to struggle, and may be further challenged by changing conditions (Barton, Hales, Waldbusser, Langdon, & Feely, 2012; Feely, Klinger, Newton, & Chadsey, 2012; Feely, Sabine, Hernandez-Ayon, Ianson, & Hales, 2008). For instance, large interannual variability in larval recruitment and frequent recruitment failures were recently reported (Wasson *et al.*, 2016; Kimbro, White & Grosholz, 2019). This variability is presumably related to inconsistent spawning success, larval survival, and retention, and governed predominantly by local conditions (Kimbro, White &

Grosholz, 2019). It is unknown how the intensity, timing, and duration of local environmental conditions can predict recruitment failure (Wasson *et al.*, 2016). If winter conditions significantly influence recruitment through direct changes to adult reproductive capacity or timing, or indirect changes through parental carryover effects, population densities and distributions will inevitably shift with conditions.

Another consideration in this study was the genetic composition of test organisms. *Ostrea lurida* exhibits varying phenotypes among distinct populations (Silliman, 2019), which can influence their sensitivity to environmental stressors (Bible & Sanford, 2016; Bible, Evans & Sanford, 2019). Indeed, the two groups to measure the response of *O. lurida* larvae to ocean acidification found contrasting results – no effect (Waldbusser *et al.*, 2016), and slower growth (Hettinger *et al.*, 2012, 2013) – possibly a result of local adaptation. The source population used for experimental studies may therefore be a critical factor influencing climate-related findings. Furthermore, testing genetically diverse organisms could reveal cryptic genetic variation, alleles that confer stress resilience only under certain settings (Paaby & Rockman, 2014; Bitter *et al.*, *preprint*), which has implications for how wild populations are restored. Therefore, we tested three phenotypically distinct Puget Sound populations (Heare, Blake, Davis, Vadopalas, & Roberts, 2017; Silliman, Bowyer, & Roberts, 2018), which were hatchery-reared in common conditions to adulthood, to account for intraspecific variation while controlling for within-generation carryover effects (Hettinger *et al.*, 2012, 2013).

Our study is the first to assess the combined effects of elevated winter temperature and pCO_2 on reproduction, and to explore intergenerational carryover in an *Ostrea* spp. We exposed adult *O. lurida* to elevated temperature (+4°C), followed by elevated pCO_2 (+2204 µatm, -0.51 pH). Gonad development, reproductive timing, and fecundity were assessed for the adults in the laboratory, and offspring performance was assessed in the field. Elevated winter temperature was expected to impede gametogenic quiescence, presumably a critical annual event, subsequently reducing larval production. This prediction was in part based on observations of low larval yields in an *O. lurida* restoration hatchery (Ryan Crim, *unpublished*) following the winter 2016 marine heat wave in the Northeast Pacific Ocean (Gentemann, Fewings, & García-Reyes, 2017). Similarly, we predicted that high pCO_2 exposure would result in negative impacts due to increased energy requirements for calcification and cellular maintenance. Finally, we predicted that negative impacts would be amplified upon exposure to both conditions. By assessing the effects of winter warming and acidification on reproduction and offspring viability in multiple Olympia oyster populations, we provide an ecologically relevant picture of how the species will respond to ocean change.

3.3 METHODS

3.3.1 *Adult oyster temperature and pCO_2 exposures*

Four cohorts of adult *Ostrea lurida* were used in this study. Three of the cohorts were first-generation hatchery-produced (F1) oysters (32.1 ± 5.0 mm), all hatched in Puget Sound (Port Gamble Bay) in 2013 (Heare *et al*., 2017). The broodstock used to produce these F1 oysters were wild, harvested from Fidalgo Bay in North Puget Sound (F), Dabob Bay in Hood Canal (D), and Oyster Bay in South Puget Sound (O-1) (O in Figure 3.1). These populations are considered phenotypically distinct subpopulations (Heare *et al.*, 2017; White, Vadopalas, Silliman, & Roberts, 2017). The fourth cohort (O-2, 21.9 ± 3.3 mm) was second-generation, hatchery-produced in 2015 from the aforementioned Oyster Bay F1 cohort, from a single larval release pulse and thus likely one family (Silliman, Bowyer, & Roberts, 2018). The O-2 cohort was included to examine whether reproductive and offspring traits were consistent across generations of a population, with the O-2 cohort being closely related to each other (siblings) and 2 years younger than the other cohorts. Prior to the experiment, all oysters were maintained in pearl nets in Clam Bay (C) for a minimum of 500 days.

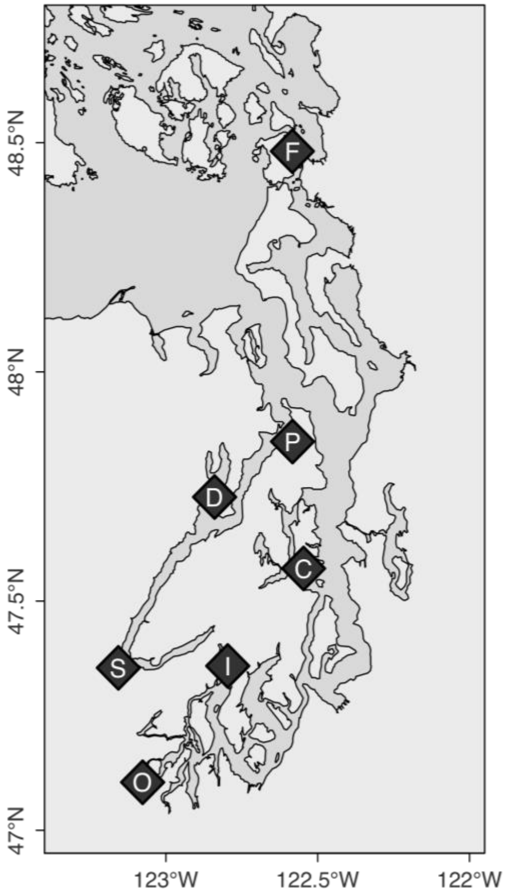

Figure 3.1. Locations where *O. lurida* populations' progenitors were collected (F, D, O), where oysters were housed prior to and during the experiment (C), and where offspring were deployed (F, P, S, I): Fidalgo Bay (F), Port Gamble Bay (P), Dabob Bay (D), Clam Bay (C), Skokomish River Delta (S), Case Inlet (I), Oyster Bay (O).

3.3.2 *Temperature treatment*

Oysters were moved from Clam Bay (C) to the Kenneth K. Chew Center for Shellfish Research and Restoration for the temperature and pCO_2 experiments. Oysters were held in one of two temperature regimes (6.1±0.2°C and 10.2±0.5°C) for 60 days beginning December 6, 2016 (Figure 3.2). The temperatures correspond to historic local winter temperature (6°C) in Clam Bay, and anomalously warm winter temperature (10°C) as experienced during 2014-2016 (Gentemann *et al.*, 2017). For the temperature exposure, oysters from each cohort (100 for O-1 and F cohorts, 60 for D, and 300 for O-2) were divided into four bags, two bags per temperature, in two flow-through experimental tanks (50L - 1.2-L/min). Temperature in the 6°C treatment was maintained using an aquarium chiller, and unchilled water was used for the 10°C treatment. Temperatures were recorded continuously with water temperature data loggers.

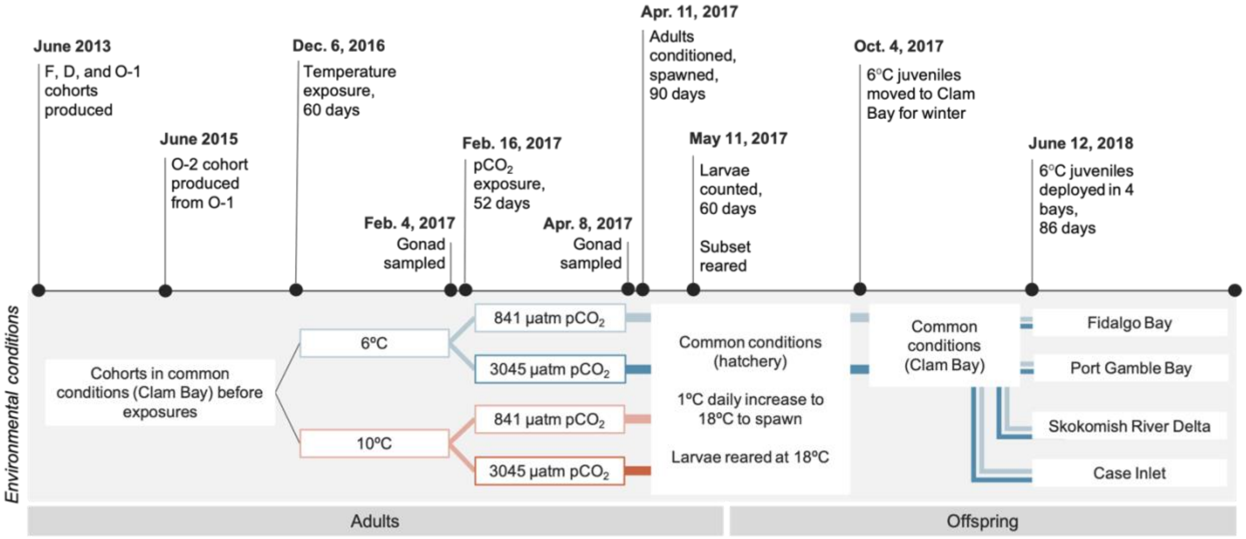

Figure 3.2. Experimental timeline. Four cohorts of adult *O. lurida* (F, D, O-1, O-2) were sequentially exposed to two winter temperatures (6.1±0.2°C, 10.2±0.5°C) then two pCO_2 levels (841±85 µatm, 3045±488 µatm). They were returned to ambient pCO_2 conditions to volitionally spawn. Larvae were collected and reared by cohort x temperature x pCO_2. Juveniles (~1 year) from 6°C-Ambient pCO_2 and 6°C-Low pCO_2 adults were deployed in 4 bays in Puget Sound.

3.3.3 *High pCO_2 treatment*

A differential pCO_2 exposure was carried out after the temperature treatment ended. Following a 10-day gradual temperature increase for the 6°C treatment to 10°C, oysters were further divided and held at ambient pCO_2 (841±85 µatm, pH 7.82±0.02) or high pCO_2 (3045±488 µatm, pH 7.31 ± 0.02) (Appendix A) for 52 days (February 16 to April 8, 2017, Figure 3.2). Animals were housed in six flow-through tanks (50-L - 1.2-L/min), with three replicate tanks per pCO_2 treatment and oyster cohort. High pCO_2 treated water was prepared using CO_2 injection. Filtered seawater (1µm) first recirculated through a reservoir (1,610-L) with a degassing column to equilibrate with the atmosphere, then flowed into treatment reservoirs (757-L) recirculating through venturi injectors. Durafet pH sensors and a Dual Input Analytical Analyzer monitored pH in treatment reservoirs with readings every 180 seconds. Using solenoid valves, CO_2 gas was injected through lines at 15 psi in 0.4 second pulses if pH exceeded the 7.22 set point. Water pH was continuously monitored in experimental tanks using Durafet pH sensors, and temperature (10.4 ± 0.4°C) was measured using water temperature data loggers. Twice weekly, water samples (1-L) were collected from experimental tanks, and temperature (°C), salinity (PSU), and pH (mV, converted to pH_T) were measured immediately using a digital thermometer, conductivity meter, and pH electrode, respectively. Simultaneously, discrete water samples (120-mL) were collected in duplicate from experimental tanks and preserved with HgCl (50-µL) for later total alkalinity measurements using a titrator. Standard pH curves were generated on each sampling day prior to pH measurements using TRIS buffer prepared in-house at five temperatures. Using the seacarb library in R, pCO_2, dissolved organic carbon (DIC), calcite saturation ($\Omega_{calcite}$), and aragonite saturation ($\Omega_{aragonite}$) were calculated for days 5, 33, and 48 (Appendix A).

During both temperature and pCO_2 treatments, all oysters were fed from a shared algae header tank daily with Shellfish Diet 1800® (300-500-mL, Reed Mariculture) diluted in ambient pCO_2 seawater (200-L, Helm & Bourne, 2004), dosed continuously with metering pumps. Experimental, reservoir, and algae tanks were drained and cleaned, and oysters were monitored for mortality and rotated within the experimental system twice weekly.

3.3.4 *Adult reproductive development*

A subset of oysters was sampled for gamete stage and dominant sex immediately before and after pCO_2 treatments (Figure 3.2) to capture developmental differences among treatments. Puget Sound *O. lurida* reportedly enter reproductive quiescence and resorb residual gametes when temperatures are below 12.5°C (Hopkins 1936, 1937), however recent evidence of low-temperature brooding in Puget Sound (10.5°C, Barber *et al*. 2016) suggests that reproductive activity may occur during warm winters. Therefore, gonad tissue was sampled to estimate the following: 1) whether residual gametes were resorbed or developed during winter treatments; 2) whether temperature and pCO_2 influenced winter activity; 3) if male and female gametes responded similarly; and 4) if gonad responses correspond with fecundity. Prior to pCO_2 exposure, 15 oysters were sampled from O-1, O-2, and F cohorts, and 9 from the D cohort. After pCO_2 exposure, 9, 6, and 15 oysters were sampled from each treatment for O-1/F, D, and O-2 cohorts, respectively (distributed equally among replicates tanks). Whole visceral mass was excised and preserved in histology cassettes using the PAXgene Tissue FIX System, then processed for gonad analysis by Diagnostic Pathology Medical Group, Inc. (Sacramento, CA).

Adult gonad samples were assigned sex and stage using designations adapted from (da Silva, Fuentes, & Villalba, 2009). Sex was assigned as indeterminate (I), male (M), hermaphroditic primarily-male (HPM), hermaphroditic (H), hermaphroditic primarily-female (HPF), and female

(F). Gonad sex was collapsed into simplified male and female designations for statistical analyses (hermaphroditic-primarily male = male, hermaphroditic-primarily female = female). For stage assignment, male and female gametes were assigned separately due to the high frequency of hermaphroditism (50.8%). Dominant gonad stage was then assigned based on the sex assignment. The da Silva gonad stages were applied for early gametogenesis (stage 1), advanced (stage 2), and ripe (stage 3). Departures from da Silva's stage 0, stage 4 (partially spawned), and stage 5 (fully spawned/resorbing) were as follows: stage 0 in this study represents empty follicles, or no presence of male or female gonad tissue; stage 4 represents both spawned and resorbing gonad; this method did not include a separate stage 5, due to the very high frequency of residual gametes, and no distinct partially spawned oysters.

Treatment effects on gonad tissue were assessed for all cohorts combined in 4 gonad metrics: 1) gonad stage of dominant sex, 2) male gonad tissue when present, 3) female gonad tissue when present, and 4) gonad sex-collapsed (Chi-square test of independence). To assess the effects of elevated winter temperature alone, gonad metrics were compared between 6°C and 10°C treatments prior to pCO_2 treatment. To determine the effect of pCO_2 exposure, gonad metrics were compared between ambient and high pCO_2 after 52 days in pCO_2 treatments, including temperature interaction effects. To estimate whether gonad changed during pCO_2 treatment, metrics were compared before and after ambient and high pCO_2 treatments, including temperature interaction effects. P-values were estimated using Monte-Carlo simulations with 1,000 permutations, and corrected using the Benjamini & Hochberg method and α=0.05 (Benjamini & Hochberg, 1995).

3.3.5 *Larval production*

Following pCO_2 exposure, adult oysters were spawned to assess impacts of winter treatment on larval production timing and magnitude. Beginning on April 11, 2017 (Figure 3.2), oysters were reproductively conditioned by raising temperatures gradually (~1°C/day) to 18.1 ± 0.1°C and fed live algae cocktail at 66,000 ± 12,000 cells/mL. Oysters spawned in the hatchery for 90 days volitionally, i.e. naturally releasing gametes without chemical or physical manipulation. Six spawning tanks were used for each temperature x pCO_2 treatment: 6°C-high pCO_2, 6°C-ambient pCO_2, 10°C-high pCO_2, and 10°C-ambient pCO_2. Within the six tanks per treatment, two spawning tanks contained the F cohort (14-17 oysters), two tanks the O-1 cohort (14-17 oysters), one tank the D cohort (9-16 oysters), and one tank the O-2 cohort (111-126 oysters). More O-2 oysters were used due to their small size. Olympia oysters release sperm, but have internal fertilization and release veliger larvae following a ~2 week brooding period (Coe ,1931; Hopkins, 1937). Therefore, production was assessed by collecting veliger larvae upon maternal release. Spawning tank outflow was collected in 7.5-L buckets using 100 µm screens made from 15.25 cm polyvinyl chloride rings and 100 µm nylon mesh.

Larval collection was assessed for differences in spawn timing and fecundity. Larvae, first observed on May 11, 2017 (Figure 3.2), were collected from each spawning tank every one or two days for 60 days. The number of larvae released was estimated by counting and averaging triplicate subsamples of larvae homogenized in seawater. The following summary statistics were compared between temperature x pCO_2 treatments: total larvae released across the 90-day period, average number of larvae collected on a daily basis (excluding days where no larvae were released), maximum larvae released in one day, date of first release, date of maximum release, and number of substantial release days (greater than 10,000 larvae). The total and daily release values were normalized by the number of broodstock * average broodstock height (cm), which can impact fecundity. Distributions were assessed using qqp in the car package for R (Fox & Weisberg, 2011), and log-transformed to meet normal distribution assumptions, if necessary. Differences between treatments were assessed using linear regression and Three-Way ANOVA (cohort was included as a covariate) with backwards deletion to determine the most parsimonious models. Tukey Honest Significant Differences were obtained using TukeyHSD to assess pairwise comparisons (R Core Team, 2016). Dates of peak larval release were also estimated for each pCO_2 x temperature treatment by smoothing using locally weighted regression, with geom_smooth in the ggplot package (Wickham, 2017), with span=0.3 and degree=1.

3.3.6 *Offspring survival in a natural setting*

To assess potential carryover effects of parental pCO_2 exposure, offspring from parents in 6ºC-ambient pCO_2 and 6ºC-high pCO_2 treatments were reared then deployed in the natural environment. To focus on the effect of parental pCO_2 exposure, only offspring from 6ºC parents were tested in the field (Figure 3.2). Larvae were collected between May 19 and June 22, 2017, separated by parental pCO_2 exposure and cohort, and reared in common conditions for approximately 1 year (Figure 3.2). On June 12, 2018 the juveniles were placed in four bays in Puget Sound — Fidalgo Bay, Port Gamble Bay, Skokomish River Delta, and Case Inlet — with two sites per bay, for a total of eight locations (Figure 3.1). Autonomous sensors collected continuous water quality data at each location for pH, salinity (via conductivity), dissolved oxygen, temperature, and chlorophyll. For the F/D and O-1/O-2 cohorts, respectively, 30 and 10 oysters were placed at each location. Initial shell height and group weight were measured, then oysters were enclosed in mesh pouches and affixed inside shellfish bags to exclude predators. At the end of three months, survival, shell height and group weight were measured for live oysters.

Juvenile oyster survival was compared among bays and parental pCO_2 exposure with a binomial generalized linear mixed model (glmm) using glmer from the lme4 package (vs. 1.1-19). Chi-square tests compared survival differences among factors using the car package Anova function (Fox & Weisberg, 2011). Mean shell growth was determined by subtracting pre-deployment mean height from post-deployment mean height (not including dead oysters). Both mean shell growth and mass change were compared among factors using ANOVA and F-statistics to test differences by bay, parental pCO_2, and cohort.

All data analysis was performed in R version 3.3.1 using the RStudio interface (R Core Team, 2016). Code for statistical analyses can be found in the associated Github repository (Spencer *et al.*, 2019).

Table 3.1. Environmental data during offspring field trial. Environmental data was collected from locations where offspring were deployed for 3 months from June through August 2018. Mean±SD of continuously monitored environmental data are shown for periods of tidal submergence only (tidal height >0.3m), collected at two deployment locations within each bay.

	Fidalgo Bay	**Port Gamble Bay**	**Skokomish River Delta**	**Case Inlet**
Temperature (°C)	15.4±1.5	15.0±1.0	16.2±2.7	16.8±1.7
DO (mg/L)	10.6±2.4	10.5±1.9	10.2±3.9	11.2±2.8
Salinity (PSU)	28.5±3.9	31.9±2.0	29.6±1.3	24.6±1.7
pH_T	8.07±0.15	7.86±0.17	8.01±0.20	8.01±0.16
Chlorophyll (µg/L)	2.27±4.09	2.25±1.45	5.72±15.36	3.31±6.13

3.4 RESULTS

3.4.1 *Adult reproductive development*

After 60 days in temperature treatments (6.1±0.2°C and 10.2±0.5°C), gonad stage of the dominant sex differed significantly between temperatures (Table 3.2). The 10°C oysters had more instances of advanced gametogenesis (stage 2), and fewer that were resorbing/spawned (stage 4) (Figure 3.3). This difference was influenced strongly by more advanced male gametes in 10ºC oysters, but there were no differences in female gamete stages. No differences in sex ratio were observed between temperature treatments (Figure 3.4).

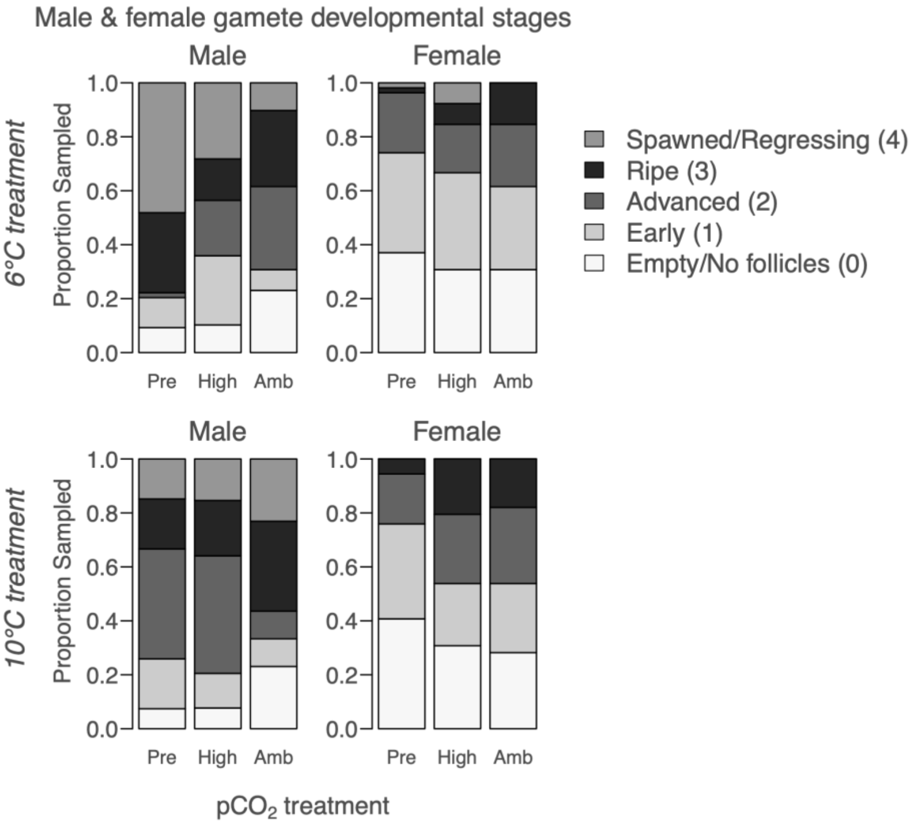

Figure 3.3. Gonad developmental stages for male and female gametes, after 60-days in temperature treatments but before pCO_2 treatments (“Pre”, n=54) and after 52 days in high pCO_2 (3045±488 µatm, n=39) and ambient pCO_2 (841±85 µatm, n=39), which indicates that sperm development was influenced by elevated winter temperature (more advanced) and high pCO_2

(less advanced, 10°C treatment only), but oocyte development was not. All oysters were assigned both male & female stages; if no oocytes were present, for example, that oyster was designated as female stage 0.

After 52 days in pCO_2 treatments, gonad stage of the dominant sex differed significantly between ambient and high pCO_2 in the oysters previously held in 10°C (Table 3.2). More mature gametes (stage 3) were found in 10°C-ambient pCO_2 (49%) compared to 10°C-high pCO_2 (33%). This difference was strongly influenced by oysters that were predominantly male, as male gamete stage tended to differ between pCO_2 treatment, but female gamete stage did not (Table 3.2, Figure 3.3). In 6°C-treated oysters, there were no pCO_2 effects on gonad stage of the dominant sex, male gamete stage, or female gamete stage. No gonad stage or sex ratio differences were detected among oysters from 10°C-high pCO_2 (combined stressors) and 6°C-ambient pCO_2 (no stressors). Gonad sex did not differ significantly among treatments, however oysters tended to contain fewer male-only and more female-only gonad tissues in the riper, ambient pCO_2-treated groups than male-only tissues (Figure 3.4).

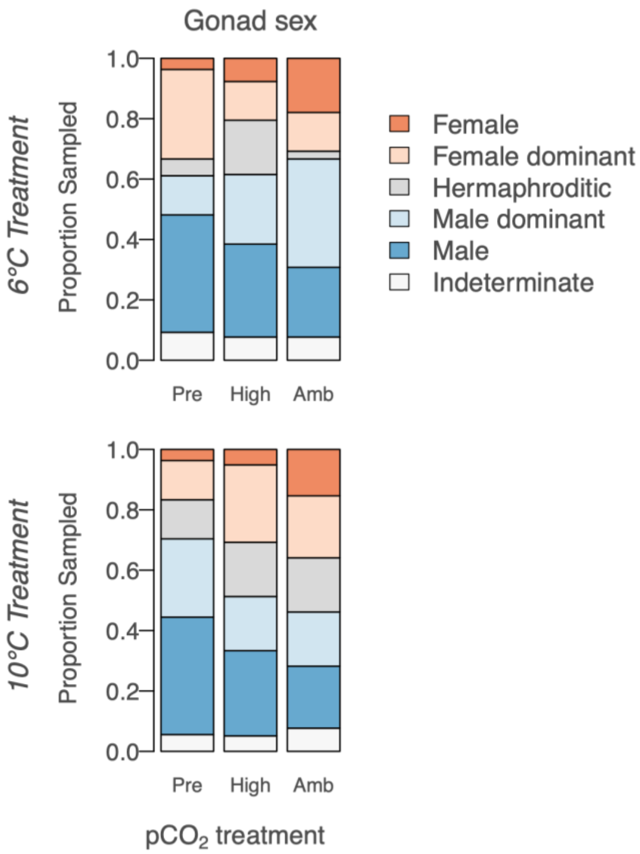

Figure 3.4. Gonad sex, after 60-days in temperature treatments but before pCO_2 treatments ("Pre", n=54) and after 52 days in high pCO_2 (3045±488 µatm, n=39) and ambient pCO_2 (841±85 µatm, n=39). Winter conditions did not significantly influence gonad sex ratios.

Compared to oysters before pCO_2 exposure, those exposed to high pCO_2 did not differ in gonad sex, stage of the dominant sex, or female gamete stage. Male gametes in the 6°C treated oysters developed while in the high pCO_2 exposure, but there was no change in the 10°C treated oysters. Oysters held in ambient pCO_2 had significantly more advanced gonad compared to before CO_2 exposure regardless of temperature, again influenced strongly by changes in male gamete stage (Table 3.2).

No sampled oysters contained brooded embryos or larvae.

3.4.2 *Larval production*

Adults exposed to 10°C produced more larvae on a daily basis (excluding days where no larvae were released) than those exposed to 6°C in ambient pCO_2-exposed oysters (p=0.040), but not in high pCO_2-exposed oysters (p=0.66) (Figure 3.5, pCO_2:temperature interaction: (F(2,8)=5.1, p=0.037). Total larvae released over the 90-day spawning period tended to differ by treatment, but not significantly (temperature:pCO_2 interaction (F(2,8)=4.0, p=0.063) (Figure 6). Temperature and pCO_2 as single factors did not affect total larvae released or daily averages.

The date of first larval release differed by temperature regardless of pCO_2 (Figure 3.5 & Figure 3.6, F(1,8)=11.9, p=0.0087), and pCO_2 had no effect on timing (not retained in model). Onset was on average 5.2 days earlier in the 10°C treatment. Timing of peak larval release also differed by temperature treatment regardless of pCO_2 (Figure 3.5, F(3,19)=6.7, p=0.018), occurring on average 8.3 days earlier in 10°C oysters. The 10°C treated oysters produced more large pulses of larvae, on average 2 additional days, than 6°C (F(1,8=7.25, p=0.027).

In total, 18.5 million larvae were collected from 767 oysters. Total larvae produced by each treatment was 3.1M, 4.8M, 5.9M, and 4.5M for 6°C-ambient pCO_2, 6°C-high pCO_2, 10°C-ambient pCO_2, and 10°C-high pCO_2, respectively. Based on reports of approximately 215,000 larvae produced per adult *O. lurida* of shell height 35 mm (Hopkins, 1936), the number of oysters that spawned as female in this study was approximately 86, with 14.3, 22.5, 27.6, and 21.0 from the 6°C-ambient pCO_2, 6°C-high pCO_2, 10°C-ambient pCO_2, and 10°C-high pCO_2 treatments, respectively. This estimate is likely low across all treatments, due to the smaller D and O-2 cohorts (mean length in F, D, O-1 and O-2 was 35.7 mm, 29.8 mm, 35.7 mm, and 20.0 mm, respectively), therefore the total number of oysters that spawned as female and released larvae is likely higher than 86.

Table 3.2. Gonad stage and sex comparisons among treatments. Gonad was sampled after temperature treatment but before pCO_2 (6°C Pre and 10°C Pre, n=54), and after pCO_2 treatment (Amb=841±85 µatm, n=39; High= 3045±488 µatm, n=39). Pearson's chi-square statistics are shown with p-adj in parentheses for gonad sex, stage of the dominant sex, male gametes when present, and female gametes when present. Cells with * and in bold indicate significant differences between comparison; blank cells=not tested; % of mature = % of sampled oysters that contained stage 3 male or female gametes, per treatment.

Temperature		**6°C**			**10°C**			**6°C**			**10°C**		
	pCO_2	**Pre**	**Amb**	**High**	**Pre**	**Amb**	**High**	**Pre**	**Amb**	**High**	**Pre**	**Amb**	**High**
6°C	**Pre**	-						-					
	Amb	0.8 (0.93)	-			*Sex Ratio*		***16.5 (0.013)**	-		*Stage of the dominant sex*		
	High	4.6 (0.34)	5.4 (0.29)	-				4.6 (0.48)	9.7 (0.090	-			
10°C	**Pre**	5.9 (0.26)			-			***15.8 (0.017)**			-		
	Amb				6.8 (0.18)	-					***12.7 (0.038)**	-	
	High		5.3 (0.29)		3.8 (0.46)	0.6 (0.94)	-		2.8 (0.78)		5.2 (0.44)	***12.5 (0.038)**	-

6°C	**Pre**	-						-					
	Amb	***24.2 (1.6e^{-3})**	-		*Male gametes*			6.3 (0.18)	-		*Female gametes*		
	High	***15.2 (0.013)**	9.0 (0.071)	-				3.6 (0.47)	4.4 (0.36)	-			
10°C	**Pre**	***31.1 (1.6e^{-3})**			-			2.1 (0.78)			-		
	Amb				***11.2 (0.038)**	-					4.2 (0.26	-	
	High		1.7 (0.78)		0.6 (0.95)	9.5 (0.084)	-		0.8 (0.9)		5.5 (0.17)	0.15 (1.0)	-
% mature		30%	28%	15%	19%	33%	21%	2%	15%	8%	6%	18%	21%

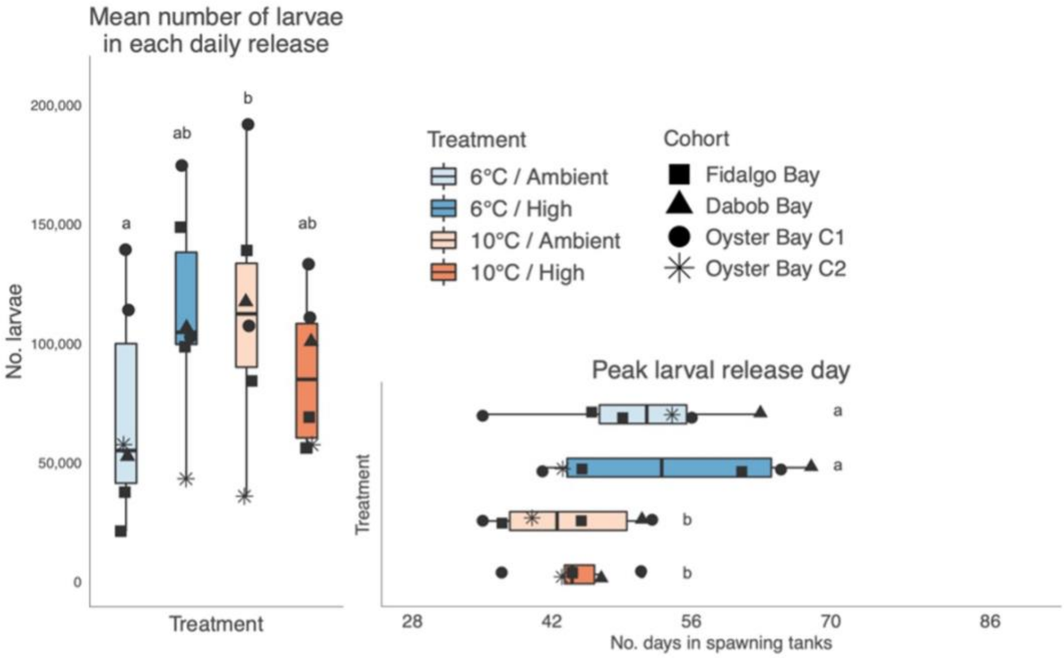

Figure 3.5. Left: average number of larvae collected on a daily basis (excluding days where no larvae were released). Daily pulses of larvae were larger in 10°C than 6°C, but only in oysters exposed to ambient pCO_2. For statistical analysis, data was normalized by number of oysters * average oyster height (cm) (data shown is not normalized). Right: number of spawning days until larval release peaked; peak release occurred on average 8.3 days earlier in 10°C treated oysters. Letters (a, ab, b) indicate differences among treatments. Boxes contain values lying within the interquartile range (IQR), with medians indicated by lines in the middle of boxes. Whiskers extend to the largest value no greater than 1.5*IQR.

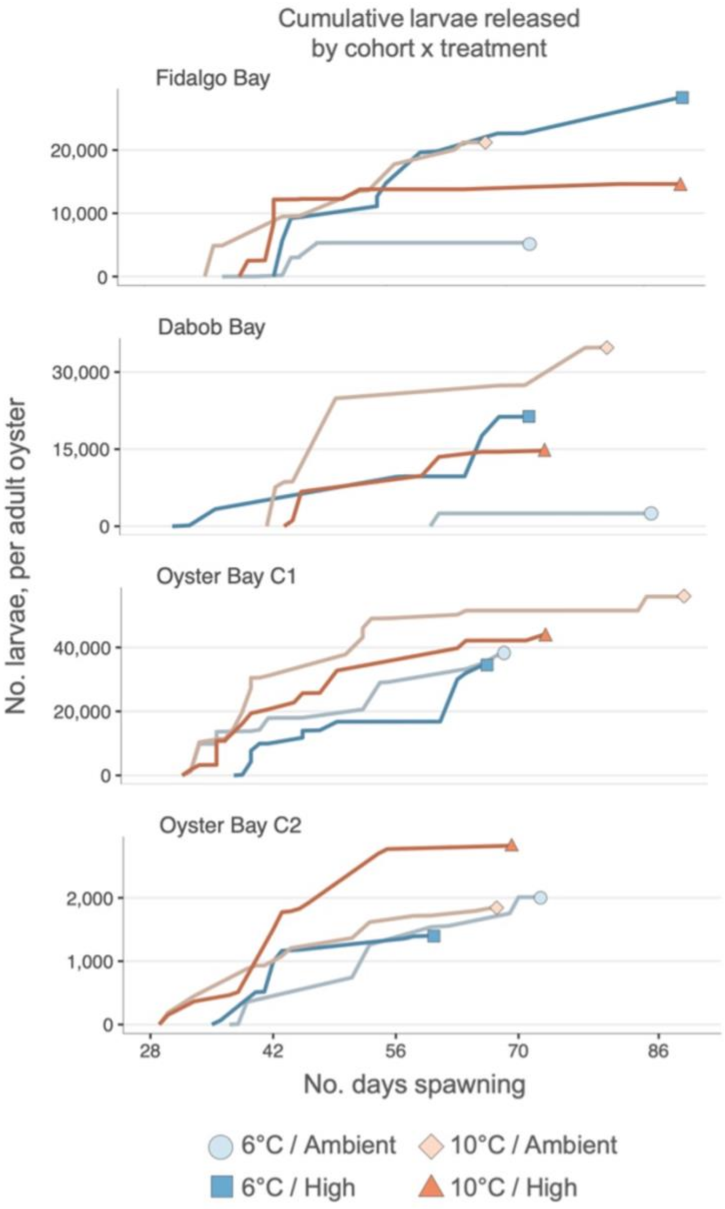

Figure 3.6. Cumulative larvae released over 90 days of continuous volitional spawning under hatchery conditions, normalized by the number of adult oysters. Each of the four panels represent a cohort, and lines are color coded by winter temperature and pCO_2 treatments, where ambient pCO_2 = 841 µatm (7.8 pH), and high pCO_2 = 3045 µatm (7.31). Reproductive conditioning and spawning occurred at 18°C, in ambient pCO_2, and with live algae at a density of 66,000 ± 12,000 cells/mL.

3.4.3 *Offspring survival in a natural setting*

Juvenile survival after three months in the field was on average 15% higher in cohorts from high pCO_2 exposed parents than from ambient pCO_2 parents (44±37%, and 29±27%, respectively, χ^2=10.6, p=0.0011). The influence of parental pCO_2 on survival varied by bay (bay:parental pCO_2 interaction χ^2=15.3, p=$1.6e^{-3}$), and by cohort (cohort:parental pCO_2 interaction χ^2=23.5, p=$3.2e^{-5}$) (Table 3.3).

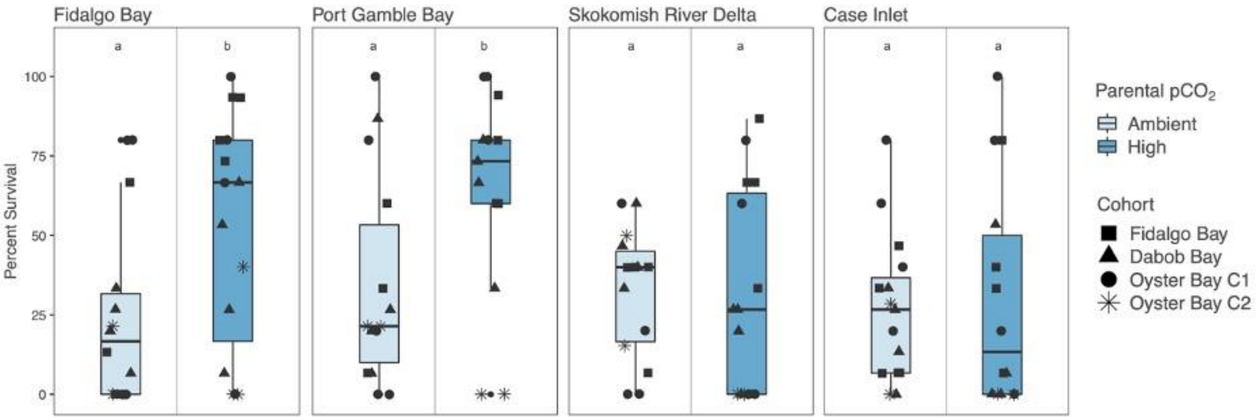

Figure 3.7. Percent survival of juvenile offspring in the field. The four panels each represent survival in one bay (Fidalgo Bay, Port Gamble Bay, Skokomish River Delta, Case Inlet). Within each panel, boxplots are separated by parental pCO_2 exposure (Ambient=841 µatm, High=3045 µatm). Points indicate % survival in each deployment pouch, and symbols indicate cohort (Fidalgo Bay, Dabob Bay, Oyster Bay Cohort 1, and Oyster Bay Cohort 2). Letters (a, b) indicate survival differences among parental pCO_2 exposure within each bay. Boxes contain values lying within the interquartile range (IQR), with median survival indicated by lines in the middle of boxes. Whiskers extend to the largest value no greater than 1.5*IQR.

Survival in offspring from high pCO_2 parents was higher in the Fidalgo Bay and Port Gamble Bay locations (χ^2=17.7, p= $2.6e^{-5}$; χ2=10.0, p=$1.6e^{-3}$, respectively), but this was not the case in Skokomish River Delta or Case Inlet. Survival in the F cohort was 38% higher in oyster from pCO_2 parents than those from ambient pCO_2 parents across all deployment bays (χ^2=28.1, p=$4.6e^{-7}$), and within the Fidalgo Bay location (χ^2=17.6, p-adj=0.0001). Survival in the D and O-

1 cohorts did not differ significantly between parental pCO_2 across all bays (D: χ^2=0.4, p=1, O-1: χ^2=2.5, p=0.44), or within individual bays. More O-2 juveniles with ambient pCO_2 parents survived across all bays (χ^2=9.1, p=0.010), and within the Skokomish River Delta (χ^2=8.9, p=0.011).

Table 3.3. Offspring survival in the field. 1-year old juveniles were deployed for 3 months in four bays in Puget Sound, Washington, in 2 sites per bay. Percent survival ± SD is shown by cohort x bay x parental pCO_2 treatment (Amb=841±85 µatm, High= 3045±488 µatm). Only offspring from 6°C-treated adults were deployed. Significant survival differences were detected between parental pCO_2 treatment within the Fidalgo Bay and Oyster Bay F2 cohorts (*), and across all cohorts (+).

Cohort →	Fidalgo Bay (F)		Dabob Bay (D)		Oyster Bay F1 (O-1)		Oyster Bay F2 (O-2)		All cohorts	
pCO_2 → Bay ↓	Amb	High	Amb	High	Amb	High	Amb	High	Amb	High
Fidalgo	***20 ±32%**	***85 ±10%**	22 ±12%	38 ±25%	40 ±46%	62 ±43%	11 ±15%	13 ±23%	$^{+}$**25 ±30%**	$^{+}$**51 ±37%**
Port Gamble	***33 ±27%**	***74 ±17%**	35± 35%	63 ±21%	40 ±47%	93 ±12%	21 ±0%	0%	$^{+}$**34 ±33%**	$^{+}$**64 ±34%**
Skokomish	32 ±17%	51 ±23%	45 ±11%	18 ±13%	20 ±28%	35 ±41%	***33 ±24%**	***0%**	32 ±21%	31 ±33%
Case Inlet	20 ±19%	40 ±30%	18 ±15%	15 ±26%	50 ±26%	50 ±48%	14 ±20%	0%	27 ±23%	30 ±35%
All Bays	***27 ±22%**	***62 ±29%**	30 ±22%	34 ±28%	38 ±37%	58 ±41%	***20 ±16%**	***4 ±13%**	$^{+}$**29 ±27%**	$^{+}$**44 ±37%**

Without considering parental pCO_2, more oysters survived in Port Gamble Bay (mean 49±36%) and Fidalgo Bay (39±36%) than in Case Inlet (mean 29±29%, p=0.012 & p=0.037, respectively) (bay factor, χ^2=18.5, p=3.4e^{-4}). Survival at Skokomish River Delta did not differ significantly from other locations (32±27%). No interaction between cohort and bay was detected (χ^2=9.8, p=0.37) (Figure 3.7, Table 3.3).

Shell length was not affected by bay, cohort or parental pCO_2. The mass per oyster (compared to before deployment) differed by cohort (F(3,76)=15.9, p=4.0e^{-8}), due to Dabob Bay cohort growing less than the other three cohorts (Δ g/oyster: D=0.5, F=1.2, O-1=1.6, & O-2=1.0). Mass change also differed by bay (F(3,76)=4.8, p=3.9e^{-3}) due to less growth in oysters placed at Fidalgo Bay than in Port Gamble Bay and Case Inlet (Δ g/oyster: FB=0.7, PGB=1.0, CI=1.1, SK=0.8).

3.5 DISCUSSION

Ocean acidification and ocean warming potentially threaten marine organisms, particularly ectothermic calcifiers (Hoffman *et al.* 2010). An organism's genotype, complete environmental history, and the timing and magnitude of environmental perturbations may all determine its fitness in future ocean conditions. To begin teasing apart these complex factors in the Olympia oyster, this study examined four adult cohorts with distinct genetic structure but known, shared histories. Elevated winter temperature resulted in increased gonad development, which corresponded with earlier and more frequent larval release (on average 5.2 days earlier, 2 additional days). High pCO_2 exposure negatively influenced gonad maturation state, but did not affect subsequent fecundity. Offspring from parents exposed to elevated pCO_2 had higher overall survival upon deployment. Differences in juvenile survival among bays and cohorts indicate that carryover effects are dependent upon the environment and genotype, and reinforce the importance of using multiple sources of test organisms in stress-response studies.

3.5.1 *Reproduction*

We expected elevated winter temperature to reduce fecundity, based on predictions that changes to reproductive quiescence and metabolism would be deleterious to spring reproduction. Counter to this prediction, warm winter temperature positively affected larval production. Oysters in elevated temperature contained more developed male gametes after treatment, and subsequently began releasing larvae earlier and produced more larvae per day compared to cold-treated oysters. We find no evidence that cold winters are critical for spring reproduction, but rather elevated winter temperature may elongate the *O. lurida* spawning season. In comparison, a 29-year dataset of *M. balthica* reproduction showed that as winter temperature increased, spring spawning began earlier and fecundity declined (Philippart *et al.*, 2003). However, the present study was conducted in a hatchery setting, with ample phytoplankton, and did result in a temperature shift during spawning. In the wild numerous additional abiotic and biotic factors will contribute to *O. lurida* fitness, and warmer winters may result in earlier and longer reproductive seasons only if nutritional requirements are met. Whether larvae released earlier in the spring can survive to recruitment will greatly depend on many factors including food availability and predation. Those modeling larval recruitment (*e.g.* Kimbro, White & Grosholz, 2019; Wasson *et al.*, 2016) should consider including winter temperature as a factor influencing spatiotemporal recruitment patterns.

We predicted that high pCO_2 exposure would redirect energy away from storage to maintenance processes, resulting in delayed gametogenesis and poor fecundity in the spring. After exposure to 3045 µatm pCO_2 (pH 7.31), fewer oysters contained ripe or advanced male gonad tissue than in ambient pCO_2, signaling reduced spermatogenic activity. Female gonad, sex ratios, and subsequent fecundity were not affected by sole exposure to high pCO_2. Similar impacts on gametogenesis during exposure were observed in the Sydney rock (*S. glomerata*) and Eastern (*C. virginica*) oysters, but with varying pCO_2 thresholds. Parker *et al.* (2018) found *S. glomerata* gametogenesis to slow in 856 µatm (pH 7.91), and Boulais *et al.* (2017) found normal rates at 2260 µatm (pH 7.5), delay at 5584 µatm (pH 7.1), and full inhibition at 18480 µatm (pH 6.9) in *C. virginica*. Together, these studies indicate that high pCO_2 slows the rate of gametogenesis, but the level at which pCO_2 affects gametogenesis appears species-specific, and likely reflective of variable physiological mechanisms and reproductive strategies.

The combined effects of sequential elevated temperature and pCO_2 treatments did not act synergistically to delay gonad development, but instead resulted in oysters with gonad stage and fecundity no different from the untreated oysters. Similarly, combined simultaneous temperature and high pCO_2 exposures did not affect *S. glomerata* fecundity (Parker *et al.*, 2018). We did detect a pCO_2 dependent effect of temperature on the average number of larvae released per day. Oysters that had previously been exposed to 10°C produced more larvae than 6°C, but only after ambient pCO_2 exposure, which may reflect a general reproductive arrest that occurs when exposed to high pCO_2. Despite experimental differences (*e.g.* sequential vs. simultaneous exposures) which can influence outcomes (Bible *et al.* 2017), both Parker *et al.* (2018) and the present study indicate that high pCO_2 slows gametogenesis, elevated temperature accelerates it, and these two environmental drivers act antagonistically on gonad development if occurring in the same reproductive season. An important factor not included in either study is ecologically relevant variability. Temperature and pCO_2 oscillations, driven by tides and diurnal photosynthesis, could offer daily refuge or expose oysters to dynamic changes, altering how combined stressors interact (Cheng *et al.* 2015).

In contrast to prior studies, temperature and pCO_2 did not impact *O. lurida* sex ratios, whereas in high pCO_2 *C. virginica* skewed male (Boulais *et al.*, 2017), and *S. glomerata* skewed female (Parker *et al.*, 2018). This observation may be explained by very low incidence of total reproductive inactivity in our *O. lurida* cohorts — only four out of the 108 oysters that were sampled prior to pCO_2 treatment contained empty follicles — and thus sex ratios may be different if pCO_2 exposure occurs earlier in life during initial sex differentiation. Furthermore, high pCO_2 exposure only occurred in winter, prior to spawning. If high pCO_2 persists during oocyte maturation and spawning, *O. lurida* fecundity may be reduced similar to *C. virginica* and *S. glomerata*. Future research should examine *O. lurida* sexual development during the initial switch from male to female, which can occur the first winter after settlement (Moore *et al.*, 2016), and across a range of pCO_2 to determine conditions in which gametogenesis and sex determination are affected.

3.5.2 *Offspring*

Abiotic parental stressors can be beneficial, neutral, or detrimental to offspring viability (Donelson *et al.*, 2018). We explored carryover effects of adult exposure to winter pCO_2 on offspring by testing survival in the field. Offspring with high pCO_2 parental histories performed better in two of four locations, Fidalgo Bay and Port Gamble Bay. Carryover effects of parental high pCO_2 exposure may therefore be neutral, or beneficial, to offspring depending on the environmental conditions. Port Gamble Bay and Fidalgo Bay are more influenced by oceanic waters, which could explain cooler observed temperatures. These locations are also typically less stratified than the Skokomish River Delta and Case Inlet. In Port Gamble Bay, where pCO_2 parental history most significantly correlated with offspring survival across cohorts, mean pH was considerably lower than the other deployment locations (-0.17 pH units), and mean salinity was higher (+3.8 PSU). Given the experimental design we are able to clearly demonstrate that manifestation of carryover effects in Olympia oysters is dependent on environmental conditions. Specifically, there is a greater likelihood of beneficial carryover effects when parents are exposed to stressful conditions. Overall, carryover effects of parental pCO_2 treatment were positive, however negative effects were observed in the O-2 cohort. This discrepancy could relate to unique O-2 juvenile characteristics, as they were bred from siblings, and were 3rd-generation hatchery produced. The complex interactions among parental exposure, bay, and cohort indicate that offspring viability is influenced by ancestral environment history, environmental conditions, and genotype.

Our results contrast with a similar study that exposed *C. gigas* oysters to high pCO_2 during the winter, and found fewer hatched larvae 18 hours post-fertilization from exposed females, with no discernable paternal effect (Venkataraman, Spencer & Roberts, 2019). Hatch rate was not directly measured in this study due to the *O. lurida* brooding behavior; however, no difference in daily and total larvae released suggest that hatch rate was unaffected by pCO_2. The different responses seen in Venkataraman, Spencer & Roberts (2019) and the present study may reflect variability among species and spawning method. *C. gigas* gametes were collected artificially by stripping gonad, whereas *O. lurida* late-stage veliger larvae were collected upon release from the brood chamber. For instance, volitionally-spawned gamete quality and fertilization rates could vary between the natural versus artificial settings to influence larval viability. Larval brooding may also be a mechanism by which sensitive larvae are acclimatized to stressors, as the *O. lurida* brood chamber pH and dissolved oxygen can be significantly lower than the environment (Gray *et al.*, *in press*).

Beneficial parental carryover may also be linked to the male-specific gonad effects, and the conditions in which the adult oysters were held. During high pCO_2 exposure, oocyte stage and prevalence did not change, which indicates that oogenesis did not occur. Negative intergenerational carryover effects are commonly linked to variation in oocyte quality, which can be affected by the maternal environment during oogenesis (Utting & Millican, 1997). In the Chilean flat oyster (*Ostrea chilensis*), for instance, egg size and lipid content positively correlate with juvenile growth and survival (Wilson, Chaparro, & Thompson, 1996). If high pCO_2 exposure were to coincide with oocyte proliferation and growth, *O. lurida* egg quality and larval viability could be compromised. In contrast, male gonad stage advanced significantly during pCO_2 exposure. Intergenerational and transgenerational carryover effects are increasingly linked to the paternal environment in other taxa, such as inheritance of epigenetic changes to the male germ line (Rodgers, Morgan, Bronson, Revello, & Bale, 2013; Anway, 2005; Soubry, Hoyo, Jirtle, & Murphy, 2014). Positive carryover effects of environmental stressors observed in this and other marine invertebrate taxa may be due to paternal epigenetic effects, but this link has not yet been observed.

3.6 CONCLUSION

This study clearly demonstrates that exposure to elevated winter temperature and altered carbonate chemistry impacts reproduction and offspring viability in the Olympia oyster. Furthermore, we report the first observations of intergenerational plasticity in an *Ostrea* species, that is dependent on offspring environmental conditions and population. The observed context-dependent carryover effects could have a substantial impact on species resilience. Combined with previous reports of resilience to environmental stressors (Waldbusser *et al* 2016; Cheng *et al.* 2017) and intraspecific variability (Bible, Evans & Sanford, 2019; Maynard, Bible, Pespeni, Sanford, & Evans, 2018; Silliman, Bowyer, & Roberts, 2018; Heare, Blake, Davis, Vadopalas, & Roberts, 2017), the Olympia oyster may be more capable than other marine bivalve species to withstand and adapt to unprecedented ocean change. Furthermore, conserving and restoring *O. lurida* in a variety of settings — including hypoxic, warmer, and less alkaline areas — could increase the probability that future populations are equipped for challenging conditions through selection or intergenerational carryover.

As temperatures rise and ocean acidification progresses, there may be profound and unexpected seasonal changes across marine taxa. Accurate predictions will need to consider parental carryover effects, as they can impart neutral, beneficial, or detrimental characteristics to offspring, which depend on complex interactions among parental exposure timing, reproductive strategies, species plasticity, and standing genetic structure. With these considerations, future biological response studies need to be aware of three possible factors influencing results: 1) source population; 2) environmental history (within-lifetime carryover effects); and 3) ancestral environmental history (inter- and transgenerational carryover effects). Controlling for, or at minimum recognizing and recording these factors, will provide important context for those predicting ecosystem response to environmental change.

3.7 ACKNOWLEDGEMENTS

Our gratitude to the following people who assisted with this project: Grace Crandall, Kaitlyn Mitchell, Olivia Smith, Megan Hintz, Rhonda Elliott, Lindsay Alma, Duncan Greeley, Beyer and Jackson Roberts, and Ian Davidson helped with oyster husbandry and sampling; Alice Helker advised on husbandry and larval rearing system engineering; Emily Kunselman helped manage the field deployment; Sam White and Hollie Putnam contributed to the carbonate chemistry analysis; Katherine Silliman and Jake Heare produced (and saved) the experimental oysters; the NOAA Manchester Research Center and Puget Sound Restoration Fund provided facilities and materials; committee members Jackie Padilla-Gamiño and Rick Goetz advised and supported this extended project. Thank you to David Kimbro and an anonymous reviewer for constructive comments on the manuscript.

This work was supported in part by the National Science Foundation Graduate Research Fellowship Program, the National Shellfisheries Association Melbourne R. Carriker Student Research Grant, Washington State Department of Natural Resources, and a grant from Washington Sea Grant, University of Washington, pursuant to the National Oceanic and Atmospheric

Administration Award No. NA14OAR4170078; Project R/SFA-8. The views expressed herein are those of the author(s) and do not necessarily reflect the views of any funding agency.

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Chapter 4. POPULATION-SPECIFIC EFFECTS OF OCEAN ACIDIFICATION IN THE OLYMPIA OYSTER

4.1 ABSTRACT

Populations of marine species that respond differently to ocean acidification offer natural reservoirs of biodiversity that can be leveraged for conservation efforts and to sustain marine food systems. The molecular and physiological traits associated with tolerance to acidification must first be identified. This study leveraged oysters from three phenotypically-distinct populations of the Olympia oyster, *Ostrea lurida*, but that were bred and reared in common conditions for four years. We assessed their growth, reproductive development, and transcriptional response to acidification within and across generations. Responses reveal energetic trade-offs that reflect unique physiotypes previously observed among populations. The population with the slowest growth but high survival rates, oysters from Dabob Bay, mounted the largest transcriptional response to acidification without effects to growth and reproduction. A moderate response was observed in the population with fastest growth rate but lowest fecundity (Fidalgo Bay). Oyster Bay, the population with highest fecundity but lowest survival rates, did not respond at the transcript level. Oyster Bay was also the only population for which acidification negatively affected growth and reproductive development. While exposure to acidification did not affect gene expression in the next generation's larval stage, it did result in larger larvae in the Oyster Bay population, which could partially alleviate negative effects of acidification in the wild for that population. Given the distinct transcriptional response of the Dabob Bay population to acidification and its high survival rates in previous studies, we then identified genes that were uniquely expressed in Dabob Bay oysters compared to the other populations. Genes involved in

antibacterial and antiviral processes, metabolism, growth, and reproduction were uniquely expressed in Dabob Bay, and many similar functions were identified in both adults and larvae, which provides insight into the mechanisms behind a stress-tolerant oyster population. The population-specific physiotypes and responses to acidification illustrate the diversity of physiological strategies in *O. lurida* that balance the energetic demands of growth, reproduction, cellular maintenance, and offspring viability. Taken together this study reveals that there are distinct physiotypes among marine invertebrate populations on small geographic scales with implications for species resilience to acidification and other environmental stressors.

4.2 INTRODUCTION

Following observations of shifting ocean conditions (IPCC, 2019) an enormous scientific effort has explored the response of marine species to ocean acidification (Riebesell & Gattuso, 2014). Empirical data has established that many species are vulnerable to ocean conditions projected for this century, particularly calcifying invertebrates, affecting a range of physiological processes over the lifetime of an organism, including development, recruitment, growth, reproduction, and survival (Gazeau et al., 2013; Kroeker et al., 2013; Lemasson et al., 2017; Melzner et al., 2019). However, these studies also indicate that biological responses are quite variable, related to an organism's genetic and environmental ancestries (Eirin-Lopez & Putnam, 2019; He & Silliman, 2019; Przeslawski et al., 2015; Sunday et al., 2014). Some species are more tolerant to the effects of acidification than others (Branch et al., 2013; Figuerola et al., 2021), as are some populations within species (Bitter et al. 2019; Swezey et al. 2020; Kelly et al. 2013; Vargas et al. 2017). There is also evidence of intergenerational (spanning one generation) and transgenerational (spanning 2+ generations) plasticity, which may buffer future populations against challenging conditions (Salinas et al., 2013; Zhao et al., 2020). Ultimately, there will be a spectrum of responses to shifting ocean chemistry, dependent on species' capacity to mitigate, acclimatize, and adapt to shifting conditions. For effective conservation and management of marine calcifiers, it is critical to identify the genotypes, physiotypes, and molecular mechanisms that impart tolerance to acidification, as well as quantify the range of responses within taxa.

Impacts of acidification to oysters were among the earliest observations of negative biological effects. Now, in an effort to build resilient commercial and wild stocks, breeding programs and researchers are increasingly seeking to identify oyster species and populations that are tolerant to acidification and other stressors. One such group of oysters appears to be species from the genus *Ostrea*, which includes the Olympia (*O. lurida*), European flat (*O. edulis*), Chilean flat (*O. chilensis*), and Australian flat oyster (*O. angasi*). Multiple studies have reported little to no effects of acidification in *Ostrea* spp. at the adult (Lemasson et al., 2018; Lemasson et al., 2019; Lemasson & Knights, 2021), juvenile (Navarro et al., 2020), and larval stages (Cole et al., 2016; Pereira et al., 2019; Waldbusser et al., 2016). In one study *O. edulis* larval growth and survival responded positively to acidification exposure (Prado et al., 2016). Unique *Ostrea* spp. life history traits, in particular brooding of veliger larvae in the maternal pallial cavity, may contribute to the species' relative tolerance to acidification, as pH can quickly decrease to levels as low as 6.96 during periods of valve closures (Chaparro et al., 2009; Gray et al., 2019). There are, however, other studies that have observed negative effects of acidification in *Ostrea*, such as decreased larval growth in *O. lurida* that persist to the juvenile stage (Hettinger et al., 2012, 2013; Sanford et al., 2014). Contrasting effects could be explained by population-specific responses, which are commonly observed in marine invertebrate taxa (Barber et al., 1991; Macdonald & Thompson, 1988). Indeed, populations of *O. lurida* from northern California have diverged salinity tolerances, which is facilitated by distinct physiological responses at the cellular level (cell death regulation, mantle ciliary activity) (Maynard et al., 2018). Molecular strategies of *Ostrea* spp. need closer examination to understand the functions that enable tolerance to acidified conditions in some, but not all, populations (Melzner et al., 2009).

Previous studies have identified a suite of molecular functions in marine calcifiers that are sensitive to ocean acidification, which has been thoroughly reviewed by Melzner et al. (2019) and Strader et al. (2020). Interestingly, the directionality and magnitude of molecular responses can differ by species and/or populations within the same species. Opposing changes in antioxidants and molecular chaperones have been reported for acidification-tolerant (upregulated) and wild-type (downregulated) Sydney rock oysters in acidified conditions, which then reverse in subsequent generations (Goncalves et al., 2016, 2017). Changes to transcripts associated with energy production provide evidence for both metabolic depression and increased metabolic demand in response to acidification (Strader et al., 2020). In some cases the degree of transcriptional plasticity varies in response to acidification (Kenkel & Matz, 2017). For instance, upon exposure to acidification, transcripts for genes related to ATP production increased in two populations of urchins, but the magnitude of increase was more pronounced in the population that experiences more frequent periods of low pH compared to those from more stable pH environments (Evans et al., 2017). These studies highlight the variety of molecular responses to acidification that can occur among populations of the same species. It is vital to identify the fundamental molecular signatures of acidification-tolerant oysters to inform breeding and conservation efforts.

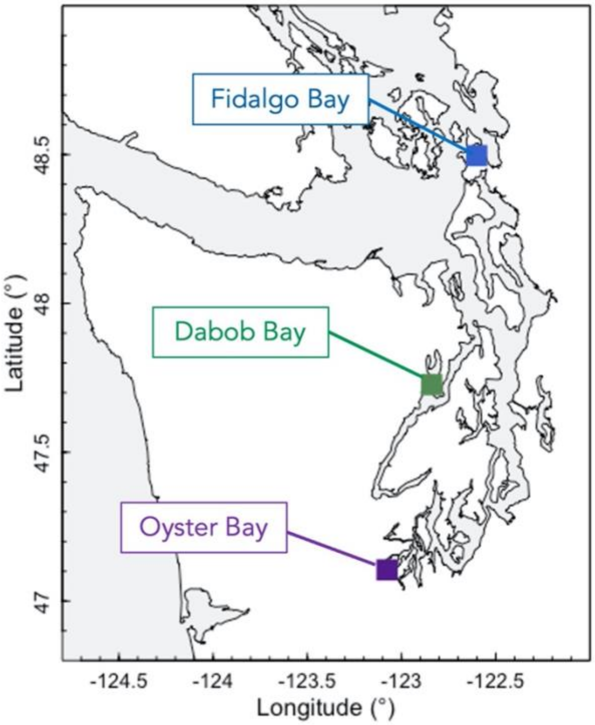

Figure 4.1. Location of the focal *O. lurida* populations in the greater Puget Sound estuary, Washington, USA

In the present study we examine effects of acidification on multiple populations of the Olympia oyster, *Ostrea lurida*. *O. lurida* is native to the North American Pacific Coast, inhabiting dynamic estuarine environments that are influenced by coastal upwelling and ocean acidification (Feely et al., 2010; McGraw, 2009; Reum et al., 2014). We build upon previous studies that have identified unique fitness traits in three populations from disparate regions of greater Puget Sound in Washington State (Figure 4.1, Table 4.1) (Heare et al., 2017; Heare et al., 2018; Silliman et al., 2018; Spencer et al., 2020; White et al., 2017). Oysters derived from Oyster Bay in the South Puget Sound are highly fecund, and compared to other populations require fewer degree-days to begin reproducing (Heare et al., 2017; Silliman et al., 2018; Spencer et al., 2020). Oysters from Dabob Bay in the Hood Canal basin consistently display slower growth than other populations but have higher survival rates in field testing and during hatchery rearing (Heare et al., 2017; Spencer et al., 2020, Ryan Crim, *pers. comm.*). Those from Fidalgo Bay, a small basin in the northern reaches of greater Puget Sound, grow faster and are less fecund or have delayed reproduction (Heare et al., 2017; Silliman et al., 2018; Spencer et al., 2020).

Table 4.1. Traits that are characteristic of the focal *O. lurida* populations from the greater Puget Sound region, summarized from previous studies of the same populations: (1) Heare et al. 2017 (2) Heare et al. 2018 (3) Silliman et al. 2018 (4) Spencer et al. 2020 (5) Ryan Crim *pers. comm*. Additionally, genetic differentiation among populations was characterized by White et al. (2017).

Location of *O. lurida* population	**Growth rate, size at maturity**	**Reproductive output, timing**	**Performance in field & hatchery trials**	**Immediate transcriptional response to stress**
Fidalgo Bay	Fast growth, large at maturity[1,3]	Low/Moderate fecundity[1,3,4]	Moderate survival[1,4]	No response[2]
Dabob Bay	Slow growth, small at maturity[1,3,4]	Moderate fecundity[1,3,4]	High survival in field, high larval survival in hatchery[1,4,5]	No response[2]
Oyster Bay	Moderate growth, medium/large at maturity[1,3,4]	Very high fecundity, early to reproduce[1,3,4]	Low/moderate survival in field[1,4]	Altered expression of targeted genes 1-hr following heat & mechanical stress[2]

Here, we use high-throughput sequencing to perform the first transcriptional characterization of an *Ostrea* species to ocean acidification. Expression analyses are paired with biometric data (gonad development, growth) to capture system-wide changes in energy allocation due to acidification exposure (Sokolova et al., 2012). By examining oysters from the aforementioned Olympia oyster populations (Dabob Bay, Fidalgo Bay, and Oyster Bay) we leverage a comprehensive understanding of the traits characteristic of each population (Table 4.1) (Heare et al., 2017; Heare et al., 2018; Silliman et al., 2018; Spencer et al., 2020), and by using individuals that were bred and grown in common conditions we control for within-generation carryover effects (Hettinger et al., 2012, 2013). Given the possible influence of intergenerational exposures on an organism's physiology (Goncalves et al., 2016, 2017), we also extend the analysis to include a second generation (larval offspring) to examine the potential impacts of parental exposures and population-of-origin on basal functions (larval size, gene expression). By fully describing its molecular and physiological response to acidification across distinct populations we show that *O. lurida* is a good candidate for aquaculture investment & conservation.

4.3 METHODS

4.3.1 *Adult oyster source and history*

Experimental oysters were bred in 2013 as described in Heare et al. (2017) in common conditions from three populations of wild broodstock, which were harvested from Dabob Bay in Hood Canal, Oyster Bay in South Puget Sound, and Fidalgo Bay in North Puget Sound. Oysters were then maintained in common conditions in a pearl net adjacent to the Kenneth K. Chew Center for Shellfish Research and Restoration in central Puget Sound for approximately 3.5 years. Upon entering experimental conditions, shell height for each population was on average 29.8 ± 4.6mm, 35.7 ± 4.5mm, and 35.7 ± 4.4mm for Dabob Bay, Oyster Bay, and Fidalgo Bay, respectively. Experimental oysters were therefore mature adults that had been produced and reared in common conditions, but had distinct genetic heritage.

4.3.2 *Adult treatments and larval collection*

Beginning February 16, 2017 adults were exposed to two pCO_2 treatments for 52 days (control pCO_2: 841 ± 85 µatm, pH 7.82 ± 0.02; high pCO_2: 3,045 ± 488 µatm, pH 7.31 ± 0.02), as described in Spencer et al. (2020) and Venkataraman et al. (2019) (Appendix A). Following experimental exposures, adults from all populations and pCO_2 treatments were returned to common conditions, reproductively conditioned, and spawned to produce offspring. Because *O. lurida* are viviparous spermcasters and brood larvae to the veliger stage, larvae were captured upon maternal liberation, which commenced on May 14th and persisted for 57 days. Details of the experimental and spawning conditions are described in (Spencer et al., 2020).

Prior to the pCO_2 treatments adults were also exposed to two temperature regimes for 60 days. These represent what would be considered normal or ambient temperature (6.1 ± 0.2°C) and a temperature that would be considered elevated at the experimental site (10.2 ± 0.5°C) (see Spencer et al. 2020). Given that temperature did not interact with pCO_2 to affect the focal characteristics in adults or larvae, only effects of parental pCO_2 exposure were further examined for this study.

4.3.3 *Tissue sampling*

Adult ctenidia tissue was collected immediately upon terminating pCO_2 treatments from Dabob Bay, Fidalgo Bay, and Oyster Bay. Nine oysters from each population and pCO_2 treatment were sacrificed and ctenidia tissue was collected and flash-frozen at approximately -116°C using a solution of ethanol and dry-ice then preserved at -80°C. Gonad tissue was collected from the same individuals, preserved in histology cassettes using the PAXgene Tissue FIX System (PreAnalytiX, Hombrechtikon, Switzerland), then processed for reproductive development analysis by Diagnostic Pathology Medical Group (Sacramento, California, USA).

Larval offspring were sampled at the veliger stage upon maternal liberation. For each group of larvae that was released, a portion were reared as described in Spencer et al. (2020) and the remaining were preserved by rinsing with fresh water into microcentrifuge tubes, removing water, then placing directly into -80°C freezer. Given the variable reproductive rates, the number of larval groups preserved for downstream analysis from control pCO_2 and high pCO_2 varied, and was 7 and 10 for Fidalgo Bay, 5 and 7 for Dabob Bay, and 17 and 13 for Oyster Bay, respectively. Each larval sample contained thousands of larvae (on average 170k), and consisted of siblings that were released from the same female on the same day. However, given that adults were continuously spawning and releasing larvae, it is possible that some larval samples contained a mix of multiple families from the same population and treatment.

4.3.4 *Adult growth and reproductive development*

Adult oysters were measured for shell height before and after pCO_2 treatments (n=9 per population x pCO_2 treatment) using digital calipers (mm), defined as the maximum distance from the umbo along the dorsal/ventral axis. Shell height after pCO_2 treatments were compared among population and pCO_2 treatments using 2-way ANOVA. Since we were most interested in assessing effects of high pCO_2 exposure on growth rate for each population, population-specific differences in size between high-, control- and pre-pCO_2 treatments were tested using 1-way ANOVA, and Tukey honest significant difference tests were performed to assess pairwise comparisons.

Gonad samples (n=9 per population x pCO_2 treatment) collected before and after pCO_2 treatments were assigned sex and stage as described in Spencer et al, (2020). For each population, contingency tables were constructed for gonad sex, developmental stage of sperm, and developmental stage of eggs, and differences between pCO_2 treatments were compared using Chi-Square or Fisher Exact Tests (depending on the number of males or females present within a population) and P-values were estimated using Monte-Carlo simulations with 1,000 permutations.

4.3.5 *Larval offspring size*

Newly liberated veliger larvae were measured using a Nikon eclipse Ni microscope and the NIS-Elements BR imaging and measuring software (version 4.60). Mean shell height (distance from hinge to margin, perpendicular to hinge), and mean shell width (longest distance parallel to hinge) were estimated from at minimum 48 larvae per collection from each tank. Mean shell height and width were compared among population and parental treatments using 2-Way ANOVA and Type II Sums of Squares with the car package (Fox & Weisberg, 2018). Then, since we were most interested in assessing population-specific responses, effects of parental pCO_2 on larval size were assessed for each population using 1-Way ANOVA with Type II Sums of Squares.

4.3.6 *Library construction and sequencing*

RNA was isolated from 52 frozen adult ctenidia samples (n=8 or 9 per population x pCO_2 treatment) and 61 pooled whole-body larvae (5-13 per population x parental pCO_2 treatment) (Table 4.2). Each sample was homogenized in liquid nitrogen with a stone mortar and pestle and isolated following the RNAzol® RT protocol for Total RNA Isolation (Molecular Research Center, Inc., Cincinnati, OH). RNA pellets were resuspended in DEPC-treated water, residual DNA contamination was removed using the Turbo DNase kit (Life Technologies, Carlsbad, CA), then RNA was quantified using the Qubit RNA assay with Qubit 3.0 Fluorometer (2.0ul of each sample for quantification, Life Technologies, Carlsbad, CA), and quality was assessed for a subset of RNA isolates using the Bioanalyzer RNA 6000 Pico Chip assay (Agilent Technologies, Santa Clara, CA).

Library preparation was performed following the QuantSeq 3’ mRNA-Seq protocol (v.015UG009V0251, Lexogen, Vienna, Austria), also known as TagSeq, which generates cDNA from the 3’ end of mRNA strands and only generates one fragment per mRNA transcript, allowing for accurate gene expression data at a reduced cost (Meyer et al., 2011). Briefly, Total RNA (350 ng) was used to generate single-stranded DNA using reverse transcription (oligoT priming), which binds to the poly(A) tail and includes the read 1 adapter; RNA was removed, then the second DNA strand + Illumina adapter was synthesized by random priming; the double stranded cDNA + adapters were purified using magnetic beads, then an aliquot (1uL) of each library was amplified using qPCR to determine the optimal number of cycles needed for Endpoint PCR, which ranged from 14 to 17; the libraries were then amplified and indexed with unique barcodes, then re-purified. Prior to sequencing, all libraries were quantified using Qubit High Sensitivity DNA kits (Life Technologies, Carlsbad, CA), and the quality (e.g. fragment length) was assessed for a subset using a Bioanalyzer High Sensitivity DNA chip kit (Agilent Technologies, Santa Clara, CA). Single-end sequencing with 100-bp read length was conducted on a NovaSeq platform (Illumina, San Diego, CA) by the University of Washington's Northwest Genomics Center, who also demultiplexed the raw sequencing data.

Table 4.2. The number of QuantSeq libraries examined per population and parental pCO_2 treatment for adult and larval tissues.

	Adult ctenidia *individuals*		Larval whole-body *pooled by maternal liberation group*	
	Control pCO_2	High pCO_2	Control pCO_2	High pCO_2
Fidalgo Bay	9	8	5	10
Dabob Bay	9	8	5	5
Oyster Bay	9	9	13	11

4.3.7 *Expression data processing & analysis*

Raw reads were inspected for quality using FastQC (Andrews 2010) and MultiQC (Ewels et al., 2016), trimmed to remove Illumnina adapters, poly(A)- and poly(G)-tails, and quality filtered (≥20 read length, >15 quality score) using the Cutadapt toolkit v2.10 (Martin, 2011), then aligned to the draft *O. lurida* genome v081 (GenBank accession GCA_903981925.1) using Bowtie2 v2.4.1 (Langmead & Salzberg, 2012) and local alignment with the pre-set --sensitive-local option. Average alignment rate was 75.7±4.4% for adult libraries and 70.7±8.2% for larval libraries. From the resulting alignment files (.bam), the number of reads that uniquely mapped to each *O. lurida* gene was determined using featureCounts v2.0.0 (Liao et al., 2014) and a gene annotation file that was adjusted to extend the 3' end by 2kb, as libraries were generated at the 3' end of the original mRNA transcripts.

Differential gene expression analysis was performed using DESeq2 (Love et al., 2014) in R v4.0.4 using RStudio interface v1.3.1093 (R Core Team, 2021; RStudio Team, 2020). Read counts were first filtered to remove genes with fewer than 10 total counts across all samples (1,229 and 7,725 genes were discarded for adult ctenidia and larval pools, respectively). For adults the number of reads retained for analysis ranged from 1.18M to 3.30M per sample and averaged 1.99M ±SD442K, and for larvae total reads ranged from 653K to 2.04M and averaged 1.32M ±SD332K. The total number of genes and per-sample average retained for analysis was 30,981 and 28,110±607 for adult ctenidia, and 24,485 and 23,114 ±SD1,259 for larvae. Gene counts were assessed for differential gene expression for adult ctenidia between pCO_2 exposure within populations, and for larvae between parental pCO_2 exposures within and across populations. DESeq2 uses raw count data to generate generalized linear models and internally corrects for library size, therefore counts were not normalized prior to differential expression analysis.

Given the high performance of the Dabob Bay population in previous lab and field trials (Heare et al. 2017; Spencer et al. 2020; Ryan Crim *pers. comm.*), we examined genes that were constitutively expressed at unique levels in Dabob Bay. Pairwise differences in expression among Dabob Bay and the other two populations were identified in DESeq2 using transcriptomes from adults held in control conditions (n=9 libraries per population), and separately using transcriptomes from all larval samples (Table 4.2). For each life stage (adult and larvae), genes from the two pairwise comparisons (Dabob vs Fidalgo, Dabob vs. Oyster) were clustered into two gene sets: 1) genes that were more abundant, and 2) genes that were less abundant than the other two populations. Overlapping genes uniquely abundant in both adults and larvae from Dabob Bay were then interrogated for relative expression patterns (i.e. higher or lower than the other populations).

Differentially expressed genes were merged with the *O. lurida* genome to generate lists of Uniprot IDs from annotated genes. Enriched biological processes of differentially expressed genes sets were determined using the Gene-Enrichment and Functional Annotation Tool from DAVID v6.8, and were defined as those with modified Fisher Exact P-Values (EASE Scores) <0.1.

4.4 RESULTS

4.4.1 *Effects of acidification on adult growth, sex ratio*

Adult oyster growth rate was reduced by acidification in the Oyster Bay population only: shell size did not differ after the high pCO_2 exposure, whereas those that were exposed to control pCO_2 were larger after treatment (Figure 4.2, Table 4.3). The Dabob Bay and Fidalgo Bay populations did not differ in size after either pCO_2 treatment compared to before (Table 4.3). Shell height differed by population before (F(2,72)=3.44, p=0.034) and after (F(2,84)=16.5, $p=9.28e^{-7}$) pCO_2 treatments, with the Dabob Bay population significantly smaller than both Fidalgo Bay and Oyster Bay, particularly after pCO_2 treatments.

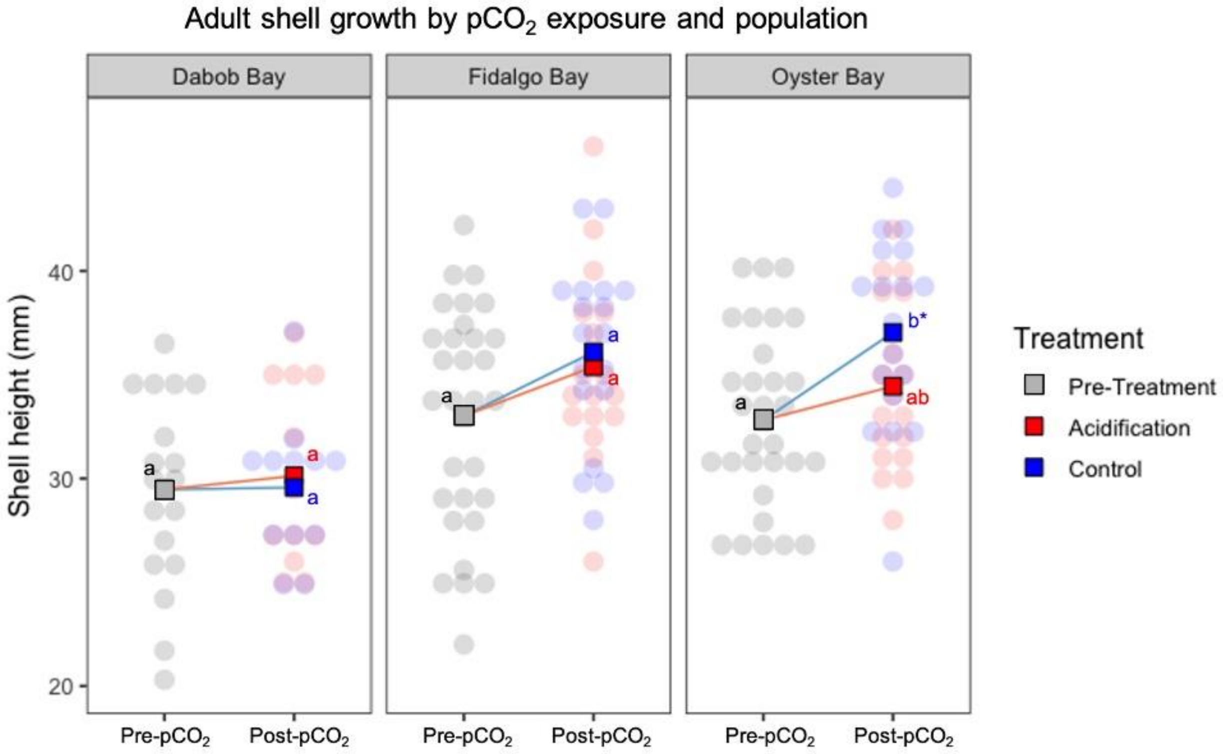

Figure 4.2. Adult shell height before pCO_2 treatment ("Pre-pCO_2") and after 52 days in acidification ($pCO_2 = 3045 \pm 488$ µatm: pH = 7.31 ± 0.02) and control conditions ($pCO_2 = 841 \pm 85$ µatm; pH = 7.82 ± 0.02) for each population. Transparent points each represent one adult oyster color-coded by pCO_2 treatment, and overlaid square points indicate mean shell height. Those exposed to control pCO_2 were larger than before the pCO_2 treatments in the Oyster Bay population only.

Table 4.3: Shell size statistics for each population. 1-way ANOVA compared shell height of oysters before treatment (Pre-Treatment) and after acidification and control treatments. Tukey multiple comparison of means was used to test for significant growth of oysters during the 52-day exposure to control conditions and acidification.

Population	ANOVA statistics	Tukey comparison of means: *Control vs. Pre-treatment*	Tukey comparison of means: *Acidification vs. Pre-treatment*
Dabob Bay	F(2,39)=0.09, p=0.91	Δ=0.10, p-adj=1.0	Δ=0.66, p-adj=0.91
Fidalgo Bay	F(2,63)=0.26, p=0.079	Δ=3.08, p-adj=0.094	Δ=2.38, p-adj=0.24
Oyster Bay	**F(2,63)=5.35, p=0.0071***	**Δ=4.22, p-adj=0.0049***	Δ=1.64, p-adj=0.42

Progression from one sex to the other in these hermaphroditic oysters was affected by acidification in the Oyster Bay population only: the ratio of predominantly-females:predominantly-males differed significantly between pCO_2 treatments (p-sim=0.015), with fewer females present after exposure to high pCO_2 (Figure 4.3). Additionally, compared to before pCO_2 treatment, the sex ratio differed following control pCO_2 exposure in Oyster Bay (p-sim= $8.0e^{-4}$) and Fidalgo Bay (p-sim=$1.3e^{-3}$), but did not differ following high pCO_2 exposure in any population, or between any comparison in the Dabob Bay population.

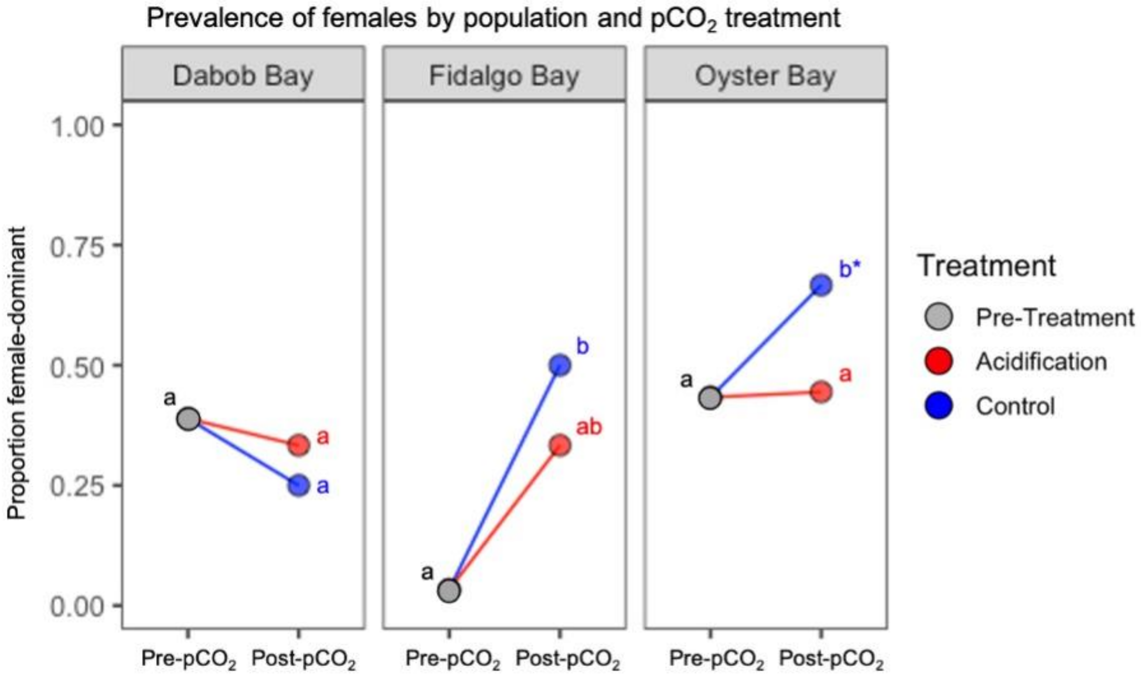

Figure 4.3. Proportion of oysters that were female or female-dominant before pCO_2 treatment ("Pre-pCO_2") and after 52 days in acidification ($pCO_2 = 3045 \pm 488$ µatm: pH = 7.31 ± 0.02) and control conditions ($pCO_2 = 841 \pm 85$ µatm; pH = 7.82 ± 0.02) for each population. The prevalence of females differed among pCO_2 treatments in the Oyster Bay population only.

The developmental stages of sperm and eggs (when present) did not differ between control and high pCO_2 treatment in any population. Sperm development did not change during the 52-day exposure period in any population. The Fidalgo Bay population exposed to control conditions contained more advanced- and late-stage oocytes following the 52-day exposure compared to before (p-sim=$1.0e^{-4}$), but this was not the case for Fidalgo Bay oysters exposed to acidification, or for any other population.

4.4.2 *Differential expression in adults upon direct exposure to acidification*

Of 32,210 genes in the draft *O. lurida* genome, we detected expression in 30,981 genes in adult ctenidia tissue. Within populations, 132 and 76 genes were differentially expressed in Dabob Bay and Fidalgo Bay in response to pCO_2 treatments, respectively (Appendix B). No expression differences were detected in the Oyster Bay population upon exposure to pCO_2 treatments. The annotated Dabob Bay and Fidalgo Bay DEGs were enriched for 25 and 6 biological functions (Figure 4.4). Four genes were differentially expressed in both Dabob Bay and Fidalgo Bay, which code for Cytochrome P450 2B4 (CYP2B4, p-adj=0.021), Cytochrome P450 2U1 (CYP2U1, p-adj=0.015), Fatty acid-binding protein (FABP4, p-adj=0.032), and Thyroxine 5-deiodinase (DIO3, p-adj=0.028), all of which were more abundant in oysters exposed to high pCO_2.

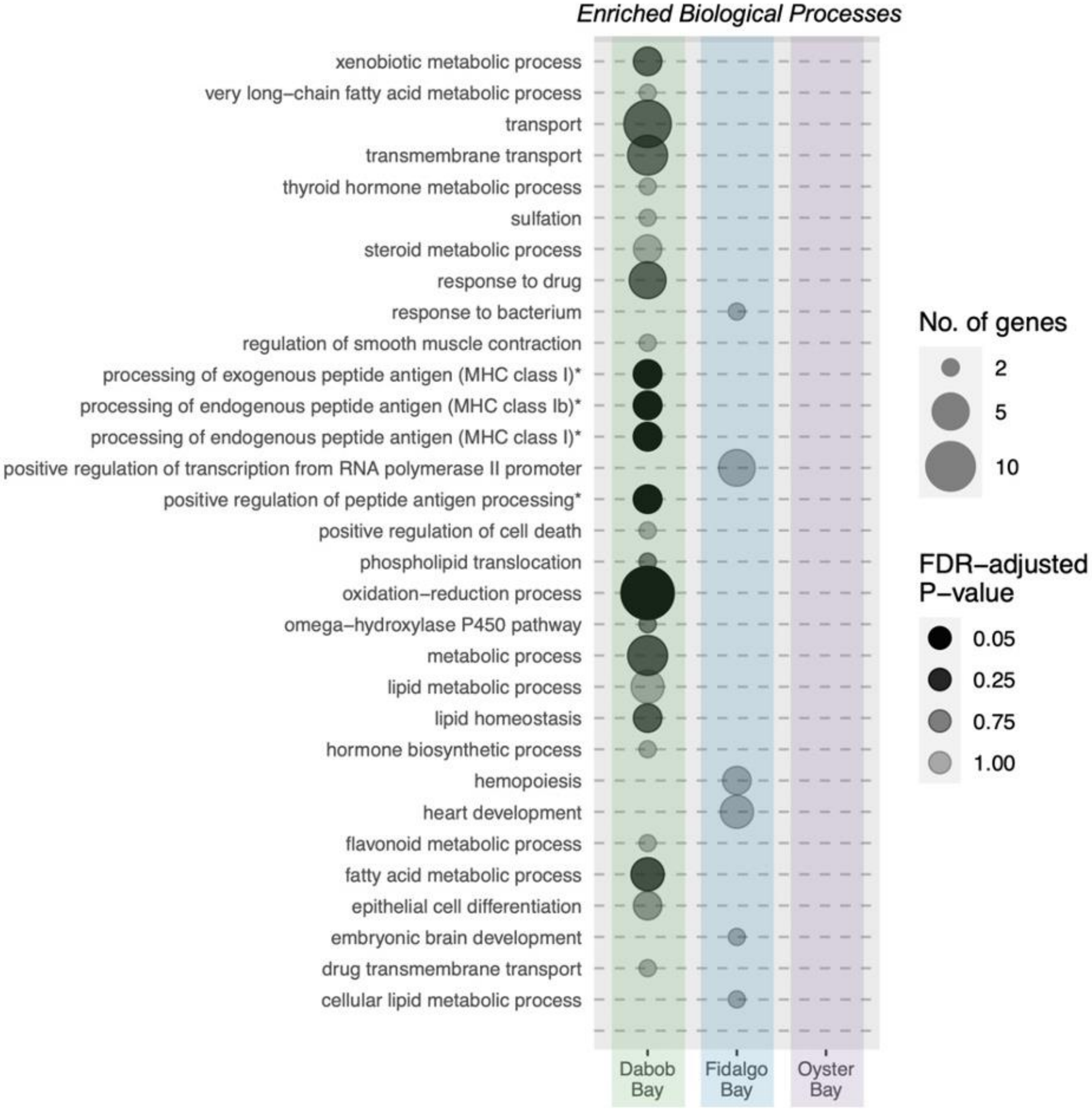

Figure 4.4. Enriched biological processes of differentially expressed genes upon exposure to high pCO_2 in three populations with different bays-of-origin. No genes were differentially expressed in the Oyster Bay population, and thus no processes were enriched. Asterisks indicate GO terms that have been edited for length.

4.4.3 *Impacts of parental exposures on larval offspring size*

Within populations, larval shell width and height differed by parental pCO_2 in the Oyster Bay population only (width: F(1,29)=5.46, p=0.027; height: F(1,29)=4.56, p=0.041), but did not differ significantly among parental pCO_2 in Fidalgo Bay (width: F(1,15)=0.0071, p=0.93; height: F(1,159)=0.14, p=0.71), or Dabob Bay (width: F(1,10)=0.71, p=0.49; height: F(1,10)=0.10, p=0.76) (Figure 4.5).

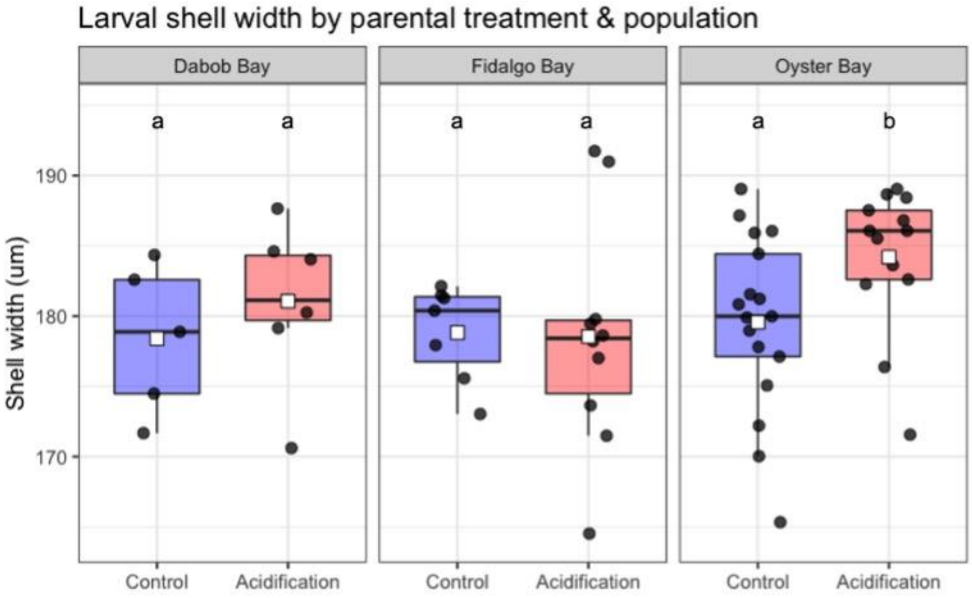

Figure 4.5. Laval shell size upon maternal liberation was significantly affected by parental exposure to high pCO_2 in the Oyster Bay population only. Shell width is shown here. Each point represents the mean shell width of 40+ larvae from a release group. Treatments were terminated prior to adult reproductive conditioning and spawning, so larvae were never directly exposed to treatments.

4.4.4 *Larval offspring gene expression*

Larvae were pooled by maternal release pulse and assessed for gene expression differences by parental exposure to acidification. We detected expression of 24,485 genes in larval samples. Within populations, one gene was differentially expressed by parental pCO_2 in the Fidalgo Bay population, “OLUR_00020618” (log2 fold change = -1.6, p-adj = 0.011), which was not annotated. Across all populations no expression differences were detected between parental pCO_2 exposures.

4.4.5 *Constitutive expression differences in the high-performing Dabob Bay population*

Of the 30,981 genes examined in adults, 280 differed between Dabob Bay and Oyster Bay (0.9% of genes), and 379 differed between Dabob Bay and Fidalgo Bay (1.2% of genes), 89 of which were identified by both comparisons. Of these genes uniquely expressed in Dabob Bay, 220 were annotated (Appendix B) and were enriched for 16 biological processes. There were 31 genes that were both constitutively expressed at different levels in Dabob Bay compared to other populations and were differentially expressed in response to acidification. Of these overlapping genes, eleven were less abundant than the other populations constitutively, but in response to acidification they increased in abundance. Twenty were more abundant constitutively, but in response to acidification they became less abundant.

Of the 24,485 genes examined in larval offspring, 260 and 452 differed between Dabob Bay and Fidalgo Bay and Oyster Bay, respectively (0.86% and 0.88%), 59 of which were identified by both comparison and 25 of which were annotated (Appendix B).

Forty-two genes were differentially expressed in both adult and larval stages among Dabob Bay and the other populations, which largely followed the same constitutive expression patterns in both life stages: 26 genes were more abundant in Dabob Bay adults and larvae compared to the other populations, and 14 were less abundant in both stages.

4.5 DISCUSSION

This study explored the within- and intergenerational response of *O. lurida* to acidification in three populations that had been reared in common conditions, but had distinct genetic ancestries. Each population demonstrated unique physiologies, which were evident in growth, gonad development, and transcriptional responses to pCO_2 treatments. In the Dabob Bay population there was no growth or change in sex ratio regardless of treatment. However, Dabob Bay oysters demonstrated a robust transcriptional response to high pCO_2 enriched for oxidation-reduction/detoxification, lipid metabolism, and other key processes. In stark contrast, no transcriptional response was detected in Oyster Bay oysters, but growth rate and prevalence of females were both negatively affected by acidification. There was a moderate transcriptional response in the Fidalgo Bay population, with no change in growth rate and minor effects to sex ratio as a result of acidification. This study also explored intergenerational carryover effects of adult exposure to acidification on basal gene expression and size of larvae upon maternal liberation across populations. Larvae were larger from adults exposed to high pCO_2 in the Oyster Bay population only. Counter to predictions we found no signature of parental exposure to acidification in larval transcriptomes. The unique responses to acidification support previous observations of varying stress-tolerance in the same populations.

4.5.1 *Population-specific effects of acidification on growth and reproduction are associated with performance*

Growth rate varies among Puget Sound *O. lurida* populations (Heare et al., 2017; Heare et al., 2018; Silliman et al., 2018; Spencer et al., 2020), and this study indicates that it also responds to acidification in a population-specific manner. Shell growth was stunted by acidification in the adults from Oyster Bay, as they grew in control conditions but did not in acidified conditions. In contrast, adults from Dabob Bay and Fidalgo Bay did not grow during the 52-day experiment regardless of pCO_2 treatment. Previous studies characterized Dabob Bay as the slowest growing population, but also found that those oysters also performed best in hatchery and field trials. Heare et al. (2017) and Silliman et al. (2018) both observed slowest growth in the Dabob Bay population and fastest growth in the Fidalgo Bay population. Heare et al. (2017) also observed highest deployment survival in Dabob Bay progeny. In Spencer et al. 2020 the Dabob Bay population was slowest to reach the eyed larval stage (~18 days) (Fidalgo Bay was the fastest, ~14 days), and had the highest survival during the larval stage (the lowest survival was in Oyster Bay population). Together, these studies indicate that *O. lurid*a from Dabob Bay may prioritize stress resilience and survival at the cost of growth rate and size at maturity.

Acidification affected reproductive processes in a population-specific manner. The natural male-female sexual progression was significantly altered by acidification in the Oyster Bay population only. Reproductive traits appear to be heritable in Puget Sound *O. lurida*, with notable differences in the Oyster Bay population. In a reciprocal transplant study, Heare et al. (2017) found that Oyster Bay had considerably higher incidents of brooding, and reached maximum percent brooding 20-30 days earlier than Dabob Bay and Fidalgo Bay populations (145-159 degree days). Silliman et al. (2018) and Spencer et al. (2020) also found the Oyster Bay population to be the most reproductively active, and in Spencer et al. (2020) Oyster Bay oysters began releasing larvae on average 9.9 days earlier than Fidalgo and Dabob Bays (99 degree days earlier). Here, we find that Oyster Bay was the only population for which pCO_2 exposure impacted reproductive processes, resulting in fewer females. A populations' reproductive capacity is limited by the number of oysters spawning as females, therefore the productivity of oysters from Oyster Bay may be uniquely impacted by acidification.

If we look beyond our focal populations, individuals that grow slowly and are not highly fecund may be more capable of withstanding high pCO_2 environments. Waldbusser *et al.* compared the response of *O. lurida* from Oregon with the faster growing Pacific oyster (*Crassostrea gigas*) to acidification, and suggested that slow shell secretion (a measure of growth rate) in *O. lurida* is a beneficial trait, contributing to their resilience to acidified conditions (Waldbusser et al., 2016). The growth rate of acidification-tolerant abalone is considerably lower than those sensitive to acidification, resulting in juveniles that are up to 80% smaller at 3-months old (Swezey et al., 2020). Whether growth rate is a proxy for resilience to acidification may not be applicable to all calcifiers, as faster growth is linked to ocean acidification resilience in selectively bred Sydney rock oysters (*Saccostrea glomerata*) (Parker et al., 2011; Stapp et al., 2018; Thompson et al., 2015). While no previous studies have associated reproductive investment with tolerance to acidification, a selectively bred line of Pacific oysters that are less sensitive to summer mortality and hypoxia also allocate less energy to reproductive tissue (Samain et al. 2007). Ultimately, the substantial and consistent resilience of the slow growing, moderately fecund Dabob Bay population in this study indicates that it is important to maintain a diversity of phenotypes, including those of lower fecundity and of smaller size. Oyster hatcheries routinely cull slow-growing larvae to maximize survival through metamorphosis, a practice that could be consistently removing stress-resilient genotypes. Highly fecund females and populations can also become overrepresented in cohorts of oyster seed. For long-term resilience to acidification and other stressors, commercial and restoration hatcheries should consider retaining slow-growing larvae and breed adults from a variety of sizes and productivity levels.

4.5.2 *Transcriptional response of O. lurida populations acclimated to acidification*

4.5.2.1 Population-specific upregulation of some, but not all, detoxification genes

The transcriptional response of *O. lurida* acclimated to ocean acidification varied considerably by population, ranging from a robust response in Dabob Bay to no significant measurable response in Oyster Bay. The pronounced response of Dabob Bay to acidification could reflect that populations' higher tolerance to stressors, and its ability to maintain altered homeostasis during the prolonged (52-day) exposure to acidification. For instance, Dabob Bay adults exposed to acidification contained a pronounced increase in transcripts from genes involved in detoxification, including several Cytochrome P450 (CYP), cytosolic sulfotransferases (SULT), and Glutathione transferases (GST). CYPs and SULTs are detoxification enzymes which metabolize both endogenous products (e.g. fatty acids, hormones) and xenobiotics (Coughtrie 2016; Snyder, 2000). Glutathiones are important cellular antioxidants which scavenge reactive metabolites, including hydrogen peroxide, and have been found at higher levels in many bivalves in response to acidification (Matozzo et al., 2013; Timmins-Schiffman et al., 2013), which may be related to their ability to protect proteins from oxidative stress (Abele et al., 2011; Sandamalika et al., 2019; Tomanek, 2015). Enhanced expression of GST and other intracellular stress mechanisms have similarly been observed in acidification-tolerant Sydney rock oysters upon intergenerational exposure to acidification (Goncalves et al., 2017), and direct exposure to thermal stress (McAfee et al., 2018). Two genes that codes for S-crystallins were also more prevalent in Dabob Bay oysters acclimated to high pCO_2. S-crystallins are known to be structural components of cephalopod eyes, however they are apparently derived from GSTs (Tomarev & Piatigorsky, 1996) as they have very similar amino acid sequences, and while they are generally thought to lack enzymatic activity they are induced by heat shock (Lang et al., 2009). This could indicate that the Dabob Bay population activates additional detoxicant pathways for a more robust response to environmental stressors.

Interestingly, none of the typical enzymatic antioxidants (catalase, superoxide dismutase, peroxiredoxin) were differentially expressed in response to acidification in any population, despite detection and sufficient coverage. Opposing expression patterns have previously been reported for GST and enzymatic antioxidants in a transgenerationally acclimated acidification-tolerant line of Sydney rock oysters (Goncalves et al., 2016), which the authors suggested reflects distinct detoxification systems and/or stimuli. The glutathione scavenging system is also uniquely upregulated in a heat-tolerant species of vent polychaete compared to a heat-sensitive congener (Dilly et al., 2012). GST and related proteins may therefore act as the main cellular antioxidants in populations of oysters and other invertebrates tolerant to acidified conditions and other abiotic stressors.

4.5.2.2 Population-specific changes in metabolism and energy production

Acidification altered expression of genes involved in lipid metabolism and transport in a population-specific pattern similar to the detoxification genes such that they ranged from highly upregulated in Dabob Bay to nearly unresponsive in Oyster Bay. These included genes that code for proteins in the peroxisome, which is an organelle that produces phospholipids, a major component of cell membranes, and metabolize long-chain fatty acids for energy production. The peroxisome proliferator-activated receptor gene, for instance, was much more active in the Dabob Bay population, which indicates the need for synthesis of peroxisome organelles. The co-activation of lipid metabolic and oxidation-reduction processes, most notably in Dabob Bay, could reflect enhanced mobilization of energy stores to support increased detoxicant synthesis (Goncalves et al., 2016; Hochachka & Somero, 2002; Mayor et al., 2015; Sokolova et al., 2012).

There are previous reports of acidification-induced increases in intracellular energy production, particularly in organisms tolerant to acidification and following long-term exposure (reviewed by Strader et al., 2020), and coinciding with activation of antioxidant defenses (Goncalves et al., 2016).

Several other genes involved in mitochondrial energy production were altered in acidification-acclimated Dabob Bay oysters, suggesting increased energy production. This included the Acadl gene which codes for the Long-chain specific acyl-CoA dehydrogenase, a mitochondrial protein involved in production of energy from fats (specifically, fatty-acid beta-oxidation), and the si:dkey-18l1.1 gene (von Willebrand factor) which is involved in mitochondrial ATPase activity, and the alxA gene, which codes for alternative oxidase and which may increase mitochondrial respiration when the cytochrome respiratory pathway is restricted. There were also population-specific changes to a clustered mitochondria protein homolog (AAEL000794) which is involved in the distribution of mitochondria in the cytoplasm. Basal levels were fewer in Dabob Bay than other populations (not significantly), but were then substantially higher in Dabob Bay in high pCO_2. The same expression pattern was observed in atad3-a (ATPase family AAA domain-containing protein 3-A), a protein that is essential for mitochondrial organization. Taken together, the basal and induced expression patterns in Dabob Bay could reflect a unique shift in mitochondrial efficiency and density in response to high pCO_2 to produce sufficient energy to maintain cellular homeostasis in prolonged acidification exposure.

4.5.3 *Constitutive expression differences in the Dabob Bay population*

We explored constitutive expression in Dabob Bay in an effort to understand why that population is unique in its transcriptional response to acidification, and how that might relate to its track record of high survival and stress tolerance (Heare et al., 2017; Silliman et al., 2018; Spencer et al., 2020). Many of the annotated genes that were uniquely expressed in control Dabob Bay adults were involved in immune functions (e.g. the complement system) and were enriched for antimicrobial and antiviral processes (e.g. cellular response to interferon-gamma), indicating that the Dabob Bay population is uniquely equipped to combat pathogens. Metabolic, growth, and reproduction processes were also overrepresented in Dabob Bay adult expression, and many of the same gene families were also uniquely expressed in Dabob Bay larvae. These annotated gene sets unique to Dabob Bay adults and larvae (220 and 25, respectively, Appendix B) provide insight into the mechanisms behind the population's unique energy distribution across life stages, and should be validated in other stress-tolerant, high-performing marine invertebrate populations.

Given its slow growth rate and relatively low/moderate fecundity, the Dabob Bay population was theorized to allocate more resources towards stress-response processes than other populations by constitutively expressing higher levels of genes that respond to acidified conditions. In this way, Dabob Bay would be uniquely "primed" for acidification by maintaining more transcripts of beneficial genes under typical conditions, which then become even more prevalent in acidification-acclimated oysters. Contrary to this prediction, those genes that were at higher levels in Dabob Bay constitutively become less abundant in response to acidification, and conversely those that were less abundant constitutively increased in acidification. The annotated genes that were depressed constitutively but were activated in response to acidification were largely involved in lipid transport and energy production, in addition to protein stabilization and cell migration. The annotated genes that were more active constitutively but then decreased in response to acidification were involved in immune function, cell cycle, and reproduction. Our findings suggest that populations of marine invertebrates tolerant to long-term acidification exposure are capable not necessarily because they are more prepared at the transcript level, but rather because they can mount then sustain a shift in resources to maintain lipid metabolic function, while down-regulating functions that are not critical to the acidification response, such as reproduction, immune function, and cell cycle.

4.5.4 *Population-specific carryover effect of parental exposure to acidification*

Parental exposure to acidification resulted in larger larval offspring in the Oyster Bay population only. Oyster Bay larvae from parents exposed to acidification were on average 5µm (3%) larger than those from control parents. Larval size is positively associated with lipid content, growth rate, and feeding ability in many bivalves, and can reduce predation risk (Bailey, 1984; Gonzalez Araya et al., 2012; Helm et al., 1973; Marshall & Keough, 2007; Wilson et al., 1996). Increased larval size following intergenerational exposure to acidification and warming could therefore benefit some *O. lurida* populations in the wild, particularly those that also encounter environmental stressors (Gibbs et al., 2021). Our results align with previous studies showing that parental exposure to acidification can influence the physiology of invertebrate larval offspring. This was first detected in the Sydney rock oyster (Parker et al., 2012), as parental exposure to acidification resulted in higher larval survival and growth rates. Subsequently, some studies also reported positive intergenerational and transgenerational carryover effects (reviewed in Zhao et al., 2020), while others found negative effects (Parker et al., 2017; Venkataraman et al., 2019), or no signal of parental exposure (Clements et al., 2021). In the present study, carryover effects were only detected in one of three populations, indicating that parental priming may only be triggered by acidification in some genotypes or epigenotypes.

Furthermore, populations unable to acclimatize directly to acidification (i.e. Oyster Bay in this study) may instead invest in parental priming.

The variety of intergenerational responses observed here and in previous studies could also stem from the mechanisms by which offspring are affected, which are theorized as changes to maternal provisioning, gamete mRNAs, and epigenetic changes (Eirin-Lopez & Putnam, 2019). If so, one would expect associated signals in larval transcriptomes, reflecting either energetic differences, remnant maternal mRNA transcripts, or regulatory shifts due to epigenetic changes (Gavery & Roberts, 2010, 2013). Curiously, despite size differences associated with parental treatments in the Oyster Bay population, there were no differences in gene expression. Differing sizes may therefore not be due to variable growth rate, as that would likely have been reflected in expression profiles (Meyer & Manahan, 2010; Pace et al., 2006). It is possible that cryptic intergenerational transcriptome plasticity could be induced by high pCO_2 or other environmental stressors, but that was not revealed under ambient conditions in which larvae were reared. It remains unclear why intergenerational acidification exposure increases larval size in some populations and species.

4.5.5 *Puget Sound O. lurida populations have unique physiotypes which may be adaptive*

To date, this and five other studies have characterized Puget Sound *O. lurida* with Fidalgo Bay, Dabob Bay, and Oyster Bay heritage (Heare et al., 2017; Heare et al., 2018; Silliman et al., 2018; Spencer et al., 2020; White et al. 2017). The three populations are close proximally (all are within the greater Puget Sound estuary), yet they represent distinct physiotypes in how they prioritize energy allocation constitutively, and when responding to an environmental stressor. Remarkably, in contrast to the other populations, high pCO_2 elicited no transcriptional response in Oyster Bay, which raises the question as to where energy typically allocated towards reproduction and growth was utilized in Oyster Bay oysters. A previous study on the same populations provides insight into Oyster Bay population's response. Heare et al. (2018) measured expression of targeted genes involved in the immediate (1-hr) stress-response following acute heat and mechanical shock, and reported that Oyster Bay was the only population for which a transcriptional response was detected. The discrepancy between Heare et al. (2018) and the present study could reflect unique responses to short-term vs. long-term abiotic stress. Specifically, those populations that are not capable of homeostatic stress-response over long periods could display the most pronounced short-term, acute response (i.e. Oyster Bay). We therefore suggest that the Oyster Bay population's aerobic scope shifted nearer to or into the pessimus range compared to the other populations (Sokolova et al. 2012), and was not capable of maintaining its regulatory response for the full 52-day exposure to acidification. Time-series expression analysis would improve our understanding of populations' varying abilities to respond to and maintain cellular functions over the course of prolonged exposure to acidification.

As suggested by Heare et al. (2017) and Silliman et al. (2018), the bay of origins' distinct environments may explain varying physiotypes observed in Puget Sound *O. lurida*. Dabob Bay is located within the Hood Canal, which is a notoriously challenging environment for marine organisms. As a highly stratified, silled fjord with long residence times, it experiences slow turn-over, periods of hypoxia, and elevated temperature (Babson, Kawase, & MacCready, 2006; Banas et al., 2015; Khangaonkar et al., 2018; Newton et al., 2007). Thus, the slow-growing, transcriptionally responsive physiotype observed in Dabob Bay oysters may have arisen due to selection for genotypes that allocate a high proportion of energy to cellular maintenance and chronic stress response. Fidalgo Bay is located in the Puget Sound's North Basin, and is more heavily influenced by tidal exchange and coastal oceanographic conditions. Fidalgo Bay characteristics (faster growth, larger mature size, low/moderate reproduction and transcriptional response) could reflect an ancestral population that has not experienced extreme selection events, and therefore represents a less specialized physiotype. Oyster Bay is located in Southern Puget Sound, which is a system of shallower finger-like basins that are highly productive, mixed, and experience large seasonal temperature swings (Moore et al., 2008). South Puget Sound is well suited for both wild and farmed shellfish, and may have preferentially selected for individuals that are highly fecund but lack the ability to acclimatize to acidification. Given that population-of-origin was also a dominant factor influencing gene expression in larvae, the genetic or epigenetic contributions to diverse physiologies should not be underestimated for populations of *O. lurida* and related species, even in small geographic scales.

4.6 CONCLUSION

This is the first study to assess the transcriptional response of an oyster from the genus *Ostrea* to ocean acidification. In doing so, we greatly expand our understanding of how different oyster species will respond to shifting ocean conditions. There is increasing evidence that *Ostrea* spp. may be more tolerant than other oysters to acidification (Cole et al., 2016; Gray et al., 2019; Spencer et al., 2020; Waldbusser et al., 2016). Exploring cellular strategies in stress-tested larvae, a highly vulnerable stage, is an important avenue for future research. Furthermore, a physiological response spectrum was observed in three *O. lurida* populations exposed to ocean acidification. Given previous observations of stress tolerance in oysters from Dabob Bay we suggest that the robust transcriptomic changes in acclimated oysters and slow growth rate represent the more acidification-tolerant physiotype. However, observations of positive carryover in Oyster Bay indicates that intergenerational plasticity could improve the outlook for future generations of less tolerant physiotypes.

4.7 ACKNOWLEDGEMENTS

Our gratitude to the following people who assisted with this project's many daily tasks: Grace Crandall, Kaitlyn Mitchell, Olivia Smith, Megan Hintz, Rhonda Elliott, Lindsay Alma, Sam White, Hollie Putnam, Brent Vadopalas, Bayer and Jackson Roberts, my husband Ian and my mom Anne. This project would not be possible without the support and encouragement of the Puget Sound Restoration Fund, which housed the experiment and whose staff contributed expert knowledge of Olympia oyster reproduction and husbandry: Ryan Crim, Stuart Ryan, Alice Helker, Brian Allen, Betsy Peabody, Josh Bouma, and Joth Davis. Thank you to my committee members Rick Goetz, Jacqueline Padilla-Gamiño, Jennifer Ruesink, and Steven Roberts.

This work was supported by the National Science Foundation Graduate Research Internship Program, the University of Washington College of the Environment Hall Conservation Research Award, and the National Shellfisheries Association Melbourne Carriker Award.

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APPENDIX A

Chapter 3 and Chapter 4 carbonate chemistry parameters.

Table 1: Chapter 3 and Chapter 4 carbonate chemistry parameters for three time points during the pCO_2 treatments, which are averages (± SE) from three replicate tanks per treatment. All parameters except for total alkalinity differed significantly between control/ambient (Amb.) and experimental/high (High.) tanks (One-way ANOVA). More details are available in *Venkataraman et al., 2019.*

Day	**pH*** F(1,16) = 5838, p = 6.12e-22		**Total Alkalinity (µmol/kg)** F(1,16) = 1.38, p = 0.257		**pCO_2 (µatm)***** F(1,16) = 235, p = 5.44e-11		**DIC (µmol/kg)*** F(1,16) = 7.12, p = 0.0168		**$\Omega_{calcite}$***** F(1,16) = 529, p = 1.10e-13		**$\Omega_{aragonite}$***** F(1,16) =527, p = 1.14e-13	
	Amb.	**High**	**Amb.**	**High**	**Amb.**	**High**	**Amb.**	**High.**	**Amb.**	**High**	**Amb.**	**High**
5	7.82 ± 0.004	7.33 ± 0.002	2307.41 ± 25.45	2332.36 ± 31.05	747.51 ± 13.94	2481.23 ± 29.83	2233.41 ± 25.29	2408.51 ± 31.76	1.86 ± 0.02	0.62 ± 0.01	1.16 ± 0.012	0.58 ± 0.007
33	7.81 ± 0.005	7.31 ± 0.004	2747.00 ± 21.13	2917.60 ± 18.36	912.22 ± 12.69	3309.52 ± 7.22	2664.57 ± 19.99	3020.99 ± 17.99	2.23 ± 0.03	0.77 ± 0.02	1.40 ± 0.020	0.48 ± 0.014
48	7.82 ± 0.015	7.29 ± 0.004	2611.40 ± 31.01	2808.39 ± 12.24	863.47 ± 42.42	3343.89 ± 49.49	2533.28 ± 35.45	2920.52 ± 15.11	2.13 ± 0.06	0.68 ± 0.01	1.32 ± 0.035	0.42 ± 0.004

Literature Cited:

Venkataraman, Y. R., Spencer, L. H., & Roberts, S. B. (2019). Adult low pH exposure influences larval abundance in Pacific oysters (Crassostrea gigas). *Journal of Shellfish Research*. https://par.nsf.gov/biblio/10082500

APPENDIX B

Ostrea lurida gene sets that were differentially expressed in response to ocean acidification in some populations (Table 1), and were uniquely expressed in the a stress-tolerant population (Dabob Bay) at the adult stage (Table 2) and veliger larval stage (Table 3).

Table 1. Genes that were differentially expressed in response to ocean acidification in *Ostrea lurida* populations, Dabob Bay and/or Fidalgo Bay. Positive Log2-FC values indicate abundances were higher in oysters exposed to acidification.

O. lurida Gene ID	**Log2 Fold Change**	**p-adj**	**Uniprot SPID**	**Similar Annotated Gene**	**Population with DEG**
OLUR_00030878	0.87	2.41E-02	Q17N71	AAEL000794: Clustered mitochondria protein homolog	Dabob Bay
OLUR_00016317	0.78	6.54E-03	Q99758	ABCA3: ATP-binding cassette sub-family A member 3	Dabob Bay
OLUR_00013619	1.93	6.82E-03	P08183	ABCB1: Multidrug resistance protein 1	Dabob Bay
OLUR_00016960	1.59	4.24E-02	P06795	Abcb1b: Multidrug resistance protein 1B	Dabob Bay
OLUR_00011049	2.7	1.48E-03	P21440	Abcb4: Phosphatidylcholine translocator ABCB4	Dabob Bay
OLUR_00000769	1.05	2.10E-02	Q93YS4	ABCG22: ABC transporter G family member 22	Fidalgo Bay
OLUR_00022318	0.73	5.56E-03	P51174	Acadl: Long-chain specific acyl-CoA dehydrogenase%2C mitochondrial	Dabob Bay
OLUR_00024728	2.03	2.89E-02	Q9R0H0	Acox1: Peroxisomal acyl-coenzyme A oxidase 1	Dabob Bay
OLUR_00012845	2.27	4.20E-02	Q5R4M8	ALDH18A1: Delta-1-pyrroline-5-carboxylate synthase	Fidalgo Bay
OLUR_00011263	1.85	9.64E-04	Q9P959	alxA: Alternative oxidase, C mitochondrial	Dabob Bay
OLUR_00000533	-0.9	3.40E-03	Q8N264	ARHGAP24: Rho GTPase-activating protein 24	Fidalgo Bay
OLUR_00022323	1.44	4.15E-02	Q58E76	atad3-a: ATPase family AAA domain-containing protein 3-A	Dabob Bay
OLUR_00004651	0.47	4.31E-02	Q99933	BAG1: BAG family molecular chaperone regulator 1	Dabob Bay
OLUR_00001152	-1.3	2.80E-02	A8Y1P7	bre-4: Beta-1%2C4-N-acetylgalactosaminyltransferase bre-4	Fidalgo Bay
OLUR_00029448	-0.8	3.31E-02	Q24157	brn: Beta-1%2C3-galactosyltransferase brn	Dabob Bay
OLUR_00021130	-2.94	8.88E-03	Q8CFR0	C1ql2: Complement C1q-like protein 2	Dabob Bay
OLUR_00026389	2.94	2.30E-02	Q4ZJM9	C1ql4: Complement C1q-like protein 4	Fidalgo Bay
OLUR_00013332	0.88	2.80E-02	Q62717	Cadps: Calcium-dependent secretion activator 1	Fidalgo Bay
OLUR_00015091	-0.54	4.80E-02	P97864	Casp7: Caspase-7	Fidalgo Bay
OLUR_00017184	-2.36	5.56E-03	P49817	Cav1: Caveolin-1	Dabob Bay
OLUR_00001710	-2.25	5.56E-03	P32320	CDA: Cytidine deaminase	Dabob Bay
OLUR_00008459	-0.68	4.72E-02	B7Z0W9	CG42265: Proton channel OtopLc	Dabob Bay
OLUR_00005263	-1.35	2.97E-02	Q8R4G9	Chrna3: Neuronal acetylcholine receptor subunit alpha-3	Dabob Bay
OLUR_00002411	-2.08	2.21E-05	A2AX52	Col6a4: Collagen alpha-4	Dabob Bay
OLUR_00019549	0.61	4.82E-02	P17886	crn: Protein crooked neck	Dabob Bay
OLUR_00014282	0.73	3.62E-02	Q01406	CTTN1: Src substrate protein p85	Dabob Bay
OLUR_00025713	1.57	3.61E-02	Q64583	Cyp2b15: Cytochrome P450 2B15	Dabob Bay
OLUR_00012237	1.74	2.10E-02	P00178	CYP2B4: Cytochrome P450 2B4	Dabob Bay, Fidalgo Bay
OLUR_00016688	3.34	9.20E-03	P24470	Cyp2c23: Cytochrome P450 2C23	Fidalgo Bay
OLUR_00008469	1.9	3.91E-03	P10632	CYP2C8: Cytochrome P450 2C8	Dabob Bay
OLUR_00024274	2.08	4.97E-02	P11714	Cyp2d9: Cytochrome P450 2D9	Dabob Bay
OLUR_00026774	1.69	6.54E-03	P24461	CYP2G1: Cytochrome P450 2G1	Dabob Bay

OLUR_00020244	1.41	4.97E-02	P52786	CYP2J1: Cytochrome P450 2J1	Dabob Bay
OLUR_00018748	2.88	1.24E-03	Q6VVW9	Cyp2r1: Vitamin D 25-hydroxylase	Dabob Bay
OLUR_00027367	2.8	1.50E-02	Q0IIF9	CYP2U1: Cytochrome P450 2U1	Dabob Bay, Fidalgo Bay
OLUR_00003331	1.49	3.62E-02	Q7Z449	CYP2U1: Cytochrome P450 2U1	Dabob Bay
OLUR_00008711	1.03	4.80E-02	Q6NT55	CYP4F22: Cytochrome P450 4F22	Fidalgo Bay
OLUR_00014014	-0.49	4.80E-02	Q80T85	Dcaf5: DDB1- and CUL4-associated factor 5	Fidalgo Bay
OLUR_00002100	2.44	1.65E-03	P49894	DIO1: Type I iodothyronine deiodinase	Dabob Bay
OLUR_00002101	2.02	2.80E-02	O42412	DIO3: Thyroxine 5-deiodinase	Dabob Bay, Fidalgo Bay
OLUR_00021120	-2.33	1.43E-02	Q9UGM3	DMBT1: Deleted in malignant brain tumors 1 protein	Dabob Bay
OLUR_00005690	-0.71	4.80E-02	A3R064	DOK3: Docking protein 3	Fidalgo Bay
OLUR_00012281	0.96	4.31E-02	P47823	EIF2B5: Translation initiation factor eIF-2B subunit epsilon	Dabob Bay
OLUR_00009872	-0.86	1.10E-02	P10160	EIF5A: Eukaryotic translation initiation factor 5A-1	Fidalgo Bay
OLUR_00012745	2.52	3.20E-02	P48035	FABP4: Fatty acid-binding protein%2C adipocyte	Dabob Bay, Fidalgo Bay
OLUR_00031779	-2.38	3.30E-02	Q8R508	Fat3: Protocadherin Fat 3	Fidalgo Bay
OLUR_00028383	-1.62	1.10E-02	Q3UMF9	Faxc: Failed axon connections homolog	Fidalgo Bay
OLUR_00025242	-1.16	3.40E-02	O42127	fgfr3: Fibroblast growth factor receptor 3	Fidalgo Bay
OLUR_00021504	-1.23	2.10E-02	O61491	Flo1: Flotillin-1	Fidalgo Bay
OLUR_00003007	-2.12	3.70E-02	Q6AYS4	Fuca2: Plasma alpha-L-fucosidase	Fidalgo Bay
OLUR_00031928	-3.91	2.64E-02	Q9NUV9	GIMAP4: GTPase IMAP family member 4	Dabob Bay
OLUR_00014547	1.86	3.61E-02	P34277	gsto-2: Probable glutathione transferase omega-2	Dabob Bay
OLUR_00013606	1.9	2.43E-02	Q9Z339	Gsto1: Glutathione S-transferase omega-1	Dabob Bay
OLUR_00000433	-1.21	3.42E-02	Q9WVI4	Gucy1a2: Guanylate cyclase soluble subunit alpha-2	Dabob Bay
OLUR_00018610	4.14	6.60E-03	E9QAM5	Helz2: Helicase with zinc finger domain 2	Fidalgo Bay
OLUR_00013228	3.36	1.80E-02	Q9BYK8	HELZ2: Helicase with zinc finger domain 2	Fidalgo Bay
OLUR_00014040	2.7	2.25E-02	D3YXG0	Hmcn1: Hemicentin-1	Dabob Bay
OLUR_00018477	2.24	2.70E-02	A2AJ76	Hmcn2: Hemicentin-2	Fidalgo Bay
OLUR_00001007	-1.61	1.12E-02	Q9D4G2	Hsf2bp: Heat shock factor 2-binding protein	Dabob Bay
OLUR_00011628	-1.65	2.70E-03	O43301	HSPA12A: Heat shock 70 kDa protein 12A	Dabob Bay
OLUR_00015765	-1.65	4.97E-02	C8VK14	hxnY: 2-oxoglutarate-Fe	Dabob Bay
OLUR_00018945	-0.99	4.70E-02	E9PY46	Ift140: Intraflagellar transport protein 140 homolog	Fidalgo Bay
OLUR_00026123	1.3	4.72E-02	O95069	KCNK2: Potassium channel subfamily K member 2	Dabob Bay
OLUR_00010601	0.83	2.64E-02	Q9U518	L-asparaginase	Dabob Bay
OLUR_00002203	0.78	2.70E-02	Q61805	Lbp: Lipopolysaccharide-binding protein	Fidalgo Bay
OLUR_00002201	-2.04	4.80E-02	Q96WM9	lcc2: Laccase-2	Fidalgo Bay
OLUR_00007747	-2.02	1.32E-02	P09849	LCT: Lactase-phlorizin hydrolase	Dabob Bay
OLUR_00004194	1.6	4.70E-02	B3EWZ5	MAM and LDL-receptor class A domain-containing protein 1	Dabob Bay
OLUR_00019130	1.59	4.15E-02	B3EWZ6	MAM and LDL-receptor class A domain-containing protein 2	Dabob Bay
OLUR_00016827	2.65	6.22E-04	B3EWZ6	MAM and LDL-receptor class A domain-containing protein 2	Dabob Bay
OLUR_00005789	-1.12	3.31E-02	Q16820	MEP1B: Meprin A subunit beta	Dabob Bay
OLUR_00001931	-1.67	4.97E-02	P08472	Mesenchyme-specific cell surface glycoprotein	Dabob Bay
OLUR_00013617	-1.82	2.48E-03	P08472	Mesenchyme-specific cell surface glycoprotein	Dabob Bay
OLUR_00030728	-2.28	5.00E-02	Q24400	Mlp84B: Muscle LIM protein Mlp84B	Fidalgo Bay
OLUR_00011332	0.98	3.20E-02	Q95NT6	mth2: G-protein coupled receptor Mth2	Fidalgo Bay
OLUR_00009450	-0.63	5.00E-02	Q62725	Nfyc: Nuclear transcription factor Y subunit gamma	Fidalgo Bay
OLUR_00014514	-1.07	2.43E-02	O07552	nhaX: Stress response protein NhaX	Dabob Bay
OLUR_00012374	1.55	4.80E-02	Q04721	NOTCH2: Neurogenic locus notch homolog protein 2	Fidalgo Bay

OLUR_00016842	2.03	2.43E-02	Q8CJ26	Nradd: Death domain-containing membrane protein NRADD	Dabob Bay
OLUR_00000214	0.83	2.80E-02	Q6KEQ9	PCDH11X: Protocadherin-11 X-linked	Fidalgo Bay
OLUR_00005074	0.71	2.25E-02	Q9Y5K3	PCYT1B: Choline-phosphate cytidylyltransferase B	Dabob Bay
OLUR_00004258	-1.54	4.80E-02	P86854	Perlucin-like protein	Fidalgo Bay
OLUR_00000551	-2.14	6.22E-04	Q96UX3	pkaR: cAMP-dependent protein kinase regulatory subunit	Dabob Bay
OLUR_00007563	1.19	7.87E-03	P47713	Pla2g4a: Cytosolic phospholipase A2	Dabob Bay
OLUR_00010581	0.93	1.80E-02	Q08BB2	pm20d1.2: N-fatty-acyl-amino acid synthase/hydrolase PM20D1.2	Fidalgo Bay
OLUR_00030589	-1.71	7.66E-04	A6NIZ1	Ras-related protein Rap-1b-like protein	Dabob Bay
OLUR_00025558	1.4	4.22E-02	P18426	S-crystallin SL11	Dabob Bay
OLUR_00027159	1.44	4.07E-02	P18426	S-crystallin SL11	Dabob Bay
OLUR_00006587	-0.97	3.20E-02	Q54KA7	secG: Ankyrin repeat%2C PH and SEC7 domain containing protein secG	Fidalgo Bay
OLUR_00012491	-0.99	2.39E-02	Q92008	shha: Sonic hedgehog protein A	Dabob Bay
OLUR_00011339	0.98	3.54E-02	B0R0T1	si:dkey-18l1.1: von Willebrand factor A domain-containing protein 8	Dabob Bay
OLUR_00022480	-1.04	4.40E-02	Q7ZVK3	sirt2: NAD-dependent protein deacetylase sirtuin-2	Fidalgo Bay
OLUR_00016903	-1.07	5.56E-03	Q7TM99	Slc16a9: Monocarboxylate transporter 9	Dabob Bay
OLUR_00020381	1.89	5.56E-03	P43006	Slc1a2: Excitatory amino acid transporter 2	Dabob Bay
OLUR_00022102	1.21	4.30E-02	Q9Z2J0	Slc23a1: Solute carrier family 23 member 1	Fidalgo Bay
OLUR_00010175	-2.11	2.65E-02	Q9Z2J0	Slc23a1: Solute carrier family 23 member 1	Dabob Bay
OLUR_00028935	-1.83	4.15E-02	O35488	Slc27a2: Very long-chain acyl-CoA synthetase	Dabob Bay
OLUR_00004448	0.99	2.43E-02	A0JPN2	Slc39a4: Zinc transporter ZIP4	Dabob Bay
OLUR_00012891	0.58	3.62E-02	Q07837	SLC3A1: Neutral and basic amino acid transport protein rBAT	Dabob Bay
OLUR_00024710	-1.02	2.10E-02	Q24524	sn: Protein singed	Fidalgo Bay
OLUR_00027313	0.91	3.08E-02	Q91Z69	Srgap1: SLIT-ROBO Rho GTPase-activating protein 1	Dabob Bay
OLUR_00020487	0.57	2.85E-02	Q90ZB9	stard3: StAR-related lipid transfer protein 3	Dabob Bay
OLUR_00010835	1.36	2.80E-02	P08842	STS: Steryl-sulfatase	Fidalgo Bay
OLUR_00025850	-1.5	3.67E-02	Q9HAC7	SUGCT: Succinate--hydroxymethylglutarate CoA-transferase	Dabob Bay
OLUR_00002962	4.12	1.72E-03	O43704	SULT1B1: Sulfotransferase family cytosolic 1B member 1	Dabob Bay
OLUR_00005785	3	2.94E-04	Q95JD5	SULT1B1: Sulfotransferase family cytosolic 1B member 1	Dabob Bay
OLUR_00000570	2.22	2.28E-04	F1QYJ6	sult6b1: Sulfotransferase 6B1	Dabob Bay
OLUR_00026074	3.23	6.20E-05	Q02858	Tek: Angiopoietin-1 receptor	Fidalgo Bay
OLUR_00000288	1.55	4.64E-02	Q7Z0T3	Temptin	Dabob Bay
OLUR_00002071	-1.87	3.20E-02	P08953	Tl: Protein toll	Fidalgo Bay
OLUR_00001769	-1.93	4.19E-02	Q9JIQ8	Tmprss2: Transmembrane protease serine 2	Dabob Bay
OLUR_00020170	-1.13	2.80E-02	V5NAL9	Toll-like receptor 4	Fidalgo Bay
OLUR_00013623	0.37	3.20E-02	P62997	Tra2b: Transformer-2 protein homolog beta	Fidalgo Bay
OLUR_00000822	-1.2	2.66E-02	E1BD59	TRIM56: E3 ubiquitin-protein ligase TRIM56	Dabob Bay
OLUR_00028050	2.05	4.97E-02	O74549	ubc12: NEDD8-conjugating enzyme ubc12	Dabob Bay
OLUR_00000751	0.74	2.10E-02	P62840	ube2d2: Ubiquitin-conjugating enzyme E2 D2	Fidalgo Bay
OLUR_00007224	-1.43	1.47E-02	P74897	Universal stress protein in QAH/OAS sulfhydrylase 3'region	Dabob Bay
OLUR_00020423	1.32	2.64E-02	P41824	Y-box factor homolog	Dabob Bay

Table 2**:** Genes that were constitutively expressed in Dabob Bay adults at different levels compared to other populations.

***O. lurida* gene ID**	**Gene**	**Uniprot SPID**
OLUR_00032068	AADAC: Arylacetamide deacetylase	Q0P5B7
OLUR_00017044	ABCA1: ATP-binding cassette sub-family A member 1	O95477
OLUR_00016317	ABCA3: ATP-binding cassette sub-family A member 3	Q99758
OLUR_00022745	ABCC3: Canalicular multispecific organic anion transporter 2	O15438
OLUR_00001814	Abcf3: ATP-binding cassette sub-family F member 3	Q8K268
OLUR_00022318	Acadl: Long-chain specific acyl-CoA dehydrogenase, C mitochondrial	P51174
OLUR_00024957	ADAMTS13: A disintegrin and metalloproteinase with thrombospondin motifs 13	Q76LX8
OLUR_00000647	adat1: tRNA-specific adenosine deaminase 1	Q28FE8
OLUR_00022969	Adgrb1: Adhesion G protein-coupled receptor B1	Q3UHD1
OLUR_00031677	AHCY: Adenosylhomocysteinase	Q3MHL4
OLUR_00031199	AHCY: Adenosylhomocysteinase	Q3MHL4
OLUR_00011263	alxA: Alternative oxidase, C mitochondrial	Q9P959
OLUR_00002441	amy: Alpha-amylase	P29957
OLUR_00002241	Anapc4: Anaphase-promoting complex subunit 4	Q91W96
OLUR_00029061	ANGPT2: Angiopoietin-2	A0A8J8
OLUR_00018984	ANK1: Ankyrin-1	P16157
OLUR_00011963	Ank1: Ankyrin-1	Q02357
OLUR_00002563	ANKRD50: Ankyrin repeat domain-containing protein 50	Q9ULJ7
OLUR_00002438	Aoah: Acyloxyacyl hydrolase	O35298
OLUR_00006201	Aopep: Aminopeptidase O	P69527
OLUR_00019355	ARI1: Probable E3 ubiquitin-protein ligase ARI1	Q949V6
OLUR_00022769	ARSJ: Arylsulfatase J	Q5FYB0
OLUR_00002790	Aspartate aminotransferase	P23034
OLUR_00005128	Atad5: ATPase family AAA domain-containing protein 5	Q4QY64
OLUR_00010015	ATPsyngamma: ATP synthase subunit gamma, C mitochondrial	O01666
OLUR_00004651	BAG1: BAG family molecular chaperone regulator 1	Q99933
OLUR_00025267	BCO1: Beta, Cbeta-carotene 15, C15'-dioxygenase	Q9I993
OLUR_00007641	Beta-1, C3-glucan-binding protein	Q8N0N3
OLUR_00021318	Beta-1, C3-glucan-binding protein	Q8N0N3
OLUR_00014400	Bsx: Brain-specific homeobox protein homolog	Q810B3
OLUR_00021824	BTBD2: BTB/POZ domain-containing protein 2	Q9BX70
OLUR_00008482	C1qc: Complement C1q subcomponent subunit C	Q02105
OLUR_00023445	C1ql2: Complement C1q-like protein 2	Q8CFR0
OLUR_00021130	C1ql2: Complement C1q-like protein 2	Q8CFR0
OLUR_00023682	C1ql4: Complement C1q-like protein 4	Q4ZJM9
OLUR_00016044	C1ql4: Complement C1q-like protein 4	Q4ZJM9
OLUR_00002843	C1qtnf4: Complement C1q tumor necrosis factor-related protein 4	Q8R066
OLUR_00029319	C38H2.2: Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1	Q18515
OLUR_00017184	Cav1: Caveolin-1	P49817
OLUR_00006811	ccdc169: Coiled-coil domain-containing protein 169	Q3KPT0
OLUR_00015839	CD151: CD151 antigen	P48509
OLUR_00022562	Cd209e: CD209 antigen-like protein E	Q91ZW7
OLUR_00001710	CDA: Cytidine deaminase	P32320
OLUR_00030502	CEMIP: Cell migration-inducing and hyaluronan-binding protein	Q8WUJ3

OLUR_00002764	CHDH: Choline dehydrogenase, C mitochondrial	Q8NE62
OLUR_00023309	Chrna6: Neuronal acetylcholine receptor subunit alpha-6	P43143
OLUR_00005622	CHRNA7: Neuronal acetylcholine receptor subunit alpha-7	P54131
OLUR_00009646	CHRNA9: Neuronal acetylcholine receptor subunit alpha-9	Q9PTS8
OLUR_00030281	chs-2: Chitin synthase chs-2	G5EBQ8
OLUR_00005393	CLEC17A: C-type lectin domain family 17, C member A	Q6ZS10
OLUR_00002411	Col6a4: Collagen alpha-4	A2AX52
OLUR_00027711	COL8A2: Collagen alpha-2	P25067
OLUR_00027741	COLEC12: Collectin-12	Q2LK54
OLUR_00004033	Collagen alpha-2	P27393
OLUR_00022598	Cradd: Death domain-containing protein CRADD	O88843
OLUR_00005907	CYP17A1: Steroid 17-alpha-hydroxylase/17, C20 lyase	Q92113
OLUR_00007286	CYP17A1: Steroid 17-alpha-hydroxylase/17, C20 lyase	Q29497
OLUR_00010095	CYP17A1: Steroid 17-alpha-hydroxylase/17, C20 lyase	Q9GMC7
OLUR_00010564	Ddx39b: Spliceosome RNA helicase Ddx39b	Q63413
OLUR_00009085	DDX58: Probable ATP-dependent RNA helicase DDX58	O95786
OLUR_00021120	DMBT1: Deleted in malignant brain tumors 1 protein	Q9UGM3
OLUR_00012599	drpr: Protein draper	Q9W0A0
OLUR_00004620	drpr: Protein draper	Q9W0A0
OLUR_00009894	DUR3: Urea-proton symporter DUR3	F4KD71
OLUR_00008003	DYNC1LI1: Cytoplasmic dynein 1 light intermediate chain 1	Q90828
OLUR_00008906	DZIP3: E3 ubiquitin-protein ligase DZIP3	Q86Y13
OLUR_00007341	EGFL8: Epidermal growth factor-like protein 8	A5A8Y8
OLUR_00006099	Eprs: Bifunctional glutamate/proline--tRNA ligase	Q8CGC7
OLUR_00021382	Ercc8: DNA excision repair protein ERCC-8	Q8CFD5
OLUR_00004310	exog: Nuclease EXOG, C mitochondrial	Q502K1
OLUR_00014579	F54H12.2: Uncharacterized protein F54H12.2	P34456
OLUR_00017820	FAM111B: Protein FAM111B	Q6SJ93
OLUR_00006320	FAT1: Protocadherin Fat 1	Q14517
OLUR_00029612	FBN1: Fibrillin-1	P98133
OLUR_00028174	fbxl15: F-box/LRR-repeat protein 15	E6ZHJ8
OLUR_00002961	FBXL4: F-box/LRR-repeat protein 4	Q9UKA2
OLUR_00025242	fgfr3: Fibroblast growth factor receptor 3	O42127
OLUR_00001763	G-protein coupled receptor GRL101	P46023
OLUR_00017370	G-protein coupled receptor GRL101	P46023
OLUR_00000777	Gal3st1: Galactosylceramide sulfotransferase	Q9JHE4
OLUR_00006985	GALNT13: Polypeptide N-acetylgalactosaminyltransferase 13	Q8IUC8
OLUR_00009688	gbpC: Cyclic GMP-binding protein C	Q8MVR1
OLUR_00001930	Gigasin-3a	P86786
OLUR_00031928	GIMAP4: GTPase IMAP family member 4	Q9NUV9
OLUR_00031158	GIMAP7: GTPase IMAP family member 7	Q8NHV1
OLUR_00010854	Gld: Glucose dehydrogenase [FAD, C quinone]	P18172
OLUR_00022856	GLRA2: Glycine receptor subunit alpha-2	P23416
OLUR_00011563	gltX: Glutamate--tRNA ligase	A4XA62
OLUR_00030833	GPx: Glutathione peroxidase	G9JJU2
OLUR_00002127	Heavy metal-binding protein HIP	P83425
OLUR_00025178	Heavy metal-binding protein HIP	P83425
OLUR_00011069	Hebp2: Heme-binding protein 2	Q9WU63

OLUR_00011115	HELZ2: Helicase with zinc finger domain 2	Q9BYK8
OLUR_00013228	HELZ2: Helicase with zinc finger domain 2	Q9BYK8
OLUR_00019943	Helz2: Helicase with zinc finger domain 2	E9QAM5
OLUR_00018610	Helz2: Helicase with zinc finger domain 2	E9QAM5
OLUR_00002821	Hemagglutinin/amebocyte aggregation factor	Q01528
OLUR_00000188	Hmcn1: Hemicentin-1	D3YXG0
OLUR_00014040	Hmcn1: Hemicentin-1	D3YXG0
OLUR_00024505	HMCN2: Hemicentin-2	Q8NDA2
OLUR_00001007	Hsf2bp: Heat shock factor 2-binding protein	Q9D4G2
OLUR_00006095	HSPA12A: Heat shock 70 kDa protein 12A	O43301
OLUR_00030423	IARS: Isoleucine--tRNA ligase, C cytoplasmic	P41252
OLUR_00013653	IFI44: Interferon-induced protein 44	Q8TCB0
OLUR_00025548	IFI44L: Interferon-induced protein 44-like	Q53G44
OLUR_00009305	Ift88: Intraflagellar transport protein 88 homolog	Q61371
OLUR_00006016	inlI: Internalin I	Q8YA32
OLUR_00020497	KALRN: Kalirin	O60229
OLUR_00018338	Kif9: Kinesin-like protein KIF9	Q9WV04
OLUR_00003139	Ky: Kyphoscoliosis peptidase	Q8C8H8
OLUR_00015061	Lectin BRA-3	P07439
OLUR_00022461	Lectin BRA-3	P07439
OLUR_00022071	LGMN: Legumain	Q95M12
OLUR_00019596	LHFPL3: LHFPL tetraspan subfamily member 3 protein	Q86UP9
OLUR_00022033	LRP8: Low-density lipoprotein receptor-related protein 8	Q98931
OLUR_00003482	lrrc58: Leucine-rich repeat-containing protein 58	Q32NT4
OLUR_00015101	Lrrc69: Leucine-rich repeat-containing protein 69	Q9D9Q0
OLUR_00018351	LRRK2: Leucine-rich repeat serine/threonine-protein kinase 2	Q5S007
OLUR_00005454	MAM and LDL-receptor class A domain-containing protein 1	B3EWZ5
OLUR_00005238	Mbtps1: Membrane-bound transcription factor site-1 protease	Q9WTZ2
OLUR_00027937	MDGA2: MAM domain-containing glycosylphosphatidylinositol anchor protein 2	Q7Z553
OLUR_00009020	ME1: NADP-dependent malic enzyme	P40927
OLUR_00010931	MED34: Mediator of RNA polymerase II transcription subunit 34	Q9FT73
OLUR_00006121	MEGF10: Multiple epidermal growth factor-like domains protein 10	Q96KG7
OLUR_00019978	Megf11: Multiple epidermal growth factor-like domains protein 11	Q80T91
OLUR_00024832	Megf6: Multiple epidermal growth factor-like domains protein 6	O88281
OLUR_00021337	MEGF6: Multiple epidermal growth factor-like domains protein 6	O75095
OLUR_00001931	Mesenchyme-specific cell surface glycoprotein	P08472
OLUR_00019627	METTL27: Methyltransferase-like protein 27	Q8N6F8
OLUR_00005473	mfsd1: Major facilitator superfamily domain-containing protein 1	Q32LQ6
OLUR_00006189	mfsd4b: Sodium-dependent glucose transporter 1	A4QN56
OLUR_00030728	Mlp84B: Muscle LIM protein Mlp84B	Q24400
OLUR_00014585	Mme: Neprilysin	Q61391
OLUR_00019226	MPPED1: Metallophosphoesterase domain-containing protein 1	O15442
OLUR_00015401	mpv17: Protein Mpv17	Q5TZ51
OLUR_00016396	Mrc1: Macrophage mannose receptor 1	Q61830
OLUR_00005132	MRPL30: 39S ribosomal protein L30, C mitochondrial	Q4R6U7
OLUR_00000053	Msed_2001: 3-hydroxypropionyl-coenzyme A dehydratase	A4YI89
OLUR_00019682	MTR: Methionine synthase	Q4JIJ3
OLUR_00006430	MYORG: Myogenesis-regulating glycosidase	Q6NSJ0

OLUR_00020139	NOTCH2: Neurogenic locus notch homolog protein 2	Q04721
OLUR_00001182	Nrg: Neuroglian	P20241
OLUR_00007852	ntr-2: Nematocin receptor 2	O62169
OLUR_00023494	Nup98: Nuclear pore complex protein Nup98-Nup96	P49793
OLUR_00012950	OIH: Ovoinhibitor	P10184
OLUR_00009826	Orct: Organic cation transporter protein	Q9VCA2
OLUR_00014246	Osbpl8: Oxysterol-binding protein-related protein 8	B9EJ86
OLUR_00007085	pats1: Probable serine/threonine-protein kinase pats1	Q55E58
OLUR_00010618	pats1: Probable serine/threonine-protein kinase pats1	Q55E58
OLUR_00015409	PDIA4: Protein disulfide-isomerase A4	P13667
OLUR_00010297	PELP1: Proline-, C glutamic acid- and leucine-rich protein 1	Q1W1Y5
OLUR_00004258	Perlucin-like protein	P86854
OLUR_00024841	Perlucin-like protein	P86854
OLUR_00031580	pes1: Pescadillo homolog	Q7ZY69
OLUR_00020391	PHF24: PHD finger protein 24	Q9UPV7
OLUR_00006733	PHKA1: Phosphorylase b kinase regulatory subunit alpha, C skeletal muscle isoform	P18688
OLUR_00006923	PI4KA: Phosphatidylinositol 4-kinase alpha	O02811
OLUR_00007722	PIF1: ATP-dependent DNA helicase PIF1	Q9H611
OLUR_00000551	pkaR: cAMP-dependent protein kinase regulatory subunit	Q96UX3
OLUR_00011428	PLG: Plasminogen	P06868
OLUR_00000837	PLG: Plasminogen	Q5R8X6
OLUR_00010581	pm20d1.2: N-fatty-acyl-amino acid synthase/hydrolase PM20D1.2	Q08BB2
OLUR_00008613	Pm20d2: Peptidase M20 domain-containing protein 2	A3KG59
OLUR_00006738	PNLIPRP2: Pancreatic lipase-related protein 2	A5PK46
OLUR_00003156	pnr: GATA-binding factor A	P52168
OLUR_00026215	Polyenoic fatty acid isomerase	Q8W257
OLUR_00012063	Ppfibp1: Liprin-beta-1	Q8C8U0
OLUR_00003429	Prokineticin Bm8-f	Q8JFX8
OLUR_00006792	PSMD2: 26S proteasome non-ATPase regulatory subunit 2	P56701
OLUR_00003910	Ptchd3: Patched domain-containing protein 3	Q0EEE2
OLUR_00000063	Rab35: Ras-related protein Rab-35	Q5U316
OLUR_00030952	RF_0381: Putative ankyrin repeat protein RF_0381	Q4UMH6
OLUR_00021095	RF_0381: Putative ankyrin repeat protein RF_0381	Q4UMH6
OLUR_00016450	RF_0381: Putative ankyrin repeat protein RF_0381	Q4UMH6
OLUR_00005336	RHBDF1: Inactive rhomboid protein 1	A9L8T6
OLUR_00006945	Rilpl1: RILP-like protein 1	D3ZUQ0
OLUR_00006249	Rnf213: E3 ubiquitin-protein ligase RNF213	E9Q555
OLUR_00000762	rnf8: E3 ubiquitin-protein ligase rnf8	Q803C1
OLUR_00017233	roco5: Probable serine/threonine-protein kinase roco5	Q1ZXD6
OLUR_00017405	RpL10: 60S ribosomal protein L10	O61231
OLUR_00031369	RPL17: 60S ribosomal protein L17	P37380
OLUR_00015500	RPS10: 40S ribosomal protein S10	O77302
OLUR_00032073	RpS15Aa: 40S ribosomal protein S15a	Q6XIM8
OLUR_00011247	rsp-7: Probable splicing factor, C arginine/serine-rich 7	O01159
OLUR_00016425	ruvbl1: RuvB-like 1	Q9DE26
OLUR_00013331	SACS: Sacsin	Q9NZJ4
OLUR_00019326	Sacs: Sacsin	Q9JLC8
OLUR_00018676	Scarf1: Scavenger receptor class F member 1	Q5ND28

OLUR_00024690	Sephs2: Selenide, C water dikinase 2	P97364
OLUR_00015749	SKIV2L: Helicase SKI2W	Q15477
OLUR_00010108	slc16a12: Monocarboxylate transporter 12	Q6P2X9
OLUR_00004850	Slc17a9: Solute carrier family 17 member 9	Q8VCL5
OLUR_00007946	slc22a6-a: Solute carrier family 22 member 6-A	Q66J54
OLUR_00022102	Slc23a1: Solute carrier family 23 member 1	Q9Z2J0
OLUR_00031094	Slc4a7: Sodium bicarbonate cotransporter 3	Q9R1N3
OLUR_00020573	Slc7a11: Cystine/glutamate transporter	Q9WTR6
OLUR_00006436	SLC7A5: Large neutral amino acids transporter small subunit 1	Q01650
OLUR_00000291	SLC7A9: b	Q9N1R6
OLUR_00010465	SMOX: Spermine oxidase	Q9NWM0
OLUR_00024710	sn: Protein singed	Q24524
OLUR_00029494	SPBC16D10.01c: Probable assembly chaperone of rpl4	Q1MTN8
OLUR_00009680	SPOCK3: Testican-3	Q9BQ16
OLUR_00027313	Srgap1: SLIT-ROBO Rho GTPase-activating protein 1	Q91Z69
OLUR_00020487	stard3: StAR-related lipid transfer protein 3	Q90ZB9
OLUR_00016331	Stx7: Syntaxin-7	O70439
OLUR_00025850	SUGCT: Succinate--hydroxymethylglutarate CoA-transferase	Q9HAC7
OLUR_00007158	SUPV3L1: ATP-dependent RNA helicase SUPV3L1, C mitochondrial	Q5ZJT0
OLUR_00001624	Syt11: Synaptotagmin-11	Q9R0N3
OLUR_00011931	TBK1: Serine/threonine-protein kinase TBK1	Q9UHD2
OLUR_00006736	Thap1: THAP domain-containing protein 1	Q5U208
OLUR_00017777	THAP9: DNA transposase THAP9	Q9H5L6
OLUR_00006555	Thyrostimulin beta-5 subunit	A0A0F7YZI5
OLUR_00018271	tmem97: Sigma intracellular receptor 2	Q6DFQ5
OLUR_00026489	TNKS: Tankyrase-1	O95271
OLUR_00008939	TPST2: Protein-tyrosine sulfotransferase 2	Q5ZJI0
OLUR_00012411	TRAF3IP2: Adapter protein CIKS	O43734
OLUR_00004783	trim71: E3 ubiquitin-protein ligase TRIM71	E7FAM5
OLUR_00006456	TRPM3: Transient receptor potential cation channel subfamily M member 3	Q9HCF6
OLUR_00007164	TRPM6: Transient receptor potential cation channel subfamily M member 6	Q9BX84
OLUR_00002194	TSNAXIP1: Translin-associated factor X-interacting protein 1	Q2TAA8
OLUR_00020564	Tspan33: Tetraspanin-33	Q8R3S2
OLUR_00017521	TT10: Laccase-15	Q84J37
OLUR_00022795	Tubulin alpha-1 chain	P02552
OLUR_00017099	ubiG: Ubiquinone biosynthesis O-methyltransferase	Q820B5
OLUR_00012780	UCP2: Mitochondrial uncoupling protein 2	O97562
OLUR_00027608	unc93a: Protein unc-93 homolog A	Q6DDL7
OLUR_00005827	VASH2: Tubulinyl-Tyr carboxypeptidase 2	Q86V25
OLUR_00019157	vps29: Vacuolar protein sorting-associated protein 29	Q7ZV68
OLUR_00027063	VWDE: von Willebrand factor D and EGF domain-containing protein	Q8N2E2
OLUR_00025976	Vwde: von Willebrand factor D and EGF domain-containing protein	Q6DFV8
OLUR_00028052	WRN: Werner syndrome ATP-dependent helicase	Q14191
OLUR_00014688	xpnpep1: Xaa-Pro aminopeptidase 1	Q54G06
OLUR_00002918	Xpnpep2: Xaa-Pro aminopeptidase 2	B1AVD1
OLUR_00026221	ZNF862: Zinc finger protein 862	O60290
OLUR_00021017	ZNF862: Zinc finger protein 862	O60290
OLUR_00015491	ZNF878: Zinc finger protein 878	C9JN71

Table 3**:** Genes that were constitutively expressed in Dabob Bay larvae at different levels compared to other populations.

***O. lurida* gene ID**	**Gene**	**Uniprot SPID**
OLUR_00010887	FMO5: Dimethylaniline monooxygenase [N-oxide-forming] 5	P49326
OLUR_00013658	At5g10370: ATP-dependent RNA helicase DEAH1, 2C chloroplastic	F4KGU4
OLUR_00013228	HELZ2: Helicase with zinc finger domain 2	Q9BYK8
OLUR_00004343	FAT2: Protocadherin Fat 2	Q9NYQ8
OLUR_00016966	DDB_G0292642: Uncharacterized protein DDB_G0292642	Q54CX4
OLUR_00022057	MSMEG_3950: Universal stress protein MSMEG_3950/MSMEI_3859	A0QZA1
OLUR_00025230	ghrA: Glyoxylate/hydroxypyruvate reductase A	Q8FIT1
OLUR_00000611	L-proline trans-4-hydroxylase	R9UTQ8
OLUR_00029178	ucpB: Mitochondrial substrate carrier family protein ucpB	B0G143
OLUR_00004362	bmp2: Bone morphogenetic protein 2	Q804S2
OLUR_00019157	vps29: Vacuolar protein sorting-associated protein 29	Q7ZV68
OLUR_00031354	DDB_G0287015: TM2 domain-containing protein DDB_G0287015	Q54KZ0
OLUR_00003900	AAO: L-ascorbate oxidase	Q40588
OLUR_00004324	UNC93A: Protein unc-93 homolog A	A2VE54
OLUR_00003482	lrrc58: Leucine-rich repeat-containing protein 58	Q32NT4
OLUR_00013735	cep290: Centrosomal protein of 290 kDa	P85001
OLUR_00018610	Helz2: Helicase with zinc finger domain 2	E9QAM5
OLUR_00019426	K02F3.12: Putative ATP-dependent DNA helicase Q1	Q9TXJ8
OLUR_00022209	Ectin (Fragment)	B3EWZ8
OLUR_00020882	UBA6: Ubiquitin-like modifier-activating enzyme 6	A0AVT1
OLUR_00005664	ABCC1: Multidrug resistance-associated protein 1	Q5F364
OLUR_00001519	HET-E1: Vegetative incompatibility protein HET-E-1	Q00808
OLUR_00025496	HSPA12B: Heat shock 70 kDa protein 12B	Q96MM6
OLUR_00019143	NADSYN1: Glutamine-dependent NAD(+) synthetase	Q5ZMA6
OLUR_00010983	CLEC3A: C-type lectin domain family 3 member A	O75596

VITA

Laura Spencer obtained her B.S. in Chemical Oceanography from the University of Washington in 2011. She began her career encouraging Seattle residents to make their homes more energy efficient while working for SustainableWorks, a non-profit dedicated to reducing energy consumption, where she held various positions over three years, culminating in Program Manager of their solar energy program. She then spent a year as a Research Associate with Ross Strategic, a Seattle-based environmental consulting firm, assisting with a variety of projects ranging from salmon recovery to air quality on tribal lands. During that year she was also scheming to return to the marine sciences and pursued a variety of volunteer positions with the Seattle Aquarium, Ocean Inquiry Project, and Washington Sea Grant. A chance meeting with the Puget Sound Restoration Fund (PSRF) led to an incredibly fun and inspiring summer internship working with the Olympia oyster and Pinto abalone. She remained with PSRF for a year, learning how to culture shellfish and microalgae, and to run hatchery-based shellfish experiments. She joined the Roberts Lab in 2016. Laura lives in White Center (near West Seattle) with her husband, son, one cat and six chickens.