Developing Tools and Resources for Maturation Control in *Bivalvia*

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Abstract

Developing Tools and Resources for Maturation Control in *Bivalvia*

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The significance of environmental stress and the physiology of bivalve mollusks is investigated under multiple- stressor conditions. The primary study animals are Pacific oysters (*Crassostrea gigas*) and geoduck clams (*Panopea generosa*) because they are the primary species of concern for the Aquaculture industry on the West Coast of the US. A laboratory study was performed with diploids and chemically induced triploids Pacific oysters under three increasingly stressful environmental conditions. There was an elevated response of the metabolic enzyme citrate-synthase during multiple-stress treatment. Differential mortality between triploid and diploid oysters was also observed in conjunction with field studies and farm observations. To investigate alternatives to triploidy for enhanced growth and marketability in the shellfish aquaculture industry, genomic and proteomic annotations were generated for Pacific geoduck leveraging

previous resources from five other clam species: *Mercenaria mercenaria, Archivesica marissinica, Ruditapes philippinarum, Spinsula solida*, and *Mactra quadrangularis*. Reproductive genes with high homology were the focus of this study for potential gene silencing applications. In addition, the feasibility of using electroporation and Morpholino-oligomers for molecular delivery in both Pacific oysters and geoduck clams was investigated. This research provides valuable insights into stress tolerance, reproductive genetics, and gene silencing potential in these economically and ecologically significant shellfish species.

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ACKNOWLEDGEMENTS

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DEDICATION

This thesis is dedicated to my mother, Laurel Hensel. Sometimes dreams do come true.

Chapter 1. Citrate-Synthase Response and Multiple Stress in Pacific Oysters (*Crassostrea gigas*)

1.1 Abstract

The Pacific oyster, *Crassostrea gigas*, an intertidal species that has colonized parts of the West Coast of the US and Canada, has significant commercial, cultural, and ecological importance. Currently, *C. gigas* is experiencing several increasing environmental and physiological stressors such as rising air and water temperatures. Triploid (3n) Pacific oysters experience heightened mortality in high temperature environments, also known as summer 'triploid mortality' events. In this study, we investigated the effect of chromosome set duplication (polyploidy) on physiological performance and survival during single heat stress (30°C) and multiple heat stress (30°C + 44°C). Physiological performance was measured by quantifying levels of citrate-synthase, a rate determining enzyme in the citric acid cycle involving mitochondria. Citrate-synthase activity was influenced by multiple heat stress but not polyploidy or oyster size. Survival of diploid (2n) oysters did not vary between single stress and multiple stress treatments. However, more triploids died when exposed to the multiple versus single heat stress treatment. This is one of the first studies to investigate the impact of polyploidy on oyster stress tolerance following a multiple stress exposure and explores the potential role of citrate-synthase in measuring energy metabolism in oysters.

1.2 Introduction

The Pacific oyster *Crassostrea gigas* is the most commonly farmed shellfish species in the world (Washington Sea Grant, 2015). Pacific oysters were introduced into the west coast of the

United States as a new aquaculture species due to their ease of culture and rapid growth (Cheney, Daniel P., Thomas F. Mumford, 1986). Pacific oyster culture has expanded throughout the NE Pacific and globally, especially in Asia. From 2000 to 2017, the rate of oyster aquaculture grew by 30% in China as opposed to 3.5% in the US (Naylor et al., 2021).

The Pacific oyster, as an intertidal species, typically experiences a wide range of seasonal temperatures from 4°C to 24°C; spawning often takes place above 19°C (Quayle, 1969). Pacific oysters undergo maturation during the spring and summer along the NE Pacific when the market for oysters is highest (Washington Sea Grant, 2015). Those reared in warm, eutrophic embayments often die in the summer months due to reproductive stress and or disease along the US west coast (Friedman et al., 1991). In addition to its importance as an agricultural industry, oyster aquaculture is increasingly recognized for its ecosystem services as well as its potential to support global nutritional security (Naylor, 2016). It is vital that we better understand the reproductive physiology of this commercially, culturally, and ecologically important species.

Production of sterile oysters, in which spawning is markedly reduced or absent, has been a popular method to reduce or eliminate the summer spawning period (Stanley et al., 1984). Additionally, sterile oysters experience increased growth rates thereby reducing the time from plant to harvest, (Barber & Mann, 1991). The development of triploid oysters, those with three chromosome copies or 3n, effectively reduces gamete production and enhances growth (Nell, 2002).

Triploids are produced by inhibiting the release of polar body i or ii during meiosis using a variety of methods; the two most common methods use chemicals or breeding (P. Wadsworth

et al., 2019; Yamamoto & Sugawara, 1988). Once fertilization occurs, the eggs resume the maturation process and after about 2–3 weeks, swimming larvae go through metamorphosis and transform into sessile juveniles, and continue to grow into adults, (Gosling, 2015). Currently, triploid seed production in most commercial hatcheries uses eggs from normal diploids (2n) that are fertilized with sperm from tetraploids (4n). To ensure predictable spawning success and avoid contamination, gamete collection for triploid seed production is performed using the strip spawning method, which is applicable for most oyster species (Z. Wang et al., 2002).

Triploid oysters have enhanced growth rates and increased marketability compared to diploids because triploids grow linearly through the period of reproduction, whereas diploids grow little during spawning (Allen & Downing, 1986). In Virginia, triploid Eastern oysters make up 80-95% of total oysters grown from 2008 to 2014 (Murray & Hudson, 2015) while on the West coast, triploid Pacific oysters make up ~50% of total oyster production (FAO, 2018).

Pacific oysters have been experiencing increased susceptibility to environmental perturbations such as elevated summer low tide temperature, variable salinities, and disease, (P. C. Wadsworth, 2018). Since 2008, oyster mortality events (>20%) have become regular summer occurrences, (Ashton et al., 2020). The cause of mortality events is inconclusive and can be location specific. High summer temperatures coincide with sexual maturation in diploid oysters, suggesting that they use energy for gametogenesis and there is less energy available for immune defense (Gagnaire et al., 2006). Due to reduced gametogenesis in triploid oysters, it is expected that they would experience lower mortality in the summer. However, several studies comparing mortality rates in triploid and diploid oysters found contrasting conclusions.

Along the U.S. West Coast, Allen & Downing (1986) found greater survival in their triploid versus diploid Pacific oyster stocks. In France, Pernet et al., (2012) found that diploid Pacific oysters died twice as often as triploid oysters in summer and autumn months, while Garnier-Gere et al., (2002) found no differential survival between diploids and triploids. On the other hand, Cheney et al., (2000) found greater mortality rates in triploid Pacific oysters than diploid oysters during periods of elevated air and water temperature. More recently, Wadsworth (2018) and Matt et al., (2020) found higher triploid mortality in the Eastern oyster, *C. virginica*, during field studies. Samain (2011) hypothesized that the main reasons for oyster mortality events are temperature, reproduction, or metabolic stress. The difference in triploid versus diploid performance could also be indicative of partial sterility in triploid oysters where the energy and biomass normally required for spawning is reallocated to somatic growth and results in a higher glycogen content, (Yang et al., 2016). Understanding the physiological mechanisms underlying observed differential performance between triploid and diploid oysters may aid in reducing the occurrences of summertime mass mortalities.

Comparison of relative energy production in oysters before a mass-mortality event may augment our understanding of stress physiology in triploid oysters. One way to measure energy metabolism in oysters is to measure the activity of the metabolic enzymes of citrate-synthase. Citrate-synthase is a homodimeric protein conventionally designated as the first enzyme of the citric-acid cycle, (Weitzman & Danson, 1976). Fuel molecules such as glucose, amino acids and fatty acids are oxidized, and ATP is produced during the citric acid cycle. Mitochondrial citrate-synthase is the key enzyme in aerobic energy production and is a useful indicator of general physiological status of *C. gigas* (García-Esquivel et al., 2001; Pernet et al., 2012).

Citrate-synthase activity (nmol min-1 mg-1) can also be used to predict an organism's tolerance to climate change, (Angonese et al., 2022). In *C. gigas*, citrate-synthase activity reflects the mitochondrial oxidation capacity and has been shown to be positively correlated with respiration rate and mortality (Pernet et al., 2012). Additionally, in *C. gigas*, citrate-synthase activity has also been shown to be positively correlated with OsHv-1 infection caused by the Warburg effect, which is an atypical metabolic shift towards aerobic glycolysis that provides energy for rapid cell division (Delisle et al., 2018). In other marine organisms such as larval zebrafish, Van de Pol et al., (2021) found that both temperature and ploidy affected the expression of genes related to metabolic processes (citrate-synthase specifically). In this study, we leverage citrate-synthase enzyme activity to better understand summer mortality events in triploid oyster stocks, and their metabolic capacity while experiencing variable environmental stress.

1.3 METHODS

Triploid and diploid Pacific oysters (*Crassostrea gigas*), (3n=250, 2n=250) were obtained from Coast Seafoods Co.'s Shellfish Hatchery, located on Quilcene Bay, Quilcene, Washington (47°48'N 122°52'W). Triploid oysters were produced by larval exposure to thermal shock according to the methods of Yamamoto et al., (1988). Oysters were received in March of 2021 and transferred to the Jamestown S'Klallam Point Whitney Shellfish Hatchery, Quilcene, Washington (47°45"N 122°50'W). A sub-sample of the animals (3n=15, 2n=15) were sacrificed for histological analysis of gonadal sections. Sampled gonad tissue was placed within a histology cassette and fixed using the PAXgene tissue fixative and Stabilizer system (Qiagen, Hilden, Germany). Images of diploid and triploid gonad sections were assigned to

reproductive stages in accordance with ploidy-specific metrics as outlined by (Ezgeta-Balić et al., 2020) and (Matt & Allen, 2021) respectively. Ploidy analysis revealed that 100% of oysters tested corresponded to their presumed ploidy. All other oysters were labeled by affixing numbered wire tags to the right shell value using cyanoacrylate. The animals received 20 days of laboratory acclimation at 20°C. After the acclimation period, the remaining oysters were submerged or placed in either a *control* (20°C, n=112), *single-stress* (30°C, n=112), or *multiple-stress* (30°C + 44°C, n=112) treatment baths or desiccation tub, each with 50-50 ratio of triploids to diploids (Figure 1.1). The treatment groups are as follows: diploid-control (2n-C), triploid-control (3n-C), diploid heated (2n-H), triploid heated (3n-H), diploid heated and desiccated (2n-HD), triploid heated and desiccated (3n-HD).

For the elevated temperature groups (heated, heated and desiccation) the water temperature was increased by 2°C per day till 30°C at day 25. After the initial temperature ramp to 30°C for the heated group, the heated + desiccation group of oysters were subjected to an additional stressor of dry heat at 44°C for 6 hours using three ceramic heat lamps, simulating a summertime low-tide heatwave. After the dry heat stress at 44°C, treated animals were placed back in the heated bath through day 60 to monitor survival. One day after their treatment the oysters were sacrificed for citrate-synthase activity analysis. The sampling occurred on day 26 for the control group (n=12) and heated group (n=12) and on day 27 for the desiccation group (n=12). Gill tissue was excised from each oyster, frozen in liquid nitrogen and transferred to a -80°C freezer at the University of Washington in Seattle for future analysis. Survival was monitored through day 60 in the remaining oysters (n=100 per group). Maximum shell length,

width and height of each oyster was recorded using vernier calipers to the nearest mm on day 1 and day 60. Shell volume was estimated from the measurements using the equation of ellipses.

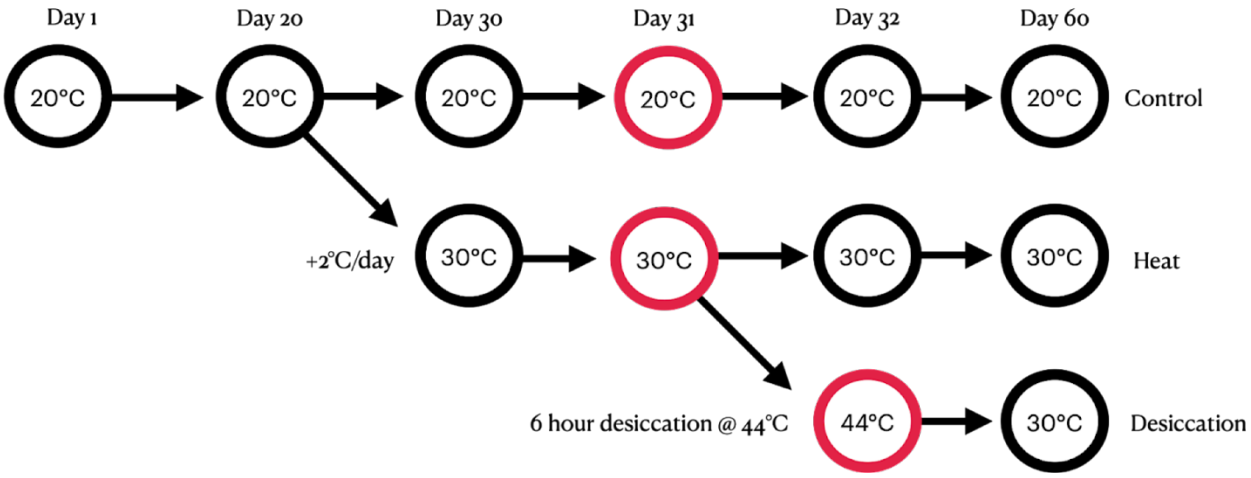

Figure 1.1: *Schematic of experimental design*. All oysters began at 20°C and spent 20 days acclimating to hatchery conditions. At day 20 the groups were split into three groups (n=112 each): control, heated (30°C) and desiccation (44°C) with the heated and desiccation groups acclimated to warmer water at a rate of 2°C per day. Temperature of the treatments indicated inside the circles. Tissue samples were taken on day 31 for the control and heated treatments and on day 32 for the desiccation treatment (in red). All oysters were followed through day 60 for mortality.

Citrate-synthase enzyme activity was determined on day 26 and 27 for a total of 72 samples (n=12 per treatment group), (Figure 1.1). Gill tissue (10-20mg) was homogenized with a handheld homogenizer for 60 seconds per sample in 350 uL of ice cold citrate-synthase Assay Buffer (ab239712, Abcam PLC, Cambridge, UK). Once homogenized, tissue supernatant was processed in a microplate standard assay according to the Bovine Serum Albumin Assay (500-0201, Bio-Rad Laboratories, Inc., Hercules, CA, USA) to determine protein concentration. The BSA standard regression line (BSA protein (ug/mL)= 2300*A - 1500) was fitted along the

linear portion of the line (0, 1000 ug/mL BSA) where A is the absorbance. Samples were measured using Perkin Elmer 1420 Multilabel Counter spectrophotometer at 570 nm in triplicates and averaged.

Relative citrate-synthase enzyme activity was obtained using a 50uL sample volume and 50uL reaction mix (43uL buffer, 5uL developer, and 2uL substrate) according to the Citrate-synthase Assay Kit (abc239712, Abcam PLC, Cambridge, UK). A standard linear regression line (Eq. 2) was fitted with standard citrate-synthase enzyme concentration using Glutathione Stock Solution (GSH) from 0 nmol to 40 nmol in response to optical density (a measure of absorbance) at 405 nm on the spectrophotometer. Samples were measured in triplicate at 405 nm for 45 minutes (ΔT) at 25°C and the change in optical density (ΔOD) was measured as the maximum absorbance (ODmax) subtracted from the minimum absorbance (ODmin) per sample. Background control wells (citrate-synthase activity = 0 nmol/min/mg) were run in parallel to citrate-synthase sample wells for one plate and did not respond to the substrate mix and therefore the citrate-synthase wells alone were used in the final calculations. To obtain the equivalent nmol of the citrate-synthase enzyme per sample, input the citrate-synthase enzyme concentration (nmol) from the citrate-synthase kit and the relative protein concentration, P (mg/uL), (after conversion from ug/mL to mg/uL) from the BSA kit into Eq. 3 to calculate the individual sample activity of citrate-synthase (nmol min^{-1} mg^{-1}). As the oysters experience heat and/or multiple-stress, the expectation is that citrate-synthase response will increase and that there should be a significant difference between treatment groups and ploidy levels as the animals respond to increasing heat stress.

Equation 1:

$$BSA(ug/mL) = 2300(A - 1500)$$

Equation 2:

$$GSHnmol = (33 \times \Delta OD) + 2.2$$

Equation 3:

$$CS(nmol/min/mg) = \frac{GSHnmol}{\Delta T(min) * V(uL)} \times \frac{1}{P(mg/uL)}$$

All statistical analyses were performed using RStudio IDE v.2022.02.3.492 "Prairie Trillium" Release in R v.4.1.1. To check if the data need transformation, a Shapiro-Wilk test for normality on Citrate Synthase Activity (p=0.575) allowed for the data to remain untransformed. All citrate-synthase data points with a Cook's distance > 0.5 were removed as an outlier (n=1). For each of the six treatment group tested (2n-C, 2n-H, 2n-HD, 3n-C, 3n-H, 3n-HD), the effect of the combined treatment (ploidy and heat) on citrate-synthase activity was evaluated by two-way Analysis of Variance (ANOVA) following the protocol of Angonese et al., (2022). A post hoc Tukey (Honestly Significant Difference) HSD test established which treatment effect was different from the controls. In addition, due to unbalanced data, a multiple variant comparison was run to determine significance codes (Figure 1.2).

One-way analysis of Variance (ANOVA) of the morphometric data where SS(shell height, shell width, shell length, calculated dry weight) was done to determine if a response variable other than our stressors (heat or ploidy) affects citrate-synthase activity. Removing the morphometric data from the model and focusing on a smaller model where SS(ploidy | heat,

ploidy*heat), a two-way ANOVA (Table 1.1) was run to determine if any interactive effects of ploidy and heat stress existed. To determine if citrate-synthase activity increased with increasing stress, a post-hoc Tukey Honestly Significantly Different (HSD) test (Table 1.2) on the treatment response variable to determine if citrate-synthase activity increased with increasing stress (ploidy, heat or desiccation). In addition, a Kaplan-Meier survival analysis examined mortality patterns during the experiment (60 days). Examining differences in survival was used as an additional assessment of an oyster's ability to handle stress. We hypothesized that triploid oysters would experience lower survival than diploid oysters.

1.4 RESULTS

Citrate-synthase activity ranged from 0.689 to 6.737 (nmol/min/mg), (Figure 1.2). Neither protein content, nor tank placement, nor shell volume, nor sample date correlated with citrate-synthase activity, (Table S1). There was no correlation between citrate-synthase activity and amount of protein measured in the sample (Figure S2). Ploidy (triploids vs diploids) did not influence citrate-synthase activity ($p = 0.537$), (Table 1.1). Citrate-synthase activity varied in oysters under different treatments ($p < 0.0005$). Citrate-synthase activity increased across the treatments, with average values for control, single stress and multiple stress as 1.83, 1.99 and 3.45 (nmol/min/mg) respectively. No difference between citrate-synthase activity between the heated and the control groups was observed, ($p = 0.95$). The most influential treatment was in the multiple-stress group, which had elevated citrate-synthase activity relative to both the control and the single-stress group ($p = 0.0002$ and 0.0001 respectively).

Table 1.1. *Two-Way ANOVA where multiple groups were compared.* Single stress and multiple stress groups are the only significant results at the 0.05 level.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
ploidy	1	0.34	0.337	0.385	0.5371	
single stress	1	4.77	4.767	5.449	0.0229	*
multiple stress	1	17.07	17.075	19.517	4.24E-05	***
ploidy x single stress	1	2.63	2.627	3.003	0.0882	.
ploidy x multiple stress	1	0.23	0.232	0.265	0.6088	
Residuals	60	52.49	0.875			

Table 1.2 *Tukey Honestly Significantly Different (HSD) test* on temperature groups. Multiple stress group is the only group significantly different from control (p = 0.0002).

	diff	lwr	upr	p adj	
multiple stress: control	1.1829	0.5105	1.8552	0.0002	***
single stress: control	-0.0805	-0.7610	0.5999	0.9565	
single stress: multiple stress	-1.2634	-1.9512	-0.5756	0.0001	***

Survival varied among the six treatment groups (2n-C, 2n-H, 2n-HD, 3n-C, 3n-H, 3n-HD), ($p < 0.0001$), and ranged from 63-94%. The highest survival was observed in the diploid control group and lowest in the triploid heat + desiccation group (Figure 1.3). Survival did not vary between the single heat stress treatments (2n-H, 3n-H) and the controls (2n-C, 3n-C, $p=0.400$). However, Survival varied between the single heat stress and multiple stress (2n-HD, 3n-HD, $p < 0.0001$) regardless of ploidy. Ploidy alone was sufficient to produce differential survival ($p < 0.05$). No differential survival existed between diploid oysters exposed to a single stress or multiple stresses ($p = 0.06$), while more triploids oysters died in those exposed to multiple relative to single stressors ($p < 0.0001$).

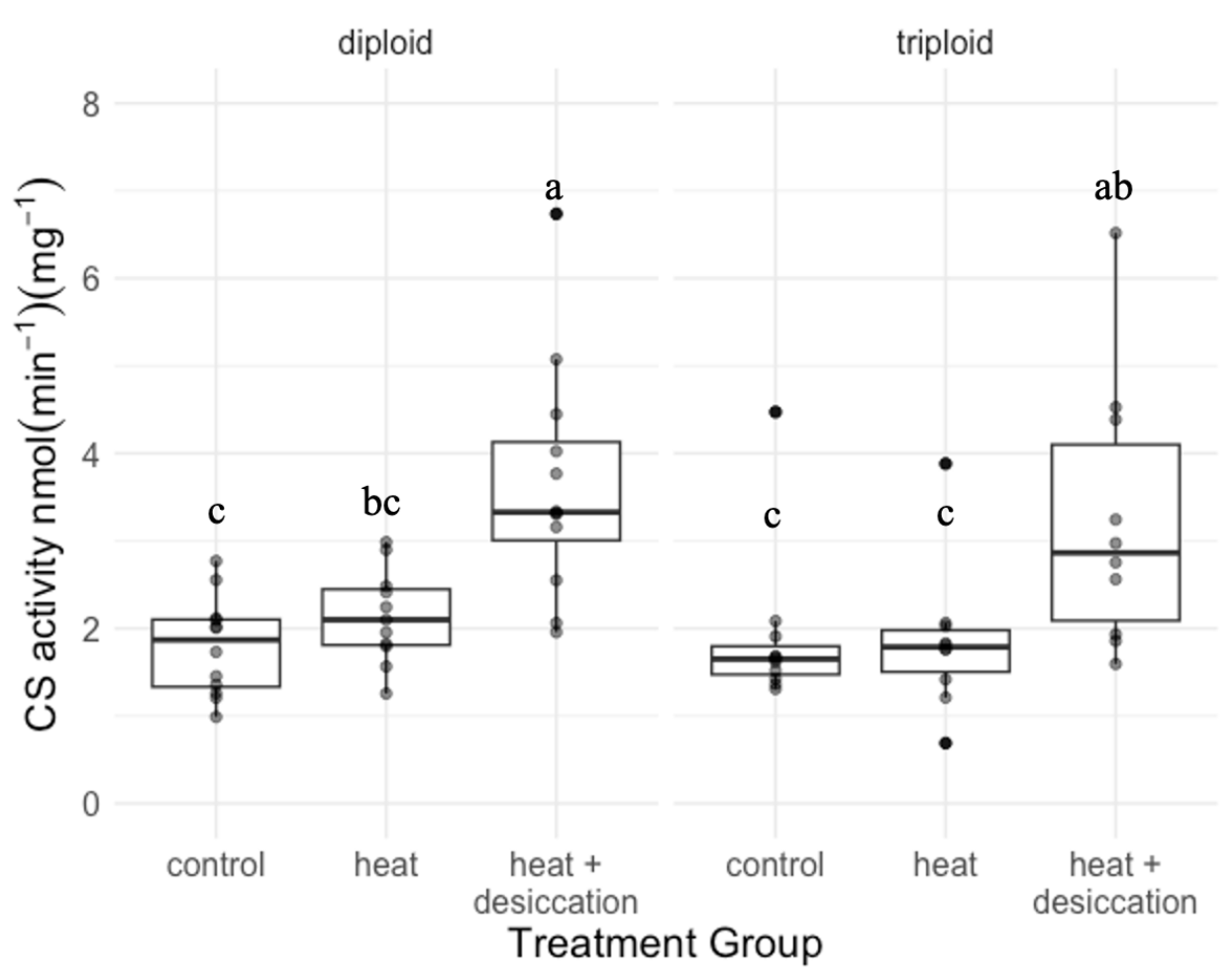

Figure 2: *Citrate-Synthase Response by Ploidy and Temperature*. Citrate-synthase activity (nmol/min/mg) by heat group or ploidy level (diploid/triploid). No differences between ploidy were detected but the heat + desiccation group had elevated CS activity.

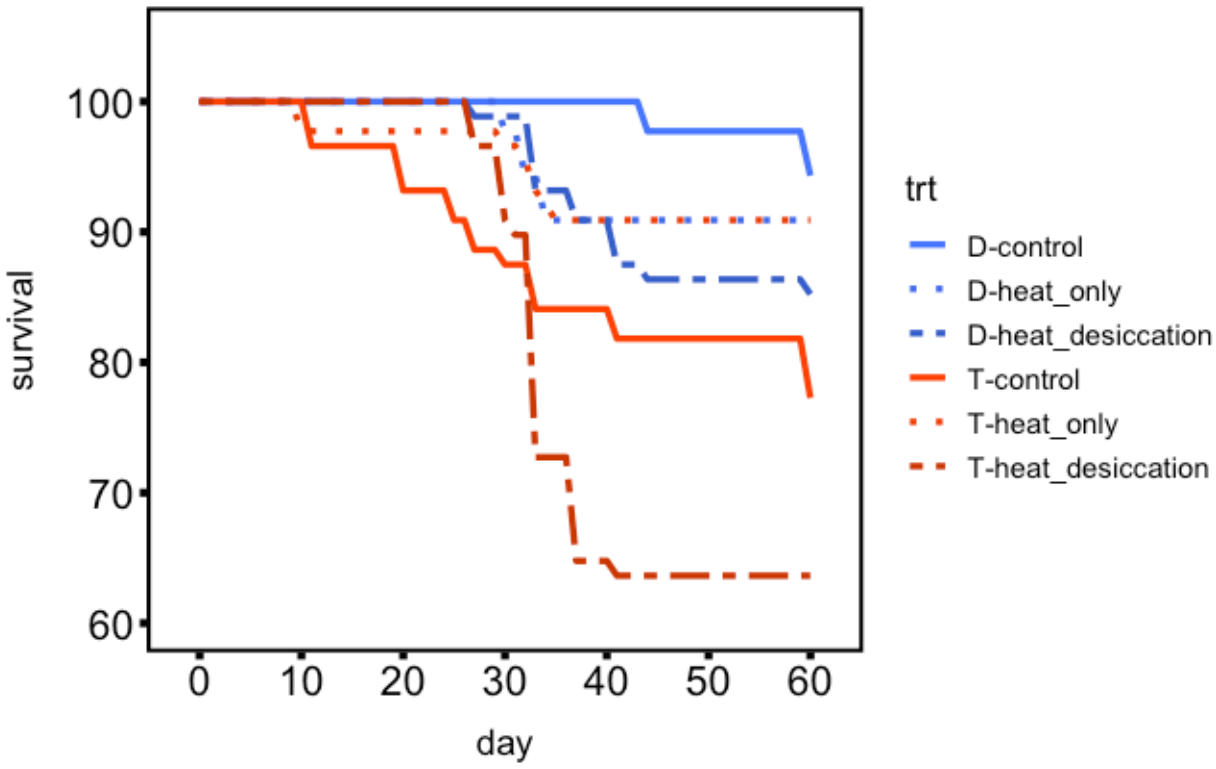

Figure 3: *Kaplan-Meier Survival Curve***.** Blue lines are diploids (2n) and orange lines are triploids (3n). Dotted lines are treatment groups and solid lines are control groups.

1.5 Discussion

The present study investigated the effect of various stressors on the citrate-synthase activity and survival of diploid and triploid oysters. We found differential mortality in triploids versus diploids, similar to the mass mortality events seen in the field with diploids outperforming the triploids by 13% (238 diploids versus 204 triploids survived out of 264 total oysters per treatment). We also found elevated citrate-synthase activity in the multiple-stressed group (30°C + 44°C) over the single-stress group (30°C) and the control (20°C). Surprisingly, there

was no differential citrate-synthase activity between triploid and diploids, as would be expected from a stress assay where the treatment animals experienced differential survival.

Regarding survival analysis, this study found significant differences in survival rates among the different treatment groups. The triploid desiccation group experienced the lowest survival rate, while the diploid control group experienced the highest survival. Ploidy alone was found to influence survival rates, with diploid oysters having better survival rates than triploid oysters. These results agree with reports from farmers who have documented triploid mortality events for over a decade, (Matt et al., 2020). Furthermore, there is a significant difference in survival rates between the single heat stress and multiple stress groups, with the latter showing lower survival rates regardless of ploidy.

The results showed that citrate-synthase activity does not correlate with protein concentration, tank placement, shell volume or sample date. Additionally, ploidy did not have any significant effect on citrate-synthase activity. However, there was a difference in citrate-synthase activity among the different heat treatment groups, with the multiple-stress group experiencing the most elevated response. The lack of differential results between triploids and diploids is surprising because multiple studies have demonstrated differential physiological responses between triploids and diploids, (Ashton et al., 2020; Cheney et al., 2000; X. Y. Li & Zhou, 2014; Pernet et al., 2012). One of the first studies to notice differential performance of triploid and diploids in the field, Cheney et al., (2000) set out to characterize the relationship between summer mortality of Pacific oysters and infectious disease. Instead, they observed high mortality rates of triploids to elevated temperatures. Pernet et al., (2012) did a similar field

study in the Mediterranean Sea where they were looking at the effect of farming sites and ploidy levels on mortality and pathogens of Pacific oysters. In contrast to our results, they found that mortality coincided with infections involving OsHV-1 and *Vibrio splendidus* when seawater temperatures were between 19°C and 24°C, with elevated diploid mortality when temperatures were above 24°C. Li & Zhou (2014) also studied the energy budgets of triploids versus diploids and they found that the influence of ploidy and temperature was significant. Their results demonstrated that triploids, which did not reproduce and only had limited gonadal development, spent more energy for growth and were more efficient in using energy than did diploids. Another European study by Ashton et al., (2020) studied oyster summer mortality events in Lough Foyle in Northern Ireland where large mortality events correlated with early summer temperature rises above 16°C. The authors suggested mitigation strategies based on their findings such as: planting oysters at lower densities and at lower tidal positions to increase survival. The findings previously listed and results from this study suggest that multiple stressors have a greater impact on the metabolic activity of oysters than single stressors with ploidy not appearing to affect the activity of mitochondria per unit of protein.

Pacific oysters experiencing more than one stressor are more likely to experience mass mortality events. Our results still raise the question of how to explain a lack of differential mitochondrial activity between diploids and triploids when we saw differential survival? The new information presented in this study suggests that differential mortality is not induced by polyploidy alone that is causing mortality but rather, under multiple stress, the triploids will perform worse than the diploids, (Cheney et al., 2000; X. Y. Li & Zhou, 2014; Y. Li et al., 2022a). Besides temperature, other studies on oysters have included disease, turbidity salinity,

and substrate as other stressors that produce differential results between triploid and diploids (Haberkorn et al., 2010; Pernet et al., 2012; P. C. Wadsworth, 2018). It has also been suggested that reproduction is also reduced during heat stress (Nash et al., 2019). The reason we observed differential triploid mortality and did not see differential triploid citrate-synthase activity is because triploids have a more accessible energy store in the form of glycogen that diploids lack during summer months when diploids are actively reproductive, (Yang et al., 2016). It may be that triploids expend this type of energy store quickly when stressed and deplete excess energy whereas diploids can use reserves in the form of gametes.

Taken together, these findings suggest that multiple stressors have a greater impact on both citrate-synthase activity and survival of oysters than do single stressors. Moreover, ploidy status plays a significant role in determining the survival of oysters under stress, with triploid oysters showing a lower survival rate than diploid oysters. The results of this study have important implications for understanding the impact of stress on oyster physiology and survival and will inform strategies for mitigating the effects of stress in oyster aquaculture.

Chapter 2. Comparative Genomic Analysis of Geoduck Clam (*Panopea generosa*)

2.1 Abstract

Pacific geoduck (*Panopea generosa*) is a subtidal clam species with a range from Alaska to Baja California. In this study, five transcriptomic libraries from three tissue types (gonad, heart, ctenidia) and two different life stages (larvae, juvenile) were assembled and annotated with biological ontological information. A particular emphasis in this study were reproductive genes and the gonad library, as the most likely location for reproductive candidate genes for gene editing. In addition, a comparative genomic approach was used to look for homologous genes across the *Venerida* clade. This effort represents an establishment of an important genomic resource for Pacific geoduck that will be valuable in the improvement of sustainable aquaculture.

2.2 Introduction

Farming of geoduck clams, *Panopea generosa*, in the cold, nutrient-rich, and clean waters of the Pacific Northwest is a long-standing tradition and important cultural, economic, and ecological part of the coastal communities, (Feldman et al., 2004). Geoduck clams live deep in the intertidal zone (-2.0 feet and below) but have been observed as deep as 360 feet (WDFW, 2023). Geoduck clams are large, long-lived, and fecund. They are reproductive as males as early as 2 years old and are often considered market size at 5 years old. In the wild, geoducks are found between 18-80 feet deep and take 15 years to reach full maturation (~7 lbs.). The oldest recorded living geoduck was 173 years old and can be sexually mature up to 50 years

(Edge et al., 2021). Due to their long-lives and sexual asynchronicity, they have a low effective population size (Vadopalas et al., 2015).

Geoduck clams are an increasingly important fishery and aquaculture product for the Eastern Pacific coast of the US from Baja California to Alaska. The geoduck industry consists of a small number of private operators committed to harvesting, processing, and marketing their product. Geoduck meat is sold primarily outside of the US; siphon meat goes to Japan and Taiwan while body meat is sold in California and the East Coast, (Cheney, Daniel P., Thomas F. Mumford, 1986). Geoduck aquaculture is considered the most economically important clam fishery in North America (Hoffman et al., 2000), bringing in $24.5 million in sales and over $1 million in state revenue in 2013 (Washington Sea Grant, 2015). Geoduck aquaculture also supports local oyster farming as well due to its high price per square acre. Recent evidence also suggests that geoduck aquaculture gear can support the recovery of the threatened cockle species in Washington State (Dimond et al., 2022).

Geoducks are primary consumers of phytoplankton by filter feeding. As filter feeders, geoducks provide essential ecosystem services as well by removing algae, organic matter, and excess nutrients from the water column (Cubillo et al., 2018). In addition, when geoducks are harvested excess nutrients such as nitrogen and phosphorus are removed from the marine ecosystem. Geoducks are a keystone species in the subtidal zones by removing organic matter from the environment and providing a food source for the declining populations of sea otters and crabs.

Wild populations have been threatened by overharvesting and poaching (KUOW, 2015). Due to concerns of genetic mixing between hatchery and wild populations, most aquaculture of

geoduck is done using wild broodstock, but the potential for genetic mixing still exists. Farmed geoducks may become reproductively mature as early as two years old (Vadopalas et al., 2015). Due to these concerns, a key area of research focuses on the reproductive biology of geoduck clams. The use of triploid geoduck clams may help alleviate reproductive maturation in farmed clams, akin to research done on ploidy in Pacific oysters (Allen & Downing, 1986). Due to issues with triploidy, there is a need to better understand the reproductive genes responsible for sexual maturation to provide resources for future work developing sterility approaches. This work could include the development of gene knock-down strategies.

The main objective of this study is to build annotated reference transcriptome libraries. There were three specialized tissue types: gonad, ctenidia, and heart. There were two tissue samples from pooled larvae and pooled juveniles as well. In addition, this study also leveraged tools and resources from previously published genome and transcriptome studies on clams from *Venerida*.

The publication of a fully annotated juvenile *P. generosa* reference genome by Putnam et al., (2022), along with previous studies focusing on geoduck genomics and gene expression in response to environmental stress, contributes to a comprehensive understanding of geoduck and mollusk genomics while shedding light on the role of DNA methylation in environmental acclimatization. Previous work on geoduck genomics focuses on gene expression in response to environmental stress. Work on the *P. globose* juvenile transcriptome exposed to chronic and acute thermal stress demonstrated that there were similar gene expression patterns between stress and non-stressed animals, (Juárez et al., 2018). In the same study there was also a high degree of expression genes related to DNA repair and transcription regulation in chronically

exposed juveniles where protective genes against oxidative stress were highly expressed in acutely exposed juveniles. Timmins-Schiffman et al., (2017), published the first proteomic study of three maturation stages in males and female geoduck clams using gonad proteins. They showed that gonad proteins became increasingly divergent between males and females as maturation progressed. Venkataraman et al., (2019) investigated sex-specific broodstock response and differential gene expression in *P. generosa* in response to low pH. Temperature and dissolved oxygen increases corresponded to differences in protein abundance patterns such as heat shock protein 90-α. In larvae, Timmins-Schiffman et al., (2020) looked at the proteomics of larval *P. generosa* with ciliate infection to investigate the molecular underpinnings of the innate immune response of the larvae to a pathogen. Ciliate response proteins included many associated with ribosomal synthesis and protein translation, suggesting the importance of protein synthesis during larval immune response. In juvenile *P. generosa,* Gurr et al., (2020, 2022) conditioned the animals before testing them with elevated pCO2 (~2400 utam). Following the secondary exposure, neither elevated nor ambient pCO2 altered juvenile respiration rates, indicating ability for metabolic recovery under subsequent conditions. Recently, Putnam et al., (2022), published an annotated *P. generosa* reference genome as part of a larger common-garden ocean acidification study. They looked at the role of DNA methylation on environmental acclimatization. Functional enrichment analysis of differentially methylated genes revealed regulation of signal transduction that influences cell growth, proliferation, tissue and skeletal formation, and cytoskeletal change. Putnam's work, as well as this study, will greatly aid in the collective understanding of not only geoduck genomics but overall mollusks genomics. In this study, five RNA-seq transcriptome libraries from three geoduck tissue types (gonad, heart, ctenidia) and two different life stages (larvae,

juvenile) were assembled and annotated with biological Gene Ontology information. A particular emphasis in this study were reproductive genes and the gonad library, as the most likely location for reproductive genes.

In addition to the five geoduck RNA-seq libraries described in this study, there is also value in leveraging publicly available data for a comparative clam species transcriptome study. Mun et al., (2017), published a transcriptome of the Manila clam (*Ruditapes philippinarum*) as part of a greater effort in selective breeding and disease control. They reported 41,275 annotated sequences in the *de novo* whole transcriptome assembly of *R. philippinarum* across three different tissues (foot, gill, and adductor muscle). Wang et al., (2016), was the first to publish an annotated transcriptome of the hard clam *Mercenaria mercenaria*. It was part of the work investigating the parasite QPX in hard clams. A *de novo* assembly was constructed and a consensus transcriptome of 62,980 sequences were functionally annotated. A total of 3,131 transcripts were identified as differentially expressed in healthy versus infected tissues. Comparative analysis of annotated genes can reveal the conserved molecular mechanisms between mollusks, such as genes with high homology expressed across the Venerida clade.

Genome resources for clams in the Venerida clade are more abundant than transcriptome or proteomic resources. A reference genome is available in *R. philippinarum* (Mun et al., 2017) and *M. mercenaria* (Wang et al., 2016) as part of the research mentioned above. To leverage even more clam genomic resources, the genomes of *Spinsula solida*, *Mactra quadrangularis* and *Archivesica marissincia* were also compared to *P. generosa* for functional analysis. The assembly of the surf clam, *S. solida*, was based on Hi-C data generated as part of the Darwin Tree of Life Project. The other surf clam, *Mactra quadrangularis* (or *Mactra veneriformis*),

also has a recently assembled genome as part of the efforts of Sun et al., (2022). Low natural yields in *M. quadrangularis* in China lead to this recent effort to better understand surf clam genomic resources. Using Hi-C assembly, a total of 29,315 protein-coding genes were predicted. From this study, a genome-level phylogenetic tree was constructed demonstrating that *M. quadrangularis* and *R. philippinarum* diverged around 231 million years ago. In the Northern quahog clam, *Mercenaria mercenaria*, Farhat et al., (2022) published the first publicly available genome. Due to high environmental variability on the East Coast of the US, and a desire to understand mass mortality events, the genome of *M. mercenaria* needed to be assembled. Genome annotation yielded 34,728 predicted protein-coding genes, the most of all the *Venerida* so far. Using these previously published genomic resources by running a comparative genomic analysis, will provide an important resource for future comparative work.

Limited studies in bivalve genomics have investigated sexual maturation through differentially gene expression in various tissue types. One study, Dheilly et al., (2012), looked at the basis of sex differentiation in Pacific oysters (*Crassostrea gigas*) using a microarray assay. Gene expression was studied in the gonad over a yearly reproductive cycle. There were 2,482 genes found to be differentially expressed between males and females during gametogenesis. The expression of 434 genes could be localized to the germ cells or somatic cells of the gonad and between the sexes. Maturation analysis processes like this study can reveal the conserved and diverged genes between males and female gonads.

Transcriptomics is an important field of study that provides insight into the complex gene expression patterns of various organisms. Comparative transcriptomics allows for a deeper understanding of the differences and similarities in gene expression between different species. In addition to a functional annotation of the geoduck transcriptome, the focus on this study will be to investigate and characterize five different geoduck tissue types (gonad, heart, ctenidia, larvae and juvenile). We compared the transcriptomes of Manila clams (*R. philippinarum*), Mercenaria clams (*M. mercenaria*) and Pacific oysters (*Crassostrea gigas*), against the geoduck (*P. generosa*) transcriptome, focusing on the most commonly expressed and overexpressed Gene Ontology (GO) terms and genes. Genomes of five clam species were also compared to the *P. generosa* genome, looking for genes with high homology. As more clam species are sequences and genomes assembled, the overall gap in knowledge will decrease and more functional applications for aquaculture can be developed.

2.3 METHODS

There is a reference genome of juvenile *P. generosa* recently published by Putnam et al., (*2*022). They used the Proximo Hi-C process (Phase Genomics) resulting in 18 chromosome scaffolds containing 1.42 Gpb of sequence (64.53% of the corrected assembly). Juicebox correction resulted in a scaffold N50 of 57,743,597 bp. Genome annotation identified 34,947 genes and 236,960 coding sequence regions which corresponds to 38,326 mRNA features. Genome feature tracks included genes, exons, introns, repetitive sequences, and CG motifs (Roberts & Gavery, 2018). Annotation yielded 16,899 tRNAs with a mean and median length of 75 bp in the range of 53-314 bp. CG content was determined to be 33.78% and a total of 15,712,294 CG motifs are present in the genome. The assembled genome is available on the

National Center for Biotechnology Information website (NCBI) under GCA_902825435.1. Sequences were annotated by comparing contiguous sequences to the UniProtKB/Swiss-Prot database (http://uniprot.org) using the BLASTn algorithm (Altschul et al., 1997) with a 1.0E-20 e-value threshold. Based on the Swiss-Prot values, there were 14,672 protein coding sequences in the *P. generosa* genome that had gene ontology characterization information such as GO enrichment analysis.

Total RNA was extracted from adult, juvenile and pooled larvae of *P. generosa*. The adult tissue was isolated using the PAXgene Tissue RNA Kit (Qiagen) based on manufacturer's instructions. The adult tissue was separated into three different types by function: gonad, ctenidia and heart. Five RNA-seq libraries were constructed from pooled mRNA and sequenced at the University of Washington High Throughput Genomics Unit (HTGU) on the Illumina Hi-Seq 2000 platform (Illumina, San Diego, CA, USA). Each library was run on a single lane. Raw sequence reads were quality trimmed using Trim Galore v0.4.0, and the sequence data was quality assessed using FastQC (Andrews, 2010).

Sequences were annotated by comparing contiguous sequences to the UniProtKB/Swiss-Prot database (http://uniprot.org) using the BLASTn algorithm with a 1.0E-20 e-value threshold. Genes were then classified according to their biological processes that were determined by their Gene Ontology (GO) information and are classified into one or more of 72 parent categories (GO slims). The full dataset is available in Supp. Table 2. Genes were classified into their RNA-seq library (ctenidia, gonad, heart, juvenile or larvae) and any gene with a transcript per million (tpm) greater than zero was removed from further analysis. Gene Ontology terms

were then characterized relative to all Gene Ontology terms (GO slims) present in the *P. generosa* genome. A new term was calculated by taking the proportion of a single GO slims present in an RNA-seq library over the proportion of that same GO slim present in the entire *P. generosa* gene set. We coined this new term "Gene Ontology Proportional Value" where, for example, when the proportional value equals one then that GO slim in the RNA-seq library is representative to the GO slim in the entire gene repertoire.

M. mercenaria and *R. philippinarum* transcriptomes were annotated by comparing sequences to the entire gene list of *P. generosa* and given Gene Ontology Proportion Values for inter-species comparison. Transcriptome libraries were obtained from NCBI. (*R. philippinarum* GenBank accession number: GCA_026571515.1 and *M. mercenaria* accession number: GCF_021730395.1). Gene lists were annotated by using BLASTn with e-value of 1E-20 and associated Gene Ontology terms classified and counted by transcriptome library (either *M. mercenaria* or *R. philippinarum*). Gene Ontology terms were then classified using the same process as the geoduck sequence analysis above. Instead, the Gene Ontology Proportional Value was calculated using the proportion of GO slims present in either *M. mercenaria* or *R. philippinarum* transcriptome libraries relative to the proportion of that same GO slim present in the entire *P. generosa* transcriptome library.

In order to see if there was a functional difference between genes with high homology, five different clam genomes in the *Venerida* family were annotated. Clam genomes include *Archivesica marissinica* (GenBank: GCA_014843695.1), *Mactra quadrangularis* (GCA_025267735.1), *Mercenaria mercenaria* (GCF_021730395.1), *Ruditapes philippinarum*

(GCA_026571515.1) and *Spinsula solida* (GCA_947247005.1). All five clam genomes were annotated by comparing contiguous sequences to the *P. generosa* gene database using the BLASTn algorithm with a 1.0E-20 e-value threshold.

Characterization of reproductive genes in *P. generosa* was done in two ways. The first way was to gather a list of genes expressed in the gonad. This produced a list of reproductive genes in the *P. generosa* adult gonad that code for proteins related to the "reproductive process" functions (Table S3).

Dheilly *et al.* (2012), investigated the temporal variation of gene expression during oyster gonad differentiation and development in *C. gigas*. The genes identified in their oyster study are differentiated by sex and stage of development: somatic tissues and oocytes. Differentially expressed oyster gonad and oocyte genes were pulled from their Supp. Table 3 from Dheilly et al., (2012). Genbank accession numbers were gathered from clusters 1-10 and were annotated against the *P. generosa* gene database using BLASTn algorithm with a 1.0E-10 e-value threshold. The focus of this study will be the female reproductive genes from developmental stage 0 to stage 3 and compared to the reproductive genes found in the RNA-seq gonad library.

2.4 RESULTS

After leveraging the genomic resources from Putnum et al., (2022) to compare to the Swiss-Prot database, a fully annotated genome linked transcriptome was produced. This genome has 34, 947 annotated protein coding sequences. Of those, 2,180 are expressed only in the juveniles. The RNA-seq library for larvae geoduck returned 19,449 genes with 868 genes found only in the larvae transcriptome library. In the heart library, there were 17,479 genes representing only 371 genes unique to the heart. In the ctenidia, there were 17, 479 genes representing 340 genes found only in the ctenidia tissue library. Most crucially for this study on reproduction, the gonad RNA-seq library had the fewest genes with only 13,682. Of those genes, 119 were found uniquely expressed in the gonad. The full annotation of the 5 RNA-seq libraries is found in Supp. Table 2. A pairwise comparison between RNA-seq libraries was produced as from the unique genes list above, represented as count of biological gene ontology terms (Supp. Figure 2).

Expanding out from gene characterization, the Gene Ontology Proportion Value is descriptive of the abundance of biological Gene Ontology processes per library relative to the entire *P. generosa* genome (Figure 2.1). In the *P. generosa* genome, there are 17, 611 GO slims representing 34, 947 genes. In the juvenile, there are 17, 277 GO slims representing 19, 449 genes. In the larvae, there are 16,632 GO slims representing 19,449 genes. In the heart there are 16, 021 GO slims representing 17, 602 genes. In the ctenidia, there are 15, 911 GO slims representing 17, 479 genes. Finally, in the gonad there are 14, 715 GO slims representing 13, 682 genes.

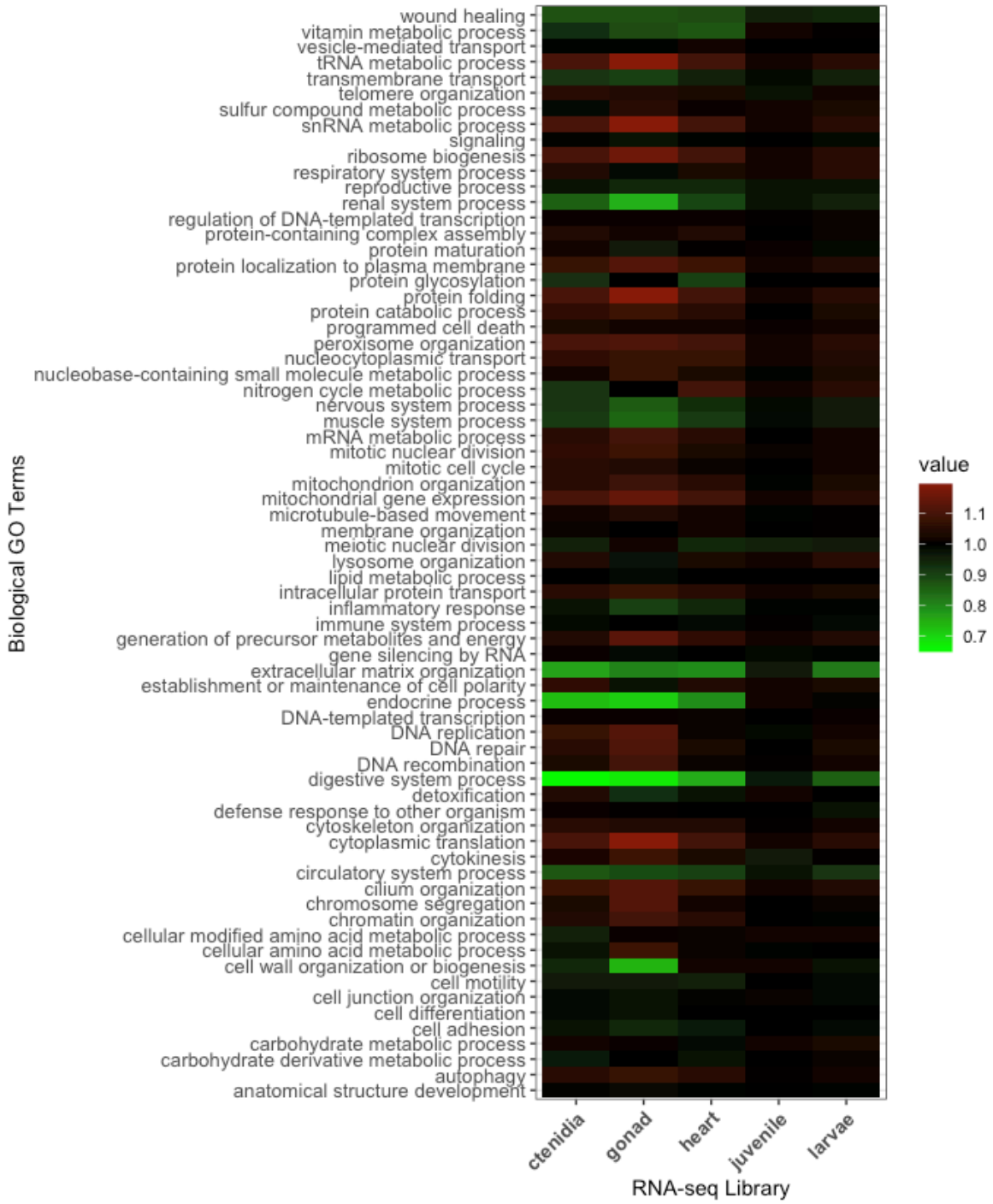

Figure 2.1: *Relative abundance of gene ontology terms compared to the entire geoduck genome.* Where value = Gene Ontology proportion value. Values highlighted in green or red represent an under or over abundant Biological Gene Ontology Term relative to the entire geoduck gene repertoire respectively.

Annotations from the *M. mercenaria* and *R. philippinarum* transcript libraries against the *P. generosa* gene list revealed a strong phylogenetic correlation between *P. generosa* and *M. mercenaria*. Of the 34,947 mRNA features in the *P. generosa* transcriptome, 5,099 (14.6%) were found in *M. mercenaria* transcriptome. In contrast, only 657 (1.8%) mRNA features were found in the *R. philippinarum* transcriptome, (Table 2.1). The transcript libraries of *M. mercenaria* and *R. philippinarum* were annotated with Gene Ontology Proportion Values. In *M. mercenaria* transcript libraries there are 7,027 GO slims while in *R. philippinarum* there are only 1,390 GO slims. The top 20 GO slim terms ranked by their Gene Ontology Proportional Values (Table S4). The most abundant GO slim categories were anatomical structural development, signaling and cell differentiation. For example, of the 456 genes shared between *P. generosa* and *M. mercenaria,* the most abundant were related to anatomical structural development while only 67 genes related to anatomical structural development were shared between *P. generous* and *R. philippinarum*.

Table 2.1 *NCBI BLASTn transcriptome annotation:* Transcriptomes are gathered off NCBI and blasted against the *P. generosa* (GCA_902825435.1). Total hits represent genes matched from M. mercenaria or *R. philippinarum* to the *P. generosa* gene list. M. mercenaria has almost x10 the number of genes matched to *P. generosa* than *R. philippinarum.*

Species	Total Hits	GenBank Accession
Mercenaria mercenaria	5,099	GCF_021730395.1
Ruditapes Philippinarum	657	GCA_026571515.1

Annotations from other clam genomes, against the *P. generosa* genome, further highlighted genomic homology. The *M. mercenaria* genome is 1.9 Gb and the CG content was determined to be 34.5% (Table 2.2). Of the 34, 947 genes in *P. generosa*, 8,736 genes were matched in *M. mercenaria*, the most matches of all five genomes. The *R. philippinarum* genome is 1.4 Gb large and the CG content was determined to be 32%. 2,263 genes found in *P. generosa* were matched in *R. philippinarum*. The *A. marissinica* genome is 1.5 Gb and the CG content was

determined to be 39%. Of those genes in *P. generosa*, 3,268 were matched to *A. marissinica*. The *S. solida* is a smaller genome at 932 Mb. *S. solida* CG content was determined to be 35.5% and 1,629 genes were matched to the *P. generosa* genome. The *M. quadrangularis* annotated genome is the smallest of the five with a size of only 979 Mb, a CG content of 33% and only returning 874 gene matches to the *P. generosa* gene set.

Table 2.2 *Comparative Genome Annotation Summary*: *M. mercenaria* has the largest shared genes (total hits) with *P. generosa* as well as the largest genome. *M. quadrangularis* has the fewest shared genes with *P. generosa*, as well as one of the smallest genomes.

Species	Total Hits	Genome Size (Mb)	GenBank Accession
M. mercenaria	8,736	1,900	GCF_021730395.1
M. quadrangularis	874	979	GCA_025267735.1
R. philippinarum	2,263	1,400	GCA_026571515.1
A. marissinica	3,268	1,500	GCA_014843695.1
S. solida	1,629	932	GCA_947247005.1

Characterization of reproductive genes in *P. generosa* was done in two ways. The first way was to gather a list of genes expressed in the gonad. This produced a list of reproductive genes in the *P. generosa* adult gonad that coding for proteins related to the "reproductive process" functions (Table S3). Of the 34, 947 genes in the *P. generosa* gene list, 640 genes are involved with the reproductive process (1.8% reproductive).

Leveraging Dheilly et al., (2012) results from 32 individual gonad samples of *C. gigas*, they identified 2,482 genes differentially expressed between gametogenesis stages. Of those differentially expressed, 511 genes were found to be expressed in major expression stage 0 of development (neither male nor female). Of those 511 oyster reproductive genes, 7 geoduck reproductive genes were found in the *P. generosa* genome. Key genes from stage 0 are Ttn, BMP2 and ZNF107. In the next developmental stage, major expression stages 1-3 for females, there were 197 genes differentially expressed in oysters. Of those 197 genes, 5 candidate genes

were found in *P. generosa*. The only candidate gene from stages 1-3 is Rusf1. In the later developmental stages 2-3, Dheilly et al., (2012) found 312 candidate reproductive genes and of those 312, we found 13 candidate genes. Key candidate genes from this developmental stage are SMC5, Cep57, KCTD5, ATF7IP, and TPST1. In the final developmental stage 3, Dheilly et al., (2012) reported 222 reproductive genes. Our annotation revealed 14 *P. generosa* reproductive genes including STOX1, SPPL3, Wdr20, and Rere. There were also two *P. generosa* genes found in cluster 9 (female and male differentially expressed gametogenesis stages) that were specific to the female gonad tissue. Those genes were SUMO3 and ARHGAP11A. The full results from this analysis are found in Table 2.3, including the Dheilly et al., (2012) cluster information as well as the results from the male reproductive candidate gene investigation.

Table 2.3 *Characterization of Reproductive Development Genes*: *P. generosa* reproductive gene annotation summary using Dheilly et al., (2012) gene clusters (1-10) and major expression stages. *P. generosa* hits are the genes matched from C. gigas reproductive candidate genes by either predominantly female or male expression stages.

Female Reproduction

Major Expression Stage	*P. generosa hits*	Dheilly et al. 2012 cluster	Reproductive Candidate Genes
Stage 0	7	1	Ttn, BMP2, ZNF107
Stage 1-3	5	4	Rusf1, Foxl1
Stage 2-3	13	3	SMC5, Cep57, KCTD5, ATF7IP, TPST1
Stage 3	14	2	STOX1, SPPL3, Wdr20, Rere
Stage 1-3	7	9	SUMO3, ARHGAP11A

Male Reproduction

Major Expression Stage	*P. generosa hits*	Dheilly et al 2012 cluster	Reproductive Candidate Genes
Stage 0	7	1	Ttn, BMP2, ZNF107
Stage 1-3	4	6	PDS5B, CREM, ELAVL2
Stage 3	8	5	Spag6
Stage 1-2	2	10	CSRP3, PCR3
Stage 1-3	7	9	CBX3, PIPOX, CBX3, mcm7
Stage 2-3	1	8	H2A. F/Z
Stage 3	13	7	DGKE

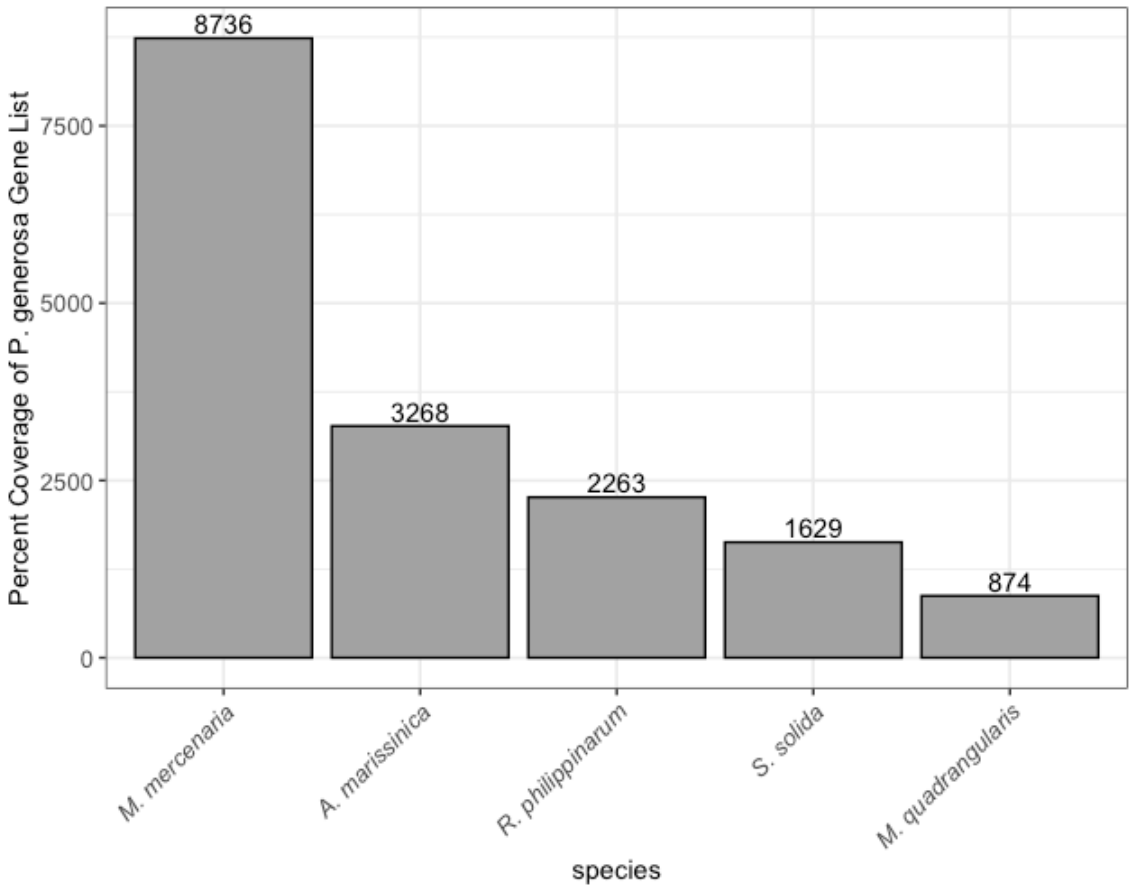

Figure 2.2: *Percent Coverage of P. generosa Gene list:* represented in the 5 clam genomes; *Mercenaria mercenaria, Archivesica marissinica, Ruditapes philippinarum, Spinsula solida*, and *Mactra quadrangularis*

2.5 DISCUSSION

The generation of five fully characterized RNA-seq libraries, provides comprehensive insights into gene expression patterns, ultimately advancing our understanding of Pacific geoduck reproductive biology and gene expression profiles. These transcript libraries are beneficial to describing individual tissue functions as the unique sets of genes found in each tissue type are consistent with their specialized functions. For example, the heart tissue had a unique set of genes involved in muscle and renal system processes, which are important for its role in circulation (Drake et al., 2012). The ctenidia tissue had a unique set of genes involved in DNA repair, which is likely important for its role in filtering and cleaning the water that the geoduck lives in (Juárez et al., 2018). The larvae library had a unique set of genes involved in nucleobase-containing small molecule metabolic processes and cytoskeleton organization. The juvenile library was the largest with 67.01% genes expressed relative to the entire gene list of geoduck, representing almost all of the relevant biological processes.

We found that each tissue type exhibited a distinct set of expressed genes, indicating unique functional roles. These genes are categorized into their biological gene ontology (GO) processes by their Gene Ontology Proportional Value into over abundant or under abundant relative to the entire *P. generosa* genome. The most commonly overabundant GO processes distinct to all tissue types were related to metabolism, gene expressions and protein making, (Figure 2.1). Indicating that metabolism is more important at the specialized tissue level for geoduck. The most common under abundant GO processes were related to specialized system processes such as renal, endocrine, digestive, and circulatory systems. This is unsurprising as digestive biological processes are going to be under expressed in tissue types that don’t

specialize in digestion. Most of the overabundant GO processes unique to specific tissue types were functional to their specific needs. For example, in the gonad, the top overabundant GO processes were related to mitochondria and metabolism while in the larvae, the top overabundant GO processes were related to movement and energy allocation systems. For the heart, the top overabundant GO processes were related to nitrogen cycle metabolism, which would agree with its main function as a mechanical pump as well as its electrical activity (Drake et al., 2012). For the ctenidia, the top overabundant GO processes were related to cytoplasmic translation. Cytoplasmic translation refers to the process by which mRNA molecules are translated into proteins in the cytoplasm of the cell, rather than in ribosomes. This process is important in many cells, including those in the ctenidia of oysters, because it allows for rapid and efficient protein synthesis,(McLean & Whiteley, 1974). The juvenile tissue types did not have any highly overabundant genes due to their later developmental stage.

Investigating the annotated transcriptome libraries shared between *P. generosa* and *M. mercenaria* or *R. philippinarum,* further illuminate foundational gene expression of *P. generosa*. Genes involved with translation, microtubule-based movement, and cilium organization are all highly expressed in both the *M. mercenaria* and the *R. philippinarum* libraries. Interestingly, there is a unique group of conserved genes in *M. mercenaria* and *P. generosa*, but not found in *R. philippinarum*. The genes were related to metabolism and cellular organization. These differences, as well as *M. mercenaria* having more shared genes than *R. philippinarum*, may be due *M. mercenaria* being a closer phylogenetic relation to *P. generosa* than *R. philippinarum*, (Chen et al., 2011). The inter-species analysis is valuable in

determining gene orthologs important for reproduction investigations. Cross species library comparison is also useful for future studies on gene function in marine bivalves outside of reproductive control, such as studies involving disease tolerance.

In the *P. generosa* genome from Putnam et al., (2022), they found the geoduck genome to be almost 2 times larger in size than oyster genomes with twice as many putative chromosomes. This trend reversed when comparing *P. generosa* to other clam genomes. *R. philippinarum, M. mercenaria* and *A. marissinica* all have genomes larger than *P. generosa* and an additional chromosome. Interesting, regardless of genome size, the relative number of genes was approximately the same (~30,000). The GC content was also highly conserved across species with 32-33%, (except for *A. marissinica* at 39%). Comparing the percent coverage of *P. generosa* gene list to the query sequences of the other five clam genomes, *M. marissinica , R. philippinarum,* and *S. solida* have shared gene lists with *P. generosa* of 3268, 2263 and 1629 respectively. *M. mercenaria* has the most shared genes with *P. generosa* with 8736 and *M. quadrangularis* the least with only 874. (Figure 2.2). Looking at the phylogenetic relationship between *M. mercenaria* and *R. philippinarum* (Chen et al., 2011) reveals that *R. philippinarum* more basal than *M. mercenaria*. As more genomes are fully annotated and made available on NCBI, then comparative studies like this will be more robust.

A key emphasis of this study was to describe reproductive genes in geoduck. Our first approach, using the gonad RNA-seq library, produced a moderately large set of genes (n = 640) with gene expression patterns related to the reproductive process. This is also consistent with previous studies (Timmins-Schiffman et al., 2017) that have shown that reproductive

tissues often have unique gene expression profiles. Leveraging the results of Dheilly et al., (2012), provided a list of reproductive genes found in geoducks (74) that are linked to a major expression stage by sex and developmental stage. Looking at highly conserved reproductive genes across species gives us confidence that these are homologous genes for controlling reproduction in *Bivalvia*. Dheilly et al., (2012), described reproductive genes related to mitosis and meiosis regulation including centromere proteins and kinesin related proteins. In *P. generosa* transcriptional dataset, reproductive genes related to centromere and kinesin related proteins were: Kinesin-like protein 6, KIF18A, and KIF9, inner centromere protein, centromere protein zw10 homolog, and centromere associated proteins S, E and X. Dheilly et al., (2012) identified genes associated with the female specific processes such as oogenesis. Those included: vitellogenin, cd63, mitotic apparatus, p62, forkhead box L2 and caveolin. In *P. generosa*, reproductive genes related to oogenesis were: putative vitellogenin receptor (protein yolkless), forkhead box protein C1 and J3, and RecQ-mediated genome instability protein 1 (M-caveolin).

Between the two approaches for identifying key reproductive genes in *P. generosa*, there are two genes found using both: Foxl1 and Cep57. The Fox genes, which code for forkhead class transcription factors, are classical orthologs involved in sex determination/differentiation, (Broquard et al., 2021). Dheilly et al., (2012) found forkhead box L2 genes in *C. gigas.* Many studies on bivalves have found this gene to be involved in reproduction and have been found in the *C. gigas* and the pearl oyster *Pinctada fucata*, as well as other bivalves (Matsumoto et al., 2013). The Cep57 gene, which codes for centrosome- and midbody-associated proteins, is not commonly studied for its implications in reproduction in bivalves. A similar ortholog, Cep55

has been documented to be involved in embryonic development in zebrafish (Jeffery et al., 2015), and should be the focus of study in bivalve reproduction going forward. In particular, genes related to vitellogenin, caveolin, foxhead class transcription factors, and Cep55/57 all appear to be highly conserved across *Bivalvia* and are very closely related to reproductive development.

Our results provide a foundation for future studies aimed at understanding the molecular basis of geoduck (*Panopea generosa*) physiology and development. Comparative analysis with *Mercenaria* clam and Manila clam transcript libraries provided insights into gene orthologs and conserved functions across species. Annotated RNA-seq libraries facilitated the identification of tissue-specific genes, including a significant number of previously undiscovered reproductive genes in geoduck. Specifically, Foxhead and Cep55/57 genes are key genes found. Each tissue type exhibited distinct sets of expressed genes, reflecting their specialized functions. Overabundant biological gene ontology (GO) terms in all tissue types were related to metabolism, gene expression, and protein synthesis, highlighting the importance of these processes at the tissue level. The study also focused on identifying reproductive genes for potential gene editing efforts, highlighting key genes involved in sex determination, oogenesis, and embryonic development. Overall, these findings lay the groundwork for future studies investigating geoduck physiology, development, and reproductive control, as well as broader investigations into gene function and disease tolerance in marine bivalves. By identifying these tissue-specific gene expression patterns, we can begin to unravel the complex molecular networks that underlie geoduck biology.

Chapter 3. Molecular Delivery and Gene Silencing

Chapter three is a smaller chapter dedicated to the attempted application of molecular delivery methods for future efforts in Pacific geoduck or Pacific oyster gene silencing.

3.1 INTRODUCTION

In recent years, Pacific oysters (*Crassostrea gigas)* have become an important model species for ecological, evolutionary, and developmental studies because of its ability to survive in extreme environmental conditions as a sessile filter feeder and its classical mosaic pattern of development. Recently, there is strong evidence of differential oyster morality in the field and in the laboratory due to triploidy (Li et al., 2022b). Due to this differential triploid mortality, there is a need to shift approaches for developing sterile oysters and clams by creating an alternative to sterility. An emerging technology for producing sterile shellfish is through gene modification. Only recently has gene modification systems been cost effective, as these tools become more highly researched and popularized. The majority of the gene modification work has been done using oysters as the model species. In Pacific oysters, much of the work on gene silencing has been done using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR- associated protein 9 (Cas9) system via direct injection into fertilized eggs. Yu et al., (2019) targeted the genes myostatin (MSTN) and Twist and was successful at inducing mutations ranging from 1-24 base pairs in those two genes. Li et al., (2022b) followed up this work with their own direct injection delivery of the CRISPR Cas9 system in Pacific oysters. They injected embryos with the Cas9 system containing extensive indel mutations to knock out the myosin light chain gene (CgMELC), thereby producing a mutant larva. In Fujian oysters (*Crassostrea angulata*), Jin et al., (2021) used the CRISPR Cas9 system to deliver edits

to the Pitx gene that governs body symmetry and is a highly conserved homeobox gene family.

Gene delivery is an important part of genome-editing technology. Instead of direct injection of the CRISPR Cas9 system into the cell, Jin et al., (2021) investigated the feasibility of using electroporation and as a means of molecular delivery. Electroporation designates the use of short high-voltage pulses to overcome the barrier of the cell membrane. By applying an external electric field, which just surpasses the capacitance of the cell membrane, transient and reversible breakdown of the membrane can be induced (Gehl, 2003). Despite a large number of larvae becoming malformed during the process of electroporation, Jin et al., (2021) was able to successfully substitute a single-base pair in D-hinge larvae from electroporated oocytes. In sea anemones, *Nematostelle vectensis*, Karabulut et al., (2019) demonstrated successful application of electroporation protocol for shRNA delivery into eggs. An alternative to electroporation for gene delivery is using an Endo-Porter chemical to induce endocytosis. Common practice is to pair the Endo-Porter with a morpholino oligomer instead of the CRISPR Cas9 system for gene editing. Morpholinos are typically used to block translation of mRNA and to block splicing of pre-mRNA, though they can block other interactions between biological macromolecules and RNA (Moulton, 2006). First used in shellfish in Pearl oysters, Fang et al., (2012) used an Endo-Porter paired with a morpholino to knock-down the expression of PfN23 in adult Pearl oysters. In other marine biota, Jeffery et al., (2015) used a morpholino to edit the gene cep55 in zebrafish, which regulates embryonic growth and development and could be a cool candidate gene to start editing *C. gigas*.

In this study, we investigated the feasibility of using Electroporation and Morpholinos for molecular delivery and gene editing in Pacific oysters (*C. gigas*). We designed and tested gene knockdown of candidate genes involved in primordial germ cell (PGC) specification in Pacific oysters and Pacific geoducks. We could not use direct injection due to concerns over the ability to scale up for gene editing at the hatchery scale. CRISPR Cas9 is not the focus of this study but is still the most promising application of gene editing technologies. Before any gene modification can be done, basic research into developmental timing, plating protocols and chemical tolerances need to be established.

3.2 METHODS

All Pacific oyster gametes were obtained from Taylor Shellfish hatchery (Dabob Bay, Quillcene, Washington, 47.818306, -122.823361). Experiments were conducted over the course of a few months during summer of 2022 (June-September). Three males and three female fertile Pacific oysters were collected at the same time as Taylor conducted their large hatchery spawns. Oysters were stripped for their oocytes or sperm and placed in 15mL Falcon tubes on ice and taken directly to the laboratory at the University of Washington (Seattle, WA). Oocytes were hydrated with filtered sea water for one hour before fertilization. Oocytes were then fertilized with the sperm in "hatchery style", in a 1L beaker with ~ 10 sperm/oocyte. Oyster embryos were plated in 6-well plates (5mL wells) and allowed to develop for at least 48 hours in the incubator at 20°C, until they reached D-hinge.

Electroporation is a powerful, and chemical free, way of introducing foreign molecules into a cell. Using electricity instead of direct injection allows for multiple genes to be knocked down

in hundreds of animals at a time. Testing to see if we could successfully deliver molecules into oyster embryos via electroporation without deforming the cells is a key step. First, we needed a molecule to deliver to test the electroporation method that produces a positive control that can be visualized via fluorescent microscopy. We used a fluorescence labeled dextran molecule to explore the electroporation parameters. Parameters include, dextran concentration, pulse shape, pulse time, number of pulses, buffer conductivity, pulse voltage and density of embryos. Indicators of success are the percentage of fluorescent embryos and the size and shape of those embryos post pulse.

Another molecular delivery method we tested was using Endo-Porter endocytosis, which is paired with a morpholino oligomer for gene editing. To deliver a substance into cells simply add that substance to the medium covering the cells, followed by addition of the pre-formulated Endo-Porter solution and swirl to mix (Summerton, 2005). For our study, we tested the efficiency of delivery of fluorescence labeled dextran into the cell via a range of Endo-Porter concentrations as well as using Endo-Porter to deliver a Vasa labeled morpholino. Dextran was reconstituted with 20mg/mL of molecular grade water. DEPC cannot be used as it interacts with the morpholino. We used the morpholino delivery protocol from the pearl oyster paper (Fang et al., 2012) as a guide, but due to the difference in species, the protocol had to be optimized.

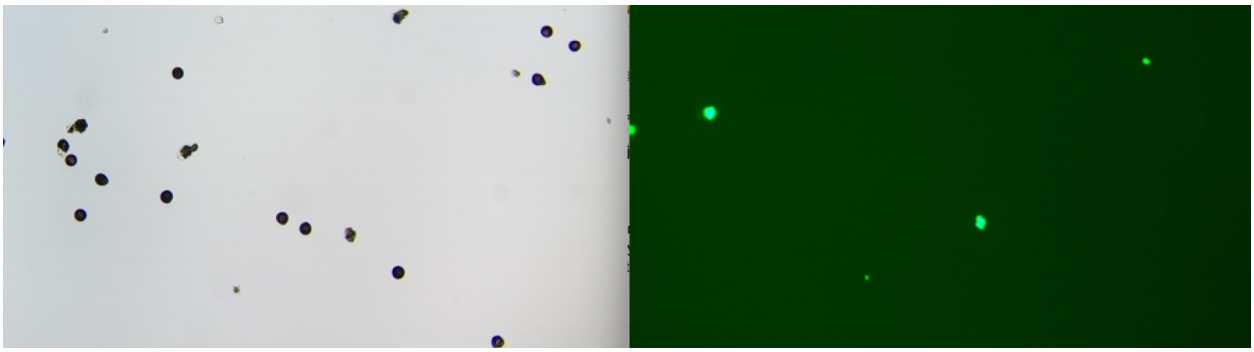

Figure 3.1 *Fluorescent microscopy images of Pacific oyster oocytes after electroporated delivered dextran into the cell*. Many cells fluoresce, but many healthy cells did not absorb the dextran despite electroporation.

3.3 DISCUSSION

A large part of this study was developing and optimizing procedures for gene modification protocols. Ideal developmental timing, plating densities and chemical concentrations were the main outcomes of this study. Using 6-well plates, the ideal stocking densities of oyster embryos is 5,000/mL in 1 mL volumes. This provides a single layer of cells that can be easily visualized under a fluorescent microscope attached with a high-definition microscope camera. First, Dextran needed to be added to the system. First, a working solution using salt water was made by adding 50uL of the 20mg/mL dextran to 450uL of filtered sea water. Then, the dextran solution was mixed with concentrated sperm or at a ratio of 1:1 (1uL gametes to 1uL dextran at 2mg/mL). After the dextran is mixed, the molecular delivery partner is added.

For electroporation, the solution containing the fertilized embryos and the fluorescence labeled dextran is added to a cuvette. For oyster embryos, 80uL total volume of the 0.4cm cuvette. The

ideal concentration for electroporation is 10,000 embryos/mL. Two different protocols for electroporation were used. The first protocol is from the pearl oyster paper (Jin et al., 2021). For electroporation, the dextran solution (with a final concentration of 1 μg/mL) was added, and the mixture was incubated for 5 min at room temperature (21–25°C). Then, the mixture was transferred into a 0.4 cm electroporation cuvette (Bio-Rad Laboratories). Electroporation was performed in a Bio-Rad Gene Pulser Xcell system (Bio-Rad Laboratories) using a square wave pulse (100 V, with 15 ms pulse duration and four pulses separated by 100 ms pulse interval). After electroporation, the eggs or blastula larvae were washed in seawater and returned to the 6-well plate for development. Based on this procedure, it was obvious that the electroporation protocol had exploded a lot of the embryos. On the other hand, there was fluorescence under the microscope (Figure 3.1). The second protocol is from a sea anemone gene knockdown experiment (Karabulut et al., 2019). Optimal parameters were determined to be 50V delivered in a single 25 ms pulse in 4mm cuvettes (Mirus bio) using an Electroporator ECM830 (Genetronics, Inc.). This protocol was gentler than the pearl oyster protocol and produced fewer exploded cellular material. Going forward, the Karabulut et al., (2019) paper would be the preference for electroporation parameters.

For determining morpholino and Endo-Porter concentrations, our main reference was following the protocols of Moulton (2006) and Fang et al., (2012). We primarily used the Basic Protocol 3 (Moulton, 2006), where the optimized concentration of Endo-Porter is 6uM of 1 mM Endo-Porter solution into 1mL aliquots of cell culture and the morpholino concentration is 10uL of 1mM stock per 990uL of cell culture. When using Endo-Porter, allow for endocytic uptake to occur over a period of 16-24 hours before washing off the morpholino solution. It

was very difficult to wash the solution containing the morpholino as there was still fluorescence in a negative control trial (no Endo-Porter) when all the fluorescent material should be washed off. Using magnesium chloride at 25mM solution to relax the developed larvae was helpful in the washing protocol.

In conclusion, we took a step forward in optimizing the procedure for gene editing in Pacific oysters. The goal was to use this technology and implement it in not just Pacific oysters, but Pacific geoduck as well. Molecular delivery by electroporation can be successful in shellfish, but it has limited ability to scale up to hatchery level production because of the large majority of oocytes that are deformed during the process. The most promising technology in this study is molecular delivery via Endo-Porter, as it does not interfere with the development of larvae oysters, and it does not produce deformed cells. The next step in this study is to test different custom morpholinos using the Endo-Porter at 6uM. We already began testing the Vasa morpholino effectiveness at gene modification but will require quantitative PCR for gene editing confirmation. Leveraging results from previous studies on reproduction and shellfish, *Foxl1* and *Cep55* genes can be the next set of candidate genes tested using morpholino control. The implication of this and future studies like this are incalculable for the future of the aquaculture industry, paving the way for improved genetic modification in *Bivalvia* at large.

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