Physiological and molecular traits associated with resilience to ocean acidification in *Crassostrea virginica*

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Michelle Barbosa

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Michelle Barbosa

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Bassem Allam – Thesis Advisor
Marinetics Endowed Professor, School of Marine and Atmospheric Sciences

Emmanuelle Pales Espinosa – Second Reader
Research Associate Professor, School of Marine and Atmospheric Sciences

Christopher Gobler – Third Reader
Endowed Chair of Coastal Ecology and Conservation, School of Marine and Atmospheric Sciences

Shannon Meseck – Fourth Reader
Research Chemist, NOAA/NMFS Milford Laboratory

This thesis is accepted by the Graduate School

Eric Wertheimer
Dean of the Graduate School

Abstract of the Thesis

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Ocean acidification (OA) is considered to be a major threat to the future of our oceans. There is a substantial gap in knowledge regarding the roles of acclimation versus adaptation for resilience to ocean acidification (OA) in bivalves. This study combines physiological assays with next generation sequencing to assess the potential for recovery and acclimation of the eastern oyster (*Crassostrea virginica*) to OA and to evaluate the underlying molecular features for resilience. In a reciprocal transplant experiment, larvae transplanted from elevated (pCO_2 ~1400ppm, pH 7.5) to ambient pCO_2 (~350ppm, pH 8.1) conditions demonstrated significantly lower mortality and larger size post-transplant than oysters remaining under elevated pCO_2 and near equal mortality compared to controls under ambient pCO_2. The recovery after transplantation to ambient conditions demonstrates the ability for larvae to rebound quickly and suggests phenotypic plasticity and acclimation contribute to resilience. In a food limitation

experiment, OA or OA and starvation did not significantly affect the survival of juvenile oysters as compared to those under ambient conditions. There was a trend for smaller size in juvenile oysters under elevated pCO_2, however, the trend was not significant. Juveniles were robust to the effects of OA which may have obscured evidence of an energy reallocation mechanism for resilience. An investigation of transcriptomes supported the hypothesis that acclimation contributes to resilience as genes were differentially regulated under OA stress in larvae and juveniles. Interestingly, the transcriptomic profiles of transplanted and non-transplanted larvae terminating in the same final pCO_2 converged, further supporting the idea that acclimation underlies resilience. Several private alleles were present in animals maintained under elevated versus ambient pCO_2 suggesting selection and adaptation contribute to resilience in combination with acclimation. Genes differentially expressed and containing private alleles included some that function in cell differentiation and development, biomineralization, ion exchange, and a few genes that are calcium dependent. Together these results support the hypothesis that acclimation and adaptation together serve as modes of resilience in the eastern oyster under OA stress; however, juvenile oysters may be more robust to the effects of OA as compared to vulnerable larvae. In addition to providing a more comprehensive understanding of the effects of OA on bivalves, the identification of genes associated with resilience can serve as a valuable resource for the aquaculture industry that would enable marker-assisted selection of OA-resilient stocks which would protect an economically and ecologically important species.

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List of Abbreviations

OA - ocean acidification

AA - larvae that remained in ambient pCO_2

EE - larvae that remained in elevated pCO_2

AE - larvae transplanted from ambient to elevated pCO_2

EA - larvae transplanted from elevated to ambient pCO_2

AF - juveniles under ambient pCO_2 and fed conditions

EF - juveniles under elevated pCO_2 and fed conditions

AS - juveniles under ambient pCO_2 and starved conditions

ES - juveniles under elevated pCO_2 and starved conditions

AFB - juveniles under ambient pCO_2, fed conditions, and exposed to bacteria

EFB - juveniles under elevated pCO_2, fed conditions, and exposed to bacteria

ASB - juveniles under ambient pCO_2, starved conditions, and exposed to bacteria

ESB - juveniles under elevated pCO_2, starved conditions, and exposed to bacteria

NGS - next generation sequencing

DIC - dissolved inorganic carbon

ROD - *Roseovarius* oyster disease

DEG - differentially expressed gene

SNP - single nucleotide polymorphism

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Chapter I: Background

The Earth's climate is changing at an unprecedented rate. Since the invention of the combustion engine which spurred the Industrial Revolution at the start of the 19th century, the burning of fossil fuels has led to a major increase in the concentration of carbon dioxide in the atmosphere (EPA, 2019). Ocean acidification, the reduction of seawater pH as a result of increasing partial pressure of CO_2 (pCO_2), has rapidly gained recognition as a major threat for ocean organisms and ecosystems.

For the past 800,000 years, atmospheric CO_2 concentrations have remained within a range of 172 to 300ppm (Lüthi et al., 2008). However, following the start of the Anthropocene, carbon dioxide emissions have risen beyond those seen in the last million years at a rate one order of magnitude faster than has ever been observed in Earth's geological past (Doney et al., 2009). Since the 19th century, carbon dioxide concentrations have increased from 280ppm to over 400ppm, and the Intergovernmental Panel on Climate Change (IPCC) predicts concentrations will reach 800-1000ppm by the end of the century (IPCC, 2014). Carbon dioxide concentrations in the atmosphere are rapidly approaching those predicted by the IPCC as each year a new record high is set; as of May 2019, atmospheric CO_2 reached a record peak of 415ppm (Munroe, 2019). With the predicted increase in atmospheric CO_2, the pH of seawater is also predicted to decrease from 8.2 to 7.8 adding to a 0.1 decrease that has already occurred since the Industrial Revolution (IPCC, 2014). The reduction in pH is largely due to ocean-atmosphere coupling which results in the uptake of one-third of all atmospheric CO_2 emissions by the ocean

(Doney et al., 2009). The acidification of the oceans will have major consequences for seawater chemistry and consequences for marine ecosystems, the latter of which remain unknown and uncertain.

In coastal areas acidification is exacerbated by land-based sources of pollution. Excessive use of fertilizers in coastal regions can run off into coastal waterways leading to high nutrient loading. Nutrient loading promotes massive increases in algal productivity, known as algal blooms, but the high algae biomass eventually exhaust available nutrients leading to their collapse. Following the collapse of these blooms, decaying algae promotes microbial decomposition and respiration, which leads to the production of carbon dioxide and the eventual decline in seawater pH. Studies have demonstrated that coastal waterways around Long Island, NY have already reached low pH conditions predicted for the end of the century (pH <7.4) partially as a result of these blooms (Wallace et al., 2014). The Long Island Sound is an extremely socially vulnerable region and is predicted to reach the mean annual chemical threshold for disruption of calcification and development of larval bivalves as early as 2071-2099 (Ekstrom et al., 2015). Coastal and shelf ecosystems are home to an estimated 15,000 species in the US alone (Fautin et al., 2010), and the altered water chemistry that results from acidification has major implications for vulnerable near-shore organisms, especially those that rely on calcification for growth.

For calcifiers, the uptake of CO_2 by the ocean has the effect of reducing the saturation states of calcium carbonate. The calcification process is predicated on the saturation state of two biogenic forms of calcium carbonate ($CaCO_3$), aragonite and calcite. By the year 2100, saturation states are predicted to decline by about 40% (Gattuso and Lavigne, 2009). A reduction in saturation states limits the deposition of material for organisms with $CaCO_3$ skeletons or shells,

such as bivalve mollusks, and can also lead to the dissolution of shell material (Parker, 2013; Ries et al., 2009). If animals cannot develop their shells properly, poor development could affect normal physiological function, weaken hosts to pathogens, or increase vulnerability to threats from predators. The exact implications of ocean acidification on marine organisms has only recently began being investigated.

In the recent decade, a number of studies have documented the adverse effects OA will have on the metabolic activity of calcifying marine taxa. Though not all taxa respond in the same manner to elevated pCO_2, many bivalve mollusks exhibit metabolic depression (Calosi et al., 2013) under experimental ocean acidification conditions. This depression manifests as reduced growth (Kapsenberg et al., 2018; Frieder et al., 2017), survival (Talmage and Gobler, 2009), development (Waldbusser et al., 2015), immunity (Asplund et al., 2013; Liu et al., 2016; Schwaner et al., 2020), and reproduction (Boulais et al., 2017). OA may also indirectly impact bivalve survival as the abundance of the pathogenic bacteria *Vibrio* is predicted to increase with elevated pCO_2 (Zha et al., 2017). The eastern oyster (*Crassostrea virginica*) has been identified as a potentially vulnerable species under future climate conditions. Numerous studies have demonstrated that larval stages are most susceptible to the adverse effects of OA (Kurihara, 2008; Gobler and Talmage, 2014; Schwaner et al., 2020). In this context, hatchery production of seed, a main component of bivalve aquaculture growth, has been seriously hampered by acidification in some of the major hatcheries in the U.S. (Barton et al., 2012) and the impact of acidification on bivalve recruitment in embayments has been more dramatic than initially thought.

Fortunately, some individuals are expected to persist as "winners" in the face of climate change, however, what confers resilience in these individuals is relatively unknown. To date, our

understanding of the effects of OA on bivalves has almost exclusively focused on the physiological effects low pH will have on animal health and survival; this somewhat superficial understanding does not probe the processes that enable bivalves to survive and tolerate OA or OA in combination with a secondary stressor such as microbial infection. In addition, the ability for oysters exposed to OA to "recover" physiological function in normal pH conditions is also unknown. This knowledge could be critical to determining the extent to which bivalve stocks are at risk in future ocean conditions.

Some studies have just begun to explore the means for resilience of calcifying species to OA. To date, most studies focus on either the role of acclimation (i.e. phenotypic plasticity) or adaptation for conferring resilience. Within the field of ocean acidification there is some debate as to the relative roles of acclimation versus adaptation for resilience to environmental stress. Under the umbrella of acclimation, several physiological mechanisms have been proposed such as an energy availability and allocation mechanism and changes in gene expression. With regard to adaptation, many OA studies suggest selection of advantageous variants that increase the fitness of an individual in the acidified environment. To note, acclimation and adaptation may not be mutually exclusive modes for resilience and likely are not; in other words, the ability and extent to which an organism can acclimate is encoded in its genome and some individuals may be more or less able to acclimate. In addition, acclimation, or phenotypic plasticity, can be acted on by different modes of selection and thus be described by two different cases: classical plasticity (Franks and Hoffman, 2012) and phenotypic buffering (Reusch, 2014). In the case of ocean acidification where animals are faced with increased stress due to environmental change, selection may be for increased tolerance and phenotypic buffering where an animal maintains normal function because of plasticity (Sunday et al., 2014). This form of acclimation enhances

the tolerance range for environmental stress and would include responses such as differential expression of important genes. As an added layer of complexity, acclimation and adaptation may differ in relative importance when considering acute versus chronic stress. With this in mind, determining which mechanism or molecular process is conferring resilience will require a holistic approach to understanding the effects of ocean acidification on an organism. Knowledge on the contributors to resilience will provide a more comprehensive understanding of the effects of ocean acidification and the degree to which species may persist under the predicted future climate. Without the ability to tolerate OA, ecosystems and economies would be severely impacted.

Within coastal and estuarine ecosystems, oysters are important both ecologically and economically. Known as ecosystem engineers, oysters provide vital ecosystem services including enhancing water quality via controlling phytoplankton populations and reducing suspended sediment, controlling nutrient cycling, benthic-pelagic coupling, and providing critical habitat (Newell, 2004; Tolley et al., 2005; Coen et al., 2007). Oyster reefs are considered one of the most threatened marine habitats (Beck et al., 2011) due in part to anthropogenic effects and disease (Powell et al., 2008), with habitat extent decreasing 64% and oyster biomass decreasing by 88% just within the 20th century (zu Ermgassen et al., 2012). Ocean acidification may further these declines and could potentially result in the complete loss of species by direct or indirect means via an effect on the eastern oyster or its associated pathogens.

The loss of oysters as a result of changes in ocean chemistry could have profound effects on global economies. Aquaculture alone produces 14M tons of bivalves annually across the globe (Wijsman et al., 2018). Shellfish, and oysters in particular, represent a major part of marine resources in the US. In 2017, eastern oyster landings in the US amounted to over $220 million

(NMFS, 2019). In addition, the seafood industry in New York supports approximately 40,000 jobs (NOAA Fisheries, 2017). A decline in the abundance of the eastern oyster could result in severe damage to national and global economies.

The objective of this thesis is to identify the mechanisms and molecular processes underlying the resilience demonstrated by the eastern oyster (*Crassostrea virginica*) under experimental ocean acidification. This work further expands upon studies that aim to characterize the relative roles of acclimation versus adaptation. Experiments combine physiological assays, which examine the potential for recovery and the impact of trophic resources, and next generation sequencing (NGS) to investigate the processes at play in larval and juvenile oysters under OA stress. This design enables us to identify whether survival is purely due to acclimation (e.g. an energy reallocation mechanism) or if this mechanism is associated with underlying changes in gene expression and/or adaptation of the genome itself. *C. virginica* serves as an excellent model for such studies as the early life stage larvae and juveniles are considered to be particularly sensitive to the effects of OA (Gobler and Talmage, 2014; Schwaner et al., in prep) and are susceptible to a number of ubiquitous microbial pathogens (Romalde et al., 2014). In addition, the annotated genome of the oyster is available (NCBI Accession GCA_002022765.4) for use as a reference in understanding the role of identified genes. The identification of these processes will be vital for selecting traits necessary for the survival of wild populations and aquaculture stocks under OA.

Chapter II: Investigating rebound potential in *Crassostrea virginica* larvae under ocean acidification: a reciprocal transplant experiment

Background

To date, little is known about the recovery potential of calcifiers exposed to OA. Many studies have described the effects on the physiology of these organisms including growth, metabolism, immunity, and reproduction (Beniash et al., 2010; Thor and Dupont, 2015; Frieder et al., 2017; Kapsenberg et al., 2018; Schwaner et al., 2020), however, whether this effect is the result of phenotypic plasticity and is reversible is unknown for many species including the eastern oyster. Phenotypic plasticity is one means by which it is suggested that marine organisms tolerate ocean acidification. Phenotypic plasticity can be defined as "the ability of an organism of one genotype to produce more than one phenotype when exposed to different environmental conditions" (Foo and Byrne, 2016). Plasticity can give way to acclimation which is a term used to describe many ways in which an animal achieves homeostasis with either adaptive or non-adaptive means.

Of the few studies examining plasticity under OA, some make use of a reciprocal transplant design. The benefit of the reciprocal transplant design is that it allows for the identification of plasticity with simple physiological assays that examine recovery, while also enabling the use of RNAseq and DNAseq technologies that can identify molecular changes. If individuals retain some plasticity for variable pH environments and acclimation is an important mechanism for resilience, animals may recover once transplanted from OA to ambient

conditions. If an individual's genotype exhibits plasticity, then they may tolerate a wider range of suboptimal pH at the expense of normal physiological function. Thus, returning oysters to optimal, pH 8.1, conditions after OA stress may result in a rebound in growth and survival.

This has been demonstrated in a study with two species of tropical sea urchins, *Lytechinus variegatus* and *Echinometra luccunter,* in which OA-exposed animals were re-exposed to normal conditions and partially recovered phagocytic activity (Figueiredo et al., 2016). In *Pseudocalanus acuspes*, a calanoid copepod, reciprocal transplant from elevated to ambient pCO_2 conditions resulted in higher egg production rates and larger clutch sizes at lower pCO_2 (Thor and Dupont, 2015). Authors describe these results as suggestive of phenotypic plasticity in the copepod species, however, transgenerational effects became more important than plasticity at higher pCO_2 levels (i.e. 1550 versus 900 µatm).

Reciprocal transplantation has not been done thus far with larval eastern oysters that were reared in acidified conditions and then transplanted to normal pCO_2 conditions. The current experiment expands upon the research published for other sensitive species by serving to understand whether recovery of the eastern oyster following exposure to OA is possible. In this chapter, recovery and phenotypic plasticity are observed through the lens of physiological assays, and later the underlying molecular processes are explored. The objective of this chapter is to identify whether the effects of OA on viability and growth of the larval eastern oyster are reversible following a reciprocal transplant to ambient pCO_2 conditions.

Methods

Seawater chemistry

Seawater was collected from Stony Brook Harbor, NY (40° 55' 15.1428" N, 73° 9' 0.6696" W) and prepared for use by filtering through a 1µm filter and sterilizing via UV light.

Ambient and elevated pCO_2 conditions were maintained according to the guidelines established by EPOCA (Gattuso et al., 2010). The ambient condition was achieved by bubbling in ambient air to maintain a pH of 8.1 (pCO_2 of ~350ppm). The elevated condition was achieved by bubbling in a mixture of 5% CO_2 (balanced in air) and air to maintain a pH of 7.5 (pCO_2 of ~1400ppm) via the use of a gas proportioner (Cole Parmer® Flowmeter system, multitube frame). The target low pH was selected based on end-of-the-century predictions by the IPCC (IPCC, 2014) and based on the current pH conditions in the Long Island Sound where this species inhabits (Wallace et al., 2014). The pH was monitored daily using an Ohaus ST300 Portable pH Meter (precision of 0.01 pH). Bubbling of CO_2 or air began approximately 24 hours before the start of the experiment to ensure the correct and stable treatment pH. Samples for DIC analysis were collected and read using a VINDTA 3D (Versatile Instrument for the Determination of Total inorganic carbon) delivery system coupled with a UIC Inc. (Joliet, IL, USA) coulometer (model CM5017O) (available through the laboratory of Dr. Christopher Gobler (Stony Brook University)). Bicarbonate standards were used and for quality assurance certified reference material was analyzed (provided by Andrew Dickson, Scripps Institution of Oceanography) with a 99.99% recovery during every run. pCO_2, $\Omega_{aragonite}$, $\Omega_{calcite}$, DIC, CO_3, and alkalinity were calculated from pH, temperature, and salinity using the *seacarb* package (https://cran.r-project.org/web/packages/seacarb/index.html) for R statistical software v3.6.1 following parameters recommended by Dickinson et al. (2017) with known first and second dissociation constants of carbonic acid in seawater (Millero, 2010).

Animal Husbandry and Experimental Methods

For the reciprocal transplant experiment, adult *C. virginica* were collected from four wild populations to increase genetic diversity of experimental offspring: Northport, NY (40.9009° N,

73.3432° W); Southampton, NY (40.8840° N 72.4414° W); Mount Sinai Harbor, NY (40.9577° N, 73.0335° W); Wellfleet, MA (41.9305° N, 70.0310° W). Broodstock were conditioned at 18°C on a diet of live-culture microalgae (*Isochrysis* spp. and *Tetraselmis* spp.) delivered via a drip system over a period of eight weeks. Adults were induced to spawn together via a single thermal shock (i.e. temperature brought up to 28°C in a period of <15 minutes) under ambient pCO_2 (~350ppm, pH 8.1) at Great Atlantic Shellfish Farm (Islip, NY (40.7059° N, 73.1946° W)). Individuals that released eggs were identified as female and placed into a separate sea table consisting only of females for temporary holding and egg collection. Individuals that released sperm were identified as males and were left in the spawning sea table for the collection of sperm. After all individuals had released gametes (approximately 7 females and 10 males), sperm was added to the water in which the females had released eggs. The addition of the mixed sperm to the eggs ensured genetic mixing and homogeneity. After allowing sufficient time for fertilization, embryos were then collected and immediately (i.e. <2 hours post fertilization) exposed to either ambient (pCO_2 of ~350ppm, pH 8.1) or elevated pCO_2 (pCO_2 of ~1400ppm, pH 7.5) with 4 replicates per condition.

Larvae were initially held in standing systems in 43-liter buckets at 28 ppt and 20°C in Islip, NY (40.7059° N, 73.1946° W) for the first 7 days following spawning. Larvae were maintained at a stocking density of 10 larvae ml^{-1} (common stocking density in aquaculture (Helm and Bourne, 2004)) and received a complete (100%) water change every other day. During water changes, animals were passed over a mesh sieve (25µm), rinsed, and resuspended in 0.2 µm filtered-seawater equilibrated to the appropriate pH, temperature, and salinity. Larvae were fed *ad libitum* a mixture of live algae including *Tisochrysis lutea* and *Isochrysis* spp. grown from semi-continuous culture using f/2 medium.

After the 7 days, the larvae were transferred to the Marine Animal Disease Laboratory at Stony Brook University and moved to 18-liter buckets (held at 28ppt, 20℃, 10 larvae ml^{-1}, and following the same water change and feeding procedures described in the previous paragraph) where they were then transplanted to the alternative pCO_2 condition. Half of the larvae in ambient pCO_2 were transplanted to elevated pCO_2 and half of the elevated pCO_2 larvae were transplanted to ambient pCO_2; the remaining half in each condition were maintained in their original pCO_2 condition to serve as non-transplanted controls. The number of sea tables available to serve as temperature-regulated water baths was limited to two, and so replicate treatment buckets were randomly assigned to one of the two sea tables. Larvae were maintained under pre-transplant and post-transplant conditions for one-week time periods to allow enough time to discern acclimation from an acute stress response and in order to be able to assess changes in physiology. Experiments terminated before larvae began metamorphosizing.

Larvae were subsampled for viability and growth analysis before transplantation and at the end of the experiment (one-week post-transplantation) by preservation in 1% glutaraldehyde. For viability, larvae were assessed microscopically (range of 350 to 630 larvae per treatment); larvae that lacked internal complexity or had indistinct tissue structure were counted as dead and all other larvae were counted as live prior to preservation. Size was measured using image analysis (ImageJ, NIH) for a range of 190 to 470 larvae per treatment. The remaining larvae were preserved at -80℃ for RNA extraction. Viability and growth data were analyzed for significant differences between treatments as described below.

Statistics

All statistical analyses were conducted using R statistical software v3.6.1. Statistically significant differences in viability between treatments were determined with a G-Test.

Significant differences were concluded if $p<0.05$ (correcting for multiple comparisons was deemed unnecessary according to Perneger (1998)). For growth data, outliers were removed (outliers identified as data points more than 1.5 interquartile ranges below the first quartile or above the third quartile), a Shapiro-Wilk test was used to check for normality, and, if the data did not conform to a normal distribution, a Kruskal-Wallis rank sum test was performed followed by Mann-Whitney U test, or if the data were normal, a Student t-test was used for pairwise comparisons with $p<0.05$ denoting significance.

Results

Dissolved Inorganic Carbon

Complete details of water chemistry conditions are provided in Table 1. Carbonate chemistry matched the target pCO_2 for the ambient and elevated treatments. pH levels in the treatment were approximately 8.1 and 7.5 for the ambient and elevated treatments, respectively. In the elevated pCO_2 condition aragonite was undersaturated.

Table 1 Seawater chemistry values ± SD for the reciprocal transplant experiment. * denotes values calculated with *seacarb.*

Treatment	pH	pCO_2 (ppm)*	$\Omega_{aragonite}$*	$\Omega_{calcite}$*	DIC*	CO_3(µmol/kg)*	ALK(µeq/kg)*
Ambient	8.14±0.08	322.57±78.74	3.36±0.54	5.20±0.83	1981.30±35.41	209.07±33.58	2268.11±67.95
Elevated	7.49±0.09	1600.23±346.23	0.83±0.15	1.28±0.24	2060.09±44.85	51.61±9.49	2084.47±46.61

PRE-TRANSPLANT

Viability

Initial pCO_2 had a significant effect on mortality of larval oysters before transplantation into the alternative pCO_2 treatment (G-test of Independence, G=47.588, $p<0.001$; Figure 1).

Percent mortality was significantly higher for oysters under elevated pCO_2 (10.70%) compared to oysters under ambient pCO_2 (4.37%).

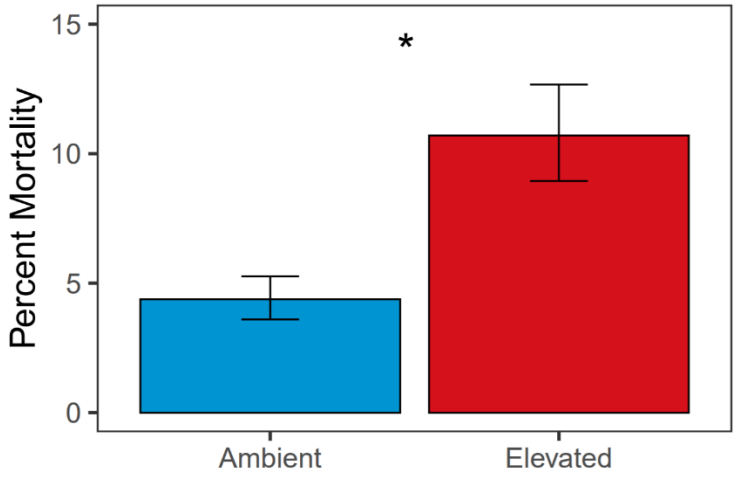

Figure 1 Percent mortality for 7 day old oyster larvae before transplantation into the alternative pCO_2 treatment (* denotes significant difference, G-test of Independence, $p<0.001$; $n=2$, with a minimum of 100 larvae per replicate). Error bars are the 95% confidence intervals.

Growth

Size of oysters maintained under either ambient or elevated pCO_2 prior to transplantation was significantly different between treatments. Oysters under the ambient pCO_2 treatment were significantly larger than those under the elevated pCO_2 treatment (54.20 ± 0.498 and 52.11 ± 0.654 µm, respectively; Mann-Whitney U test, n=2, p<0.001) (Figure 2).

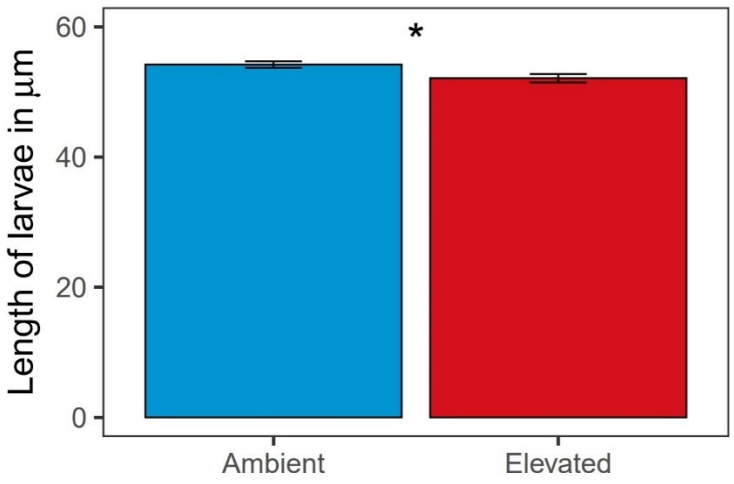

Figure 2 Length in micrometers for 7 day old oyster larvae before transplantation into the alternative pCO_2 treatment in the reciprocal transplant experiment (* denotes significant difference, Mann-Whitney U test, p<0.001; n=2, with a minimum of 65 larvae per replicate). Error bars denote standard error of the mean.

POST-TRANSPLANT

Viability

Final, post-transplantation pCO_2 had a significant effect on mortality of larval oysters (G-test of Independence, G=131.91, p<0.001; Figure 3). Percent mortality was significantly higher for non-transplanted elevated pCO_2 oysters (EE, 46.35%) compared to ambient pCO_2 oysters (AA, 25.36%; G-test of Independence, p<0.001). Oysters transplanted from elevated to ambient pCO_2 conditions (EA) had significantly lower mortality (21.51%) compared to EE (G-test of Independence, p<0.001) and similar mortality compared to the control AA oysters (G-test of Independence, p=0.174). Oysters transplanted from ambient to elevated pCO_2 conditions (AE) had significantly higher percent mortality (48.22%) compared to AA (G-test of Independence, p<0.001) and similar mortality compared to the EE oysters (G-test of Independence, p=0.559).

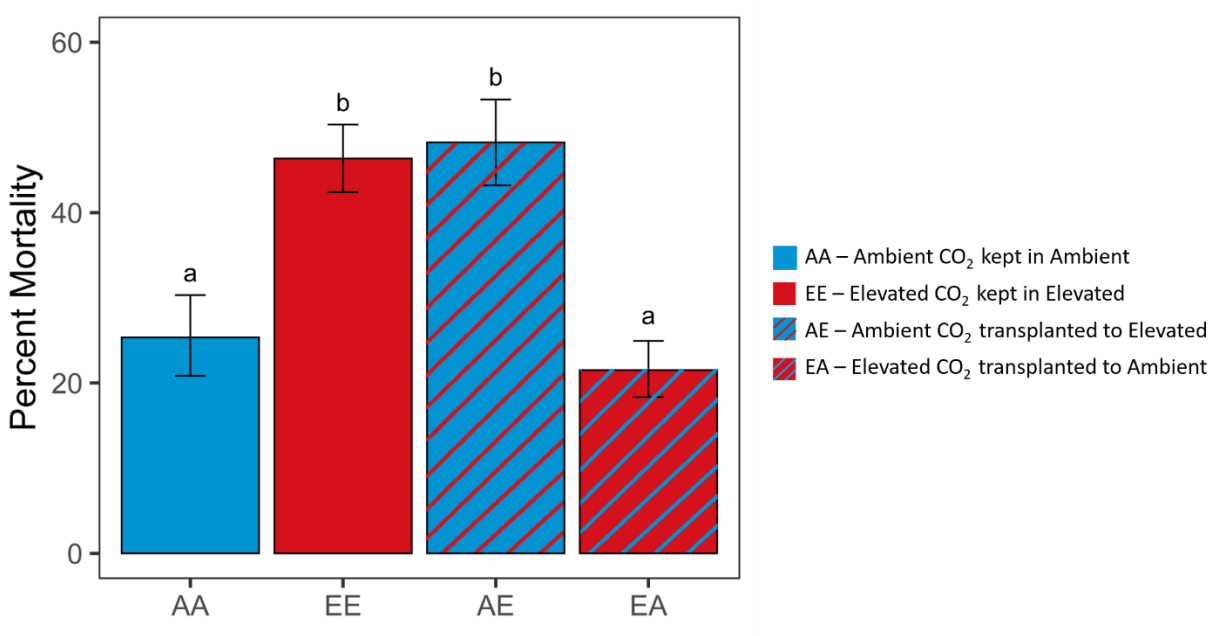

Figure 3 Reciprocal transplant experiment percent mortality (different letters represent significant difference, G-test of Independence, $p<0.001$; $n=4$, with a minimum of 340 larvae per replicate). Error bars are the 95% confidence intervals.

Growth

Oysters in the control ambient pCO_2 treatment (AA) were significantly larger than those in the non-transplanted elevated treatment (EE) (128.84 ± 0.442 and 113.91 ± 0.199 µm, respectively; Mann-Whitney U test, n=2, p<0.001) (Figure 4). Oysters transplanted from the elevated treatment to the ambient pCO_2 treatment (EA) were significantly larger (117.43 ± 0.271 µm) than EE oysters (Mann-Whitney U test, n=2, p<0.001). Alternatively, oysters transplanted from the ambient to the elevated pCO_2 treatment (AE) were significantly smaller (116.99 ± 1.340 µm) than the AA oysters (Mann-Whitney U test, n=2, p<0.001). There was no significant difference in size between oysters from the two transplanted treatments (AE vs. EA; Mann-Whitney U test, n=2, p>0.05).

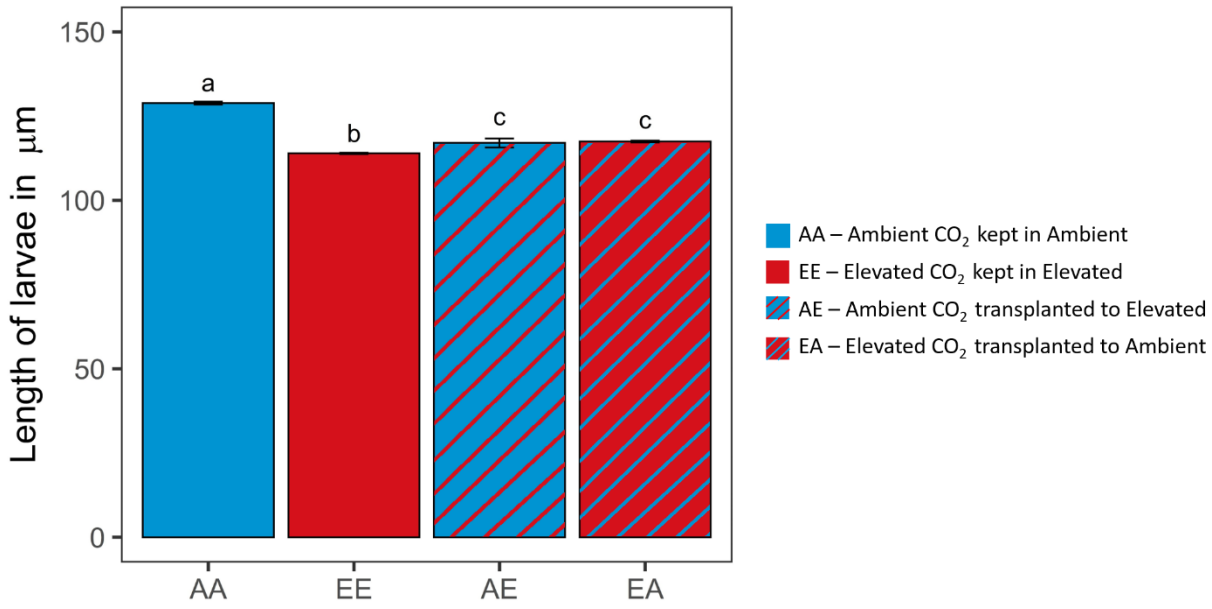

Figure 4 Length in micrometers for 15 day old oyster larvae transplanted (diagonal lines) and kept as controls (solid) in the reciprocal transplant experiment (different letters denote significant difference, Kruskal-Wallis rank sum test, p=0.013; n=4, with a minimum of 180 individuals per replicate). Error bars denote standard error of the mean.

Discussion

The results of this study suggest phenotypic plasticity and acclimation contribute to resilience to ocean acidification. The potential for recovery holds promise for the success of aquaculture management in the future. Ocean pH is not expected to rebound rapidly in such a way that the recovery demonstrated here could occur in the wild, however, the reciprocal transplant design is a means for providing insight on mechanisms for resilience. Our understanding of the ability for calcifying species exposed to OA conditions to rebound and recover normal physiological function is poor. The results of the reciprocal transplant experiment presented here suggest survival under OA conditions is due, at least in part, to phenotypic plasticity that enables larvae to succeed under stressful conditions. The results also suggest that acclimation plays a role in governing resilience to elevated pCO_2 which may enable the individual to tolerate the changing environmental conditions. This conclusion also sheds some

light on the mechanisms larval oysters use to cope with cyclic periods of low pH, especially during summer periods with frequent and extreme fluctuations.

Viability and growth for the non-transplanted treatments before and after transplantation demonstrated higher mortality and smaller size in the oysters under elevated pCO_2 versus those under ambient pCO_2 (EE and AA, respectively, after transplantation) as expected based on previous studies (Gobler and Talmage, 2014; Schwaner et al., in prep). Oyster larvae transplanted from elevated to ambient pCO_2 conditions demonstrated significantly lower mortality and larger size than oysters remaining under elevated pCO_2. On the contrary, larvae transplanted from ambient to elevated pCO_2 conditions demonstrated significantly higher mortality and smaller size than oysters remaining under ambient pCO_2.

The recovery of both viability and growth after transplantation in ambient conditions demonstrates the ability for larvae to rebound quickly (i.e. in one week) under normal conditions. The recovery is suggestive of phenotypic plasticity of the eastern oyster, which has been demonstrated in other species with experiments of a similar design (Gobler and Talmage, 2013; Thor and Dupont, 2015; Figueiredo et al., 2016). Bay scallops, *Argopecten irradians*, exposed to OA exhibited smaller size than those under normal conditions, however, individuals demonstrated compensatory growth after transplantation from elevated pCO_2 to ambient conditions (Gobler and Talmage, 2013) which is similar to the recovery of growth demonstrated in *C. virginica* here. Recovery has also been shown by Calosi et al. (2013) in the polychaete species of *Amphiglena mediterranea*. Following transplantation of *A. mediterranea* from a natural CO_2 vent system to ambient pH, metabolic rates increased back to levels equal to those of a population living outside the vent system after just 5 days. Calosi et al. (2013) describe the results as indicative of phenotypic plasticity, however, results were species-specific. For

instance, *Platynereis dumerilii* (also a polychaete) individuals living within and outside of the vent system exhibited near equal metabolic rates, but transplant to ambient CO_2 conditions from elevated resulted in an increase in rates above those of both non-transplanted populations. This suggests that *P. dumerilii* genetically adapted to living within a high CO_2 vent environment as the vent population was able to cope with elevated CO_2 conditions. Here, *C. virginica* demonstrate a pattern more similar to *A. mediterranea* when transplanted to ambient pCO_2 conditions from elevated. This recovery would not have been observed had selection/adaptation been the mode of survival. If elevated pCO_2 led only to the selection of individuals that tolerate low pH and resulted in a population that would be stressed under ambient conditions, oysters would not have rebound following transplantation to ambient pCO_2. Instead, these results demonstrate phenotypic plasticity at a significant cost to normal physiological function (i.e. growth). The idea of costs or "trade-offs" between resilience traits is well-described in OA research and has been demonstrated in a number of species (Wood et al., 2008; Kelly et al., 2016; Schwaner et al., 2020). Often survival under OA is described as the result of several trade-offs in physiological functions (e.g. growth, reproduction, immunity), but phenotypic plasticity can also be associated with trade-offs as it can result in the reallocation of energy budgets away from some functions (Pörtner, 2008). However, if plasticity or acclimation becomes too "costly" then selection may act to eliminate these genotypes in favor of more fit phenotypes and an adaptive mechanism for resilience would take over. For this reason, the mechanism that confers resilience to OA in marine species is not only species-specific but may also change over time with chronic stress.

A common question surrounding OA research asks about the rate with which these changes (i.e. phenotypic plasticity and acclimation) are expected to take place as compared to the

pace of climate change. The present study begins to answer this question. If larval oysters can acclimate to a new pH regime within a period of approximately one week then there may be hope for their success under future ocean conditions. However, though this experiment demonstrated oysters exhibit phenotypic plasticity under OA stress, this leads to further questions regarding the mechanisms and underlying molecular features for resilience and whether other, less energetically costly adaptive mechanisms are possible. As described previously, the ability to acclimate does not mutually exclude an adaptive mechanism and the physiological assays presented here do not probe the molecular processes for acclimation. Thus, further investigation into underlying molecular processes is needed to comprehensively describe oyster resilience to OA. An initial investigation into the potential for an energy re-allocation mechanism, changes in gene expression, and/or changes to the genome itself are described in the following chapters.

Chapter III: The effect of trophic resources on resilience of *Crassostrea virginica* to ocean acidification

Background

A physiological mechanism that has been suggested to confer resilience to OA in calcifiers is the reallocation of energy budgets (Pansch et al., 2014). Under homeostasis an organism's system is in energetic equilibrium; however, stress on the system may cause energetic disequilibrium in which some processes becoming much more energetically demanding for the organism. This disequilibrium may be acute, as in the case of most attacks on the immune system during infections, in which case equilibrium is returned once the immune system has finished mounting a response and successfully eliminated the infectious agent, or the disequilibrium may be chronic, such as the stress imposed by living in suboptimal habitat with temperature, salinity, or pH outside of the optimal range for a species. This energetic disequilibrium can cause harm to the organism if managing the stressor drains energy away from vital physiological processes. Thus, how an organism allocates its energy budget may determine its overall fitness within a stressful environment.

The traditional view of energy budgets examines the total energy reserves of an individual based on energy intake (i.e. food consumption) and partitions the reserves into processes critical to life: metabolism, growth, and reproduction. Under this "budget" concept, a mechanism for survival under stress could involve an organism reallocating energy reserves towards metabolism and away from growth and immunity. Given the data which suggests

reduced growth, immunity, and reproduction under OA (Bibby et al., 2008; Liu et al., 2016; Sun et al., 2017; Boulais et al., 2017; Frieder et al., 2017; Kapsenberg et al., 2018; Schwaner et al., 2020), the energy reallocation mechanism is one mechanism that has been hypothesized for conferring tolerance to OA (Pansch et al., 2014). In addition, studies have demonstrated higher oxygen consumption rates and thus metabolic rates amongst *C. virginica* exposed to elevated pCO_2 which suggests higher energetic demands under acidification conditions (Beniash et al., 2010; Barbosa et al., 2019). It is predicted that this increase in demand is associated with the increased costs of calcification and acid-base homeostasis (Thomsen and Melzner, 2010; Harvey et al., 2016).

Food-limiting conditions may reinforce the energy reallocation mechanism and further reduce tolerance thereby reducing growth and immunity. However, abundant food could mitigate the effects of a mechanism that prioritizes survival and thus mask the energy reallocation mechanism resulting in an organism with normal physiology. Calcification and organic shell production in bivalves have been demonstrated to be energetically demanding with 31-60% of the total energy budget invested in shell growth (Sanders et al., 2018). Thus, food availability is vital to the normal growth and physiological function of calcifiers especially under acidification stress. To date the effect of food availability on resilience to OA has only been examined in a limited number of studies though most demonstrate food availability is of greater importance for growth and calcification than pCO_2 concentration (Thomsen et al., 2013). A population of *Amphibalanus (Balanus) improvisus* naïve to elevated pCO_2 (1000 μatm) was able to maintain normal growth under elevated pCO_2 when food availability was high, but growth was reduced when food was limited (Pansch et al., 2014), however, these effects were population-specific. In the Olympia oyster *Ostrea lurida* high food availability partially offset the reduction in growth,

total dry weight, and metamorphic success in larvae that resulted from elevated pCO_2 exposure (Hettinger et al., 2013). Thomsen et al. (2013) found juvenile *Mytilus edulis* growth and calcification depended more on food availability than pCO_2 concentration at concentrations below 3350 μatm in the lab. The study also demonstrated *in situ* growth rates were seven times higher in mussels in elevated pCO_2 habitats compared to low pCO_2 primarily as a result of high particulate organic carbon concentrations.

Given the studies that propose primary productivity will decline in some shelf regions (Gregg et al., 2003; Behrenfeld et al., 2006; Gröger et al., 2013), it is especially important to understand how constraints on energy availability may compound the effects of ocean acidification on bivalves. Studies have demonstrated the importance of food availability and the potential for an energy reallocation mechanism's role in conferring resilience to OA for various calcifiers but none have examined the potential in the eastern oyster. In addition, there are no studies examining the importance of an energy reallocation mechanism under combined OA and immune stress. The objective of this chapter is to identify whether food availability significantly impacts the eastern oyster's ability to cope with ocean acidification stress. In this chapter, juvenile oysters are exposed to OA in combination with food limiting conditions and exposure to a major microbial pathogen to determine whether an energy reallocation mechanism confers resilience.

Experimental Methods

Bacteria Culture

Aliiroseovarius crassostreae (causative agent of Roseovarius Oyster Disease (ROD), formerly Juvenile Oyster Disease (JOD)) was maintained in culture grown on Difco™ Marine Agar (Thermo Fisher Scientific, Wilmington, Delaware) at ~20°C and subcultured monthly. One

week prior to the start of the pathogen challenge for the food limitation experiment, *A. crassostreae* was subcultured to target the growth phase for the challenge. The relationship between bacterial growth phase and infectivity is unknown for *A. crassostreae*, therefore two cultures, one at 4-days-old and one at 6-days-old were used in the exposure for the pathogen challenge to enhance disease development. The colonies growing on the plates were collected in 25 ppt 0.22µm-filtered artificial seawater and total number of CFUs was determined by spectrophotometry at 500nm. Target CFU/ml was divided in two and the required amount of each of the cultures was added to the bacteria challenged treatment vessels.

Animal Husbandry and Experimental Methods

For the food limitation experiment juvenile oysters approximately 2-3 months old (shell length: 5mm ± 1.5mm, n=1,600) were procured from Great Atlantic Shellfish Farm from hatchery stock. Juveniles were held in standing systems at 28 ppt and 25°C (optimal temperature for ROD infection) at a stocking density of ~10 juveniles/liter on average throughout the experiment (within range for the abundance of spat in estuaries (Bergquist et al., 2006)). Seawater chemistry was maintained by bubbling according to the methods described in the previous chapter. The number of sea tables available to serve as temperature-regulated water baths was limited to two, and so replicate treatment buckets were randomly assigned to one of the two sea tables. Juveniles received partial (50%) water changes twice a week. During water changes, animals were passed over a mesh sieve (40µm), rinsed, and resuspended in 0.2 µm filtered-seawater equilibrated to the appropriate pH, temperature, and salinity.

Juvenile experiments were fully factorial and represented every possible combination of the three factors: pCO_2, food regime, and pathogen challenge. All conditions were represented by 4 replicates. For Phase 1 of the experiment, juvenile oysters were acclimated to either ambient or

elevated pCO_2 conditions (ambient pCO_2 of ~350ppm, pH 8.1; elevated pCO_2 of ~1400ppm, pH 7.5; 4 replicates per condition) for 7 days at a stocking density of 200 oysters in 10 liters (7 day acclimation was deemed sufficient based on results from the previous chapter). Juveniles were obtained from hatchery stock that had only ever been exposed to ambient pCO_2 and so a pH-acclimation period was necessary. During acclimation, oysters were fed a combination of a commercial Shellfish Diet 1800 (Reed Mariculture, Campbell, California) and live algae (*T. lutea, Isochrysis* spp., and/or *T. suecica* grown from semi-continuous culture using f/2 medium) *ad libitum*. After 7 days, Phase 2 began and half of the oysters from each of the pCO_2 conditions were moved into either the starved (no algae received at all) or fed (*ad libitum*) condition resulting in 4 total conditions (Ambient/Fed, Elevated/Fed, Ambient/Starved, Elevated/Starved) with 4 replicates each. Oysters remained under these conditions for 10 days before Phase 3 began. For Phase 3, the total number of oysters in each replicate bucket and treatment was divided in half again and moved into either the control, non-ROD-exposed treatment or the ROD-exposed treatment (in combination with the continued fed or starved treatment and pCO_2 condition) resulting in 8 total conditions (Ambient/Fed/Control, Elevated/Fed/Control, Ambient/Starved/Control, Elevated/Starved/Control, Ambient/Fed/+Bacteria, Elevated/Fed/+Bacteria, Ambient/Starved/+Bacteria, Elevated/Starved/+Bacteria) with 4 replicates each. ROD was added initially at a total concentration of $5x10^5$ CFU/ml; half of the total concentration of bacteria added was from a 4-day-old culture and the other half was from a 6-day-old culture for reasons described above. Oysters received repeat exposure to ROD twice weekly following partial water changes after which the new bacteria was added to the fresh seawater at the required final concentration. After 2 weeks the total concentration of bacteria was doubled to $1x10^6$ CFU/ml due to overall low mortality. The experiment was concluded after 4

weeks of phase 3 (Day 45 of the experiment) when there was approximately 50% mortality in at least one of the conditions. Samples for DIC measurement were taken prior to the introduction of animals and at the conclusion of the experiment and samples were analyzed according to the methods described in the previous chapter.

During each of the 3 phases, viability was monitored daily and dead oysters (determined based on gaping shells) were discarded. At the end of each phase, all animals were photographed and images were processed for growth analysis of length using ImageJ (Version 1.44, NIH). Cumulative mortality and growth data from the final time point in the experiment (range of ~60 to 130 juveniles measured per treatment) were analyzed for significant differences between treatments as described in the previous chapter under the "Statistics" section. Briefly, statistically significant differences in viability were assessed with a G-Test with $p<0.05$ denoting significance and a Mann-Whitney U test was performed on the growth data. At the end of the experiment, all survivors were preserved at -80°C for tissue RNA extraction.

Results

Dissolved Inorganic Carbon

Complete details of water chemistry conditions are provided in Table 2. Carbonate chemistry matched the target pCO_2 for the ambient and elevated treatments. pH levels in the treatment were approximately 8.1 and 7.5 for the ambient and elevated treatments, respectively. pCO_2 and pH were relatively unchanged between the start and end of the experiment, between fed and starved conditions, or between control and ROD-exposed conditions.

Table 2 Seawater chemistry values ± SD at the start and end of the food limitation experiment. * denotes values calculated with *seacarb.*

Treatment	pH	pCO_2 (ppm)*	$\Omega_{aragonite}$*	$\Omega_{calcite}$*	DIC*	CO_3(µmol/kg)*	ALK(µeq/kg)*
Before the introduction of animals							
Ambient	8.11±0.01	303.78±14.46	2.69±0.04	4.20±0.07	1749.15±21.14	166.45±2.77	1982.20±15.98
Elevated	7.50±0	1403.03±35.39	0.75±0.02	1.17±0.03	1875.96±46.82	46.24±1.24	1899.57±47.02
At the completion of the experiment							
Ambient/ Starved/ Control	8.16±0.04	301.29±38.48	3.58±0.25	5.53±0.39	2003.35±40.25	223.74±15.79	2312.64±15.80
Ambient/ Fed/ Control	8.14±0.03	328.67±60.01	3.55±0.16	5.49±0.25	2073.82±232.13	222.08±10.37	2377.40±235.41
Elevated/ Starved/ Control	7.56±0.01	1420.12±66.65	1.05±0.02	1.62±0.03	2219.89±31.49	65.61±1.24	2268.62±27.24
Elevated/ Fed/ Control	7.54±0.04	1410.96±99.01	0.95±0.12	1.47±0.18	2104.08±59.44	59.73±7.42	2146.17±72.44
Ambient/ Starved/ +Bacteria	8.16±0.01	313.59±30.06	3.74±0.10	5.78±0.15	2089.68±125.67	234.00±6.14	2409.08±128.08
Ambient/ Fed/ +Bacteria	8.09±0.01	358.11±43.49	3.03±0.28	4.67±0.43	1974.73±204.52	189.16±17.47	2235.74±219.81
Elevated/ Starved/ +Bacteria	7.56±0.06	1453.46±136.60	1.07±0.21	1.65±0.33	2241.21±119.68	66.70±13.31	2290.32±141.30
Elevated/ Fed/ +Bacteria	7.53±0.01	1445.27±39.60	0.94±0.04	1.45±0.06	2103.90±9.81	58.90±2.48	2144.11±14.64

Viability

In control treatments there was no significant effect of pCO_2 treatment in fed oysters (G-test of Independence, p>0.05; Figure 5), however, there was significantly higher mortality in non-ROD-exposed ambient pCO_2 oysters in the starved condition as compared to the starved non-ROD-exposed elevated pCO_2 condition (35.37% and 25.31%, respectively; G-test of Independence, p=0.048). In the bacteria challenged treatments, there was no significant difference in mortality under the fed condition for both pCO_2 treatments with regard to the control treatments or compared to each other (G-test of Independence, p>0.05). In the starved and pathogen-challenged treatments there was significantly higher mortality in the ambient pCO_2 oysters (62.35%) compared to those under elevated pCO_2 (51.88%; G-test of Independence, p=0.057) and with respect to all other treatments (G-test of Independence, p<0.05 for all).

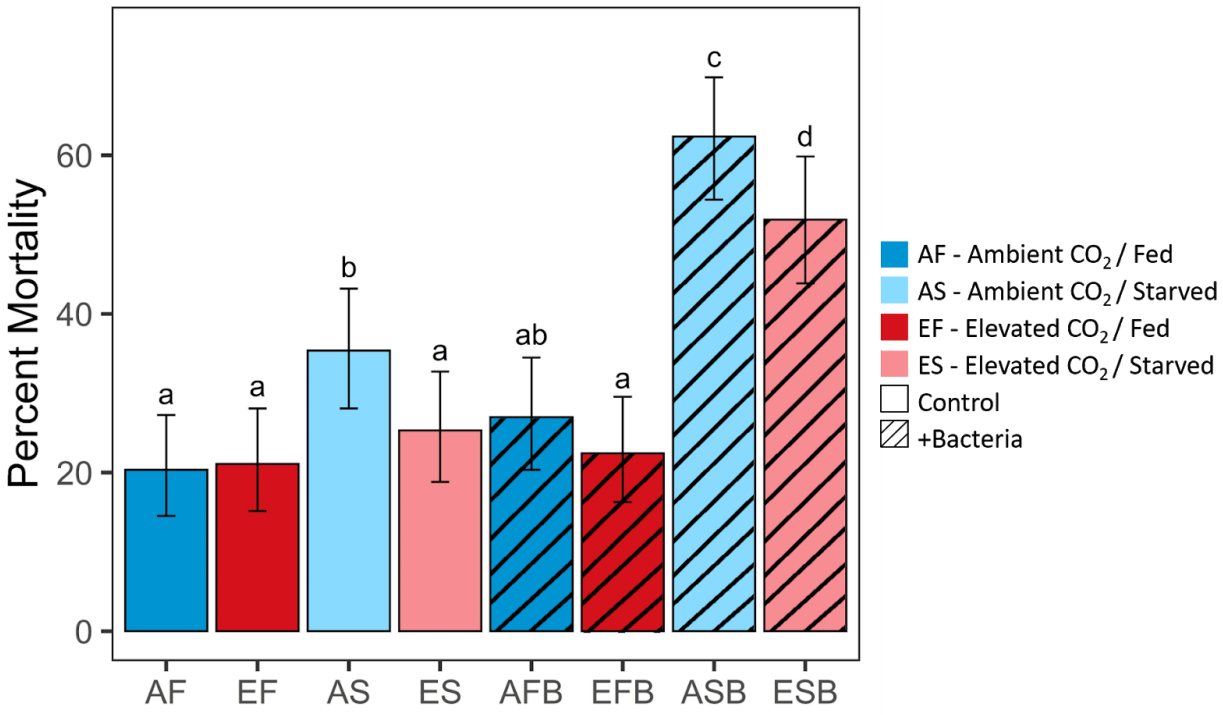

Figure 5 Juvenile food limitation experiment and pathogen challenge percent mortality (different letters denote significant difference, G-test of Independence, p<0.001; n=4, with a minimum of 160 juveniles per treatment). Error bars are the 95% confidence intervals.

Growth

Food availability, but not pCO_2, had a significant effect on size (Kruskal-Wallis rank sum test, $p<0.001$; Figure 6). Ambient pCO_2/Fed oysters measured 7.04 ± 0.12 mm, ambient pCO_2/Starved oysters measured 6.15 ± 0.13 mm, elevated pCO_2/Fed oysters measured 6.52 ± 0.20 mm, and elevated pCO_2/Starved oysters measured 5.98 ± 0.05 mm. Ambient pCO_2/Fed/+Bacteria oysters measured 6.92 ± 0.10 mm, ambient pCO_2/Starved/+Bacteria oysters measured 5.53 ± 0.27 mm, elevated pCO_2/Fed/+Bacteria oysters measured 6.95 ± 0.22 mm, and elevated pCO_2/Starved/+bacteria oysters measured 6.27 ± 0.17 mm. Fed oysters were significantly larger than starved oysters within their respective pH treatment (Mann-Whitney U test, n=2, p=0.009 for ambient pCO_2 fed versus starved, p=0.045 for elevated pCO_2 fed versus starved). Though there was a trend for smaller oysters in the elevated pCO_2 treatment for both fed and starved, control (non-ROD-exposed) conditions, there was no significant difference in size when comparing within each of the two pCO_2 treatments (Mann-Whitney U test, n=2, $p>0.05$). Exposure to ROD did not significantly affect size within a respective pCO_2 and food treatment (Mann-Whitney U test, n=2, $p>0.05$ for all comparisons).

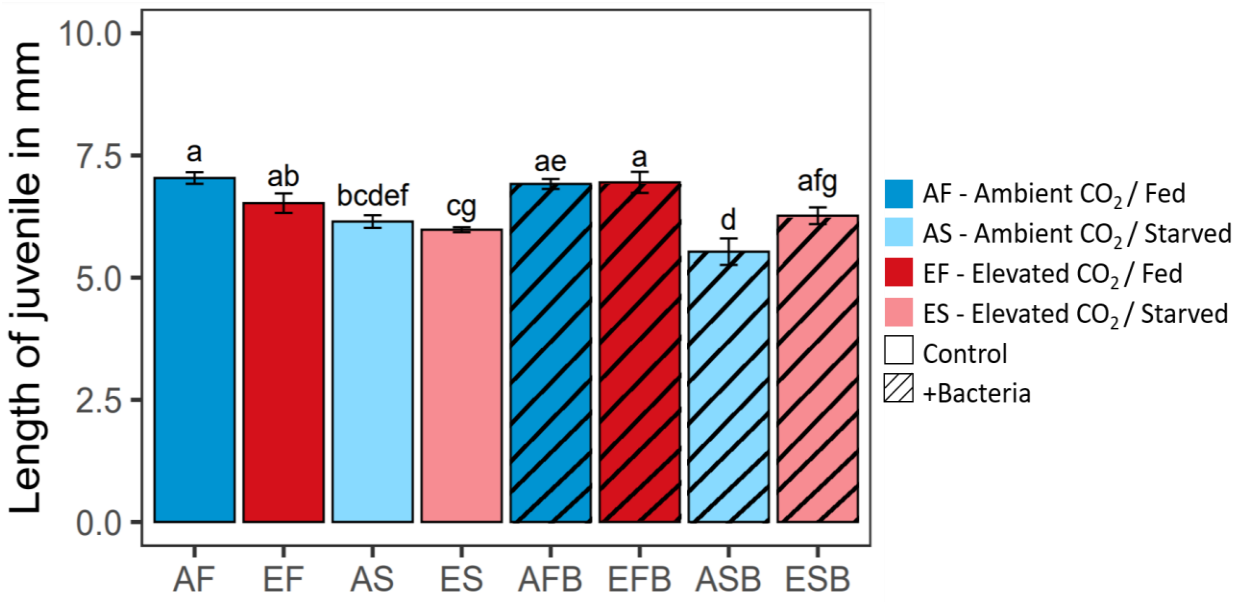

Figure 6 Length in millimeters for juvenile oysters from the food limitation experiment and pathogen challenge following 45 days in pH treatment and 38 days in fed or starved conditions (different letters denote significant difference, Kruskal-Wallis rank sum test, $p<0.001$; n=4, with a minimum of 160 individuals measured per treatment). Error bars denote standard error of the mean.

Discussion

An energy reallocation mechanism has been proposed to be one way calcifiers resist the effects of OA (Pansch et al., 2014) though to date few studies have directly examined the impact of food availability on bivalve resilience to OA. As ocean warming is expected to reduce surface water nutrient availability and thus primary productivity in some regions by the end of the century (Gregg et al., 2003; Behrenfeld et al., 2006; Ramírez et al., 2017), understanding how food limitation impacts resilience is important to predicting the ability for species to persist under future climates. This research is the first to investigate the presence of an energy reallocation mechanism in juvenile *C. virginica* under combined OA and pathogen stress. The results of the food limitation experiment presented here suggest that overall juvenile eastern oysters are more resilient to OA, with regard to viability and growth, than larval oysters. These

results agree with the majority of research demonstrating that larval bivalves are more sensitive to the effects of OA compared to later life stages (Gobler and Talmage, 2014; Schwaner et al., in prep). Though previous studies suggested food availability may be a mechanism for resilience to OA for some populations (Thomsen et al., 2013; Pansch et al., 2014), the results of this study demonstrate that even under complete starvation for 38 days, juvenile oysters in OA conditions do not exhibit significantly higher mortality as compared to those fed or starved in the ambient condition. On the contrary, juvenile oysters in the ambient pCO_2 condition and starved had the highest percent mortality compared to all other control (non-ROD-exposed) conditions. Though these results contradict studies demonstrating reduced viability under combined OA and food limitation stress, they still offer important insight into how juvenile oysters may react to future stressors.

With regard to immunity, starved oysters were more susceptible to infection by ROD, but again the oysters in ambient conditions were more susceptible to infection than oysters in elevated pCO_2 conditions. The difference in mortality for fed versus starved oysters under pathogen challenge was expected as lower food availability would reduce energy available to maintain normal immune function (Delaporte et al., 2006). However, lower mortality in OA exposed oysters relative to ambient pCO_2 oysters was unexpected. These results suggest the juvenile oysters overall are more robust to the stress of OA and combined pathogen stress. Though the majority of studies point to immunosuppression under OA stress (Bibby et al., 2008; Liu et al., 2016; Sun et al., 2017; Schwaner et al., 2020), few have shown no effect on some immune parameters (Wang et al., 2016; Ellis et al., 2015). Ellis et al. (2015) demonstrated that while immune function was impaired in *M. edulis* prior to exposure to *Vibrio tubiashii*, following exposure hemolymph bactericidal activity was restored. This suggests immunosuppression may

be a temporary physiological trade-off and can be reversed if needed. If this is possible in *C. virginica* juveniles then it may support the results that seemingly contradict the susceptibility to bacterial pathogens demonstrated in previous studies. As there was no large mortality event at the beginning of the experiment following exposure to OA, one can rule out the possibility that the oysters in the OA treatment exposed to ROD have been selected to be the most resilient individuals. Combined with the viability results for control, non-ROD-exposed oysters, the results of the pathogen challenge do not point at an energy reallocation mechanism for resilience to OA under our experimental conditions.

Viability results indicate that juveniles are more resilient under OA, however, the results from growth analysis show different trends. Though the differences are not statistically significant, in the fed, control (non-ROD-exposed) condition there is a trend for smaller size in oysters held under elevated pCO_2 conditions. It is likely that if the experiment lasted longer larger differences in size over the 45 day period may have presented and those differences may have been significant. The difference in length between the ambient and elevated starved conditions is smaller although the trend for smaller size in elevated conditions is still present. The difference between the two starved conditions is likely not statistically significant due to slow overall growth as a result of lack of energy for shell biogenesis. Interestingly, exposure to ROD did not affect growth of oysters in any ROD-exposed treatment with regard to their respective control, non-exposed treatment despite the energetic demands an active immune system would have. The slight decrease in growth for juveniles fed and under elevated pCO_2 compared to those fed and under ambient pCO_2 still does suggest metabolic depression to some extent under OA. However, resilience or survival under metabolic depression may not be

sustained by energy reallocation, but instead by some molecular process that is not demonstrated with the physiological assays described in this chapter.

As described earlier, Pansch et al. (2014) demonstrated that high food availability partially mitigated the reduction in size due to exposure to elevated pCO_2 in a population of *A. improvisus* barnacles derived from the Tjärnö Archipelago in Sweden. However, this effect was population-specific and within the same study authors documented the contrary for a population of barnacles from Germany. Results similar to those demonstrated here were shown for a population of *A. improvisus* from Kiel Fjord, Germany in which survival and growth was unaffected by elevated pCO_2 under both normal and food limited conditions (Pansch et al., 2014). These results both support the work here and also serve to demonstrate the presence of population-specific effects of OA. The difference in susceptibility to OA demonstrated by oysters in other studies and the resilience of juveniles demonstrated here could be the result of differences in susceptibility between populations. Durland et al. (2019) demonstrated differences in survival and growth of spat between 'wild' and selectively bred stocks of *C. gigas* exposed to elevated pCO_2. Juvenile oysters used here were derived from hatchery stock and as so were selectively bred to enhance the qualities desired by the industry (i.e. fast-growth, resistance to pathogens, etc.); however, little else is known about the adaptive history of this stock with regard to pH or the precise pH range the hatchery experiences. Selective breeding of this cultured line may have directly or indirectly affected resilience to elevated pCO_2 in this stock, and as a result juveniles used in this study may in fact differ in resilience from wild stocks. Population, therefore, is a major consideration when designing OA studies and could also impact the identification and relative importance of resilience mechanisms.

Alternatively, the slow shell building shown here in the growth trends may have alleviated some of the energetic demand that resulted from OA stress. Waldbusser et al. (2016) demonstrated that the larvae of the Olympia oyster *O. lurida* showed no negative response to OA and attributed this resilience to the relatively slow calcification rate and shell building. Under this hypothesis, the trend for slow growth in oysters exposed to elevated pCO_2 may have played a key role in the energy reallocation mechanism which was not evident by looking at viability alone. Additionally, research into fluctuating versus static pH regimes has demonstrated fluctuating regimes to be more energetically costly for bivalves (Mangan et al., 2017). Here oysters were maintained on static systems which may have been less energetically costly thus resulting in no effect of pCO_2 on survival in both control and ROD-exposed conditions and only a trend for a decrease in growth under elevated pCO_2. Therefore, pH regime is likely another important consideration when comparing the effects of OA between studies.

In many cases/publications it may be unclear as to whether or not animals in a study are receiving adequate food resources as data on the recommended food levels for some species may be limited. As such, many studies may be indirectly documenting the effect of OA on food-limited individuals either unknowingly or without directly informing the reader. Alternatively, animals maintained *in situ* or on flow-through systems may have ample food available and so the effects of OA documented in these studies may differ from studies where animals are fed cultured algae. Food availability may be another reason for which there is such high variability from publication to publication documenting the effect of OA on the same species, and, in experiments where food availability was limited, the effects of OA may be inflated (Ramajo et al., 2016).

Recent OA work has shifted from documenting the physiological and physical impacts of OA to understanding why such differences are often species- and population-specific and how this may shed light on the mechanisms for resilience. Our ability to identify specific processes and mechanisms important for resilience can often be limited by the scope of physiological assays and natural variability. Here, an energy reallocation mechanism for resilience to OA is investigated, but results are inconclusive due to the tolerance demonstrated by juveniles under OA. These results suggest either that juvenile *C. virginica* are, overall, resilient to the effects of OA physiologically, or that other, hidden mechanisms may underlie the resilience owing to the inability to distinguish the energy reallocation mechanism based on the physiological assays alone. In order to discern the true reason behind the demonstrated resilience, the physiological assays are combined with next-generation sequencing so that underlying molecular mechanisms for resilience may be uncovered. The presence of a molecular mechanism (e.g. changes in gene expression or selection at the genomic level) is further evaluated in the following chapters to provide a complete view of the potential mechanisms underlying resilience to OA.

Chapter IV: Transcriptomic signature of acclimation to ocean acidification in *Crassostrea virginica*

Background

Acclimation refers to the suite of physiological changes that occur within an organism with the intent to achieve homeostasis in response to a change in environment. Acclimation is often reversible, occurs on the level of the individual, and can include processes such as maternal effects, epigenetic changes, and changes in gene expression among others (De Wit et al., 2016). Changes in gene expression levels have been suggested to be one method by which organisms acclimate to and tolerate OA stress (Hofmann et al., 2008). As gene expression is considered to be highly plastic in response to changing environmental conditions (Wolf, 2013), assessing changes in expression makes for an excellent choice when studying the effects of OA on bivalves and investigating the role of acclimation in conferring resilience. Under stress an organism may modulate mRNA transcript abundance within a cell and thus gene expression and protein production will be altered. This change can result in observed phenotypic changes in growth and immune function but may also have unobservable consequences. Comparing the relative expression levels between populations under control and stressed conditions can lead to the identification of genes either affected by the stress or vital to the resilience of the organism or both.

Changes in the cellular transcriptome can be measured using the next generation sequencing (NGS) method of whole-transcriptome shotgun sequencing or RNA sequencing

(RNAseq). The advantage of transcriptome sequencing is that the RNAseq data are derived from only the functional, protein-coding genes (Wolf, 2013). Sequence information is generally derived in the form of short read sequence data; by counting the number of reads that align to the genome at a specific gene/transcript, the transcript abundance can be measured and used for gene expression comparisons between populations. The use of RNAseq is simplified when the reference genome of the study species is already sequenced which, fortunately, is the case for *C. virginica* (NCBI Accession GCA_002022765.4).

A growing number of studies have begun to examine shifts in gene expression in response to environmental stress and OA specifically. Studies have demonstrated variability in *C. virginica* gene expression associated with changes in pH with some genes exhibiting higher expression at low pH and some exhibiting lower expression (Chapman et al., 2011). Additional studies in other bivalve species have shown differential regulation of genes related to biomineralization and immunity which has been suggested to function in frontloading the immune system (Ertl et al., 2016). In the bivalve *Laternula elliptica,* exposure to low pH resulted in up-regulation of the enzyme chitin synthase (an enzyme associated with synthesizing shell material) (Cummings et al., 2011). In the blood clam, *Tegillarca granosa,* low pH conditions led to the down-regulation of genes responsible for NF-kappa β and toll-like receptor pathways thus reducing immune response (Liu et al., 2016). The genes affected under OA and the degree to which the expression of those genes are upregulated or downregulated as an associated "cost" appear to be species-specific and age-specific, however, acclimation via gene expression plasticity shows promise as a mechanism for resilience to OA.

While the use of RNAseq is beginning to be employed more in OA research to understand resilience and acclimation potential, few studies have examined the changes in gene

expression level for multiple life history stages of *C. virginica* exposed to OA stress. Furthermore, even fewer studies have the benefit of pairing physiological assays with NGS RNAseq technology. By combining RNAseq with the experimental design of the reciprocal transplant experiment (i.e. transplanted treatments), it will be possible to truly identify acclimation and plasticity if non-transplanted and transplanted larvae exhibit similar patterns of expression under the same, final pCO_2. Investigations of terrestrial and freshwater species have combined reciprocal transplants with genetic studies to identify local adaptation, and, more specifically, genes that confer adaptive resilience to local habitat conditions (Lohman et al., 2017; Gould et al., 2018), however this technique is relatively unexplored in ocean climate change research. In addition, few studies have examined whether there are molecular features underlying the physiological mechanisms for resilience. Many studies suggest physiological mechanisms (e.g. shifting energy budgets or an energy reallocation mechanism) (Pansch et al., 2014; Ventura et al., 2016; Waldbusser et al., 2016) for conferring resilience in calcifiers under OA stress, however, it is more than likely that these mechanisms have underlying molecular features and thus molecular processes also comprise the mechanisms for resilience.

The objective of this chapter is to identify genes differentially expressed in OA-tolerant larvae and juveniles after transplantation to the alternative pH condition, at different food conditions, and under pathogen challenge. If resilience to OA is due to plasticity in expression of transcripts/genes then, between oysters under ambient versus elevated pCO_2, we would expect the differential expression of genes considered to be involved in response to environmental stress. This investigation will provide a more holistic approach to understanding the mechanisms and molecular features underlying resilience to OA.

Methods

RNA Sample Preparation

For the reciprocal transplant experiment, RNA from samples of pooled larvae stored at -80 °C (one sample per bucket; 4 replicate buckets per treatment) was extracted using the NucleoSpin® RNA Plus RNA isolation kit (Machery-Nagel, Düren, Germany). For the food limitation experiment, RNA from three juvenile oysters per replicate bucket previously stored at -80 °C was extracted individually using the NucleoSpin® RNA Plus RNA isolation kit and equal quantities of extracted RNA from the three individuals were pooled resulting in one sample per bucket (4 replicate buckets per treatment). RNA quantity and quality was checked with a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware). Library preparation, sequencing, and initial analyses of RNA from the reciprocal transplant and food limitation experiments were performed by Novogene Corporation (UC Davis, Sacramento, California). RNA degradation and contamination were monitored on 1% agarose gels, RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA), and RNA integrity and quantitation were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). For library preparation, 1 µg RNA per sample was used as input material and sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) with cDNA fragments of 150~200 bp in length preferentially selected for. Library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was

assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) before sequencing on an Illumina platform where 125 bp/150 bp paired-end reads were generated.

RNAseq Library Preparation, Sequencing, and Analysis

For RNAseq data, Novogene performed quality control, read mapping, quantification of gene expression level, differential expression analysis, GO enrichment analysis of differentially expressed genes, and KEGG pathway enrichment analysis of differentially expressed genes for RNAseq data. In the quality control step, clean reads were obtained by removing reads containing adapters, reads containing ploy-N, and low quality reads from raw data. Q20, Q30, and GC content of the clean data were calculated. Cleaned data was used in downstream analyses. The reference genome and gene model annotation files were obtained from NCBI (NCBI Accession GCA_002022765.4) and the index of the reference genome was built using hisat2 2.1.0. Paired-end clean reads were mapped to the reference using HISAT2. HTSeq v0.6.1 was used to count the number of reads mapped to each gene and FPKM (Fragments Per Kilobase of transcript per Million mapped reads) was calculated using the length of the gene and the number of reads mapping to that gene. Differential expression analysis was performed using the DESeq R package (1.18.0) which uses a model based on the negative binomial distribution for determining differential expression. P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR) and genes with a p-value < 0.05 were considered differentially expressed. Genes identified as differentially expressed were annotated using UniProtKB and data were explored for promising genes/proteins related to processes that may be important for resilience (e.g. biomineralization, homeostasis, development) and compared to genes found in similar studies. Gene Ontology enrichment analysis was possible as

the *C. virginica* transcriptome has been previously annotated. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias was corrected and GO terms with corrected p-value less than 0.05 were considered significantly enriched. KOBAS software was used to test the statistical enrichment of differentially expressed genes in KEGG pathways.

Results

RECIPROCAL TRANSPLANT EXPERIMENT

Differential Gene Expression Analysis

An average of 607,989 ± 262,546 trimmed reads per sample mapped to the reference genome corresponding to an average of 1.40 ± 0.69% mapping rate. Pearsons's correlation coefficient comparisons demonstrated a moderate correlation ($R^2 > 0.314$) for samples derived from the same treatment (Figure 7). After cluster analysis, treatments clustered based on the final pCO_2 condition (i.e. non-transplanted ambient pCO_2 samples (AA) and samples transplanted from elevated to ambient pCO_2 (EA) clustered most closely and non-transplanted elevated pCO_2 samples (EE) and samples transplanted from ambient to elevated pCO_2 (AE) clustered together) (Figure 8). The total number of differentially expressed genes (DEGs) between treatments ranged from 1 to 49 (A full list of DEGs can be found in supplementary data Table S1). The greatest number of DEGs were found to be between AA versus EE (the two control treatments), with a total of 49 genes of which 21 were upregulated (i.e. relatively higher expression in EE) and 28 were downregulated (i.e. relatively lower expression in EE). The fewest number of DEGs were between AE and EA treatments with a total of 1 gene which was upregulated in the AE treatment. AA versus AE and EE versus EA both had intermediate numbers of DEGs with a total of 6 and 8 DEGs, respectively. Of the 49 DEGs between AA and EE, 32 (65%) were annotated.

In the comparison of AA versus AE, 4 of the 6 DEGs were annotated (66.7%). Of the 8 DEGS between EE versus EA, 5 genes were annotated (62.5%). The single gene differentially expressed between AE and EA was annotated.

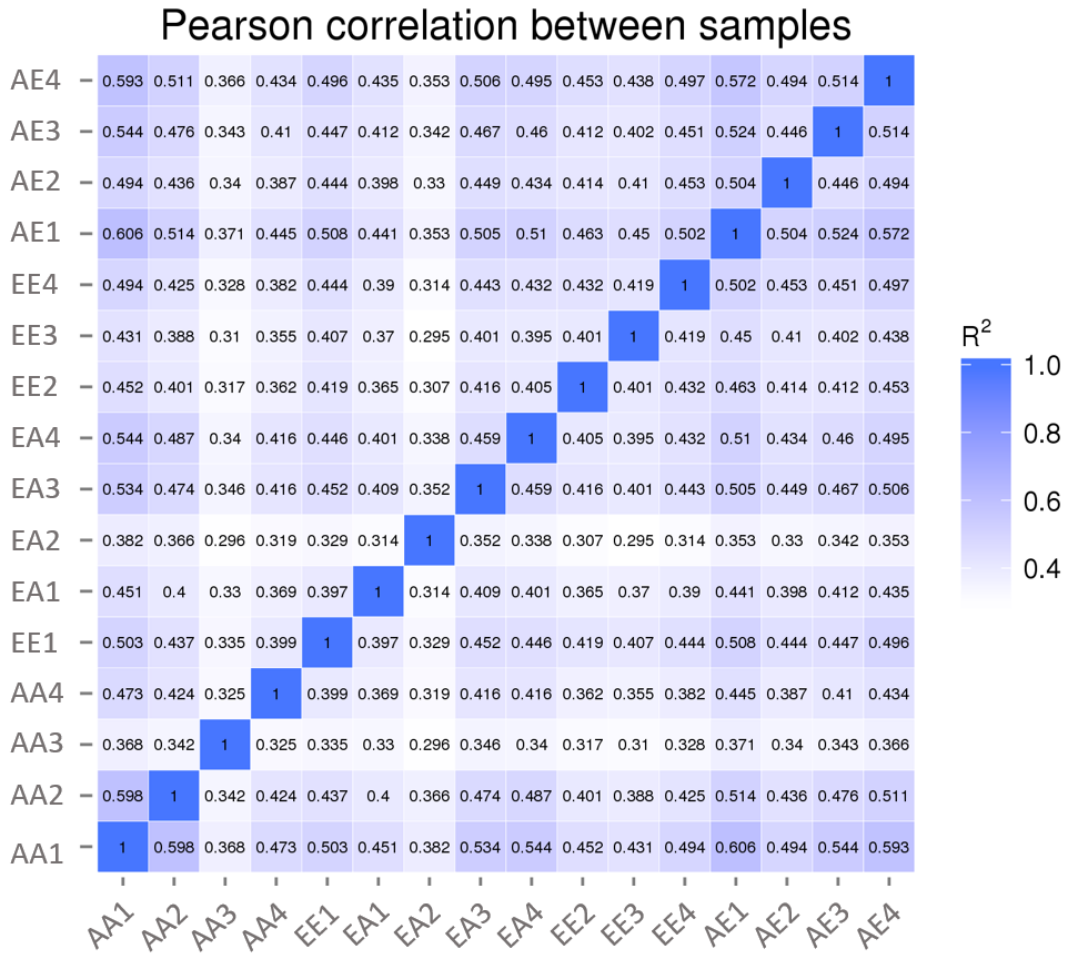

Figure 7 Pearson's correlation coefficient matrix for the reciprocal transplant experiment. Sample code names: First letter signifies original pCO_2 treatment (A: Ambient, E: Elevated); Second letter signifies final pCO_2 treatment (first and second letters are different for transplanted treatments); Number: replicate number

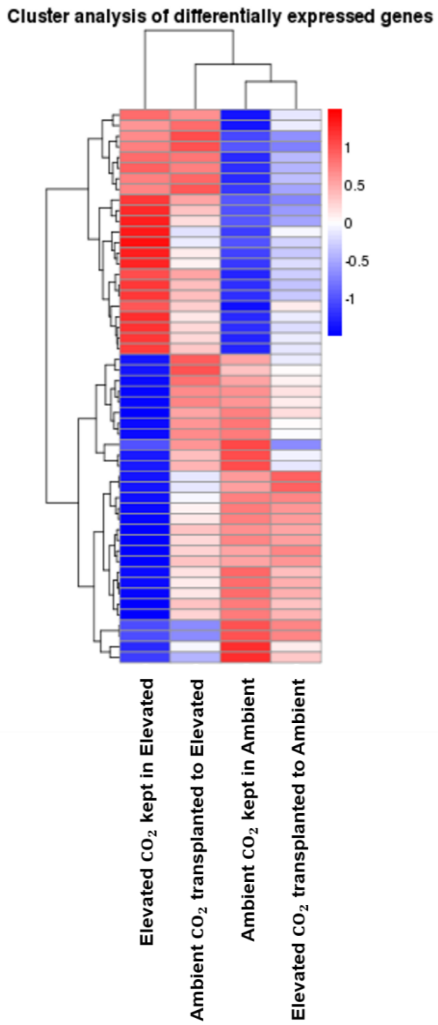

Figure 8 Cluster analysis of DEGs for the reciprocal transplant experiment. Red denotes a gene is overexpressed in a treatment and blue denotes a gene is under expressed in a treatment. Data are normalized by average expression in all samples.

Twelve genes up- or downregulated in a non-transplanted treatment were similarly expressed in larvae from a transplanted treatment (Supplementary data Table S2). Nine of the eleven genes were annotated and had functions related to fibropellin, the mitochondrial ATP synthase beta subunit, MAM and LDL-receptor class, tubulin beta chain, coadhesin, hemicentin, transforming growth factor-beta-induced protein ig-h3, and two had hyaluronidase function.

Gene Ontology Analysis

A comparison of significant GO terms enriched between AA and EE yielded one term broadly related to molecular function and two terms related to biological processes, all three of which were upregulated in EE (Supplementary data Table S3). The GO term with molecular function was found to be involved in arabinosyltransferase activity (hypergeometric test, p=0.00063). The GO terms involved in biological processes were found to function in actinobacterium-type cell wall biogenesis (hypergeometric test, p=0.00063) and peptidoglycan-based cell wall biogenesis (hypergeometric test, p=0.048). There were no significantly over-represented GO terms for all other comparisons (hypergeometric test, p>0.05) (AA vs AE, EE vs EA, or AE vs EA).

KEGG Pathway Enrichment Analysis of DEGs

Several pathways were significantly enriched in the DEGs for all treatment comparisons (Supplementary data Table S4). A comparison of KEGG pathways enriched between AA and EE yielded 3 significantly enriched pathways: ribosome biogenesis in eukaryotes, phagosome and oxidative phosphorylation. The former was upregulated in EE and the two latter pathways were downregulated. A comparison of KEGG pathways enriched between EE and EA yielded 2 significantly enriched, upregulated pathways: phagosome and oxidative phosphorylation. Between AA and AE KEGG pathway enrichment analysis yielded 2 significantly, upregulated enriched pathways: dorso-ventral axis formation and notch signaling pathway. A comparison of KEGG pathways enriched between AE and EA also yielded 2 significantly enriched, upregulated pathways: dorso-ventral axis formation and notch signaling pathway. For the latter two comparisons (AA vs AE and AE vs EA), the genes that related to those two specific pathways, dorso-ventral axis formation and notch signaling pathway, were the same gene (Gene ID 111113165).

FOOD LIMITATION EXPERIMENT

Differential Gene Expression Analysis

An average of 35,289,420 ± 8,052,916 trimmed reads per sample mapped to the *C. virginica* reference genome corresponding to an average of 76.99 ± 4.19% mapping rate. Pearsons's correlation coefficient comparisons demonstrated a high correlation ($R^2 > 0.858$) for samples derived from the same treatment and also high similarity in transcription profiles for all populations ($R^2 > 0.758$) (Figure 9). After cluster analysis, treatments clustered based on the feeding condition (i.e. fed treatments clustered most closely and starved treatments clustered together) independent of pCO_2 treatment or bacteria exposure (Figure 10). The total number of DEGs between treatments ranged from 1 to 1,355 (A full list of DEGs can be found in supplementary data Table S5). The greatest number of DEGs was found between Elevated pCO_2/Fed oysters (EF) and Elevated pCO_2/Starved oysters (ES), with a total of 1,014 genes upregulated under starvation (i.e. relatively higher expression in Elevated pCO_2/Starved oysters) and 341 genes downregulated (i.e. relatively lower expression in Elevated pCO_2/Starved oysters). The fewest number of DEGs were between Elevated pCO_2/Starved oysters (ES) and Elevated pCO_2/Starved/+Bacteria oysters (ESB) with a total of 1 gene which was downregulated in the Elevated pCO_2/Starved/+Bacteria treatment. Between Ambient and Elevated pCO_2 fed oysters (AF and EF, respectively) 8 genes were differentially expressed with 1 gene upregulated and 7 genes downregulated under elevated pCO_2. Between Ambient and Elevated pCO_2 starved oysters (AS and ES, respectively) 17 genes were differentially expressed with 6 genes upregulated and 11 genes downregulated under elevated pCO_2. On average, 51% of the identified DEGs in each of the comparisons were annotated.

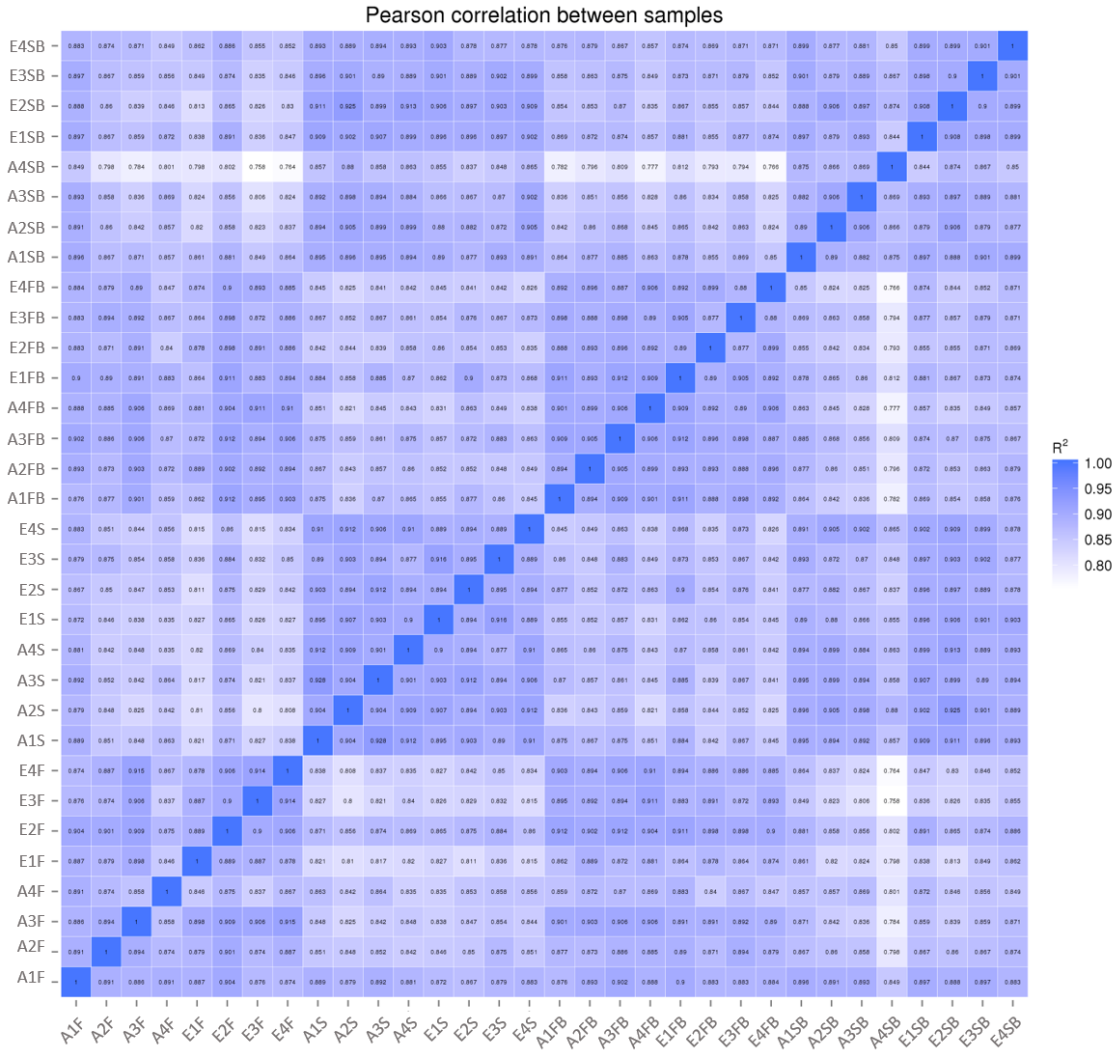

Figure 9 Pearson's correlation coefficient matrix for the food limitation experiment. Sample code names: First letter signifies original pCO_2 treatment (A: Ambient, E: Elevated); Number signifies replicate number; Second letter signifies food treatment (F: Fed; S: Starved); Samples with the letter B were exposed to bacteria (i.e. ROD).

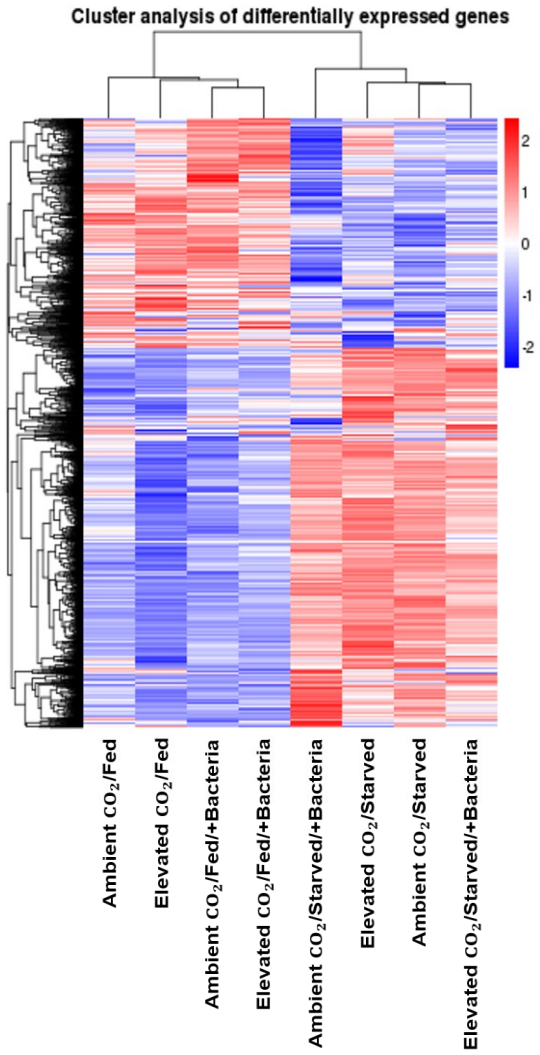

Figure 10 Cluster analysis of DEGs for the food limitation experiment. Red denotes a gene is overexpressed in a treatment and blue denotes a gene is under expressed in a treatment. Data are normalized by average expression in all samples.

Gene Ontology Analysis

Four of the twelve comparisons performed in this study yielded significantly enriched GO terms between treatments (Supplementary data Table S6). Between Ambient pCO_2/Fed and Ambient pCO_2/Starved oysters (AF and AS, respectively), 17 terms were significantly upregulated under starved conditions and 29 were downregulated. Of those upregulated under starvation, 1 related to cellular components, 6 belonged to biological processes, and 10 related to

molecular functions (hypergeometric test, $p<0.05$ for all). Of those downregulated, 19 related to cellular components, 8 belonged to biological processes, and 2 related to molecular functions (hypergeometric test, $p<0.05$ for all). Between Elevated pCO_2/Fed and Elevated pCO_2/Starved oysters (EF and ES, respectively), 39 terms were significantly upregulated under starvation and 17 were downregulated. Of those upregulated, 4 were broadly related to cellular components, 14 belonged to biological processes, and 21 related to molecular functions (hypergeometric test, $p<0.03$ for all). Of those downregulated under elevated CO_2 and starvation, 5 were broadly related to cellular components, 10 belonged to biological processes, and 2 related to molecular functions (hypergeometric test, $p<0.007$ for all). Between Ambient pCO_2/Fed/+Bacteria and Ambient pCO_2/Starved/+Bacteria oysters (AFB and ASB, respectively), 3 terms were significantly upregulated in the starved oysters and 4 were significantly downregulated. Of those terms significantly upregulated under starvation, all 3 terms were related to molecular function (hypergeometric test, $p<0.04$ for all). Of those terms significantly downregulated, 2 were broadly related to cellular components, 1 belonged to biological processes, and 1 related to molecular functions (hypergeometric test, $p<0.004$ for all). Between Elevated pCO_2/Fed/+Bacteria and Elevated pCO_2/Starved/+Bacteria oysters (EFB and ESB, respectively), 7 terms were significant between treatments of which all were upregulated under starvation. Of those significant terms, 1 was related to cellular components, 5 were broadly related to biological processes, and 1 related to molecular functions (hypergeometric test, $p<0.003$). There were no significantly over-represented GO terms for all other comparisons (hypergeometric test, $p>0.05$) (AFvsEF, AFvsAFB, EFvsEFB, ASvsES, ASvsASB, ESvsESB, AFBvsEFB, and ASBvsESB).

KEGG Pathway Enrichment Analysis of DEGs

Several pathways were significantly (hypergeometric test, $p<0.05$) enriched in the DEGs for eight of the twelve treatment comparisons (Supplementary data Table S7). A comparison of KEGG pathways enriched between AF and EF yielded 5 significantly enriched pathways: porphyrin and chlorophyll metabolism, metabolic pathways, spliceosome, protein processing in endoplasmic reticulum, and endocytosis with the two former pathways being upregulated in EF and the later three downregulated. A comparison of KEGG pathways enriched between AF and AS yielded 6 significantly enriched pathways that were upregulated in AS: starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, ECM-receptor interaction, lysosome, tyrosine metabolism, and metabolic pathways. One pathway related to ribosome function was downregulated in AS compared to AF. A comparison of KEGG pathways enriched between EF and ES yielded 5 significantly enriched, upregulated pathways in ES: ECM-receptor interaction, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, dorso-ventral axis formation, and lysosome. Two pathways were downregulated in ES compared to EF: ribosome and arachidonic acid metabolism. For AS compared to ASB, one pathway related to amino sugar and nucleotide sugar metabolism was upregulated in ASB. A comparison of KEGG pathways enriched between AFB and EFB yielded 2 significantly enriched pathways: glycerolipid metabolism and metabolic pathways. Between AFB and ASB one pathway, related to phagosome function, was upregulated in ASB and two pathways, related to the phosphatidylinositol signaling system and glycerolipid metabolism, were downregulated. A comparison of KEGG pathways enriched between EFB and ESB yielded two significantly enriched, upregulated pathways in ESB: ECM-receptor interaction and tyrosine metabolism. For ASB compared to ESB, one pathway related to ribosome function was downregulated in ESB.

Discussion

Analysis of gene expression levels in both the reciprocal transplant and food limitation experiments revealed a shift in steady state gene expression in oysters exposed to elevated pCO_2. These results suggest acclimation as a mode for resilience to OA stress and demonstrate that *C. virginica* possesses the genetic repertoire needed for plasticity under extreme environmental conditions. Overall, the trends demonstrate that larval transcriptomes respond to OA by downregulating genes involved in energy/metabolism, the cytoskeleton, and genes dependent on calcium while juveniles appear to respond with a general stress response largely via regulation of chaperone proteins (e.g. heat shock proteins). This further supports the results demonstrated by the physiological assays which suggest juveniles are more robust to the effects of OA as there is little compensation either in growth or gene expression levels of energy/metabolism or biomineralization genes. Nevertheless, guided by prior studies (Chapman et al., 2011; Cummings et al., 2011; Moya et al., 2012; Pespeni et al., 2012; Koh et al., 2015; Liu et al., 2016) on related taxa, the differentially expressed genes (DEGs) between each treatment comparison were broken down into a small number of categories which highlight the role they may play in resilience to ocean acidification. To understand the reasons for which each gene is either up or downregulated under OA, the function of each protein can be investigated. Here only a select few genes are described as these appear to be the most interesting genes for acclimation to OA. It is important to note that a single gene can have many functions and so the functions discussed here may or may not be the reason for which a particular gene is up- or downregulated. Furthermore, the majority of proteins mentioned here have only been described in vertebrates and so their function may differ in invertebrate bivalves. Thus, the discussion here highlights just one of potentially many reasons a gene is differentially regulated under OA.

RECIPROCAL TRANSPLANT EXPERIMENT: *Interpreting DEGs*

Most DEGs found in the reciprocal transplant experiment fit into one of four categories, many of which are related to biomineralization: (1) “energy/metabolic processes”, (2) components of the “extracellular matrix”, (3) the “cytoskeleton”, or (4) “immunity”. Several genes of interest were shared between the dataset presented here and with previous OA studies with RNAseq components. As expected, the greatest number of DEGs was between the control ambient pCO_2 condition (AA) and the control elevated pCO2 condition (EE) and the fewest differences in gene expression were between the two reciprocally transplanted conditions (AE and EA) which is likely due to the fact that many acute stress related genes may be shared and similarly expressed between these 2 groups. When comparing larvae from non-transplanted treatments in the larval experiment, larvae predominantly downregulated genes under OA exposure (AA vs EE). Multiple genes encoding proteins involved in cell differentiation and proliferation, including two cell surface hyaluronidases (Yamamoto et al., 2017) and nucleolar GTP-binding protein, were upregulated in EE versus AA which may be an attempt to maintain growth and differentiation under elevated pCO_2 stress. However, as a note, the difference in the expression of growth-related genes may also be related to differences in growth stages between treatments. Slowed metamorphosis has shown to be associated with slowed growth under OA exposure (Talmage and Gobler, 2009), and differences in larval stages (e.g. veliger to pediveliger) can be associated with differences in gene expression. Here metamorphic stage was not assessed and so a cautious note is placed on prescribing differences in expression of growth-related genes to exposure to OA. Two genes with proteins involved in ion exchange were also found to be upregulated in EE versus AA. Of these two genes, one, a 60kDa neurofilament protein, was found to have a domain function in sodium/calcium exchange and the other, prestin, was found to encode a sulfate anion transporter. Evans et al. (2013) and Todgham and Hofmann

(2009) found sodium/calcium exchangers to be upregulated under elevated pCO_2 in larval purple sea urchins, *Strongylocentrotus purpuratus,* and suggest this may be in an effort to promote calcification. Ramesh et al. (2019) also identified the upregulation of sulfate-anion transporters in blue mussel larvae under OA and suggest this may be related to the need to uptake sulfate during shell biogenesis for the production of macromolecules in the organic matrix of the shell. With regard to immune-related genes, a gene encoding soma ferritin was found to be upregulated in EE versus AA. Soma ferritin serves in the oxidative stress response (Castillo et al., 2017) and functions in metal binding and, under low pH, when reactive oxygen intermediate (ROI) production is high (Tomanek et al., 2011), ferritin can mitigate the harmful effects on the system (Zhou et al., 2008). Alternatively, two genes associated with energy production (i.e. ATP synthesis) were downregulated in EE larvae when compared to AA larvae. A gene encoding an ATP synthase beta subunit protein and a gene encoding an adenosylhomocysteinase A both encode proteins that function in aerobic and anaerobic energy metabolism and ATP synthase. A number of studies (Hüning et al., 2013; Harney et al., 2016; Thompson et al., 2016) found the downregulation of ATP synthase-related genes under elevated pCO_2 which suggests an altered metabolism in calcifiers under OA stress.

Interestingly, all genes identified as DEGs for the 3 other comparisons (AA versus AE, EE versus EA, and AE versus EA) were represented in the AA versus EE comparison with the exception of one gene which encoded fibropellin (which was identified as a DEG in AA versus AE and also AE versus EA). This leads to an interesting pattern in the data: twelve genes that were either up or downregulated in a non-transplanted treatment were similarly expressed in larvae from a transplanted treatment. To this extent, the differential regulation of some genes in the AE transplanted treatment converges with the regulation of the EE treatment with regard to

the baseline expression in AA; in other words the expression level of a select few genes is the same for both treatments terminating in elevated pCO_2 (EE and AE) with regard to the baseline expression in AA. The same is true for the regulation of some genes in the EE treatment with regard to the baseline expression in both AA and EA; in other words the expression of some genes is the same for both treatments terminating in ambient pCO_2 (AA and EA) with regard to the expression in EE. Thus, transplanted larvae had expression profiles that resembled the patterns of expression demonstrated by non-transplanted larvae within one week of transplantation. This is supported by the cluster analysis in which treatments clustered most closely based on final pCO_2 treatment. In a study performed by Ghalambor et al. (2015) a similar pattern of shared expression was found for transplanted versus native guppies though the study looked at patterns of expression in the absence of predators and not environmental stress. The study transplanted guppies adapted to living in the presence of predatory cichlids to a predator-free habitat and compared gene expression with a guppy population that evolved in the absence of predators. The authors found 135 transcripts that exhibited up- or down-regulation in the same direction as the native cichlid-free population. The authors conclude that this is demonstrative of rapid changes in plasticity. Similarly, Lohman et al. (2017) found convergence of gene expression profiles for transplants and natives in a reciprocal transplant experiment in which three-spined stickleback (*Gasterosteus aculeatus*) were transplanted to foreign environments. Authors also suggest that this convergence suggests expression plasticity, but they note that fish from different habitats still differed in survival rates and infection rates after transplant.

Of the genes in common between comparisons AA versus EE and AA vs AE three genes, which encode two cell surface hyaluronidases and the transforming growth factor-beta-induced protein ig-h3, are upregulated in EE and AE with respect to AA. Hyaluronidase is a component

of the connective tissue matrix and functions in cleaving high-molecular-weight hyaluronan into an intermediate-size hyaluronan. Hyaluronan functions in several roles within the intracellular matrix including in water and plasma protein homeostasis. Production of hyaluronan increases in proliferating cells and plays an important role in development and differentiation. In humans in a state of severe stress after blood loss, burns, post surgery, and in a state of shock the level of circulating hyaluronic acid increases (Kogan et al., 2008). This suggests that hyaluronidase levels may be tangentially related to stress in humans and could play a similar role for oysters under stress. Interestingly, TMEM2 hyaluronidase (now CEIP2) is dependent on calcium as a cofactor (Yamamoto et al., (2017). The importance of hyaluronidase during acclimation to OA is unclear, however, upregulation of the gene may be the result of an increase in hyaluronan in an attempt to mitigate the effects on development under OA stress. Studies have documented delayed development in calcifiers (Waldbusser et al., 2015), but the upregulation of this gene may be an attempt to achieve homeostasis in larvae under acidic conditions. The second of the two types of upregulated genes is a gene that encodes transforming growth factor-beta-induced protein ig-h3 which is a fascilin protein and a member of a family of cell adhesion molecules. Fascilin shares features with immunoglobulins, cadherins, integrins, and selectins. The function of fascilin in oysters is unknown, however, the purpose of the upregulation of the gene encoding the protein may be hypothesized based on the function of other cell adhesion molecules. Immunoglobulins, integrins, and selectins all play important functions in the cell, but, under OA stress, cadherins may be the most interesting molecules to examine. Cadherins are cell adhesion molecules dependent on calcium and play a role in morphogenesis and homeostasis. This is particularly interesting given the upregulation of hyaluronidase, another calcium dependent molecule with function in development and differentiation. Together these genes may be

important in sustaining the development of larvae even if development is disrupted or slowed under OA.

Of the genes in common between comparisons EE versus AA and EE vs EA four genes, which encode the mitochondrial ATP synthase beta subunit, tubulin beta chain, coadhesin, and hemicentin, are downregulated in EE with respect to both AA and EA. The reasons for which some genes are downregulated under elevated pCO_2 is more clear for some genes than it is for other genes, however, overall downregulated genes may be interpreted as "costs" under OA. The gene encoding the mitochondrial ATP synthase beta subunit is important for aerobic and anaerobic energy production by functioning in the synthesis of ATP, the molecule responsible for storing and transporting chemical energy in cells (Jonckheere et al., 2012). As suggested in Harney et al. (2016) in which larval *C. gigas* demonstrated lower expression of ATP synthase under OA, decreased expression suggests altered metabolism under acidification. A gene encoding tubulin beta chain was also downregulated under EE with respect to AA and EA. Tubulin is a major constituent of microtubules and is associated with the cytoskeleton (Müller et al., 2018). In Müller et al. (2018), under exposure to diesel fuel water-accommodated fraction, *Crassostrea brasiliana* differentially regulated the expression of alpha and beta tubulin. This differential expression was cited to be the result of oxidative stress which could explain the downregulation in larvae here. Coadhesin has been found to be a component of the skeletal organic matrix of the staghorn coral *Acropora millepora* (Ramos-Silva et al., 2014) and thus the downregulation of the gene encoding this protein here may be related to the smaller size demonstrated in the physiological assay. The fourth downregulated gene, which encodes hemicentin, is a gene for which the function is only described for vertebrates and so drawing conclusions about the cost of the protein function under OA is difficult. In mice, hemicentin

plays a role in the architecture of adhesive and flexible epithelial cell junctions (Xu et al., 2007) which may be one reason for which hemicentin is downregulated. Additionally, one of the GO-molecular functions associated with hemicentin is calcium-ion binding which may provide even further information as to why the specific gene is downregulated under OA stress.

Though hemicentin may have calcium dependency, interestingly, most of the calcium-dependent enzymes identified as DEGs were found to be upregulated under elevated pCO_2 conditions. The CEIP2 gene encoding a cell surface hyaluronidase and the gene encoding the transforming growth factor beta-induced ig-h3 were upregulated in both EE and AE versus AA and identified as calcium dependent. Additionally, the EGF1 gene which encodes the protein Fibropellin-1 was upregulated in AE when compared to both the AA and EA conditions. Fibropellin-1 is characterized by a calcium-binding EGF-like domain which results in a protein dependent on calcium for proper biological function (Stenflo et al., 2000). These results suggest not only that larval oysters are responding to their present environment by means of a plastic response (as is the case with the expression of CEIP2 in treatments terminating in elevated pCO_2), but also that one of the key systems important in tolerating the stress of OA is dependent on the presence of calcium.

FOOD LIMITATION EXPERIMENT: *Interpreting DEGs*

In the juvenile food limitation experiment, the greatest differences in gene expression were ultimately the result of food condition and not initial or final pCO_2 treatment. However, as expected, there were slightly more differentially expressed genes between ambient and elevated pCO_2 conditions when animals were starved versus fed which was expected based on the additional stress resulting from limited energy availability. In juveniles under elevated pCO_2 and in the fed condition the response to OA appeared to be mainly a general stress response (e.g.

downregulation of heat shock proteins, though this opposes the general trend in which these proteins increase under stress (Sørensen et al., 2003)) rather than a specific response to acidification. Many biomineralization genes (e.g. genes encoding cadherin, c-type lectins, tyrosinase, calmodulin (Koh et al., 2015), galaxin, and carbonic anhydrase (Moya et al., 2012)) that were identified as differentially regulated under OA in other species/studies were also found here, however, differential expression of these genes was found only between fed and starved treatments regardless of pCO_2 condition. This suggests that juveniles overall are more robust to the effects of OA and combined OA and pathogen stress which supports the previous conclusions drawn from the physiological assays in the previous chapter.

Similar to the pattern demonstrated in larvae in the reciprocal transplant experiment, juveniles predominantly downregulate expression of DEGs under OA exposure, however, when exposed to ROD juveniles under OA predominantly upregulate gene expression (AFB versus EFB). For most DEGs in AFB versus EFB, the function of the associated proteins has only been described in vertebrates and so the functional importance of these genes in oysters under OA stress is unknown (as mentioned above). In addition, many of these genes have not been mentioned in the literature within the last two decades, so there has been little progress made in documenting their function. To this extent few conclusions can be drawn about only 2 of the 5 annotated, upregulated DEGs between AFB and EFB; the genes encoding porphobilinogen deaminase and "multiple epidermal growth factor-like domains protein" have been described as functioning as transport proteins (Lu et al., 2020) and in calcium ion binding (Nakayama et al., 1998), respectively, however little else is known about these proteins.

Similarly, although more than half of the DEGs identified in AS versus ES were annotated, the proteins have only been described in humans. Therefore, the functional

importance of "multiple epidermal growth factor-like domains protein 11", multimerin-2, a 32 kDa beta-galactoside-binding lectin, and FH2 domain-containing protein 1 are unknown with regard to OA and bivalves. For the remaining genes, the function can be speculated based on what is known for other organisms, but the role in resilience to OA is only a hypothesis. One such gene encodes a von Willebrand factor D and EGF domain-containing protein. In general, von Willebrand factors have been shown to be overexpressed in invertebrates under pathogen challenge (Goncalves et al., 2014; Hartman et al., 2018); however, there are many different types of von Willebrand factors and von Willebrand Factors D and EGF Domains (VWDE) has been functionally described the least. Studies suggest VWDE may be involved in blastemas which function in the regeneration of limbs and fins in regenerative species (Leigh et al., 2019). The trend for a decrease in growth exhibited by juveniles under OA and starvation may be explained by the downregulation of this gene, however, it may have an entirely different role in oysters.

If the functional importance of this gene is in fact related to immunity, this adds to an interesting trend observed in the data: several genes found to be differentially regulated under OA and starvation (ES versus AS) (e.g. encoding proteins such as fucolectin which was upregulated in ES, and CD209 antigen-like protein and collectin-12 which were downregulated in ES) appear to function in immunity. However, this does not support the physiological assay results as the immune processes of these animals were not any more compromised with regard to juveniles under ambient pCO_2 conditions in the physiological assay (ESB versus ASB). Cao et al. (2018) examined mRNA transcript abundance of several genes related to aerobic metabolism in adult *C. gigas* exposed to OA and *Vibrio splendidus* and found no significant difference in expression between OA and non-OA exposed treatments exposed to bacteria. A similar pattern can be seen here between AFB and EFB which exhibited the second fewest number of DEGs

between all treatment comparisons (tied with AF versus EF). This may suggest unaltered metabolic pathways in juvenile eastern oysters and further support the resilience demonstrated in juveniles.

Similar to the results found for the larvae, genes encoding calcium-binding proteins were upregulated under elevated pCO_2. When starved animals under either ambient or elevated pCO_2 were compared (AS versus ES), those under OA upregulated a gene responsible for the production of fucolectin which, as described previously, is involved in the innate immune system but also binds calcium. This upregulation of calcium-related proteins was demonstrated amongst other calcifying species (Evans and Watson-Wynn, 2014) and is suggested to represent an adaptive response that promotes calcification in carbonate-limited seawater (Evans et al., 2013). In the context of the juvenile oysters, where ocean acidification had no noticeable effect on viability, immunity, or growth, the upregulation of this gene under starvation and acidification indicates it assists in maintaining homeostasis under stress.

Gene Ontology Analysis

To date Gene Ontology (GO) categories are not optimized for eastern oysters which results in gene groups that seemingly have no relation to oyster homeostasis or development. For this reason, it can be difficult to relate the GO categories identified as significant to a specific function that confers resilience under acidification stress. In the reciprocal transplant experiment the three significant GO terms related to arabinosyltransferase activity, actinobacterium-type cell wall biogenesis, and peptidoglycan-based cell wall biogenesis. The significance of these terms may not be related to the effects of OA on larvae, but as an indirect result of a relatively high abundance of biological contaminants (e.g. Euplotes and other ciliates, algae, etc.) in the samples. A major challenge throughout the experiment was the appearance and persistence of

biological contaminants. The contaminants, namely Euplotes ciliates, were approximately the same size as the larvae which prevented their elimination by sieving. The presence of these contaminants likely resulted in not only a low read mapping percentage, but also significant GO terms that were associated primarily with non-oyster species. In this case, GO may not be the most informative, however, for the juvenile food limitation experiment where as many as 1,300 DEGs were identified for a single comparison (i.e. EF versus ES) GO and KEGG may be more informative than investigating the role of each individual gene/protein. Most significant GO terms in the food limitation experiment related to the differential regulation of broad metabolic processes and peptidase activity under starvation and starvation with combined exposure to ROD. No significant GO terms were identified for comparisons between treatments with different pCO_2 levels. This analysis serves to continue to support the conclusion that juveniles are robust to the effects of OA and combined OA and pathogen stress.

KEGG Pathway Enrichment Analysis

Similar to the GO analysis, KEGG is not optimized for the eastern oyster and so many enrichment pathways have seemingly unrelated function; however, some investigation of the pathways is still possible. In the reciprocal transplant experiment, between AA and EE the oxidative phosphorylation pathway was downregulated in EE oysters and was represented by two DEGs encoding ATP synthase. The downregulation of this pathway is likely related to the altered metabolism suggested for calcifiers under OA stress (Hüning et al., 2013; Harney et al., 2016; Thompson et al., 2016). KEGG pathway enrichment in the food limitation experiment demonstrated two pathways enriched between ambient pCO_2 and elevated pCO_2 fed oysters (AF versus EF) of which one related broadly to metabolic processes. In addition, ribosomal pathways were enriched in AF versus AS and EF versus ES. Higher ribosomal gene expression in fed

versus starved oysters is likely the driving mechanism behind the significant decrease in size when oysters are starved, independent of pCO_2 condition, as higher expression results in faster growth (Pespeni et al., 2012). Looking at the results together, most KEGG functions were broadly related to metabolism which is not unusual given the predicted effects of OA on calcifiers.

Summary of RNAseq Findings

Differences in gene expression may provide some information as to the genes affected under environmental stress, however, the degree to which stressed and control populations differ does not always indicate tolerance versus susceptibility (Kelly, 2019). In some species, the more tolerant species exhibits greater differential expression between stressed and control treatments (e.g. the seagrass *Zostera marina*; Franssen et al., 2011; spiny damselfish *Acanthochromis polyacanthus*; Schunter et al., 2016) whereas in other species it's the more sensitive population that exhibits greater differential expression when stressed (e.g. coral; Barshis et al., 2013). Therefore, adaptive pressure can act in different ways on different species to result in changes in the ability of a species/population to acclimate via changes in gene expression. To this degree, caution must be taken when discussing the results of gene expression studies as interpreting the results can be complicated. Here, the relatively higher number of DEGs in larvae versus juveniles is taken as demonstrative of higher susceptibility in the younger life stage as this is supported by the results of the physiological assay (decreased viability and growth) demonstrated earlier. In addition, this conclusion is supported by the types of genes regulated in the different life stages; in larvae changes in expression are associated with alteration of basic functions such as ATP synthase, fascilin proteins, ion transporters, and immune-related proteins, thus larvae are deemed susceptible overall. In juveniles, DEGs between AF and EF primarily related to

chaperone proteins (e.g. heat shock proteins and epidermal growth factors) which function in a protective role to promote resilience. Despite the complexity in interpreting DEGs, transcriptional plasticity can be an important component underlying resilience and acclimation to stress. Ultimately the modification of plastic responses can lead to adaptation to climate change if the stress of OA leads to directional selection on expression level of DEGs. It can, therefore, be difficult to parse the roles of acclimation and adaptation when the modes of resilience can select for one another.

This chapter serves to identify the potential for acclimation (via changes in gene expression) to OA in multiple life stages of the eastern oyster. Work here supports the results demonstrated in the physiological assays and in previous studies (Gobler and Talmage, 2014; Schwaner et al., in prep) which suggest larvae are more susceptible to the effects of OA and that resilience in surviving larvae is due, in part, to changes in gene expression. Multiple studies investigating DEGs between two populations under environmental stress can often differ in their results due to biological variability, differences in experimental methods (e.g. age of individuals, source population, and time of sample collection; Wolf, 2013), and sensitivity of NGS methods, but many genes identified here including ATP synthase, sodium/calcium exchangers, and heat shock proteins were also identified in other OA studies employing RNAseq (Todgham and Hofmann, 2009; Evans et al., 2013; Hüning et al., 2013; Moya et al., 2015; Harney et al., 2016; Thompson et al., 2016). These genes and the proteins they encode may be considered the most promising genes for resilience under OA stress and represent targets for the next step in understanding mechanisms for resilience. Identifying the presence of selection at the genome level (e.g. single nucleotide polymorphisms, SNPs) can serve to enhance and complement our understanding of the molecular features associated with resilience to OA in the larval and

juvenile eastern oyster. As acclimation potential has a genetic component (Kelly, 2019), investigating the genome of *C. virginica* under OA stress for changes in allele frequency may be crucial to understanding resilience in this species.

Chapter V: A transcriptomic approach to detecting allelic variants associated with resilience to ocean acidification in *Crassostrea virginica*

Background

At the molecular level, adaptation is another means by which marine organisms may tolerate ocean acidification. Adaptation is defined as a shift in genotype that evolves through natural selection often in response to prolonged environmental pressure. If resilience to OA is under selection, individuals with advantageous phenotypes would result in a resilient population. A number of recent studies have begun to suggest an adaptive mechanism for survival under environmental stress, however, the potential is population and species-specific (Kelly et al., 2012; Reusch, 2014). The variability is primarily a result of standing variation, i.e. the potential for evolutionary rescue is constrained by the degree of standing variation (Reusch, 2014). Though adaptation is generally considered a slow process, especially in comparison to acclimation, standing genetic variation can enable rapid response under rapid environmental change (Hermisson and Pennings, 2005). Fortunately, members of the phylum Mollusca have been demonstrated to possess highly polymorphic genomes with active transposable elements and expansion of gene families involved in stress response (Zhang et al., 2012; Powell et al., 2018). The high standing variation present in mollusks can potentially afford them some resiliency in the face of ocean acidification. A large degree of variation can be attributed to single nucleotide polymorphisms (SNPs) (Gaudet et al., 2009). SNPs are at the basis of a number of disease traits and are major contributors to susceptibility to environmental factors (Song et al.,

2015). SNPs may occur within or outside coding regions of the genome and can result in either synonymous or nonsynonymous changes in amino acid sequences.

Next generation sequencing has improved the sequencing of genomes and enabled the rapid identification of variants from genomic and transcriptomic data. Through the use of transcriptomic data, information may be collected on both gene expression levels and nucleotide variation from a single dataset (Ekblom and Galindo, 2011). Nucleotide variation may include single nucleotide polymorphisms/variants (SNPs or SNVs), multi-nucleotide variants (MNVs), insertions, or deletions. Alternatively, many OA studies employ the use of restriction site-associated DNA sequencing (RADseq) which reduces the complexity of the genome and thus reduces sequencing costs (Liu et al., 2020). Regardless of the method, identifying variants can be important for uncovering the genes that are under selective pressure.

Recently an increasing number of studies have begun to explore the adaptive potential of sensitive marine organisms under future OA conditions. These studies have determined that selection at the genomic level does significantly contribute to the resilience of a population. A study by Pespeni et al. (2013b) suggested the role of genome-wide selection for populations of purple sea urchins (*S. purpuratus*) resistant (no change in growth) to the effects of OA. This study looked primarily at changes in allele frequencies, in which selection on SNPs results in individuals with potentially resistant phenotypes. Selection occurred primarily at loci associated with biomineralization, lipid metabolism, and ion homeostasis (processes known to play a role in internal pH maintenance). This may also be true for the juveniles in the food limitation experiment described earlier where there was no significant effect of pCO_2 level on viability or growth despite the literature that describes *C. virginica* as a vulnerable species. In the copepod species *Pseudocalanus acuspes* approximately 1,500 SNPs significantly differed in allele frequency under

elevated pCO_2 (De Wit et al., 2016). SNPs were enriched in genes related to RNA transcription, ribosomal function, and ion transport. The investigation into DEGs presented in the previous chapter demonstrated an upregulation of ion transport proteins in larvae maintained in elevated versus ambient pCO_2 and an investigation into variants in these samples may also demonstrate SNPs in genes with similar functions. In the red abalone *Haliotis rufescens*, three populations from regions with varying aragonite saturation states significantly differed by approximately 700 SNPs in genes involved in biomineralization, energy metabolism, heat tolerance, and disease tolerance (De Wit and Palumbi, 2013). In the mediterranean mussel *Mytilus galloprovincialis* 88 outlier loci were found to be specific to animals maintained under low pH (Bitter et al., 2019). Number of variants differ between species, however, a large number of studies have identified SNPs under selective pressure in OA conditions.

A number of mechanisms and molecular processes for resilience to OA have been demonstrated in other studies (Pansch et al., 2014; Ventura et al., 2016; Waldbusser et al., 2016) and here however, these mechanisms likely have underlying genetic components that determine the functionality of that mechanism. For example, some genotypes may possess more plasticity and if these genotypes are heritable then adaptive evolution will occur. Some studies suggest nongenetic short-term changes can combine with long-term genetic changes to rapidly facilitate evolution (Ghalambor, 2007; Sunday et al., 2014). For this reason, a holistic approach is needed to understand the mechanisms underlying resilience, and, therefore, an investigation of variants in *C. virginica* under OA would complement the work done here in previous chapters (Donelson et al., 2018). In order to assess the potential for *C. virginica* to adapt to OA stress it is necessary to identify regions of the genome/transcriptome that are under selection. The objective of this chapter is to localize variants enriched in OA-tolerant larvae and juveniles after transplantation

to the alternative pH condition and at different food conditions. If resilience to OA is due to genetic selection for advantageous variants, then I hypothesize the analysis of the transcriptome will reveal the presence of variants (e.g. SNPs) at specific loci associated with tolerance. The results are analyzed with a focus on identifying genes and proteins associated with resilience and potential trade-offs under OA in *C. virginica* larvae. This work will further contribute to the holistic approach to understanding the mechanisms and molecular features underlying resilience to OA in the eastern oyster. In addition, identifying these variants will provide a valuable resource for the aquaculture industry that would enable marker-assisted selection of OA-resilient stocks.

Methods

Variant detection was performed using CLC Genomics Workbench v12.0.3 (CLC Bio, Aarhus, Denmark) on transcriptome data for both the reciprocal transplant and food limitation experiments. Treatment-specific SNPs were identified by first importing RNAseq BAM files of reads mapped to the *C. virginica* reference (provided by Novogene Corporation (UC Davis, Sacramento, California)) into CLC Genomics Workbench. Mapped reads were then passed to the "Indel and Structural Variants" tool to identify structural variants (e.g. insertions, deletions, inversions, translocations and tandem duplications) using the following default parameters: p-value threshold: 0.0001 for unaligned end breakpoints; maximum number of mismatches: 3 for unaligned end breakpoints; minimum quality score: 0 for calculation or unaligned end consensus; minimum relative consensus coverage: 0 for calculation or unaligned end consensus; ignore broken pairs. The result file from the indel and structural variant detection and the BAM file were used in the "Local Realignment" tool to improve the read mapping using the following default settings: realign unaligned ends; multi-pass realignment: 2; maximum guidance-variant

length: 200. Variants were detected from the locally realigned reads using the "Low Frequency Variant Detection" tool with default settings: required significance: 1%; ignore positions with coverage above: 100,000 for reference masking; ignore broken pairs; ignore non-specific read matches; minimum coverage: 10; minimum count: 2; minimum frequency: 1%; relative read direction filter significance: 1%. The low frequency tool was chosen as it is suitable for mixed-tissue samples where variants may be present at a low frequency. SNPs between two treatments were identified using FDR-corrected Fisher Exact Test implemented in CLC Genomics Workbench with a corrected p-value of $p<0.05$ as significant. SnpEff (Cingolani et al., 2012) was used to annotate SNPs and enumerate transcript effects based on genomic position using protein characterization information from NCBI. As a single gene may have multiple different transcripts, SnpEff counts the number of possible transcript effects an allele in a gene may have. In the computational analysis a variant is only described by chromosome, position, reference allele, and alternate allele; this does not include any information regarding the transcript or isoform for which the variant is present. Therefore, to comprehensively annotate the variant from this simple information, SnpEff annotates a variant for all known transcripts of a gene at that position. Furthermore, transcript effects may vary from a start lost, stop gained, a missense variant, a synonymous variant, etc. Thus, the number of transcript effects is often greater than the number of variants. Genes were investigated for their potential role for resilience to OA and for overlap with genes identified in the previous chapter as differentially expressed. The data was also explored for private alleles present in all samples of one treatment but absent in all samples of the alternative treatment in the paired comparison. Here private alleles are examined in both samples for the AE versus EA comparison which yields 5 "comparisons" for investigating

private alleles in the reciprocal transplant experiment as opposed to the 4 comparisons for the RNAseq investigation.

Results

RECIPROCAL TRANSPLANT EXPERIMENT

There was no significant shift in allele frequency for all four treatment comparisons (Fisher Exact Test, $p>0.05$ for all variants). The investigation into private alleles demonstrated 87 variants present in all EE samples (i.e. replicates) but absent in all AA samples, 181 variants present in all AE samples but absent in all AA samples, 13 variant present in all EA samples but absent in all EE samples, 218 variants present in AE samples but absent in EA samples, and 0 variants present in EA but absent in AE (Table 3).

For private allele analysis, annotation with SnpEff yielded 217 possible transcript effects for the private alleles in EE samples versus AA samples of which 105 were annotated in the NCBI *C. virginica* database (Table 3). Between AA and AE, 533 possible transcript effects were identified for the private alleles present in all AE samples but absent in all AA samples of which 254 were annotated. Between EE and EA, 69 transcript effects were identified with SnpEff for private alleles in all EA samples but absent in all EE samples of which 27 were annotated. Between AE and EA, 218 possible transcript effects were identified for the alleles present in AE samples but absent in EA samples of which 313 were annotated.

Table 3 Number of private alleles identified using CLC Genomics Workbench, number of possible transcript effects identified by SnpEff, and number of annotated transcript effects for the reciprocal transplant experiment. The numbers correspond to the alleles/transcript effects present in the treatment in parenthesis and absent in the alternative treatment in the comparison.

	AA vs (EE)	AA vs (AE)	EE vs (EA)	EA vs (AE)	AE vs (EA)
# Private alleles	87	181	13	218	0

# SnpEff possible transcript effects	217	533	69	655	N/A
# NCBI annotated transcript effects	105	254	27	313	N/A

FOOD LIMITATION EXPERIMENT

There was no significant shift in allele frequency for all twelve treatment comparisons (Fisher Exact Test, $p>0.05$ for all variants). The total number of private alleles in a paired treatment comparison ranged from 834 variants present in all EFB samples (i.e. replicates) but absent in all AFB up to 10,993 variants present in all ES samples but absent in EF (Table 4). All other treatment comparisons showed moderate numbers of private alleles. Of note, between AF and EF, 1,035 variants were present in all EF samples but absent in all AF samples, 2,488 variants were present in ES but absent in AS, and 7,320 variants were present in AS and absent in AF.

For private allele analysis, annotation with SnpEff yielded 5,449 possible transcript effects for the private alleles in EF samples versus AF samples of which 2,694 were annotated in the NCBI *C. virginica* database (Table 4). Between AS and ES, 10,970 possible transcript effects were identified for the private alleles present in all ES samples but absent in all AS samples of which 5,408 were annotated. Between AF and AS, 19,878 transcript effects were identified with SnpEff for private alleles in all AS samples but absent in all AF samples of which 9,836 were annotated. Between EF and ES, 26,174 possible transcript effects were identified for the alleles present in ES samples but absent in EF samples of which 12,919 were annotated. Between AFB and EFB, 4,187 possible transcript effects were identified for the alleles present in EFB samples but absent in AFB samples of which 2,066 were annotated.

Table 4 Number of private alleles identified using CLC Genomics Workbench, number of possible transcript effects identified by SnpEff, and number of annotated transcript effects for the food limitation experiment. The numbers correspond to the alleles/transcript effects present in the treatment in parenthesis and absent in the alternative treatment in the comparison.

	AF vs (EF)	AF vs (AS)	AF vs (AFB)	EF vs (ES)	EF vs (EFB)	AS vs (ES)	AS vs (ASB)	ES vs (ESB)	AFB vs (EFB)	AFB vs (ASB)	EFB vs (ESB)	ASB vs (ESB)
# Private alleles	1035	7320	5869	10993	4560	2488	2070	1476	834	3158	4512	2698
# SnpEff possible transcript effects	5449	19878	20387	26174	16585	10970	10008	6853	4187	11704	14811	10576
# NCBI annotated transcript effects	2694	9836	10077	12919	8173	5408	4943	3376	2066	5773	7298	5228

Discussion

Analysis of the transcriptomes of larval and juvenile *C. virginica* of both the reciprocal transplant and food limitation experiments reveal the potential for adaptation under elevated pCO_2. To determine whether resilience was associated with selection at the genomic level, genome-wide shifts in allele frequency and the presence of private alleles were assessed from RNAseq data of larvae and juveniles exposed to elevated pCO_2. No significant changes in allele frequencies were identified between any conditions in either the reciprocal transplant or food limitation experiments for any variants, however, a relatively large number of private alleles were present in OA-exposed treatments. These results contradict those demonstrated by Pespeni et al. (2013a, 2013b), De Wit et al. (2016), De Wit and Palumbi (2013), and Bitter et al. (2019) in which sensitive marine organisms demonstrated significant changes in allele frequency under elevated pCO_2. However, for some species, studies conclude that there is low adaptive potential under OA. In the tropical sea urchin *Echinometra* sp. C only low numbers of outlier SNPs (6-19) were observed in larvae living near a high CO_2 vent (Uthicke et al., 2019). Authors conclude that

this suggests little potential for adaptation in this species of the sea urchin especially given the SNPs were mostly silent or synonymous substitutions. These results are in stark contrast to the more than 300 SNPs identified in the purple sea urchin by Pespeni et al. (2013a). This variability between organisms within the same phylum may explain why *C. virginica* differed in adaptive potential (i.e. number of significant SNPs) as compared to other organisms within the phylum Mollusca (De Wit and Palumbi, 2013; Bitter et al., 2019).

Alternatively, the lack of significant differences in the genome of oysters from elevated versus ambient pCO_2 may be the result of several factors related to the experimental design. It is possible that the parental populations were not sufficiently diverse enough to yield larvae that had different genomes. This could be because the broodstock population was not as genetically diverse as expected, or it could be that the broodstock pool of individuals was too small resulting in oysters with essentially the same parents. In addition, pooling samples may substantially reduce the chances of identifying a significant shift in allele frequency which may have had a large impact on the ability to draw conclusions here. To this extent, it is also important to mention that a non-significant result does not indicate "no effect" of the pCO_2 treatment, but rather that the results are inconclusive (Nakagawa & Foster, 2004). Further studies with different populations of *C. virginica* or multiple generations under elevated pCO_2 may uncover genes under selective pressure.

Though they were not identified as significant here, examining private alleles (alleles that are present in all samples of one treatment and absent in all samples of another) can be one way to begin to uncover genes that may be important for resilience and adaptation. To date, this is the only known study which examines the presence of private alleles underlying resilience to OA in any marine species. The results of the investigation into private alleles in the reciprocal

transplant experiment demonstrate trends that oppose those identified in the RNAseq results with regard to the number of differences between comparisons. Between the two, non-transplanted treatments, larvae in EE appear to have a moderate number of private alleles as compared to AA, which is unlike the results demonstrated in the chapter previously where AA and EE had the largest number of DEGs. Here, animals transplanted to elevated pCO_2 from ambient (AE) have the highest number of private alleles compared to a treatment that was not transplanted (AA). This shift in the genome may be associated with the high mortality demonstrated in the physiological assay in which there may have been a selection event leading to the relatively high number of private alleles. Interestingly, the animals transplanted from elevated pCO_2 to ambient (EA) had the fewest number of private alleles when compared to those that remained in elevated pCO_2 (EE) which may also relate to the decrease in mortality rate and lack of selection. This also supports the notion that the elevated pCO_2 condition selected for animals tolerant of a larger range of pCO_2 rather than just lower pH as demonstrated in juvenile *Mercenaria mercenaria* (Schwaner et al., 2020). Another interesting trend is the high number of private alleles present in AE but absent in EA versus the complete absence of private alleles in EA with respect to AE; this trend follows the hypothesis that exposure to elevated pCO_2 results in a selection event that leads to the selection of private alleles that enable larvae to tolerate a wide range of pCO_2. These results, together, suggest adaptation via the selection of private alleles plays an important role in resilience under OA in addition to acclimation via changes in gene expression. The results also suggest oysters may adapt to OA by increasing the number of private alleles in a population without decreasing the potential for populations to withstand normal, ambient pCO_2.

Further investigation into the variants revealed that some occurred in genes that were identified as DEGs in the previous chapter. A SNP in a gene encoding a cell surface

hyaluronidase was present in EE and AE but absent in AA and the same gene was found to be upregulated in EE with respect to AA. Though not one of the DEGs described previously, a private allele present in AE but absent in AA related to a protein that functioned in inducing cell migration and binding hyaluronan, the substrate of hyaluronidase. The presence of private alleles in these genes further stresses the importance of hyaluronidase for resilience under OA and that resilience may be related to cell differentiation and development. Additionally, several SNPs were identified in EE and AE in a GTP binding protein and a gene with similar GTP binding function was upregulated in EE versus AA. Interestingly a private allele in a gene encoding fibropellin was present in EE and AE but absent in AA, and the same gene was downregulated in treatments terminating in ambient pCO_2 compared to AE. Fibropellin has been demonstrated to be integral to the biomineralization process (Flores and Livingston, 2017) and so the downregulation of this gene and a variant within the gene demonstrate a clear cost to survival under OA. In AE there were 3 SNPs in a gene encoding a 60 kDa neurofilament protein that was absent in EA and the same gene was upregulated in EE with respect to AA. In AE, a SNP was located in a gene encoding a transforming growth factor-beta-induced protein ig-h3 which functions in a similar way to a protein that was upregulated in EE and AE with respect to AA. These results are particularly interesting because not only are they genes that are also DEGs in the same samples and sample comparisons described in the previous chapter, but many of the private alleles are present in both of the treatments that terminate in the same pCO_2 (e.g. variants present in the same genes in EE and AE). This further supports the conclusion that larvae respond quickly (i.e. in less than one week) to OA via acclimation and the selection of private alleles in key genes that we can conclude are highly associated with resilience to OA in larvae.

Similar to the trend demonstrated for DEGs described in the previous chapter, juveniles possessed more private alleles as compared to larvae for all treatment comparisons. With regard to trends within the food limitation experiment, the number of private alleles between comparisons was similar to but not exactly the same as the trends in DEGs. The treatment comparison with the greatest number of private alleles and DEGs was the comparison between EF and ES. This indicates that, although there was no effect demonstrated in the physiological assays, juveniles under OA and food limited conditions are impacted by the stress and, as a result, their transcriptomes and genomes are modified. However, as described in the previous chapter, the greatest differences in number of private alleles was ultimately the result of food condition and not pCO_2 treatment. The comparisons AF vs EF, AS vs ES, and AFB vs EFB, were ranked the 3 out of the 4 lowest in terms of number of private alleles. This trend is similar with regard to number of DEGs where those three comparisons were ranked 3 out of the 5 lowest. These trends are in stark comparison to those demonstrated in larvae in which the trends between number of DEGs and private alleles opposed each other. This suggest molecular processes differ in relative importance for different life history stages of *C. virginica*; in the juvenile, adaptation and acclimation appear to play equally important roles in resilience, however, in the larval stage acclimation is relatively more important for chronic stress and adaptation is more important under acute stress. This conclusion is based on the fact that animals that remained under chronic elevated pCO_2, EE oysters, responded with the highest number of DEGs when compared to AA and the second lowest number of private alleles with regard to all other paired comparisons. When AE oysters are compared to AA (where transplanted animals are exposed to elevated pCO_2 on a relatively shorter, and thus, acute time scale as compared to EE animals) there is a moderate number of DEGs but a high number of private alleles. This

opposes most traditional population genetics theory which suggests adaptation is a mode of resilience under prolonged, chronic environmental stress (Messer et al., 2016), and thus provides interesting insight into the relative roles and importance of molecular processes for resilience to OA. However, as a note, the difference in the number of private alleles could also be related to the age at which larvae are exposed to OA (i.e. from fertilization or at after ~1-week post-fertilization). Interestingly, juveniles also demonstrated a number of private alleles one to two orders of magnitude higher than the number documented in larvae despite lower mortality rates over a longer period of time. This may suggest higher capacity to tolerate the accumulation of variants in juveniles as compared to sensitive larvae. Alternatively, this may actually be an artifact of the technical differences between the methods employed for juveniles versus larvae. For both age classes, individuals were pooled to generate one sample per replicate, but whereas RNA from a set number of juveniles (i.e. three per replicate) was pooled per replicate, for larval samples pools were made up of approximately 1,000 individuals but the exact numbers differed between replicates. Pooling likely limited the ability to call variants in both the larval and juvenile samples. Ideally, larval and juvenile samples would not have been pooled but instead sequenced individually, however, the technology remains cost prohibitive. Furthermore, the number of reads mapped to the reference differed substantially between larvae and juveniles (approximately 600,000 and 35,000,000 for larvae and juveniles, respectively) which may have been due to the high abundance of contaminants in larval samples and relatively low abundance of oyster larvae. For these reasons, some caution needs to be exercised when comparing the absolute number of private alleles between juveniles and larvae, but the trends remain interesting nonetheless.

An investigation into the specific function of genes in which variant private alleles were present demonstrated genes that varied in function from common housekeeping genes to genes that have been described as being of importance under OA. Several private alleles in genes relating to biomineralization were present in EF and ES and absent in AF and AS, respectively, including alleles in genes encoding cadherins, protocadherins, tyrosinase, fibropellin, and gigasin (Koh et al., 2015; Hüning et al., 2016; Flores and Livingston, 2017). In ES with respect to AS, several perlucin transcripts contained private alleles. Perlucin has been described as a C-type lectin that functions in the shell biomineralization pathway in bivalves by nucleating calcium carbonate layers and through its incorporation into calcium carbonate crystals (Blank et al., 2003; Wang et al., 2008). If the variant in the perlucin gene in ES resulted in a non-synonymous amino acid change, it may have resulted in the relatively small size of ES as compared to the length of AS and EF oysters and would also demonstrate a change in the biomineralization pathways in these animals. In addition, private alleles were present in a sodium/calcium exchanger in ES but absent in AS. As described previously, Evans et al. (2013) and Todgham and Hofmann (2009) hypothesized that the modification of sodium/calcium exchangers under elevated pCO_2 may be in an effort to promote calcification. Here, if a SNP resulted in a non-synonymous change it may have been advantageous in the function of this protein thereby enhancing resilience in acidified water. Furthermore several private alleles were present in calcium dependent genes in EF and ES (as compared to AF and AS, respectively) including those that encode calponin, calmodulin, calpain, a calcium-responsive transcription factor, and a protein with an EF-hand calcium-binding domain (el-Mezgueldi, 1998; Perrin and Huttenlocher, 2002; Koh et al., 2015).

As in the larvae from the reciprocal transplant, several private alleles in the juveniles were present in genes with a similar function as those that were up or downregulated and DEGs. In EF several private alleles were present in heat shock proteins which were downregulated in EF with respect to AF. In ES versus AS a gene encoding a von Willebrand factor protein was downregulated in ES and here several private alleles were found in genes encoding von Willebrand factor proteins. Similarly, a variant was found in a gene encoding the calcium-binding protein fucolectin in ES which was also upregulated in ES with respect to AS. Of note, in EF many private alleles were located in genes that encode ubiquitin. Ubiquitin is a highly conserved protein in eukaryotes that is responsible for the degradation of short-lived, regulatory proteins (Hershko and Ciechanover, 1998). Ubiquitin has been hypothesized to play an important role in the immune response, development, programmed cell death, and transcriptional regulation, among other pathways. In particular, in brine shrimp, mussels, and insects ubiquitin aides in the response to environmental stress (Mykles, 1998). The high presence of private alleles in juvenile oysters under elevated pCO_2 and high food availability may just be the result of random mutations in a conserved, core gene, or, more interestingly, the presence may be related to ubiquitin's role in development and response to stress. Without additional confirmation methods such as gene silencing, it is difficult to predict the true effects of this change in the ubiquitin-encoding gene and other genes mentioned here.

This chapter serves to identify the potential for adaptation (via changes in allele frequency and selection of private alleles) to OA in multiple life history stages of the eastern oyster. Work here regarding changes in allele frequency is inconclusive as there were no significant changes in either larvae or juveniles from the reciprocal transplant or food limitation experiments; however, an investigation of private alleles present in animals under OA stress

demonstrates adaptation is possible, but accumulation of enough genetic divergence to successfully adapt may take several generations. These results, in combination with the results from previous chapters, suggest the mechanisms for resilience to OA in the eastern oyster are multi-faceted and the relative importance of acclimation versus adaptation may differ between life stages. It is important to note that the use of RNAseq data in variant detection limits the ability to identify SNPs throughout the entire genome as the reads are derived from the coding regions of the genome only. SNPs may arise in intergenic regions which may have unknown consequences for the organism. However, the results here still present a novel identification of private alleles associated with resilience to OA in *C. virginica* larvae and juveniles. The next step in this analysis is to identify the predicted effects of these private alleles for protein function. The most interesting alleles would result in nonsynonymous amino acid changes that would alter protein function. These genes would be targets in follow-up analyses to identify how those proteins may contribute to resilience or how they may actually be the result of a potential trade-off for survival under OA.

Chapter VI: Overall Thesis Conclusions

Changes in ocean chemistry associated with the acidification of seawater are predicted to have major consequences for bivalve species. The dual importance of bivalves in an ecological and economic context places the group as a keystone taxon in estuarine environments and drives the need for research into their capacity to persist. Our ability to forecast the potential for *C. virginica* to tolerate OA and persist under future ocean conditions is limited by our knowledge on their mechanisms for resilience. This thesis examined the potential mechanisms and molecular features associated with resilience to ocean acidification in the eastern oyster by examining whether resilience is the result of a plastic response, an energy allocation mechanism, regulation of gene expression, and/or selection at the genome level. The work here demonstrates the larval stage of the eastern oyster, the most vulnerable life stage, is capable of rebounding after transplantation from elevated to ambient pCO_2, acclimating and adapting to elevated pCO_2 via changes in gene expression and selection of private alleles in key genes associated with cell morphogenesis and development. The results of the physiological assays support the concept that the larval stage acts as the "bottleneck" in the species' survival under OA, and, once larvae settle out of the water column, juveniles will be robust to the effects of OA and OA in combination with pathogen challenge (as evidenced by the low levels of gene expression and robust selection of private alleles).

In the recent years during which this work was being designed and completed several new studies have been published that shed light on the complexity surrounding experimental OA.

To this extent, this study was designed to provide a holistic view of the mechanisms and molecular processes for resilience to OA in the eastern oyster at the most basic level; several factors which are just starting to be accepted as important elements to consider within OA research were not included within the study. The following discussion will highlight important notes about the animals used in the experiments and ways in which this study could be built upon by incorporating these recent insights.

One such insight surrounds the concept that the effects of OA can vary within a species across populations and also between wild and hatchery-raised stock. The juvenile oysters used in this study were derived from hatchery stock that has been selectively bred to enhance the qualities desired by the aquaculture industry (i.e. fast-growth, resistance to pathogens, etc.). Work on the Sydney rock oyster demonstrated that selectively bred oysters were more resilient to OA as a whole than wild oysters (Fitzer et al., 2019) and their proteomic responses also varied with regard to wild populations in response to OA (Thompson et al., 2015). Thus, under this experimental design the effect of OA on larvae versus juveniles should be prudently compared, and the conclusions derived from the results presented here should be applied to other populations of the eastern oyster cautiously. Initial trials for experiments on this project sourced broodstock animals from several populations around New York and Massachusetts, however, resulting larvae failed to metamorphosize and thus were not good candidates for these studies. The failure of many spawning attempts necessitated the need to derive juveniles from hatchery stock. The initial design which incorporated genetically diverse broodstock would have been a more robust way to measure the true effects of OA on this species without the artifact of population-specific resilience. Additional studies with animals sourced from different populations are needed to provide a more comprehensive view of the impacts on this species.

Furthermore, a growing number of publications have described the effect of diel fluctuations in pH for some species of bivalves. In scallops, clams, oysters, and abalone, fluctuations have magnified the adverse impacts of high pCO_2 on survival (Clark and Gobler, 2016; Onitsuka et al., 2018) though effects vary by species. The importance of fluctuations may become more clear with further research. The studies presented here do not consider fluctuating pH regimes. These methods may limit the extent to which the results can be applied to wild populations, however, as described by Kapsenberg et al. (2018) for early life stages of some bivalves it is the mean pH that has the largest effect on the organism. In addition, a number of recent studies have been published describing carry over effects from parent to offspring (Parker et al., 2012; Dupont et al., 2013; Griffith and Gobler, 2017). Trans-generational studies demonstrate variable results, however, this may be due to differences in experimental design (e.g. length of acclimation, number of spawning events prior to experimentation) and broodstock source. The role of carry-over effects is not examined here but could be a major determinant for resilience under OA.

Though acclimation potential has been demonstrated in the eastern oyster, if CO_2 emissions continue to increase, oysters (and other calcifiers) may fall into a Red Queen's race, in which they must continually acclimate/adapt to survive or risk extinction. The threshold after which these animals can no longer acclimate or adapt has not yet been defined but could be seen in the next few centuries. The results here demonstrate the mechanisms and molecular processes which some "winners" may use to tolerate the ocean conditions predicted for the end of the century. However, the upwards acceleration of pCO_2 concentration with time will only serve to push the limits of resilience in the eastern oyster even further. Our knowledge of the potential for the survival of the eastern oyster species is additionally limited by our ability to forecast the effects over the entire lifetime of the individual. Chronic stress can impact organisms in a

number of ways including shortening the lifespan of an individual (Pickering et al., 2013). As the eastern oyster can live up to 20 years (Buroker, 1983), the effect of OA on the lifespan of *C. virginica* cannot be studied in the lab. Acclimation or adaptation may come at a cost to the oyster and, while the prognosis for the species is positive here, we cannot predict how these changes may impact the life history of the oyster.

While there have been a few studies identifying the molecular features underlying resilience to ocean acidification, the body of knowledge remains incomplete. Even fewer studies examine the features under a factorial, multiple stressor experimental design. The study presented here fills in many of those missing pieces but also uncovers additional questions surrounding the underlying mechanisms for resilience. Future work on this project could incorporate the most recent advances in OA research including examining effects in wild versus aquaculture oysters, incorporating an acclimation period for broodstock, and identifying the effects of static versus fluctuating pCO_2 regimes. In addition, a time series experiment for larvae and post sets of different ages under elevated pCO_2 could provide some novel information on how the different age classes respond to environmental stress through different developmental stages (which also have different demands on the system regarding calcification). To directly follow up on the results of this project, future work could validate the results of the RNAseq and variant detection work presented here by means of targeted gene silencing methods. Knockdown of the genes encoding hyaluronidase, which were upregulated in both the EE and AE treatments with respect to AA and possessed variants in EE, would be the primary targets of interest as the protein is likely used for acclimation to elevated pCO_2. Our knowledge of the features associated with resilience would also benefit from an investigation into the epigenomes of the eastern oyster. Methylation state of DNA may be important for conferring resilience and is not covered

here. The differential expression demonstrated here may actually be the result of changes in methylation state which could be another important missing piece for the puzzle regarding mechanisms for resilience. Methylation state may explain the convergence of expression profiles for animals that remained under elevated pCO_2 versus those transplanted from ambient to elevated pCO_2 in the transplant experiment. Dimond and Roberts (2020) showed that the DNA methylation profiles of the reef coral *Porites astreoides* obtained from different habitats converged after one year in a common garden setting indicating an acclimation response. Understanding how methylation may work in combination with SNP selection and changes in gene expression for resilience could come closer to providing the complete story for how eastern oysters may survive ocean acidification.

This thesis provides novel information regarding the mechanisms and molecular features underlying resilience to OA in *C. virginica*. Acclimation via changes in gene expression appears to alleviate the stress caused by OA in larvae and likely enable them to tolerate the stress for long enough to pass through the bottleneck and settle out into OA-tolerant juveniles. Though significant changes in allele frequency were not identified here, the presence of private alleles within animals exposed to OA suggests adequate potential to adapt to the stress of climate change. The information gained in this study regarding genes likely associated with resilience can serve to provide tools for a sustainable bivalve aquaculture industry that is robust to the predicted effects of OA to ensure the survival of the industry and species.

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