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The Impact of CO2-related Ocean Acidification on the Molecular Regulation of Shell Development in the Eastern Oyster (Crassostrea virginica).

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THE IMPACT OF CO₂-RELATED OCEAN ACIDIFICATION ON THE MOLECULAR REGULATION OF SHELL DEVELOPMENT IN THE EASTERN OYSTER (CRASSOSTREA VIRGINICA).

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Renewable Natural Resources

in

The School of Renewable Natural Resources

by

Mackenzie Leigh Richards
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ABSTRACT

Eastern oysters (*Crassostrea virginica*), native to the Gulf of Mexico, are keystone species in estuarine ecosystems and are economically valued. Current research indicates that ocean acidification adversely affects the physiology and morphology of larval oysters, but the molecular mechanisms of this impact remain unstudied. Ocean acidification is contributed to by elevated atmospheric CO$_2$ due to increased anthropogenic activities, causing heightened partial pressure of CO$_2$ (pCO$_2$), and eutrophication from land-based runoff in the Gulf. The objective of this work was to determine the genomic response of the eastern oyster in Louisiana to simulated ocean acidification. In this study four biomineralization-related genes were cloned in *C. virginica*: caltractin (*cetn*), calmodulin (*calm*), calreticulin (*calr*), and calnexin (*canx*). The relative expression of these genes in response to changes in environmental pCO$_2$ concentrations was analyzed both *in vivo* utilizing larval oysters and *in vitro* mantle cell culture models. Results revealed that larval oysters cultured in increased CO$_2$ environments had reduced mean shell length and survival in comparison to those reared in ambient conditions. Expression levels of all four calcium-binding protein genes were altered in both larvae and mantle cells exposed to elevated pCO$_2$, or hypercapnia conditions. Relative expression of calcium-binding proteins was representative of gene expression in both larvae and cells. The expression profiles of the calcium-binding protein encoding genes were correlated to the changes of pCO$_2$ concentrations in the environment, which suggests critical roles of these proteins in the early biomineralization in *C. virginica* in response to ocean acidification. This study also validated the use of primarily cultured mantle cells as an effective model for investigating the impacts of environmental stressors on biomineralization mechanisms in *C. virginica* on the molecular level to predict the physiological responses of these organisms to future acidified conditions in the wild.
CHAPTER 1: INTRODUCTION

1.1 Background

Indigenous to the Atlantic Coast and Gulf of Mexico (hereafter Gulf), eastern oysters (Crassostrea virginica) are critical to estuarine habitats, provide numerous ecosystem services, and are valuable in aquaculture and fisheries. On both a national scale and within the Gulf, the eastern oyster is recognized as a leading aquaculture bivalve, holding great commercial value. The oyster fishery is comprised of public and private oyster lands, and is profitable recreationally and commercially, especially in Louisiana. C. virginica alone accounts for 75% of the total oyster harvest in the nation, making it the second most valued bivalve industry, where Louisiana is the top producer contributing $317 million annually (Wallace 2001; FAO 2004; LDWF 2014).

Oysters are keystone species that filter large volumes of water daily, suspending and facilitating the availability of nutrients for other organisms, while also reducing toxins and turbidity in the water column (FAO 2004; LDWF 2014). Eastern oysters are euryhaline osmoconformers, meaning they match their internal conditions to those of their surroundings, allowing the organism to inhabit an exceptionally wide range of stressful estuarine ecosystems that are constantly experiencing fluctuating conditions. Their range stretches up to the eastern coast of Canada and extends all the way to Argentina’s Atlantic coast (Wallace 2001). Populations of C. virginica endemic to Louisiana’s Gulf coast flourish in unique estuarine habitats, differing from ecosystems along the Atlantic coast, that receive extremely high volumes of freshwater discharge, variable by season, from the Mississippi and Atchafalaya River systems (Guo, et al. 2012). In addition to high water discharge, massive river basins input large nutrient loads and sediment into Louisiana estuaries exported from land-based runoff, contributing to alterations in carbonate chemistry and stressful conditions within the ecosystem (IPCC 2007;
Doney, et al. 2009). Established oyster reefs, created from clustered shells, provide settling substrate, shelter and protection for various organisms thriving in estuaries. Additionally, complex oyster reef structures stabilize shorelines against erosion, sheltering coastal areas from large storm events (Galtsoff 1964; Ward, et al. 1994; Kurihara, et al. 2007), demonstrating the eastern oyster’s important relationship to humans as well.

*C. virginica*, along with various other calcifying species, begin the life cycle as free-floating larvae and metamorphose into sessile benthic dwellers (Figure 1.1), where they remain settled in the same location for the entirety of their adult life. In early veliger stages, shell deposition is vital for successful settlement and survival into adulthood (Wallace 2001). Biomineralization is the process by which larval and adult oysters utilize forms of calcium carbonate in seawater to form their calcified shells (Zhang, et al. 2012). In this process, the oyster mantle secretes a skeletal, organic fluid into the extrapallial cavity (Figure 1.2), where calcification occurs (Furuhashi, et al. 2009). While there are several studies focused on calcification in oysters, the mechanisms and process of biomineralization are still widely unknown (Wang, et al. 2013). Although adult eastern oysters are resilient in a wide range of fluctuating environmental factors in estuarine ecosystems, maturing larvae are especially vulnerable to stressors during the early phases of biomineralization through metamorphosis (Kurihara, et al. 2007; Miller, et al. 2009; Parker, et al. 2011).

Global climate change is predicted to induce larger fluctuations in environmental factors within estuarine and marine ecosystems, leaving larval calcifying organisms susceptible to more extreme stressors (Kurihara, et al. 2007; Parker, et al. 2009; Gazeau, et al. 2010; Barton, et al. 2012; Timmins-Schiffman, et al. 2013). Rising temperatures might have drastic impacts on various marine bivalves that rely on temperature cues to initiate spawning. Ocean temperatures
are predicted to increase 1.8-6.4˚C by the year 2100 (Doney, et al. 2009). In marine ecosystems, combined impacts from increasing temperatures and acidification contributes to decreased tolerance of marine calcifying organisms and higher susceptibility to stress. Decreased fertilization, development, and increased shell abnormalities of Sydney rock oyster (*Saccostrea glomerata*) D-veligers were observed at high temperature and pCO$_2$ (Parker, et al. 2009). Low salinity (< 15ppt) is another environmental variable that, in combination with elevated pCO$_2$, inhibits biomineralization, growth and survival rates in *C. virginica* (Dickinson, et al. 2012). Environmental fluctuations of pH, temperature, and salinity could contribute to substantial declines in populations of calcifying marine organisms. One of the greater threats to calcifying organisms as a consequence of global climate change is ocean acidification in coastal environments.

Figure 1.1: The oyster life cycle, detailing the stages of early development and shell formation through settlement, into adulthood (Wallace 2001).
Due to anthropogenic activities in energy consumption and deforestation, the global atmospheric concentration of CO$_2$ has dramatically increased in past decades (IPCC 2007). Increases in atmospheric CO$_2$ alters the carbonate chemistry of surface seawaters when carbon dioxide in the atmosphere reacts with water molecules at the surface interface forming carbonic acid. A result of this reaction is the freeing of hydrogen ions from bicarbonate and carbonate in the seawater (Doney, et al. 2009):

$$
\text{CO}_2^{\text{atmospheric}} \leftrightarrow \text{CO}_2^{\text{aqueous}} + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^- \leftrightarrow 2\text{H}^+ + \text{CO}_3^{2-}
$$

The increase in hydrogen ions in seawater results in rising dissolved CO$_2$ concentrations, declining pH, and ultimately in facilitating acidification of the ocean (Miller, et al. 2009; Feely, et al. 2010). Since the industrial revolution, the pH of surface ocean waters has decreased by 0.1 units (Orr, et al. 2005) and, according to moderate scenario climate change models, oceans will experience an additional decline in pH by 0.3-0.4 units and an increase in the pCO$_2$ from 400ppm to 1000ppm by the year 2100 (Caldeira and Wickett 2003; IPCC 2007). Surface ocean
waters, including estuarine ecosystems, sequester ~30% of this anthropogenic carbon dioxide from the atmosphere, resulting in elevated pCO$_2$ in seawater (Caldeira and Wickett 2003; Feely, et al. 2004; Beniash, et al. 2010). In addition to altering the pH, elevated carbon dioxide concentrations in the ocean restrict the availability or saturation of carbonate and calcite minerals utilized by calcifying organisms for shell formation. Estuarine larval organisms, especially calcifiers, are particularly susceptible to this acidification caused by elevated pCO$_2$. However, responses to future ocean acidification of calcifying species across taxa and of individuals of the same species in differing populations are variable.

Previous studies have reported an adverse effect on growth, calcification rates, mechanical shell structure, metabolism, homeostasis regulation, metamorphosis, and survival in various larval calcifying species in response to CO$_2$-induced ocean acidification (Parker, et al. 2009; Gazeau, et al. 2010; Lannig, et al. 2010; Talmage and Gobler 2010; Barton, et al. 2012). Exposure to predicted, elevated pCO$_2$ decreased survival and developmental rates and reduced calcification in early _C. virginica_ veligers (Miller et al. 2009; Gobler and Talmage 2014). Additionally, elevated pCO$_2$ negatively impacted pacific oyster (_Crassostrea gigas_) larvae by causing shell abnormalities in addition to significantly smaller and thinner shells (Kurihara, et al. 2007; Timmins-Schiffman, et al. 2013). Proper shell formation in early developmental phases are vital to the development and survival of the organism into full maturity, emphasizing the importance of understanding the mechanisms involved with biomineralization to make predictions of future impacts on marine calcifiers (Orr, et al. 2005; Gazeau, et al. 2007; Beniash, et al. 2010).

The biomineralization process is poorly described in marine invertebrates, and while it is a complex multi-organ mechanism, previous studies suggest the involvement of genes encoding
calcium-binding proteins in mantle cells (Gong, Li, et al. 2008; Zhang, et al. 2012). These calcium-binding proteins identified in mantle cells are responsible for calcification, ion and acid-base regulation, and maintaining homeostasis both intracellularly and extracellularly. Xiang et al. (2014) suggested that the calcification process is initiated intracellularly in mantle cells, where calcium-binding proteins aid in the storage of calcium ions that are later transported to the site of biomineralization in the cells. Biomineralization-related genes and linked calcium-binding protein are differentially expressed between various marine calcifying species and within distinctive populations of a specific species when exposed to stress induced by ocean acidification (Woo et al. 2010; Dineshram et al. 2015; Ertl et al. 2016; Goncalves et al. 2017). Therefore, it is important to investigate reactions of species in unique populations to determine an organism’s susceptibility and potential adaptability to future climate change.

This study is the first to clone the four specific biomineralization-related genes from C. virginica. Additionally, this study utilizes a primary cell culture model, derived from mantle tissue, to investigate the expression profiles of these biomineralization-related genes in response to ocean acidification. Investigating the molecular mechanisms of biomineralization and determining the genomic responses of larval eastern oysters to acidified conditions allows for understanding of morphological and physiological effects, as well as adaptability, of ocean acidification on biomineralization in calcifying organisms.

1.2 Research Objectives

The objective of this master’s thesis project is to investigate the impact of CO\(_2\)-related ocean acidification, on the molecular regulation of shell development in eastern oysters by (1) cloning and characterizing the biomineralization-related genes in C. virginica, (2) analyzing the genetic responses of C. virginica early stage veligers (\textit{in vivo}) exposed to elevated pCO\(_2\).
conditions and (3) utilizing primarily cultured mantle cells as a model to identify the productions of calcium-binding proteins in response to acidified conditions.
CHAPTER 2: RESPONSE OF LARVAL EASTERN OYSTERS EXPOSED TO ELEVATED CARBON DIOXIDE IN SEAWATER: AN IN VIVO PILOT STUDY

2.1 Introduction

The global atmospheric concentration of carbon dioxide (CO₂) has dramatically increased in past decades due to increased anthropogenic activities in energy consumption and deforestation. As a result, dissolved CO₂ in seawater has risen causing associated pH to decline, contributing to ocean acidification. The pH of surface waters has decreased by 0.1 units (Orr, et al. 2005) since the industrial era and, according to moderate scenario climate change models, oceans will experience an additional decline in pH by 0.3-0.4 units and a growth in the pCO₂ from 400ppm to 1000ppm by the year 2100 (Caldeira and Wickett 2003; IPCC 2007). Eastern oysters (Crassostrea virginica) indigenous to the Gulf of Mexico, are crucial estuarine species in their natural ecosystem and are economically important aquaculture bivalves. Louisiana oysters are euryhaline osmoconformers with an innate tolerance to variation and are ideal species for both aquaculture practices and wild recruitment for restoration (Cameron 1990; Gobler and Talmage 2014). Seventy-five percent of the total oyster harvest in the nation is comprised of C. virginica, making the species a highly valued bivalve industry, where Louisiana is the top producer contributing $317 million annually (Wallace 2001; FAO 2004; LDWF 2014). In addition, oysters are keystone species that suspend and facilitate the availability of nutrients for other organisms through filter feeding, while also reducing toxins and turbidity in the water column.

Although adult eastern oysters are resilient in a wide range of fluctuating environmental factors in estuarine ecosystems, maturing larvae are especially vulnerable to stressors in the early phases of biomineralization through metamorphosis (Kurihara, et al. 2007; Miller, et al. 2009; Parker, et al. 2011). However, a prior study where several species across taxa were exposed to
hypercapnia conditions suggested that calcifying organisms have species-specific responses and tolerances towards acidification. Adult *C. virginica* survival and growth was unchanged by hypercapnia conditions, and similar responses were observed in the mature hard clam (*Mercenaria mercenaria*), blue mussel (*Mytilus edulis*), and bay scallop (*Argopecten irradians*), all of which are marine calcifiers (Ries, et al. 2009). These results suggest that calcifying adults appear more tolerant to CO$_2$ stress. However, other studies have demonstrated that shell calcification rates and growth in adult *C. virginica*, pacific oysters (*Crassostrea gigas*) and *M. edulis* declined with increasing pCO$_2$ concentrations and metabolic expenditure increased due to reduced carbonate ions in hypercapnic seawater (Gazeau, et al. 2007; Talmage and Gobler 2009; Beniash, et al. 2010; Thomsen and Melzner 2010). Variations in growth response and biomineralization rates of marine calcifying organisms in hypercapnia may also be reliant upon duration and severity of exposure to elevated pCO$_2$ (Miller, et al. 2009). Furthermore, survival, growth rates, metabolism and shell calcification rates were altered in hypercapnia conditions in larval *C. virginica* and *C. gigas* during metamorphosis, which are less robust than fully formed adults (Beniash, et al. 2010; Dickinson, et al. 2012).

While estuaries innately experience environmental stress, global climate change has the potential to exacerbate stressors in these ecosystems, creating conditions intolerable by specific calcifying species (Gazeau, et al. 2007; Miller, et al. 2009; Lannig, et al. 2010; Thomsen, et al. 2013), especially in early developmental stages. Previous studies reported an adverse effect on growth and shell formation in various larval calcifying organisms in response to CO$_2$-induced ocean acidification (Feely, et al. 2004; Orr, et al. 2005; Yates and Halley 2006; Gazeau, et al. 2007; Kurihara, et al. 2007; Miller, et al. 2009; Gazeau, et al. 2010; Talmage and Gobler 2010). Additionally, adverse effects on developing larval morphology have previously been attributed to
decreased saturation of calcium carbonate and calcium ions, specifically aragonite, in elevated
CO$_2$ marine environments (Gazeau, et al. 2007; Ries, et al. 2009). Increasing pCO$_2$ in surface
waters, including estuarine ecosystems, alters the carbonate chemistry by decreasing the
available carbonate (CO$_3^{2-}$) ion concentration and increasing the abundance of free hydrogen
ions, driving the pH down (Weiss, et al. 2002). Increased hydrogen ion concentration in seawater
results in acidified conditions capable of degrading fully deposited or developing shells in
calcifying organisms, creating morphological deficiencies that could impede their survival
(Talmage and Gobler 2010). The reduction of carbonate ions lowers the calcium carbonate
saturation in seawater, impeding the biomineralization process of marine calcifiers that bind
calcium ions with an organic matrix secreted from mantle cells and extrapallial fluid to deposit
2013).

The biomineralization process begins about 24 hours post-fertilization in early stage
larval oysters (Zhang, et al. 2012) and metamorphosis is initiated after 14-21 days of
development. Understauration of aragonite in acidified seawater resulted in significantly
decreased growth and calcification in two-day old $M$. edulis larvae (Gazeau, et al. 2010). In
addition, a significant negative relationship was found in $C$. gigas between aragonite saturation
and spawning success as well as development in older veligers, indicating that insufficient
aragonite saturation due to increased pCO$_2$ environments could affect larvae thoughout

Reduced growth, decreased calcification and impaired metabolism in mature and
developing marine bivalves are potential products of higher energy demands, resulting from
acidified environments. In calcifying organisms exposed to CO$_2$ stress, energy expenditure is
diverted away from processes like biomineralization and directed towards essential mechanisms involved in maintaining homeostasis, such as balancing intracellular and extracellular acid-base equilibrium, or metabolic regulation (Lannig, et al. 2010; Thomsen and Melzner 2010; Thomsen, et al. 2013; Li, et al. 2016). Until recently, many studies have only observed the physiological changes of shell development in oysters in response to ocean acidification (Galtsoff 1964), with little research on the impacts at the molecular level. Investigating the mechanisms involved with biomineralization in early stage bivalves, and its response to environmental stressors may produce a basis for predicting susceptibility of eastern oyster populations in Louisiana to climate change.

The regulatory mechanisms of the biomineralization process is understudied, especially in *C. virginica* veligers (Zhang, et al. 2012; Wang, et al. 2013). Research has indicated that increasing acidification of oceans negatively impacted the development, especially biomineralization, of larval oysters and other marine calcifiers (Feely, et al. 2004; Orr, et al. 2005; Yates and Halley 2006; Gazeau, et al. 2007; Kurihara, et al. 2007; Miller, et al. 2009; Gazeau, et al. 2010; Talmage and Gobler 2010), yet the genomic and morphological responses of *C. virginica* veligers in Louisiana remains largely unknown. The *C. gigas* genome was recently sequenced by Zhang et al. (2012) and approximately 259 proteins were identified in the pacific oyster likely to be associated with shell formation and similar proteins are thought to exist in the eastern oyster. Of these 259 identified genes, 214 were determined as mantle specific and 39 biomineralization-related genes were believed to be conserved across species, highly expressed in mantle tissue (Zhang, et al. 2012). Calcium-binding proteins, and associated shell development genes are the primary types of proteins believed to be involved with the formation of calcium carbonate shells in bivalves and these specific genes respond to stressors in the
environment, especially in maturing larvae. Following convention, genes will be denoted in italics, and proteins will be capitalized. Calr is a calcium-binding protein located in mantle tissue of C. virginica that buffers calcium ion homeostasis and Calm aids in regulating the biological processes that rely on intracellular calcium ions (Woo, et al. 2010). Cetn is another protein that is associated with calcium-binding in the biomineralization process and is functionally and structurally analogous to Calm (Chazin 2011). Calr and Canx are believed to be associated with a “Calr/Canx” cycle that regulates and maintains calcium homeostasis within the mantle tissue of calcifying organisms (Gelebart, et al. 2005). All four of these proteins and associated genes were identified in C. virginica and utilized as genomic markers for studying the early biomineralization process.

The objective of this study was to determine the impact of CO₂-related ocean acidification on the molecular regulation of shell development in Louisiana eastern oysters by: (1) identifying and sequencing four target genes caltractin (cetn), calmodulin (calm), calreticulin (calr), and calnexin (canx) and associated calcium-binding proteins related to the biomineralization process in C. virginica and (2) analyzing the morphological and genomic responses of C. virginica early stage veligers post exposure to elevated pCO₂ (1000ppm) conditions.

2.2 Materials and Methods

2.2.1 Facility and System Design

Larval eastern oysters were spawned and then maintained for the first six days of early development in culture buckets bubbled with CO₂ enriched air to create either an ambient condition of 400 ppm or a treatment condition of 1000 ppm pCO₂ concentration. The concentrations of environmental CO₂ created in this experiment represented the current surface
seawater condition (400 ppm) and the predicted condition (1000 ppm) for the year 2100, according to models projected by the Intergovernmental Panel on Climate Change (IPCC 2007). The experiment was conducted at the Michael C. Voisen Oyster Hatchery in Grand Isle, Louisiana. The hatchery facility used water extracted directly from Bayou Rigaud where it was filtered through 2-inch 20µm disc filters and stored in an 30283L tank. Stored water was then filtered through a 10µm filter after passing through a propeller bead filter. A UV filter sterilized the water, foam fractioned to remove organic matter, and buffered with soda ash to attain a pH of 8.25. Finally, the stored water was filtered through cartridge filters with 1-micron pore size before use by the hatchery.

The system designed for this study (Figure 2.1) has been previously used in an earlier study (Mallozzi 2017). One week before the experiment was initiated, reservoirs were created by filling up two 189L tanks with hatchery water. Water in the tanks were bubbled with a working-class-certified AirGas mixtures to alter the inorganic carbonate chemistry and generate 400 or 1000 ppm of pCO$_2$ in water. Upon refilling, reservoirs were continuously bubbled for 2 days to recondition the water before the next use. Both treatments (400 ppm and 1000 ppm pCO$_2$) were run in triplicates in three closed polyethylene buckets for each treatment. Buckets, or culture vessels, were filled with 15-L of pre-conditioned water from the respective reservoir (400 or 1000 ppm pCO$_2$) and the pCO$_2$ concentrations in culture vessels were maintained by bubbled through fine glass frits, with the identical AirGas mixtures previously stated (ambient or elevated) prior to larval inoculation.

2.2.2 Oyster Larval Culture and Sampling

Oysters were spawned from a diploid brood stock originating from Lake Calcasieu, collected in the spring of 2016. The brood stock individuals that were used for spawning were
collected from suspended oyster cages, located at the hatchery. The selected gravid individuals were previously maintained in ambient temperatures, then exposed to 34°C bay water to thermally induce spawning. As the individuals began to spawn, they were divided into separate containers to collect and segregate the gametes. Once the spawning event was complete, the isolated eggs and sperm were pooled and thoroughly mixed to initiate fertilization. Successfully fertilized eggs, determined by light microscopy, were used for the experiment two hours post fertilization, and culturing vessels were inoculated with fertilized eggs. Following fertilization, the larval density in each of the six pre-conditioned culture buckets was maintained at 100 eggs/ml. After 48 hours, the larvae were restocked at 50 larvae/ml to meet the culture standards.

The trial was conducted for six days (0h, 24h, 48h, 96h, and 120h), where 0h represented the day eggs were fertilized and stocked and at 120h trial was terminated. Initiated 24 hours after inoculating the culture buckets, larvae were fed daily with 40,000cells/ml of an algal diet consisting of *Tisochrysis lutea, Cisochrysis galbana, Chaetoceros calcitrans, Chaetoceros muelleri* and *Pavlova luthen*, a mixture produced by the hatchery for successful bivalve growth and survival. In addition to feeding, temperature, salinity and pH of the water within the culture buckets was recorded daily. Temperature and pH were measured using a pH meter (Orion 370 PerpHecT benchtop pH/ORP/ISE/T meter, Thermo Fisher Scientific, Waltham, MA). Total alkalinity was measured from water that had been treated with supersaturated 0.02% mercuric chloride and frozen prior to analysis. Briefly, total alkalinity samples were titrated with 0.097N hydrochloric acid (HCl) to achieve a pH of 3.5 using a Schott Titroline easy titrator (General Laboratory Supply, Pasadena, TX) (Dickenson, et al. 2007). Samples were then left to de-gas for 3 minutes and titrated step-wise at 20 second intervals in 0.05ml increments until pH 3.0 to create a Gran Line, using the SeaCarb (http://CRAN.Rproject.org/package=seacarb) program in
R Studio (Boston, MA). Water changes were conducted every other day (48h and 96h) to maintain water quality by draining 90% of the bucket through a 35µm screen and re-suspending the larvae in 500ml of fresh pre-conditioned water from the reservoir. The bottom 10% of water was discarded as waste. Buckets were then scrubbed clean, rinsed and refilled with reservoir water and promptly restocked with the larvae.

Figure 2.1: Experimental system design showing the alteration of the inorganic carbonate chemistry for both reservoirs and culture vessels conditioned at present day pCO₂ concentrations (400 ppm) and elevated pCO₂ conditions (1000 ppm). Within each treatment there were three closed replicate vessels, 15L in volume, to culture oyster larvae (Mallozzi 2017).

Oyster larvae samples were collected daily for shell length measurements and survival by filtering a known volume of seawater from the sample buckets through a 35µm screen and re-suspending the larvae in 50ml of water. The 50ml sample was then continuously mixed to equally suspend and distribute the larvae into three 100µl aliquots for larval counts and shell length. Counts from the three aliquots were averaged to calculate the percent survival for each bucket within both pCO₂ treatments. The larval shell size, or length, was measured lengthwise across the longest part of the shell of the oyster larvae (n=10) for each replicate bucket within both treatments. Starting from 24h, larval samples for molecular analysis were collected by
straining a known volume of water from each of the culture buckets through a 25µm screen. Using a micropipette, larvae were collected from the screen and allocated into a 1.5ml microcentrifuge tube with 1000µl of TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA) and stored in -20°C for future processing and analysis. Additionally, an aliquot of larvae samples was collected and fixed in 70% ethanol for whole mount immunofluorescence analysis.

2.2.3 RNA Isolation and cDNA Synthesis

Chloroform was added to the thawed samples in TRIzol® Reagent to isolate RNA and remove proteins (Gong, Li, et al. 2008; Gong, Ma, et al. 2008; Xiang, et al. 2014; Goncalves, et al. 2017). Samples were shaken thoroughly, incubated in room temperature and centrifuged for 15 minutes. The supernatant was carefully removed and placed into a new microcentrifuge tube with equal parts 100% isopropanol and held in -20°C overnight to precipitate out RNA. Precipitated RNA was centrifuged to create a pellet that was washed with 1mL 70% ethanol and the supernatant was removed, leaving the pellet to air dry.

DNA was dissolved with a TURBO DNA-free™ Kit, where the 50µl master mix consisting of 44µl nuclease-free water (Ambion, Thermo Fisher Scientific, Waltham, MA), 5µl 10x TURBO DNase buffer, and 1µl TURBO DNase was aliquoted to each sample and placed in 37°C water bath for half an hour. Samples were then removed from the water bath and 5µl of DNase inactivation reagent was added to isolate RNA. Finally, samples were centrifuged and the supernatant was collected and distributed to a new microcentrifuge tube. A 2.5µl aliquot of RNA was measured by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) to determine the concentration and quality of the isolated RNA from each sample.

Complementary DNA (cDNA) was synthesized from the isolated RNA using SuperScript IV Reverse Transcriptase (Life Technologies Corporation, Grand Island, NY). The samples were
further processed in compliance with the manufacturer’s instructions. The cDNA synthesis was completed at 65°C for 5 minutes, 4°C for 20 minutes, 55°C for 20 minutes, 80°C for 10 minutes. Once removed, 180µl of molecular grade water was added in each reaction.

2.2.4 Primer Design and Validation

Gene specific primers were designed for cloning and later qPCR analysis (Table 2.1) of gene expression in *C. virginica* of elongation factor 1-α (*ef1α*), caltractin (*cetn*), and calmodulin (*calm*) using gel electrophoresis. The gene, *ef1α*, a commonly used housekeeping gene, was obtained from previous studies on *Crassostrea virginica* in GenBank (GenBank accession number BG624869.1). The genes, *cetn* (GenBank accession number CD648584) and *calm* (GenBank accession number CD526718), were sequenced with the tool Integrated DNA Technologies (Coralville, IA) software using existing expressed sequences tags (ESTs) for the genes in *C. virginica*. The target genes *calr* and *canx* in *C. virginica* did not previously exist in GenBank, but the homologous sequences for these genes are available for the Pacific oyster (*Crassostrea gigas*). Primers for *calr* and *canx* were designed with NCBI nucleotide BLAST, which references and compares conservative areas within sequences of genes from homologous species (accession numbers AB262086 and AB262085, respectively). These primers were then utilized for cloning partial sequences of the target gene; once isolated, the sample was sequenced at the Louisiana State University School of Veterinary Medicine BioMMED Cores (Baton Rouge, LA). Specified primers for the two target genes (*calr* and *canx*) were then created for the partially sequenced genes and they were eventually fully sequenced.

To test the primer pairs for each shell development-related gene (*cetn*, *calm*, *calr* and *canx*), fragments of the four target genes were amplified with PCR and products were validated with gel electrophoresis. Each primer pair was run with a positive control, containing *C.
virginica cDNA, and a negative control that contained molecular grade water. First, RNA was isolated from C. virginica mantle tissue with TRIzol® Reagent, followed by DNA removal using a TURBO DNA-free™ Kit, then cDNA was synthesized from isolated RNA using SuperScript IV Reverse Transcriptase according to manufacturer instructions. PCR was conducted with the primer pairs and sample cDNA to create a product for the positive control. For each primer pair, 8µl of master mix containing 5.5µl molecular grade water, 2µl 5x GoTaq PCR reaction buffer, 0.2µl 10mM dNTP, 0.1µl 50 µM forward primer, 0.1µl 50 µM reverse primer, 0.1µl GoTaq DNA polymerase (0.5 unit) was aliquoted to 2µl of sample cDNA. PCR ran one cycle at 50˚C for 2 minutes, 94˚C for 2 minutes, followed by 40 cycles at 94˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 30 seconds.

Positive controls for each primer pair were prepared with a master mix comprised of 2µl 4x gel loading buffer, 1µl 10x SYBR green, 5µl of PCR product, while negative controls used molecular grade water instead. Samples were run on a 7% polyacrylamide-TBE gel alongside a DNA ladder, as a reference, at 150 volts for 45 minutes. Gels were analyzed with an E-Gel Imager with UV Light base (Life Technologies Corporation, Grand Island, New York) to confirm the primers were amplifying a specific gene fragment in C. virginica cDNAs.

2.2.5 Gene Cloning and sequencing

Gene cloning was conducted to generate specific primers for the two target genes (calr and canx) that did not previously exist in GenBank for C. virginica. The total RNA was isolated from homogenized C. virginica mantle tissues using TRIzol® Reagent followed by DNA removal with TURBO DNA-free™ Kit. The total RNA was then used for cDNA synthesis with SuperScript IV Reverse Transcriptase according to manufacturer’s instructions. Fragments of the four calcium-binding protein genes were amplified using the gene
cloning primers and regular PCR cycles (94°C 5 min followed by 32 cycles of 94°C 30s-
55°C 30s-72°C 30s, and a final extension step at 72°C for 7 min). For all primer pairs, a 24µl
PCR master mix was prepared consisting of 17.75µl of molecular grade water, 5µl of GoTaq 5x
reaction buffer, 0.5µl 10mM dNTP, 0.25µl 50 µM forward primer, 0.25µl 50 µM reverse primer,
0.25µl GoTaq DNA polymerase (1.25 units) and mixed with 1µl of sample cDNA (total volume
25µl). PCR products were tested with vertical gel electrophoresis. Preparation of the master mix
included 15µl 4x gel loading dye, 5µl 10x SYBR green, and 50µl of each sample cDNA product,
with 23µl of the total solution allocated into the wells of a 7% polyacrylamide-TBE gel. Each
cDNA product ran in duplicate for 60 minutes at 150 volts and the products were visualized on
an E-Gel Imager with UV Light base (Life Technologies Corporation, Grand Island, NY). The
targeting band of each gene on the gel were visualized under UV light and collected. The
collected gel pieces containing targeting DNAs were then transferred to sealed dialysis bags
containing 300-400µl 1x TAE buffer. Dialysis bags were submerged in 1x TAE buffer in a
horizontal gel electrophoresis machine for 2 hours at 150 volts. Following, the machine’s electric
field was reversed and allowed to run for an additional 2 minutes at the same voltage to ensure
that all DNAs from the gel pieces were suspended in the buffer solution within the dialysis bags.
The DNAs in each gel piece were purified using DNA dialysis collection method following the
standard protocol.

The purified PCR products were cloned to the pGEM-T Easy vector using T4 DNA
Ligase System Ligation (0.5µl pGEM-Teasy vector, 0.5µl T4 DNA ligase, 5µl 2x rapid ligation
buffer, and 4µl of sample DNA) (Promega, Madison, WI). Thereafter, plasmids were
transformed into DH5α competent Escherichia coli following a standard protocol. In the
transformation process, 5µl of the plasmid mixture from each primer pair was combined with
50µl of *E. coli* DH5α strain (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) and cells were incubated on ice for ~15 minutes, quickly transferred to 42°C water bath for 45 seconds and then immediately placed back on ice for 10 minutes. Following, 450µl of Luria-Bertani broth (LB) (1% bacto tryptone, 0.5% yeast extract, and 1% sodium chloride) was added to cells and incubated in a shaking incubator at 37°C, 250rpm for 30-60 minutes. The cell suspension was then spread on an LB-agar plate containing 100 µg/ml ampicillin and incubated at 37°C overnight, allowing the bacterial colonies to proliferate.

After incubation, plasmids were isolated from three randomly selected colonies on each of the four petri-dish plates and inoculated in 3ml of LB broth and 100µl 1x ampicillin in a shaking incubator at 37°C, 250rpm for 4 hours in darkness. Inoculation tubes were removed from the incubator and 3 ml of each sample was allocated into separate microcentrifuge tubes, centrifuged, the supernatant was discarded and the step was repeated, leaving a pellet. The pellet was re-suspended with 100µl GTE buffer (25mM Tris, 50mM glucose, and 10mM EDTA), 200µl SDS lysis buffer was added and solution was inverted gently for 5 times. Then, 150µl of the third lysis buffer was allocated into the sample solution, inverting gently for 5 times to mix thoroughly, and centrifuged. The supernatant, for each sample, was collected and transferred to a microcentrifuge tube with 1µl of RNase and incubated in 37°C water bath for 60 minutes.

Once removed from the water bath, 3M sodium acetate (pH 5.5) at 10% the original volume of the sample and 2.5-3x original volume of 100% ethanol was added to each sample solution and held in -20°C overnight. Samples were then centrifuged, the supernatant was discarded, and they were washed with 1ml 70% ethanol. Again, samples were centrifuged and the supernatant was discarded, leaving a pellet of DNA. DNA was re-suspended with 25µl of molecular grade water and DNA concentrations were measured with a GeneQuant pro
Spectrophotometer (GE Healthcare Biosciences, Pittsburgh, PA). To identify bacteria with plasmid containing the target product, PCR with gene cloning primers and gel electrophoresis were conducted using standard protocol. Samples were sequenced at the Louisiana State University School of Veterinary Medicine BioMMED Cores (Baton Rouge, LA). The purified plasmids were sent to the Louisiana State University School of Veterinary Medicine Gene Lab (Baton Rouge, LA) for sequencing. For plasmids isolated from each bacterial inoculate, the inserts of the plasmids were sequenced from two directions with T7 and SP6 primers, respectively. The two reads of each insert were aligned using BLASTn. The aligned sequences were trimmed with VecScreen to remove the sequence of pGEM-T Easy vector.

2.2.6 Full sequence gene cloning

With the obtained fragment of calr and canx, new primers were designed using the primer design software provided by IDT DNA website. These specific primers were used for both full-length gene cloning and qPCR. All gene specific primers were tested by PCR with cDNA synthesized from C. virginica mantle RNA. The program used for this PCR was 2 min at 50, 2 min at 94°C, and 40 cycles of 94°C (30s) - 55°C (30s) – 72°C (30s). The PCR products were run on a polyacrylamide gel in TBE buffer and visualized by Gel Documentation System.

Cloning of full length sequences of the four genes was performed using the Rapid Amplification of cDNA Ends (RACE) system from Invitrogen. The cDNAs for 3’- and 5’-RACE assay were synthesized based on the steps in the manufacturer’s instruction. Briefly, the first stranded cDNA was synthesized by Superscript II Reverse Transcriptase led by the 3’-anchor primer (3-AP) (Table 1). The 3’ of each gene was amplified by PCR using the abridged universal amplification primer (AUAP) and the forward primer of each gene. The cycling program for 3’-end PCR was regular PCR cycles with slight modification (94°C 3 min followed
by 32 cycles of 94°C 30s-55°C 30s-72°C 60s, and a final extension step at 72°C for 7 min). Full length sequence of 5’-end of each calcium-binding protein genes was also cloned using 5’ RACE system. The first stranded cDNA was synthesized using 3’-AP and Superscript II Reverse Transcriptase. A homopolymeric tail was then added to the 3’-end of the cDNA using dCTP and terminal deoxynucleotidyl transferase (TdT). The first PCR amplification was accomplished using the universal amplification primer (UAP) and a deoxyinosine-containing abridged anchor primer (5’-AAP) with the following thermocycler program: 2 min at 94°C, 30 cycles of 94°C (60s) - 55°C (60s) – 72°C (60s), and a final DNA extension step at 72°C for 7 min. The PCR product was diluted 50-fold with molecular grade water. The diluted PCR product was used for a nested PCR with the reverse gene-specific primer and the AUAP primer. The program set for the nested PCR was the same to that used for the first PCR of 5’ RACE. PCR products for 3’ or 5’ end gene fragments were loaded and run on a 0.8% agarose TAE gel. The PCR products were purified using Zymoclean Gel DNA Recovery Kit (Zymo Research) following the instructive steps in manufacturer’s manual. The purified PCR products were submitted for sequencing at LSU Gene Lab using the gene specific primers or AUAP for both directions for each gene.

2.2.7 Full length gene sequence analyses

The obtained 3’ and 5’ end sequences of each gene along with the originally cloned fragment in step 2 were assembled using BLASTn for two sequences. The protein sequence of each gene was predicted by the mRNA translation tool in Expert Protein Analysis System (ExPaSy) (Artimo, et al. 2012). The open reading frame (ORF) of each gene was identified based on the length of predicted ORF and the length of the ORFs in homologous sequences in GenBank database. The translated protein sequence for each gene was first submitted to GenBank for homologous sequence searching using BLASTp. This identified the proteins in
primary structure level. Secondly, motifs and signature sequences of each protein were predicted using the simple modular architecture research tool (SMART) (Schultz, et al. 1998; Letunic, et al. 2015). This annotated the functional domains of each protein. Finally, the three-dimensional structure of each protein was predicted based on protein structure homology-modelling system (SWISS-MODEL) (Arnold, et al. 2006; Kiefer, et al. 2009; Biasini, et al. 2014). This allowed us to further confirm the identity of each calcium-binding protein.

Table 2.1: Primers used for gene cloning and qPCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer ID</th>
<th>Roles</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ef1α</td>
<td>Cv01 (F)</td>
<td>qPCR</td>
<td>5’-ATC AAC TTC CAC TGG CCA TC-3’</td>
</tr>
<tr>
<td></td>
<td>Cv02 (R)</td>
<td>qPCR</td>
<td>5’-TTT TTC CAT CTC AGC TGC TT-3’</td>
</tr>
<tr>
<td>cten</td>
<td>Cv29 (F)</td>
<td>Cloning/qPCR</td>
<td>5’-GAA GCA AGG CAA TCT CCA AAG-3’</td>
</tr>
<tr>
<td></td>
<td>Cv30 (R)</td>
<td>Cloning/qPCR</td>
<td>5’-CCG TCC GAA TTA GCA TCA CA-3’</td>
</tr>
<tr>
<td>calm</td>
<td>Cv31 (F)</td>
<td>Cloning/qPCR</td>
<td>5’-ACA GAT GAA GAG GTA GAT GAA ATG A-3’</td>
</tr>
<tr>
<td></td>
<td>Cv32 (R)</td>
<td>Cloning/qPCR</td>
<td>5’-GCT ATA CAA GCA GTT GCC ATT C-3’</td>
</tr>
<tr>
<td>calr</td>
<td>Cv33 (F)</td>
<td>Cloning</td>
<td>5’-ATT GAC TGT GGG GGT GGA TA-3’</td>
</tr>
<tr>
<td></td>
<td>Cv34 (R)</td>
<td>Cloning</td>
<td>5’-ACC TTC TTG GTG CCT GGT C-3’</td>
</tr>
<tr>
<td></td>
<td>Cv51 (F)</td>
<td>qPCR</td>
<td>5’-CCC AAA GAT CTC GAC CAG AAA-3’</td>
</tr>
<tr>
<td></td>
<td>Cv52 (R)</td>
<td>qPCR</td>
<td>5’-GGT GCC TGG TCC ACA AAT A-3’</td>
</tr>
<tr>
<td>canx</td>
<td>Cv35 (F)</td>
<td>Cloning</td>
<td>5’-CCT CAA GTC CAA AGC AAA GC-3’</td>
</tr>
<tr>
<td></td>
<td>Cv36 (R)</td>
<td>Cloning</td>
<td>5’-AGG CTC CTC CAC AGT CGA TA-3’</td>
</tr>
<tr>
<td></td>
<td>Cv57 (F)</td>
<td>qPCR</td>
<td>5’-CCA GAC AAA TGT GGA TTG GAT AAC-3’</td>
</tr>
<tr>
<td></td>
<td>Cv58 (R)</td>
<td>qPCR</td>
<td>5’-CTT CCA GCA GTG ATC CTT CAT-3’</td>
</tr>
</tbody>
</table>

Primers for RACE

<table>
<thead>
<tr>
<th>3'-AP</th>
<th>3'-RACE cDNA synthesis</th>
<th>5’-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT T-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-AAP</td>
<td>5’-RACE primary PCR</td>
<td>5’-GGC CAC GCG TCG ACT AGT ACG GGI GGG II GGG II-3’</td>
</tr>
<tr>
<td>UAP</td>
<td>5’-RACE first PCR</td>
<td>5’-CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC-3’</td>
</tr>
<tr>
<td>AUAP</td>
<td>3’-RACE PCR and 5’-RACE nested PCR</td>
<td>5’-GGC CAC GCG TCG ACT AGT AC-3’</td>
</tr>
</tbody>
</table>

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2.2.8 Quantitative PCR Analysis of Larval Samples

Quantitative PCR (qPCR) was utilized to analyze the relative expression levels of the target genes (cetn, calm, calr and canx) in larvae. The total RNA was isolated from C. virginica larvae using TRIzol® Reagent followed by DNA removal with TURBO DNA-free™ Kit. The total RNA was then used for cDNA synthesis with SuperScript IV Reverse Transcriptase according to manufacturer’s instructions. For each primer pair, including the housekeeping gene, a master mix consisting of 4.8µl of molecular grade water, 2µl of GoTaq 5x colorless reaction buffer, 0.2µl 10mM dNTP, 0.1µl 50 µM forward primer, 0.1µl 50 µM reverse primer, 0.5µl 10x SYBR green, 0.2µl ROX and 0.1µl GoTaq DNA polymerase (0.5 unit) was prepared. In a 384-well plate, 8µl of master mix was mixed with 2µl of each cDNA sample. The plate was run at 50˚C for 2 minutes, 94˚C for 2 minutes, followed by 40 cycles at 94˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 30 seconds in a 7900HT Real-Time PCR System (Applied Biosystems, Grand Island, NY).

2.2.9 Whole Mount Immunofluorescence

The relative expression levels of the calcium-binding proteins were also estimated at the protein level using whole mount immunofluorescence. Larval samples were collected daily from each treatment during the trial period and were fixed in 70% ethanol. Samples from 48h and 96h of each treatment were removed with a 25µm screen and re-fixed in 4% paraformaldehyde in CMFSS buffer overnight at 4 °C. Larvae were washed three times through a 25µm screen with 500µl 1x phosphate-buffered saline (PBS) and allocated into a 12-well plate on a shaker for 5 minutes. Samples were then blocked for 2 hours at room temperature with 300µl of 10% lamb serum in PBS. After the block, larvae were washed 3 times with 1x PBS and submerged in 300µl of the primary antibody (mouse-anti-human antibody) diluted 1:200 in PBS at 4˚C overnight.
Following, the secondary antibody (goat-anti-mouse IgG conjugated with either Alexa 594 (red) or Alexa 488 (green)) diluted 1:500 in PBS was allocated into the wells, the plates were covered in foil and placed on a shake plate for 2 hours. Samples were washed 3 times, keeping the plates covered with foil and were visualized with a Nikon Eclipse TI microscope (Nikon Instruments, Melville, NY) and analyzed with ImageJ software (Schneider et al. 2012). Imaging was completed by visualizing the average intensity of positive expression in calcium-binding proteins within larvae.

2.2.10 Statistical Analysis

Daily larval data, such as pH, total alkalinity, salinity, and temperature were averaged across replicates for each treatment per day, starting 24h post fertilization. The daily percent survival (or stocking density) of the larvae for each treatment was calculated by averaging the number of larvae per 100μl aliquot (abundance) for each replicate and multiplying it by the volume of solution the larvae were re-suspended in divided by the original volume of sample collected. Mean larval shell length was determined by averaging the lengths of larvae ($n=10$) within each replicate for each treatment.

Statistical significance was determined between treatments within each time point and between the difference of treatments for each time point in comparison to 24h for pH, total alkalinity, salinity, temperature, shell length and abundance using a linear model with residual maximum likelihood (REML) estimation (PROC MIXED, SAS 9.4, Cary, NC). Normal distribution of residuals was confirmed before the model was implemented as best fit for each variable of interest. Linear models were also used to establish if mean shell length and survival had a significant relationship with pH.
Relative expression for each gene was analyzed using ΔΔCt method described previously (Rao, et al. 2013) for larvae at 24h, 48h, and 72h post fertilization. Mortality in larvae was too high to conduct analysis on relative expression at 96h and 120h post fertilization. The Cycle threshold (Ct) values of the four calcium-binding encoding protein gene and a housekeeping gene, elf1α, were generated from the results of qPCR. Briefly, the Ct value of each reaction was normalized using the Ct value of elf1α generated from the sample in C. virginica larvae during early developmental stages exposed to elevated pCO₂ concentrations. Values residing outside of the 95% confidence interval of the mean were removed. Each statistical test was conducted using a linear model (PROC MIXED, SAS 9.4) with restricted maximum likelihood (REML) estimation, where the residuals were tested and confirmed as normally distributed by a Shapiro-Wilk test and variance was constant. The first statistical analysis of each gene examined the significant difference between the relative expression of pCO₂ treatments (400ppm and 1000ppm) for each time point (24h-72h), while the second analysis tested for significance of the difference in relative expression of the treatments for each time point after 24h (48h-72h) in comparison to 24h post fertilization. Statistical significance was determined by \( p \leq 0.05 \).

2.3 Results

2.3.1 Water Quality and Larval Development

Water quality measurements such as pH, total alkalinity, salinity, and temperature were analyzed for statistically significant differences between pCO₂ treatments (400ppm and 1000ppm) within time point and between the difference of treatments for each time point in comparison to 24h. There was a statistically significant difference in the mean pH throughout the trial period between the 400ppm and 1000ppm treatment, where the pH in the ambient 400ppm treatment was higher (8.47 ± 0.04SD) than the elevated pCO₂ 1000ppm treatment (8.32 ±
0.12SD) (Figure 2.3). As expected, the average total alkalinity (TA) did not fluctuate drastically and was not statistically significantly different between the treatments or throughout the trial period. Temperature and salinity remained consistent across time points and between treatments, with no significance detected (Table 2.2).

Despite larvae cultured in the 400ppm pCO₂ experiencing larger shell length overall (69.91 ± 0.75µmSD) than larvae cultivated in 1000ppm pCO₂ (69.84 ± 0.60µmSD), there was not statistically significant difference between the treatments (Table 2.2). There was no indication of statistically significant correlation between pH and shell length (F₁,₂₈= 1.05, p= 0.32) or survival (F₁,₂₈= 0, p= 0.10) in early larval development. At 24h post fertilization, an initial standard mortality event reduced the abundance of early veligers to ~40% the original density (100 larvae/ml; ~1.5 million larvae/ bucket) for both pCO₂ treatments. However, in the following time periods (48h-96h) the survival increased between ~40-70% in 400ppm pCO₂ and ~30-70% in 1000ppm pCO₂ (Figure 2.4).

Table 2.2: The mean ± SD and test statistics for water quality measurements and shell length of larvae for all replicates in both control (400 ppm pCO₂) and elevated treatment (1000 ppm pCO₂).

<table>
<thead>
<tr>
<th>Target pCO₂ (ppm)</th>
<th>400</th>
<th>1000</th>
<th>F₁,₂₈</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.47 ± 0.04</td>
<td>8.32 ± 0.12</td>
<td>23.49</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Total Alkalinity (µmol/kg)</td>
<td>2593.30 ± 87.74</td>
<td>2517.64 ± 129.99</td>
<td>3.53</td>
<td>0.07</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>27.73 ± 1.22</td>
<td>27.34 ± 1.35</td>
<td>0.07</td>
<td>0.79</td>
</tr>
<tr>
<td>Salinity (ppt)</td>
<td>14.2 ± 0.41</td>
<td>14.2 ± 0.83</td>
<td>0.70</td>
<td>0.41</td>
</tr>
<tr>
<td>Larval shell length (µm)</td>
<td>69.91 ± 0.75</td>
<td>69.84 ± 0.60</td>
<td>0.02</td>
<td>0.89</td>
</tr>
</tbody>
</table>

2.3.2 Primer Validation

The positive and negative products for specified primer pairs of each shell development-related gene (cetn, calm, calr and canx) were examined with PCR and gel electrophoresis on a 7% polyacrylamide-TBE gel. Positive controls for each primer pair resulted in a single product,
verifying that the primers were locating the correct sequence for the targeted genes in *C. virginica*, while the negative controls displayed no product (Figure 2.5).

Figure 2.2: Fluctuation of mean pH in each pCO$_2$ treatment (400ppm and 1000ppm) for the trial period, beginning 24 hours post fertilization ($F_{1,28}= 23.49, p \leq 0.0001$).

Figure 2.3: Mean ± SE percent survival of larvae in each treatment (400ppm and 1000ppm pCO$_2$) for 0h-120h post fertilization in development ($F_{1,28}= 0, p = 0.96$). 0h is the time stage eggs were fertilized and culture buckets were inoculated with larvae (100 larvae/ml; ~1.5 million larvae/bucket).
2.3.3. Structural Analyses of the Calcium Binding Proteins and Encoding Genes

Full sequences of the four calcium-binding protein genes were obtained through cloning and fragment assembly. All four gene sequences have been submitted to NCBI GenBank database with accession numbers (MG029428- MG029431) with detailed information of the sequences in Table 2.3. The homologous sequences of the four calcium-binding protein genes were identified by GenBank database searching using BLASTx. Their homologues were listed in Table 2.3. The translated protein sequences of calcium-binding proteins were screened in ScanProsite for signature domain analyses (de Castro, et al. 2006). The proteins Cetn and Calm both include four EF-hand motifs, and have similar overall structure, while Calr and Canx appeared to be more analogous. Relative expression of genes associated with early shell development (cetn, calm, calr and canx) in oyster veligers were variable at 1000ppm pCO$_2$ and throughout early developmental phases, but remained consistent in control conditions (400ppm). Similar results were displayed in the calcium-binding proteins Calm, Calr, and Canx.
structure analyses with the four protein sequences were performed using homology-modelling method through SWISS-MODEL (Arnold, et al. 2006).

![a. Caltractin](image)
![b. Calmodulin](image)

![c. Calreticulin](image)
![d. Calnexin](image)

Figure 2.5: Prediction of Functional domains and three dimensional structures of the calcium-binding proteins. EFh: EF-hand that is classified as a calcium-binding motif.

Table 2.3: Sequencing information of *C. virginica* calcium-binding protein genes and their homologues. ORF: open reading frame; UTR: untranslated region; E-values indicate the probabilities of mismatching.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession #</th>
<th>Full Length (bp)</th>
<th>ORF (bp)</th>
<th>Protein (aa)</th>
<th>5' UTR (bp)</th>
<th>3' UTR (bp)</th>
<th>Homologues (accession #)</th>
<th>Identities (%)</th>
<th>Expectation value (E-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cv-calr</td>
<td>MG029428</td>
<td>1779</td>
<td>1314</td>
<td>438</td>
<td>16</td>
<td>449</td>
<td><em>C. gigas calr</em> (XP_022318558)</td>
<td>99</td>
<td>0.0</td>
</tr>
<tr>
<td>Cv-canx</td>
<td>MG029429</td>
<td>3156</td>
<td>1380</td>
<td>460</td>
<td>95</td>
<td>1681</td>
<td><em>C. gigas canx</em> (EKC32723)</td>
<td>82</td>
<td>0.0</td>
</tr>
<tr>
<td>Cv-cetn</td>
<td>MG029430</td>
<td>1023</td>
<td>417</td>
<td>139</td>
<td>148</td>
<td>458</td>
<td><em>C. gigas cetn</em> (XP_011447406)</td>
<td>92</td>
<td>2x10^{-16}</td>
</tr>
<tr>
<td>Cv-calm</td>
<td>MG029431</td>
<td>1069</td>
<td>450</td>
<td>150</td>
<td>115</td>
<td>504</td>
<td><em>Polistes Canadensis calm</em> (XP_014613282)</td>
<td>99</td>
<td>3x10^{-99}</td>
</tr>
</tbody>
</table>

2.3.4 Quantitative PCR Analysis

Mean relative expression values in *C. virginica* larvae for each target gene (*cetn, calm, calr and canx*) at 24h, 48h, and 72h post fertilization were not significantly difference between treatments (400ppm and 1000ppm pCO₂) for each time period (Table 2.4). Additionally, there
was no indication of statistical significance in relative expression of the mean difference in treatments for time period post fertilization in comparison to 24h for each gene (Table 2.4).

The mean relative expression of the control pCO₂ (400ppm) treatment was consistent from 24h-72h (~1) for each shell development-related gene in oyster larval samples (Figure 2.6). In contrast, expression in the increased pCO₂ treatment varied across days and between genes in oyster larvae. The genes, cetn and calm, exhibited similar expression in oyster larvae in both pCO₂ treatments over the trial period. At 24h post fertilization, the mean relative expression of cetn indicated nonsignificant downregulation in larvae exposed to acidified conditions (1000ppm pCO₂). However, cetn displayed increasing expression from 24h-72h in early development. In elevated pCO₂ conditions, calm expression was initially downregulated in larvae at 24h and at 48h, then downregulated at 72h. calr was upregulated during the first two days (24h-48h) of oyster development in larvae cultured in 1000ppm pCO₂, and downregulated at 72h. Inversely, the mean relative expression of canx in larvae cultivated in increased pCO₂ conditions is downregulated the first two developmental days (24h-48h) and upregulated on the third day.

Figure 2.6: Mean ± SE relative expression of shell development-related genes in C. virginica larvae at 400ppm (control) and 1000ppm (elevated) pCO₂ treatments for 24, 48 and 72 hours post fertilization, where (a) cetn, (b) calm, (c) calr and (d) canx.
Table 2.4: Test statistics for the difference in relative gene expression in larvae between treatments (Treatment Effect) and the difference in relative gene expression at 48 or 72 hours post fertilization in comparison to 24 hours post fertilization in larvae.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment Effect</th>
<th>48h Post Fertilization</th>
<th>72h Post Fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>cetn</td>
<td>$F_{1,21} = 1.19, p = 0.29$</td>
<td>$F_{4,7} = 1.68, p = 0.73$</td>
<td>$F_{4,7} = 1.68, p = 0.16$</td>
</tr>
<tr>
<td>calm</td>
<td>$F_{1,21} = 2.40, p = 0.14$</td>
<td>$F_{4,7} = 1.21, p = 0.29$</td>
<td>$F_{4,7} = 1.21, p = 0.27$</td>
</tr>
<tr>
<td>calr</td>
<td>$F_{1,22} = 0.34, p = 0.56$</td>
<td>$F_{4,8} = 1.38, p = 0.80$</td>
<td>$F_{4,8} = 1.38, p = 0.40$</td>
</tr>
<tr>
<td>canx</td>
<td>$F_{1,23} = 0.16, p = 0.70$</td>
<td>$F_{4,9} = 0.39, p = 0.92$</td>
<td>$F_{4,9} = 0.39, p = 0.10$</td>
</tr>
</tbody>
</table>

Figure 2.7: Whole mount immunoflorescence of calcium-binding proteins (a) calm: red, (b) calr: green and (c) canx: green linked to shell development-related genes in C. virginica larvae 72h post fertilization of early development.

2.3.5 Whole Mount Immunofluorescence Visualization

Relative expression and distribution of calcium-binding proteins (Calm, Calr and Canx) linked to shell development-related genes (calm, calr and canx) in C. virginica larvae during early developmental stages were generated by whole mount immunofluorescence visualization, but were not quantified. As seen in Figure 2.7, Calm and Calr showed greater relative expression, or florescence, in larvae at 48h of development in elevated pCO$_2$ (1000ppm), while Canx exhibited lower expression in these conditions. Relative expression of genes on the calcium-binding protein level detected in the whole mount immunofluorescence analysis.
correlated with expression of shell development-related genes at the RNA level in oyster larvae at 48h post fertilization. In Figure 2.6, Calm and Calr presented greater expression (or were upregulated) in larvae at elevated pCO₂ conditions in comparison to control, but Canx is downregulated in larvae exposed to identical conditions. Visualization of the expression of proteins in relationship to the relative expression of genes linked to those proteins creates a more complete understanding of mechanisms involved with shell development in larvae.

2.4 Discussion

The current study exposed native Louisiana oyster larvae to ambient pCO₂ conditions (400ppm) and predicted acidified pCO₂ conditions (1000ppm) determining that the mean shell length, abundance and survival of larvae were not influenced by reduced pH in an elevated pCO₂ environment during early development and biomineralization. However, larval shell length and abundance were greater, although not statistically significantly greater, in 400 pCO₂ environments as opposed to larvae cultivated in elevated conditions predicted for the future century. Eastern oyster larvae cultured in both pCO₂ treatments experienced depressed shell lengths in comparison to those projected by hatchery culturing standards and expression of biomineralization-related genes were altered in elevated pCO₂ environments. Overall, developing larval *C. virginica* cultivated in elevated pCO₂ environments exhibited genomic impacts, suggesting alterations to the biomineralization mechanism in early development. Influences of stressful acidified environments on early development in oyster veligers could potentially have substantial consequences on metamorphosis, settlement success, and future juvenile or adult populations (Miller, et al. 2009; Parker, et al. 2009; Gazeau, et al. 2010; Lannig, et al. 2010; Talmage and Gobler 2010; Barton, et al. 2012). Data collected provides insight on
potential responses of larval oysters in Louisiana to future acidified conditions on molecular and morphological scales.

To understand impacts of stressors on larval development in marine bivalves, it is optimal to observe the primary stages of biomineralization through metamorphosis when veligers are especially vulnerable (Kurihara, et al. 2007; Miller, et al. 2009; Parker, et al. 2011; Timmins-Schiffman, et al. 2013; Gobler and Talmage 2014). In the current study, developing oyster veligers cultured in elevated pCO₂ conditions exhibited reduced mean shell length compared to larvae cultivated in current pCO₂ environments during the first 72h post fertilization (Table 2.2). Evidence in previous studies investigating the development of larvae exposed to both current and amplified CO₂ environments determined that adverse morphological and physiological impacts occurred in early life-stage larvae as pCO₂ concentrations increased. Earlier studies found that early veligers propagated and survived in both pCO₂ treatments and were not significantly impacted at elevated CO₂ conditions; however mean growth rates and overall survival of larvae were lower in elevated pCO₂ conditions (Miller, et al. 2009; Talmage and Gobler 2009). In another trial, survival between elevated 1000ppm and control 400ppm pCO₂ in larvae of a homologous species, the pacific oyster, was not different between treatments, but larval growth was significantly diminished and development was delayed in hypercapnia conditions (Timmins-Schiffman, et al. 2013). There was no significant difference in larval survival between current pCO₂ and future pCO₂ conditions, but in extreme elevated pCO₂ environments (1500ppm), an earlier study revealed early C. virginica veligers displayed significantly decreased survival and developmental rates and reduced biomineralization (Gobler and Talmage 2014). Decreased shell length in maturing C. virginica larvae cultured in hypercapnia conditions suggests that the biomineralization mechanism was inhibited within the first 72h post fertilization, generating
lower abundance and survival. Where successful calcification of shells is vital for early larval development, improper shell deposition or shell malformation due to alterations in processes involved with biomineralization may also contribute to negative impacts on larval survival.

The targeting calcium-binding protein encoding gene sequences in this study were not previously sequenced based on our GenBank database searching, so it was critical to obtain the full sequences of these genes and fully characterize them using bioinformatics analyses. Once the genes were fully sequenced, they could be utilized for analyzing gene expression responses in elevated pCO₂ conditions of *C. virginica* early stage veligers to better understand how this stressor impacts the biomineralization mechanism. Zhang *et al.* (2012), responsible for sequencing the *C. gigas* genome, determined that the majority of biomineralization-related genes and proteins were located in the EF-hand domain in mantle tissue. Projected structures of the proteins associated with the fully sequenced shell development-related genes included EF-hand motifs, indicative of calcium-binding sites (Lakowski, et al. 2007). Therefore, Ctn, Calm, Calr and Canx are probable calcium-binding proteins, located in the mantle tissue of *C. virginica* and are involved with the biomineralization process within early development. Upregulated and downregulated expression within genes in *C. virginica* larvae exposed to stressful or elevated CO₂ conditions indicated a possible influence on the molecular mechanism involved with shell formation in early development.

The calcifying process in marine invertebrates is energy demanding. Approximately 24 hours post fertilization at initiation of shell formation, *cetn, calm* and *canx* displayed nonsignificant downregulated relative expression in larvae cultivated in low pH (~8.32) and high pCO₂ (1000ppm) compared to control (pH~8.47, 400ppm pCO₂). Downregulated expression in stressful hypercapnia conditions might indicate that the biomineralization mechanism is being
repressed or energy is being redirected towards essential mechanisms involved in maintaining homeostasis, such as balancing intracellular and extracellular acid-base equilibrium, or metabolic regulation. This energy tradeoff reduces calcification rates in larvae (Beniash, et al. 2010; Lannig, et al. 2010; Thomsen, et al. 2010; Gobler and Talmage 2014). The biomineralization process in developing larvae was potentially impacted by increased pCO$_2$ in this study, suggesting shells were not formed properly, which could contribute to higher energy demands for feeding, instead of calcification. While aragonite saturation was not measured in this study, downregulation of calm and similarly structured cetn may suggest decreased calcium ion saturation in the elevated pCO$_2$ treatment. When calcium-binding proteins were not activated, and the linked genes were not expressed, there was likely less calcium extracellularly for those proteins to interact with. Alternatively, decreased relative expression of the target genes associated with shell formation in the preliminary stages of development in marine calcifiers suggests that biomineralization was not entirely initiated. In the current study, as larval development continued in C. virginica, an upregulation in expression of cetn (48h-72h), calm (48h), and canx (72h) was observed in oyster veligers cultivated in high pCO$_2$. Larval Crassostrea hongkongensis exposed to high pCO$_2$ expressed upregulation of genes and proteins associated with calcification, including cetn and calm, suggesting biomineralization processes were sped up (Dineshram, et al. 2015). Upregulated expression in calcium-binding proteins suggested that biomineralization mechanisms in specific calcifying larval organisms, while effected, are sustainable in acidified conditions.

Relative gene expression of calr in C. virginica, as indicated by results from this study, was upregulated 24h-48h post fertilization in CO$_2$ stress. Calr is a calcium-binding protein responsible for calcium ion regulation and homeostasis that is predicted to be involved with canx
in a “calreticulin/celnexin cycle” (Gelebart, et al. 2005). The predicted structures of the calcium-binding proteins of Calr and Canx are similar and it appears that, at each time point, larval expression of calr and canx are inversely related. While calr is upregulated in the first 48h of development, canx has repressed relative expression, but at 72h canx expression becomes upregulated. Kawabe and Yokoyama (2010) found similar results in stressed C. gigas, where canx was induced at 72h in mantle mRNA. Upregulation of expression may suggest that calcium-binding proteins involved with biomineralization were compensating in response to an imbalance in calcium ion regulation or stress. As relative expression increases from 24h to 72h post fertilization, this may suggest the progression or higher activation of the early biomineralization mechanisms in veligers. Overall, differences in relative expression and genomic responses are attributed to by variation in individuals within the larvae pooled for sampling in each treatment. Biomineralization-related genes and linked calcium-binding protein were differentially expressed between various marine calcifying species and within distinctive populations of a specific species when exposed to stress induced by elevated pCO2. In both cases, genetic variation within a species contributes to its genomic response to environmental stressors (Goncalves, et al. 2017). Therefore, investigating species specific and population specific reactions on the molecular level is important to determine an organism’s susceptibility and potential adaptability to future climate change.

In this study, the culture system was designed to imitate ambient Louisiana estuarine pCO2 conditions and predicted future elevated pCO2. There was a significant difference in average pH, but it was difficult to maintain consistent pH conditions within the culture vessels. pH manipulation in this study produced a pH value of ~8.47 and lower values of ~8.32 for 400ppm and 1000ppm pCO2, respectively. Previous studies that employing similar
methodologies for the manipulation of pH with CO₂-enriched air, ambient pCO₂ conditions had an associated pH of ~8.1-8.2 and future conditions, due to predicted increased atmospheric CO₂, produced ~7.9-7.95 pH (Parker, et al. 2009; Ivanina, et al. 2013; Ivanina, et al. 2014). There were limitations to the culture system where CO₂ was lost to the atmosphere through air-gas exchange in non-air tight vessels, solubility of CO₂ in seawater decreased with high temperatures (~27-28°C), and the manipulation of pH by CO₂-enriched air was inhibited by buffered hatchery seawater (Barton, et al. 2012). Reduced disparity between treatments, as well as greater mean pH values in both treatments, may have contributed to the nonsignificant morphological and molecular response of oyster veligers.

Genes identified and sequenced in the current study can be utilized as molecular markers of stress response in early stage bivalves for prospective selective breeding programs in both culture and wild populations. Potential future larval studies should implement chronic elevated pCO₂ exposure from early development through settlement and should focus on alternative genes that are molecular markers for stress in bivalves or related to shell formation processes. Acute elevated pCO₂ concentrations and associated low pH could affect biomineralization in early stage C. virginica veligers on a molecular and physiological scale, and while Louisiana populations appear more resilient, Atlantic populations, along with other marine bivalves, are vulnerable to future ocean acidification.
CHAPTER 3: SHELL DEVELOPMENT GENE EXPRESSION IN CELLS PRIMARILY CULTURED FROM EASTERN OYSTER MANTLE TISSUE EXPOSED TO INCREASED CONCENTRATIONS OF CARBON DIOXIDE: IN VITRO

3.1 Introduction

Acidified conditions are likely to affect organisms thriving in estuarine habitats, especially marine calcifying organisms depending on the availability, or saturation, of calcium ions in their external environment for biomineralization in early developmental stages. Biomineralization is the process by which calcifying organisms utilize forms of calcium ions, such as aragonite and calcite, in seawater to deposit their shells, initiated in extremely early stages of development (Zhang, et al. 2012). In this process, marine calcifiers secrete an organic matrix comprised of various polysaccharides, proteins and phospholipids from their mantle cells and extrapallial fluid that interacts with calcium ions in their environment, forming a rigid outer shell (Addadi, et al. 2006; Gong, Li, et al. 2008; Furuhashi, et al. 2009; Jeffroy, et al. 2013). Previous in vivo studies have discovered an adverse effect on growth rates, shell length, calcification rates, mechanical shell structure, metabolism, homeostasis regulation, metamorphosis and survival in various larval calcifying species in response to CO$_2$-induced ocean acidification (Miller, et al. 2009; Parker, et al. 2009; Gazeau, et al. 2010; Lannig, et al. 2010; Talmage and Gobler 2010; Barton, et al. 2012). While there are several studies focusing on shell formation in early veliger oysters, the mechanisms of biomineralization is still widely unknown in marine bivalves (Wang, et al. 2013).

An alternative to examining the impacts of ocean acidification on the preliminary biomineralization mechanism in vivo, where manipulation of pCO$_2$ and associated pH may be difficult, is implementing an in vitro model utilizing primarily cultured mantle cells to study genomic and related physiological responses of calcifiers in hypercapnia conditions. Cell models
have been shown to be an innovative technique that allows for studying complex physiological mechanisms, such as biomineralization, that are difficult to observe in organisms. Compared to in vivo studies, cell models are easily manipulated and permit more controlled conditions. (Yoshino, et al. 2013). While this practice is understudied, there are findings supporting the application of primary cell cultures as effective models for investigating ocean acidification in marine invertebrates (Domart-Coulon, et al. 1994; Gong, Li, et al. 2008; Gong, Ma, et al. 2008; Xiang, et al. 2014). Cell lines for marine invertebrates are not currently available, but primary cell culture of organ-specific cells have been successful and prove to be viable for short and long-term studies that rely on precise conditions (Domart-Coulon, et al. 1994; Chen and Wen 1999; Poncet, et al. 2000; Rinkevich 2005; Suja and Dharmaraj 2005; Suja, et al. 2007; Gong, Li, et al. 2008).

Multiple methods for cultivating cells from specific tissues within marine invertebrates have been developed. One common method allows the cells to migrate from explants derived from the tissue of interest, such as mantle tissue (Domart-Coulon, et al. 1994; Chen and Wen 1999; Suja and Dharmaraj 2005). Alternatively, utilizing enzymatic disassociation of targeted tissue to collect cells for primary culturing and allowing the cells to proliferate has previously been successful in marine invertebrates. In both cases, prior studies have revealed that cells are easily damaged and contaminated, thus creating limitations to the extent of time cells remain viable and the rate of proliferation, where (Chen and Wen 1999; Yoshino, et al. 2013). Primary cell culture in marine invertebrates requires the use of proper culturing medium, techniques implementing disassociating enzymes to ensure cell viability and propagation, and protocol to prevent microbial contamination (Chen and Wen 1999; van der Merwe, et al. 2010; Yoshino, et al. 2013).
The current study implemented primarily cultured cells derived from the mantle epithelial tissue of *C. virginica* to study genomic responses in early stage shell synthesis. Previous studies have determined that the mantle in marine bivalves is the principal organ involved with biomineralization and insight into the complex processes of shell formation can be explored through primarily cultured cells derived from mantle tissue (Rinkevich 2005; Gong, Li, et al. 2008; Jeffroy, et al. 2013; Wang, et al. 2013). Primary cell culture from tissues or organs of marine invertebrates offers a model for investigating the mechanisms involved with the functionality of the target tissue within an organism and can be utilized to monitor the impacts of environmental stressors on the cells within that specific tissue (Yoshino, et al. 2013). Several prior studies have had success in primary cell culture specifically derived from mantle tissue in various aquatic species such as the pearl oyster *Pinctada fucata* (Gong, Li, et al. 2008; Gong, Ma, et al. 2008), zebra mussel *Dreissena polymorpha* (Sud, et al. 2001; Quinn, et al. 2009), the abalone *Haliotis tuberculata* (Poncet, et al. 2000; Poncet, et al. 2002), *Haliotis varia* (Suja and Dharmoraj 2005; Suja, et al. 2007), *Haliotis midae* (Yoshino, et al. 2013), and the pacific oyster *Crassostrea gigas* (Chen and Wen 1999). Gong, Li, et al. (2008) within mantle cells of *P. fucata* concluded that tissue cells maintain their biomineralization functions *in vitro*. This work confirms the benefits of utilizing cell models for biomineralization studies on the molecular level. Active mantle cells in primary culture have also been shown to secrete organic substances related to the shell deposition mechanisms *in vitro* (Gong, Ma, et al. 2008), further indicating that cells remain functional *in vitro*. Proteins associated with the regulation of shell formation mechanisms are centrally located in mantle cells and the response of these proteins *in vitro* provides genomic information about shell formation (Xiang, et al. 2014).
Variation in expression and activation of calcium-binding proteins linked to biomineralization-related genes determines the regulation of shell development mechanisms in marine calcifiers and can be identified in mantle cells. The calcium-binding proteins identified in mantle cells are responsible for calcification, ion and acid-base regulation, and maintaining homeostasis both intracellularly and extracellularly. Genes and proteins linked to the biomineralization mechanism are differentially expressed between various marine calcifying species and within distinctive populations of a specific species when exposed to stress induced by ocean acidification (Woo, et al. 2010; Ertl, et al. 2016; Dineshram, et al. 2015; Zhao, et al. 2012; Li, et al. 2016). Genetic variation within a species contributes to its specified genomic and physiological susceptibility to environmental stressors and potential adaptability to future climate change (Goncalves, et al. 2017). The calcification process is initiated intracellularly in mantle cells, where calcium-binding proteins aid in the storage of calcium ions that are later transported to the site of biomineralization in the cells (Xiang, et al. 2014). Numerous studies have observed morphological and physiological effects in developing oysters in response to ocean acidification (Galtsoff 1964), while few studies have investigated genomic responses in larval mechanisms involved with biomineralization.

In the current study, the activities of caltractin (*cetn*), calmodulin (*calm*), calreticulin (*calr*), and calnexin (*canx*) were examined *in vitro* via primary cell culture from *C. virginica* mantle tissue and incubated in oyster medium at various increasing CO₂ concentrations (1%, 1.5%, and 2.5%) to induce a reaction and relative expression was analyzed. Mantle cells were utilized as a model to determine genomic responses in the biomineralization process to further predict morphological and physiological impacts on calcifying organisms in acidified conditions.
3.2 Materials and Methods

3.2.1 Oysters

Adult eastern oysters \((n=40)\) originated from Caillou Lake on the west side of Terrebonne Bay and were obtained from the Michael C. Voisen Oyster hatchery, where they were maintained in off bottom culture cages. Oysters were then transferred to the School of Animal Sciences at Louisiana State University to become acclimated before use in primary cell culture. In the laboratory, oysters were sustained in flow through systems at 25ppt salinity and 20°C.

3.2.2 pH Proxy for Acidification

Oyster medium used in primary oyster cell culture possesses the ability to buffer itself against pH swings, created by fluctuating CO\(_2\) concentrations, within a confined range (~0-2.5% CO\(_2\)). Past its threshold, the medium does not retain a buffering capacity, generating an unstable environment, including low pH, for successful cell growth. The oyster basal cell culture media consists of 1x Leibovitz- L15, 345.6mM NaCl, 7.2mM KCl, 5.4mM CaCl\(_2\), 4.0mM MgSO\(_4\)·7H\(_2\)O, 19.2mM MgCl\(_2\)·6H\(_2\)O, 4.0mM L-glutamine and is adjusted to a pH of 7.6, filtered to 0.2\(\mu\)m, 10% chicken (Gallus gallus domesticus) serum is added and filtered again to 0.2\(\mu\)m (Deuff, et al. 1994; Domart-Coulon, et al. 1994; Chen and Wang 1999; Quinn, et al. 2009; van der Merwe, et al. 2010). Trials were conducted to determine the maximum CO\(_2\) concentration that primarily cultured oyster mantle cells can remain viable, and proliferate, in the oyster medium. The correlating pH values acted as a proxy for acidification of the medium, much like the acidification of surface seawaters. Employing an incubator set at 27°C, oyster medium was exposed to preliminary treatments of 0% (control), 2.5%, 5%, 7.5%, and 10% CO\(_2\) and associated pH values were measured (Figure 3.1).
To measure pH values, 15ml of oyster medium containing penicillin-streptomycin was allocated into four petri dishes. All four dishes were then incubated at 27°C for 24 hours at the target CO₂ concentration. After incubation, medium from each petri dish was transferred into separate 50ml tubes and, using a calibrated pH probe (Beckman Coulter Life Sciences, Indianapolis, IN), the pH values of the medium in each tube was measured. The previous steps were repeated for each of the target CO₂ treatment concentrations. Using pH as a proxy determined the CO₂ treatment threshold (~2.5%), where the medium became too acidified for cell viability and proliferation. To confirm this threshold, a cell viability assay was conducted to compare cell survival in the control and the maximum CO₂ concentration of 2.5% to ensure apoptosis was not due to unstable culture conditions within the media.

Figure 3.1: The associated pH values of oyster cell culture medium after exposure to preliminary CO₂ treatments, where the 2.5% threshold is marked by the dotted line.

3.2.3 Primary cell culture

All tools and surfaces were sterilized prior to use to reduce contamination. Adult oysters were cleaned with 70% ethanol and soaked in CMFSS buffer (436.0mM NaCl, 10.7mM KCl, 21.2mM Na₂HPO₄, 16.7mM glucose, 12.0mM HEPES filtered to 0.2 µm) with 1% penicillin
streptomycin overnight in 4°C (Perkins and Menzel 1964; Domart-Coulon, et al. 1994; Chen and Wang 1999). Mantle tissue was then excised, washed with CMFSS buffer 2-3 times for 30-60 minutes, and minced to prepare for digestion. Minced tissue was enzymatically digested in 50ml tubes containing 10ml CMFSS buffer with 0.2% collagenase I (Gibco, Thermo Fisher Scientific, Grand Island, NY) on an orbital shaking platform at room temperature overnight. In preparation for plating cells the following day, 24-well plates were coated with 300µl in each well of 0.01% Poly-L-lysine in CMFSS buffer to aid in cell adhesion and incubated in 4°C overnight (or room temperature for 2 hours) (Poncet, et al. 2000; van der Merwe, et al. 2010). Coated plates were removed, solution was drained with a vacuum pump, and the wells were washed with 500µl CMFSS buffer and incubated at 27°C before culture use.

After digestion, 20ml of fresh oyster medium was allocated into each 50ml tube and passed through a 100µm cell strainer (VWR International, Radnor, PA) into a new 50ml tube and centrifuged at 400g for 10 minutes. The supernatant was removed and cells were re-suspended with 10ml of fresh oyster medium and centrifuged. Again, the supernatant was removed, cells from the same individual oyster were combined into one 50ml tube and mixed with 24ml of oyster medium. The 24-well plates were obtained from the incubator and 500µl of cell solution (~13.6 million cells/ml) from each individual was transferred into each well (2 plates per individual oyster). The plates were incubated in control conditions for 24 hours at 27°C without additional input of CO₂. After 24 hours of incubation, the old medium in each well was replaced with 500µl of fresh oyster medium and incubated for an additional 24 hours in control conditions. Cells were then observed under a microscope to ensure attachment and to confirm the cell density was ~80% (Figure 3.2). Then, medium was replaced and the cells were treated in their respective CO₂ condition. Mantle cells from one individual in a 24-well plate acted as
control and were incubated for 24 hours at 27°C in 0% CO₂. The second plate, containing the same individual’s cells, was incubated for the same time period at 27°C in the assigned CO₂ treatment (1%, 1.5%, or 2.5%). Plates were then removed from incubation and washed with 1x PBS buffer before cells were harvested for RNA isolation using TRIzol® Reagent as outlined by protocol in Chapter 2.

Figure 3.2: Primary culture of *C. virginica* mantle cells successfully attached to the well at ~80% density, indicating the cells were ready for harvest. Scale bar: 50 μm.

3.2.4 Cell Viability and Proliferation Tests

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for cell viability assays (ATCC, Manassas, VA) were conducted on mantle cells cultured from three individual oysters to compare the viability of the cells incubated in control conditions and increased (2.5%) CO₂ conditions. 2.5% CO₂ was selected as the increased CO₂ treatment because it was the maximum exposure in the trial; therefore, if viability persists at this treatment then it should be viable at lower CO₂ concentrations. Before running the assay, oyster mantle cells were cultured following the protocol detailed in section 3.2.3 with minor modifications. However, for this assay, cells were allocated into two 96-well microtiter plates, a control plate that was incubated
at 0% CO₂ and a treatment plate. The first and last column consisted of blank wells in both plates, while 40 wells contained mantle cells from one individual and another 40 wells contained mantle cells from the second individual. Each well, excluding blanks, contained 100µl of cells in oyster medium and the entire plates were incubated at 27°C, 0% CO₂ for 24 hours to allow for mantle cells to be activated and metabolizing (Gong, Ma, et al. 2008).

Cell density and proliferation were verified, the supernatant from each well was removed, and replaced with 100µl of fresh oyster medium and incubated for an additional 24 hours in either control or elevated CO₂ (2.5%) conditions. Once removed, 20µl of MTT reagent (thiazolyl blue tetrazolium bromide) in PBS was allocated into each well, including blanks, and incubated at 27°C, 0% CO₂ for 3 hours until purple precipitate was visible (Figure 3.3). Reagent was then removed and 100µl of detergent reagent (4mM HCl, 0.1% Nondet P-40 in isopropanol) was added to each well, covered with foil and placed on a shake plate for 10 minutes. A Synergy 2 multi-mode microplate reader (BioTek, Winooski, VT) was utilized to analyze the absorbance levels at 570nm and Gen5 1.5 data analysis software (BioTek, Winooski, VT) was utilized to quantify the results. Analysis from the MTT assay served as quality control for mantle cell health in primary culture conditions for both treatments (Domart-Coulon, et al. 1994; Poncet, et al. 2000).

Immunofluorescence was used to visualize and quantify the proliferation of mantle cells in both the control and treatment with Ki-67, a universal cell proliferation marker. Oyster mantle tissue was excised, digested, and cells were cultured using the protocol outlined in section 3.2.3 with modifications. Cells were treated as either control or elevated CO₂ (2.5%) in an incubator for 24 hours at 27°C to compare the proliferation rates between the treatments. Similar to the MTT assay that assessed viability, comparisons in proliferation provided quality control for the
fitness of cells in both conditions. Treated cells, instead of being harvested, were then fixed in 4% paraformaldehyde in CMFSS buffer for 30 minutes at room temperature. Cells were washed three times with 500µl 1x phosphate-buffered saline (PBS) for each well in a 12-well plate and allowed to shake for 5 minutes. Samples were then blocked for 2 hours at room temperature with 300µl of 10% lamb serum in PBS in each well. After the block, cells were washed 3 times with 1x PBS and submerged in 300µl of the primary antibody (mouse-anti-human antibody) diluted 1:1000 in PBS at 4˚C overnight. Following, the secondary antibody, goat-anti-mouse antibody conjugated with either Alexa flour 594 (red) or Alexa flour 488 (green) dye, diluted 1:500 in PBS was allocated into the wells, the plates were covered in foil and placed on a shake plate for 2 hours. Samples were washed 3 times, keeping the plates covered with foil. Finally, 300µl of DAPI (4',6-diamidino-2-phenylindole) diluted 1:10,000 in PBS was allocated into each sample well and was allowed to shake for 10 minutes. After 3 washes in PBS, mantle cell immunofluorescence samples were visualized with microscope and analyzed with ImageJ software to quantify the expression of Ki-67 by overlaying the total number of DAPI stained cells with fluorescing positive cells.

Figure 3.3: MTT viability assay of oyster mantle cells from one individual in control conditions (left) and elevated 2.5% CO2 conditions (right).
3.2.5 Quantitative PCR Analysis of Larval Samples

Quantitative PCR (qPCR) was utilized to analyze the relative expression levels of the target genes (*cetn, calm, calr* and *canx*). As outlined in Chapter 2, the total RNA was isolated from cells primarily cultured from *C. virginica* mantle tissue using TRizol® Reagent followed by DNA removal with TURBO DNA-free™ Kit. The total RNA was then used for cDNA synthesis with SuperScript IV Reverse Transcriptase according to manufacturer’s instructions.

Immunofluorescence was conducted to analyze the expression of the calcium-binding proteins (Calm, Calr, and Canx) linked to the shell development-related genes in the mantle cells. Following previously described protocol, oyster mantle cells were cultured, treated as either control or elevated CO₂ (2.5%) in an incubator for 24 hours at 27°C, fixed, washed and then blocked for 2 hours at room temperature with 300µl of 10% lamb serum in PBS in each well. After the block, cells were submerged in 300µl of the primary antibody (mouse-anti-human antibody) diluted 1:200 in PBS at 4°C overnight. Following, the secondary antibody, goat-anti-mouse antibody conjugated with Alexa flour 488 (green) dye, was allocated into the wells, the plates were allowed to shake for 2 hours. Finally, 300µl of DAPI was allocated into each sample well and left to shake for 10 minutes. After being washed 3 times in PBS, mantle cell immunofluorescence samples were visualized with microscope and analyzed with ImageJ software to quantify the protein expression of Calr and Canx.

3.2.6 Statistical analysis

Mean absorbance at 570nm generated by the MTT assay to assess and quantify cell viability between control and elevated CO₂ (2.5%) conditions, were calculated by normalizing the treatment values to the corresponding control values. A Wilcoxon two-sample test was
performed to determine statistical significance between viability of cells cultured in the control and treatment CO₂ (PROC NPAR1WAY, SAS 9.4, Raleigh, NC).

To analyze immunofluorescence, ImageJ software was implemented to quantify the relative expression of the calcium-binding proteins Calr and Canx, as well as percent proliferation with the molecular marker Ki-67 in oyster mantle cells cultured in control and treatment (2.5% CO₂). In ImageJ, the DAPI cell images were overlaid with the signal cell images. Images were adjusted, set to the standard threshold (125-225), made binary, and the watershed action was applied. Signal particles within the cells were quantified at sizes 100-1000 and circularity 0.2-1. For calcium-binding proteins, the average intensity value of all positive cells were quantified for both treatments. Ki-67 was quantified by analyzing the average percent positive cells out of the total number of cells. Percent proliferation of oyster mantle cells in primary culture exposed to both the control CO₂ treatment and the elevated CO₂ treatment was quantified. The relative expression of two calcium-binding proteins in mantle cells of three individual oysters exposed to both the control and elevated (2.5%) CO₂ treatment was visualized and quantified with ImageJ. The calcium-binding protein expression in mantle cells exposed to elevated CO₂ was normalized to correlating protein expression of mantle cells cultured in control conditions, producing relative expression values for each protein at control and treatment (2.5% CO₂). To test for significance between relative expression in treatment and control, a Wilcoxon two-sample test was performed (PROC NPAR1WAY, SAS 9.4, Raleigh, NC).

Quantitative PCR data yielded Cₜ values that quantified gene expression for each of the four target genes (cetn, calm, calr and canx) in C. virginica mantle cells cultured in control and elevated CO₂ conditions (1%, 1.5%, and 2.5% CO₂). Cₜ values for each sample were normalized and then analyzed with the ΔΔCₜ method, removing values residing outside of the 95%
confidence interval of the mean. For each target gene, the statistical analysis examined the relative gene expression values of samples exposed to elevated CO₂ treatments (1%, 1.5% and 2.5% CO₂) in comparison to the correlating expression values of control samples for the respective treatment using the restricted maximum likelihood (REML) estimation in a linear model (PROC MIXED, SAS 9.4, Cary, NC), where the residuals were tested and confirmed as normally distributed by a Shapiro-Wilk test with constant variance. All statistical significance was determined by $p \leq 0.05$.

3.3 Results

3.3.1 Cell Viability and Proliferation

There was no statistically significant difference between the viability of *C. virginica* mantle cells cultured in control or acidified conditions, with invariable values between control and treatment (Wilcoxon test statistic = 14,162, $p = 0.17$) (Figure 3.4). Percent cell proliferation was very similar between treatments, with small marginal error, where the control treatment demonstrated ~85% proliferation and ~88% in the CO₂ treatment when implementing a complimentary immunofluorescence assay with Ki-67 (Figure 3.4).

3.3.2 Quantitative PCR Analysis

Relative expression of *cetn* in mantle cells exposed to elevated CO₂ concentrations was not statistically significantly different from the control (Table 3.1). The gene, *cetn*, was upregulated at 1% and 2.5%, but downregulated at 1.5% CO₂ treatment. In the shell development-related gene *calm* there was a significant difference of the mean relative expression in cells incubated in 1% CO₂ ($F_{5,136} = 3.20, p=0.022$) and the respective control, where gene expression was upregulated by a factor of ~3. *calm* was also upregulated in mantle cells cultured in more acidified conditions (2.5%), but was downregulated at 1.5% CO₂. *Cetn* and *calm*
presented similarly in oyster mantle cells exposed to increasingly acidified conditions. In mantle cells, statistical significance was also detected in \textit{calr} expression between 2.5\% CO\textsubscript{2} and the respective control (\(F_{5,127}= 6.68, p<0.0001\)), where expression was upregulated by a factor of 9.4. As CO\textsubscript{2} increased and conditions became more acidified, \textit{calr} gene expression exhibited an upregulating trend, while expression in control conditions were consistent. \textit{canx} relative gene expression in \textit{C. virginica} mantle cells across CO\textsubscript{2} treatments remained invariably upregulated (Figure 3.5), with no statistically significant difference in comparison to control (Table 3.1).

![Figure 3.4](image-url)

**Figure 3.4:** Mean ± SE of (a) absorbance levels determining cell viability of \textit{C. virginica} mantle cells cultured in control and elevated CO\textsubscript{2} (2.5\%) treatment (Wilcoxon test statistic= 14,162, \(p= 0.17\)) and (b) percent of cells proliferating analyzed with Ki-67 immunofluorescence of mantle cells cultured in control and elevated CO\textsubscript{2} (2.5\%) treatment.

<table>
<thead>
<tr>
<th>Gene</th>
<th>F value</th>
<th>1% CO\textsubscript{2}</th>
<th>1.5% CO\textsubscript{2}</th>
<th>2.5% CO\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{cetn}</td>
<td>(F_{5,114}= 1.37)</td>
<td>(p= 0.68)</td>
<td>(p= 0.47)</td>
<td>(p= 0.19)</td>
</tr>
<tr>
<td>\textit{calm}</td>
<td>(F_{5,136}= 3.20)</td>
<td>(p= 0.02)</td>
<td>(p= 0.99)</td>
<td>(p= 0.18)</td>
</tr>
<tr>
<td>\textit{calr}</td>
<td>(F_{5,127}= 6.68)</td>
<td>(p= 0.45)</td>
<td>(p= 0.18)</td>
<td>(p\le 0.0001)</td>
</tr>
<tr>
<td>\textit{canx}</td>
<td>(F_{5,133}= 0.47)</td>
<td>(p= 0.53)</td>
<td>(p= 0.20)</td>
<td>(p= 0.80)</td>
</tr>
</tbody>
</table>
3.3.3 Immunofluorescence of Calcium-binding Proteins

Statistical significance was detected for the difference in Calr (Wilcoxon test statistic= 12,567, \(p = 0.02\)) relative expression in mantle cells between 2.5% CO\(_2\) treatment and control, but no statistical significance was found in Canx relative expression (Wilcoxon test statistic= 1.966, \(p = 0.98\)). Protein expression of Calr was significantly greater in the acidified 2.5% CO\(_2\) treatment in comparison to the control in oyster mantle cells, which was also represented in the quantification of relative expression between treatment and control (Figure 3.6). The quantification shows the average expression levels of calcium-binding proteins are different between control and CO\(_2\) treatment. The calcium-binding protein Canx exhibited similar relative expression values for both control and elevated CO\(_2\) treatment to those displayed by Calr. At elevated CO\(_2\) concentrations, Canx had higher fluorescence and quantified relative expression in cells compared to control (Figure 3.7). Overall, calcium-binding proteins Calr and Canx in eastern oyster mantle cells responded similarly to elevated CO\(_2\), or acidified, conditions.

![Figure 3.5: Mean ± SE relative expression of shell development-related genes in *C. virginica* mantle cells cultured at control (0%) and elevated CO\(_2\) treatments (1%, 1.5% and 2.5%), where (a) cetn, (b) calm, (c) calr and (d) canx. *Statistical significance detected between control and 1% CO\(_2\) in calm (F\(_{5,136}\) = 3.20, \(p = 0.022\)) and control and 2.5% CO\(_2\) in calr (F\(_{5,127}\) = 6.68, \(p \leq 0.0001\)).](image-url)
Figure 3.6: Immunofluorescence (green) visualization of relative expression of the calcium-binding protein Calr in *C. virginica* mantle cells cultured in control and elevated CO$_2$ (2.5%) treatment (left) and the quantified mean ± SE relative expression of Calr in *C. virginica* mantle cells cultured in control and elevated CO$_2$ (2.5%) treatment (Wilcoxon test statistic= 12,567, $p= 0.02$) (right). Scale bar: 50 µm.

Figure 3.7: Immunofluorescence (green) visualization of relative expression of the calcium-binding protein Canx in *C. virginica* mantle cells cultured in control and elevated CO$_2$ (2.5%) treatment (left) and the quantified mean ± SE relative expression of Canx in *C. virginica* mantle cells cultured in control and elevated CO$_2$ (2.5%) treatment (Wilcoxon test statistic= 1.966, $p= 0.98$) (right). Scale bar: 50 µm.
3.3 Discussion

Primary cell culture in marine invertebrates is an evolving field where techniques and protocols are widely undeveloped, especially in bivalve mantle cells. The larval system designed for manipulating pCO$_2$ concentrations and pH had difficulty to controlling and maintaining conditions, even in acute exposures (Chapter 2). Instead, to investigate ocean acidification’s impacts on the genomic responses of the early biomineralization process in *C. virginica* an *in vitro* model generated from cells primarily cultured from mantle tissue was developed. In this study, cells were primarily cultured from the mantle tissue of *C. virginica*, exposed to elevated CO$_2$ concentrations (1%, 1.5% and 2.5%). Overall expression of biomineralization-related genes in both the control and elevated CO$_2$ treatment verified that the targeted calcium-binding proteins associated with the biomineralization mechanism were present in the mantle cells of *C. virginica*. pH of oyster culturing medium incubated in each CO$_2$ treatment was implemented as a proxy for acidification and results revealed that all four target genes were altered by increased CO$_2$ conditions in comparison to controls. Although pH was low (~7.62) in oyster medium incubated in elevated CO$_2$ (2.5%) in comparison to future acidified ocean conditions, after 24h there was no significant effect on mantle cell viability or proliferation between cells incubated in control and CO$_2$ conditions. These results ensure that the oyster medium was sufficient for mantle cell metabolic activity and that cell health was not compromised in the most extreme CO$_2$ concentration (Domart-Coulon, et al. 1994; Poncet, et al. 2000). The implementation of the mantle cell model allowed for easier manipulation, control, and sterility of conditions. In addition, genomic results in response to elevated CO$_2$ produced from the cell model contributed to a clearer understanding of mechanisms involved with biomineralization. Studying the shell
formation process on the molecular level provides insight into the physiological responses of calcifying organisms to impending ocean acidification and additional environmental stressors.

*C. virginica* primary culture mantle cells remained viable and proliferated, according to MTT reduction assays and immunoflouresce of Ki-67, in medium cultured at each elevated CO$_2$ concentration. MTT assays, in prior primary cell culture studies of marine invertebrates, have been utilized as a prominent tool for detecting cell viability and metabolic activity, even when cells are no longer proliferating (Domart-Coulon, et al. 1994; Poncet, et al. 2000). This study determined that there was no significant difference in viability between treatments, suggesting that the medium environment was sufficient for culturing and elevated CO$_2$ conditions did not impact overall cell health. Previous studies have reported that primarily cultured mantle cells were an effective model for analyzing the impact of ocean acidification on the biomineralization mechanisms in marine invertebrates: *C. gigas* and *P. fucata* (Domart-Coulon, et al. 1994; Gong, Li, et al. 2008). *In vivo* studies of ocean acidification’s impacts on calcifiers are limited, where trials require chronic exposures to generate a response, sample sizes are smaller, individual genetic variation is lower, and complicated physiological mechanisms are often difficult to observe. Primary cell cultures, such as this study, create a method for analyzing immediate genomic responses to acute environmental stressors that are indicative of physiological impacts, making cell models beneficial for studying the complex mechanisms involved with biomineralization. Ideally, these models can be utilized to predict the impacts stressors have on marine calcifying organisms in the wild.

While biomineralization is a complex multi-organ mechanism, previous studies suggest that it is regulated by expression of genes and calcium-binding proteins in the mantle cells (Gong, Li, et al. 2008). *In vitro*, calcium-binding proteins are involved with the intracellular
uptake and storage of calcium ions by mantle cells, essential for biomineralization. Calcium ions are then transported to the site of mineralization for use in shell deposition. Calcium-binding proteins are also responsible for regulating ions and maintaining acid-base homeostasis, crucial to the survival of marine calcifiers in fluctuating ecosystems (Wang, et al. 2013; Xiang, et al. 2014; Goncalves, et al. 2017). In this study, the relative expression of four newly sequenced shell formation-related genes (cetn, calm, calr and canx) and linked calcium-binding proteins were evaluated after 24 hours of incubation in various elevated CO₂ concentrations. After harvest, cultivated mantle cells, were ~48-72h old. Gene expression of cetn in C. virginica mantle cells was upregulated at 1% and 2.5%, while calm was upregulated across all elevated treatments and significantly different from the control at 1% CO₂. Similarly, Dineshram et al. (2015) found gene expression upregulated in both cetn, calm and various other proteins associated with calcification in Crassostrea hongkongensis larvae exposed to stressful CO₂ conditions. In the current study, the upregulation of calr and canx expression at both the RNA and protein level in mantle cells appeared to be a consistent response to increased CO₂.

Upregulation in expression of biomineralization-related genes and calcium-binding proteins indicated that the biomineralization mechanisms were still active in mantle cells exposed to elevated CO₂ conditions. However, in comparison to normal expression of the target genes in controlled conditions, upregulation in cells cultivated in increased CO₂ indicated that there were still impacts to the shell deposition mechanisms. Cells within the mantle tissue of adult C. virginica may have induced expression to combat or compensate for environmental stress. The expression profiles of the calcium-binding protein genes identified in larval oysters showed upregulations of calr, calm, cetn, and canx at 24-48h, 48h, 72h, and 72h post fertilization, respectively. Within similar time frames of development, both C. virginica early stage veligers
and mantle cells presented similar upregulated expression of target genes. It appears that the genomic results from primary cell culture are complimentary to the larval trial (Chapter 2), both of which were utilized to study the impacts of acidification on the molecular regulation of the biomineralization process. In addition, the cell culture study provides an adequate model for observing the genomic, and possible physiological responses of *C. virginica*.

The primary cell culture model reflects real world processes. Oyster culturing medium has a buffering capacity much greater than that of seawater, so to initiate genomic responses in cultured mantle cells, the medium required incubated in extremely elevated atmospheric CO₂ conditions. Past a certain CO₂ threshold, the medium was no longer a suitable environment for viable cell culture. In control conditions with no CO₂ input, the pH of the medium was ~7.78 and at the highest treatment concentration (2.5%) pH was ~7.62. Past this 2.5% CO₂ threshold, culture medium changed color, representative of compromised medium, and pH was too low to culture viable, proliferating mantle cells (Figure 3.1). Ambient ocean pH at pCO₂ of 400ppm and 1000ppm is ~8.1-8.2 and ~7.9-7.95, respectively, which is higher than the pH in the control medium. However, Louisiana oysters are frequently subject to fluctuating and stressful environmental factors, such as pH, dissolved oxygen, temperature, salinity and nutrient input, in their estuarine habitats (Kurihara, et al. 2007; Miller, et al. 2009). The cell model generated pH values lower than ambient pH in most estuaries, which is caused by the buffering capacities of cell culture medium and seawater. The condition created for mantle cell culture system is to imitate the possible effects of ocean acidification to make “real world” predictions.

The four previously unstudied shell development-related genes revealed upregulation in *C. virginica* mantle cells cultured in hypercapnia environments exhibiting that *in vitro* biomineralization mechanisms were functioning and possibly overcompensating due to stress.
Genomic responses generated by this novel model, utilizing primarily cultured mantle cells cultured in acidified conditions, display potential adaptability and rapid evolution of genes involved with the shell development mechanism in early Louisiana *C. virginica*. The target genes, newly sequenced for the species, are suitable molecular markers for determining the effects of stress in eastern oysters, however, prospective studies investigating various other biomineralization-related genes in the species is essential to fully understanding the consequences of future stressors in estuarine ecosystems. Future studies should conduct a functional analysis of the targeted calcium-binding proteins and molecular pathway analysis to generate a fuller understanding the complex biomineralization mechanism in both developing and adult eastern oysters. Implications of examining the impact of species specific molecular mechanisms associated with biomineralization in marine calcifiers provides a foundation for determining the potential morphological and physiological effects ocean acidification will have on these organisms. Implemented by this trial, primarily cultured mantle cells provided an applicable model to analyze the impact of ocean acidification on biomineralization mechanisms at the molecular level. Genomic responses of the shell deposition process in *C. virginica* generated by the model can be utilized to predict the morphological and physiological impacts acidified conditions will have on future oyster populations. Additionally, this study presents a set of molecular markers that can be utilized for future selective breeding and restoration of oysters in both cultured and wild populations susceptible to climate change.
REFERENCES


Talmage SC, Gobler CJ. 2009. The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (Mercenaria mercenaria), bay scallops (Argopecten irradians), and Eastern oysters (Crassostrea virginica). Limnology and Oceanography 54:2072-2080.


VITA

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