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Effects Of Environmental Stress From A Changing Climate On Populations Of Commercially Important Bivalves Along The Eastern Coast Of The United States

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Effects of environmental stress from a changing climate on populations of commercially important bivalves along the eastern coast of the United States

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ABSTRACT

This dissertation evaluates population-level variability in physiological tolerances to environmental stressors in the eastern oyster, *Crassostrea virginica*, and the hard clam, *Mercenaria mercenaria*, two ecologically and economically important bivalve species that inhabit the eastern coast of the United States. In Chapter 2, an assessment of the acidification tolerance of larval eastern oysters spawned from two reefs in Chesapeake Bay revealed physiological differences under control and acidified conditions. Differences observed at control conditions indicate potential variations in basal metabolic processes, while the variations in acidification tolerance illustrate the potential for some oyster populations to possess greater resilience to ongoing acidification. More population-level assessments are needed as variability among other populations across larger spatial scales is likely present. In Chapter 3, stress tolerances to elevated temperatures and low salinities were evaluated for juvenile clams from five populations along the US East Coast. Differences in nonlethal temperature stress responses were minimal among populations, although the two most southernly distributed populations had the highest survival under an extreme high temperature. Further population-level variability was observed in response to low salinity stress; however, all populations showed a similar low salinity tolerance limit below 15. More multifaceted assessments are needed to better capture population-level differences in stress response mechanisms. Following Chapter 3, three population crosses were conducted to assess if physiological tolerances could be modified (Chapter 4). Nonlethal temperature tolerances were similar among juveniles from all crosses. Juveniles from outcrosses of Pocomoke Sound, VA with Wachapreague, VA and Bogue Sound, NC with Cape Cod, MA showed higher survival compared to a Wachapreague, VA self-cross under an extreme high temperature. Interestingly, all three crosses showed marked declines in oxygen consumption below a salinity of 20, which was not seen in any parent population until below a salinity of 15. This variability between experiments indicates that genetic variations in salinity tolerance may exist within study populations. Elevated temperature and low salinity tolerances of larvae produced from the same three crosses were also evaluated (Chapter 5). Larvae from the Wachapreague, VA self-cross showed the largest decline in survival under elevated temperature stress, as seen in juvenile cross assessments. When exposed to low salinity stress, larvae from the Bogue Sound, NC and Cape Cod, MA cross showed the highest survival; however, larvae from all crosses showed a steep drop in cellular energy reserves. Alongside this physiological assessment, larval microbiomes were sequenced to provide insight into microbial community structures and to explore the impacts of environmental stress on these communities. Larval microbiomes from the Bogue Sound, NC and Cape Cod, MA cross were clearly different from those of the other crosses, demonstrating the influence parental microbiomes can have on their offspring. Lastly, in all crosses, low salinity stress resulted in the greatest shifts in microbial community composition observed here. Overall, this dissertation provides deeper insights into physiological stress mechanisms in early life stages of *C. virginica* and *M. mercenaria*. Population-level variability will likely play an important role in the long-term persistence of eastern oysters and hard clams as climate changes continues.

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Chapter 1

Introduction

Global climate change is of increasing concern as human activities continue to modify the global atmosphere and the world's oceans. As humans burn fossil fuels, atmospheric concentrations of greenhouse gases, such as carbon dioxide (CO_2) and methane, rise. These elevated concentrations contribute to many changes in environmental conditions, such as ocean warming, ocean acidification, and changes in precipitation patterns (Latif, 2007; Cai et al., 2021; Masson-Delmotte et al., 2021). As greenhouse gases accumulate in the atmosphere, they act as insulators, trapping heat in the form of long-wave radiation from the sun, which thus drives ocean warming (Latif, 2007; Cheng et al., 2019). However, CO_2 not only accumulates in the atmosphere, but also diffuses into the ocean along its concentration gradient, with estimates of up to one third of all anthropogenic CO_2 emissions being absorbed by the oceans each year. Once CO_2 dissolves into seawater, it reacts to form carbonic acid before dissociating and releasing free hydrogen ions in the process known as ocean acidification (Latif, 2007; Doney et al, 2009; Mangan et al. 2017; Ilyina and Heinze 2019; Masson-Delmotte et al., 2021; Cai et al., 2021). While the dissolution of anthropogenic CO_2 does drive acidification, there are other processes that also can contribute to acidification that are more region specific, such as bacterial respiration in eutrophied systems and input of freshwater with naturally lower buffering capacity in coastal regions (Doney et al, 2009; Masson-Delmotte et al., 2021; Cai et al., 2021). As climate modifications continue, patterns of precipitation are also projected to change, with certain regions experiencing increases in annual precipitation totals, while others experience decreases in precipitation totals (Portmann et al, 2009; Donat et al, 2016; Masson-Delmotte et al., 2021). Due to the variability in how climate change will impact specific regions, it is first necessary to explore the projected changes for the Mid-Atlantic region of the United States where much of the proceeding work was conducted. Specifically, the focus of Chapter 2 is Chesapeake Bay, while

components of Chapters 3, 4, and 5 span from the Mid-Atlantic up into the Northeastern United States.

Water temperature, pH, and precipitation patterns are all projected to change in the future in the study regions that are the focus of this work (Masson-Delmotte et al., 2021). For Chesapeake Bay specifically, ongoing ocean warming is primarily driven by atmospheric CO_2; however, both circulation patterns in the Mid-Atlantic region and riverine warming within specific tributaries of Chesapeake Bay also contribute to ocean warming in this region (Shearman and Lentz, 2010; Karmalkar and Horton, 2021; Hinson et al., 2022). Moving northward, the waters of the northeastern United States have been found to be an ocean warming hotspot due to changes in Atlantic Meridional Overturning Circulation and atmospheric circulation pattern changes associated with the North Atlantic Oscillation (Karmalkar and Horton, 2021). Coastal regions throughout the Mid-Atlantic and Northeastern United States are more susceptible to acidification as freshwater input can naturally lower buffering capacity, which can lead to more rapid decreases in pH (Doney et al, 2009; Masson-Delmotte et al., 2021; Cai et al., 2021). Furthermore, in coastal regions with high nutrient inputs from land runoff, such as many of the tributaries in Chesapeake Bay, further acidification occurs as a result of elevated bacterial respiration from the digestion of the more abundant phytoplankton communities supported by the increased nutrient load (Masson-Delmotte et al., 2021; Cai et al., 2021). Lastly, precipitation is projected to increase along the entire east coast of the United States, with storm intensities projected to increase as well, which will drive more intense low salinity events (Portmann et al, 2009; Donat et al, 2016). It is important to note, however, that projected changes in precipitation patterns have greater uncertainty and that projected increases in sea level may offset the impacts to salinity from changes in precipitation rates and storm

intensities within shallow coastal systems specifically (Walsh et al., 2016; Muhling et al., 2018; Ross et al., 2021). Since these wide-ranging changes are occurring at a rapid pace, there is a pressing need to understand how ecologically and economically important species within these regions will respond to these various stressors in order to anticipate their persistence in the future and appropriately manage our natural resources (Boyd et al., 2015).

The eastern oyster, *Crassostrea virginica*, and the hard clam, *Mercenaria mercenaria* are two ecologically and economically important bivalve species in the Mid-Atlantic region that will likely be impacted by climate change. *C. virginica* is a fast growing, reef building species that reaches sexual maturity within approximately 1 year and ranges from the east coast of Canada down into the Gulf of Mexico (Reeb and Avise, 1990; Powell et al., 2013; Bayne, 2017). *M. mercenaria* does not form reefs and instead is an infaunal suspension feeder that burrows into soft sediments. This species reaches sexual maturity within 1 to 3 years and has a similar geographic range to *C. virginica* that spans from the Gulf of St. Lawrence, Canada to the Florida Keys (Kraeuter and Castagna, 2001; Baker et al., 2008; Pernet et al., 2008). Both of these species provide important ecosystem services to the environments they inhabit such as water filtration, nutrient cycling, and, in the case of the eastern oyster, reef structure that provides habitat to a whole host of other organisms (Kraeuter and Castagna, 2001; Parker et al, 2013; Murphy et al. 2015; Darrow et al. 2017; Lemasson et al, 2017). Furthermore, as these two species are both fast growing, they have become crucial aquaculture species in Virginia, where in 2018 alone the oyster aquaculture industry had a farm gate value of $14.5 million while the hard clam aquaculture industry had a farm gate value of $38.8 million (Hudson, 2019). Due to the importance of each of these species, it is vital to understand how they will be impacted by future climate change. In particular, improved understanding of the sensitivities of these species to

environmental change as well as the limits of their physiological stress mechanisms are needed to better project how they will persist in the future.

Marine invertebrates possess a variety of mechanisms for responding to environmental fluctuations, from behavioral changes to changes in cellular regulation. For more sessile species, including life stages of many marine bivalves, cellular mechanisms of stress tolerance are of utmost importance as moving out of a stressful environment is not possible, and therefore the organism is subject to various physiological impacts based on its changing external environment (Dupont et al., 2010; Talmage and Gobler, 2010; Crim et al., 2011). The general cellular stress response is activated in the face of any environmental stressor and focuses on modulation of the cell cycle, repair of protein and DNA, and removal of irreparably damaged cellular components (Kultz, 2003; Kultz, 2005). Beyond the generalized cellular stress response, there are more specific cellular mechanisms that are activated by individual stressors. Increasing water temperatures for example, are known to alter metabolic rate, which has widespread impacts on cellular processes, resulting in changes to growth and development patterns of marine bivalves (Ivanina et al, 2013; Matoo et al, 2013). As metabolic rate increases but oxygen levels decrease due to lower oxygen solubility at higher water temperatures, the aerobic scope of a species narrows to a species-specific thermal window, which directly impacts an organisms' ability to respond to additional environmental stressors while maintaining proper growth and development (Pörtner, 2012). Ocean acidification can also impact growth due to the increased rate of calcium carbonate dissolution under more corrosive water conditions, which results in a larger energy investment needed to produce the same amount of new shell (Ivanina et al, 2013; Matoo et al, 2013; Stevens and Gobler, 2018). Both acidification and low salinity stress impact cellular ionic regulation, which increases energy demand for cellular regulation in order to maintain

homeostasis, which results in a reduced energy pool for growth and reproduction (Pörtner, 2012; Sokolova et al, 2012). Low salinity events have also been linked to both the cessation of growth in bivalves as well as a marked increase in mortality (Dickinson et al, 2013; Goodwin et al, 2021). Lastly, all three of these environmental stressors have been linked to increases in energy demand, which can result in increased cellular oxidative stress as reactive oxygen species (ROS) are produced as a byproduct of increased ATP production. ROS are highly reactive and can damage cellular macromolecules, which can further reduce the energy pool available to an organism for growth and reproduction due to elevated maintenance costs (Kultz, 2005; Rivera-Ingraham and Lignot, 2017; Duran et al, 2018).

In addition to simply understanding the regulatory potential of sessile species in response to environmental stress, it is necessary to understand how stress tolerance can vary across a species' biogeographic range. Environmental conditions vary along latitudinal gradients, meaning that local adaptation could occur among populations leading to differential stress tolerance. Populations whose adaptations include more narrow tolerance windows would be more sensitive to changing environmental conditions than populations with greater tolerance to variable conditions (Osovitz and Hofmann, 2007; Sanford and Kelly, 2011). Population-level evaluations of stress physiology are not commonplace for most species, and therefore it is difficult to know what, if any, variability in stress tolerance may exist across the geographic range of any given species. In the case of *C. virginica*, few studies examine population-level variability, and those that do are not focused on future climate shifts (Dittman et al., 1998; Cherkasov et al., 2010; Pierce et al., 2016). While genetic differences are known to exist between geographically distinct populations of *M. mercenaria*, in the context of shifting environmental stressors, no comprehensive studies have been conducted and many challenges

persist when trying to assemble the results of multiple studies on separate populations into one unified, population-level understanding (Baker et al., 2008; Gaylord et al., 2015; Grear et al., 2020; Ropp 2021). The existence of variable stress tolerances across a species range would have broad implications for whether or not a species as a whole will persist in coming centuries, and therefore is an important area of further research.

Both the ecological and economic significance of eastern oysters and hard clams fuel the need to understand how these species will respond to and cope with future climate change conditions. Therefore, this work will explore both whole-organism and cellular-level physiological responses to increased temperatures, lower salinities, and ongoing ocean acidification in order to deepening our understanding of the physiological limitations of each species while also informing future strategies to support aquacultural operations as well as restoration efforts.

Chapter 2 evaluated acidification tolerance at both the whole-organism and cellular level in larval eastern oysters spawned from adults of two separate populations within Chesapeake Bay. Different metrics for measuring acidification stress in *C. virginica* larvae varied in their effectiveness across a range of future ocean conditions. In particular, shell length was a more sensitive indicator of mild acidification stress, but under more moderate and severe conditions, total protein and triglyceride content better captured the extent and severity of acidification stress. Results of the population comparison revealed physiological differences in survival and growth under control conditions as well as unique sensitives to future ocean acidification. This work serves to inform our understanding of acidification tolerance in larval eastern oysters, while also demonstrating the need for further population-level assessments in order to understand the extent to which climate change will impact this species.

Chapter 3 explores population-level variability in response to elevated temperatures and lower salinities among juvenile hard clams from five different populations ranging from Massachusetts to Virginia. Changes in respiration rates of juvenile clams were measured as a proxy for changes in metabolic rate in order to assess sub-lethal physiological stress at the whole-organism level. Minimal changes in respiration rate among populations at various elevated temperatures were detected, which agreed with several previous studies, further supporting the idea that thermotolerance in *M. mercenaria* is not directly dependent on changes in respiration rate. Conversely, respiration rates did fluctuate among all populations in response to low salinity stress. Differences were detected between populations across several of the low salinity conditions examined, indicating that salinity tolerance may vary among populations. However, all populations showed a similar low tolerance limit, where all populations performed equally poorly under the most extreme low salinity exposure. This work helps inform our understanding of population-level variability in hard clams, while also providing evidence that certain populations of hard clams may be better candidates for future selective breeding programs as a tool for potentially producing more low salinity tolerant broodstock lines.

Chapter 4 further explores population-level variability in physiological tolerances of juvenile hard clams. This project expands upon the results of Chapter 3 through the evaluation of controlled population crosses to investigate whether any of the physiological differences previously observed in the parent populations could be manipulated across generations. If the previously observed differences were heritable traits, then there would be potential for improved fitness in offspring, but if these traits were the result of acclimatization processes that occurred in the native habitat of each population, they would not be heritable, and therefore, no improvement would be expected in offspring. Changes in oxygen consumption were measured in juvenile

clams produced from three population crosses in order to assess environmental stress tolerance. Two population outcrosses were assessed alongside a self-cross of native Virginia clams, which served as a baseline for comparison. Minimal differences in oxygen consumption rates among population crosses exposed to elevated temperatures were detected, which is in agreement with results from Chapter 3. Slight differences were observed in a mortality trial at a more extreme temperature, where both outcrosses showed improved tolerance compared to the self-cross. When clams were exposed to a salinity of 20, both outcrosses had higher oxygen consumption rates compared to the self-cross, potentially demonstrating greater aerobic scope. Interestingly, all three crosses performed worse at a salinity of 15 compared to the population assessment in Chapter 3 that was conducted at that same low salinity level. While poorer performance of outcrosses could indicate outbreeding depression, the difference in performance of the self-cross between study years may be the result of genetic variations in the parents involved in spawning each year. Assessing only the first generation of offspring cannot conclusively show either improved or diminished environmental tolerance in outcrosses, but the evidence of improved performance from the outcrosses warrants further research.

Chapter 5 assesses physiological tolerance of larval hard clams spawned from the same population crosses in Chapter 4, in order to determine if larval tolerances also varied between crosses in response to either an elevated temperature or low salinity condition. Larval growth and survival were assessed over the first week of life alongside various cellular markers for growth, energy accumulation, and cellular stress level. Alongside this physiological assessment, the microbial communities associated with larval clams were also evaluated to explore both how these microbial communities changed in the face of environmental stress as well as if microbial community composition was influenced by parental lineage. This study helps to inform our

understanding of larval clam physiology at the cellular level, while also exploring how microbial communities associated with hard clam larvae may vary under climate change conditions. As microbes can provide a diverse array of benefits to their host organism, it is important to deepen our understanding of how climate change can impact larval clam microbiomes and how that may affect their tolerance to future ocean conditions.

Chapter 6 summarizes all findings on bivalve physiological stress tolerance and population-level variability. Impacts of this work are discussed with a focus on future research directions needed to further the understanding of how climate change will impact *C. virginica* and *M. mercenaria*.

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Chapter 2

Differences in larval acidification tolerance among populations of the eastern oyster, *Crassostrea virginica*

Abstract

The eastern oyster, *Crassostrea virginica*, is an ecologically and economically important species that is threatened by ongoing coastal ocean acidification. Oyster larvae are known to be more susceptible to acidification than either juvenile or adult life stages, but less is known about threshold levels of acidification that induce a stress response and how this stress response changes with increased acidification. Furthermore, little is known about population-level variability in acidification tolerance in *C. virginica*, making predictions of how this species will respond to future environmental scenarios difficult. To address these knowledge gaps, both whole animal and cellular metrics were used to quantify the acidification response of larvae produced by adults collected from Page Rock reef and Parrot's Rock reef, two spatially distinct reefs in adjacent tributaries of Chesapeake Bay. A comparison of Page Rock reef larvae among four acidification scenarios (pH 7.8, 7.5, 7.2, 7.0) revealed differences in shell length, total protein content, and triglyceride content, with growth increasingly impacted as acidification intensity increased. However, the sensitivity of these traits to acidification varied, where shell length was similarly impacted between pH 7.5 and 7.2, while protein and triglyceride content continued to decrease between these acidification scenarios. Triglyceride accumulation was most severely impacted under the lowest pH tested (pH 7.0), signaling that acidification could ultimately reduce the number of individuals successfully recruiting into the adult population if adequate energy stores cannot be accumulated for metamorphosis. A comparison of larvae between the two reefs at pH 7.8 and 7.2 revealed differences in survival, growth, and energy accumulation that suggest clear underlying physiological variations in larvae between reefs and unique sensitivities to acidification stress. These findings demonstrate that acidification tolerance within *C. virginica* can vary by population, which will have important implications for

industry and conservation efforts as more resilient populations would make better candidates for future selective breeding efforts as well as restoration initiatives.

Keywords: *Crassostrea virginica*, oyster larvae, ocean acidification, population, Chesapeake Bay, environmental stress

1. Introduction

The eastern oyster, *Crassostrea virginica*, is native to the east and gulf coasts of the United States and provides a host of both ecological and economic benefits to coastal communities. Ecologically, *C. virginica* provides important ecosystem services such as water filtration, nutrient cycling, and reef structures that provide habitat to a whole host of other organisms (Parker et al, 2013; Darrow et al. 2017; Lemasson et al, 2017). Economically, this species can help to protect coastal properties from storm damage through wave attenuation, provides for a wild harvest fishery, and serves as the basis for a growing aquaculture industry, which had a farm gate value of $14.5 million in 2018 in the state of Virginia alone (Lemasson et al, 2017; Hudson, 2019). While adult oysters provide these wide-ranging benefits to both the ecosystems they inhabit and coastal fishing economies, it is their larval stages that are most susceptible to environmental stress, and therefore of greatest concern with respect to ongoing anthropogenic climate change (Dupont et al., 2010; Talmage and Gobler, 2010; Crim et al., 2011).

Bivalve larvae are at increasing risk from continuing acidification as energy requirements for calcification increase, which can result in decreased growth, overall health, and even higher mortality rates (Talmage and Gobler, 2010; Pörtner, 2012; Waldbusser et al., 2013; Waldbusser

et al, 2015a; Waldbusser et al, 2015b; Frieder et al, 2017; Mangan et al., 2017). As anthropogenic carbon dioxide (CO_2) emissions increase, more CO_2 dissolves into the oceans, driving the process of ocean acidification. Once dissolved, CO_2 rapidly reacts with the surrounding water and releases free hydrogen ions, thus lowering pH. While low pH alone can trigger stress responses, the increased concentration of free hydrogen ions drives the formation of bicarbonate through the reaction with free carbonate ions, which are one of the necessary building blocks for bivalve shells (Doney et al, 2009; Waldbusser et al., 2013; Waldbusser et al., 2015b; Mangan et al. 2017; Masson-Delmotte et al., 2021; Cai et al., 2021). Acidification stress thus increases the energetic demand necessary for larvae to produce their first shell, prodissoconch I, which typically occurs in the first 24-48 h of life, as more energy is required to scavenge the same amount of carbonate ions from surrounding seawater (Talmage and Gobler, 2010; Pörtner, 2012; Waldbusser et al., 2013; Waldbusser et al, 2015a; Waldbusser et al, 2015b; Frieder et al, 2017; Mangan et al., 2017). Furthermore, elevated pCO_2 can delay the onset of larval feeding, which can lead to an additional restriction of larval energy budgets (Gray et al., 2017). In coastal regions, ocean acidification can also be exacerbated by the decreased buffering capacity of freshwater inputs as well as CO_2 additions from bacterial respiration as a result of eutrophication, a process termed coastal ocean acidification that creates an even greater challenge for coastal bivalves (Doney et al, 2009; Masson-Delmotte et al., 2021; Cai et al., 2021). Diminished energy budgets due to increased acidification could potentially result in insufficient energy stores necessary for metamorphosis to the juvenile life stage. In this way, acidification can threaten the persistence of *C. virginica*, as well as other calcifying species, if new individuals fail to recruit into their respective regional populations over successive years (Waldbusser et al, 2015b; Thomsen et al, 2017). As acidification is projected to worsen in the

coming century and beyond, improving the understanding of how oyster larvae will respond to a range of acidified conditions is of growing importance.

To generate this understanding, it is necessary to evaluate a wide range of cellular mechanisms in order to characterize how future acidification may impact oyster larvae, as acidification is a complex, multi-faceted stressor (Gobler and Talmage, 2014; Waldbusser et al., 2015a; Barbosa et al., 2022). Many acidification studies have focused on the effects of one projected future acidification level in order to assess how a species will respond to climate change (Ivanina et al., 2013; Matoo et al., 2013; Durland et al., 2021; Barbosa et al., 2022; McNally et al., 2022). Of these studies, those focused on *C. virginica* have assessed broader physiological metrics such as shell size, survival, and metabolic rate, which have provided insight into how larval oysters will generally respond to future conditions (Miller et al., 2009; Clark and Gobler, 2016; Richards et al., 2018; Stevens and Gobler, 2018; Clements et al., 2021; McNally et al., 2022). While this approach has provided insight into general impacts of acidification, these past studies have not identified the level of acidification at which the onset of stress occurs or the shape of the exposure-response relationship across a range of acidified scenarios. Some acidification studies have evaluated responses to a wider range of acidified conditions; however, few have specifically examined *C. virginica* with respect to its cellular functioning (Miller et al., 2009; Ivanina et al., 2013; Matoo et al., 2013; Gobler and Talmage, 2014; Waldbusser et al., 2015b; Ventura et al., 2016; McNally et al., 2022). Improving our understanding of the cellular acid-base mechanisms that function in response to acidification stress will be necessary to determine the limits of these responses before available larval energy reserves are exceeded. Determining which underlying cellular processes are impacted at varying

levels of acidification will better inform projections of when oyster larvae will be most at risk due to continuing acidification.

Additionally, evaluating population-level variability in physiological tolerance to acidification stress is needed for making meaningful generalizations about species-level responses. In order to explore population-level variability, the definition of population must first be established. While there is no consensus on a unifying definition of population (Wells and Richmond, 1995; Berryman, 2002; Luck et al., 2003; Schaefer, 2006; Waples and Gaggiotti, 2006; Millstein, 2009), common lines of evidence for delineating populations are spatial disjunctions, demographic disjunctions, and genetic disjunctions (reviewed in Wells and Richmond, 1995). For the purposes of this study, the two populations assessed here are defined by their spatial disjunction, as they inhabit separate tributaries within Chesapeake Bay, with potential demographic and genetic disjunctions based on estimates for larval dispersal distances and evidence of genetic variation over small spatial scales throughout the range of *C. virginica* (Buroker, 1983; Rose et al., 2006; Galindo-Sánchez et al., 2008; North et al., 2008; Eierman and Hare, 2013; Eierman and Hare, 2016; Varney et al., 2016; Bernatchez et al., 2018). Previous studies in other marine invertebrates have noted variations in physiological tolerance across both large and small geographic spatial scales, illustrating the need for further physiological assessments at the population level (Ivanina et al., 2009; Sanford and Kelly, 2011; Sorte et al., 2011; Range et al., 2014). However, studies evaluating population-level stress tolerance across a variety of different environmental stressors are lacking. This knowledge gap further compounds the difficulty in understanding how any particular species will persist with ongoing climate change. For *C. virginica* specifically, the few studies assessing physiological differences between populations have focused on natural variability and responses to environmental

pollutants in primarily adult oysters (Dittman et al., 1998; Cherkasov et al., 2010; Eierman and Hare, 2013; Pierce et al., 2016). Expanding our understanding of population-level differences with respect to continuing acidification as well as the examination of stress tolerance in earlier life stages will enable resource managers to make better informed decisions to best maintain eastern oysters in the future.

The goals of this study were therefore to understand the onset and extent to which acidification can impact *C. virginica* larvae as well as evaluate physiological variability between study populations. To accomplish this, oyster larvae spawned from spatially distinct adult populations were exposed to different ranges of acidified conditions. Specifically, this project examined larvae spawned from two different oyster reefs within lower Chesapeake Bay. Furthermore, sampling focused on larvae within their first week of life, as this is a developmental time period when they are most susceptible to environmental stress (Talmage and Gobler, 2010; Pörtner, 2012; Waldbusser et al., 2013; Waldbusser et al, 2015a; Waldbusser et al, 2015b; Frieder et al, 2017; Mangan et al., 2017). Similar to previous studies, larval growth and survival were monitored throughout each experimental treatment to assess the overall health of larvae. In addition to measuring changes in shell length as a metric for growth, total protein content was measured as a proxy for changes in somatic tissue growth. Multiple cellular mechanisms were assessed to further understand how larval oysters respond to acidification stress, including two oxidative stress markers as well as Na^+/K^+-ATPase activity, an enzyme known to function in ionic regulation necessary to support acid-base regulation (Pörtner, 2008; Havird et al, 2013). Additionally, total triglyceride (TG) content was assessed in order to understand any fluctuations in energy reserves in response to acidification stress as triglycerides are known to be the primary energy storage mechanism for marine invertebrate larvae (Moran

and Manahan, 2004; Genard et al., 2011; Prowse et al., 2017; Gibbs et al., 2021). By exploring both the thresholds at which different cellular parameters are impacted by acidification stress as well as population-level variability, this study aims to improve the understanding of what acidification levels will be most threatening to *C. virginica* in the future.

2. Materials and Methods

2.1 Adult oyster collections

In February of 2021, adult eastern oysters were collected from two reefs within Chesapeake Bay. The first reef, Parrot's Rock reef, is located near the mouth of the Rappahannock River, VA (37.605837°N 76.421667°W), while the second reef, Page Rock reef, is located several miles upstream of the mouth of the York River (37.273383°N 76.579733°W) (Fig. 1). Once collected, adults from Parrot's Rock reef were held in floating cages at the Virginia Institute of Marine Science (VIMS) Kauffman Aquaculture Center, which is located on the Rappahannock River within close proximity to Parrot's Rock reef itself. Adults from Page Rock reef were held in an on-bottom bag and rack system near the VIMS Pier, just downstream from their collection site in the York River. Adults at each location were checked routinely to monitor gonad development until strip spawning was conducted in June 2021. Adults from Page Rock reef were spawned first on June 7th and the spawn for Parrot's Rock reef followed on June 17th.

2.2 Strip spawning

For both reefs, adults were cleaned to remove any dirt or debris before each individual was shucked and sexed. Males and females were separated, and each individual's gametes were stripped into separate beakers. Any hermaphroditic individuals were discarded. Gametes were assessed for quality, and in the case of sperm, scored based on activity level. Any males with minimal to no sperm activity were also discarded. Eggs from females were scored based on the presence of a clearly defined nucleus as well as overall shape to ensure the selection of high-quality eggs. Eggs of all females were high quality and therefore pooled together, cleaned, and counted to confirm that enough eggs had been obtained to achieve the desired stocking densities (30,000 embryos/L) for all research aquaria. Pooled eggs were then aliquoted to smaller volumes, and aliquots of sperm from each male were added to initiate fertilization. This ensured each male had the opportunity to fertilize eggs from all females in order to maximize the genetic diversity captured by each spawn. Fertilization was then monitored for the following 1 to 1.5 h to ensure cell division was occurring. The fertilized embryos were rinsed with filtered seawater to remove excess sperm before being added to research aquaria at a density of 30,000 embryos/L. From Page Rock reef, a total of 13 females and 4 males were stripped spawned to yield approximately 40 million fertilized embryos. From Parrot's Rock reef, a total of 10 females and 5 males were stripped to yield approximately 40 million fertilized embryos.

2.3 Experimental conditions

Embryos from Page Rock reef were divided among four different pH treatments: 7.8 (control), 7.5, 7.2, and 7.0. To determine potential population-level variability, embryos from Parrot's rock reef were exposed to two of these pH levels, 7.8 (control) and 7.2. pH ranges were selected to approximate the present-day summer average carbonate chemistry for these reefs

(Chesapeake Bay Program Data Hub, Tidal Water Quality Monitoring Program, monthly measurements from station LE 4.2 for Page Rock reef and station LE 3.4 for Parrot's Rock reef) and more extreme average carbonate chemistry predictions for the end of the century for Chesapeake Bay (Cai et al, 2017). Control pH values were specifically matched to the water conditions from which adults were removed in their respective tributaries before strip spawning in order to reduce any potential pH shock on either the gametes or newly hatched larvae. Within each experiment, there were three technical replicates of each pH treatment level, and the exposures lasted for six days. To monitor treatment conditions, pH and temperature were recorded every minute at the individual aquarium level by GF Signet DryLoc pH and ORP Electrodes (3-2724.090). If pH levels deviated from the target pH, pure CO_2 or CO_2 free air were bubbled to either decrease or increase pH, respectively, via an automated dosing system. Although multiple carbonate chemistry system parameters differed among experimental treatments, pH was the only parameter monitored in real time in order to control treatment levels in each experimental aquaria, and therefore, treatments will be referred to by pH level for the purposes of this paper. All pH exposures were conducted at 25°C, which matched water temperature at each holding site when adults were brought in for strip spawning. To maintain temperature, each tank was submerged in a water bath where temperature was autonomously controlled using heat exchangers. Salinity for all tanks was also matched to the salinity recorded at the holding site on the day the adult oysters were brought into the lab and spawned. Salinity was adjusted in a large reservoir for the entire aquarium system using 1 μm mechanically filtered seawater and deionized water as needed. As our seawater source was at the mouth of the York River where our Page Rock reef adults were held, no dilution was necessary to achieve the target

salinity of 18; however, dilution was necessary to reach the target salinity of 13 for the Parrot's Rock reef trial.

During each experiment, larvae were fed live *Pavlova* daily based on best practices of the VIMS Aquaculture Genetics & Breeding Technology Center (ABC) shellfish hatchery, starting at 20,000 cells/L with an increase of 5,000 cells/L per day. Larvae were maintained in static cultures with small recirculating aquarium pumps to evenly maintain treatment conditions. Pumps were isolated from larvae using custom built banjo screens to ensure no larvae were lost to the pump intake. Each 100 L research aquarium was drained and cleaned on days 2, 4, and 6, which is also when larval samples were collected for later analysis. On each sampling day, larvae were collected on fine mesh screens, concentrated into a 1 L beaker, and counted on a Sedgewick-Rafter slide in triplicate using a Leica DM1000 LED light microscope to determine survival rates. Images were also acquired during sampling using a Leica MC170 HD camera for later shell growth analysis using ImageJ (Schneider et al., 2012). Shell length was measured for ten larvae that were randomly selected from these images for each tank replicate. Larval samples were also collected from the concentrated stock and preserved at -80°C for later analysis of total protein content, total non-enzymatic antioxidant potential, malondialdehyde content, total triglyceride content, and sodium potassium ATPase activity level (7,500-20,000 larvae per sample, based on the sensitivity of each assay).

2.4 Water quality assessment

During both trials, pH and temperature were monitored in real time in each experimental aquarium as described in section 2.3. Temperature, pH, conductivity, and dissolved oxygen were also measured daily in each aquarium with a YSI Pro Plus (6050000). Water samples were

collected during sampling on day 6 and immediately analyzed using a spectrophotometric pH method following Dickson et al. (2007) with calculations following Douglas and Byrne (2017) using molar absorbance ratios from Liu et al. (2011) to serve as a means of assessing the accuracy at which our experimental system maintained our target pH levels. Water samples were also collected from the reservoir of filtered seawater used to fill all research aquaria before the start of each experiment and preserved for later total alkalinity titrations following methods by Dickson et al., 2007. On the final day of sampling for the Page Rock reef experiment, additional water samples were taken from each individual research aquarium to assess how total alkalinity was impacted by the additions of live algal feed. Direct measurements of temperature, salinity, spectrophotometric pH, and initial total alkalinity were used to calculate pCO_2 and Ω_{Ar} using CO2SYS (Lewis and Wallace, 1998) in order to fully compare each experimental condition across the full suite of carbonate chemistry parameters. For these calculations the following constants were used: K_1 and K_2 for carbonic acid from Lueker et al. (2000), K_{SO4} from Dickson et al. (1990), K_F from Perez and Fraga (1987), and total boron from Lee et al. (2010).

2.5 Biochemical assays

Total protein content of oyster larvae was assessed using the Pierce BCA protein assay kit following the manufacture's guidelines (Thermo Scientific, 23225). Each sample from individual aquarium replicates was measured in triplicate. Larval samples were homogenized on ice using a Sonic Dismembrator with a 1/8-inch probe (Fischer Scientific, base unit: FB-505, probe: FB4418) set to output four 1 second pulses at 20% amplitude with a 10 second break in between each pulse to prevent heating of the sample. These settings were used for all larval homogenizations in this study. All protein samples were homogenized in Milli-Q ultrapure

water (Millipore Sigma). The provided protein standard was run alongside each batch of samples in duplicate standard curves ranging from 0 to 2000 μg/mL and averaged before being used to calculate the total protein per larvae in each sample as a proxy for tissue growth. Assays were read at the recommended wavelength of 562 nm using a SpecratMax iD3 plate reader (Molecular Devices). Triplicate technical replicates were averaged for each aquarium.

Total non-enzymatic antioxidant potential was measured using the ferric reducing/antioxidant potential (FRAP) assay following Griffin and Bhagooli (2004). FRAP measurements were made on separate aliquots taken from the larval homogenate used to quantify total protein (described above) with the addition of 1:100 protease inhibitor cocktail (Millipore Sigma, P8340). In brief, the absorbance of FRAP working reagent [300 mM acetate buffer, 10 mM 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) dissolved in 40 mM HCL, and 20 mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1, respectively] at 37°C was first read at 595 nm using a SpectraMax iD3 plate reader. Sample homogenate was then added to the respective wells containing the working reagent and incubated for 5 minutes at 37°C before a second reading was taken at 595 nm. To account for the absorbance of the working reagent, the first reading was subtracted from the second absorbance measurement for each corresponding well. The FRAP value was calculated using duplicate iron (III) chloride standard curves ranging from 0 to 1000 μm on each plate. FRAP measurements of technical triplicates were averaged for each larval sample and then normalized to total protein content to prevent bias due to differences in larval sizes among experimental treatments.

Malondialdehyde (MDA) content was assessed as a marker for cellular damage from oxidative stress using the Lipid Peroxidation Malondialdehyde Assay kit (Colorimetric/Fluorometric) from Abcam (118970). Samples were homogenized in the provided

lysis buffer containing 1:100 2,6-Di-tert-butyl-4-methylphenol (BHT). Each sample was run in technical triplicates, and MDA content was calculated using duplicate standard curves of the provided MDA standard ranging from 0 to 20 μM on each plate. The assay was carried out according to the manufacturers specified guidelines for the fluorometric version of the assay to accommodate the low concentrations of MDA contained in larval samples. Assay fluorescence was measured using an excitation wavelength of 513 nm and an emission wavelength of 553 nm (SpectraMax iD3). Data was normalized to total protein content in each sample to avoid bias due to variations in size of larvae among treatment groups.

Total triglyceride (TG) content was assessed using the Triglyceride Assay Kit from Abcam (65336) following manufacturer's guidelines for the colorimetric version of this assay. Each sample was homogenized in 5% NP-40 following manufactures guidelines. Absorbance of all samples was measured at 570 nm using a SpectraMax iD3 plate reader. For this assay, each sample was run in technical duplicates. An aliquot of each replicate received the enzyme lipase, which cleaves TG into free fatty acids and the glycerol that is ultimately quantified by the assay, and a second aliquot of each replicate received assay buffer as a blank in order to assess background glycerol levels. The absorbance of aliquots containing assay buffer was subtracted from the absorbance of aliquots containing lipase, for each replicate pair, such that only glycerol cleaved from TG was quantified in our analysis. Samples were run alongside the provided TG standard in duplicate ranging from 0 to 10 nmol/well. TG per larvae was then calculated to assess energy reserves at the individual level.

Na^+/K^+-ATPase (NKA) activity was quantified as the ouabain-sensitive rate of inorganic phosphate production from ATP following Esmann (1988) based on the colorimetric determination of phosphate from Fiske and Subbarow (1925). Optimization of this assay for

oyster larvae follows from Pan et al., 2016 based upon modifications from Leong and Manahan, 1997. In brief, samples were homogenized in buffer containing 10% sucrose, 50 mM imidazole, and 5 mM EDTA. Homogenates were centrifuged at 4000 g for 30 seconds to remove larval shell fragments. The reaction buffer contained 130 mM NaCl (including the sodium from ATP sodium salt addition), 20 mM KCl, 10 mM $MgCl_2$, 50 mM imidazole, and 5 mM ATP. For each sample, the reaction was performed with and without 13.5 mM ouabain in order to determine the ouabain sensitive fraction of the total ATPases. This ouabain concentration was determined through serial testing as the lowest concentration at which maximum ATPase inhibition consistently occurred in test samples. All samples were run such that each technical replicate reaction contained 2.5 μg of total protein based upon previously measured total protein content. More concentrated samples were diluted with autoclaved ultrapure water before each reaction as necessary. Once the sample homogenates were combined with the reaction buffer with and without ouabain, they were incubated for 30 minutes in the dark at 25°C. The reaction was then halted by addition of 5% ice-cold trichloroacetic acid. Samples were then centrifuged to remove any potential protein precipitate from the acid addition before 2.5% ammonium molybdate dissolved in 4 N HCl was added. Lastly, 1-amino-2-naphthol-4-sulfonic acid (ANSA) was added, following the preparation by Peterson, 1978, and the reactions were incubated in the dark for 30 minutes at 20°C to allow color development before absorbance was read at 700 nm using a SpectraMax iD3 plate reader. Reaction blanks were run alongside samples, and the average absorbance from these blanks was subtracted from the absorbance of reactions containing sample homogenate. Samples were run in technical triplicates, each with and without ouabain. All samples were run alongside duplicate standard curves of a phosphate standard, NaH_2PO_4, ranging from 0 to 1000 μM. Tests were run to determine if the addition of alamethicin was

necessary to reveal latent ATPase activity, however these tests revealed no change in ATPase activity across a range of alamethicin concentrations, so it was excluded from sample analysis (Leong and Manahan, 1999). Activity data were normalized to protein content to account for differences in enzyme abundance associated with differences in larval biomass alone.

2.6 Statistics

All data were analyzed using GraphPad Prism 9 (San Diego, California, USA). To assess differences in physiological responses among pH conditions within each reef, full two-way analysis of variance (ANOVA) models were used, with time and pH as independent categorical factors. For the reef comparison, data from pH 7.8 and 7.2 were compared in a full three-way ANOVA model with pH, time, and reef as independent categorical variables. Multiple comparison tests (MCT) were used to assess *a priori* contrasts for all ANOVA models. The Sidák test was selected due to its more conservative correction for alpha inflation as many contrasts were examined with a fairly low sample size. This approach helps to minimize the Type I error rate to prevent drawing false conclusions. The alpha threshold for all ANOVAs and MCTs was set at $P < 0.05$. For each two-way ANOVA model, pH levels were compared to one another at each time point to determine if the four levels of pH had different impacts on oyster larvae compared to one another. The contrasts evaluated for each three-way ANOVA model focused on comparing the control and low pH treatments of the respective reefs to one another at each time point in order to determine if larvae from each reef responded in a similar way to acidification stress. Additionally, effect size was calculated for all variables in each ANOVA table to assess the proportion of total variance each effect accounted for in the model (Olejnik and Algina, 2000). ω^2 (omega squared) was selected as the effect size metric for use in this

study as it is less positively biased than the more commonly used η^2 (eta squared) while also being more resilient to sampling error due to small sample sizes (Olejnik and Algina, 2000; Ferguson, 2009; Lakens, 2013) Effect size was calculated as ω^2 following equations from Olejnik and Algina (2000) and Lakens (2013) for between-subject designs. See supplementary material for full statistical tables for each test reported to the appropriate significant digits based on the precision of each specific measurement method. All nonsignificant digits were truncated.

3. Results

3.1 Water quality

Average target pH in all treatments of Page Rock reef larvae was maintained within 0.07 pH units (Table 1). Spectrophotometric pH measurements from all tanks taken at the end of the experiment were first converted from the total pH scale to the NBS pH scale before comparison with average pH (NBS) as measured by the GF Signet pH electrodes in each tank. These values were in close agreement (Table 1), demonstrating that the pH targets were accurately maintained. The starting total alkalinity for all aquaria was 1148 µmol/kg; however, on day 6, total alkalinity of all tanks regardless of pH condition was 1623 ± 13 µmol/kg (mean ± SD; Table S1), indicating that the addition of live algae did indeed impact the carbonate chemistry during experimentation. Salinity and temperature were also consistent across all aquaria with a salinity of 18.04 ± 0.46 and a temperature of 25.1 ± 0.3°C (mean ± SD). The slightly higher average temperature observed in the pH 7.0 treatment is due to one tank, which had an average temperature of 25.7ºC. Data from this warmer tank were not excluded from the analysis, as its average temperature was minimally higher than intended, and there were no observable

differences in growth rate or survival of larvae compared to the other replicates of this treatment (Table S1).

For Parrot's Rock reef, average target pH for both treatments was maintained within 0.04 pH units (Table 1). Similar to the Page Rock reef experiment, spectrophotometric pH measurements taken from all aquaria on the final day of experimentation and converted to the NBS pH scale were found to be in agreement with average conditions monitored by the electrodes within each aquarium (Table S1). Total alkalinity of the filtered seawater used to fill all tanks at the start of experimentation was 1115 μmol/kg. Salinity and temperature were consistently maintained for the duration of experimentation across all aquaria with a salinity of 12.96 ± 0.42 and a temperature of 25.0 ± 0.2°C (mean ± SD).

3.2 Survival

Under all pH conditions, larvae from Page Rock reef showed a large decline in percent survival from 0 to 2 days postfertilization (dpf), followed by a much slower decline from 2 dpf onward (Fig. 2A). This observed decline was significantly affected by both time and pH [ANOVA, time: $F(3,32)=226.4$, $P<0.0001$, $\omega^2=0.8990$, pH: $F(3,32)=9.741$, $P=0.0001$, $\omega^2=0.0349$] (Table S2), although time accounts for the majority of the variance in the model as seen by the calculated effect sizes. At 2, 4, and 6 dpf, larvae raised under pH 7.2 had significantly higher percent survival than larvae raised at pH 7.0 [Sidák MCT, 2 dpf: $t=3.265$, $P=0.0156$, 4 dpf: $t=4.194$, $P=0.0012$, 6 dpf: $t=3.269$, $P=0.0154$] (Table S5).

Larvae from Parrot's Rock reef did not exhibit the same large decrease in survival during their first 48 h of life as that observed for Page Rock reef larvae, but instead showed a slower, but more consistent decline in percent survival over time (Fig. 2B). Similar to Page Rock reef

larvae, survival of larvae from Parrot's Rock reef were significantly affected by time and pH [ANOVA, time: $F(3,12)=20.39$, $P<0.0001$, $\omega^2=0.6570$, pH: $F(1,12)=10.70$, $P=0.0067$, $\omega^2=0.1095$] (Table S3), with time also accounting for the majority of the variance in the model, although pH had a larger effect on larvae from Parrot's Rock reef than those from Page Rock reef. However, potentially due to low sample size or high variability within the data set, no significant contrasts were detected between pH 7.8 or 7.2 at any time point for larvae from this reef (Table S6).

When comparing percent survival between reefs, a significant interaction was detected between reef and pH [ANVOA, $F(1,28)=19.18$, $P=0.0002$, $\omega^2=0.0514$] (Table S4), indicating that larvae from the two reefs responded differently across pH levels. That said, the effect size of this interaction is small and therefore may not be very influential on survival. Of the tested *a priori* contrasts, the only significance detected was between the reefs under control conditions at 2 dpf [Sidák MCT, $t=4.403$, $P=0.0169$] (Table S7).

3.3 Shell Length

Larvae from Page Rock reef grew over time under all pH conditions (Fig. 3A); however, larvae exposed to the more extreme low pH conditions showed smaller shell lengths on average. Results from the statistical model showed that the interaction of time and pH had a significant effect on shell length for these larvae [ANOVA, $F(6,348)=9.959$, $P<0.0001$, $\omega^2=0.0372$] (Table S2), indicating that the distinct pH levels tested here had different impacts on larval growth over time. The effect size of this interaction term is quite small, however, indicating that it may not be particularly influential. By 2 dpf, larvae raised under both pH 7.8 and 7.5 were larger than larvae raised at pH 7.0 [Sidák MCT, 7.8 v 7.0: $t=4.200$, $P=0.0002$, 7.5 v 7.0: $t=3.188$, $P=0.0094$]

(Table S5). These significant differences in larval size remained at day 4 [Sidák MCT, 7.8 v 7.0: t=7.451, P<0.0001, 7.5 v 7.0: t=5.233, P<0.0001] with additional significant differences between larvae at pH 7.8 and 7.2, and larvae at pH 7.2 and 7.0 [Sidák MCT, 7.8 v 7.2: t=4.081, P=0.0003, 7.2 v 7.0: t=3.370 P=0.005]. On day 6, shell size differed among all treatment groups [Sidák MCT, all comparisons: P<0.0001], except between pH 7.5 and 7.2.

For larvae spawned from Parrot's Rock reef, similar growth trends were observed at both pH conditions (Fig. 3B). Both time and pH were found to have significant effects on shell length, with time having a larger effect on shell length as compared to pH [ANOVA, time: F(2, 144)=175.0, P<0.0001, ω^2=0.6622, pH: F(1, 144)=26.24, P<0.0001, ω^2=0.0480] (Table S3). The results of *a priori* multiple comparisons revealed that larvae raised under a pH of 7.8 had significantly greater shell lengths at both 2 and 4 dpf, but by 6 dpf this difference was no longer present [Sidák MCT, 2 dpf: t=2.919 P=0.0122, 4 dpf: t=4.440, P<0.0001] (Table S6).

When comparing shell length between reefs at pH 7.8 and 7.2, the three-way interaction of time, pH, and reef was found to have a significant effect on shell length, although the size of this effect was quite small and likely has little practical effect [ANOVA, F(2,318)=6.499, P=0.0017, ω^2=0.0062] (Table S4). *A priori* comparisons were run to assess if larval shell length between reefs varied within each pH treatment on each day. At 4 dpf, larvae from Page Rock reef were significantly larger than their respective Parrot's Rock reef counterparts under control conditions [Sidák MCT, Parrot's v Page pH 7.8: t=3.436 P=0.0432] (Table S7). This significant difference was still present at 6 dpf, where larvae from Page Rock reef were now significantly larger under both pH conditions [Sidák MCT, pH 7.8: t=10.74 P<0.0001, pH 7.2: t=4.848 P=0.0001].

3.4 Total protein content

Total protein content increased more rapidly over the course of the experiment under higher pH conditions compared to lower pH conditions in larvae from Page Rock reef (Fig. 3C). When tested, the interaction of time and pH was found to have a significant effect on the observed variable protein accumulation rates across the experimental conditions [ANOVA, $F(6,24)=6.159$, $P=0.0005$, $\omega^2=0.1313$] (Table S2). While the effect size of this interaction is small, it shows importance in the model indicating that as the exposures progressed the effect of pH varied by treatment. Results from the planned contrasts detected no significant differences between any pH treatments at 2 or 4 dpf, but by 6 dpf many differences were observed. While protein content in larvae at 6 dpf raised under pH 7.8 and 7.5 were not significantly different, both of these groups had significantly higher protein content than larvae at pH 7.2 [Sidák MCT, 7.8 v 7.2: $t=4.145$, $P=0.0022$, 7.5 v 7.2: $t=3.026$, $P=0.0343$] and pH 7.0 [Sidák MCT, 7.8 v 7.0: $t=7.243$, $P<0.0001$, 7.5 v 7.0: $t=6.127$, $P<0.0001$] (Table S5). Additionally, larvae at pH 7.2 had greater protein content than those at pH 7.0 by 6 dpf [Sidák MCT, $t=3.098$, $P=0.0291$].

Protein content also increased over time in larvae from Parrot's Rock reef under both the control and low pH conditions, with larvae under the control condition tending to be larger on average at each time point (Fig. 3D). Both time and pH had significant effects on these larvae, where time clearly had a greater effect on protein content [ANOVA, time: $F(2,9)=60.92$, $P<0.0001$, $\omega^2=0.8160$, pH: $F(1,9)=13.87$, $P=0.0047$, $\omega^2=0.0876$] (Table S3). No significant contrasts were detected between either pH condition at any time point (Table S6).

When comparing reefs, larvae from Page Rock reef grew faster from 4 to 6 dpf under control conditions compared to their Parrot's Rock reef counterparts. A significant three-way interaction of time, reef, and pH was detected; however, the size of this effect is quite small and

likely of little practical significance [ANOVA, $F(2,21)=3.493$, $P=0.0490$, $\omega^2=0.0121$] (Table S4). While no significant contrasts were found between reefs in the low pH condition, Page Rock reef larvae had significantly greater protein content than Parrot's Rock reef larvae at 6 dpf under control conditions [Sidák MCT, $t=4.204$ $P=0.0260$] (Table S7).

3.5 Nonenzymatic antioxidant potential

Few significant effects were detected within either reef for nonenzymatic antioxidant potential (Fig. 4A and B). While both populations showed decreasing trends in nonenzymatic antioxidant potential over time, this trend was only significant in larvae from Page Rock reef [ANOVA, $F(2,24)=7.018$, $P=0.0040$, $\omega^2=0.2631$] (Table S2). Beyond this effect, nonenzymatic antioxidant potential was similar among pH treatments for both reefs.

However, there were significant effects of both time and reef observed when comparing both populations, with time having a moderate effect and reef having a small effect on antioxidant potential [ANOVA, time: $F(2,21)=13.96$, $P=0.0001$, $\omega^2=0.3650$, reef: $F(1,21)=13.42$, $P=0.0014$, $\omega^2=0.1750$] (Table S4). The observed difference in reefs may stem from the initially higher average antioxidant potential observed in Page Rock reef larvae; however, no significant contrasts were detected between reefs (Table S7).

3.6 Malondialdehyde (MDA) content

MDA content in larvae from Page Rock reef (Fig. 4C) decreased over time in all pH treatment groups [ANOVA, $F(2,24)=9.307$, $P=0.0010$, $\omega^2=0.3351$] (Table S2), but did not vary significantly with pH. Larvae at pH 7.0 did have slightly higher levels of MDA at 4 and 6 dpf compared to the other treatments; however, this trend was not significant.

Larvae from Parrot's Rock reef showed a different pattern in MDA content, where MDA content increased over time for both pH 7.8 and 7.2 (Fig. 4D). Time had a large significant effect on MDA content [ANOVA, $F(2,9)=25.72$, $P=0.0002$, $\omega^2=0.7580$] (Table S3), while no effect of pH was detected.

When comparing reefs, significance was detected for the interaction of time and reef, which accounted for a large portion of variance within the model [ANOVA, $F(2,21)=51.55$, $P<0.0001$, $\omega^2=0.6032$] (Table S4). Interestingly, larvae from Page Rock reef had significantly higher MDA content at 2 dpf under control conditions compared to Parrot's rock reef [Sidák MCT, $t=4.456$, $P=0.0143$], but at 4 and 6 dpf, Parrot's Rock reef larvae in both pH treatments had significantly higher MDA levels compared to their Page Rock reef counterparts [Sidák MCT, 4 dpf: pH 7.8: $t=5.416$, $P=0.0015$, pH 7.2: $t=4.475$, $P=0.0137$, 6dpf: pH 7.8: $t=4.511$, $P=0.0126$, pH 7.2: $t=6.472$, $P=0.0001$] (Table S7).

3.7 Na^+/K^+-ATPase (NKA) activity

NKA activity was highly variable across the pH treatments for larvae from Page Rock reef at 2 and 4 dpf (Fig. 5A). Despite this, the interaction of time and pH was still found to have a significant effect [ANOVA, $F(6,24)=3.050$, $P=0.0232$, $\omega^2=0.1380$] (Table S2). The only significant differences observed between pH conditions was on day 2 where larvae in pH 7.8 had higher activity levels than larvae in either pH 7.5 or 7.0 [Sidák MCT, pH 7.5: $t=2.893$, $P=0.0470$, pH 7.0: $t=3.046$, $P=0.0329$] (Table S5). Additionally, a non-significant trend was observed at 4 and 6 dpf where larvae reared at pH 7.0 had a higher average NKA activity compared to all other pH conditions.

NKA activity in larvae from Parrot's Rock reef (Fig. 5B) was less variable and decreased significantly over time [ANOVA, $F(2,9)=36.24$, $P<0.0001$, $\omega^2=0.7819$] (Table S3). No significant contrasts were detected, and larvae from both pH conditions showed decreases in NKA activity down to similar levels by the end of the experiment (Table S6).

When comparing reefs, NKA activity significantly differed only with time [ANOVA, $F(2,21)=4.451$, $P=0.0245$, $\omega^2=0.1743$] (Table S4) and no significant differences were observed when comparing the pH treatment groups from each reef to one another (Table S7).

3.8 Total triglyceride (TG) content

TG content of Page Rock reef larvae varied significantly with the interaction of time and pH, although this effect was small [ANOVA, $F(6,24)=3.087$, $P=0.0220$, $\omega^2=0.0232$] (Table S2). Average TG content increased through time under all pH conditions with clear separation between pH treatments by the end of the exposure (Fig. 6A). While the differences between pH conditions begin to emerge at 4 dpf, significant differences were not detected until 6 dpf, on which larvae raised at pH 7.8 had significantly higher TG content than larvae raised at either pH 7.2 or 7.0 [Sidák MCT, pH 7.2: $t=3.240$, $P=0.0207$, pH 7.0: $t=4.846$, $P=0.0004$] (Table S5). Additionally, larvae from the pH 7.5 condition also showed significantly higher TG content than larvae raised at pH 7.0 at 6 dpf [Sidák MCT, $t=4.028$, $P=0.0029$].

Larvae from Parrot's Rock reef (Fig. 6B) showed an increase in TG content over time, with minor separation of the pH groups occurring after 2 dpf. The main effects of time and pH were significant, while the interaction term was not [ANOVA time: $F(2,9)=19.23$, $P=0.0006$, $\omega^2=0.5896$, pH: $F(1,9)=7.212$, $P=0.0250$, $\omega^2=0.1005$] (Table S3). Time was found to have a

greater effect on TG content in Parrot's Rock reef larvae than pH. No significant contrasts were detected between pH treatments (Table S6).

When comparing reefs, a significant three-way interaction term was detected; however, the effect size of this term was quite small [ANOVA, $F(2,21)=4.011$, $P=0.0335$, $\omega^2=0.0232$] (Table S4). TG was accumulated at a faster rate and to a greater extent in larvae from Page Rock reef compared to those from Parrot's Rock reef under both pH conditions. More specifically, differences between reefs began to emerge at 4 dpf, where larvae from Page Rock reef had significantly higher TG content under control conditions compared to larvae from Parrot's Rock reef [Sidák MCT, $t=4.808$ $P=0.0062$] (Table S7). By 6 dpf, larvae from Page Rock reef had significantly higher TG content than larvae from Parrot's Rock reef under both pH regimes [Sidák MCT, pH 7.8: $t=9.056$, $P<0.0001$, pH 7.2: $t=4.503$, $P=0.0128$].

4. Discussion

This study examined the onset and extent of the impact of acidification stress on larval oysters from Chesapeake Bay VA. Additionally, two spatially disjunct populations were compared to assess potential population-level variability in physiological tolerance to coastal ocean acidification that could further our understanding of how oysters will be affected by ongoing climate change.

4.1 Onset of acidification stress

High mortality levels were seen in all pH treatments for Page Rock reef larvae, although minimal impact from acidification stress was observed. The mortality assessment for Page Rock

reef larvae across all four pH conditions revealed a large decline in survival within the first 48 h of the exposure followed by a slow but steady decline over the remaining duration of the experiment. This follows many previous reports that describe similarly high mortality rates in early life stages across numerous marine bivalves, even in the absence of environmental stress (Kennedy, 1996; Plough and Hedgecock, 2011; Bitter et al., 2019; McFarland et al., 2020). Minimal differences in mortality were detected between pH treatment groups, although larvae raised at pH 7.0 showed the lowest overall survival. This demonstrates that mortality may not be a robust metric for assessing minimal to moderate larval acidification stress in some populations of *C. virginica*.

Both growth metrics (shell length and total protein content) were better able to detect differences among Page Rock reef larvae exposed to the three low pH treatment groups. Variations in shell length were detected as early as 2 dpf, where larvae exposed to pH 7.0 were already significantly smaller than control larvae. Further separation between the pH treatments continued over the course of the experiment, with control group larvae being larger than all other groups and pH 7.0 larvae being smaller than all other groups by 6 dpf. Interestingly, shell lengths were similar between pH groups 7.5 and 7.2, revealing a potential limitation when resolving sublethal effects on oyster larvae. However, when biomass accumulation was assessed via total protein content, differences could be detected between Page Rock reef larvae raised at pH 7.5 compared to larvae at pH 7.2. This finding demonstrates that while shell growth is sensitive to mild pH stress, it does not have the resolution necessary to differentiate between varying levels of moderate acidification stress, therefore pairing it with another growth metric, such as protein content, can help to better elucidate the effects of acidification on *C. virginica* larvae. Variability in the sensitivity of these growth metrics was also observed in larvae from

Parrot's Rock reef, where differences in shell length were observed at 2 dpf between the control and low pH conditions that disappeared by 6 dpf. Total protein content, however, followed the opposite trend where no differences were observed initially, but protein content in larvae at pH 7.2 was significantly lower than the control group at 6 dpf. The observed variation in when and to what extent shell length and protein content are affected by varying degrees of acidification stress provides insight to future studies on how best to capture changes in growth of *C. virginica* larvae subjected to acidified conditions. When applying both growth metrics together within this study, it is clear that shell growth was impacted first when larvae were subjected to mildly acidified conditions, but as conditions worsen, growth of somatic tissue was also stunted. Under more extreme acidification stress, pH 7.0 in this study, both shell and tissue growth were dramatically reduced by 6 dpf.

While neither oxidative stress marker revealed distinctions between pH levels as clearly as the aforementioned growth metrics, they do still offer some insight into the cellular response of this species to acidification stress. Oxidative stress markers were assessed within this study due to previous reports of increased oxidative stress in response to acidified conditions in several marine invertebrates (Tomanek et al., 2011; Deschaseaux et al., 2015; Dineshram et al., 2015; Wang et al., 2016). Both nonenzymatic antioxidant potential and MDA content decreased with increasing larval age in Page Rock reef larvae at all pH levels. As these trends were observed within the control group as well, these changes in oxidative stress markers may be indicative of a natural process related to larval maturation or the background culture conditions. However, larvae at pH 7.0 showed increased total nonenzymatic antioxidant potential and MDA content by 6 dpf compared to all other pH levels. While this trend was slight, it could indicate the beginning of increased oxidative stress and cellular damage due to prolonged exposure to more

extreme acidification, which warrants further exploration. Based on recent projections, pH 7.0 is not an unreasonable estimate for average future conditions in the low salinity regions of Chesapeake Bay, and, if this observed increase in oxidative stress at pH 7.0 persists through the remainder of larval development, could pose a threat to future survival and recruitment of eastern oyster larvae in these regions (Cai et al., 2017; Cai et al., 2021).

Another line of evidence of increased stress in larvae at pH 7.0 compared to those at all other pH levels is the observed trend in NKA activity. Similar to the oxidative stress parameters, while NKA activity decreased in all pH treatment groups over time, it was marginally elevated in larvae from pH 7.0 compared to all other groups at both 4 and 6 dpf. Elevated variation between tank replicates within each treatment group makes it difficult to elucidate if acidification stress alters NKA activity, but because this enzyme is known to function in regulating disruptions in the ion gradient necessary for maintaining acid-base balance, it follows that increased NKA activity would be necessary to compensate for more acidic conditions (Pace et al, 2006; Pörtner, 2008; Havird et al, 2013). Recent work examining the role of NKA activity in the response to acidification stress of another oyster species has shown that upregulation of a gene coding for NKA can occur; however, more work is needed to assess if *C. virginica* larvae specifically have a similar capability (Wright-LaGreca et al., 2022).

While clear differences between pH treatment groups were difficult to detect using the specific cellular mechanisms assessed within this study, the reduced accumulation of energy reserves was apparent at both pH 7.2 and pH 7.0 for larvae from Page Rock reef. As triglycerides are a primary energy source for larvae, this reduced accumulation is indicative of elevated metabolic costs in response to acidification stress as larvae under all pH conditions were fed an optimized diet for growth (Moran and Manahan, 2004; Genard et al., 2011; Prowse et al.,

2017; Gibbs et al., 2021). If adequate energy reserves are not accumulated, then larvae could either fail to successfully complete metamorphosis into their juvenile forms, or the larval stage itself could be prolonged exposing larvae to continued environmental stress and risk of predation (Moran and Manahan, 2004; Ko et al., 2014; Torres and Gimenez, 2020). Either of these scenarios could pose a serious risk to the continued persistence of oyster reefs as acidification intensifies over time.

Overall, the differences observed between pH treatments for the parameters assessed here reveal that careful attention should be paid when selecting metrics to quantify the future impacts of coastal ocean acidification on *C. virginica* larvae. As shell growth was impacted earliest in larval development, this metric may be better suited for assessing mild acidification stress, while metrics such as protein and triglyceride content may prove more insightful in moderate to severe acidification scenarios. However, as will be discussed in detail below, generalizing responses from an assessment of one population may be insufficient for anticipating the impacts acidification may have on other populations of *C. virginica*.

4.2 Population-level variability

Even before assessing the impacts of acidification on larvae from each of the two reefs examined in this study, clear physiological differences between the two study populations were observed under control conditions. When comparing survival between the control groups for each reef, larval mortality during the first 48 h was higher for Page Rock reef compared to Parrot's rock reef. Survival of larvae from each reef decreased with time beyond 2 dpf, with higher average survival seen in larvae from Parrot's Rock reef, although this difference between populations was not significant. When considered alongside the observed slower growth rate of

Parrot's Rock reef larvae, demonstrated by significantly smaller shell lengths and lower total protein content under control conditions, an apparent tradeoff between growth and survival becomes evident. Larvae from Page Rock reef grew faster but had a higher mortality rate while Parrot's Rock reef larvae grew slower with a lower mortality rate. While tributary-specific differences in selective pressures could be driving underlying genetic changes in these population-specific life history traits, studies focused on a variety of marine organisms have shown that fishing pressure can drive changes in the genetic makeup of a variety of traits, including growth rate (Swain et al., 2007; Nussle et al., 2009; Munroe et al., 2013; Marty et al., 2014; Sabolic et al., 2021). Furthermore, eastern oyster populations within Chesapeake Bay have been dramatically reduced due to a series of major disease events since the 1950s (reviewed in Jesse et al., 2021) that could have further driven genetic divergence among the fragmented surviving reefs. Regardless of the source of these apparent tradeoffs, populations that already have higher mortality rates, like Page Rock reef, could be more severely impacted by future climate change, as any stressor that further contributes to a rise in mortality rate could ultimately result in failed recruitment classes for said population.

Further evidence of differing growth strategies between these two reefs under control conditions was observed in their rates of triglyceride accumulation. While larvae from both reefs began with similar levels of triglycerides on day 2, Page Rock reef larvae accumulated triglycerides at a faster rate. While not assessed here, this faster rate of triglyceride accumulation coupled with faster overall growth rates could lead to a shorter larval developmental period, and thus less time spent in their most vulnerable life stage (Moran and Manahan, 2004; Dupont et al., 2010; Talmage and Gobler, 2010; Crim et al., 2011; Ko et al., 2014; Torres and Gimenez, 2020). In this way, even with higher initial mortality rates, populations that employ this strategy could

still have many successful recruits during each spawning seasons. However, as climate change progresses, any stressor that further increases mortality rates could threaten any population that employs this strategy and thus highlights the need for more population-level assessments throughout the range of *C. virginica*.

When comparing the impacts of future coastal ocean acidification between populations, larvae from both reefs were negatively affected, but not to the same extent. While shell length was significantly smaller at pH 7.2 in larvae from Page Rock reef by 6 dpf, shell length in Parrot's Rock reef larvae was initially greater in the control group compared to pH 7.2 at 2 dpf but by the end of the exposure this difference was gone. Shell growth of Parrot's Rock reef larvae may be sensitive to coastal ocean acidification in the first few days of life, but by the end of the first week of development, they appear to be able to acclimatize and recover a higher rate of shell growth. Impact from acidification exposure could still be seen in somatic growth for both populations though, indicating that not all impacts of acidification on Parrot's Rock reef larvae could be overcome in this time span.

Interestingly, the two populations showed opposite trends in MDA content, where MDA decreased over time in Page Rock reef larvae, indicating minimal damage from oxidative stress, but it steadily increased over time in Parrot's Rock reef larvae. While an increase in MDA concentration could be the result of higher levels of stress in Parrot's Rock reef larvae, it is unlikely as the observed increase was seen in both the control and acidified conditions, indicating that the elevated MDA concentrations were not driven by acidification stress and instead may be another underlying difference in development between these two reefs.

The most dramatic difference observed between these populations when subjected to acidification stress occurred with respect to their accumulation of energy stores in the form of

triglycerides. Under all pH conditions, including even pH 7.0, Page Rock reef larvae accumulated greater energy stores compare to Parrot's Rock reef larvae. As both populations started with similar triglyceride levels on day 2, this was unexpected. Interestingly, Parrot's Rock reef larvae at pH 7.2 started to show higher triglyceride content compared to control larvae as the experiment progressed, although this difference never crossed the significance threshold. This finding would be contrary to what would be expected of larvae experiencing stress and conflicts with results from a recent study showing decreased triglyceride content in larvae exposed to acidified conditions (Gibbs et al., 2021). While the reason for such an increase remains unclear, it stands to further demonstrate potential physiological differences that may exist between populations, and therefore deserves further attention.

As this study has demonstrated physiological differences between these two populations at both control and low pH conditions, generalizations across populations related to how *C. virginica* will respond to acidification stress should be made with caution. Given the differences in acidification tolerance of these two populations living in adjacent tributaries, generalizations of stress tolerance across this species' biogeographic range (e.g., thousands of miles) are likely inappropriate, and therefore population-level assessments are needed to characterize the physiological tolerance of *C. virginica* not only to acidification stress, but also to the wide array of other stressors this species encounters. Further work will also be needed to explore the underlying drivers of the observed physiological differences between populations, such as environmental history, underlying genetic variations, and maternal effects to name a few. The nature of these differences will be key to understanding their potential heritability, and therefore, what potential benefits they could provide to both aquacultural operations and conservation efforts (Eierman and Hare, 2013; Burford et al., 2014; Griffiths et al., 2021). This study

contributes to a growing body of research demonstrating population-level differences among marine bivalves, which likely extends to many more species that have yet to be assessed (Cherkasov et al., 2010; Sorte et al., 2011; Range et al., 2014; Liu et al., 2019). While the existence of population-level variability can complicate predictions about the impacts of climate change on a specific species, it also offers the potential for the discovery of more resilient populations that may be better adapted to future climate conditions.

5. Conclusion

Our examination of the onset and extent to which acidification stress impacts larval *C. virginica* revealed that while shell length is initially the most sensitive metric assessed here, tissue growth and energy accumulation better capture the impact of acidification stress under more moderate and severe scenarios. The identification of these parameters and the range of scenarios where they are most effective should inform future monitoring efforts on this ecologically and economically important species as climate change progresses. Coupling this analysis with a population-level assessment has clearly demonstrated both that physiological tolerance to acidification stress can vary across the range of *C. virginica* and that more work is needed in the future to understand how this species will persist with continuing climate change. While population-level variability may complicate future studies, it also could serve as a vital resource within commercially important species as the discovery of more resilient populations could help to maintain sustainable aquaculture practices through selective breeding programs to develop broodstock lines more resilient to ongoing climate change. The experimental designs employed here demonstrate the importance and necessity of evaluating stress tolerance to future climate change across a range of conditions as opposed to one future scenario as well as the need

to further explore the role population-level variability will play in the persistence of various marine invertebrate species in the future.

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Table 1. Water quality parameters over the course of each experiment. Data is shown as mean ± SD for the tank replicates within each treatment level. Total alkalinity of the filtered seawater used to fill each experimental aquarium was measured on the first day of each experiment and used to calculate representative values for pCO_2 and Ω_{Ar}. Initial alkalinity for Page Rock reef and Parrot's Rock reef were 1148 and 1115 µmol/kg, respectively.

Treatment	Temperature (°C)	Salinity	pH AQ Sys (NBS scale)	Spec pH (NBS scale)	pCO_2 (µatm)	Ω_{Ar}
Page Rock Reef						
7.0	25.2 ± 0.5	18.05 ± 0.47	7.01 ± 0.04	7.00 ± 0.04	4549 ± 465	0.09 ± 0.01
7.2	25.0 ± 0.2	18.02 ± 0.49	7.20 ± 0.02	7.24 ± 0.01	2595 ± 49	0.16 ± 0.00
7.5	25.0 ± 0.1	18.04 ± 0.48	7.49 ± 0.07	7.49 ± 0.07	1450 ± 251	0.28 ± 0.06
7.8	25.0 ± 0.1	18.04 ± 0.46	7.78 ± 0.04	7.76 ± 0.05	750 ± 91	0.50 ± 0.07
Parrot's Rock Reef						
7.2	25.0 ± 0.2	12.99 ± 0.48	7.20 ± 0.03	7.16 ± 0.12	3347 ± 869	0.11 ± 0.03
7.8	25.0 ± 0.3	12.94 ± 0.37	7.78 ± 0.04	7.69 ± 0.07	932 ± 153	0.36 ± 0.06

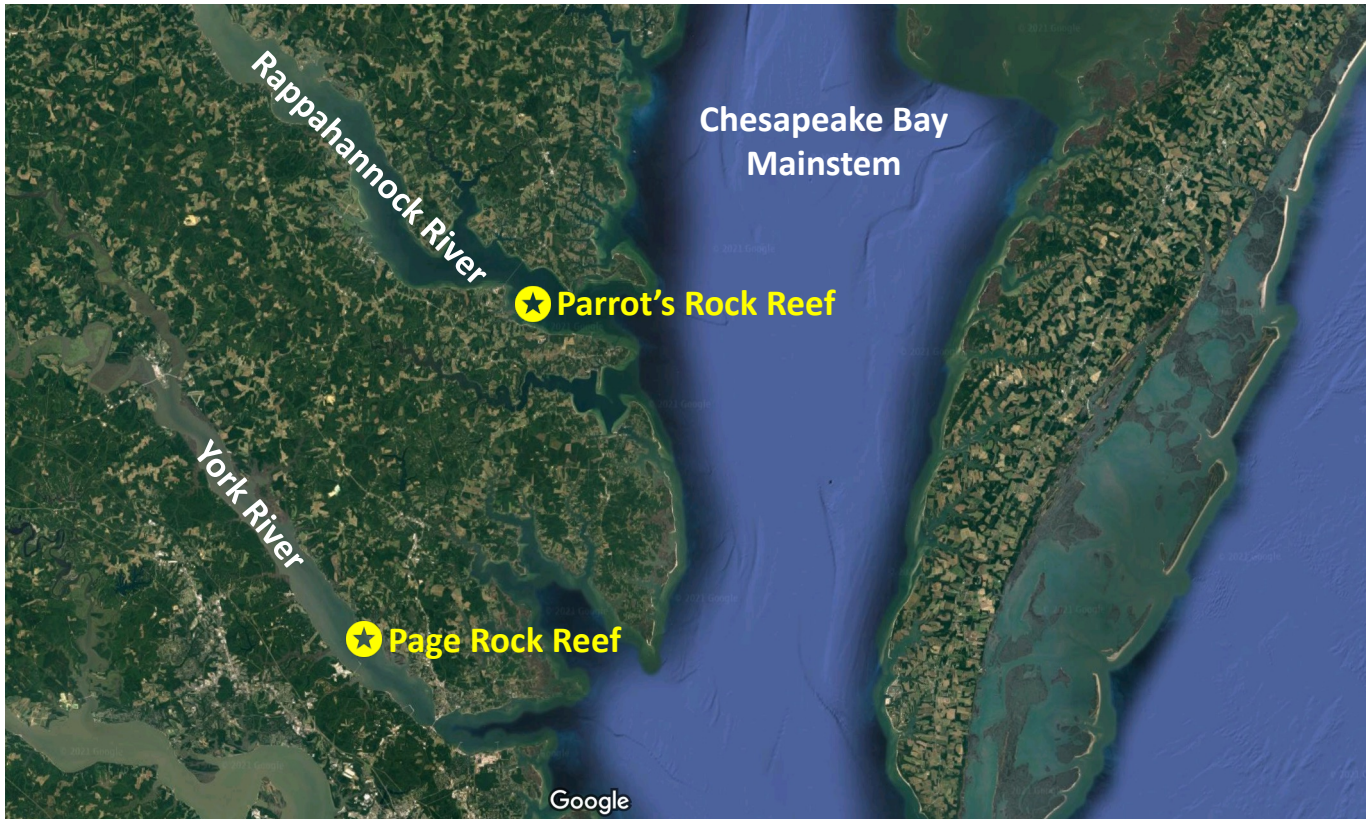

Figure 1. Map of the lower stem of Chesapeake Bay showing the two reefs where adult oysters were collected from for this study (yellow stars). Map image credit: Google.

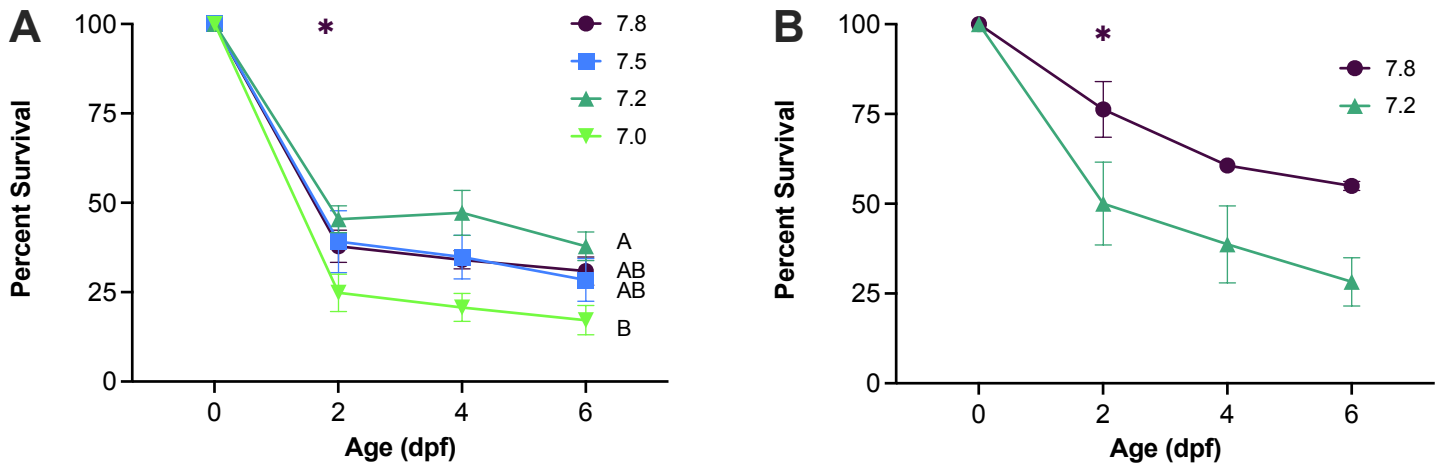

Figure 2. Percent survival at 2, 4, and 6 days postfertilization (dpf) for (A) Page Rock reef and (B) Parrot's Rock reef larvae shown at their respective pH treatments. Data shown as mean ± SEM (n=3 except Parrot's Rock reef control where n=2). Lack of visible error bars indicates they fall within the graphic illustration of the point itself. Capital letters indicate significant differences between pH treatments among Page Rock reef larvae at each time point, and color-coded asterisks indicate significant differences between populations at the corresponding pH level and time points (Sidák MCT, α=0.05).

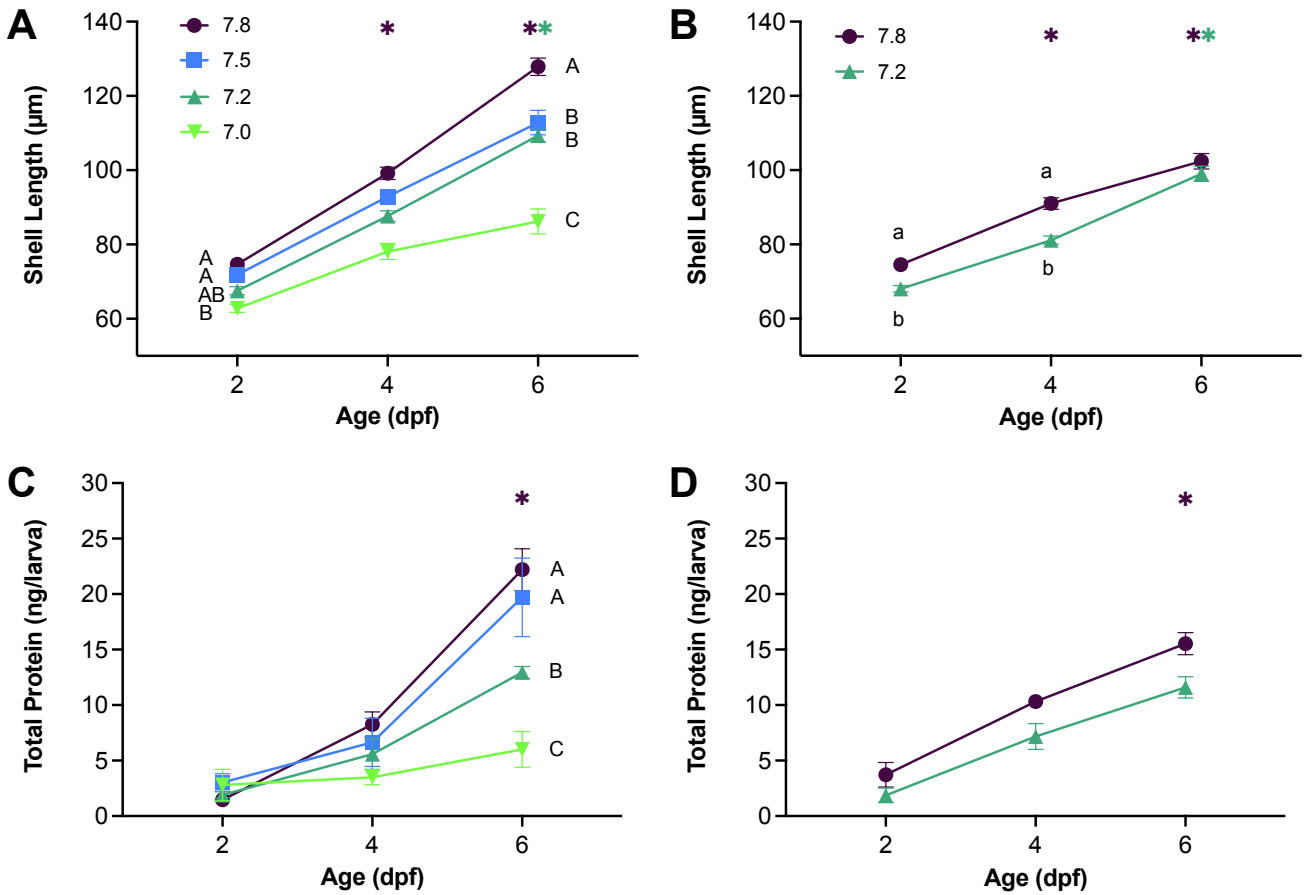

Figure 3. Growth metrics for larvae from (A and C) Page Rock reef and (B and D) Parrot's Rock reef at 2, 4, and 6 days postfertilization (dpf). (A and B) show average shell length in microns for ten larvae randomly selected from each tank replicate at each time point (n=30). (C and D) show average total protein per larva in nanograms for their respective pH treatments (n=3 except Parrot's Rock reef control where n=2). All data shown as mean ± SEM. Lack of visible error bars indicates they fall within the graphic illustration of the point itself. Capital letters indicate significant differences between pH treatments among Page Rock reef larvae at each time point, and lowercase letters indicate significant differences between pH treatments for Parrot's Rock reef at each

time point. Color-coded asterisks indicate significant differences between populations at the corresponding pH level (Sidák MCT, α=0.05). For (A), significance at day 4 not shown due to graphical constraints.

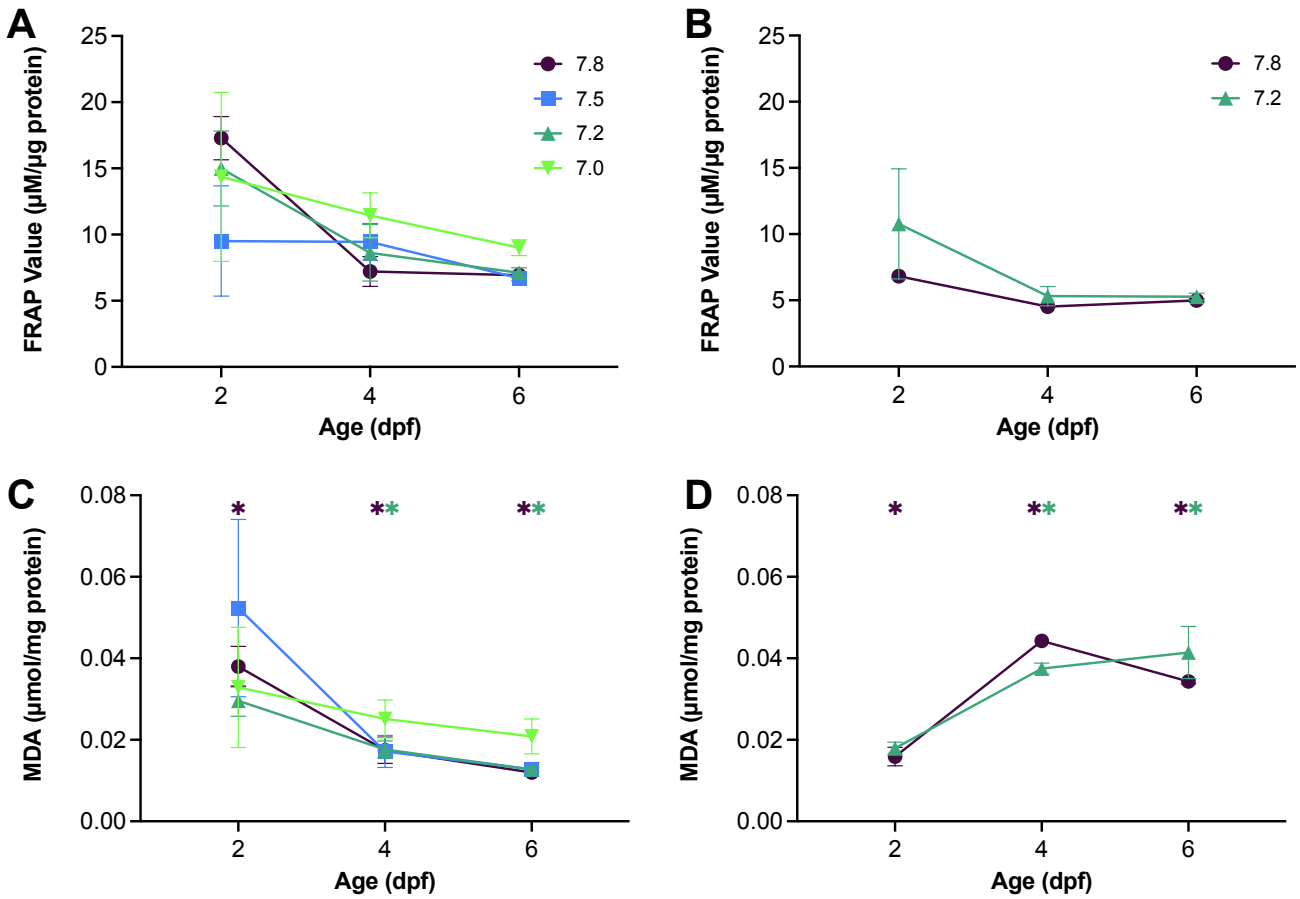

Figure 4. Nonenzymatic antioxidant potential and malondialdehyde (MDA) content for (A and C) Page Rock reef and (B and D) Parrot's Rock reef larvae at 2, 4, and 6 days postfertilization (dpf). Antioxidant potential data shown as ferric reducing/antioxidant potential (FRAP) values normalized to the protein content of each sample (µM/µg). MDA concentrations were also normalized to the total protein content within each sample (µmol/mg). Data shown as mean ± SEM (n=3 except Parrot's Rock reef control where n=2) for both metrics. Lack of visible error bars indicates they fall within the graphic illustration of the point itself. Color-coded asterisks indicate significant differences between populations at the corresponding pH level and time point (Sidák MCT, α=0.05).

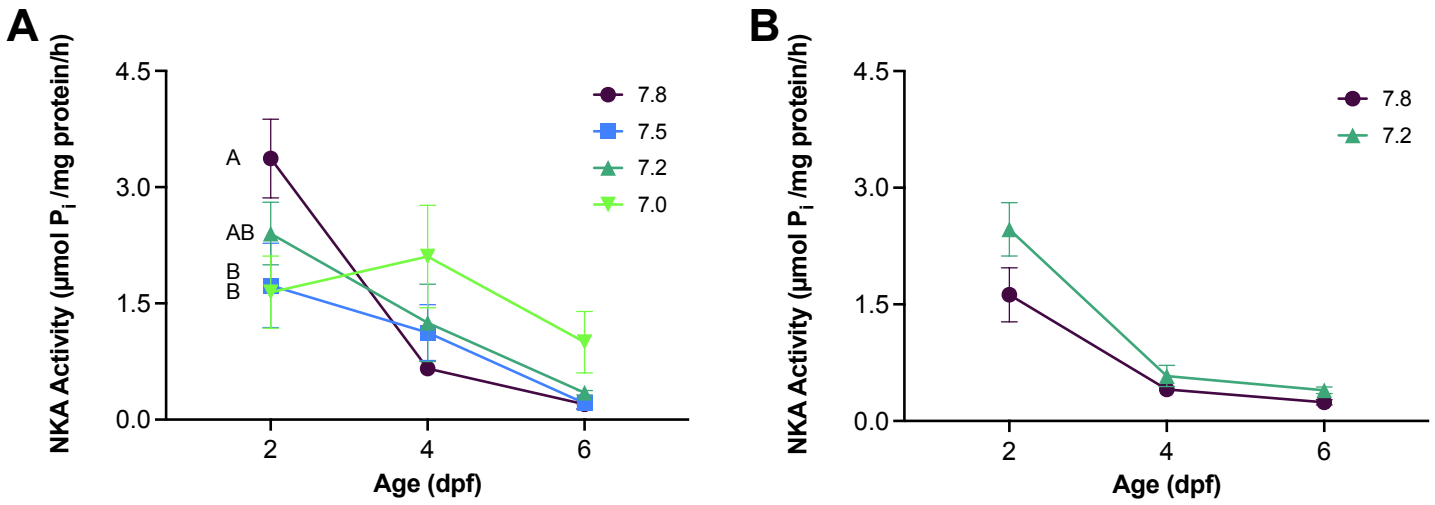

Figure 5. Na^+/K^+-ATPase (NKA) activity measured as the ouabain-sensitive rate of inorganic phosphate production per unit protein per hour the reaction was allowed to proceed (µmol/mg/h) for (A) Page Rock reef and (B) Parrot's Rock reef larvae at 2, 4, and 6 days postfertilization (dpf). Data shown as mean ± SEM (n=3 except Parrot's Rock reef control where n=2). Lack of visible error bars indicates they fall within the graphic illustration of the point itself. Capital letters indicate significant differences between pH treatments among Page Rock reef larvae at each time point (Sidák MCT, α=0.05).

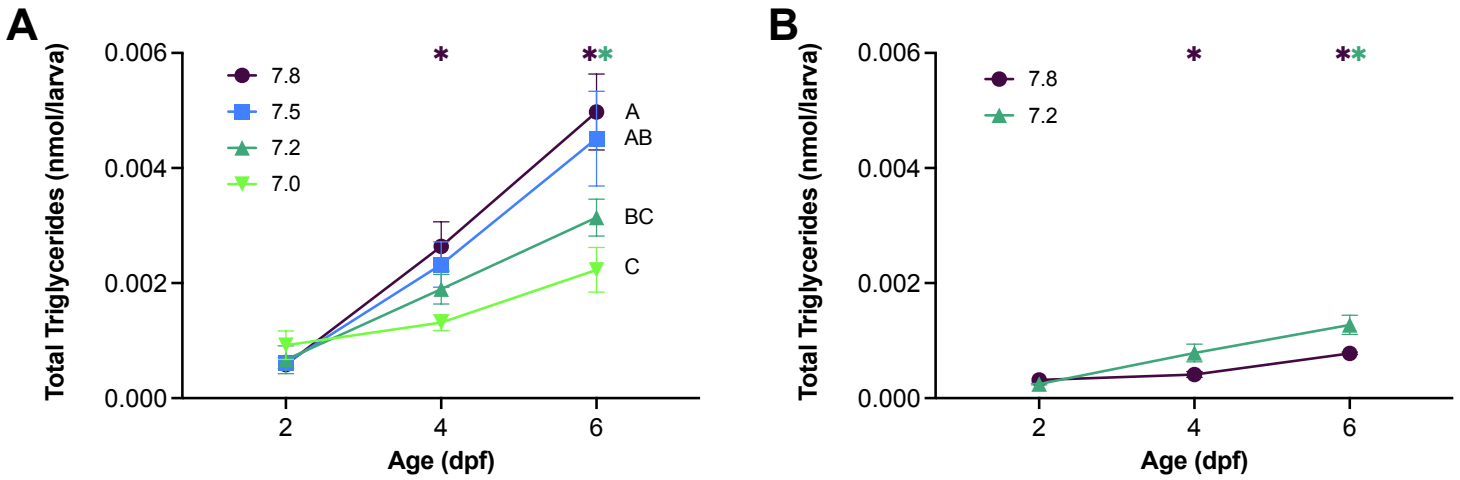

Figure 6. Total triglyceride content (TG) for larvae from (A) Page Rock reef and (B) Parrot's Rock reef shown as concentration per individual larvae (nmol/individual) at 2, 4, and 6 days postfertilization (dpf). Data shown as mean ± SEM (n=3 except Parrot's Rock reef control where n=2). Lack of visible error bars indicates they fall within the graphic illustration of the point itself. Capital letters indicate significant differences between pH treatments among Page Rock reef larvae at each time point, and color-coded asterisks indicate significant differences between populations at the corresponding pH level and time point (Sidák MCT, α=0.05).

Table S1. Water quality parameters for each individual tank replicate, denoted by capital letters, for larvae from both Page Rock reef and Parrot's Rock reef. Where applicable data shown as mean ± SD. Last three columns represent the water conditions for Page Rock reef larvae on the final day of experimentation. These values are indicative of how water conditions changed over the two days between each tank cleanings.

Treatment Replicate	Temperature (°C)	Salinity	pH AQ Sys (NBS scale)	Spec pH (NBS scale)	pCO_2 initial (µatm)	Ω_{Ar} initial	Total Alkalinity 6 dpf (µmol/kg)	pCO_2 6 dpf (µatm)	Ω_{Ar} 6 dpf
Page Rock Reef									
7.0 A	25.0 ± 0.1	18.04 ± 0.52	7.02 ± 0.02	6.99	6563	0.13	1647	6563	0.13
7.0 B	25.8 ± 0.6	18.05 ± 0.53	7.03 ± 0.05	7.04	5796	0.14	1631	5796	0.14
7.0 C	25.0 ± 0.1	18.06 ± 0.51	7.00 ± 0.03	6.96	7182	0.12	1646	7182	0.12
7.2 A	25.0 ± 0.1	18.04 ± 0.53	7.20 ± 0.02	7.23	3731	0.22	1615	3731	0.22
7.2 B	25.0 ± 0.1	18.04 ± 0.53	7.20 ± 0.03	7.24	3601	0.23	1619	3601	0.23
7.2 C	25.1 ± 0.3	17.99 ± 0.56	7.20 ± 0.02	7.24	3650	0.22	1615	3650	0.22
7.5 A	24.9 ± 0.1	18.04 ± 0.53	7.56 ± 0.04	7.57	1659	0.46	1607	1659	0.46
7.5 B	25.0 ± 0.1	18.05 ± 0.53	7.48 ± 0.04	7.42	2372	0.34	1618	2372	0.34
7.5 C	25.0 ± 0.1	18.03 ± 0.54	7.44 ± 0.05	7.47	2107	0.38	1620	2107	0.38
7.8 A	25.0 ± 0.1	18.03 ± 0.46	7.78 ± 0.03	7.80	957	0.76	1611	957	0.76
7.8 B	25.0 ± 0.1	18.06 ± 0.53	7.79 ± 0.02	7.70	1215	0.63	1629	1215	0.63
7.8 C	25.0 ± 0.1	18.05 ± 0.52	7.76 ± 0.05	7.77	1018	0.73	1620	1018	0.73
Parrot's Rock Reef									
7.2 A	25.0 ± 0.1	12.87 ± 0.12	7.20 ± 0.01	7.19	3066	0.12	-	-	-
7.2 B	25.0 ± 0.2	13.24 ± 0.82	7.21 ± 0.02	7.25	2653	0.13	-	-	-
7.2 C	25.0 ± 0.3	12.86 ± 0.11	7.18 ± 0.04	7.04	4322	0.08	-	-	-
7.8 A	25.0 ± 0.3	13.15 ± 0.66	7.79 ± 0.02	7.74	824	0.40	-	-	-
7.8 B	25.0 ± 0.2	12.88 ± 0.10	7.76 ± 0.04	7.64	1041	0.32	-	-	-

Table S2. Full two-way ANOVA results for all metrics assessed within this study for larvae from Page Rock reef. Significance threshold for all tests was set at 0.05. Effect size (ω^2) was calculated following Olejnik and Algina (2000) and Lakens (2013).

Metric	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Page Rock Reef							
Survival							
	Time x pH	9	641.7	71.3	F (9, 32) = 1.193	0.333	0.0023
	Time	3	40590	13530	F (3, 32) = 226.4	<0.0001	0.8990
	pH	3	1747	582.2	F (3, 32) = 9.741	<0.0001	0.0349
	Residual	32	1913	59.77	-	-	-
Shell Length							
	Time x pH	6	7157	1193	F (6, 348) = 9.959	<0.0001	0.0372
	Time	2	95180	47590	F (2, 348) = 397.3	<0.0001	0.5481
	pH	3	29090	9696	F (3, 348) = 80.96	<0.0001	0.1659
	Residual	348	41680	119.8	-	-	-
Total Protein Content							
	Time x pH	6	276.8	46.14	F (6, 24) = 6.159	0.0005	0.1313
	Time	2	1062	531.1	F (2, 24) = 70.89	<0.0001	0.5928
	pH	3	240.4	80.13	F (3, 24) = 10.70	<0.0001	0.1234
	Residual	24	179.8	7.491	-	-	
Nonenzymatic antioxidant potential							
	Time x pH	6	91.53	15.25	F (6, 24) = 0.7628	0.6062	-0.0311
	Time	2	280.7	140.4	F (2, 24) = 7.018	0.004	0.2631
	pH	3	42.86	14.29	F (3, 24) = 0.7144	0.553	-0.0187
	Residual	24	480	20	-	-	
MDA content							
	Time x pH	6	8.817E-04	1.469E-04	F (6, 24) = 0.7306	0.6296	-0.0326
	Time	2	3.744E-03	1.872E-03	F (2, 24) = 9.307	0.0010	0.3351
	pH	3	3.183E-04	1.061E-04	F (3, 24) = 0.5275	0.6676	-0.0286
	Residual	24	4.827E-03	2.011E-04	-	-	

NKA activity							
	Time x pH	6	8.804	1.467	F (6, 24) = 3.05	0.0232	0.138
	Time	2	20.54	10.27	F (2, 24) = 21.34	<0.0001	0.4568
	pH	3	1.485	0.4949	F (3, 24) = 1.029	0.3975	0.001
	Residual	24	11.55	0.4811	-	-	-
TG content							
	Time x pH	6	8.908E-06	1.485E-06	F (6, 24) = 3.087	0.022	0.0714
	Time	2	5.489E-05	2.745E-05	F (2, 24) = 57.07	<0.0001	0.6395
	pH	3	8.530E-06	2.843E-06	F (3, 24) = 5.913	0.0036	0.084
	Residual	24	1.154E-05	4.809E-07	-	-	-

Table S3. Full two-way ANOVA results for all metrics assessed within this study for larvae from Parrot's Rock reef. Significance threshold for all tests was set at 0.05. Effect size (ω^2) was calculated following Olejnik and Algina (2000) and Lakens (2013).

Metric	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Parrot's Rock Reef							
Survival							
	Time x pH	3	576.7	192.2	F (3, 12) = 1.224	0.3437	0.0076
	Time	3	9612	3204	F (3, 12) = 20.39	<0.0001	0.657
	pH	1	1681	1681	F (1, 12) = 10.7	0.0067	0.1095
	Residual	12	1885	157.1	-	-	-
Shell Length							
	Time x pH	2	254.5	127.3	F (2, 144) = 2.144	0.1209	0.0044
	Time	2	20770	10390	F (2, 144) = 175	<0.0001	0.6622
	pH	1	1557	1557	F (1, 144) = 26.24	<0.0001	0.048
	Residual	144	8546	59.35	-	-	-
Total Protein Content							
	Time x pH	2	2.651	1.326	F (2, 9) = 0.5774	0.5809	-0.0058
	Time	2	279.8	139.9	F (2, 9) = 60.92	<0.0001	0.816
	pH	1	31.84	31.84	F (1, 9) = 13.87	0.0047	0.0876
	Residual	9	20.66	2.296			
Nonenzymatic antioxidant potential							
	Time x pH	2	9.482	4.741	F (2, 9) = 0.3977	0.6831	-0.0778
	Time	2	45.68	22.84	F (2, 9) = 1.916	0.2027	0.1184
	pH	1	10.13	10.13	F (1, 9) = 0.8498	0.3807	-0.0097
	Residual	9	107.3	11.92	-	-	-
MDA content							
	Time x pH	2	1.185E-04	5.925E-05	F (2, 9) = 1.86	0.2107	0.0264
	Time	2	1.638E-03	8.191E-04	F (2, 9) = 25.72	0.0002	0.758
	pH	1	2.081E-06	2.081E-06	F (1, 9) = 0.06535	0.8040	-0.0143

	Residual	9	2.867E-04	3.185E-05			
NKA activity							
	Time x pH	2	0.3681	0.1841	F (2, 9) = 1.538	0.2663	0.0120
	Time	2	8.672	4.336	F (2, 9) = 36.24	<0.0001	0.7819
	pH	1	0.5479	0.5479	F (1, 9) = 4.58	0.061	0.0397
	Residual	9	1.077	0.1196	-	-	-
TG content							
	Time x pH	2	2.154E-07	1.077E-07	F (2, 9) = 3.081	0.0956	0.0673
	Time	2	1.344E-06	6.720E-07	F (2, 9) = 19.23	0.0006	0.5896
	pH	1	2.521E-07	2.521E-07	F (1, 9) = 7.212	0.025	0.1005
	Residual	9	3.145E-07	3.495E-08	-	-	-

Table S4. Full three-way ANOVA results for all metrics assessed within this study for the comparison of larvae from each reef at pH 7.8 and 7.2. Significance threshold for all tests was set at 0.05. Effect size (ω^2) was calculated following Olejnik and Algina (2000) and Lakens (2013).

Metric	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Reef Comparison							
Survival							
	Time	3	25150	8384	F (3, 28) = 91.84	<0.0001	0.7709
	Reef	1	953.7	953.7	F (1, 28) = 10.45	0.0031	0.0267
	pH	1	371.5	371.5	F (1, 28) = 4.07	0.0533	0.0087
	Time x Reef	3	639.7	213.2	F (3, 28) = 2.336	0.0953	0.0113
	Time x pH	3	172.5	57.5	F (3, 28) = 0.6299	0.6018	-0.0031
	Reef x pH	1	1751	1751	F (1, 28) = 19.18	0.0002	0.0514
	Time x Reef x pH	3	584.5	194.8	F (3, 28) = 2.134	0.1183	0.0096
	Residual	28	2556	91.29	-	-	-
Shell Length							
	Time	2	78850	39420	F (2, 318) = 584	<0.0001	0.6593
	Reef	1	5568	5568	F (1, 318) = 82.48	<0.0001	0.0461
	pH	1	7207	7207	F (1, 318) = 106.8	<0.0001	0.0598
	Time x Reef	2	4382	2191	F (2, 318) = 32.46	<0.0001	0.0356
	Time x pH	2	286.7	143.4	F (2, 318) = 2.124	0.1213	0.0013
	Reef x pH	1	679.5	679.5	F (1, 318) = 10.07	0.0017	0.0051
	Time x Reef x pH	2	877.5	438.8	F (2, 318) = 6.499	0.0017	0.0062
	Residual	318	21470	67.51	-	-	-
Total Protein Content							
	Time	2	956.1	478	F (2, 21) = 158.4	<0.0001	0.7662
	Reef	1	1.014	1.014	F (1, 21) = 0.3361	0.5683	-0.0016
	pH	1	92.76	92.76	F (1, 21) = 30.73	<0.0001	0.0724
	Time x Reef	2	53.8	26.9	F (2, 21) = 8.912	0.0016	0.0385
	Time x pH	2	47.18	23.59	F (2, 21) = 7.815	0.0029	0.0332

Reef x pH	1	1.486	1.486	F (1, 21) = 0.4922	0.4907	-0.0012
Time x Reef x pH	2	21.08	10.54	F (2, 21) = 3.493	0.049	0.0121
Residual	21	63.39	3.018	-	-	-
Nonenzymatic antioxidant potential						
Time	2	275.8	137.9	F (2, 21) = 13.96	0.0001	0.365
Reef	1	132.6	132.6	F (1, 21) = 13.42	0.0014	0.175
pH	1	4.174	4.174	F (1, 21) = 0.4226	0.5227	-0.0081
Time x Reef	2	44.26	22.13	F (2, 21) = 2.241	0.1312	0.0349
Time x pH	2	1.034	0.517	F (2, 21) = 0.05235	0.9491	-0.0267
Reef x pH	1	7.299	7.299	F (1, 21) = 0.7391	0.3997	-0.0037
Time x Reef x pH	2	19.01	9.507	F (2, 21) = 0.9626	0.3981	-0.0011
Residual	21	207.4	9.876	-	-	-
MDA content						
Time	2	1.126E-04	5.632E-05	F (2, 21) = 1.909	0.173	0.0109
Reef	1	9.105E-04	9.105E-04	F (1, 21) = 30.87	<0.0001	0.1783
pH	1	6.004E-06	6.004E-06	F (1, 21) = 0.2036	0.6565	-0.0048
Time x Reef	2	3.041E-03	1.520E-03	F (2, 21) = 51.55	<0.0001	0.6032
Time x pH	2	9.113E-05	4.556E-05	F (2, 21) = 1.545	0.2367	0.0065
Reef x pH	1	2.117E-05	2.117E-05	F (1, 21) = 0.7177	0.4064	-0.0017
Time x Reef x pH	2	1.108E-04	5.542E-05	F (2, 21) = 1.879	0.1775	0.0105
Residual	21	6.194E-04	2.950E-05	-	-	-
NKA activity						
Time	2	9.327	4.664	F (2, 21) = 4.451	0.0245	0.1743
Reef	1	1.406	1.406	F (1, 21) = 1.342	0.2597	0.0086
pH	1	0.1966	0.1966	F (1, 21) = 0.1876	0.6693	-0.0205
Time x Reef	2	1.795	0.8973	F (2, 21) = 0.8564	0.439	-0.0073
Time x pH	2	3.831	1.915	F (2, 21) = 1.828	0.1854	0.0418
Reef x pH	1	0.4358	0.4358	F (1, 21) = 0.4159	0.526	-0.0148
Time x Reef x pH	2	1.457	0.7283	F (2, 21) = 0.6950	0.5102	-0.0154

Residual	21	22	1.048	-	-	-
TG content						
Time	2	2.330E-05	1.165E-05	F (2, 21) = 45.25	<0.0001	0.3404
Reef	1	2.260E-05	2.260E-05	F (1, 21) = 87.78	<0.0001	0.3338
pH	1	6.401E-07	6.401E-07	F (1, 21) = 2.486	0.1298	0.0057
Time x Reef	2	9.625E-06	4.812E-06	F (2, 21) = 18.69	<0.0001	0.1361
Time x pH	2	6.479E-07	3.239E-07	F (2, 21) = 1.258	0.3047	0.002
Reef x pH	1	2.398E-06	2.398E-06	F (1, 21) = 9.313	0.0061	0.032
Time x Reef x pH	2	2.065E-06	1.033E-06	F (2, 21) = 4.011	0.0335	0.0232
Residual	21	5.407E-06	2.575E-07	-	-	-

Table S5. Results from Sidák multiple comparisons test on *a priori* contrasts for all metrics assessed within this study for larvae from Page Rock reef. Significance threshold for all tests was set at 0.05.

Metric	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Page Rock Reef						
Survival						
0 dpf						
	7.8 vs. 7.5	32	0	-17.7 to 17.7	0	>0.9999
	7.8 vs. 7.2	32	0	-17.7 to 17.7	0	>0.9999
	7.8 vs. 7.0	32	0	-17.7 to 17.7	0	>0.9999
	7.5 vs. 7.2	32	0	-17.7 to 17.7	0	>0.9999
	7.5 vs. 7.0	32	0	-17.7 to 17.7	0	>0.9999
	7.2 vs. 7.0	32	0	-17.7 to 17.7	0	>0.9999
2 dpf						
	7.8 vs. 7.5	32	-1.284	-18.98 to 16.42	0.203	>0.9999
	7.8 vs. 7.2	32	-7.571	-25.27 to 10.13	1.199	0.8061
	7.8 vs. 7.0	32	13.04	-4.66 to 30.74	2.065	0.2511
	7.5 vs. 7.2	32	-6.287	-23.99 to 11.41	0.996	0.9069
	7.5 vs. 7.0	32	14.32	-3.377 to 32.02	2.269	0.1679
	7.2 vs. 7.0	32	20.61	2.91 to 38.31	3.265	0.0156
4 dpf						
	7.8 vs. 7.5	32	-0.778	-18.48 to 16.92	0.123	>0.9999
	7.8 vs. 7.2	32	-13.15	-30.85 to 4.546	2.084	0.2426
	7.8 vs. 7.0	32	13.32	-4.379 to 31.02	2.11	0.2307
	7.5 vs. 7.2	32	-12.38	-30.07 to 5.324	1.96	0.3044
	7.5 vs. 7.0	32	14.1	-3.601 to 31.8	2.233	0.1806
	7.2 vs. 7.0	32	26.47	8.774 to 44.17	4.194	0.0012
6 dpf						
	7.8 vs. 7.5	32	2.472	-15.23 to 20.17	0.392	0.9992
	7.8 vs. 7.2	32	-6.917	-24.62 to 10.78	1.096	0.8623

	7.8 vs. 7.0	32	13.72	-3.981 to 31.42	2.173	0.2039
	7.5 vs. 7.2	32	-9.389	-27.09 to 8.31	1.487	0.614
	7.5 vs. 7.0	32	11.25	-6.453 to 28.94	1.781	0.4106
	7.2 vs. 7.0	32	20.63	2.936 to 38.33	3.269	0.0154
Shell Length						
2 dpf						
	7.8 vs. 7.5	348	2.862	-4.615 to 10.34	1.013	0.8938
	7.8 vs. 7.2	348	7.144	-0.3332 to 14.62	2.528	0.0694
	7.8 vs. 7.0	348	11.87	4.392 to 19.35	4.2	0.0002
	7.5 vs. 7.2	348	4.282	-3.195 to 11.76	1.515	0.5682
	7.5 vs. 7.0	348	9.007	1.530 to 16.48	3.188	0.0094
	7.2 vs. 7.0	348	4.725	-2.752 to 12.2	1.672	0.4519
4 dpf						
	7.8 vs. 7.5	348	6.267	-1.21 to 13.74	2.218	0.1525
	7.8 vs. 7.2	348	11.53	4.055 to 19.01	4.081	0.0003
	7.8 vs. 7.0	348	21.05	13.58 to 28.53	7.451	<0.0001
	7.5 vs. 7.2	348	5.264	-2.213 to 12.74	1.863	0.3245
	7.5 vs. 7.0	348	14.79	7.309 to 22.26	5.233	<0.0001
	7.2 vs. 7.0	348	9.522	2.045 to 17	3.37	0.005
6 dpf						
	7.8 vs. 7.5	348	15.03	7.553 to 22.51	5.319	<0.0001
	7.8 vs. 7.2	348	18.54	11.07 to 26.02	6.562	<0.0001
	7.8 vs. 7.0	348	41.64	34.17 to 49.12	14.74	<0.0001
	7.5 vs. 7.2	348	3.512	-3.965 to 10.99	1.243	0.7656
	7.5 vs. 7.0	348	26.61	19.14 to 34.09	9.419	<0.0001
	7.2 vs. 7.0	348	23.1	15.62 to 30.58	8.176	<0.0001
Total Protein						
2 dpf						
	7.8 vs. 7.5	24	-1.561	-7.965 to 4.844	0.6983	0.9827

	7.8 vs. 7.2	24	-0.429	-6.834 to 5.976	0.1919	>0.9999
	7.8 vs. 7.0	24	-1.33	-7.735 to 5.075	0.5951	0.9925
	7.5 vs. 7.2	24	1.132	-5.273 to 7.537	0.5064	0.9969
	7.5 vs. 7.0	24	0.231	-6.174 to 6.636	0.1032	>0.9999
	7.2 vs. 7.0	24	-0.901	-7.306 to 5.504	0.4032	0.9991
4 dpf						
	7.8 vs. 7.5	24	1.613	-4.792 to 8.018	0.7219	0.9796
	7.8 vs. 7.2	24	2.674	-3.731 to 9.079	1.197	0.812
	7.8 vs. 7.0	24	4.765	-1.64 to 11.17	2.132	0.2339
	7.5 vs. 7.2	24	1.061	-5.344 to 7.466	0.4747	0.9978
	7.5 vs. 7.0	24	3.152	-3.253 to 9.556	1.41	0.6761
	7.2 vs. 7.0	24	2.091	-4.314 to 8.496	0.9355	0.9305
6 dpf						
	7.8 vs. 7.5	24	2.494	-3.91 to 8.899	1.116	0.8553
	7.8 vs. 7.2	24	9.263	2.858 to 15.67	4.145	0.0022
	7.8 vs. 7.0	24	16.19	9.781 to 22.59	7.243	<0.0001
	7.5 vs. 7.2	24	6.768	0.3636 to 13.17	3.029	0.0343
	7.5 vs. 7.0	24	13.69	7.286 to 20.1	6.127	<0.0001
	7.2 vs. 7.0	24	6.923	0.5181 to 13.33	3.098	0.0291
Nonenzymatic antioxidant potential						
2 dpf						
	7.8 vs. 7.5	24	7.771	-2.694 to 18.24	2.128	0.2356
	7.8 vs. 7.2	24	2.297	-8.168 to 12.76	0.629	0.9899
	7.8 vs. 7.0	24	2.917	-7.548 to 13.38	0.799	0.9665
	7.5 vs. 7.2	24	-5.474	-15.94 to 4.991	1.499	0.6145
	7.5 vs. 7.0	24	-4.853	-15.32 to 5.611	1.329	0.7304
	7.2 vs. 7.0	24	0.6204	-9.844 to 11.09	0.1699	>0.9999
4 dpf						
	7.8 vs. 7.5	24	-2.231	-12.7 to 8.234	0.6109	0.9914

	7.8 vs. 7.2	24	-1.409	-11.87 to 9.056	0.3858	0.9993
	7.8 vs. 7.0	24	-4.241	-14.71 to 6.224	1.161	0.8316
	7.5 vs. 7.2	24	0.822	-9.643 to 11.29	0.2251	>0.9999
	7.5 vs. 7.0	24	-2.01	-12.47 to 8.455	0.5505	0.995
	7.2 vs. 7.0	24	-2.832	-13.3 to 7.633	0.7756	0.971
6 dpf						
	7.8 vs. 7.5	24	0.2125	-10.25 to 10.68	0.05819	>0.9999
	7.8 vs. 7.2	24	-0.1894	-10.65 to 10.28	0.05187	>0.9999
	7.8 vs. 7.0	24	-2.083	-12.55 to 8.382	0.5704	0.994
	7.5 vs. 7.2	24	-0.4019	-10.87 to 10.06	0.1101	>0.9999
	7.5 vs. 7.0	24	-2.295	-12.76 to 8.17	0.6286	0.99
	7.2 vs. 7.0	24	-1.893	-12.36 to 8.571	0.5186	0.9964
MDA content						
2 dpf						
	7.8 vs. 7.5	24	-1.434E-02	-4.753E-02 to 1.885E-02	1.238	0.7875
	7.8 vs. 7.2	24	8.413E-03	-2.477E-02 to 4.160E-02	0.7266	0.9789
	7.8 vs. 7.0	24	5.109E-03	-2.808E-02 to 3.830E-02	0.4412	0.9985
	7.5 vs. 7.2	24	2.275E-02	-1.043E-02 to 5.594E-02	1.965	0.3149
	7.5 vs. 7.0	24	1.945E-02	-1.374E-02 to 5.264E-02	1.68	0.4895
	7.2 vs. 7.0	24	-3.304E-03	-3.649E-02 to 2.988E-02	0.2854	0.9999
4 dpf						
	7.8 vs. 7.5	24	2.960E-04	-3.289E-02 to 3.348E-02	0.02556	>0.9999
	7.8 vs. 7.2	24	-1.946E-04	-3.338E-02 to 3.299E-02	0.01681	>0.9999
	7.8 vs. 7.0	24	-7.709E-03	-4.090E-02 to 2.548E-02	0.6657	0.9865
	7.5 vs. 7.2	24	-4.906E-04	-3.368E-02 to 3.270E-02	0.04237	>0.9999
	7.5 vs. 7.0	24	-8.005E-03	-4.119E-02 to 2.518E-02	0.6913	0.9836
	7.2 vs. 7.0	24	-7.514E-03	-4.070E-02 to 2.567E-02	0.6489	0.9882
6 dpf						
	7.8 vs. 7.5	24	-7.741E-04	-3.396E-02 to 3.241E-02	0.06685	>0.9999

	7.8 vs. 7.2	24	-7.395E-04	-3.393E-02 to 3.245E-02	0.06386	>0.9999
	7.8 vs. 7.0	24	-8.844E-03	-4.203E-02 to 2.434E-02	0.7637	0.9731
	7.5 vs. 7.2	24	3.458E-05	-3.315E-02 to 3.322E-02	2.986E-03	>0.9999
	7.5 vs. 7.0	24	-8.070E-03	-4.126E-02 to 2.512E-02	0.6969	0.9829
	7.2 vs. 7.0	24	-8.104E-03	-4.129E-02 to 2.508E-02	0.6999	0.9826
NKA activity						
2 dpf						
	7.8 vs. 7.5	24	1.638	0.01512 to 3.262	2.893	0.047
	7.8 vs. 7.2	24	0.9683	-0.6549 to 2.592	1.71	0.4693
	7.8 vs. 7.0	24	1.725	0.1018 to 3.348	3.046	0.0329
	7.5 vs. 7.2	24	-0.67	-2.293 to 0.9532	1.183	0.8197
	7.5 vs. 7.0	24	0.08667	-1.537 to 1.71	0.153	>0.9999
	7.2 vs. 7.0	24	0.7567	-0.8665 to 2.38	1.336	0.726
4 dpf						
	7.8 vs. 7.5	24	-0.4626	-2.086 to 1.161	0.8168	0.9627
	7.8 vs. 7.2	24	-0.5879	-2.211 to 1.035	1.038	0.8917
	7.8 vs. 7.0	24	-1.445	-3.068 to 0.1786	2.551	0.1008
	7.5 vs. 7.2	24	-0.1253	-1.749 to 1.498	0.2213	>0.9999
	7.5 vs. 7.0	24	-0.982	-2.605 to 0.6412	1.734	0.4534
	7.2 vs. 7.0	24	-0.8567	-2.48 to 0.7665	1.513	0.605
6 dpf						
	7.8 vs. 7.5	24	-0.01467	-1.638 to 1.609	0.02591	>0.9999
	7.8 vs. 7.2	24	-0.1505	-1.774 to 1.473	0.2657	>0.9999
	7.8 vs. 7.0	24	-0.8025	-2.426 to 0.8207	1.417	0.6715
	7.5 vs. 7.2	24	-0.1358	-1.759 to 1.487	0.2398	>0.9999
	7.5 vs. 7.0	24	-0.7879	-2.411 to 0.8353	1.391	0.6892
	7.2 vs. 7.0	24	-0.6521	-2.275 to 0.9711	1.151	0.837
TG content						
2 dpf						

	7.8 vs. 7.5	24	-2.825E-05	-1.651E-03 to 1.595E-03	0.04989	>0.9999
	7.8 vs. 7.2	24	-8.590E-05	-1.709E-03 to 1.537E-03	0.1517	>0.9999
	7.8 vs. 7.0	24	-3.391E-04	-1.962E-03 to 1.284E-03	0.5989	0.9922
	7.5 vs. 7.2	24	-5.765E-05	-1.680E-03 to 1.565E-03	0.1018	>0.9999
	7.5 vs. 7.0	24	-3.108E-04	-1.934E-03 to 1.312E-03	0.549	0.9951
	7.2 vs. 7.0	24	-2.532E-04	-1.876E-03 to 1.370E-03	0.4472	0.9984
4 dpf						
	7.8 vs. 7.5	24	3.151E-04	-1.308E-03 to 1.938E-03	0.5566	0.9947
	7.8 vs. 7.2	24	7.425E-04	-8.803E-04 to 2.365E-03	1.311	0.7421
	7.8 vs. 7.0	24	1.325E-03	-2.978E-04 to 2.948E-03	2.34	0.1563
	7.5 vs. 7.2	24	4.274E-04	-1.195E-03 to 2.050E-03	0.7548	0.9746
	7.5 vs. 7.0	24	1.010E-03	-6.129E-04 to 2.633E-03	1.784	0.4214
	7.2 vs. 7.0	24	5.825E-04	-1.040E-03 to 2.205E-03	1.029	0.8957
6 dpf						
	7.8 vs. 7.5	24	4.628E-04	-1.160E-03 to 2.086E-03	0.8174	0.9626
	7.8 vs. 7.2	24	1.834E-03	2.117E-04 to 3.457E-03	3.24	0.0207
	7.8 vs. 7.0	24	2.744E-03	1.121E-03 to 4.366E-03	4.846	0.0004
	7.5 vs. 7.2	24	1.372E-03	-2.511E-04 to 2.994E-03	2.422	0.1321
	7.5 vs. 7.0	24	2.281E-03	6.580E-04 to 3.903E-03	4.028	0.0029
	7.2 vs. 7.0	24	9.091E-04	-7.136E-04 to 2.532E-03	1.606	0.5401

Table S6. Results from Sidák multiple comparisons test on *a priori* contrasts for all metrics assessed within this study for larvae from Parrot's Rock reef. Significance threshold for all tests was set at 0.05.

Metric	Contrast	DF	Predicted (LS) mean diff.	95% CI of difference	t	Adjusted P Value
Parrot's Rock Reef						
Survival						
7.8 vs. 7.2						
	0 dpf	12	0	-33.46 to 33.46	0	>0.9999
	2 dpf	12	26.19	-7.265 to 59.65	2.289	0.1541
	4 dpf	12	21.95	-11.51 to 55.41	1.918	0.2811
	6 dpf	12	26.71	-6.747 to 60.17	2.334	0.1427
Shell Length						
7.8 vs. 7.2						
	2 dpf	144	6.492	1.12 to 11.86	2.919	0.0122
	4 dpf	144	9.875	4.502 to 15.25	4.44	<0.0001
	6 dpf	144	3.364	-2.009 to 8.736	1.512	0.3474
Total Protein						
7.8 vs. 7.2						
	2 dpf	9	1.848	-2.195 to 5.891	1.336	0.515
	4 dpf	9	3.145	-0.8976 to 7.188	2.274	0.14
	6 dpf	9	3.929	-0.1139 to 7.972	2.841	0.057
Nonenzymatic antioxidant potential						
7.8 vs. 7.2						
	2 dpf	9	-3.954	-13.17 to 5.258	1.255	0.5631
	4 dpf	9	-0.7906	-10 to 8.422	0.2508	0.9929
	6 dpf	9	-0.2876	-9.5 to 8.925	0.09126	0.9996
MDA content						
7.8 vs. 7.2						
	2 dpf	9	-2.019E-03	-1.708E-02 to 1.304E-02	0.3919	0.9741
	4 dpf	9	6.811E-03	-8.247E-03 to 2.187E-02	1.322	0.5232

6 dpf	9	-7.073E-03	-2.213E-02 to 7.985E-03	1.373	0.4938
NKA activity					
7.8 vs. 7.2					
2 dpf	9	-0.8422	-1.765 to 0.0807	2.667	0.0752
4 dpf	9	-0.1739	-1.097 to 0.749	0.5508	0.9337
6 dpf	9	-0.1543	-1.077 to 0.7686	0.4886	0.9521
TG content					
7.8 vs. 7.2					
2 dpf	9	7.420E-05	-4.246E-04 to 5.730E-04	0.4348	0.9653
4 dpf	9	-3.737E-04	-8.725E-04 to 1.251E-04	2.19	0.1595
6 dpf	9	-4.943E-04	-9.932E-04 to 4.471E-06	2.897	0.0521

Table S7. Results from Sidák multiple comparisons test on *a priori* contrasts for all metrics assessed within this study or the comparison of larvae from Page Rock reef and Parrot's Rock reef at pH 7.8 and 7.2. Significance threshold for all tests was set at 0.05.

Metric	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Reef Comparison						
Survival						
0 dpf						
	Parrot's 7.8 vs. Page 7.8	28	0	-34.83 to 34.83	0	>0.9999
	Parrot's 7.2 vs. Page 7.2	28	0	-31.16 to 31.16	0	>0.9999
2 dpf						
	Parrot's 7.8 vs. Page 7.8	28	38.4	3.566 to 73.23	4.403	0.0169
	Parrot's 7.2 vs. Page 7.2	28	4.634	-26.52 to 35.79	0.594	>0.9999
4 dpf						
	Parrot's 7.8 vs. Page 7.8	28	26.6	-8.228 to 61.44	3.05	0.4493
	Parrot's 7.2 vs. Page 7.2	28	-8.497	-39.65 to 22.66	1.089	>0.9999
6 dpf						
	Parrot's 7.8 vs. Page 7.8	28	24.07	-10.77 to 58.9	2.759	0.704
	Parrot's 7.2 vs. Page 7.2	28	-9.562	-40.72 to 21.59	1.226	>0.9999
Shell Length						
2 dpf						
	Parrot's 7.8 vs. Page 7.8	318	-0.1578	-8.207 to 7.891	0.06654	>0.9999
	Parrot's 7.2 vs. Page 7.2	318	0.4939	-6.706 to 7.693	0.2328	>0.9999
4 dpf						
	Parrot's 7.8 vs. Page 7.8	318	-8.149	-16.2 to -0.09981	3.436	0.0432
	Parrot's 7.2 vs. Page 7.2	318	-6.493	-13.69 to 0.7069	3.06	0.1466
6 dpf						
	Parrot's 7.8 vs. Page 7.8	318	-25.46	-33.51 to -17.41	10.74	<0.0001
	Parrot's 7.2 vs. Page 7.2	318	-10.29	-17.48 to -3.086	4.848	0.0001
Total Protein						

2 dpf					
Parrot's 7.8 vs. Page 7.8	21	2.248	-3.978 to 8.473	1.417	>0.9999
Parrot's 7.2 vs. Page 7.2	21	-0.0292	-5.597 to 5.539	0.02058	>0.9999
4 dpf					
Parrot's 7.8 vs. Page 7.8	21	2.059	-4.167 to 8.284	1.298	>0.9999
Parrot's 7.2 vs. Page 7.2	21	1.587	-3.981 to 7.155	1.119	>0.9999
6 dpf					
Parrot's 7.8 vs. Page 7.8	21	-6.667	-12.89 to -0.442	4.204	0.026
Parrot's 7.2 vs. Page 7.2	21	-1.333	-6.902 to 4.235	0.94	>0.9999
Nonenzymatic antioxidant potential					
2 dpf					
Parrot's 7.8 vs. Page 7.8	21	-10.46	-21.72 to 0.8004	3.646	0.0949
Parrot's 7.2 vs. Page 7.2	21	-4.209	-14.28 to 5.863	1.64	0.9997
4 dpf					
Parrot's 7.8 vs. Page 7.8	21	-2.687	-13.95 to 8.573	0.9368	>0.9999
Parrot's 7.2 vs. Page 7.2	21	-3.306	-13.38 to 6.766	1.288	>0.9999
6 dpf					
Parrot's 7.8 vs. Page 7.8	21	-1.931	-13.19 to 9.33	0.673	>0.9999
Parrot's 7.2 vs. Page 7.2	21	-1.832	-11.90 to 8.239	0.7142	>0.9999
MDA content					
2 dpf					
Parrot's 7.8 vs. Page 7.8	21	-2.209E-02	-4.155E-02 to -2.630E-03	4.456	0.0143
Parrot's 7.2 vs. Page 7.2	21	-1.166E-02	-2.906E-02 to 5.748E-03	2.629	0.6478
4 dpf					
Parrot's 7.8 vs. Page 7.8	21	2.685E-02	7.390E-03 to 4.631E-02	5.416	0.0015
Parrot's 7.2 vs. Page 7.2	21	1.984E-02	2.438E-03 to 3.725E-02	4.475	0.0137
6 dpf					
Parrot's 7.8 vs. Page 7.8	21	2.237E-02	2.906E-03 to 4.183E-02	4.511	0.0126
Parrot's 7.2 vs. Page 7.2	21	2.870E-02	1.129E-02 to 4.610E-02	6.472	0.0001

NKA activity					
2 dpf					
Parrot's 7.8 vs. Page 7.8	21	0.4364	-3.231 to 4.104	0.467	>0.9999
Parrot's 7.2 vs. Page 7.2	21	0.06423	-3.216 to 3.345	0.07685	>0.9999
4 dpf					
Parrot's 7.8 vs. Page 7.8	21	-0.8044	-4.472 to 2.863	0.1606	>0.9999
Parrot's 7.2 vs. Page 7.2	21	-0.6696	-3.95 to 2.611	0.2202	>0.9999
6 dpf					
Parrot's 7.8 vs. Page 7.8	21	-1.59	-5.258 to 2.078	1.701	0.9993
Parrot's 7.2 vs. Page 7.2	21	0.04778	-3.233 to 3.328	0.05717	>0.9999
TG content					
2 dpf					
Parrot's 7.8 vs. Page 7.8	21	-2.633E-04	-2.081E-03 to 1.555E-03	0.5685	>0.9999
Parrot's 7.2 vs. Page 7.2	21	-4.234E-04	-2.050E-03 to 1.203E-03	1.022	>0.9999
4 dpf					
Parrot's 7.8 vs. Page 7.8	21	-2.227E-03	-4.045E-03 to -4.088E-04	4.808	0.0062
Parrot's 7.2 vs. Page 7.2	21	-1.111E-03	-2.737E-03 to 5.154E-04	2.681	0.6053
6 dpf					
Parrot's 7.8 vs. Page 7.8	21	-4.194E-03	-6.013E-03 to -2.376E-03	9.056	<0.0001
Parrot's 7.2 vs. Page 7.2	21	-1.866E-03	-3.492E-03 to -2.395E-04	4.503	0.0128

Chapter 3

Variation in physiological tolerances among US east coast populations of the hard clam, *Mercenaria mercenaria*

Abstract

Climate change threatens the persistence of the ecologically and economically important hard clam, *Mercenaria mercenaria*. This species provides important ecosystem services while also serving as a valuable aquaculture species along the eastern coast of the United States. As climate change continues, increasing temperatures and changing precipitation patterns will expose *M. mercenaria* to more stressful conditions, which could negatively impact the benefits this species provides. However, little is known about potential population-level variability in physiological tolerance to environmental stress throughout the biogeographic range of this species, which if present could serve to lessen the impacts of climate change on the hard clam aquaculture industry. To address this knowledge gap, juvenile hard clams from five populations (Cape Cod, MA; Great Bay, NJ; Mobjack Bay, VA; Pocomoke Sound, VA; and Wachapreague, VA) were exposed to a series of elevated temperature conditions ranging from 25°C to 35°C and a series of low salinity conditions from 20 down to 10. Juvenile clams were specifically targeted as they are more susceptible to environmental stress compared to their adult life stage. Oxygen consumption rates were measured to assess overall metabolic stress under each experimental condition. An additional more extreme elevated temperature exposure (36°C) was conducted for assessments of mortality within each population. Slight increases in oxygen consumption at elevated temperatures were observed within several populations, but no significant differences were detected. Results of the mortality trial revealed population-level differences, where clams from Wachapreague and Mobjack Bay, VA were more tolerant of this more extreme exposure. When exposed to low salinity, clams from Mobjack Bay, VA maintained higher oxygen consumption rates compared to other populations, which could be a result of selection for low salinity tolerant genotypes in their native environment within Chesapeake Bay, where low

salinity conditions are more common. However, clams from all populations showed marked declines in oxygen consumption rates when exposed to salinities below 15, revealing that even in the potentially more resilient populations, low salinity will likely be a factor limiting their future persistence. These findings demonstrate the potential for population-level variability throughout the geographic range of *M. mercenaria*, but more work is needed to fully assess the extent of and the role that this variability may play in the face of a changing climate.

Introduction

The hard clam, *Mercenaria mercenaria*, is an important ecosystem engineer due to its burrowing behavior in soft sediments while also serving as one of the primary shellfish aquaculture species in Virginia (Kraeuter and Castagna, 2001; Murphy et al. 2015; Hudson, 2019). Due to the important roles this species plays, it is of growing concern how *M. mercenaria* will respond to climate change. Increasing water temperatures and more intense low salinity events are two such changes that are of significant concerns due to their impacts on the growth and survival of hard clams (Ivanina et al, 2013; Matoo et al, 2013). Furthermore, little is known about population-level variability in physiological tolerance to environmental stress within hard clams, which makes understanding how *M. mercenaria* will be impacted by climate change throughout its biogeographic range difficult.

Previous studies have already observed negative effects of elevated water temperatures on *M. mercenaria* in several key ways. First, elevated temperatures are known to increase metabolic rate, which can increase the energy demand for both active and resting metabolism (Pörtner, 2012; Matoo et al., 2013; Specht and Fuchs, 2018). As energy demand increases, more food must be ingested to maintain energy supply for stress response mechanisms alongside

normal growth and development, although it is not known how food availability will change with increasing water temperature across the geographic range of this species and, therefore, if elevating ingestion rates is a viable strategy that *M. mercenaria* could employ. Second, increased mortality rates of hard clams have been observed in response to sustained increases in water temperature, which could have serious consequences for the aquaculture industry if larger percentages of yearly crops do not survive to market size (Ivanina et al., 2013). Furthermore, elevated temperatures have been shown to increase the energetic cost of suspension feeding in *M. mercenaria*, which could make this species more susceptible to ocean warming as this additional energy allocated to filter feeding would no longer be available to combat heat stress or to support growth (Specht and Fuchs, 2018). A recent study by Hu et al. (2022) has provided new insight into cellular responses of adult hard clams exposed to temperature stress, including increased levels of oxidized lipids and the accumulation of osmolytes known to protect proteins from heat stress. While the effects of elevated temperatures on hard clams are wide ranging and warrant continued study, especially as ocean temperature projections along the eastern coast of the United States show anywhere from a 2°C to upwards of 5°C increase by the end of the century alone, it is also imperative to understand how other changes in environmental factors will impact *M. mercenaria* in the future (Muhling et al., 2018; Karmalkar and Horton, 2021; Masson-Delmotte et al., 2021).

Exposure to low salinity is another environmental stressor that is known to have deleterious effects on hard clams. While *M. mercenaria* can withstand short exposures to small reductions in salinity with minimal consequences, more extreme low salinity events as well as more prolonged exposures can result in increased mortality; however, the majority of studies conducted on juvenile hard clams have focused on commercial broodstock lines, which are

unlikely to be representative of all wild populations (Baker et al., 2005; Dickinson et al., 2013). As total annual precipitation as well as storm intensity are projected to increase along the eastern coast of the United States with continuing climate change, it is likely that hard clams will face more frequent low salinity exposures and potentially more extreme low salinity events in the future, which could threaten the viability of both aquaculture broodstock lines and wild populations (Najjar et al., 2010; Donat et al., 2016; Walsh et al., 2016; Muhling et al., 2018). However, it is important to note that uncertainty persists within many climate models with respect to changes in precipitation patterns at local scales due to the highly dynamic nature of coastal environments, especially within Chesapeake Bay region itself (Muhling et al., 2018; Ross et al., 2021). Beyond increasing mortality, more prolonged low salinity exposures have also been shown to reduce growth in commercial clam stocks even in the presence of increased food availability (Carmichael et al., 2004). Reduced growth is problematic for aquaculture operations as time to market would increase, elevating the cost of production. For wild populations, reduced growth rates would increase time to sexual maturity, and thus more individuals could be lost to predation and disease before they are able to reproduce.

While shifts in environmental parameters due to climate change are known to negatively impact hard clams, they do not equally affect all of the life stages of this species. In particular, the larval and juvenile life stages of many molluscan species are known to be more susceptible to environmental stress compared to their adult life stage (Dupont et al., 2010; Talmage and Gobler, 2010; Talmage and Gobler, 2011). If future ocean conditions exceed the stress tolerance of immature clams, then both wild and cultured clam stocks will suffer as yearly spawning classes will begin to fail, and juveniles will fail to survive to sexual maturity. Many studies on juvenile hard clams have specifically focused on assessing environmental stress responses in seed clams

obtained from commercial hatcheries, which likely limits the accuracy of predictions about how climate change may impact wild populations (Ringwood and Keppler, 2002; Carmichael et al., 2004; Baker et al., 2005; Dickinson et al., 2013; Ivanina et al., 2013; Matoo et al., 2013; Portilla et al., 2015; Specht and Fuchs, 2018; Nascimento-Schulze et al., 2021). For aquacultural operations specifically, understanding wild juvenile clam stress tolerance is vital, as wild populations can serve as the foundation for selective breeding programs for more resilient broodstock lines. While larval clams are cultured in hatcheries where water conditions could be buffered against negative impacts from climate change if needed, juveniles are planted by growers in the natural environment with fewer options for mitigation of stressful environmental conditions (Gray et al., 2022).

Juvenile hard clams from different populations are unlikely to respond to environmental stress similarly, as previous work has identified a high level of genetic variability in this species as well as variations in growth and disease resistance between different populations (Hilbish et al., 1993; Ropp, 2021 Farhat et al., 2022). As genetic variability is high, there is potential for variations to have arisen within stress response mechanisms across the geographic range of *M. mercenaria*. However, while improvements are being made in genome annotations for *M. mercenaria*, a fully annotated genome remains to be completed, and the need for genomic sequencing throughout the full biogeographic range of this species has yet to be addressed (Song et al., 2021; Farhat et al., 2022). Lacking these genomic resources limits the ability of future sequencing efforts to identify unique alleles within a population that could be responsible for improved stress tolerance. Due to these limitations, exploring physiological variation between hard clam populations is challenging on the genetic level and instead may be better approached through experimental physiology.

To this end, the following study explored if population-level variability in physiological tolerance to elevated temperatures and decreased salinities exists in juvenile *M. mercenaria*. Five geographically distinct populations were selected for analysis: Wachapreague, VA; Mobjack Bay, VA; Pocomoke, VA; Great Bay, NJ; and Cape Cod, MA. Mobjack Bay, VA and Pocomoke, VA populations persist within Chesapeake Bay and encounter both more frequent and more sustained low salinity events compared to the other three populations that were assessed. These five populations span a latitudinal gradient, which allows for the evaluation of potential latitudinal patterns in thermotolerance like those previously observed for other marine invertebrate species (Schroer et al., 2009; Byrne, 2011). To assess stress tolerance, juvenile clams from each population were exposed to a series of elevated temperature and lower salinity conditions. A six-hour exposure time for elevated temperature trials was chosen as it represents a realistic acute exposure during the hottest part of a summer day. For salinity trials, 48 h exposures were used as this duration is a relevant timescale for both the transfer of juveniles from a nursery setting to a grow-out plot, where water conditions can often differ, and the duration of a salinity depression due to a severe storm event in coastal regions where most clams are grown for aquaculture. After each exposure, oxygen consumption rates were measured as a proxy for energy demand to assess overall stress levels. By exposing clams to a range of each stressor, the degree to which this species can modulate its metabolic rate could be assessed while also monitoring for any signs of metabolic depression if its tolerances were exceeded. Lastly, mortality trials were conducted at more extreme conditions to evaluate differences in tolerance limitations among populations. Characterizing population-level differences in physiological tolerance in hard clams will be insightful for future vulnerability assessments of this species and

for future selective breeding programs seeking to produce more resilient broodstock to ensure a more sustainable harvest for the aquaculture industry.

Materials and Methods

Broodstock and spawning

Wild adult hard clams (*Mercenaria mercenaria*) were obtained from five locations along the eastern coast of the United States (Fig. 1): Cape Cod, MA (CC); Great Bay, NJ (GB); Mobjack Bay, VA (MB); Pocomoke Sound, VA (P); and Wachapreague, VA (W) by the Virginia Institute of Marine Science (VIMS) Eastern Shore Laboratory (ESL). All adult clams were acclimated to ambient water conditions in research plots near Wachapreague, VA for at least six months before spawning at the VIMS ESL.

Spawns of all clam populations were conducted by ESL staff in early spring 2019: GB clams were spawned on March 12th, W, CC, and P clams were spawned on March 26th, and MB clams were spawned on April 2nd. Adult clams were induced to naturally release gametes by raising water temperatures and by introduction of sterilized gametes into their incurrent syphons. Approximately two dozen adults from each population were induced to spawn; however, the specific numbers of males and females that successfully released gametes within each population were not tracked. Larvae were then reared under standard hatchery conditions through settlement and into their early juvenile stage. Growth was monitored weekly, and experimentation began once juvenile clams from all populations had reached at least 2 mm in shell length. All experiments were completed between May 20th and June 16th, 2019.

Experimental design

Approximately 600 juvenile clams from each population were removed from hatchery upwellers and held in mesh containers in a 300 L recirculating tank that was constantly aerated for up to three weeks. Water conditions were maintained in this holding tank at a temperature of 25°C and a salinity of 31, which matched the conditions from the upwellers at the time clams were transferred into the holding tank. Clams were fed daily with LPB Frozen Shellfish Diet (Reed Mariculture, Campbell, California, USA) following the manufacturer's recommendations for juvenile bivalves. A recirculation pump and manifold system were used to distribute water into the tops of each mesh container to ensure food reached all clams effectively. Every 48 hours, 100% water changes were performed, and mesh containers were mechanically cleaned. ~~The longest any individual clam was held within this system was three weeks.~~

Juvenile clams from each population were exposed to five different temperature (25°C, 27.5°C, 30°C, 32.5°C, 35°C) and four different salinity (31, 20, 15, and 10) conditions. The maximum temperature was representative of projected summer high water temperatures based on available climate model projections for the region at the time of experimentation (Stocker et al., 2013). Experimental salinities were chosen to span both the wide range of salinity conditions found within Chesapeake Bay as well as previously observed limitations of salinity tolerance in both a wild population and hatchery broodstock line of hard clams (Davis and Calabrese, 1964; Ringwood and Keppler, 2002). Before each experimental trial, clams were cleaned with fine paint brushes to remove any dirt or fouling organisms, and then imaged using an Olympus SZX7 stereo microscope with an Olympus DP25 camera. Shell length measurements were made on ten randomly selected clams from each population for each experimental condition using ImageJ software (Schneider et al., 2012). Shell length was tracked over the one-month duration of this

project to confirm that clams from all populations remained within a similar size class, with previous studies differentiating size classes at approximately 7-10 mm (Castagna, 1984; Kraeuter and Castagna, 1984; Bricelj et al., 2007; Zarnoch et al., 2015).

For thermotolerance trials, five clams each were loaded into microcentrifuge tubes with ten replicate tubes prepared per population. The tubes were filled with 25°C filtered seawater and placed into a programable Digital Heating/Cooling Drybath (Thermo Fisher Scientific, 88880029). Multiple clams were placed in each tube as a precaution to ensure replicates were not lost if an individual died during experimentation. The dry bath temperature increased at 0.5°C/h until the target experimental temperature was reached, a ramping rate similar to peak daily warming rates observed in summer for sites along the Eastern Shore of Virginia (data not shown; Virginia Estuarine and Coastal Observing System (VECOS) station CRS001.80). Because the length of temperature ramping differed among treatment groups, clams were held in microcentrifuge tubes at the control temperature for the necessary amount of time before heating began to ensure all clams across all treatments remained within the microcentrifuge tubes for the same length of time (26 h) with the same number of water changes. One hundred percent water changes were conducted every 12 h to prevent accumulation of waste products even though clams were starved for the duration of each exposure. Water was thermostated to the appropriate temperature before each water change to prevent any temperature shocks. Once the target temperature was reached for each trial, the exposure continued for six hours before an individual clam from each replicate microcentrifuge tube was placed into a biological oxygen demand (BOD) vial in order to measure oxygen consumption rates.

For salinity tolerance trials, ten replicate microcentrifuge tubes containing five clams each per population were held at the target salinity and control temperature for 48 h in a 10 L

water bath (PolyScience, 110-031). Water changes were conducted every 12 h with filtered seawater at the appropriate salinity and thermostated to the control temperature of 25°C. Similar to the temperature exposures, once each salinity exposure had ended, oxygen consumption rates were then determined for one clam from each replicate tube.

Respirometry

At the conclusion of the temperature and salinity exposures, an individual clam from each microcentrifuge tube was loaded into a 1 mL BOD vial containing fully oxygenated, 0.2-µm filtered treatment water. Vials were sealed with no headspace and then incubated in the dark at the appropriate temperature for 45 minutes. Alongside samples, blank vials containing only filtered seawater were also incubated in order to account for any potential background respiration within the filtered seawater. After the incubation, a water sample was removed from each vial using a gas-tight syringe and loaded into a custom, temperature-controlled glass chamber fitted with a needle-type oxygen microsensor (PreSens Precision Sensing, NTH-PSt7). Oxygen measurements were recorded after 1.5 minutes, which allowed sensor readings to stabilize. Three consecutive readings were then recorded for each sample to ensure accuracy. Individual clams were then frozen at -20ºC for later dry weight determination. The respirometry chamber was rinsed with ethanol followed by deionized water between each sample. The oxygen sensor was calibrated daily using 0% and 100% dissolved oxygen solutions. The 0% solution was a freshly prepared solution of saturated sodium sulfite in 0.2-um filtered seawater. The 100% solution was generated through aeration of filtered seawater. The three raw oxygen concentration values per sample were averaged and then multiplied by the volume of each individual vial to calculate the total oxygen concentration in micromoles within each vial. If at

least a 10% decrease in oxygen concentration was not observed in a sample vial compared to the average of blank vials, that sample was excluded from further analysis as it could not be clearly determined that the clam ever opened and actively consumed oxygen. Preliminary testing revealed that clam oxygen consumption was linear over time until oxygen saturation was reduced down to 50-55%. Any clam that reduced oxygen concentrations to below 60% were therefore excluded from further analysis as well. Total oxygen consumed was then calculated for remaining samples by subtracting oxygen concentration in the sample vial from average oxygen concentration in blank vials. The specific incubation time for each individual sample vial was then used to calculate an oxygen consumption rate. Each clam's oxygen consumption rate was normalized to its total dry weight to account for size variations among individual clams before average oxygen consumption rates were calculated for each population.

Dry weight determination

Individual clams were rinsed in deionized water to remove any salt before being loaded into preweighed aluminum weighing dishes and dried for 72 h at 65°C. Sample weights were recorded on two consecutive days using a Mettler Toledo semi-micro analytical balance (Mettler Toledo, Columbus, Ohio, USA). If consecutive weights were within 0.1 mg, samples were considered dried, and the two weights were averaged. If sample weights had not stabilized after 72 h, samples were dried for another 24 h and reweighed, repeating as necessary until consecutive weights had stabilized.

Mortality trials

Mortality trials were conducted in the same manner as the temperature trials described previously with a few modifications. Firstly, the mortality trials were conducted at 36°C, which was a temperature observed to cause mortality in most populations during preliminary testing in summer 2018 (data not shown). Secondly, after the six-hour exposure at 36°C, the temperature was then ramped back down (0.5°C/h) to 25°C. Clams were then allowed to recover for 48 h in a tank of unfiltered flow-through seawater thermostated to 25°C. After the recovery period, mortality was assessed by observing persistent shell gape, while survival was visually determined by observing filtration activity or foot movement using a microscope. For each population, ten replicate microcentrifuge tubes containing five clams each were evaluated.

A low salinity mortality trial was also attempted; however, below a salinity of 10, clams appeared moribund, and it was impossible to assess if clams were alive or dead with their valves closed.

Statistics

Data from all experimental trials were analyzed using GraphPad Prism 9 (San Diego, California, USA). To assess differences in oxygen consumption between populations exposed to either elevated temperature or low salinity, full two-way analysis of variance (ANOVA) models were used, with population and temperature (or salinity) as independent categorical factors. A Sidák multiple comparisons test (MCT) was used to assess *a priori* contrasts for both temperature and salinity in order to compare oxygen consumptions rates between each population at each treatment level. Due to variable sample sizes between populations as a result of both clams never opening and clams drawing down oxygen levels to below their linear consumption range, the Sidák MCT was chosen as it uses a more conservative correction to

prevent alpha inflation. The alpha levels for all ANOVAs and MCTs were set at $P < 0.05$. A one-way ANOVA was used to evaluate the results of the mortality trial, paired with a MCT, to assess which populations differed from one another. Effect size (ω^2) was also calculated for all variables in each ANOVA table following the same reasoning and calculations described in the *Statistics* section of Chapter 2. For full ANOVA tables see Table 1 and for MCT results see Table 2, 3, and 4 where all results are reported to the appropriate significant digits based on the precision of the measurement method used. All nonsignificant digits were truncated.

Results

Shell size during experimentation

Average shell length of the ten randomly selected clams measured from each population on each day before experimental trials began is shown in Figure 2. Over the course of this project, all populations stayed within 3-5mm in average shell length, indicating that all populations were within the same size class for all experimental trials. Interestingly, the slight but persistent differences in average shell length observed between populations on each day generally followed the timing of when each population was spawned, with GB clams being both the largest on average on most days and the first population that was spawned. Similar growth rates were observed between all population within the holding tank, indicating that any impacts of being held within this recirculating system were similar across all populations.

Elevated temperature tolerance

Effects of elevated temperature on each hard clam population were difficult to evaluate due to high inter-animal variability and high variability in sample size among populations at each treatment level. Both temperature and population had significant effects on oxygen consumption (Fig. 3) in the ANOVA model [temperature: $F(4,179)=4.320$, $P=0.0023$, $\omega^2=0.0596$, population: $F(4,179)=3.343$, $P=0.0114$, $\omega^2=0.0421$] (Table 1). There was a slight increasing trend in oxygen consumption rate with increasing temperature, and a minimal decrease in respiration at 35°C for all populations except for W clams (Fig. 3). However, calculated effect sizes for temperature and population were both small, demonstrating that minimal variance within the data set was accounted for by these main effects. There were no significant contrasts detected between populations at any temperature (Table 2).

Results of the one-way ANOVA for the mortality trial conducted at 36°C (Fig. 4) were significant, with population accounting for a large proportion of the variance in the data set [ANOVA, $F(4,45)=28.50$, $P<0.0001$, $\omega^2=0.6875$]. Results of the MCT revealed that MB and W clams had similar survival to one another, but greater survival than all other populations [Sidák MCT, MB v CC: $t=5.964$, $P<0.0001$, MB v GB: $t=9.087$, $P<0.0001$, MB v P: $t=6.532$, $P<0.0001$, W v CC: $t=4.544$, $P=0.0004$, W v GB: $t=7.667$, $P<0.0001$, W v P: $t=5.112$, $P<0.0001$] (Table 3). CC clams were also found to have significantly greater survival than GB clams, with survival of P clams falling in between these two populations [Sidák MCT, CC v GB: $t=3.124$, $P=0.0308$].

Low salinity tolerance

Oxygen consumption under low salinity conditions varied among hard clam populations (Fig. 5). A significant interaction of salinity and population was detected [ANOVA, $F(12, 150)=1.897$, $P=0.0388$, $\omega^2=0.0240$]; however, the effect size of this interaction term is quite

small, indicating it is of little practical importance in the model. Oxygen consumption varied between populations even under control conditions (S=31), where W clams had significantly higher oxygen consumption rates than either CC, GB, or P clams [Sidák MCT, W v CC: t=3.108, P=0.0223, W v GB: t=3.809, P=0.0020, W v P: t=2.862, P=0.0471] (Table 4). When exposed to low salinity stress, oxygen consumption rates in CC, GB, and P clams slightly increased at a salinity of 20 compared to a salinity of 31; however, these trends were not significant. A significant difference did emerge at a salinity of 15, where MB clams had significantly larger oxygen consumption rates compared to CC clams [Sidák MCT, t=3.582, P=0.0046]. Under the most severe low salinity conditions tested, all populations showed a reduction in oxygen consumption rates down to a similarly low level.

Discussion

Elevated temperature and low salinity tolerances were evaluated among juvenile *M. mercenaria* from five populations along the eastern coast of the United States. The goal of this study was to assess population-level variability to several levels of each stressor in order to determine how, if at all, environmental stress tolerance varies across the biogeographic range of this species. Understanding population-level variability in environmental stress tolerance will improve the ability of the scientific community to make accurate predictions about how future climate change will affect this ecologically and economically important species, while also providing a basis for potential selective breeding programs that could improve the resilience of commercial hard clam stocks.

Elevated temperature tolerance

The observed lack of sensitivity of hard clam populations to increasing temperatures agrees with results from other published studies. The slight increasing trend in oxygen consumption observed here for juvenile clams exposed to between 5 and 10°C increases in water temperature were previously observed for other wild and cultured populations (Ulrich and Marsh, 2009; Matoo et al., 2013; Stevens and Gobler, 2018). Few studies of *M. mercenaria* examining the effects of elevated temperature across a 10-degree range or more could be found, however, making it difficult to assess if the observed minor decrease in oxygen consumption rates at 35°C is also typical of other populations. If this decrease is representative of metabolic depression, then this threshold may represent the thermal limitations of the populations assessed here. Interestingly, W clams were the only population not to show this decrease in oxygen consumption from 32.5°C to 35°C, indicating that they might have a greater thermal tolerance than the other study populations. As Wachapreague, VA is located at the southern end of the biogeographic range represented in this study, this population may be more well adapted to elevated temperatures due to its more consistent exposure to them, which is a latitudinal pattern previously observed in other marine invertebrates (Schroer et al., 2009; Byrne, 2011). Average summer water temperatures in Wachapreague, VA were recently reported by Farmer et al. (2023) for 2019 and 2020 to be approximately 28°C; however, the maximum water temperature recorded during this time period was approximately 35°C (Ross and Snyder, 2020; Ross and Snyder, 2021). By comparison, summer water conditions in Cape Cod, MA, where the most northerly distributed population examined in this study originates from, are typically between 20 and 22°C (Yu and Yang, 2022). The presence of a latitudinal gradient in thermotolerance for hard clams is further supported by the results of the mortality trial, where the two most southern

populations examined in this study had the greatest percent survival. While CC and GB clams had significantly lower survival rates, P clams, a more southern population, also tolerated this stressor poorly, which demonstrates that any gradient in thermotolerance for *M. mercenaria* may be weak unless this Pocomoke, VA population is particularly aberrant. In order to better evaluate the presence or absence of a latitudinal gradient in thermotolerance within this species, more extensive testing is needed.

The pairing of oxygen consumption measurements with measurements of more specific cellular mechanisms related to metabolic processes would likely provide more insight. While the observed, albeit slight, increases in oxygen consumption could be a strategy for responding to elevated temperature stress, other documented strategies that are independent of oxygen consumption have been observed in *M. mercenaria*. Ulrich and Marsh (2009) found that while the respiration control ratios of isolated hard clam mitochondria decreased at elevated temperatures, there was no corresponding decrease in phosphorylation efficiency of adenosine diphosphate to adenosine triphosphate (ATP), like that seen in soft-shell clams (Abele et al., 2002). Furthermore, Ulrich and Marsh (2009) observed clear shifts in phosphorylation of mitochondrial regulatory proteins at higher temperatures, with the phosphorylation of some proteins being independent of ATP concentrations, which could be indicative of an alternative source of phosphate within the cell that is not impacted by changes in respiration rates. Taken together, these results show that while respiration rates in *M. mercenaria* may drop slightly at higher temperatures, their mitochondria are still capable of producing adequate levels of ATP to support cellular needs, which is not commonly observed during metabolic depression in other marine species. Typically, mitochondrial dysfunction due to increased proton leakage across the

mitochondrial membrane at higher temperatures leads to a reduction in ATP generation through oxidative phosphorylation (Pörtner, 2001; Sokolova, 2004).

Another strategy observed in hard clams is the rapid upregulation of heat shock protein genes within 2 h of exposure to elevated temperatures (Wang et al., 2016). While this rapid response itself could require an initial energy input, it could help prevent irreparable damage to cellular proteins, thus preserving cellular resources under more stressful conditions. Lastly, Ulrich and Marsh (2008) observed that clams from Lewes, DE possessed proteins in their mantle and digestive system that were stable when exposed to elevated temperatures up to 35°C. Thermal stability would help prevent proteins from denaturing at higher temperatures, which would help conserve energy within the organism as there would be less need to activate cellular repair mechanisms as less protein damage is occurring. Lower energy demand would result in minimal changes in oxygen consumption when stressed, as observed in all populations at all temperatures in this study, because the need for oxygen as the final electron acceptor necessary to produce large amounts of ATP within the cell would be lower. Therefore, even if a slight drop in mitochondrial respiration at 35ºC occurs, juvenile hard clams do not demonstrate the marked decrease in oxygen consumption at higher temperatures consistent with complete mitochondrial uncoupling, as has been seen in both marine fishes and other bivalves (Pörtner, 2001; Sokolova, 2004; Ulrich and Marsh, 2009). The presence of these various thermotolerance mechanisms coupled with known variations in optimal temperatures for growth and respiration illustrates how the response of *M. Mercenaria* to future ocean warming will be complex and requires more in-depth evaluations of cellular processes across populations to better understand how this species will persist in the future (Hibbert, 1977; Stevens and Gobler, 2018).

Low salinity tolerance

Differences in respiration rates were observed among populations in the salinity trial control treatment that were not apparent in the temperature trial control treatment. W clams had significantly higher oxygen consumption rates than CC, GB, and P clams, indicating that at resting conditions, W clams had greater energy demands. This was surprising as W clams are the only population that could not experience any genotype-environment mismatch or persistent localized acclimatization effects due to being transplanted to Wachapreague, VA, both of which could explain an increased need for cellular energy in response to a less optimal environment. The difference in oxygen consumption could be indicative of underlying genetic variability associated with resting metabolic rate within individual W clams sampled within this study, although this cannot be determined from respirometry data alone. Trends observed in oxygen consumption rates among all other populations in the low salinity trial control treatment more closely followed those observed previously in the elevated temperature control group. Interestingly, the lowest respiration rates were observed in CC and GB clams, the two most northern populations assessed within this study, which could be signs of genetic differences or transgenerational plasticity from each source population's environment that persisted through the acclimation time in common garden conditions before spawning (Marshall, 2008; Shama and Wegner, 2014; Ross et al., 2016). Other studies in various marine invertebrate species have shown both homogeneous stress tolerance across latitudinal gradients as well as persistent trans-generational variation in stress tolerance between populations, indicating more in-depth experimentation is needed to explore the source of this resting variation in the populations assessed here (reviewed in Sanford and Kelly, 2011).

Low salinity stress induced changes in oxygen consumption rates in all populations, where populations from lower salinity regions did show improved tolerance, although this characteristic was not unique to just the low salinity populations. When exposed to a salinity of 20, all populations showed an increase in oxygen consumption, indicative of an increase in energy demand, with the exception of W clams, which had the highest oxygen consumption rates in the control treatment. These increases eliminated the significant differences that were observed under control conditions. Decreasing oxygen consumption rates were observed under the two more severe low salinities and are likely a sign of metabolic depression as low salinity is known to be a limiting factor in the survival of hard clams (Baker et al., 2005; Dickinson et al., 2013). The largest reductions in oxygen consumption at a salinity of 15 were observed in CC and GB clams, while MB and W clams showed minimal changes in oxygen consumption under this experimental condition. MB clams were the only population that had a slight increase in oxygen consumption at a salinity of 15, which could indicate that the lower salinity environment inhabited by MB clams is driving the selection of more low salinity tolerant individuals. However, W clams, which do not inhabit a naturally low salinity environment, also maintained higher oxygen consumption rates at a salinity of 15 compared to clams from CC, GB, and P. P clams showed the third highest oxygen consumption rate at a salinity of 15, but their oxygen consumption rate did decrease compared to P clams exposed to a salinity of 20, which could indicate the onset of metabolic depression. Based on these results, there is potential that certain low salinity populations are more tolerant to low salinity stress; however, this tolerance is not exclusive to just lower salinity populations and could potentially be found throughout the range of *M. mercenaria*. Despite these variations among populations, all clams exposed to a salinity of 10 showed marked reductions in respiration rates down to the lowest observed in this study.

Observed activity rates in these clams were greatly reduced, as all individuals were moribund, demonstrating that a salinity of 10 is beyond the tolerance of any of these population.

Due to both high inter-animal variability within each population and variable sample sizes among populations within each experimental condition, it is difficult to draw definitive conclusions about the extent of physiological variability in environmental tolerance among these populations of *M. mercenaria*. Differential oxygen consumption rates when exposed to low salinity stress do indicate that some degree of population level variability exists in this species, but further work is needed on specific cellular stress response mechanisms to better understand the extent of this variability and if it could be used in selective breeding programs to provide support to both restoration efforts and the hard clam aquaculture industry.

Spawning design

It is important to note that the spawns conducted in this study likely only involved a small number of individuals, as previous research has shown that pooled spawning events can result in overrepresentation of only a few adults (Gaffney et al., 1992; Boudry, et al., 2002; Appleyard and Ward, 2006). Due to this limitation, it is possible that any noted differences are not in fact unique to the populations they were observed within, but instead could persist across the biogeographic range of this species. However, due to the high levels of genetic variability within hard clams, there is potential for the observed differences to be unique, population-specific adaptations (Hilbish et al., 1993; Ropp, 2021; Farhat et al., 2022). More experimentation is needed to truly conclude if population-level variability in physiological tolerances is present across the biogeographic range of *M. mercenaria*, but the results found in this study do demonstrate the potential for such differences.

Conclusions

This comparative study on populations of *M. mercenaria* did find physiological variability among populations in response to both elevated temperature and low salinity stress. Oxygen consumption rates from juvenile hard clams exposed to elevated temperature was inconclusive with respect to population-level variability; however, results of the mortality trial demonstrated that tolerance to extreme temperatures does vary between populations. Although, the noted pattern of variation in survival did not closely follow the latitudinal gradient in temperature of the native habitats for the five populations assessed here. Potential differences in low salinity tolerance among populations were detected, with Mobjack Bay clams outperforming several of the populations from higher salinity environments. These results should be viewed in an exploratory light due to potential spawning limitations; however, given the high level of genetic variation within this species, physiological variability in environmental stress tolerance among populations is possible, which could alter future projections regarding where *M. mercenaria* will persist with climate change. If clear differences are identified in future studies, population-level variability could serve as a resource to enhance restoration efforts and support the aquaculture industry through enabling the selective breeding of more resilient hard clam stocks.

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Table 1. Full ANOVA tables for each model run with calculated effect sizes (ω^2). Column headings abbreviations: degrees of freedom (DF), degrees of freedom of numerator (DFn), degrees of freedom of denominator (DFd).

Experiment	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Elevated temperature tolerance							
	Temperature x Population	16	43.8	2.738	F (16, 179) = 0.765	0.7229	-0.0168
	Temperature	4	61.8	15.45	F (4, 179) = 4.32	0.0023	0.0596
	Population	4	47.83	11.96	F (4, 179) = 3.343	0.0114	0.0421
	Residual	179	640.2	3.577	-	-	-
Low salinity tolerance							
	Salinity x Population	12	84.76	7.063	F (12, 150) = 1.897	0.0388	0.024
	Salinity	3	956.6	318.9	F (3, 150) = 85.63	<0.0001	0.5663
	Population	4	66.05	16.51	F (4, 150) = 4.434	0.002	0.0306
	Residual	150	558.6	3.724	-	-	-
Mortality trial							
	Treatment (between groups)	4	28270	7068	F (4, 45) = 28.5	P<0.0001	0.6875
	Residual (within groups)	45	11160	248	-	-	-

Table 2. Summary statistics for all contrasts of oxygen consumption rates evaluated from the elevated temperature trials using the Sidák multiple comparisons test (α=0.05). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Column headings abbreviations: degrees of freedom (DF).

Experiment	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Elevated temperature tolerance						
25°C						
	CC vs. GB	179	1.739	-1.243 to 4.722	1.653	0.6517
	CC vs. MB	179	-0.6316	-3.614 to 2.351	0.6003	0.9997
	CC vs. P	179	0.5832	-2.282 to 3.449	0.577	0.9998
	CC vs. W	179	0.4262	-2.348 to 3.201	0.4354	>0.9999
	GB vs. MB	179	-2.371	-5.466 to 0.724	2.171	0.2719
	GB vs. P	179	-1.156	-4.138 to 1.827	1.099	0.959
	GB vs. W	179	-1.313	-4.208 to 1.582	1.285	0.893
	MB vs. P	179	1.215	-1.768 to 4.197	1.155	0.9435
	MB vs. W	179	1.058	-1.837 to 3.953	1.036	0.9724
	P vs. W	179	-0.157	-2.931 to 2.617	0.1604	>0.9999
27.5°C						
	CC vs. GB	179	0.4335	-2.926 to 3.793	0.3657	>0.9999
	CC vs. MB	179	-1.733	-4.598 to 1.133	1.714	0.6029
	CC vs. P	179	0.4575	-2.408 to 3.323	0.4526	>0.9999
	CC vs. W	179	0.2785	-2.496 to 3.053	0.2846	>0.9999
	GB vs. MB	179	-2.166	-5.526 to 1.194	1.828	0.5122
	GB vs. P	179	0.02401	-3.336 to 3.384	0.02026	>0.9999
	GB vs. W	179	-0.155	-3.438 to 3.128	0.1338	>0.9999
	MB vs. P	179	2.19	-0.675 to 5.056	2.167	0.2744
	MB vs. W	179	2.011	-0.763 to 4.786	2.055	0.3443
	P vs. W	179	-0.179	-2.953 to 2.595	0.1829	>0.9999
30°C						
	CC vs. GB	179	1.447	-1.327 to 4.222	1.479	0.7813

CC vs. MB	179	-0.5015	-3.106 to 2.103	0.5457	0.9999
CC vs. P	179	0.7146	-1.89 to 3.319	0.7776	0.9968
CC vs. W	179	0.9777	-1.627 to 3.583	1.064	0.9669
GB vs. MB	179	-1.949	-4.65 to 0.753	2.045	0.3513
GB vs. P	179	-0.7326	-3.434 to 1.969	0.7686	0.9971
GB vs. W	179	-0.4694	-3.171 to 2.232	0.4925	>0.9999
MB vs. P	179	1.216	-1.311 to 3.743	1.364	0.8526
MB vs. W	179	1.479	-1.048 to 4.006	1.659	0.6467
P vs. W	179	0.2631	-2.264 to 2.790	0.2952	>0.9999
32.5°C					
CC vs. GB	179	-0.2232	-2.686 to 2.24	0.2569	>0.9999
CC vs. MB	179	-0.6388	-3.036 to 1.759	0.7553	0.9975
CC vs. P	179	-0.4526	-2.85 to 1.945	0.5352	0.9999
CC vs. W	179	1.258	-1.139 to 3.656	1.488	0.7751
GB vs. MB	179	-0.4156	-2.879 to 2.047	0.4783	>0.9999
GB vs. P	179	-0.2294	-2.692 to 2.234	0.264	>0.9999
GB vs. W	179	1.481	-0.982 to 3.944	1.705	0.6104
MB vs. P	179	0.1862	-2.211 to 2.584	0.2201	>0.9999
MB vs. W	179	1.897	-0.5004 to 4.294	2.243	0.2326
P vs. W	179	1.711	-0.687 to 4.108	2.023	0.3662
35°C					
CC vs. GB	179	-0.2526	-2.857 to 2.352	0.2749	>0.9999
CC vs. MB	179	-0.4395	-2.903 to 2.024	0.5058	>0.9999
CC vs. P	179	-0.7823	-3.245 to 1.681	0.9003	0.9900
CC vs. W	179	-0.4433	-2.97 to 2.084	0.4973	>0.9999
GB vs. MB	179	-0.1869	-2.73 to 2.356	0.2084	>0.9999
GB vs. P	179	-0.5298	-3.073 to 2.013	0.5905	0.9997
GB vs. W	179	-0.1908	-2.796 to 2.414	0.2076	>0.9999
MB vs. P	179	-0.3428	-2.74 to 2.055	0.4053	>0.9999

MB vs. W	179	-0.0038	-2.467 to 2.459	0.0044	>0.9999
P vs. W	179	0.339	-2.124 to 2.802	0.3901	>0.9999

Table 3. Summary statistics for all contrasts in percent survival evaluated from the mortality trial using the Sidák multiple comparisons test (α=0.05). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Column headings abbreviations: degrees of freedom (DF).

Experiment	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Mortality trial						
36°C						
	CC vs. GB	45	-22	-42.73 to -1.269	3.124	0.0308
	CC vs. MB	45	42	21.27 to 62.73	5.964	<0.0001
	CC vs. P	45	-4	-24.73 to 16.73	0.568	0.9998
	CC vs. W	45	32	11.27 to 52.73	4.544	0.0004
	GB vs. MB	45	64	43.27 to 84.73	9.087	<0.0001
	GB vs. P	45	18	-2.731 to 38.73	2.556	0.1319
	GB vs. W	45	54	33.27 to 74.73	7.667	<0.0001
	MB vs. P	45	-46	-66.73 to -25.27	6.532	<0.0001
	MB vs. W	45	-10	-30.73 to 10.73	1.42	0.8303
	P vs. W	45	36	15.27 to 56.73	5.112	<0.0001

Table 4. Summary statistics for all contrasts evaluated from the low salinity trials using the Sidák multiple comparisons test (α=0.05). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Column headings abbreviations: degrees of freedom (DF).

Experiment	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Low salinity tolerance						
31						
	CC vs. GB	150	0.7818	-1.883 to 3.446	0.8338	0.9945
	CC vs. MB	150	-0.9243	-3.509 to 1.661	1.016	0.976
	CC vs. P	150	-0.1646	-2.829 to 2.5	0.1755	>0.9999
	CC vs. W	150	-3.023	-5.786 to -0.259	3.108	0.0223
	GB vs. MB	150	-1.706	-4.371 to 0.958	1.820	0.5203
	GB vs. P	150	-0.9464	-3.688 to 1.795	0.9809	0.9813
	GB vs. W	150	-3.805	-6.642 to -0.967	3.809	0.002
	MB vs. P	150	0.7597	-1.905 to 3.424	0.8102	0.9956
	MB vs. W	150	-2.098	-4.862 to 0.665	2.158	0.2817
	P vs. W	150	-2.858	-5.696 to -0.02	2.862	0.0471
20						
	CC vs. GB	150	-0.4523	-3.503 to 2.598	0.4213	>0.9999
	CC vs. MB	150	-0.1808	-3.019 to 2.657	0.1810	>0.9999
	CC vs. P	150	-2	-4.838 to 0.838	2.002	0.3824
	CC vs. W	150	-0.7329	-3.571 to 2.105	0.7338	0.9981
	GB vs. MB	150	0.2715	-2.690 to 3.233	0.2605	>0.9999
	GB vs. P	150	-1.548	-4.509 to 1.414	1.485	0.7779
	GB vs. W	150	-0.2805	-3.242 to 2.681	0.2692	>0.9999
	MB vs. P	150	-1.819	-4.561 to 0.923	1.885	0.4689
	MB vs. W	150	-0.5521	-3.294 to 2.19	0.5721	0.9998
	P vs. W	150	1.267	-1.475 to 4.009	1.313	0.8801
15						
	CC vs. GB	150	-0.6416	-3.306 to 2.023	0.6842	0.9989

	CC vs. MB	150	-3.359	-6.023 to -0.695	3.582	0.0046
	CC vs. P	150	-1.944	-4.608 to 0.721	2.073	0.3346
	CC vs. W	150	-2.508	-5.093 to 0.077	2.757	0.0637
	GB vs. MB	150	-2.717	-5.459 to 0.024	2.816	0.0538
	GB vs. P	150	-1.302	-4.044 to 1.44	1.349	0.8613
	GB vs. W	150	-1.866	-4.531 to 0.798	1.990	0.3911
	MB vs. P	150	1.415	-1.326 to 4.157	1.467	0.79
	MB vs. W	150	0.8512	-1.813 to 3.516	0.9078	0.9894
	P vs. W	150	-0.564	-3.229 to 2.1	0.6017	0.9996
10						
	CC vs. GB	150	0.02978	-2.422 to 2.482	0.0345	>0.9999
	CC vs. MB	150	-0.04245	-2.495 to 2.41	0.04919	>0.9999
	CC vs. P	150	-3.929E-03	-2.456 to 2.448	0.00455	>0.9999
	CC vs. W	150	-0.1968	-2.649 to 2.255	0.2281	>0.9999
	GB vs. MB	150	-0.0722	-2.524 to 2.38	0.08369	>0.9999
	GB vs. P	150	-0.0337	-2.486 to 2.418	0.03905	>0.9999
	GB vs. W	150	-0.2266	-2.679 to 2.226	0.2626	>0.9999
	MB vs. P	150	0.03852	-2.414 to 2.491	0.04464	>0.9999
	MB vs. W	150	-0.1544	-2.607 to 2.298	0.1789	>0.9999
	P vs. W	150	-0.1929	-2.645 to 2.259	0.2235	>0.9999

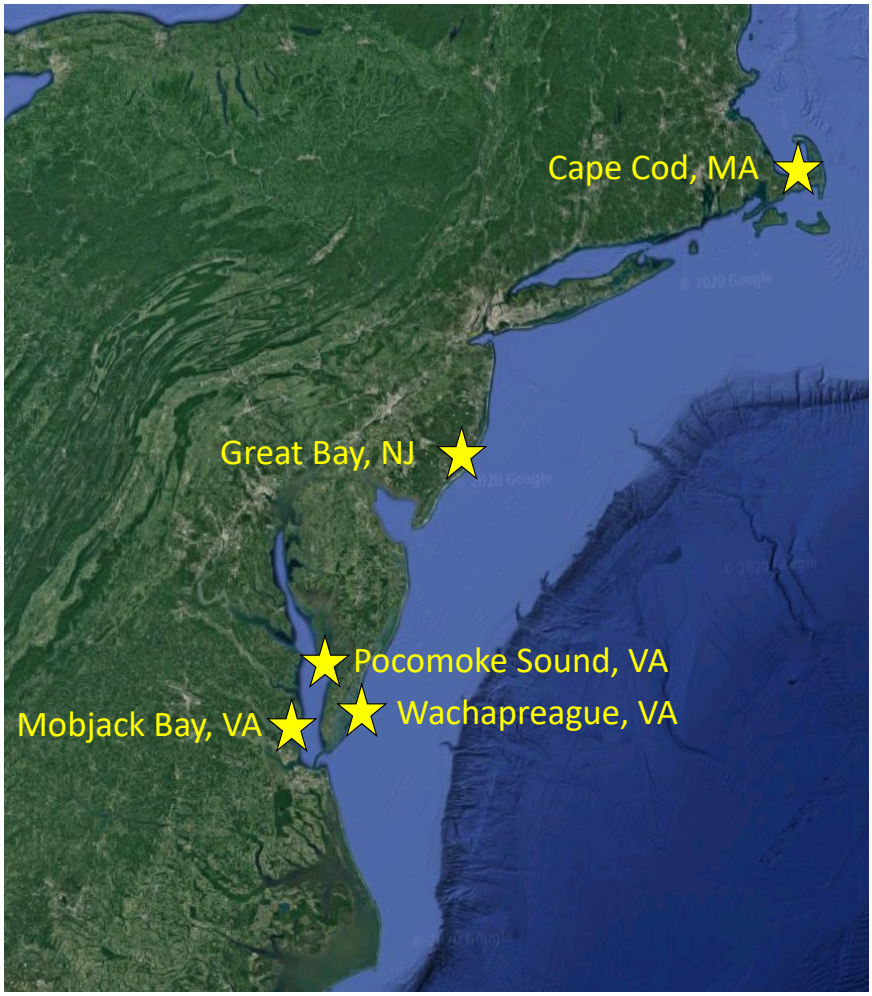

Figure 1. Map of source populations of hard clams, marked by yellow stars. Map image credit: Google.

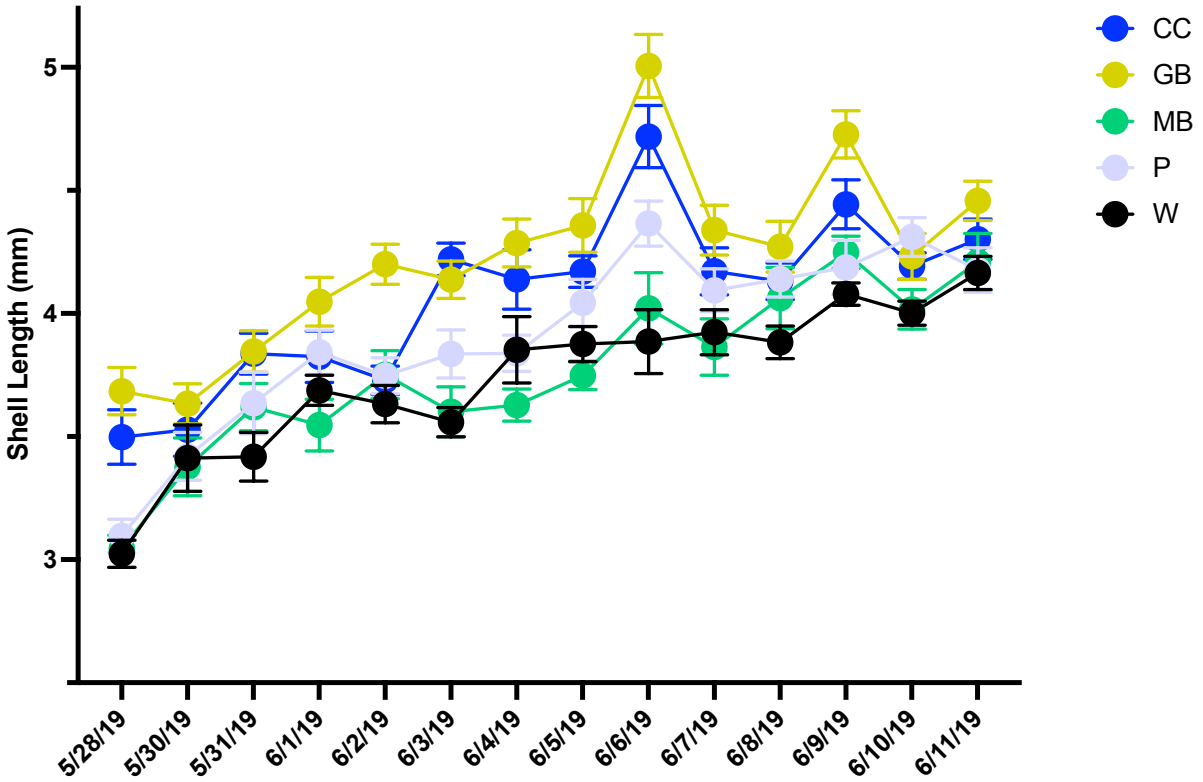

Figure 2. Shell length in millimeters for ten randomly selected clams from each population on each day experimental trials were initiated (mean ± SEM). All clams were measured after removal from nursery tanks, but before being placed into an experimental treatment. Lack of visible error bars indicates they fall within the graphic illustration of the point itself. Population abbreviations: Cape Cod MA (CC), Great Bay NJ (GB), Mobjack Bay VA (MB), Pocomoke VA (P), Wachapreague VA (W).

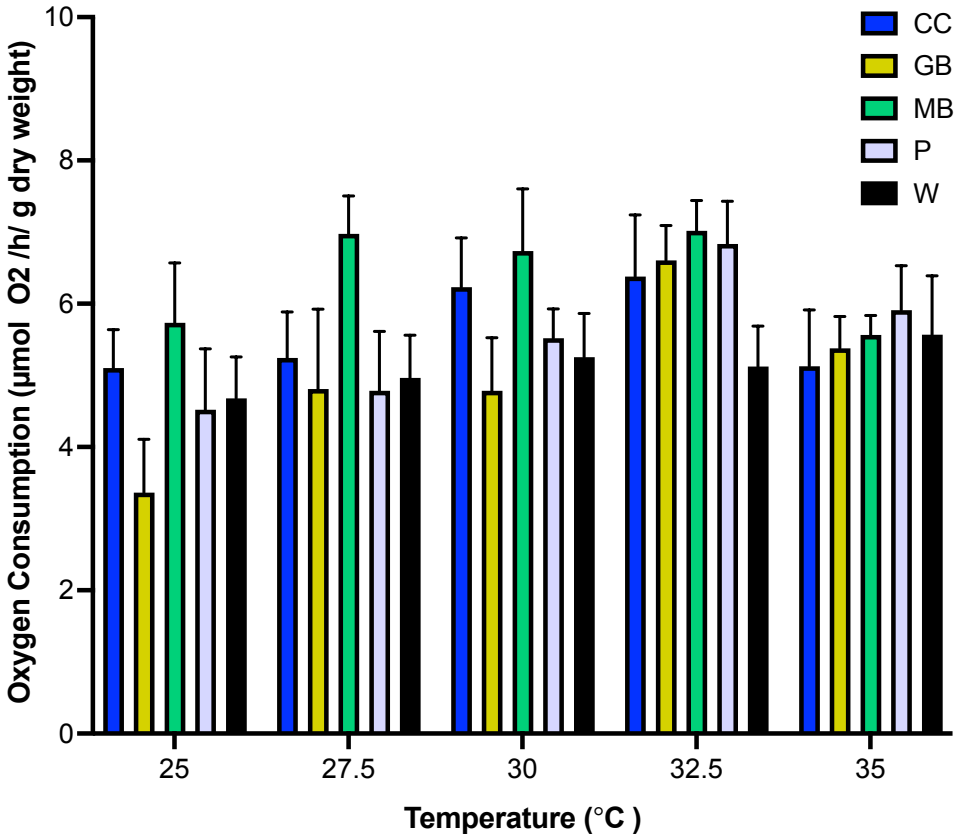

Figure 3. Juvenile clam oxygen consumption rates (n=4-10) in moles of oxygen consumed per hour per gram of dry tissue weight after each temperature exposure (mean ± SEM). Population abbreviations: Cape Cod MA (CC), Great Bay NJ (GB), Mobjack Bay VA (MB), Pocomoke VA (P), Wachapreague VA (W).

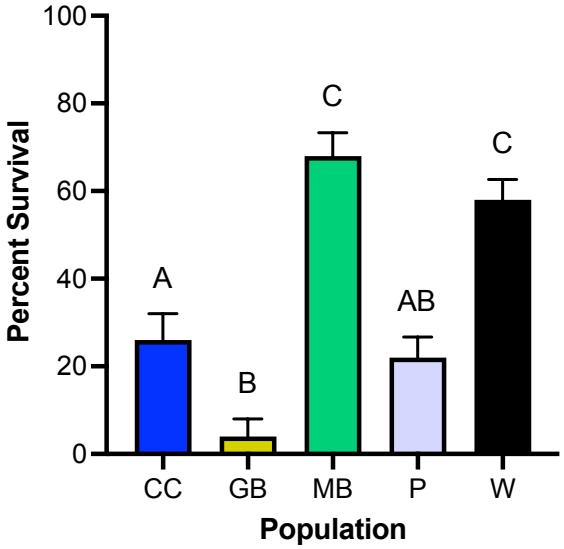

Figure 4. Percent survival of juvenile clams exposed to 36°C. Population abbreviations: Cape Cod MA (CC), Great Bay NJ (GB), Mobjack Bay VA (MB), Pocomoke VA (P), Wachapreague VA (W). Different letters denote significant differences between populations (n=10).

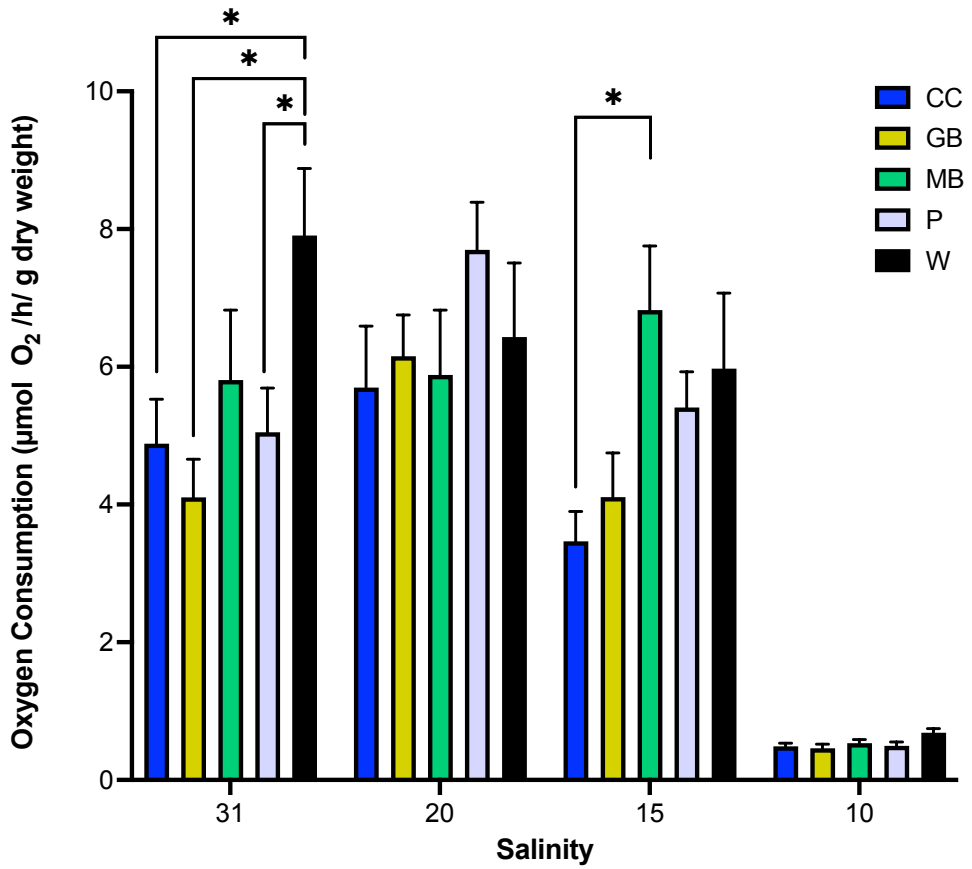

Figure 5. Juvenile clam oxygen consumption rates in moles of oxygen consumed per hour per gram of dry tissue weight after each salinity exposure (mean ± SEM). Population abbreviations: Cape Cod MA (CC), Great Bay NJ (GB), Mobjack Bay VA (MB), Pocomoke VA (P), Wachapreague VA (W). Asterisks denote significant differences between populations (n=6-10).

Chapter 4

Variations in stress tolerance of juvenile *Mercenaria mercenaria* from three controlled population crosses

Abstract

Hard clam populations from along the east coast of the United States were crossbred based on the population assessments conducted in Chapter 3 to assess if physiological tolerance to environmental stress could be manipulated. If improved physiological tolerance to environmental stressors can be selectively bred into hard clams, this strategy could serve as a valuable tool to the aquaculture industry and restoration efforts alike in the face of future climate change. The three crosses evaluated here were a self-cross of clams from Wachapreague, VA, an outcross of clams from Pocomoke Sound, VA and Wachapreague, VA, and a second outcross of clams from Bogue Sound, NC and Cape Cod, MA. Juveniles from all crosses were exposed to a range of elevated temperatures (25°C (control), 27.5°C, 30°C, 32.5°C, 35°C) and lower salinities (32 (control), 20, 15, 12) before oxygen consumption rates were measured as a proxy for changes in metabolic rate. Additionally, all crosses were exposed to a more extreme temperature (36ºC) in order to evaluate potential differences in lethal thermotolerance limits. Oxygen consumption rates of juvenile clams from all population crosses were similar across all temperature levels. Slight, but nonsignificant, differences in survival at 36ºC were observed, where each outcross had higher survival rates than the Wachapreague, VA self-cross. Overall, the results of the elevated temperature trials were similar to the results seen in Chapter 3, with potential signs of increased performance in outcrosses compared to their parent populations. At a salinity of 20, juveniles from all crosses showed an increase in oxygen consumption compared to the control salinity, followed by a large decrease at a salinity of 15. Both outcrosses showed significantly higher oxygen consumption at a salinity of 20 compared to the Wachapreague, VA self-cross. The steep decline in oxygen consumption rates observed in all crosses at a salinity of 15 was not expected based on the performance of parent populations assessed at this same salinity in

Chapter 3. While decreased performance under low salinity stress could be a sign of outbreeding depression in both outcrosses, the fact that juveniles from the Wachapreague, VA self-cross also performed poorly indicates that interannual variability in physiological tolerances among year classes may be high, potentially due to genetic differences in the adult clams spawned in 2019 compared to 2021. Improved performance in outcrosses demonstrates the potential for selectively breeding more physiologically tolerant hard clam lines, although more research is needed over successive generations to improve the understanding of how physiological tolerance in this species can be altered through selective breeding.

Introduction

Following from the physiological differences in environmental stress tolerance observed between *Mercenaria mercenaria* populations in Chapter 3, specific populations were crossbred to explore potential heritability of these observed differences. As climate change continues to threaten both wild and cultured stocks of hard clams, it is of increasing importance that an understanding of population-level variability and the potential for crossbreeding more resilient clams be gained (Baker et al., 2005; Dickinson et al., 2013; Ivanina et al., 2013; Matoo et al., 2013; Specht and Fuchs, 2018). As discussed in detail in Chapter 3, both projected increases in elevated water temperatures and more intense low salinity events due to precipitation changes can lead to increased mortality in *M. mercenaria*, which poses a clear threat to the viability of hard clam aquacultural operations as climate change progresses (Baker et al., 2005; Dickinson et al., 2013; Ivanina et al., 2013). The existence of heritable differences in stress tolerance among hard clam populations could serve as a resource for industry and conservation as more resilient clam lines could be produced.

Selective breeding of commercially important bivalves has been successfully performed many times around the world as a means to produce industry broodstock lines with desirable attributes, including size, growth, and stress tolerance (reviewed in Tan et al., 2020). In hard clams specifically, selective breeding efforts have been primarily conducted in commercial operations through successive rounds of controlled breeding to select for better growth and survival (Gallivan and Allen, 2000; Camara et al., 2006). Some studies assessing disease resistance and low temperature tolerance in *M. mercenaria* have demonstrated clear differences among wild populations as well as between wild and cultured clam stocks; although, despite the economic value of this species, relatively few studies have thoroughly explored the heritability of any specific traits in hard clams (Ford et al., 2002; Pernet et al., 2006). Furthermore, previous work has shown that crossbreeding distinct clam lines can result in offspring that are genetically distinct from both parental lines, thus serving as a tool for maintaining genetic diversity within industry broodstock lines (Manzi et al., 1991). While more work is still needed to explore selective breeding in *M. mercenaria*, recent studies on hard clam genetics have shown high levels of genetic variability among wild clam populations (Hu et al., 2019; Song et al., 2021; Farhat et al., 2022). When taken together, these studies demonstrate the potential for underlying genetic variation in physiological stress tolerance, which could be used in more targeted selective breeding programs to produce more resilient clam lines to help maintain sustainable harvests as climate change continues (Ford et al., 2002; Pernet et al., 2006; Hu et al., 2019; Song et al., 2021; Farhat et al., 2022).

While selective breeding can be a powerful tool if the desirable traits are heritable, there are other confounding effects that can make the evaluation of offspring from controlled population crosses challenging. When population crosses are performed, there are several

known phenomena that can occur that alter the fitness of offspring in either potentially beneficial or harmful ways. Two such phenomena are heterosis, also referred to as hybrid vigor, and its opposite: outbreeding depression (Hedgecock et al., 1996; Camara et al., 2006; Edmands, 2007; Frankham et al., 2010). Heterosis occurs when offspring show improved fitness compared to both parental populations and is believed to be the result of heterozygous phenotypes having enhanced fitness due to either the masking of deleterious recessive alleles or overdominance, the term used to describe when the heterozygous combination of alleles has greater fitness than either homozygous phenotype (Edmands, 2007; Birchler et al., 2010). Outbreeding depression, however, is the observed reduction in fitness of offspring often caused by a disruption in gene interactions, which can be the result of several different direct and indirect mechanisms (Edmands, 2007). Gene expression can be directly altered due to allelic changes at certain gene loci or changes in gene regulation due to the new combination of alleles present in offspring (Swain et al., 2002; Lei et al., 2015). Outbreeding depression can arise indirectly through disruption in the translation of gene products due to alterations in other cellular processes, such as growth and division (Swain et al., 2002; Lei et al., 2015). Both heterosis and outbreeding depression can further complicate selective breeding efforts as both processes can be masked in the first several generations of offspring, leading to the need for more in-depth study of selectively bred lines over at least several generations (Edmands, 2007; Frankham et al., 2010). Despite these challenges, the benefit of crossbreeding has been demonstrated in situations where inbreeding depression has occurred due to population fragmentation driven by human activities. In these situations, crossbreeding with more distant populations has restored genetic variability and improved offspring fitness in a process known as genetic rescue (Edmands, 2007; Frankham et al., 2010). As research on selective breeding in *M. mercenaria* specifically is limited, work is

needed to explore if differences in physiological tolerance among populations, as seen in Chapter 3, can be manipulated through controlled population crosses, and therefore, if this is a viable strategy to increase the resilience of hard clam stocks to future climate change.

In this study, three different controlled crosses were conducted involving four different populations of *M. mercenaria* from along the east coast of the United States to assess if tolerance of juvenile clams to elevated temperature and low salinity could be altered through outcrossing. A self-cross of adult clams from Wachapreague, VA served as a baseline for comparison for the two outcrosses conducted here: 1) Wachapreague, VA clams with Pocomoke, VA clams and 2) Cape Cod, MA clams with Bogue Sound, NC clams. The first outcross was designed to investigate whether the low salinity tolerance of Wachapreague, VA clams could be improved by crossbreeding with Pocomoke, VA clams, which live within Chesapeake Bay and experience more consistently low salinities throughout the year (Chesapeake Bay Program Data Hub, Tidal Water Quality Monitoring Program, monthly measurements from station EE 3.4). The cross of Cape Cod, MA and Bogue Sound, NC clams was selected as these populations span the greatest latitudinal distance of those available for spawning in spring 2021, and they were previously found to be genetically distinct from one another (Ropp, 2021). While juveniles were only assessed from the first generation of these crosses due to the length of time needed to reach sexual maturity in *M. mercenaria*, insight can still be gained about potential fitness changes as a result of outcrossing with respect to changes in environmental stress tolerance. Additionally, this study can provide evidence for future work to explore the roles of heterosis or outbreeding depression within these clam lines if either is apparent in the first generation of offspring.

Materials and Methods

Broodstock and spawning

Wild adult *M. mercenaria* used in this study were obtained by the Virginia Institute of Marine Science (VIMS) Eastern Shore Laboratory (ESL). Cape Cod, MA, (CC), Pocomoke Sound, VA (P), and Wachapreague, VA (W) clams were obtained from the same populations as those used in Chapter 3. For the second outcross, wild adult clams were also obtained from Bogue Sound, NC (BS). Adult clams from all populations (Fig. 1) were acclimated to ambient water conditions in research plots near Wachapreague, VA for at least six months before crosses were conducted.

ESL staff conducted all population crosses in April of 2021, where adults were induced to spawn in isolation by gently raising water temperatures and introducing previously frozen gametes in their incurrent siphons as an environmental cue to spawn as necessary. Once released, gametes were then used to sex adult clams before spawning. Adults from BS and CC were spawned first on April 1st, where one female CC clam was crossed with three BS male clams. On April 6th, both the W self-cross and the P x W outcross were conducted. For the W self-cross, eggs from two female W clams were each fertilized by sperm from the same three male W clams. Fertilized embryos were then pooled and cultured under standard hatchery conditions. For the P x W outcross, a total of three families were produced and pooled. Two of these families each consisted of one female W clam fertilized by three male P clams; however, a total of five male P clams were used across both of these families. The third family consisted of eggs pooled from two female P clams fertilized by three male W clams. Once fertilization was apparent, embryos were pooled and cultured under standard hatchery conditions through settlement to the juvenile life stage alongside the other crosses. Juvenile growth was monitored

until clams grew to an average shell length greater than 2 mm, which was the same threshold used in Chapter 3. All experimentation was conducted between June 25th and 30th, 2021.

Experimental design

While the experimental design of this project generally followed the design used in Chapter 3, several changes were made in an effort to decrease the observed issues in Chapter 3 that lead to more variable sample sizes between treatment groups. One such difference was that juvenile clams were no longer held in a recirculating system with filtered seawater, but instead were left in nursery tanks with flow-through raw seawater to maintain their natural diet and were only removed as necessary to begin each experimental trial. Juvenile clams were still cleaned of any fouling organisms before the start of each exposure. Clams were also imaged before each trial using an Olympus SZX7 stereo microscope with an Olympus DP25 camera to track how clam size changed over the course of all experimental trials in order to evaluate if clam size could be a confounding factor impacting any one cross' performance in any given trial. Shell length was later measured from these images using ImageJ software (Schneider et al., 2012).

Thermotolerance exposures were conducted following the same procedures detailed in the *Experimental design* section of Chapter 3. In brief, five clams were place into microcentrifuge tubes with filtered seawater thermostated to the control temperature of 25ºC and incubated in programmable dry baths set to increase temperature by 0.5ºC/h until the target temperature was reached. Clam were held at the target temperature for 6 h before oxygen consumption rates were measured. Water changes were still conducted every 12 h with filtered seawater thermostated to the appropriate temperature for each treatment. The same temperature levels were used for each population cross as those used to evaluate parent populations in 2019

(25°C, 27.5°C, 30°C, 32.5°C, 35°C), with the same control temperature of 25°C. The only difference between the thermotolerance trials in 2021 compared to those in 2019 came at the end of each trial where instead of loading a single clam into a 1 mL biological oxygen demand (BOD) vial from each of the ten replicate microcentrifuge tubes per population, all five clams from each tube were loaded into a 10 mL BOD vial. This change was implemented to lessen the likelihood of losing sample replicates when a clam failed to open its valves within the BOD vials over the duration of the respirometry incubation. Pooling multiple clams within each sample should also provide a more representative assessment of each cross as high inter-animal variability was noted in all trials conducted in 2019.

The low salinity exposures were more heavily altered in 2021. Instead of using microcentrifuge tubes, five clams per cross were all incubated at the same time within ten larger 2-gal aquarium tank replicates for each low salinity level. Clams were separated into small mesh bottomed plastic holders within each tank to prevent mixing of individuals among crosses. Aquarium tanks were placed within a larger water bath to maintain all tank replicates at the control temperature of 25ºC, and each tank was aerated for the duration of each exposure. Salinity levels were similar to those used in 2019; however, the lowest treatment level was raised from a salinity of 10 to 12 as juveniles from all parent populations appeared moribund at a salinity of 10. Therefore, the salinity levels that were assessed within this experiment were 32, 20, 15, and 12. The ambient salinity at VIMS ESL was slightly higher than in 2019, which is why the control salinity shows an increase from 31 to 32. Exposures were shortened compared to those in 2019 in order match the duration of the temperature exposures while still being representative of natural drops in salinity associated with either juvenile transfer to grow out sites or storm events. Clams were starved for the duration of the exposure before oxygen

consumption rates were measured. Similarly to the modified respirometry methods described in the preceding paragraph, five clams from each population cross from each of the ten tanks per salinity level were loaded into 10 mL BOD vials for respirometry.

Respirometry

Respirometry methods were conducted following the same procedure described in the *Respirometry* section of Chapter 3. In brief, a 45 min incubation in the dark was still used with the pooled clams in the 10 mL BOD vials. Blank BOD vials containing only the fully oxygenated 0.2-µm filtered treatment water used to fill each vial were still incubated alongside vials containing clams to account for potential background respiration. Due to the increased length of the 10 mL BOD vials, each vial needed to be inverted every 20 min to ensure oxygen levels did not decrease so drastically at the bottom of each vial that the clams shifted to anaerobic respiration. While this procedure certainly caused clams to close and stop respiring in response to the agitation, it was preferred to the alternative of constant mechanical stirring as these methodologies pose a much greater risk of continually disturbing individual clams, especially clams in the size class assessed in this study. End-point oxygen consumption was measured using the same respirometry system, custom glass chamber, and needle-type oxygen microsensor (PreSens Precision Sensing, NTH-PSt7) as in 2019. After oxygen levels were measured, clams from each BOD vial were placed in microcentrifuge tubes and stored at -20ºC for later dry weight determination. Oxygen consumption rates were calculated following the same calculations described previously with the same normalization to total dry weight of the pool of clams from each vial (Chapter 3: *Respirometry*).

Dry weight determination

Each group of five clams was rinsed in deionized water to remove salt before being placed into preweighed aluminum dishes. Clams were dried at 65ºC for 72 h before sample weights were recorded using a Mettler Toledo semi-micro analytical balance (Mettler Toledo, Columbus, Ohio, USA). Once weights were within 0.1 mg on consecutive days, clams were transferred to 15 mL plastic tubes and submerged in 200 µL of freshly made tissue dissolution solution [5% 1 M NaOH and 15% H_2O_2 in deionized water]. Clams remained in solution for 4 h and were agitated every 1 h to help dislodge tissue. The tissue dissolution solution was then discarded, and clams were rinsed three times with deionized water to remove any chemical residue or remaining tissue. Clams were then placed back onto preweighed aluminum dishes and dried for 72 h at 65ºC. As with the total dry weights, clam shells were weighed daily until consecutive weights were within 0.1 mg. The weight of the dried shells was then subtracted from the total dry weights to determine dry tissue weight. Respirometry data was normalized to both total dry weight and dry tissue weight to determine if the normalization method had any impact on trends observed in the data. As only slight differences were observed (data not shown), respirometry data normalized to total dry weight was used for all statistical analyses to facilitate the comparison back to the results of the parent population assessments in Chapter 3.

Mortality trial

The mortality trial was conducted identically to that described in the *Mortality trial* section of Chapter 3, but at the control salinity of 32 for 2021. In short, ten tubes containing five clams each for each population cross were ramped to 36ºC at 0.5 ºC/h before being ramped back down at that same rate to the starting temperature of 25 ºC. Clams were then transferred to a

recovery tank with flow-through raw seawater. After 48 h, mortality was determined based on the presence of persistent shell gape, and survival was determined by visual detection of active filtering or burrowing behavior.

Statistics

Data from all experimental trials were analyzed using GraphPad Prism 9 (San Diego, California, USA). Full two-way analysis of variance (ANOVA) models were used to assess the differences in respiration rates among population crosses exposed to either elevated temperature or low salinity stress. *A priori* contrasts comparing each population cross to every other cross at each treatment level were calculated using the Sidák multiple comparisons test (MCT). The Sidák MCT was chosen because although the larger BOD vials did decrease variability in sample sizes among populations, it did not eliminate this problem, and the Sidák MCT uses a more conservative correction to prevent alpha inflation that better accounts for sampling error. The alpha threshold for both two-way ANOVAs and each MCT were set at $P < 0.05$. Following from Chapter 3, results of the mortality trial were analyzed with a one-way ANOVA model paired with a MCT to assess if percent survival varied between each population cross. Effect size (ω^2) was also calculated for all variables in each ANOVA table following the same calculations described in Chapter 2. See Table 1 for full ANOVA results, and see Tables 2, 3, and 4 for MCT results where all results are reported to the appropriate significant digits based on the precision of each measurement method used in this study. All nonsignificant digits were truncated.

Results

Shell size during experimentation

Shell lengths of ten randomly selected clams were measured from each population cross on each day out of the subset of clams removed from nursery tanks to begin each experimental trial (Fig. 2). Average shell lengths among all crosses on all days were similar, ranging from 3.7 to 4.3 mm.

Elevated temperature tolerance

Rates of oxygen consumption of juvenile clams from all population crosses were similar across the range of temperatures tested in this study (Fig. 3). No significant effects in the ANOVA model or contrasts in the MCT were detected (Tables 1 and 2). As would be expected from these statistical results, oxygen consumption rates were stable for all crosses at all temperatures with no clear increasing or decreasing trends.

Results of the mortality trial at 36ºC revealed slight differences between population crosses (Fig. 4); however, none of the observed differences in percent survival were found to be significant (Tables 1 and 3). The BS x CC cross showed the highest percent survival, followed by P x W, with the W self-cross having the lowest observed survival rate.

Low salinity tolerance

Clear changes in oxygen consumption rates were observed in all population crosses when exposed to low salinity (Fig. 5). A significant interaction between salinity and population cross was detected [ANOVA, $F(6,97)=2.511$, $P=0.0266$, $\omega^2=0.0125$]; however, the effect size of this interaction is very small, indicating it has little practical effect in the model (Table 1). All populations showed an increase in oxygen consumption when exposed to a salinity of 20,

followed by a large decrease in oxygen consumption at a salinity of 15. A slight decrease in oxygen consumption rate is also observable between salinities of 15 and 12, but this decrease is minimal compared to the decrease from 20 to 15. The only significant differences observed between population crosses were seen at a salinity of 20, where both BS x CC and P x W had significantly higher oxygen consumption rates than the W self-cross [Sidák MCT, BS x CC v W x W: t=4.052, P=0.0003, P x W v W x W: t=3.639, P=0.0013] (Table 4). Oxygen consumption was similar between population crosses at the other three salinity levels evaluated in this study.

Discussion

This study assessed differences in physiological tolerance among juvenile hard clams produced from selected population crosses exposed to a series of elevated temperatures and lower salinities. By exploring if physiological tolerance can be enhanced through selective cross breeding, this study aims to provide foundational knowledge that can help to support the aquaculture industry as climate change progresses.

Elevated temperature tolerance

No differences were detected across any of the elevated temperature trials for any population cross. The consistent oxygen consumption rates observed across all temperature conditions agree with rates observed in parent populations in 2019 (Chapter 2 Fig. 3) as well as previous studies that found elevated temperatures had minimal impacts on *M. mercenaria* (Ulrich and Marsh, 2009; Matoo et al., 2013; Stevens and Gobler, 2018). These results serve to strengthen the growing body of evidence that *M. mercenaria* employs alternative means of

responding to elevated temperature stress, or that this species is particularly well adapted to the temperatures employed in this study (Ulrich and Marsh, 2008; Ulrich and Marsh, 2009; Wang et al., 2016). The need to further understand mitochondrial regulation, cellular stress response mechanisms, and protein characteristics in *M. mercenaria* are underscored here as a deeper understanding of all of these areas will be needed to explore if population-level variability in thermotolerance does exist within this species. The classical physiological techniques employed throughout the literature, and in this study specifically, did not provide much insight into how hard clams respond to elevated temperature stress so future studies focused on cellular mechanisms will be crucial to evaluating how *M. mercenaria* will fare with continuing climate change.

Interestingly, results from several parent populations in 2019 showed potential decreases in oxygen consumption at 35ºC; however, this was not observable in any of the population crosses conducted in 2021. As the 2019 decreases were slight and nonsignificant, it is possible they are mere aberrations due to the high inter-animal variability observed in all populations. Alternatively, differences between study years could also be the result of variation in spawn classes potentially due either to differences in parental provisioning, different environmental conditions experienced in nursery tanks, or genetic variation among the different parents used in each year (Miyawaki and Sekiguchi, 1999; Ahn et al., 2003; Viergutz et al., 2012; Hu et al., 2019; Song et al., 2021; Farhat et al., 2022). Much more work is needed to explore these factors and the role they may play in influencing juvenile clam performance.

Trends in percent survival showed some variation among crosses; however, these results were also nonsignificant. Additionally, the effect size for population cross was small, indicating the observed variations are not strongly linked to the cross of origin. The W self-cross in this

study showed a similar survival rate to the W self-cross conducted in 2019, indicating that interannual variability, at least for this population, may have been small between 2019 and 2021; however, more information would be needed to truly assess if this was the case. Furthermore, the survival rate observed in the P x W outcross was greater on average than the W self-crosses in both 2019 and 2021 as well as the P self-cross in 2019, which could be indicative of heterosis (Chapter 2 Fig. 4). The BS x CC outcross also showed greater survival than the CC self-cross from 2019, which also supports improved tolerance in offspring as a result of crossbreeding; however, more work is needed over successive generations to see if these trends persist before any such conclusions could be drawn (Edmands, 2007; Frankham et al., 2010). While confounding factors like variation in parental genetic makeup as well as potential differences in abiotic factors could influence percent survival, as discussed for oxygen consumption rates, some signs are present of improved physiological performance among outcrosses and, therefore, warrants further exploration.

Low salinity tolerance

Juvenile hard clams from all population crosses had similar low salinity tolerances. Increases in oxygen consumption rates were observed in all crosses when exposed to a salinity of 20; however, these increases were significantly larger in both outcrosses compare to the W self-cross. Clams from all crosses then showed large decreases in oxygen consumption at a salinity of 15, which remained similarly low at a salinity of 12. The significantly higher oxygen consumption seen in both outcrosses compare to the W self-cross at a salinity of 20 could be interpreted in one of two ways. First, this greater increase could indicate these clams possess a greater aerobic scope, and therefore have a greater capacity for increasing cellular energy

production to meet increased cost of cellular maintenance (Pörtner, 2012; Sokolova et al., 2012). Alternatively, individuals from the W self-cross may have been less stressed at a salinity of 20 and had a lesser need for increasing cellular energy production. As all population crosses were severely impacted at a salinity of 15, it is difficult to conclusively state which could be the case without further evidence at salinities above and below 20 to better capture the nature of each population cross' response. While it is challenging to understand what the increases in oxygen consumption rates at a salinity of 20 might mean among the population crosses, when comparing these results to the juvenile clam data from 2019, an interesting difference becomes apparent.

All juvenile clams tested in 2019, regardless of population, tolerated low salinity stress better than clams from each of the three population crosses assessed in this chapter. In 2019, while differences between parent populations were apparent in oxygen consumption rates at a salinity of 15, all populations had average oxygen consumption rates over twice that observed for all three of the 2021 crosses. While this could be indicative of outbreeding depression with respect to salinity tolerance for the two outcrosses, the dramatic difference observed in W clams between years may highlight instead that other factors such as parental provisioning or underlying genetic variability among the adult clams used for spawning had strong influences on low salinity tolerance among larval year classes (Swain et al., 2002; Edmands, 2007; Lei et al., 2015; Hu et al., 2019; Song et al., 2021; Farhat et al., 2022). If underlying genetic variability is indeed responsible for the drastic shift in low salinity tolerance observed between years, further research is certainly warranted as individuals with more robust low salinity tolerances would be ideal candidates for future selective breeding efforts. Furthermore, as spawning in commercial settings can often involve only a few adults, highly variable salinity tolerances could result in large changes in the number of cultured clams that survive to market size in any given year

(Gaffney et al., 1992; Appleyard and Ward, 2006). Consequently, more selective breeding studies in *M. mercenaria* are needed as, at least with respect to low salinity tolerance, interannual variability could complicate efforts in the future to produce clam lines resistant to low salinity stress. Attention will also need to be paid to preserving genetic variability within any selectively bred line through periodic outcrossing to prevent inbreeding depression. While this study demonstrates some of the potential difficulties in producing selectively bred hard clams with improved low salinity tolerances, the potential benefits to sustainable aquacultural harvest in the face of ongoing climate change should not be overlooked.

Conclusions

The assessment of selected population crosses of hard clams revealed some potential signs of improved tolerance in the two outcrosses compared to their parent populations. In particular, percent survival of Pocomoke x Wachapreague clams was greater than either parent population assessed in Chapter 3. However, signs of interannual variability in tolerance to low salinity stress are also evident from the low salinity exposures, where all three crosses showed clear signs of metabolic depression at a salinity of 15, which was not observed in any parent populations assessed previously. This difference could be a sign of outbreeding depression with respect to low salinity tolerance, but the drop in performance in the Wachapreague self-cross suggests that interannual variability or some other unknown source may instead be the driver. While outbreeding depression cannot be conclusively dismissed after a single generation, the data presented in this study provide positive indications that selective breeding for improved physiological tolerance could be a viable tool for providing support to the hard clam aquaculture industry. As climate change continues to alter habitats, there is an ever growing need to

understand how cultured shellfish species will be impacted and what resources can be developed to help support and maintain local economies reliant on them.

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Table 1. Full ANOVA tables for each model run, with calculated effect sizes (ω^2). Column headings abbreviations: degrees of freedom (DF), degrees of freedom of numerator (DFn), degrees of freedom of denominator (DFd).

Experiment	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Elevated temperature tolerance							
	Temperature x Population cross	8	9.475	1.184	F (8, 103) = 0.418	0.9082	-0.0399
	Temperature	4	26.59	6.648	F (4, 103) = 2.344	0.0597	0.0460
	Population cross	2	0.5904	0.2952	F (2, 103) = 0.104	0.9013	-0.0153
	Residual	103	292.2	2.837	-	-	-
Low salinity tolerance							
	Salinity x Population cross	6	20.35	3.391	F (6, 97) = 2.511	0.0266	0.0125
	Salinity	3	818.1	272.7	F (3, 97) = 201.9	<0.0001	0.8304
	Population cross	2	9.449	4.724	F (2, 97) = 3.498	0.0341	0.0069
	Residual	97	131	1.351	-	-	-
Mortality trial							
	Treatment (between groups)	2	5227	2613	F (2, 27) = 3.133	0.0597	0.1245
	Residual (within groups)	27	22520	834.1	-	-	-

Table 2. Summary statistics for all contrasts of oxygen consumption rates evaluated from the elevated temperature trials using the Sidák multiple comparisons test (α=0.05). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Column headings abbreviations: degrees of freedom (DF).

Experiment	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Elevated temperature tolerance						
25°C						
	BSxCC vs. PxW	103	-0.3206	-2.365 to 1.723	0.3807	0.9741
	BSxCC vs. WxW	103	-0.09751	-2.084 to 1.889	0.1192	0.9992
	PxW vs. WxW	103	0.2231	-1.763 to 2.209	0.2726	0.9902
27.5°C						
	BSxCC vs. PxW	103	0.3732	-1.505 to 2.251	0.4822	0.9496
	BSxCC vs. WxW	103	0.4945	-1.384 to 2.373	0.6391	0.8923
	PxW vs. WxW	103	0.1214	-1.707 to 1.950	0.1611	0.9979
30°C						
	BSxCC vs. PxW	103	0.2132	-1.847 to 2.273	0.2511	0.9923
	BSxCC vs. WxW	103	1.015	-0.9996 to 3.029	1.223	0.533
	PxW vs. WxW	103	0.8018	-1.076 to 2.680	1.036	0.6608
32.5°C						
	BSxCC vs. PxW	103	-0.2304	-2.505 to 2.044	0.2459	0.9927
	BSxCC vs. WxW	103	-0.2378	-2.423 to 1.947	0.2641	0.991
	PxW vs. WxW	103	-7.307E-03	-2.282 to 2.267	0.0078	>0.9999
35°C						
	BSxCC vs. PxW	103	-0.721	-3.081 to 1.639	0.7414	0.8426
	BSxCC vs. WxW	103	-1.038	-3.398 to 1.322	1.067	0.6397
	PxW vs. WxW	103	-0.3167	-2.677 to 2.043	0.3257	0.9835

Table 3. Summary statistics for all contrasts of percent survival evaluated from the mortality trial using the Sidák multiple comparisons test (α=0.05). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Column headings abbreviations: degrees of freedom (DF).

Experiment	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Mortality trial						
36°C						
	BSxCC vs. PxW	27	20	-12.87 to 52.87	1.549	0.3486
	BSxCC vs. WxW	27	32	-0.8712 to 64.87	2.478	0.0582
	PxW vs. WxW	27	12	-20.87 to 44.87	0.9291	0.7392

Table 4. Summary statistics for all contrasts evaluated from the low salinity trials using the Sidák multiple comparisons test (α=0.05). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Column headings abbreviations: degrees of freedom (DF).

Experiment	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Low salinity tolerance						
32						
	BSxCC vs. PxW	97	-0.3206	-1.732 to 1.091	0.5517	0.9272
	BSxCC vs. WxW	97	-0.09751	-1.470 to 1.275	0.1727	0.9974
	PxW vs. WxW	97	0.2231	-1.149 to 1.595	0.395	0.9713
20						
	BSxCC vs. PxW	97	-8.943E-03	-1.497 to 1.479	0.0146	>0.9999
	BSxCC vs. WxW	97	2.22	0.8887 to 3.551	4.052	0.0003
	PxW vs. WxW	97	2.229	0.7405 to 3.717	3.639	0.0013
15						
	BSxCC vs. PxW	97	-0.11	-1.373 to 1.153	0.2116	0.9953
	BSxCC vs. WxW	97	0.01124	-1.252 to 1.274	0.02162	>0.9999
	PxW vs. WxW	97	0.1212	-1.142 to 1.384	0.2332	0.9938
12						
	BSxCC vs. PxW	97	0.04462	-1.218 to 1.307	0.08585	0.9997
	BSxCC vs. WxW	97	0.1432	-1.120 to 1.406	0.2756	0.9898
	PxW vs. WxW	97	0.09861	-1.164 to 1.361	0.1897	0.9966

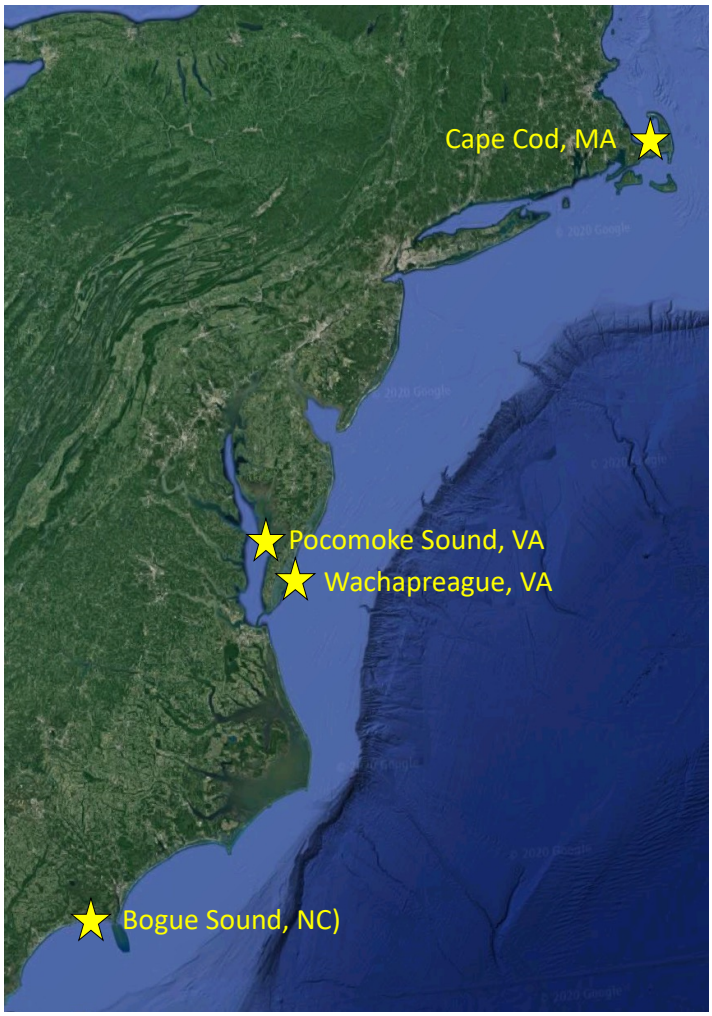

Figure 1. Map of adult hard clam source populations, marked by yellow stars, used in each cross. Map image credit: Google.

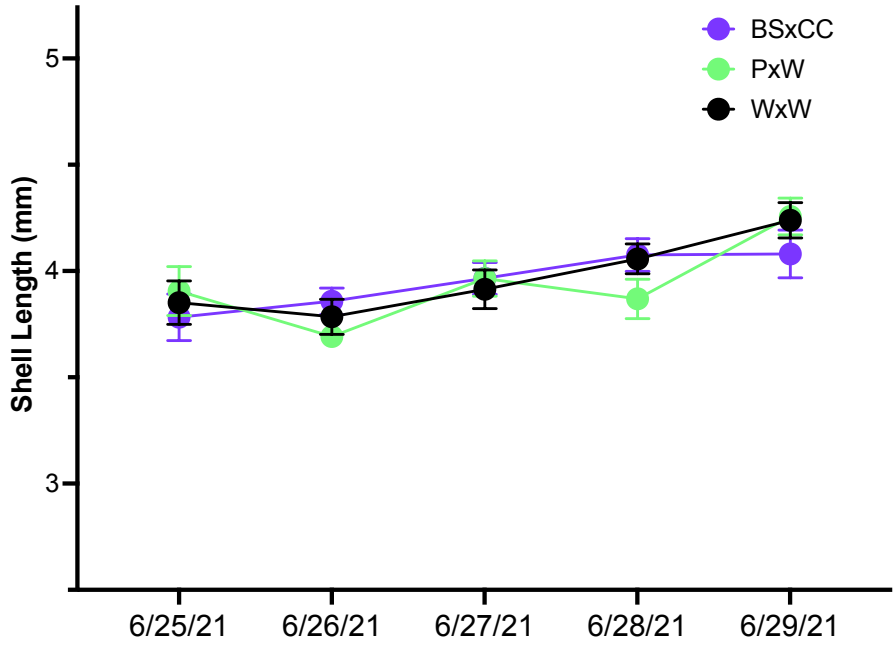

Figure 2. Shell length in millimeters from ten randomly selected clams from each population cross on each day (mean ± SEM). All clams were measured after removal from nursery tanks, but before being placed into an experimental treatment. Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Lack of visible error bars indicates that they fall within the graphic of the data point itself.

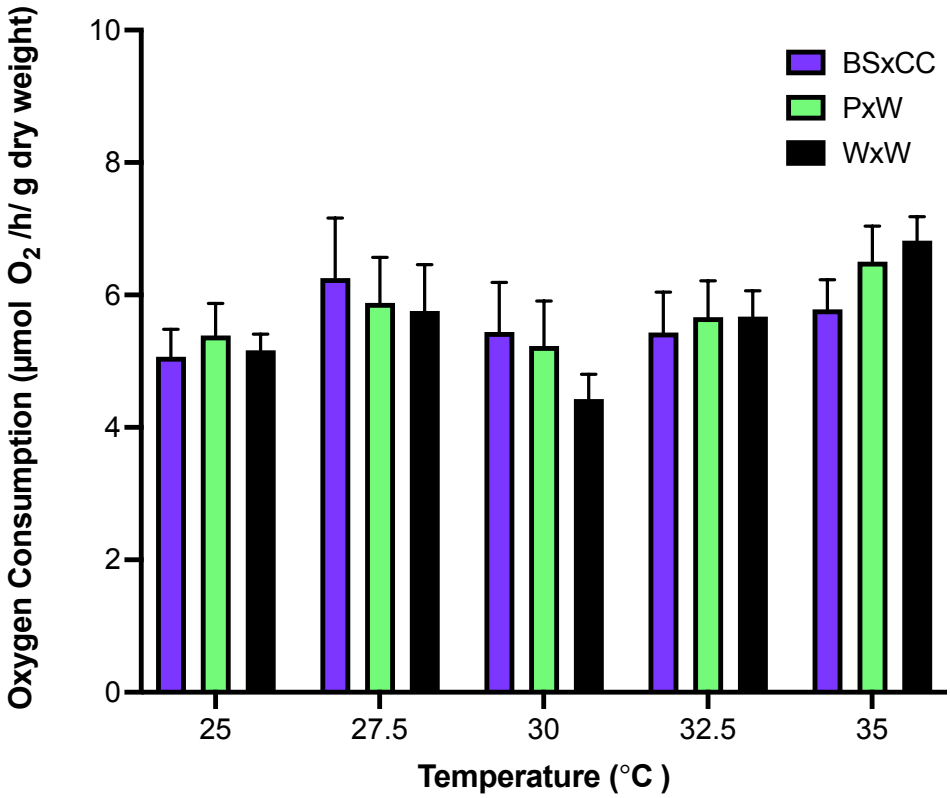

Figure 3. Oxygen consumption rates in micromoles of oxygen consumed per hour per gram total dry weight of juvenile clams (n=6-10) from three population crosses after each temperature exposure (mean ± SEM). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W).

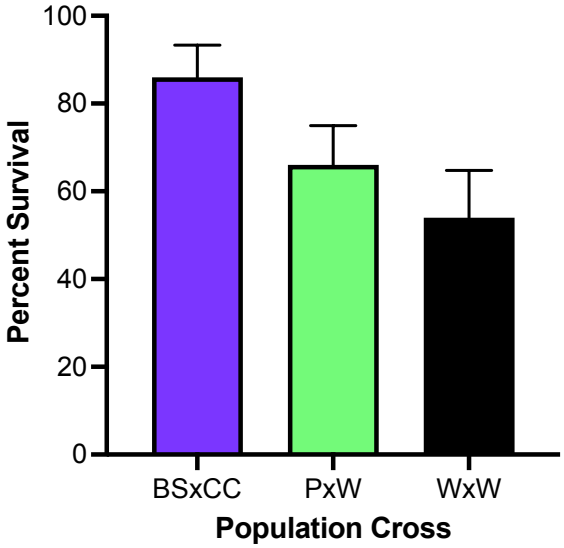

Figure 4. Percent survival of juvenile clams from three population crosses exposed to 36°C (mean ± SEM). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). For all populations, n=10.

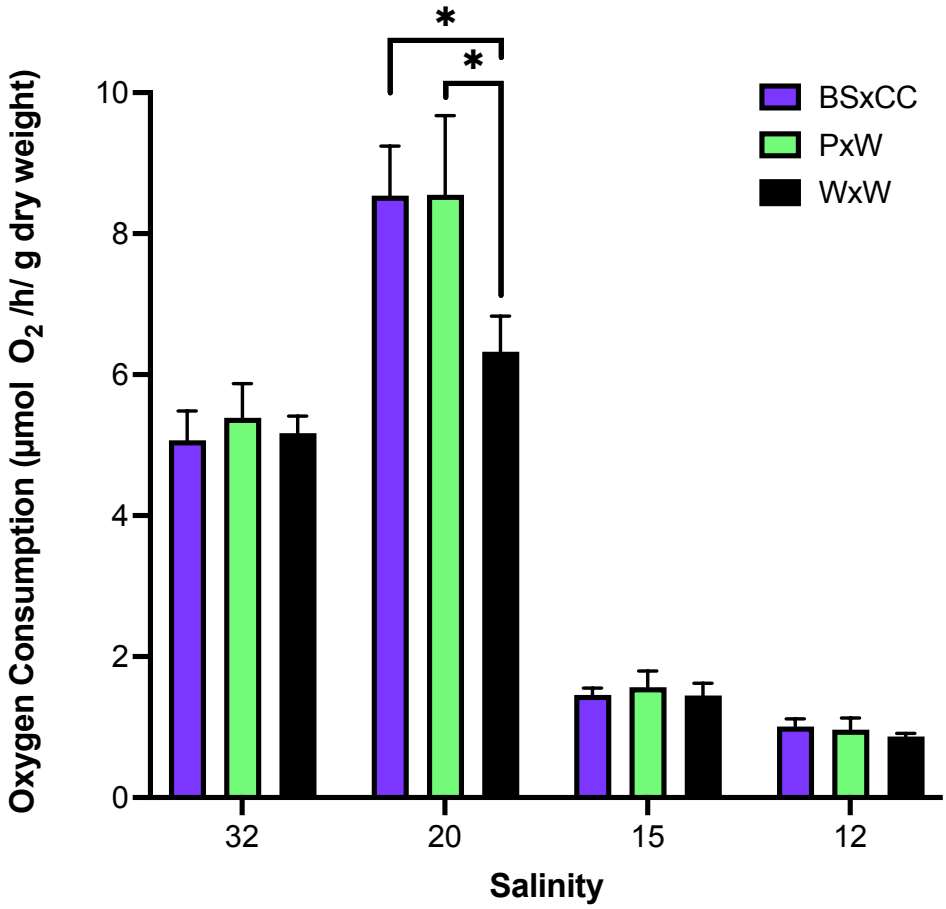

Figure 5. Oxygen consumption rates in micromoles of oxygen consumed per hour per gram total dry weight of juvenile clams (n=8-10) from three population crosses after each salinity exposure (mean ± SEM). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Asterisks denote significant differences between population crosses.

Chapter 5

A coupled physiological and microbiomic approach to understanding the effects of elevated temperature and low salinity on larval *Mercenaria mercenaria*

Abstract

The hard clam, *Mercenaria mercenaria*, is an ecologically and economically important species that inhabits the eastern coast of the United States and is threatened by projected ocean warming and increased precipitation due to climate change. In particular, the larval life stage of *M. mercenaria* is most susceptible to environmental stress, but less is known about how hard clam larvae respond to elevated temperature or lower salinity stress. Alongside the project conducted in Chapter 4, this study examined stress tolerance in a subset of larvae produced from the same three controlled population crosses from which juvenile clams were later evaluated (Cape Cod, MA clams crossed with Bogue Sound, NC clams; Pocomoke Sound, VA clams crossed with Wachapreague, VA clams; Wachapreague, VA clam self-cross). In this way, not only could larval clam tolerance be evaluated, but the potential influence of population-level variability on environmental stress tolerance could also be assessed. In addition to measuring both whole-animal and cellular level physiological responses, a larval microbiome analysis was also conducted to examine shifts in microbial communities in response to environmental stress. Assessing larval clam microbiomes provided foundational knowledge on the composition of larval microbial communities, as few previous studies have explored this subject. Larvae exposed to an elevated temperature condition (+5°C above ambient) showed increased growth, but reduced survival among all population crosses; however, larvae from the Pocomoke Sound, VA and Wachapreague, VA cross showed a smaller reduction in survival compared to each of the other two crosses. Larval microbiomes were altered by exposure to an increased temperature condition. Microbial communities from both the cross of Pocomoke Sound, VA and Wachapreague, VA clams and the Wachapreague, VA self-cross showed similar alterations under elevated temperature stress, but larvae produced from the Cape Cod, MA and Bogue

Sound, NC cross had markedly different initial microbial community structures that also experienced their own unique shifts. Lower salinity stress (salinity reduction of 5 from ambient) was more impactful on both larvae and their microbiomes compared to the elevated temperature stress assessed here. Changes in survival, growth, and antioxidant potential were observed among populations crosses. Larvae from the Cape Cod, MA and Bogue Sound, NC cross showed the highest survival at lower salinity, but also the least amount of shell growth. All crosses showed large decreases in triglyceride content, indicating that low salinity exposure was increasing cellular energy demand, which in a more prolonged exposure could reduce successful recruitment of larvae into wild populations. Larval microbiomes shifted most dramatically under low salinity as well. Both the Pocomoke, VA with Wachapreague, VA cross and the Wachapreague, VA self-cross showed similar shifts in community structure, but once again the shifts observed in the Cape Cod, MA with Bogue Sound, NC cross were unique. These results demonstrate not only that larval microbiomes are likely to shift with ongoing climate change, but that they also are influenced by parental provisioning and thus are a mechanism for transgenerational plasticity. More work is needed to better understand both larval clam stress response mechanisms as well as the role specific microbial taxa play in larval environmental stress responses.

Introduction

While Chapter 4 explored physiological tolerance to environmental stress in juvenile hard clams produced from controlled population crosses, this chapter explores tolerance in a subset of larvae produced from those same spawns. Larval tolerance to elevated temperature and low salinity were assessed, similarly to Chapter 4, as both ocean warming and the intensity of low

salinity events are projected to increase along the east coast of the United States, the study region for this project, as climate change progresses (Walsh et al., 2016; Muhling et al., 2018; Karmalkar and Horton, 2021; Masson-Delmotte et al., 2021; Ross et al., 2021). Larvae were specifically targeted for further study as previous work in various bivalve species have noted increased susceptibility to environmental stressors during the larval life stage (Talmage and Gobler, 2010; Pörtner, 2012; Waldbusser et al., 2013; Waldbusser et al, 2015a; Waldbusser et al, 2015b; Frieder et al, 2017; Mangan et al., 2017). Furthermore, little work exploring cellular responses to physiological stress has been conducted in larval *M. mercenaria*, let alone any analysis of potential population-level variability in larval stress tolerance. Only a few studies have been conducted that explore either the impacts of elevated temperature or low salinity stress on larval *M. mercenaria*, and none of these studies have assessed cellular-level metrics (Davis, 1958; Davis and Calabrese, 1964; Talmage and Gobler, 2011; Rugila, 2022). Much of the rest of the larval clam stress physiology literature has focused on stress caused by acidification, changes in dissolved oxygen concentration, and copper toxicity, where once again most studies examined whole-organism level measures with only a few cellular metrics reported (LaBreche et al., 2002; Talmage and Gobler, 2009; Talmage and Gobler, 2011; Gobler and Talmage, 2013; Gobler et al., 2017; Rugila, 2022). Similarly to the juvenile clam literature discussed in the introduction of Chapter 3, many of these larval studies used larvae supplied by a hatchery with unreported parental lineages, which is unlikely to be representative of populations throughout the entire biogeographic range of *M. mercenaria* (LaBreche et al., 2002; Talmage and Gobler, 2009; Talmage and Gobler, 2011; Gobler et al., 2017). Limited knowledge of population-level variability and cellular stress responses in larval *M. mercenaria* make accurately understanding

how climate change will impact this species difficult, as negative impacts at the larval life stage could result in reduced recruitment into adult populations.

Climate change may impact larval fitness not only through direct effects on larval physiology, but also through induced changes in larval microbiomes. However, little is understood about larval microbial communities, the benefits they provide to their larval host, and how ongoing climate change could alter these symbiotic relationships, which could potentially reduce larval fitness. Many examples exist within the marine invertebrate literature of symbiotic relationships between host species and their microbiome, which provide benefits to the host, including improved digestion, immune health, and even camouflage (Muscatine et al. 1981, McFallNgai et al. 2013; Zhang et al., 2016; Pierce and Ward, 2018; Nyholm and McFall-Ngai, 2021). Due to the vast level of diversity that exists throughout microbial phyla, little is known about the specific functions of individual microbes or how specific taxa will be impacted by environmental stress (Pierce et al., 2016; Pierce and Ward, 2018; Cavicchioli et al, 2019; Sepulveda and Moeller, 2020). Some previous studies have focused specifically on pathogenetic microbes in primarily adult bivalves, such as *Vibrio* species, but as technologies and analytical tools have improved, more broad-scale studies have begun exploring shifts in bivalve microbial communities (Beaz-Hidalgo et al., 2010; Pierce and Ward, 2018; Neu et al, 2020 Masanja et al., 2023). Recent work has shown that microbiome diversity and composition can change seasonally as well as vary geographically within bivalve species, likely in response to associated changes in environmental conditions (Pierce et al., 2016; Neu et al., 2020). Community composition in particular seems to be the factor that is most impacted by environmental fluctuations, which could change the benefits a host receives from its microbiome as certain taxa are lost (Alma et al., 2020; Neu et al., 2020; Timmins-Schiffman et al., 2021). In addition to

these environmental impacts, several studies have identified major shifts in microbial communities associated with transitions between life history stages (Dai et al., 2023; Ma et al., 2023). This further demonstrates the need to understand the role microbiomes will play in the response of larval clams to future climate change as understanding adult microbiomes, even within the same species, will likely not be representative of their earlier life history stages.

Little work has explored the microbiome of *M. mercenaria* specifically at any life stage, with the exception of a recent study by Rugila (2022) examining changes in the microbial communities of both larval and juvenile hard clams in response to acidification and dissolved oxygen stress. Rugila (2022) found significant differences in community composition across life stages as well as significant shifts in composition and diversity in relation to environmental stress. Alterations in microbial community composition in response to environmental stress are concerning, as they have been linked to decreases in digestive efficiency and impaired immune health (Muscatine et al. 1981; Holmes et al. 2011; McFallNgai et al. 2013; Apprill 2017). These potential negative impacts from shifts in microbial communities highlight the need to understand the roles that other environmental stressors, such as elevated temperature and low salinity, could play in shaping larval microbiomes.

The goals of this study were, therefore, to 1) gain insight into the underlying cellular stress mechanisms of larval *M. mercenaria* in response to elevated temperature and lower salinity stress, 2) explore if selective breeding of hard clam populations could influence larval stress tolerance and microbial communities of larvae, and 3) contribute foundational knowledge about larval microbial community structures under both ambient and stressed conditions. Larvae from the three population crosses previously described in Chapter 4 (Cape Cod, MA clams crossed with Bogue Sound, NC clams; Pocomoke Sound, VA clams crossed with Wachapreague,

VA clams; Wachapreague, VA clam self-cross) were exposed to either an elevated temperature or lower salinity condition. These experimental trials were conducted during the first week post-fertilization, when it is known that larvae are most vulnerable to various environmental stressors (Talmage and Gobler, 2010; Pörtner, 2012; Waldbusser et al., 2013; Waldbusser et al, 2015a; Waldbusser et al, 2015b; Frieder et al, 2017; Mangan et al., 2017). To assess overall health of clam larvae, percent survival was calculated over the duration of each exposure, and growth of both shell and somatic tissue were monitored by measuring shell length and total protein content, respectively. Cellular stress was evaluated by measuring two oxidative stress markers as well as changes in total triglyceride (TG) content, as triglycerides are known to be a key energy storage molecule within marine invertebrate larvae (Moran and Manahan, 2004; Genard et al., 2011; Prowse et al., 2017; Gibbs et al., 2021). Alongside these physiological metrics, 16S rRNA gene sequencing was conducted to assess changes in diversity and community composition of larval microbiomes when exposed to environmental stress. This coupled analysis of both physiological tolerance and microbial community structure in larval *M. mercenaria* from controlled population crosses will help to improve the understanding of how larval clams will respond to future ocean conditions as well as if selective breeding alters larval clam tolerances in ways potentially beneficial to the hard clam aquaculture industry.

Materials and Methods

Broodstock and spawning

The hard clam larvae examined in this chapter were a subset of larvae produced from the same spawns that generated the juvenile clams evaluated in Chapter 4. For full methodologies,

see the *Broodstock and spawning* section of Chapter 4. In brief, three population crosses were conducted using adults collected from four parent populations: Cape Cod, MA, (CC), Pocomoke Sound, VA (P), and Wachapreague, VA (W). Adults were brought to the Virginia Institute of Marine Science (VIMS) Eastern Shore Laboratory (ESL) where they were acclimated to ambient water conditions in research plots near Wachapreague, VA for at least six months before spawning. The first spawn was conducted on April 1st, 2021 and was a cross of BS clams with CC clams. The next two spawns, a W self-cross and a P x W cross, were conducted on April 6th, and larvae were assessed in parallel. All experimentation was conducted between April 3rd and April 12th, 2021.

Experimental design

M. mercenaria larvae from all spawns were raised under standard hatchery conditions until 2 days post fertilization (dpf), when larvae were transferred into either a control condition (T=23°C, S=32), elevated temperature condition (T=28°C, S=32), or lower salinity condition (T=23°C, S=27). As few previous studies on larval hard clam thermotolerance or low salinity tolerance have been conducted, exposure levels were chosen that would likely induce only a mild to moderate stress without exceeding the tolerance limits of the larvae. Additionally, as some larvae had to remain in the VIMS ESL hatchery to produce the juvenile clams assessed in Chapter 4, larval supplies were limited, and elevated mortality due to the selection of a more extreme experimental treatment could have prevented successful sampling over the duration of each exposure.

Once larvae reached 2 dpf, approximately 500,000 from each population cross were removed from the hatchery and divided into each of the three treatments. Larvae were held in

aerated 5-gal plastic buckets (from here on referred to as tanks) that were placed within larger water baths containing 100 L aquarium tank heaters that were used to maintain either the control or elevated temperature condition for each respective treatment. Larvae from each spawn were divided among two replicate tanks per treatment that were later sampled after 48 and 96 h of exposure. A YSI Pro Plus (6050000) was used to monitor temperature and salinity in each tank over the course of each experimental trial (Table 1). A water change was done 48 h into each exposure following standard hatchery practices, and since each exposure was ended at the 96-h time point, there was no need for further water changes. Treatment water was prepared in larger revisor tanks the night before it was needed to allow it to reach the appropriate temperature for each exposure. All treatments were prepared using 1 μm filtered seawater, which was diluted with dechlorinated freshwater to achieve the lower salinity treatment. Larvae from all spawns were fed live algal cultures daily following standard hatchery practices for *M. mercenaria*. On the first two days of experimentation, larvae were fed a mixture of *Pavlova* and Tahitian *Isochrysis* (T-iso) before the addition of *Chaetoceros* in the final two feedings.

Before each exposure began, a subsample of larvae from the initial stock pool of approximately 500,000 larvae was imaged using an Olympus CX41 light microscope with an Olympus DP25 digital camera for later shell length measurements. Shell length of 10 randomly selected larvae from each treatment replicate was measured using ImageJ (Schneider et al., 2012). Stock larvae were also collected and frozen at -80°C for later total protein content and non-enzymatic antioxidant potential analysis. After 48 and 96 h of exposure to each of the three conditions, larvae were collected on fine mesh screens, concentrated into a 1 L beaker, and counted on a Sedgewick-Rafter slide in triplicate using the same microscope system describe above. Larvae were also imaged for later shell length measurements at these time points, and

samples of larvae from each individual tank replicate were also frozen for total protein content and non-enzymatic antioxidant potential analysis. At the end of each exposure, all remaining larvae from each tank were collected and frozen for later malondialdehyde (MDA) content determination, TG content determination, and larval microbiome analysis.

Biochemical assays

Total protein content, non-enzymatic antioxidant potential, MDA content, and TG content were all measured following the same procedures laid out in detail in the *Biochemical assays* section of Chapter 2. As in Chapter 2, 10,000 larval clams were homogenized, and this homogenate was then split in order to measure both total protein content and non-enzymatic antioxidant potential following Griffin and Bhagooli (2004). Procedures identical to those described in Chapter 2 were followed for MDA and TG content determination, where 15,000 clam larvae per tank replicate were collected for each MDA sample, and 8,500 clam larvae per tank replicate were collected for each TG sample.

Microbiome sample processing and sequencing

DNA extractions were performed using DNeasy PowerLyzer PowerSoil kits from Qiagen (12855-100). Samples containing 8,000 larvae each were homogenized in a bead beater at 4,500 rpm for 45 s, before following the manufacturer's guidelines for the remainder of the procedure. Once DNA was isolated, quality and purity were assessed using a Thermo Scientific NanoDrop 2000 spectrophotometer. Samples with DNA concentrations below the detection range of the NanoDrop 2000 were quantified using an Invitrogen Qubit dsDNA high sensitivity kit (Q32851) and an Invitrogen Qubit 3.0 Fluorometer (Q33216). Once quantified, DNA was diluted to 0.5

ng/μL and amplified using Phusion high-fidelity DNA polymerase (New England Biolabs, M0530S) with 515F-Y and 806R primers to target the V4 variable region of 16S rRNA (Caporaso et al., 2011; Parada et al., 2016). The Phusion PCR mix per reaction consisted of 5 μL of 5x Phusion HF buffer, 1.25 μL of 10 mM 515F-Y primer, 1.25 μL 10 mM of 806R primer, 0.5 μL of 10 mM dNTPs, 0.25 μL Phusion DNA polymerase, 15.75 μL of sterile water, and 1 μL of sample DNA. The PCR protocol was as follows: 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min; then 72°C for 5 min, before cooling to 12°C. Amplification was then confirmed using gel electrophoresis, and DNA dilutions were adjusted as needed to achieve amplification in all samples. PCRs were then run using Illumina barcoded primers to allow for sample multiplexing, before the sample pools were purified using the Promega Wizard Gel and PCR Cleanup System (PR-A9281). DNA concentrations of each sample pool were then quantified again using the Agilent Technologies D1000 ScreenTape System (5067-5582) and an Agilent Technologies 2200 TapeStation. Each of the three sample pools were then sequencing using the Illumina MiSeq platform.

Sequence processing and analysis

Once samples were sequenced, all Illumina MiSeq sequencing data was processed using the DADA2 pipeline in Rstudio with R v 4.3.0 (Callahan et al., 2016; R Core Team, 2023). Only forward reads were processed because none of the reverse reads in one of the three sequencing runs passed quality control standards. Forward reads that passed the quality filter were trimmed to 200 base pairs, barcode sequences were removed, and any chimeric sequences detected were also removed. Taxonomic identification was determined using the SILVA small subunit rRNA database version 138.1 (Quast et al., 2013). Amplicon sequence variants (ASV) identified as

originating from mitochondrial DNA or chloroplast DNA were removed before further processing.

Microbial community composition and diversity were analyzed using the phyloseq package in R to calculate alpha (α) diversity indices and to perform a principal coordinate analysis (PCoA) using the Bray-Curtis dissimilarity metric (McMurdie and Holmes, 2013). Four α-diversity indices were calculated due to known biases that exist within most diversity indices: the Chao1 index gives more weight to less abundant taxa; the abundance-based coverage estimator (ACE) index reduces abundance data down to two arbitrary categories, either abundant or rare; the Shannon index gives more weight to species richness; and the inverse Simpson's index gives more weight to species evenness (Kim et al., 2017; Roswell et al., 2021). Homogeneity of dispersions among samples was tested using the betadisper function in the vegan package in R before the adonis2 function within vegan was used to run a permutational multivariate analysis of variance (PERMANOVA) in order to assess which treatment factors had significant influence on microbial community structure (Oksanen et al., 2022). Lastly, a canonical analysis of principal coordinates (CAP) calculated using the Bray-Curtis dissimilarity metric was plotted to explore the effects of specific levels of each treatment factor. All plots were generated using the ggplot2 package in R (Wickham, 2016).

Statistical analyses for larval physiology

All physiological data were analyzed using GraphPad Prism 9 (San Diego, California, USA). To assess differences in shell length, total protein content, and non-enzymatic antioxidant potential, full three-way analysis of variance (ANOVA) models were fit with time, population cross, and either temperature or salinity as independent categorical factors. For percent survival,

MDA content, and TG content, full two-way ANOVA models were used with population cross and either temperature or salinity as independent categorical factors. Larvae at 2 dpf were not included in statistical analyses due to the lack of replication at this time point. Sidák multiple comparison tests (MCT) were used to assess *a priori* contrasts for all ANOVA models. The alpha threshold for all ANOVAs and MCTs was set at $P < 0.05$. The contrasts assessed for each three-way ANOVA compared crosses at each time point within either the two salinity or temperature levels to assess if physiological tolerances differed among crosses through time. Contrasts for each two-way ANOVA compared population crosses within each treatment level to assess if physiological responses varied among crosses. Effect sizes (ω^2) were also calculated for all variables in each ANOVA table to assess the proportion of total variance each effect accounted for in the model, following the same calculations explained in the *Statistics* section of Chapter 2 (Olejnik and Algina, 2000). All statistical results shown in Tables 2 through 10 are reported to the appropriate significant digits based on the precision of each metric, respectively. All nonsignificant figures were truncated.

Results

Treatment conditions

As seen in Table 1, temperatures and salinities were well maintained over the duration of each exposure. On average, the low salinity conditions were closer to a salinity of 28 instead of the intended salinity of 27; however, since few previous studies have explored low salinity tolerance in larval *M. mercenaria* and the treatment was intended to induce a mild to moderate stress response, this does not affect the results of this study. Temperature levels were maintained

within one degree on average for both the control and elevated temperature conditions across all three population crosses.

Survival

Temperature was found to have a significant overall effect on percent survival [ANOVA, $F(1,6)=13.65$, $P=0.0101$, $\omega^2=0.4870$] with a large effect size, indicating that this variable accounted for nearly half the variance observed in this dataset (Table 2). An observed decrease in survival can be seen in each population cross by the end of the 96-h exposure; however, no significant contrasts were detected among crosses at either the control or elevated temperature condition (Fig. 1A, Table 3).

Salinity and population cross were both found to have significant effects on percent survival in the lower salinity exposure [ANOVA, salinity: $F(1,6)=12.36$, $P=0.0126$, $\omega^2=0.3558$; population cross: $F(2,6)=5.454$, $P=0.0447$, $\omega^2=0.2791$] (Table 4). Both variables have moderate effects sizes, with salinity accounting for slightly more variance in the model. All population crosses showed a decrease in percent survival when exposed to a salinity of 28, where BS x CC clams appeared to survive better than both other crosses; however, no significant contrasts were detected between crosses (Fig. 1B, Table 5).

Shell Length

Population cross, time, and temperature were all found to have significant effects on shell length in clam larvae [ANOVA, population cross: $F(2,228)=6.567$, $P=0.0017$, $\omega^2=0.0168$; time: $F(1,228)=372.3$, $P<0.0001$, $\omega^2=0.5612$; temperature: $F(1,228)=41.28$, $P<0.0001$, $\omega^2=0.0609$]. Time had a substantially larger effect size then either population cross or temperature, both of

which were of little practical significance (Table 6). Increasing trends in shell length were observed over time in both control and elevated temperature conditions for all three crosses (Fig. 2A). While no significant contrasts were detected between crosses exposed to the elevated temperature treatment at either time point, under control conditions P x W larvae were significantly larger than BS x CC larvae at 6 dpf [Sidák MCT, BS x CC v P x W: t=3.768, P=0.0137] (Table 7).

Variation in shell length was significantly influenced by the interaction of population cross and salinity [$F(2,228)$=4.452, P=0.0127, ω^2=0.0087], but the effect size of this interaction is incredibly small, and therefore, of no practical significance (Table 8). Trends in shell growth were more variable between population crosses exposed to lower salinity, where BS x CC and P x W larvae were slightly smaller on average compared to their control counterparts, but this difference was not seen in W x W larvae (Fig. 2B). In the control treatment at 6 dpf BS x CC larvae were significantly smaller than P x W larvae, as was seen in the elevated temperature ANOVA model [Sidák MCT, BS x CC v P x W: t=4.329, P=0.0015]; however, under the lower salinity condition, no such difference was detected (Table 9). Larvae from the BS x CC cross were significantly smaller than W x W larvae at both 4 and 6 dpf under the lower salinity treatment [Sidák MCT, 4 dpf BS x CC v W x W: t=4.176, P=0.0028; 6 dpf BS x CC v W x W: t=4.541, P=0.0006].

Total protein content

Total protein content was significantly affected by population cross, time, and temperature in the elevated temperature ANOVA [population cross: $F(2,12)$=4.445, P=0.0359, ω^2=0.0635; time: $F(1,12)$=61.55, P<0.0001, ω^2=0.5578; temperature: $F(1,12)$=16.56, P=0.0016,

ω^2=0.1433] (Table 6). Time had the greatest effect in the model, while temperature had a more moderate effect. The effect size of population cross was slight, and therefore, of little practical importance. Total protein content showed an increasing trend over time in both temperature treatments for all population crosses. BS x CC clam larvae did appear to be smaller on average than the other two crosses, but no significant contrasts were detected for this metric (Fig. 3A, Table 7).

When assessing the effect of salinity on total protein content, a significant interaction of population cross and time was detected [ANOVA, $F(2,12)=8.208$, $P=0.0057$, $\omega^2=0.0569$] (Table 8) although this interaction is of little practical importance in the model based on its small effect size. All population crosses showed similar total protein content compared to their control counterparts at each time point, and as seen in the elevated temperature assessment, BS x CC larvae did appear smaller on average than either of the other crosses; however, no significant contrasts were detected between any crosses (Fig. 3B, Table 9).

Non-enzymatic antioxidant potential

A significant interaction of population cross and time was detected in the non-enzymatic antioxidant potential of clam larvae from the temperature conditions [ANOVA, $F(2,12)=9.153$, $P=0.0039$, $\omega^2=0.3471$], with a moderate effect size indicating that antioxidant potential changes over time differently among population crosses (Table 6). Different changes in antioxidant potential over time can be observed within the control groups of each population cross, where BS x CC larvae show a decreasing trend over time, while the other two crosses on average maintain a consistent antioxidant potential through time (Fig. 4A). Trends between crosses differ even more under the elevated temperature treatment where BS x CC larvae still show a decreasing

trend in antioxidant potential over time, P x W larvae remain at a similar level between 4 and 6 dpf, and W x W larvae show an increasing trend from 4 to 6 dpf. Despite these varying trends, no significant contrasts were detected between population crosses at either time point (Table 7).

In the low salinity analysis for non-enzymatic antioxidant potential, the three-way interaction of population cross, time, and salinity was found to be significant [ANOVA, $F(2,12)=4.606$, $P=0.0328$, $\omega^2=0.1058$], with a small, but not inconsequential, effect size indicating that not only does antioxidant potential change over time differently between population crosses, but that the responses of crosses at the two salinity levels are also different (Table 8). Both P x W and W x W larvae showed initial decreases in antioxidant potential at 4 dpf followed by increases at 6 dpf (Fig. 4B). BS x CC larvae cultured under low salinity, however, showed a large increase in antioxidant potential at 6 dpf, which was significantly greater than the average antioxidant potential observed in either of the other two crosses [Sidák MCT, 6 dpf BS x CC v P x W: $t=6.446$, $P=0.0021$; 6 dpf BS x CC v W x W: $t=4.827$, $P=0.0270$] (Table 9).

Malondialdehyde (MDA) content

No significant differences were detected in MDA content among larvae from any cross at either temperature level (Tables 2 and 3). Larvae from all population crosses appear to have lower MDA concentrations compared to their respective control groups, although variability between treatment replicates was high (Fig. 5A).

A significant effect of salinity on MDA content was found [ANOVA, $F(1,6)=21.87$, $P=0.0034$, $\omega^2=0.5652$] that accounted for the majority of the variance within the model (Table 4). BS x CC larvae showed little difference in MDA content between the two salinity treatments,

but both P x W and W x W larvae showed lower MDA concentration when exposed to lower salinity compared to control groups (Fig. 5B). BS x CC clams also had lower MDA content under control conditions compared to the other crosses, but at the lower salinity level showed higher average MDA content compared to either of the other crosses. No significant contrasts were detected, however, between any of the population crosses (Table 5).

Total triglyceride (TG) content

Population cross had a significant effect when comparing TG content between population crosses at each of the two temperature levels [$F(2,5)=22.88$, $P=0.0030$, $\omega^2=0.7131$], with a very large effect size accounting for over two-thirds of the variance in this ANOVA model (Table 2). Under control conditions, both P x W and W x W larvae had significantly higher TG content than BS x CC larvae [Sidák MCT, BS x CC v P x W: $t=5.190$, $P=0.0105$; BS x CC v W x W: $t=3.911$, $P=0.0335$] (Table 3). W x W larvae showed a decrease in TG content under the elevated temperature condition, which was similar to TG content observed in BS x CC larvae, which did not vary much between temperature levels (Fig. 6A). P x W larvae showed the highest TG content at both temperature levels; however, TG content at the elevated temperature level is from only a single sample. No significant contrasts were detected between crosses at the higher temperature treatment, although no contrasts containing P x W were evaluated due to the lack of replication.

When assessing TG content at both salinity levels, a significant interaction of population cross and salinity was detected [ANOVA, $F(2,6)=10.85$, $P=0.0102$, $\omega^2=0.0889$] (Table 3). The effect size of this interaction term is relatively small and likely of minimal practical significance in the model. The same significant differences in TG content detect under control conditions

between BS x CC larvae and each of the other two crosses were also seen in this analysis, where both P x W and W x W larvae had significantly higher TG content than BS x CC larvae [Sidák MCT, BS x CC v P x W: t=6.922, P=0.0013; BS x CC v W x W: t=5.216, P=0.0059] (Table 5). The effect of the lower salinity treatment was similar across all population crosses, where large decreases in TG content were observed compared to each cross's respective control group (Fig. 6B). W x W larvae had slightly higher average TG content under this lower salinity condition compared to the other crosses; however, this difference was both slight and nonsignificant.

Alpha (α) diversity indices

Differences in α-diversity between crosses under each environmental condition can be seen across the four indices calculated in this study. Species richness was similar between Chao1 and ACE indices for all population crosses in all environmental conditions (Fig. 7). Both indices were higher for BS x CC larval microbiomes compared to both the microbiomes of P x W and W x W larvae regardless of treatment condition, indicating greater species richness in microbial communities of BS x CC larvae. Microbial community richness was similar between P x W and W x W larvae across all treatment conditions.

Under control conditions, the Shannon diversity index was higher in BS x CC and P x W larval microbiomes compared to W x W larval microbiomes; however, all three population crosses had lower Shannon diversity indices when exposed to lower salinity, indicating reductions in community diversity (Fig. 7). Microbial community diversity, as measured by the Shannon index, was more variable between tank replicates of the BS x CC and W x W crosses in the elevated temperature treatment. A similar pattern was observed in the inverse Simpson's index as that seen in the Shannon index for larval microbiomes under control conditions, where

both BS x CC and P x W larval microbial communities showed greater diversity compared to W x W larval microbial communities (Fig. 7). However, under low salinity conditions, the inverse Simpson's index for W x W larvae was higher compared to the control group, indicating microbial community diversity increased, while values of this index decreased in both other crosses under low salinity compared to their respective controls. Larval microbial community diversity in BS x CC and W x W larvae exposed to the elevated temperature treatment showed less variability in the inverse Simpson's index as compared to the Shannon index, indicating that elevated temperature may be impacting species richness more so than species evenness.

Family-level relative abundance of microbial communities

Taxa identified to the family level with relative abundancies greater than 1% are shown for larvae from each treatment replicate within each population cross in Figure 8. Family-level classification is shown as this was the lowest taxonomic rank that could be consistently identified in all samples. Clear differences in relative abundance can be seen in microbial community composition between BS x CC larvae and both P x W and W x W larvae. In particular, *Cellvibrionaceae* and *Alteromonadaceae* are more abundant in BS x CC larval microbiomes, while *Flavobacteriaceae* and *Rhodobacteraceae* are more abundant in P x W and W x W larval microbial communities. Differences are also apparent between treatment conditions within the microbiomes of both P x W and W x W larvae, where lower salinity exposure resulted in increases in the relative abundance of *PS1 clade*, a family within the Parvibaculales order, and *Alteromonadaceae*, alongside corresponding decreases in both *Flavobacteriaceae* and *Rhodobacteraceae*. Microbial communities within BS x CC larvae also shifted in response to low salinity, with increases seen in the relative abundances of *Alteromonadaceae* and

Rhodobacteraceae, decreases seen in *Cellvibrionaceae* and *Methylophilaceae*, and the emergences of *Nannocystaceae*, *Oxalobacteraceae*, and *PS1 clade*. Changes in relative abundances of taxa were also apparent in the elevated temperature treatments, where more taxa emerged in lower abundances that were not present in either control or low salinity conditions within all three population crosses.

While microbial taxa clearly persist at different abundances between the three different population crosses, the same core taxa predominant larval clam microbiomes regardless of population cross or environmental condition (Fig. 9). The same 10 microbial families account for at least 60% of larval clam microbial communities across all samples. Of these 10 families, seven originate within the Protobacteria phylum, two are from the Bacteroidota phylum, and the last remaining family is from the Myxococcota phylum.

Ordination analyses

Results of the PERMANOVA revealed significant effects of both population cross [F=4.468, P=0.0010] and environmental condition [F=5.158, P=0.0010], demonstrating that both factors contribute to shaping larval clam microbial communities (Table 10). PCoA results, where the first two principal coordinates account for 52% of the variation between microbial communities, also show separation based on population cross and environmental condition (Fig. 10). BS x CC larval microbiomes cluster separately from P x W and W x W larval microbiomes, which cluster more closely together. Furthermore, separation of the low salinity condition within each population cross is apparent, although larval microbial communities exposed to low salinity still cluster together for samples from the P x W and W x W crosses, separately from the low salinity BS x CC samples.

CAP analysis, where the first two canonical coordinates account for 35.9% of the dissimilarity between microbial communities, shows clustering similar to the PCoA plot, where BS x CC larval microbial communities are distinct from P x W and W x W larval microbial communities, and microbiomes from low salinity treatment groups are distinct from microbiomes of both control and elevated temperature groups within their respective crosses (Fig. 11). The BS x CC vector clearly separates BS x CC microbiomes away from those of P x W and W x W, while the salinity vector clearly separates low salinity exposed microbiomes from both control and elevated temperature groups. There was a smaller temperature vector delineating a slight shift away from control groups, and similarly a P x W vector slightly separated P x W and W x W microbial communities.

Discussion

This study examined the effects of mild elevated temperature and low salinity stress on larval hard clams, *M. mercenaria*, at both the whole-animal and cellular levels. Larvae were produced from three different populations crosses, allowing for the exploration of the influence of population-level variability on environmental stress tolerance. Additionally, a microbiome analysis was conducted to gain foundational knowledge about the composition of larval hard clam microbial communities as well as how these communities respond to environmental stress.

Larval clam thermotolerance

Elevated temperature stress negatively impacts larval hard clam survival, but population-level variability in thermotolerance may provide resilience to future ocean warming. All clam

populations showed decreases in percent survival when exposed an elevated temperature stress for 96 h. P x W clam larvae showed a slight reduction in percent survival, but both BS x CC and W x W larvae showed on average over 30% decreases in survival compared to their respective control groups. While temperature had an overall significant effect on survival of clam larvae, no significant contrasts were detected between population crosses, potentially due to the low number of tank replicates within each treatment, a factor likely reducing statistical power throughout this study. That said, W x W larvae had the lowest average percent survival in the elevated temperature treatment of all three crosses. As P x W larvae had nearly 20% higher survival than W x W, this could indicate that any trait responsible for this difference was present in the P clam population. This is further supported by the results of the juvenile clam mortality assessment (Chapter 4 Fig. 3), where P x W juvenile clams showed improved survival compared to W x W juveniles; however, this cannot be conclusively shown due to limited sample sizes in both studies. The few previous studies conducted assessing larval clam tolerance to elevated temperatures found conflicting results, where Talmage and Gobler (2011) saw a significant decline in larval clam survival after only a 4°C increase in water temperature, while Davis and Calabrese (1964) did not see an impact on larval clam survival until temperatures exceeded 30°C. A study by Wright et al (1983) also showed general decreasing trends in larval clam survival with increasing temperature; however, exposures in this study exceeded 40°C and were intended to replicate more intense temperature shock events. Different source populations were used across all of these studies, and taken together with the results presented here, indicate that population-level variability may influence larval clam survival in response to elevated temperature stress. However, no underlying thermotolerance mechanisms or genetic features

have been identified that could explain these variations, demonstrating the need for more extensive study on larval hard clam thermotolerance.

Growth of all larvae increased under elevated temperature conditions, but little differences were seen among population crosses. Both growth metrics evaluated in this study, shell length and total protein content, were significantly affected by temperature, and increasing trends were observed over the 96-h duration of the experimental trials in all population crosses. BS x CC larvae had significantly smaller average shell lengths by 6 dpf compared to P x W larvae under control conditions, even though all population crosses had similar shell lengths at 4 dpf, indicating that growth rates may fluctuate in unique ways over the course of the larval life cycle in different populations. This trend of BS x CC larvae being smaller is apparent in both growth metrics, potentially indicating underlying differences originating from their parent populations. Interestingly, no differences were detected among crosses in the elevated temperature condition, showing that BS x CC larvae were able to increase their calcification rates more dramatically under elevated temperatures compared to either of the other crosses. Somatic tissue growth was on average still lower in BS x CC larvae compared to the other crosses, however, indicating that tissue growth in this cross was not upregulated to the same extent due to the increase in temperature. Shell and somatic tissue growth in both P x W and W x W larvae were very similar, which could be a result of both of these crosses having one female W parent and two male W parents in common. Variations in how shell growth increased under the elevated temperature condition could potentially mean that either BS x CC larvae had a higher optimum temperature for growth that allowed them to keep pace with P x W and W x W once temperature increased, or that growth of P x W and W x W larvae was more stunted due to the elevated temperature stress. Results from Davis and Calabrese (1964) support the latter, as

they found larval clam growth rates increased dramatically between temperatures of 20°C and 30°C, but reductions in growth rate were not noted until larvae were exposed to temperatures greater than 30°C. As noted previously, however, this study was conducted on a different hard clam population, and therefore, the temperatures for both peak growth rate and the threshold at which temperature begins to impair growth rate are likely different from the crosses evaluated in this study. Lastly, Davis and Calabrese (1964) showed larval growth rates at various temperatures were also dependent on algal diets and, given the large geographic range of the parent populations for each cross, there is potential that due to underlying differences in digestive efficiencies, certain crosses may be better suited for rapid growth on the mixed algal diet provided in this study compared to the other crosses. Both variations in optimal temperatures for growth and optimal algal diets are mere hypotheses as little is known about larval clam physiology in general, but these are two areas of needed research as continued ocean warming will likely impair growth in some populations or broodstock lines, while others could be more well suited to rapid growth under future ocean conditions.

Beyond these differences in whole-animal level thermotolerance observed among larvae of each population cross, several underlying cellular differences were detected. Both BS x CC and P x W larvae showed decreasing trends in non-enzymatic antioxidant potential when exposed to the elevated temperature condition. W x W larvae showed an initial decrease after 48 h, but by 96 h, antioxidant potential had increased. Increased growth rates at higher temperatures would likely be indicative of increased metabolic rate, which would result in a natural increase in the production of reactive oxygen species (ROS), and therefore, an increased need for antioxidant molecules (reviewed in Soldatov et al., 2007). Interestingly, BS x CC larvae showed a decreasing trend with age under both temperature conditions, which may indicate this is a

natural change that occurs during larval development, and no additional oxidative stress is occurring in larvae from this cross due to the elevated temperature conditions. The initial decrease in antioxidant potential at 4 dpf, followed by an increase at 6 dpf in W x W larvae could indicate differences in cellular mechanisms between these two crosses. The initial decrease followed by recover at 6 dpf could show that W x W larvae's regulatory capabilities are impaired in the first few days of their life resulting in a delay in the upregulation of non-enzymatic antioxidant molecule production until 6 dpf. Following from this, the persistent decrease in antioxidant potential observed in P x W larvae could indicate that upregulation of non-enzymatic antioxidant molecules is further delayed in P clams. While non-enzymatic antioxidant potential could play an important role in neutralizing ROS, bivalve molluscs are known to have particularly robust enzymatic antioxidant responses, including catalase, superoxide dismutase, and several enzymes involved in glutathione transformation, which could be more than adequate for responding to any potential ROS produced due to the increases in growth seen in larvae from the elevated temperature exposures (reviewed in Soldatov et al., 2007). Furthermore, this could contribute to why no significant effects were detected in MDA content, a common byproduct of damage to macromolecules in the cell cause by ROS (Del Rio, et al., 2005). The lack of previous studies on oxidative stress responses in larval *M. mercenaria* makes the interpretation of non-enzymatic antioxidant potential data here challenging while also illustrating the need for more studies assessing the cellular stress mechanism within larval clams in order to better understand how climate change will impact this species in the future. While few significant differences in either oxidative stress marker were observed between crosses, clear differences were seen when cellular energy reserves were evaluated.

Differences in TG content among population crosses demonstrate clear physiological differences that correlate to differences observed in the juvenile life stage. Specifically, under control conditions, BS x CC larvae had significantly lower TG content than either P x W or W x W larvae. This difference under control conditions could be indicative of underlying developmental differences, potentially differences in digestive efficiencies related to the unique microbial communities associated with BS x CC larvae, or differences in maternal provisioning that persist throughout the first week of larval development. No decrease in TG content was detected in this population cross in response to the elevated temperature exposure, indicating that this mild temperature stress did not have a net impact on their energy reserves. Coupled with the observed increase in both shell and tissue growth in BS x CC larvae, consistent TG content between temperature treatments indicates that not only is there no negative impact on larval energy reserves from the elevated temperature treatment, but that these larvae can maintain their energy reserves even while growing more rapidly, further supporting that these larvae may have a higher optimal temperature for peak growth. Increased performance of BS x CC larvae under an elevated temperature condition agrees with juvenile clam survival data that showed BS x CC juveniles survived better than juveniles from each of the other crosses when exposed to a more extreme temperature challenge (Chapter 4 Fig. 3). Conversely, a decrease under elevated temperature stress was noted in TG content of W x W larvae, which could be related to why larvae from this cross showed the largest decrease in percent survival under elevated temperature conditions. Reduced energy reserves could also contribute to the decrease in antioxidant potential observed at 4 dpf in W x W larvae, although more data on changes in TG content over the course of the 96-h exposure would be needed to provide more insight into whether TG content decreased between 4 dpf and 6 dpf larvae, potentially following a concomitant increase

in non-enzymatic antioxidant molecules. The nature of what drives this decrease in TG content in W x W larvae is difficult to fully capture based on the metric used in this study, but as larvae from this cross show the greatest reduction in TG content, it is clear that W x W larvae are the most negatively impacted cross assessed in this study. This further agrees with the juvenile clam data in Chapter 4 as W x W juveniles showed the lowest percent survival in the more extreme temperature exposure. Overall, these results demonstrate that greater thermotolerance in hard clams is a trait that can persist across life stages, and therefore, could be beneficial for the aquaculture industry during both hatchery-based larval culturing and post-settlement relocation to grow out sites. While cellular stress mechanisms and energy reserves play an important role in larval clam stress tolerance, symbiotic microbial communities within hard clams will also be impacted by future climate change, which could alter the overall health and resilience of their host organisms as the abundance of beneficial microbes changes.

Inheritance of larval microbial communities from adult clams was clearly demonstrated in this study, even though adult clams were all conditioned to the same environment for at least 6 months. Larval microbiomes differed among crosses under control conditions, as seen in the Chao1 and Ace indices, relative abundance plots of microbial taxa, and ordination analyses where BS x CC larval communities cluster separately from W x W and P x W microbiomes, which showed slight separation between one another. The most dramatic differences were seen in BS x CC larval microbial communities, which supports transgenerational inheritance of unique microbial taxa as the cross shared neither parent population with either of the other two crosses. The slight correlation of P x W with the CAP ordination axes further supports this parental influence on larval microbial communities, as separation between P x W and W x W microbiomes was detect despite W parent being shared between both crosses. Transgenerational

transfer of microbial communities has been documented in marine invertebrates previously and is an area of expanding research as there is potential for organisms to withstand greater environmental stressors based on the benefits provided by their microbiomes (Sharp et al., 2007; Torda et al., 2018; Marangon et al., 2021 Zhou et al., 2021). While similar taxa were present at similar relative abundances under control conditions in both P x W and W x W larval microbiomes, BS x CC larvae had much higher abundances of *Cellvibrionaceae*, which are members of the class Gammaproteobacteria, one of the most diverse classes of prokaryotes that includes a wide range of functions and metabolic strategies, and *Alteromonadaceae*, a family of Gammaproteobacteria that are known obligate aerobic heterotrophs capable of growing quickly with minimal nutrients, producing secondary metabolites that could potential support larval immune health, and for having relatively high optimal growth temperature ranges (Williams et al., 2010; Lopez-Perez and Rodriguez-Valera, 2014). BS x CC larval microbial communities had much lower abundances of *Rhodobacteraceae*, a group containing primarily aerobic photo- and chemo- heterotrophs, of which some are known to play important roles in both sulfur and carbon biogeochemical cycling, compared to the microbial communities of both P x W and W x W larvae (Pujalte et al., 2014).

Elevated temperature stress altered microbial taxa abundances and microbial community diversity in larval hard clams from all crosses. The highest Shannon and inverse Simpson's diversity indices calculated in this study were from larval microbiomes exposed to elevated temperature stress. This indicates that not only can increasing ocean temperatures alter the abundances of numerous microbial taxa within larval clams, but also the total number of taxa present. If new taxa are able to colonize *M. mercenaria* as oceans warm, then there is potential for both new beneficial symbiotic interactions that could help mitigate future environmental

stress, but also the opportunity for pathogenic taxa to colonize clam larvae and reduce their overall fitness (Wendling et al., 2017; Rubio-Portillo et al., 2021; Scanes et al., 2021). Within this study, shifts in community composition due to elevated temperature stress were difficult to assess as the function of many microbial taxa are as yet unknown. That said, when exposed to elevated temperature stress, BS x CC larvae saw a reduction in *Cellvibrionaceae*, and an increase in *Nannocystaceae*, a small group of microbes known for lysing other microorganisms and producing long chains of polyunsaturated fatty acids, which could potentially contribute to larval energy needs under elevated temperature stress, and therefore, help preserve larval energy stores (Garcia and Muller, 2014). Both P x W and W x W larval microbiomes showed reductions in *Flavobacteriaceae*, a diverse group of microbes that has recently undergone large scale taxonomic reclassifications with little known of its potential functions in a host microbiome (Brady and Leber, 2018). As future advances are made into elucidating the specific functions of various microbial taxa, the understanding of what the observed shifts in microbiome structure might mean will improve, allowing this dataset to serve as a foundation for future studies on the role that microbial communities will play in the persistence of *M. mercenaria* as ocean warming continues.

Larval clam salinity tolerance

Low salinity exposure decreased larval survival, but this effect varied by population cross. BS x CC larvae showed a slight decrease in survival after the 96-h exposure at a salinity of 27, but both P x W and W x W showed larger decreases in survival, both of comparable magnitudes to the decreases seen in BS x CC and W x W larvae exposed to elevated temperature stress. Previous studies on larval *M. mercenaria* tolerance to low salinity are particularly scarce,

but the work of Davis and Calabrese (1964) did find a decrease in survival in hard clam larvae when exposed to low salinity; however, a noticeable drop in survival was not detected in their study until larvae were exposed to a nearly 10-unit reduction in salinity. Both data sets help to illustrate how larval clam tolerance may vary by population, although changes in percent survival are far from the only difference observed between each population cross when exposed to lower salinity.

BS x CC larvae showed further evidence of lower growth rates compared to each of the other crosses when exposed to low salinity. Shell length varied significantly between BS x CC and W x W larvae at both 48 and 96 -h time points under the lower salinity condition. BS x CC larvae were significantly smaller than W x W larvae, although BS x CC larvae exposed to low salinity showed minimal differences in shell length compared to their control counterparts, indicating that similarly to the elevated temperature exposures, larvae from this population cross appear to grow slower than either P x W or W x W larvae. This observation is further supported by total protein data showing that somatic tissue growth in BS x CC larvae was also lower under both control and low salinity conditions compared to larvae from the other two crosses. Slower growth in BS x CC larvae may confer an advantage as they show the highest percent survival in the lower salinity condition, potentially due to a lower energetic demand needed to maintain a lower growth rate. Further differences between population crosses in response to low salinity stress were also detected on the cellular level.

Minimal differences in nonenzymatic antioxidant potential were observed within both P x W and W x W larvae over the course of the low salinity exposure; although, slight decreases were seen in 4 dpf larvae that were no longer seen in 6 dpf larvae. BS x CC larvae, however, showed a dramatic increase in antioxidant potential by the end of the exposure. This could help

explain their increased survival compared to the other two crosses as increased antioxidant potential could help them prevent damage from oxidative stress generated from low salinity exposure. While an increase in antioxidant potential could also mean there is a greater demand for antioxidants if more ROS are being produced within BS x CC larvae, all population crosses show lower levels of MDA under lower salinity compared to their respective controls, indicating that oxidative damage was lower. As stated previously in the temperature tolerance discussion, bivalve larvae have robust enzymatic mechanisms for responding to oxidative stress that were not assessed in this study and may have contributed to the observed differences seen here between population crosses (Soldatov et al., 2007). While these oxidative stress markers showed some changes in response to low salinity, the impact of low salinity stress on clam larvae was best capture by TG content.

Cellular energy reserves in larvae from all population crosses were greatly diminished after 96 h of exposure to a mild low salinity stress, even though larvae were fed an optimized diet for growth. BS x CC larvae showed lower TG content under control conditions compared to both P x W and W x W larvae, so while a large decrease in TG content was observed in this cross, the decrease observed in both P x W and W x W larvae was more dramatic. These patterns in TG content could help explain the decrease in survival seen in each of these two crosses as such a drastic decrease in energy reserves would be indicative of a large increase in cellular energy demand in order to activate cellular stress response mechanisms. The decrease in energy reserves in BS x CC larvae could also be related to the large increase seen in non-enzymatic antioxidant potential, which could contribute to the increased percent survival seen in this cross. Further work is needed to explore what long term implications exposures like this, and subsequent depletion of energy reserves, could have on clam larvae, as increased

precipitation is projected with continue climate change for the east coast of the United States, where all of these populations persist (Masson-Delmotte et al., 2021). Reduced energy reserves increase the likelihood of larvae failing to recruit into wild populations, and thus could threaten the persistence of this species as a whole. Beyond these physiological impacts, low salinity exposure also resulted in the most dramatic shifts observed among taxa within larval clam microbiomes.

Clear shifts in microbial community structure were seen in all crosses as a result of the lower salinity exposure; however, not all population crosses showed shifts in the same taxa. Both P x W and W x W larval microbiomes showed reductions in abundance of *Flavobacteriaceae* and *Rhodobacteraceae*, with corresponding increases in abundance of *PS1 clade* and *Alteromonadaceae*. The *PS1 clade* is a group of Alphaproteobacteria that are not well understood, but *Alteromonadaceae* are known to be able to digest a diverse array of substrates which could be beneficial to their host. Although as *Alteromonadaceae* also produce various secondary metabolites whose functions are not all well understood, there is potential that this shift could be harmful to clam larvae (López-Pérez and Rodriguez-Valera, 2014). Additionally, as little is known about the function of *Flavobacteriaceae*, the implications of shifts in this family are difficult to assess. BS x CC larval microbial communities also showed an increase in the abundance of *Alteromonadaceae*, but interestingly they showed an increase in *Rhodobacteraceae* instead of the decrease seen in P x W and W x W larval microbiomes. The difference in microbial communities between BS x CC and the other two crosses helps to illustrate how underlying genetic variations in hard clam populations may help influence not only what taxa are abundant in their microbial communities, but also how shifts in taxa may vary across the biogeographic range of this species. BS x CC larval microbiomes showed decreases

in abundance of *Cellvibrionaceae* and *Methylophilaceae*, a group of Betaproteobacteria capable of using methanol and methylamine as their carbon sources (Doronina et al., 2014). The dramatic reduction in abundance of this family when exposed to low salinity could signify a loss of beneficial microbes capable of utilizing energy sources that hard clams otherwise cannot metabolize. Lastly, BS x CC larval microbial communities showed the emergence of several other taxa after exposure to lower salinity, which included *Nannocystaceae*, *Oxalobacteraceae*, and *PS1 clade*. An increase in abundance of *Nannocystaceae* could be beneficial due to their known role in producing large fatty acid chains, which could further contribute to the increased survival seen within BS x CC larvae at a salinity of 27 compared to the other two crosses. *Oxalobacteraceae* are a group of Betaproteobacteria that are known to be oligotrophic; however, some members of this family are also plant and human pathogens, so more work is needed to fully understand what increased relative abundances of these taxa mean for larval clams as climate change progresses (Baldani et al., 2014).

Implications for aquaculture

Taken together, the results of this study demonstrate negative impacts of both elevated temperature and low salinity stress on larval hard clams. However, differences between crosses were apparent. P x W larvae showed similar growth rates to W x W larvae, but had improved survival when exposed to the elevated temperature condition. While this evidence is not overwhelming, it does provide a basis for continued work to explore population level variability among populations of *M. mercenaria*. As illustrated by the BS x CC cross in comparison to P x W and W x W, variability in physiological mechanisms do exist throughout the biogeographic range of hard clams. Increased survival seen in BS x CC larvae exposed to low salinity could be

particularly beneficial to the aquaculture industry in the face of ongoing climate change. While all larvae showed reduced energy reserves in response to low salinity exposure, this increase in survival over a 96-h exposure could help buffer industry broodstocks against increased mortality experienced due to storm events (Masson-Delmotte et al., 2021). As one risk assessment shows, hard clam aquaculture may be at serious risk from climate change, so any advantage that can be gained by selectively breeding more resilient broodstock lines is worthy of continued research (Moor et al., 2022). The variability observed among larval clam microbiomes may provide a further route to enhance resiliency as it was shown here larvae can acquire unique microbial communities from their parents, even if said parents were acclimated to the same environmental conditions for several months. While more work is needed to understand the function of symbiotic microbes within *M. mercenaria*, this work has demonstrated that differences can be inherited, and therefore, if key microbial taxa that provide resistance to environmental stress can be identified, there is potential for artificially selecting a combination of the most beneficial microbes from various wild clam populations.

Conclusion

Physiological tolerances of larval clams to elevated temperature stress and lower salinity stress were found to vary among controlled population crosses. Exposure to elevated temperature conditions resulted in increased shell and tissue growth within all crosses; however, decreases in percent survival were also noted in larvae from all population crosses. P x W larvae showed the highest percent survival under this elevated temperature stress, while W x W larvae showed the largest decrease in survival as well as the largest decrease in cellular energy reserves. These differences could potentially be driven by contributions from P clams in combination with

W clams, and therefore, demonstrate the need for more population level assessment and selective breeding studies to explore potential means for supporting the production of more resilient broodstock lines for industry. Differences between population crosses were more apparent under the lower salinity stress, where BS x CC larvae showed the highest percent survival, but also the least amount of shell growth. These larvae also showed a large increase in antioxidant potential by the end of the experimental trial, potentially contributing to their improved survival. However, within all population crosses, a large decrease in cellular energy reserves was noted, indicating that projected increases in precipitation with ongoing climate change could pose a significant risk to larval clam recruitment in the future. Both elevated temperature and low salinity stress drove shifts in larval microbial community structures, with low salinity producing the most dramatic shifts in all population crosses. Interestingly, while all larval clam microbiome samples contain similar core taxa, relative abundances within BS x CC larvae were dramatically different from either P x W or W x W larval clams, demonstrating how parental provisioning of microbial taxa can have persistent effects across generations. Inheritance of beneficial microbes that persist even when transplanted to new environments is another means by which resilience could be gained in clam broodstock lines in the face of continuing climate change. Due to limited understanding of the function of specific microbial taxa, interpreting the impacts of shifts within larval clam microbiomes is difficult, demonstrating the need for more research into the symbiotic relationships between bivalves and their microbial communities. The negative consequence of environmental stress on this ecologically and economically important species at both the physiological and microbial levels could have serious consequences for both the ecosystems *M. mercenaria* inhabit and the industries it supports, highlighting the need for

further investigations into cellular stress mechanisms responsible for larval tolerance to future environmental stress.

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Table 1. Water conditions for each experimental exposure for each population cross (mean ± SD). Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W).

Treatment	Temperature (°C)	Salinity
BS x CC		
Control	22.9 ± 0.1	32.57 ± 0.52
Low Salinity	22.9 ± 0.1	28.31 ± 1.09
Temperature	28.0 ± 0.5	34.06 ± 1.51
P x W		
Control	23.8 ± 0.3	32.96 ± 0.37
Low Salinity	23.8 ± 0.2	28.83 ± 1.20
Temperature	28.2 ± 0.5	33.76 ± 0.78
W x W		
Control	23.8 ± 0.2	32.89 ± 0.31
Low Salinity	23.8 ± 0.2	28.75 ± 1.18
Temperature	28.2 ± 0.5	33.73 ± 0.77

Table 2. Full two-way ANOVA results for all metrics used to assess elevated temperature tolerance in larval clams at 6 days post fertilization. Effect sizes (ω^2) were calculated for all model terms. Column headings abbreviations: degrees of freedom (DF), degrees of freedom of numerator (DFn), degrees of freedom of denominator (DFd).

Metric	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Elevated temperature tolerance							
Survival							
	Population cross x Temperature	2	300.6	150.3	F (2, 6) = 1.1	0.3917	0.0077
	Population cross	2	427.1	213.5	F (2, 6) = 1.563	0.2841	0.0433
	Temperature	1	1865	1865	F (1, 6) = 13.65	0.0101	0.4870
	Residual	6	819.5	136.6	-	-	-
MDA content							
	Population cross x Temperature	2	1.477E-03	7.386E-04	F (2, 6) = 0.5134	0.6226	-0.0756
	Population cross	2	1.904E-03	9.521E-04	F (2, 6) = 0.6618	0.5499	-0.0526
	Temperature	1	5.073E-03	5.073E-03	F (1, 6) = 3.527	0.1095	0.1962
	Residual	6	8.632E-03	1.439E-03	-	-	
TG content							
	Population cross x Temperature	2	2.154E-06	1.077E-06	F (2, 5) = 2.101	0.2177	0.0359
	Population cross	2	2.347E-05	1.173E-05	F (2, 5) = 22.88	0.003	0.7131
	Temperature	1	2.759E-06	2.759E-06	F (1, 5) = 5.381	0.0681	0.0714
	Residual	5	2.564E-06	5.127E-07	-	-	-

Table 3. Results from Sidák multiple comparisons test on *a priori* contrasts for each metric used to assess elevated temperature tolerance in larval clams at 6 days post fertilization. Significance threshold for all tests was set at 0.05. Column headings abbreviations: degrees of freedom (DF).

Metric	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Elevated temperature tolerance						
Survival						
23°C						
	BS x CC vs. P x W	6	17.67	-20.59 to 55.93	1.512	0.4514
	BS x CC vs. W x W	6	14.96	-23.3 to 53.22	1.28	0.5745
	P x W vs. W x W	6	-2.709	-40.97 to 35.55	0.2318	0.9946
28°C						
	BS x CC vs. P x W	6	-3.911	-42.17 to 34.35	0.3346	0.9842
	BS x CC vs. W x W	6	14.25	-24.01 to 52.51	1.219	0.6085
	P x W vs. W x W	6	18.16	-20.1 to 56.42	1.554	0.4306
MDA content						
23°C						
	BS x CC vs. P x W	6	-0.0247	-0.1489 to 0.09948	0.6512	0.9021
	BS x CC vs. W x W	6	-0.0576	-0.1818 to 0.06658	1.518	0.448
	P x W vs. W x W	6	-0.0329	-0.1571 to 0.09128	0.8673	0.804
28°C						
	BS x CC vs. P x W	6	3.225E-03	-0.1209 to 0.1274	0.08504	0.9997
	BS x CC vs. W x W	6	-3.247E-03	-0.1274 to 0.1209	0.0856	0.9997
	P x W vs. W x W	6	-6.472E-03	-0.1306 to 0.1177	0.1706	0.9978
TG content						
23°C						
	BS x CC vs. P x W	5	-3.716E-03	-6.235E-03 to -1.197E-03	5.19	0.0105
	BS x CC vs. W x W	5	-2.801E-03	-5.32E-03 to -2.815E-03	3.911	0.0335
	P x W vs. W x W	5	9.155E-04	-1.604E-03 to 3.435E-03	1.279	0.5901
28°C						

BS x CC vs. P x W	-	-	-	-	-
BS x CC vs. W x W	5	-1.015E-03	-3.534E-03 to 1.504E-03	1.418	0.5172
P x W vs. W x W	-	-	-	-	-

Table 4. Full two-way ANOVA results for all metrics used to assess lower salinity tolerance in larval clams at 6 days post fertilization. Effect sizes (ω^2) were calculated for all model terms. Column headings abbreviations: degrees of freedom (DF), degrees of freedom of numerator (DFn), degrees of freedom of denominator (DFd).

Metric	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Lower salinity tolerance							
Survival							
	Population cross x Salinity	2	242.3	121.2	F (2, 6) = 0.828	0.4813	-0.0107
	Population cross	2	1596	798	F (2, 6) = 5.454	0.0447	0.2791
	Salinity	1	1808	1808	F (1, 6) = 12.36	0.0126	0.3558
	Residual	6	878	146.3	-	-	-
MDA content							
	Population cross x Salinity	2	3.365E-03	1.683E-03	F (2, 6) = 3.071	0.1206	0.1122
	Population cross	2	1.047E-03	5.237E-04	F (2, 6) = 0.9559	0.4361	-0.0024
	Salinity	1	1.198E-02	1.198E-02	F (1, 6) = 21.87	0.0034	0.5652
	Residual	6	3.287E-03	5.479E-04	-	-	
TG content							
	Population cross x Salinity	2	6.255E-06	3.128E-06	F (2, 6) = 10.85	0.0102	0.0889
	Population cross	2	9.522E-06	4.761E-06	F (2, 6) = 16.52	0.0036	0.1401
	Salinity	1	4.607E-05	4.607E-05	F (1, 6) = 159.8	<0.0001	0.7168
	Residual	6	1.730E-06	2.883E-07	-	-	-

Table 5. Results from Sidák multiple comparisons test on *a priori* contrasts for each metric used to assess lower salinity tolerance in larval clams at 6 days post fertilization. Significance threshold for all tests was set at 0.05. Column headings abbreviations: degrees of freedom (DF).

Metric	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Lower salinity tolerance						
Survival						
32						
	BS x CC vs. P x W	6	17.67	-21.94 to 57.27	1.46	0.4773
	BS x CC vs. W x W	6	14.96	-24.65 to 54.56	1.237	0.5988
	P x W vs. W x W	6	-2.709	-42.31 to 36.89	0.2239	0.9951
28						
	BS x CC vs. P x W	6	27.62	-11.98 to 67.22	2.283	0.1761
	BS x CC vs. W x W	6	36.94	-2.662 to 76.54	3.054	0.0657
	P x W vs. W x W	6	9.32	-30.28 to 48.92	0.7704	0.8514
MDA content						
32						
	BS x CC vs. P x W	6	-0.0247	-0.1013 to 0.05193	1.055	0.7019
	BS x CC vs. W x W	6	-0.0576	-0.1342 to 0.01904	2.461	0.1401
	P x W vs. W x W	6	-0.0329	-0.1095 to 0.04373	1.405	0.506
28						
	BS x CC vs. P x W	6	0.03199	-0.04464 to 0.1086	1.367	0.5267
	BS x CC vs. W x W	6	0.02211	-0.05452 to 0.09874	0.9446	0.7632
	P x W vs. W x W	6	-9.882E-03	-0.08651 to 0.06675	0.4222	0.9695
TG content						
32						
	BS x CC vs. P x W	6	-3.716E-03	-5.474E-03 to -1.958E-03	6.922	0.0013
	BS x CC vs. W x W	6	-2.801E-03	-4.558E-03 to -1.043E-03	5.216	0.0059
	P x W vs. W x W	6	9.155E-04	-8.422E-04 to 2.673E-03	1.705	0.3618
28						

BS x CC vs. P x W	6	-1.862E-04	-1.944E-03 to 1.572E-03	0.3467	0.9826
BS x CC vs. W x W	6	-8.422E-04	-2.6E-03 to 9.155E-04	1.569	0.4236
P x W vs. W x W	6	-6.56E-04	-2.414E-03 to 1.102E-03	1.222	0.6071

Table 6. Full three-way ANOVA results for all metrics used to assess elevated temperature tolerance in larval clams over time. Effect sizes (ω^2) were calculated for all model terms. Column headings abbreviations: degrees of freedom (DF), degrees of freedom of numerator (DFn), degrees of freedom of denominator (DFd).

Metric	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Elevated temperature tolerance							
Shell Length							
	Population cross	2	3038	1519	F (2, 228) = 6.567	0.0017	0.0168
	Time	1	86120	86117	F (1, 228) = 372.3	<0.0001	0.5603
	Temperature	1	9548	9548	F (1, 228) = 41.28	<0.0001	0.0608
	Population cross x Time	2	694.7	347.3	F (2, 228) = 1.502	0.2249	1.51E-03
	Population cross x Temperature	2	237.1	118.6	F (2, 228) = 0.5126	0.5996	-1.47E-03
	Time x Temperature	1	237	237	F (1, 228) = 1.025	0.3125	3.72E-05
	Population cross x Time x Temperature	2	437.6	218.8	F (2, 228) = 0.946	0.3898	-1.63E-04
	Residual	228	52740	231.3	-	-	-
Total Protein Content							
	Population cross	2	1068	534.1	F (2, 12) = 4.445	0.0359	0.0635
	Time	1	7397	7397	F (1, 12) = 61.55	<0.0001	0.5578
	Temperature	1	1990	1990	F (1, 12) = 16.56	0.0016	0.1433
	Population cross x Time	2	484.7	242.3	F (2, 12) = 2.017	0.1758	0.0187
	Population cross x Temperature	2	176.5	88.27	F (2, 12) = 0.7346	0.5001	-4.9E-03
	Time x Temperature	1	355.8	355.8	F (1, 12) = 2.961	0.1109	0.0181
	Population cross x Time x Temperature	2	10.66	5.33	F (2, 12) = 0.04436	0.9568	-0.0176
	Residual	12	1442	120.2	-	-	-
Nonenzymatic antioxidant potential							
	Population cross	2	3.971	1.985	F (2, 12) = 3.305	0.0719	0.0706
	Time	1	0.1869	0.1869	F (1, 12) = 0.3111	0.5873	-0.0106
	Temperature	1	9.879	9.879	F (1, 12) = 16.45	0.0016	0.2366
	Population cross x Time	2	11	5.498	F (2, 12) = 9.153	0.0039	0.2498
	Population cross x Temperature	2	1.267	0.6334	F (2, 12) = 1.054	0.3786	1.67E-03

Time x Temperature	1	0.8255	0.8255	F (1, 12) = 1.374	0.2639	5.73E-03
Population cross x Time x Temperature	2	4.276	2.138	F (2, 12) = 3.559	0.0611	0.0784
Residual	12	7.209	0.6007	-	-	-

Table 7. Results from Sidák multiple comparisons test on *a priori* contrasts for each metric used to assess elevated temperature tolerance in larval clams over time. Significance threshold for all tests was set at 0.05. Column headings abbreviations: degrees of freedom (DF).

Metric	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Elevated temperature tolerance						
Shell Length						
4 dpf						
	BS x CC 23°C vs. P x W 23°C	228	-3.456	-19.84 to 12.93	0.7185	>0.9999
	BS x CC 23°C vs. W x W 23°C	228	-1.801	-18.18 to 14.58	0.3744	>0.9999
	P x W 23°C vs. W x W 23°C	228	1.655	-14.73 to 18.04	0.3441	>0.9999
	BS x CC 28°C vs. P x W 28°C	228	-5.48	-21.86 to 10.9	1.14	>0.9999
	BS x CC 28°C vs. W x W 28°C	228	-4.176	-20.56 to 12.21	0.8684	>0.9999
	P x W 28°C vs. W x W 28°C	228	1.304	-15.08 to 17.69	0.2711	>0.9999
6 dpf						
	BS x CC 23°C vs. P x W 23°C	228	-18.12	-34.51 to -1.738	3.768	0.0137
	BS x CC 23°C vs. W x W 23°C	228	-10.95	-27.34 to 5.429	2.278	0.7941
	P x W 23°C vs. W x W 23°C	228	7.168	-9.216 to 23.55	1.49	>0.9999
	BS x CC 28°C vs. P x W 28°C	228	-6.917	-23.30 to 9.467	1.438	>0.9999
	BS x CC 28°C vs. W x W 28°C	228	-6.803	-23.19 to 9.581	1.415	>0.9999
	P x W 28°C vs. W x W 28°C	228	0.114	-16.27 to 16.5	0.0237	>0.9999
Total Protein Content						
4 dpf						
	BS x CC 23°C vs. P x W 23°C	12	-5.16	-54.07 to 43.75	0.4707	>0.9999
	BS x CC 23°C vs. W x W 23°C	12	1.74	-47.17 to 50.65	0.1588	>0.9999
	P x W 23°C vs. W x W 23°C	12	6.9	-42.01 to 55.81	0.6295	>0.9999
	BS x CC 28°C vs. P x W 28°C	12	-8.719	-57.63 to 40.19	0.7954	>0.9999
	BS x CC 28°C vs. W x W 28°C	12	-8.878	-57.79 to 40.03	0.8099	>0.9999
	P x W 28°C vs. W x W 28°C	12	-0.1589	-49.07 to 48.75	0.01449	>0.9999
6 dpf						

BS x CC 23°C vs. P x W 23°C	12	-20.43	-69.34 to 28.48	1.863	0.9975
BS x CC 23°C vs. W x W 23°C	12	-18.23	-67.13 to 30.68	1.663	0.9998
P x W 23°C vs. W x W 23°C	12	2.202	-46.71 to 51.11	0.2009	>0.9999
BS x CC 28°C vs. P x W 28°C	12	-21.05	-69.96 to 27.86	1.92	0.9956
BS x CC 28°C vs. W x W 28°C	12	-32.42	-81.33 to 16.49	2.958	0.5483
P x W 28°C vs. W x W 28°C	12	-11.38	-60.29 to 37.53	1.038	>0.9999
Nonenzymatic antioxidant potential					
4 dpf					
BS x CC 23°C vs. P x W 23°C	12	1.403	-2.055 to 4.861	1.81	0.9987
BS x CC 23°C vs. W x W 23°C	12	1.965	-1.494 to 5.423	2.535	0.8265
P x W 23°C vs. W x W 23°C	12	0.5619	-2.896 to 4.02	0.725	>0.9999
BS x CC 28°C vs. P x W 28°C	12	1.77	-1.688 to 5.228	2.284	0.9386
BS x CC 28°C vs. W x W 28°C	12	3.101	-0.3573 to 6.559	4.001	0.1097
P x W 28°C vs. W x W 28°C	12	1.331	-2.127 to 4.789	1.717	0.9996
6 dpf					
BS x CC 23°C vs. P x W 23°C	12	-0.3034	-3.762 to 3.155	0.3914	>0.9999
BS x CC 23°C vs. W x W 23°C	12	0.3209	-3.137 to 3.779	0.414	>0.9999
P x W 23°C vs. W x W 23°C	12	0.6243	-2.834 to 4.082	0.8054	>0.9999
BS x CC 28°C vs. P x W 28°C	12	0.5191	-2.939 to 3.977	0.6698	>0.9999
BS x CC 28°C vs. W x W 28°C	12	-1.875	-5.333 to 1.583	2.419	0.8859
P x W 28°C vs. W x W 28°C	12	-2.394	-5.852 to 1.064	3.089	0.463

Table 8. Full three-way ANOVA results for all metrics used to assess low salinity tolerance in larval clams over time. Effect sizes (ω^2) were calculated for all model terms. Column headings abbreviations: degrees of freedom (DF), degrees of freedom of numerator (DFn), degrees of freedom of denominator (DFd).

Metric	Source of Variation	DF	Sum of Squares	Mean Square	F (DFn, DFd)	P value	ω^2
Lower salinity tolerance							
Shell Length							
	Population cross	2	7928	3964	F (2, 228) = 22.62	<0.0001	0.0544
	Time	1	87430	87430	F (1, 228) = 498.8	<0.0001	0.6267
	Salinity	1	753.9	753.9	F (1, 228) = 4.301	0.0392	4.16E-03
	Population cross x Time	2	645.9	323	F (2, 228) = 1.843	0.1608	2.12E-03
	Population cross x Salinity	2	1561	780.3	F (2, 228) = 4.452	0.0127	8.69E-03
	Time x Salinity	1	310.4	310.4	F (1, 228) = 1.771	0.1846	9.7E-04
	Population cross x Time x Salinity	2	464	232	F (2, 228) = 1.324	0.2682	8.14E-04
	Residual	228	39960	175.3	-	-	-
Total Protein Content							
	Population cross	2	655.2	327.6	F (2, 12) = 15.47	0.0005	0.1143
	Time	1	4027	4027	F (1, 12) = 190.2	<0.0001	0.7469
	Salinity	1	10.26	10.26	F (1, 12) = 0.4845	0.4996	-2.03E-03
	Population cross x Time	2	347.6	173.8	F (2, 12) = 8.208	0.0057	0.0569
	Population cross x Salinity	2	29.22	14.61	F (2, 12) = 0.69	0.5204	-2.45E-03
	Time x Salinity	1	13.54	13.54	F (1, 12) = 0.6395	0.4394	-1.42E-03
	Population cross x Time x Salinity	2	5.099	2.55	F (2, 12) = 0.1204	0.8876	-6.94E-03
	Residual	12	254.1	21.17	-	-	-
Nonenzymatic antioxidant potential							
	Population cross	2	20.8	10.4	F (2, 12) = 20.28	0.0001	0.3546
	Time	1	6.296	6.296	F (1, 12) = 12.28	0.0043	0.1037
	Salinity	1	0.0111	0.0111	F (1, 12) = 0.02165	0.8855	-9E-03
	Population cross x Time	2	0.4623	0.2311	F (2, 12) = 0.4508	0.6475	-0.0101
	Population cross x Salinity	2	7.888	3.944	F (2, 12) = 7.692	0.0071	0.1231

Time x Salinity	1	8.913	8.913	F (1, 12) = 17.38	0.0013	0.1507
Population cross x Time x Salinity	2	4.723	2.362	F (2, 12) = 4.606	0.0328	0.0663
Residual	12	6.153	0.5128	-	-	-

Table 9. Results from Sidák multiple comparisons test on *a priori* contrasts for each metric used to assess lower salinity tolerance in larval clams over time. Significance threshold for all tests was set at 0.05. Column headings abbreviations: degrees of freedom (DF).

Metric	Contrast	DF	Mean Difference	95% CI of difference	t	Adjusted P Value
Lower salinity tolerance						
Shell Length						
4 dpf						
	BS x CC 32 vs. P x W 32	228	-3.456	-17.72 to 10.81	0.8254	>0.9999
	BS x CC 32 vs. W x W 32	228	-1.801	-16.06 to 12.46	0.4301	>0.9999
	P x W 32 vs. W x W 32	228	1.655	-12.61 to 15.92	0.3953	>0.9999
	BS x CC 28 vs. P x W 28	228	-12.81	-27.07 to 1.453	3.06	0.1512
	BS x CC 28 vs. W x W 28	228	-17.48	-31.75 to -3.221	4.176	0.0028
	P x W 28 vs. W x W 28	228	-4.673	-18.94 to 9.59	1.116	>0.9999
6 dpf						
	BS x CC 32 vs. P x W 32	228	-18.12	-32.39 to -3.859	4.329	0.0015
	BS x CC 32 vs. W x W 32	228	-10.95	-25.22 to 3.308	2.617	0.4665
	P x W 32 vs. W x W 32	228	7.168	-7.095 to 21.43	1.712	0.9978
	BS x CC 28 vs. P x W 28	228	-13.89	-28.15 to 0.3758	3.317	0.0675
	BS x CC 28 vs. W x W 28	228	-19.01	-33.27 to -4.747	4.541	0.0006
	P x W 28 vs. W x W 28	228	-5.123	-19.39 to 9.14	1.224	>0.9999
Total Protein Content						
4 dpf						
	BS x CC 32 vs. P x W 32	12	-5.16	-25.69 to 15.37	1.121	>0.9999
	BS x CC 32 vs. W x W 32	12	1.74	-18.79 to 22.27	0.3782	>0.9999
	P x W 32 vs. W x W 32	12	6.9	-13.63 to 27.43	1.5	>0.9999
	BS x CC 28 vs. P x W 28	12	-4.817	-25.35 to 15.71	1.047	>0.9999
	BS x CC 28 vs. W x W 28	12	-4.029	-24.56 to 16.5	0.8757	>0.9999
	P x W 28 vs. W x W 28	12	0.7878	-19.74 to 21.32	0.1712	>0.9999
6 dpf						
	BS x CC 32 vs. P x W 32	12	-20.43	-40.96 to 0.1028	4.439	0.0519

BS x CC 32 vs. W x W 32	12	-18.23	-38.76 to 2.305	3.961	0.1174
P x W 32 vs. W x W 32	12	2.202	-18.33 to 22.73	0.4785	>0.9999
BS x CC 28 vs. P x W 28	12	-17.24	-37.77 to 3.295	3.746	0.1686
BS x CC 28 vs. W x W 28	12	-19.54	-40.07 to 0.994	4.246	0.0723
P x W 28 vs. W x W 28	12	-2.301	-22.83 to 18.23	0.5	>0.9999
Nonenzymatic antioxidant potential					
4 dpf					
BS x CC 32 vs. P x W 32	12	1.403	-1.792 to 4.598	1.959	0.9936
BS x CC 32 vs. W x W 32	12	1.965	-1.23 to 5.159	2.744	0.6947
P x W 32 vs. W x W 32	12	0.5619	-2.633 to 3.757	0.7847	>0.9999
BS x CC 28 vs. P x W 28	12	2.045	-1.15 to 5.24	2.856	0.6174
BS x CC 28 vs. W x W 28	12	2.289	-0.9056 to 5.484	3.197	0.3986
P x W 28 vs. W x W 28	12	0.2439	-2.951 to 3.439	0.3406	>0.9999
6 dpf					
BS x CC 32 vs. P x W 32	12	-0.3034	-3.498 to 2.891	0.4237	>0.9999
BS x CC 32 vs. W x W 32	12	0.3209	-2.874 to 3.516	0.4481	>0.9999
P x W 32 vs. W x W 32	12	0.6243	-2.571 to 3.819	0.8718	>0.9999
BS x CC 28 vs. P x W 28	12	4.616	1.421 to 7.811	6.446	0.0021
BS x CC 28 vs. W x W 28	12	3.456	0.2615 to 6.651	4.827	0.027
P x W 28 vs. W x W 28	12	-1.16	-4.355 to 2.035	1.62	>0.9999

Table 10. Permutational multivariate analysis of variance (PERMANOVA) test results calculated using the Bray-Curtis dissimilarity metric for whole microbiome community level assessment. Column headings abbreviations: degrees of freedom (DF).

Variable	DF	Sum of Squares	R^2	F	P value
PERMANOVA					
Condition	2	1.194	0.3021	5.158	0.001
Spawn	2	1.034	0.2617	4.468	0.001
Conditon x Spawn	4	0.6822	0.1727	1.474	0.106
Residual	9	1.041	0.2636	-	-

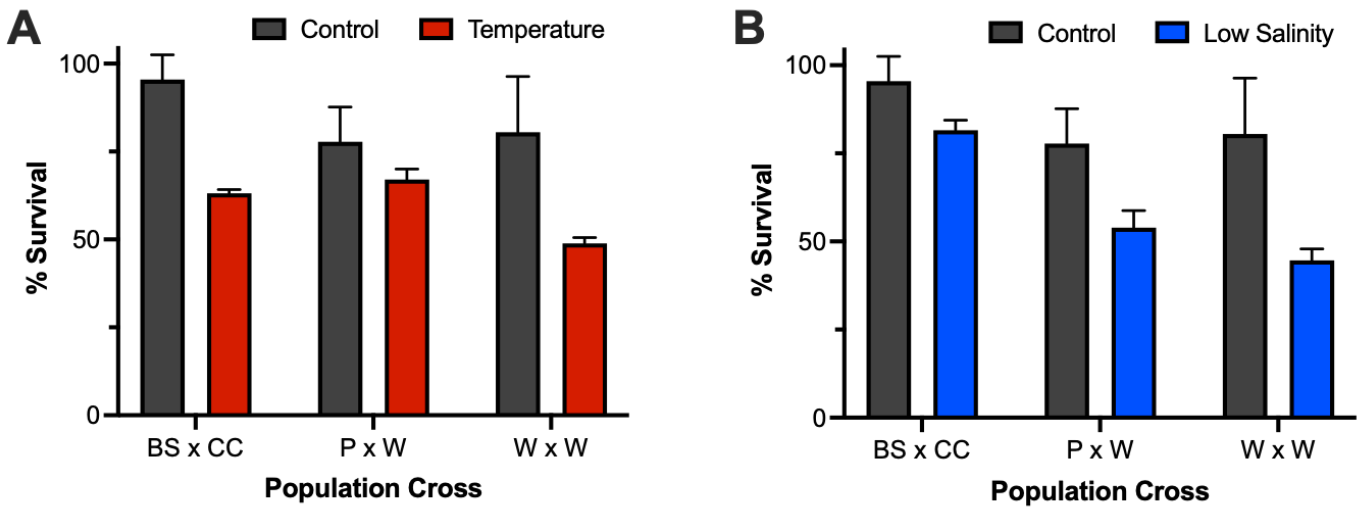

Figure 1. Percent survival of larval clams 6 days post fertilization (n=2), in response to A) an elevated temperature or B) a low salinity condition (mean ± SEM). Control conditions (gray) were maintained at 23ºC at a salinity of 32. The elevated temperature treatment (28 ºC, red) was maintained at control salinity and, likewise, the low salinity treatment (27, blue) was maintained at the control temperature. Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W).

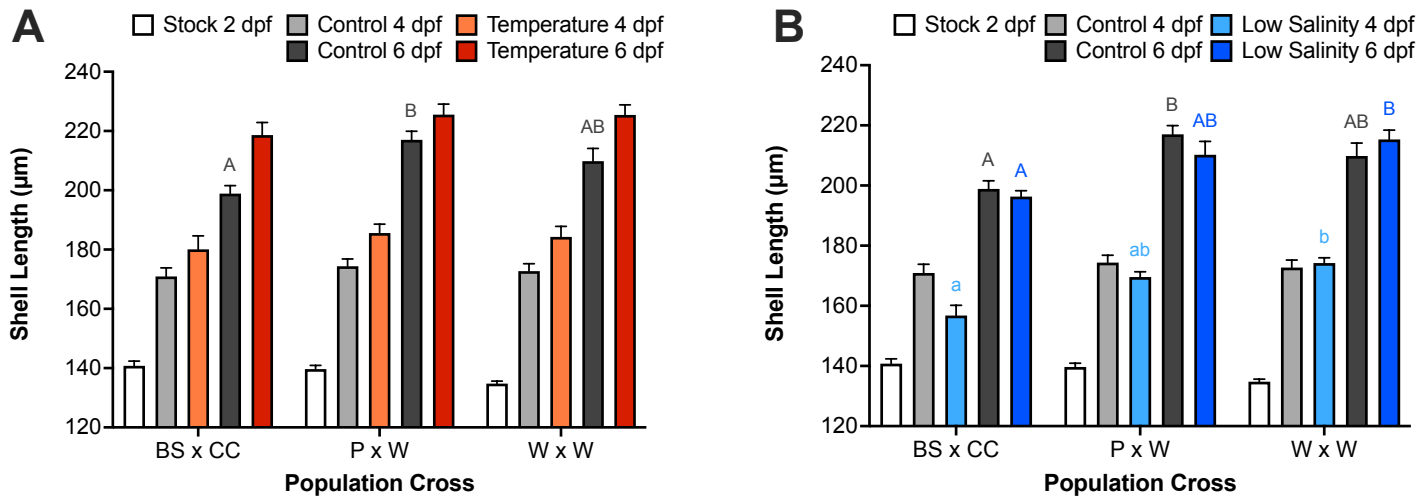

Figure 2. Shell length of clam larvae when exposed to A) an elevated temperature stress and B) a low salinity condition. Bars are mean ± SEM of measurements from ten larvae randomly selected from each tank replicate at each time point (n=20) and twenty larvae at 2 days post fertilization (dpf) from the stock used to initiate the treatment cultures (no tank replicates). Control conditions are shown in gray, elevated temperature conditions are shown in red, and low salinity conditions are shown in blue, with lighter colors representing larvae that were 4 dpf and darker colors representing larvae that were 6 dpf. Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Color-coded letters indicate significant differences between population crosses within a treatment. Lowercase letters denote significance between 4 dpf larvae while capital letters denote significance between 6 dpf larvae (Sidák MCT, α=0.05).

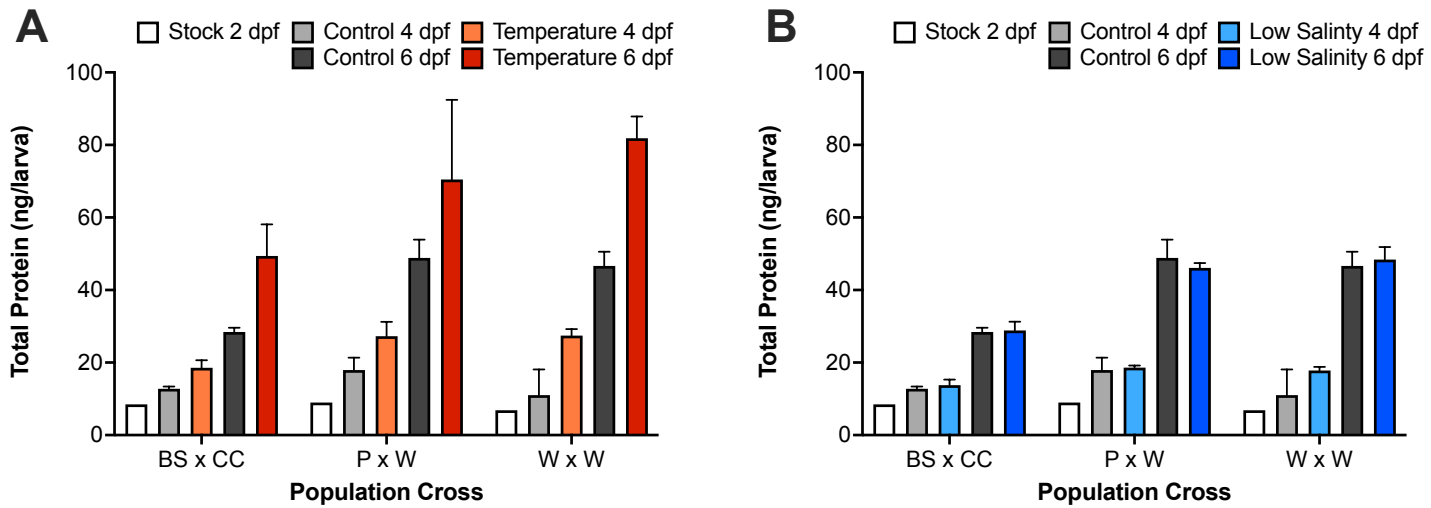

Figure 3. Total protein content of larvae from each population cross exposed to A) an elevated temperature or B) a low salinity condition. Data shown as mean ± SEM (n=2). Protein content for 2 days post fertilization (dpf) larvae have no error bars as they represent the single sample collected from each starting larval stock. Control conditions are shown in gray, elevated temperature conditions are shown in red, and low salinity conditions are shown in blue, with lighter colors representing 4 dpf larvae and darker colors representing 6 dpf larvae. Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W).

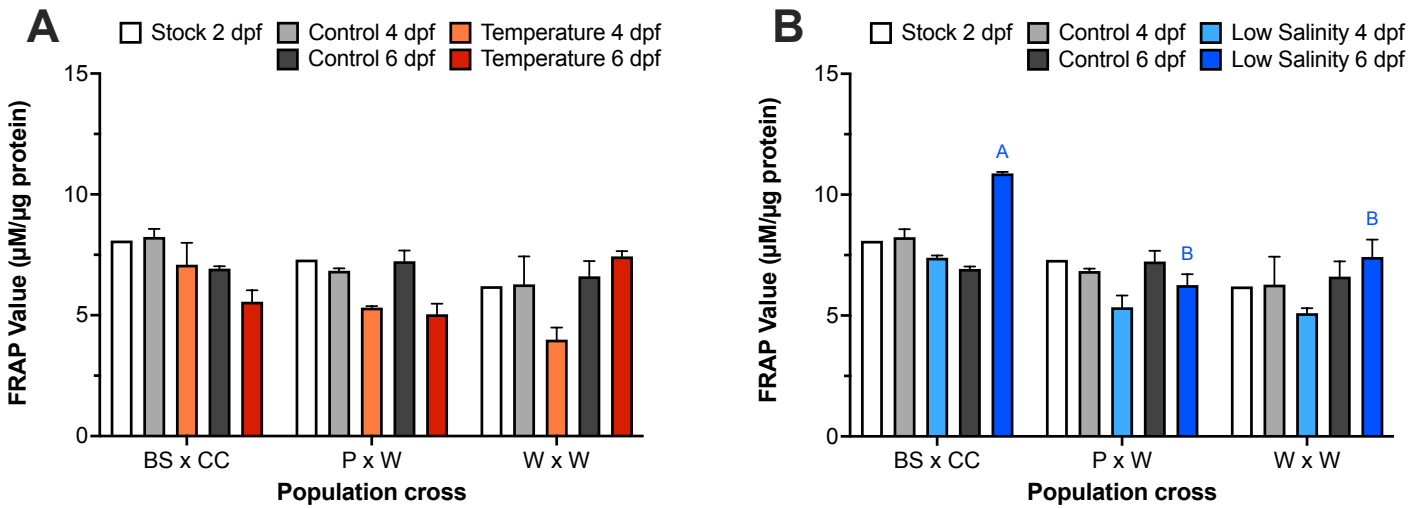

Figure 4. Nonenzymatic antioxidant potential of clam larvae form each population cross exposed to A) an elevated temperature or B) a low salinity condition. Data shown as ferric reducing/antioxidant potential (FRAP) values normalized to the protein content of each sample (µM/µg). Data shown as mean ± SEM (n=2). FRAP values for 2 days post fertilization (dpf) larvae have no error bars as they represent the single sample collected from each starting larval stock. Control conditions are shown in gray, elevated temperature conditions are shown in red, and low salinity conditions are shown in blue, with lighter colors representing 4 dpf larvae and darker colors representing 6 dpf larvae. Population abbreviations: Bogue Sound NC (BS), Cape Cod MA (CC), Pocomoke Sound VA (P), and Wachapreague VA (W). Color-coded capital letters indicate significant differences between population crosses within that respective treatment at 6 dpf (Sidák MCT, α=0.05).

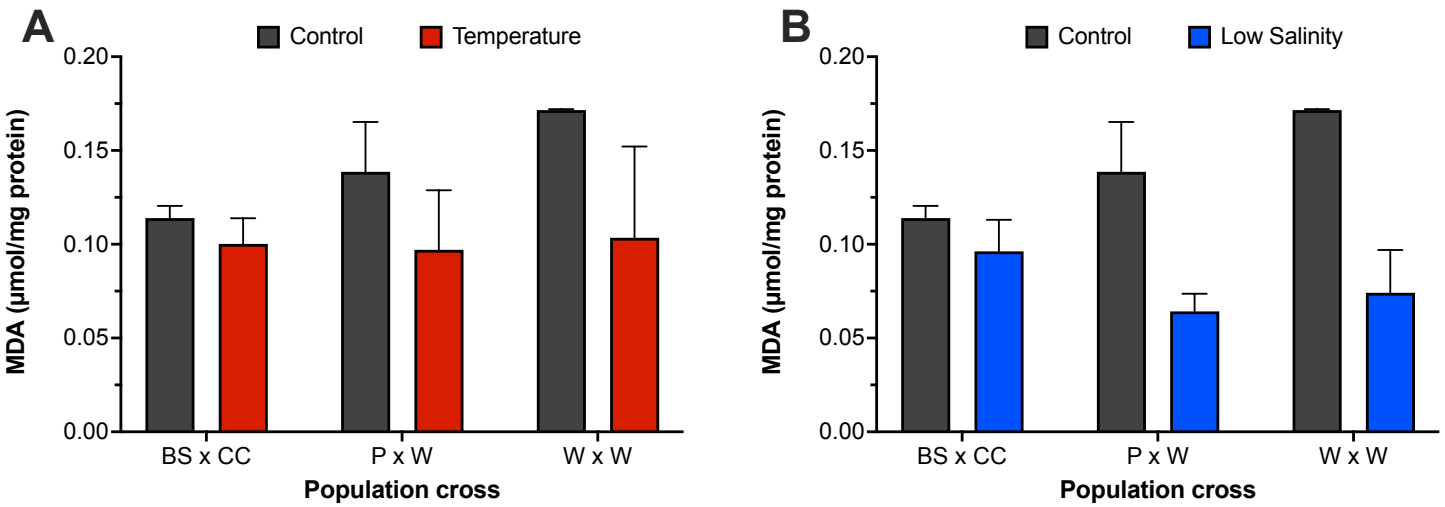

Figure 5. Malondialdehyde (MDA) content of clam larvae from each population cross exposed to A) an elevated temperature or B) a low salinity condition. MDA content was normalized to protein content within each sample. All data shown as mean ± SEM (n=2). MDA content was only assessed at the end of each experimental trial (6 days post fertilization).

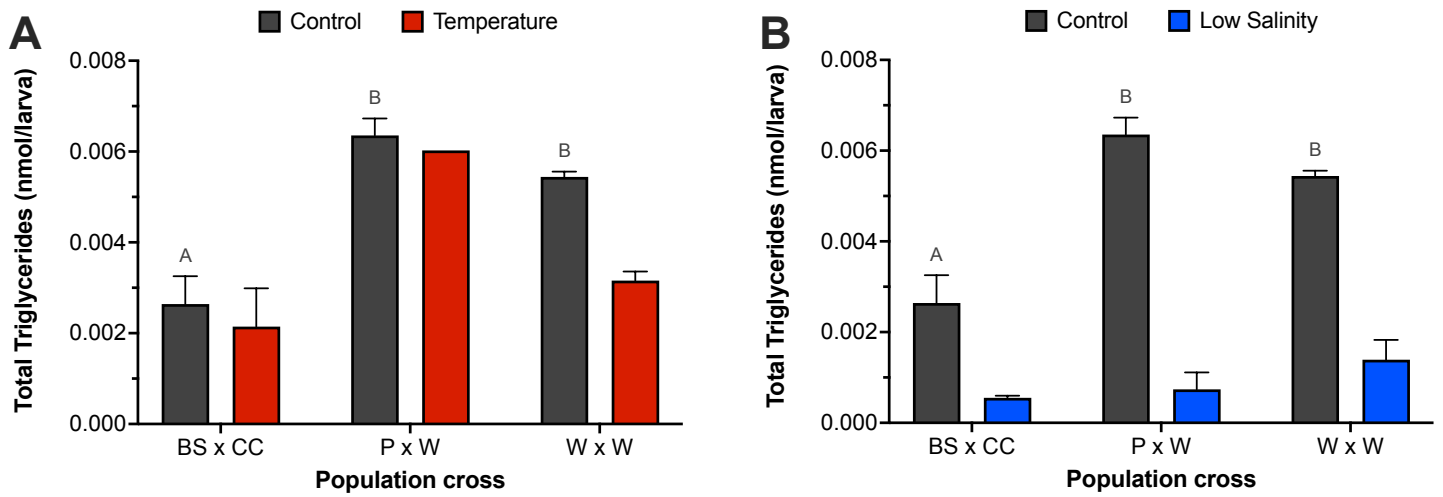

Figure 6. Total triglyceride content (TG) of clam larvae from each population cross exposed to A) an elevated temperature or B) a low salinity condition. Data shown as mean ± SEM (n=2, except for the P x W elevated temperature group where n=1). TG content was only assessed at the end of each experimental trial (6 days post fertilization). Color-coded capital letters indicate significant differences between population crosses within that respective treatment (Sidák MCT, α=0.05).

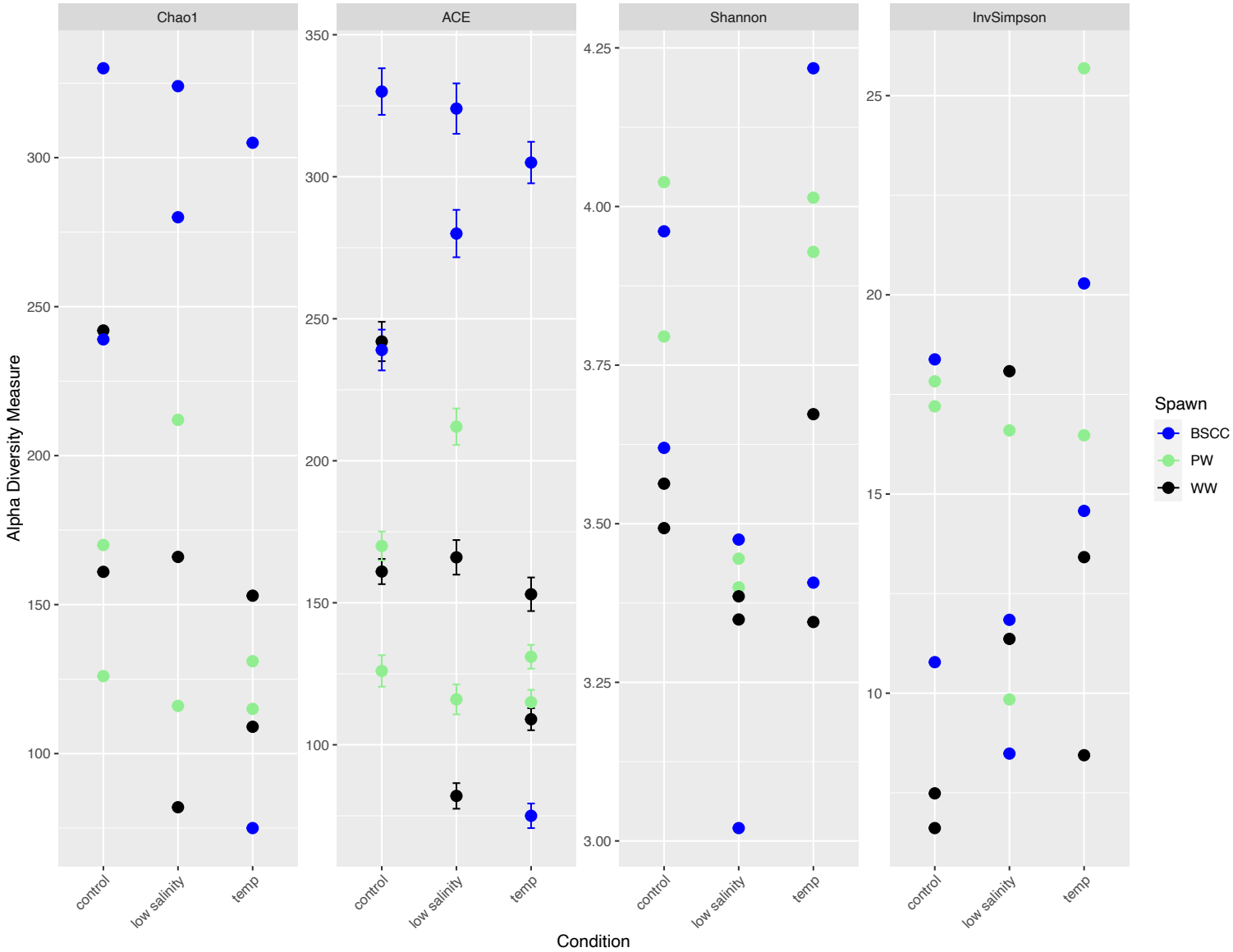

Figure 7. Alpha diversity of larval hard clam microbiomes, using four metrics: Chao1 index, abundance-based coverage estimator (ACE) index, Shannon diversity index, and the inverse Simpson's index. Individual points represent a tank replicate within each

treatment condition listed along the x-axis. ACE error bars represent standard error of the multiple permutations necessary to calculate this index. Population crosses are color-coded and abbreviated as follows: Bogue Sound, NC x Cape Cod, MA (BSCC, blue); Pocomoke Sound, VA x Wachapreague, VA (PW, green); Wachapreague, VA self-cross (WW, black).

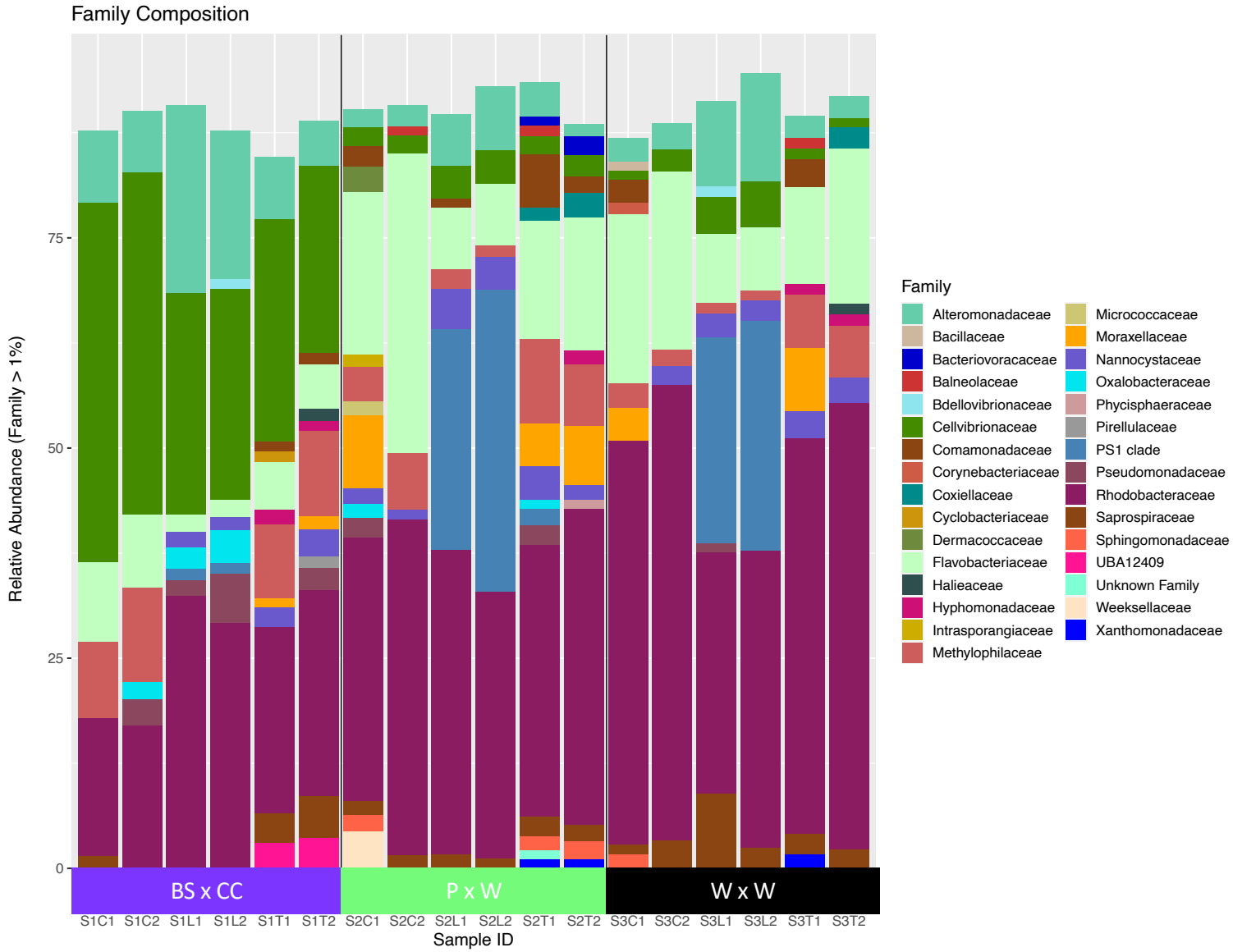

Figure 8. Family-level relative abundances of taxa present within the microbiomes of larval clams sampled at the end of each experimental trial (6 days postfertilization). Only taxa with greater than 1% relative abundance are shown. Taxa are listed in the

legend as they appear within each sample column top to bottom (if present). Sample ID abbreviations: S1 refers to BS x CC, S2 refers to P x W, S3 refers to W x W, C is the control treatment, L is the low salinity treatment, T is the elevated temperature treatment, and the numbers following each treatment abbreviation indicates the tank replicate (1 or 2).

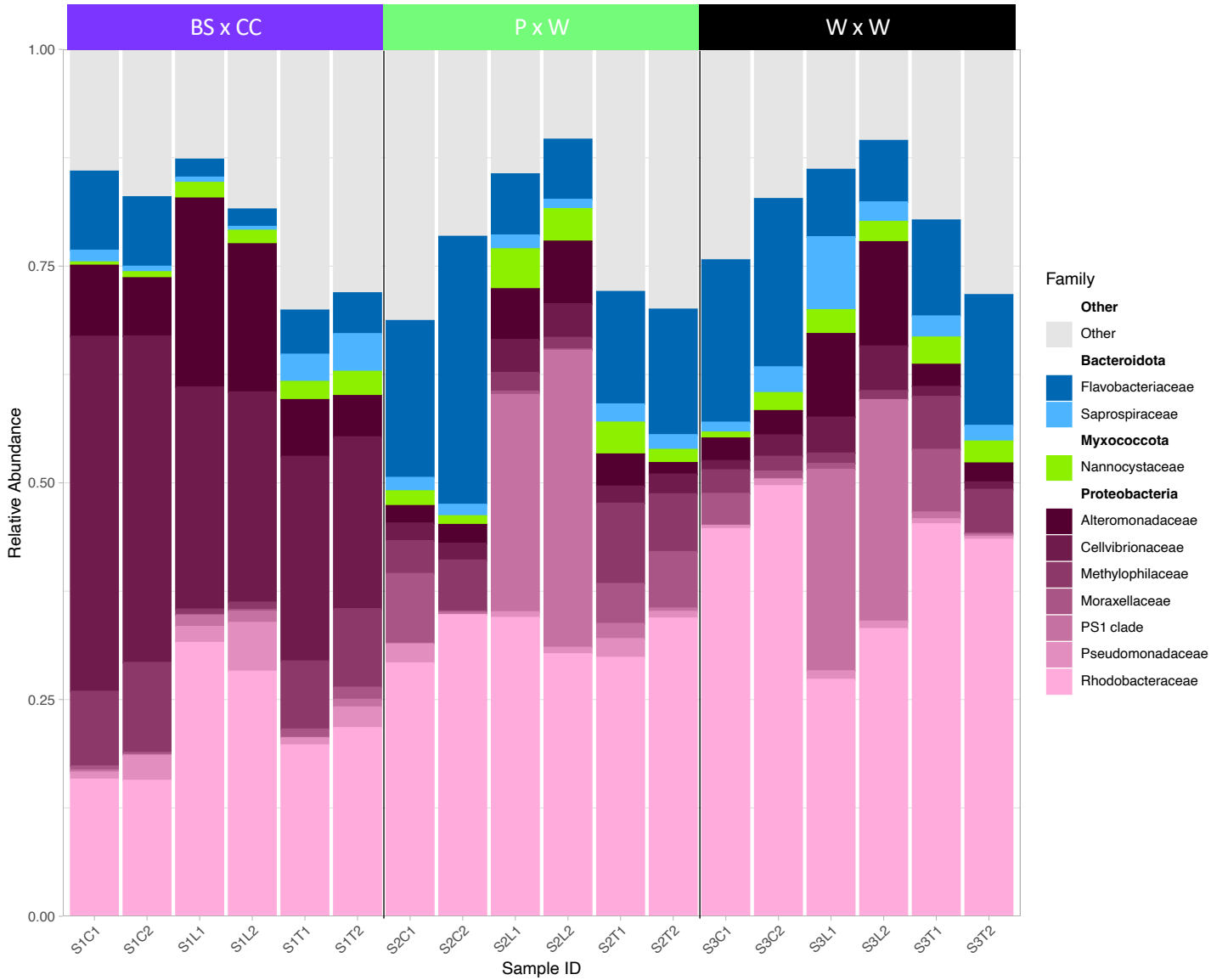

Figure 9. Relative abundance of the ten most abundant taxa at the family level across all samples. Bolded names in the legend correspond to phyla, with families listed within each phylum following a color-coded gradient. Taxa are listed in the legend as they

appear within each sample column top to bottom (if present). Sample ID abbreviations: S1 refers to BS x CC, S2 refers to P x W, S3 refers to W x W, C is the control treatment, L is the low salinity treatment, T is the elevated temperature treatment, and the numbers following each treatment abbreviation indicates the tank replicate (1 or 2).

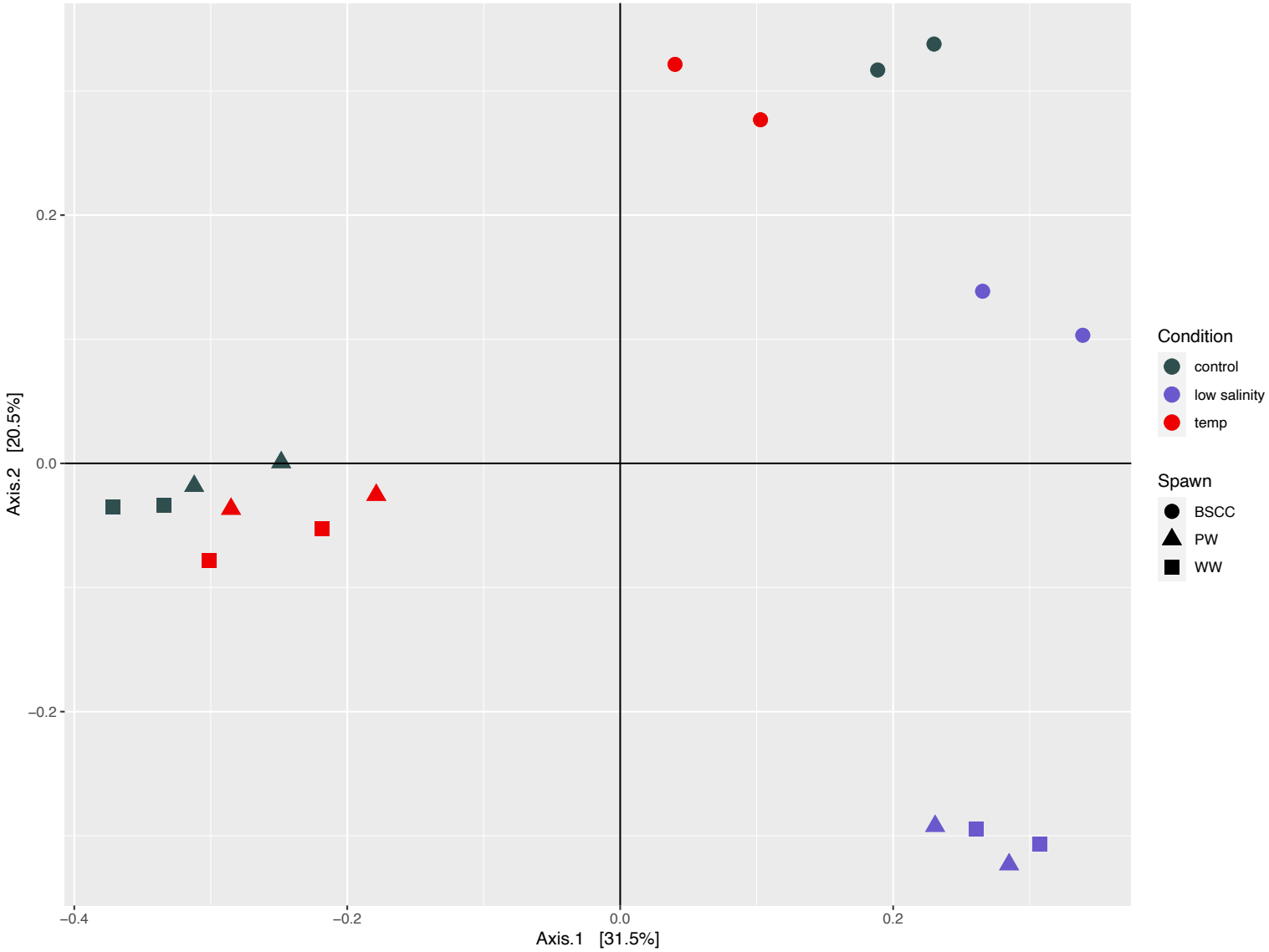

Figure 10. Principal coordinate analysis calculated using the Bray-Curtis dissimilarity metric on larval microbiomes from each treatment condition among all population crosses. Colors represent treatment conditions while shapes represent populations crosses.

Each individual point represents a larval microbiome from a single tank replicate within this study. Population cross abbreviations: Bogue Sound, NC x Cape Cod, MA (BSCC); Pocomoke Sound, VA x Wachapreague, VA (PW); Wachapreague, VA self-cross (WW).

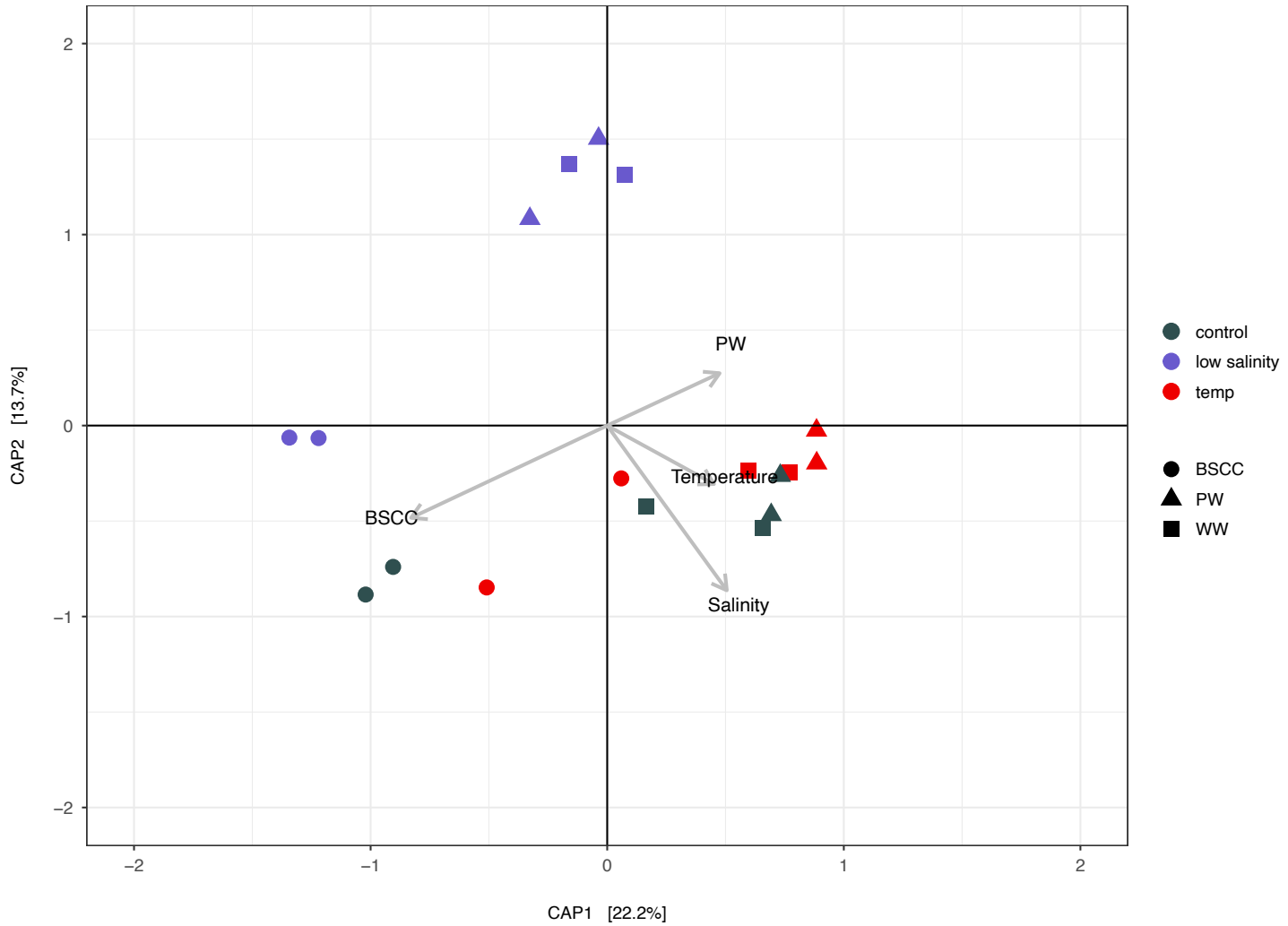

Figure 11. Canonical analysis of principal coordinates (CAP) plot calculated using the Bray-Curtis dissimilarity metric on larval microbiomes from each treatment condition among all population crosses. Colors represent treatment conditions while shapes represent populations crosses. Each individual point represents a tank replicate within this study. Vectors represent environmental

drivers shaping differences in observed microbial communities. Population cross abbreviations: Bogue Sound, NC x Cape Cod, MA (BSCC); Pocomoke Sound, VA x Wachapreague, VA (PW); Wachapreague, VA self-cross (WW).

Chapter 6

Conclusions

The work presented in this dissertation focused on two coastal bivalve species that inhabit the east coast of the United States: the eastern oyster, *Crassostrea virginica*, and the hard clam, *Mercenaria mercenaria*. These two species were of particular interest as they provide a host of ecosystem services to the regions they inhabit, while also supporting commercial fisheries and aquaculture operations. As climate change progresses, these species will continue to be threatened by increasing water temperatures, decreasing ocean pH, and increasing frequency and intensity of low salinity events, all of which could negatively impact their abundances throughout their biogeographic range (Sanford and Kelly, 2011; Masson-Delmotte et al., 2021). As any such fluctuations in these species would have wide ranging consequences both ecologically and economically, there is an ever-growing need to not only understand the extent to which these species can tolerate environmental stress, but also to what extent natural resilience to future changes may already exists within certain populations. Little work has been conducted in marine invertebrates exploring population-level variability in physiological tolerance to environmental stressors, making it especially difficult to project how ongoing climate change will impact either *C. virginica* or *M. mercenaria*. Furthermore, as earlier life stages in bivalves have been found to be more susceptible to environmental stress, it is necessary to explore population-level variations in physiological tolerance in larval and juvenile life stages as any environmental condition that exceeds the stress tolerance of younger individuals could result in reduced recruitment to the adult life stage (Talmage and Gobler, 2009; Talmage and Gobler, 2010; Talmage and Gobler, 2011; Waldbusser et al., 2013; Waldbusser et al, 2015a; Waldbusser et al, 2015b; Frieder et al, 2017; Mangan et al., 2017). To that end, this dissertation examined population level-variability in acidification tolerance of larval eastern oysters as well as elevated temperature and low salinity tolerance in both larval and juvenile hard clams in order

to deepen our understanding of population-level variability in physiological responses to future climate change conditions.

A comparison of larval tolerance to acidification stress between two oyster populations within Chesapeake Bay revealed that even over small spatial scales, physiological differences can be detected. Patterns in the accumulation of energy reserves, growth, and survival differed between larvae from Page Rock reef (York River) and Parrot's Rock reef (Rappahannock River) under both control and acidified conditions, demonstrating physiological variability in not just environmental stress tolerance, but also underlying basal metabolic processes. While physiological differences over such a small spatial scale likely do not capture the full extent of physiological variation across the range of *C. virginica*, these data do demonstrate the need for and importance of continued work examining population-level variability in stress tolerance as the potential for further variability is high. Population-level variability in environmental stress tolerance has been a powerful tool used in the past to create more resilient broodstock lines in support of industry and restoration efforts, and therefore, has the potential to be implemented to mitigate negative impacts caused by ongoing climate change (reviewed in Tan et al., 2020).

The assessments of juvenile clam stress tolerance spanned a much larger geographic range, and the data produced were more variable and difficult to interpret. When exposed to nonlethal temperature stress, juvenile clams from all five populations assessed showed minimal changes in oxygen consumption rates, which contributes to a growing body of evidence that juvenile *M. mercenaria* have alternative mechanisms for responding to heat stress beyond increasing aerobic cellular energy production (Ulrich and Marsh, 2009; Matoo et al., 2013; Stevens and Gobler, 2018). Population-level variability in percent survival under a more severe temperature condition was detected, indicating that variations in thermotolerance do exist among

populations, at least with respect to their maximal temperature tolerances. Furthermore, variability among populations was observed in oxygen consumption rate changes when juveniles were exposed to low salinity; however, clams from all populations did show a similar low salinity tolerance limit below 15. Taken together, there is some evidence of variation in physiological tolerance to environmental stress among study populations, which warrants further exploration into population-level variability in juvenile hard clam. The limitations of this study demonstrate the need for assessing stress tolerance in more multifaceted approaches in the future to better understand the nature of the variations observed here.

Building upon the differences in responses to elevated temperature and low salinity stressors observed among hard clam populations, selective cross breeding was conducted to explore if and to what extent physiological tolerances could be altered. Three controlled crosses were conducted and evaluated at both the juvenile and larval life stages. Results of the juvenile population cross assessment was similar to the parent population assessments in that no changes in oxygen consumption were detected in response to increasing temperatures; however, differences began to emerge under the more extreme temperature challenge and the low salinity exposures. The two outcrosses showed evidence of improved tolerance compared to the Wachapreague, VA self-cross, but it was not possible to evaluate the full extent of this potential hybrid vigor as only the first generation of offspring were evaluated (Edmands, 2007; Birchler et al., 2010). Furthermore, juveniles from all three crosses performed worse when exposed to low salinity than all populations previously assessed, showing greatly diminished oxygen consumption rates between salinities of 20 and 15. This difference indicates that potential variation in the genetic contribution of adult animals used for spawning between these studies may have had a greater effect on physiological tolerances than any effect noted from the

controlled crosses. The observation of such a shift between experiments does, however, demonstrate that genetic variability may exist within the study populations used in these projects and should be explored.

Larval population cross assessments showed that larvae from the Wachapreague, VA self-cross had the lowest percent survival under elevated temperature, which aligns with the results of the juvenile mortality trial at a more extreme temperature; however, the rest of the observed differences between larval crosses were not clearly reflected in the juvenile data. One key difference seen in larval physiology was that, regardless of treatment condition, larvae produced from the cross of Bogue Sound, NC and Cape Cod, MA clams grew slower compared to either of the other two crosses. Furthermore, this cross showed the highest percent survival when exposed to a lower salinity condition; although, all crosses showed large reductions in cellular energy reserves in response to low salinity exposure, indicating that low salinity is likely a significant challenge for *M. mercenaria* larvae in general.

In conjunction with this physiological assessment of hard clam larvae, larval microbiomes were sequenced to provide foundational knowledge about the composition of larval clam microbiomes and how they may vary with parent populations. This assessment across both an elevated temperature and lower salinity condition also allowed for the analysis of shifts in microbial communities associated with environmental stress and the implications that could have for larval clam health and environmental tolerance in the future. Interestingly, larval microbial communities were similar between the Wachapreague, VA self-cross and the Wachapreague, VA with Pocomoke Sound, VA cross, likely due to their shared parental lineage, while the Bogue Sound, NC and Cape Cod, MA larvae showed clear differences in species richness and community structure. Shifts in relative abundances of microbial taxa were observed in larvae

from all crosses in response to elevated temperature and lower salinity exposure, with the largest shifts occurring under lower salinity stress. As large-scale microbiome sequencing is still a relatively new technology, there is still much to be learned about the functions of specific microbial taxa, and therefore, how shifts in these communities due to environmental stress may impact larval clam health and survival in the future (Pierce and Ward, 2018; Neu et al, 2020). The documentation of shifts in microbial taxa within larval hard clams in this study demonstrates the need to further explore the role that symbiotic microbial communities play in hard clam persistence, especially in the face of ongoing climate change.

Overall, this dissertation has provided insight into specific stress response mechanisms within two ecologically and economically important coastal bivalves. Further work is still needed to fully elucidate the underlying cellular mechanisms responsible for environmental stress tolerance within the early life stages of eastern oysters and hard clams, but this work has helped to provide insight on what metrics are most appropriate for capturing physiological changes in both larval and juvenile life stages of bivalves at various levels of acidification, elevated temperature, and low salinity stress. By assessing variations in physiological tolerances at the population level, this work serves to demonstrate the importance population-level variability will likely play in the long-term persistence of eastern oysters and hard clams as climate changes continues. The finding that physiological differences can exist over small spatial scales should be encouraging as further variation in physiological tolerance across the entire range of these species is likely to exist, and therefore, could serve as a resource to produce more resilient broodstock lines in an effort to help support both the aquaculture industry and restoration efforts in the future. Microbiome data illustrating that parental populations can have strong influence on larval microbiomes is also a key finding as transgenerational transfer of

microbial communities could potentially serve as a mechanism to confer future resilience to environmental stress to offspring (Sharp et al., 2007; Torda et al., 2018; Marangon et al., 2021 Zhou et al., 2021). Further explorations of bivalve microbial communities are needed to understand both the function of specific microbial taxa within their host organisms, and what climate change driven shifts in these taxa mean for the fitness of commercially and ecologically important species.

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