Linking physiological effects of environmental stressors from cellular to whole-organismal levels in the early-life history stages of *Crassostrea virginica* (Eastern oyster)

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This Ph.D. is dedicated to all the kids who have ever said, “I want to be a marine biologist or dolphin trainer when I grow up.” I also dedicate this dissertation to my husband, family, friends, mentors, fur babies, and my future self. Last, but certainly not least, this Ph.D. is dedicated to Dr. Mark A. Wollaeger, who is greatly missed, loved, and now not the only doctor in the family.

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ABSTRACT

The Eastern oyster, *Crassostrea virginica* (Gmelin, 1791), is an ecologically and economically important species that resides in dynamic coastal ecosystems along the East and Gulf coasts of the United States. The success of oyster populations depends on the recruitment of their early life stages, which are especially vulnerable to environmental stress due to high developmental energy demands. As climate change continues, it is necessary to anticipate how the early life stages of the Eastern oyster will respond to environmental stressors under ecologically relevant scenarios. Therefore, the goal of this dissertation was to understand how the early life stages of *C. virginica* are physiologically affected by multiple global climate change stressors from a holistic perspective by incorporating local environmental data, observations across three life stages (i.e., carryover effects), responses from two important types of Eastern oysters, and physiological metrics from the cellular to whole-organism levels. To achieve this goal, chapter two observes the relative importance of three environmental tolerance mechanisms (selective mortality, carryover effects, and phenotypic plasticity) in shaping the performance of juvenile oysters in response to salinity exposures during the larval stage. Findings from this chapter indicate that typical differences in salinity among successive larval cultures in shellfish hatcheries likely do not impact performance as juveniles; rather, phenotypic plasticity likely underpins juvenile oyster performance as their physiology correlated with environmental conditions during the juvenile phase, not the larval exposures. Chapter three investigates carryover effects in more detail to explore how multiple global climate change stressors, ocean acidification and ocean warming, might affect the physiology of larval *C. virginica*, if those effects carry over to impact the performance of juvenile oysters and lastly, if those carryover effects change under different future environmental scenarios. Conditions of ocean acidification and ocean warming did affect the larval stage of the Eastern oyster and carry over into the juvenile stage, though these effects were nuanced and context dependent. Specifically, carryover effects from conditions of acidification were more persistent and negative, whereas warming had more fleeting carryover, and cross-tolerant, effects that were generally positive. Continuing to observe the effects of ocean acidification and ocean warming on the Eastern oyster, chapter four compares the larval responses of wild and selectively bred oysters. *C. virginica* larvae from wild and selectively bred oysters responded differently to conditions of acidification, but not warming. Furthermore, wild oyster larvae may be more resilient in the face of ongoing climate change. Despite exhibiting more lethal and negative effects of acidified conditions early in the larval stage, wild oyster larvae compensated for these earlier negative effects, while larvae from selectively bred oysters began showing signs of stress towards the end of the experiment. Lastly, exploring the development of carryover effects, chapter five observes how conditions of acidification during the settlement stage (i.e., settlement and metamorphosis) carried over to impact the juvenile stage. No detectable carryover effects were found, even though conditions of acidification negatively affected tissue growth at the beginning of the settlement stage. Overall, while Eastern oysters are able to withstand environmental stress to some degree, there were sub-lethal and carryover effects from multiple global climate change stressors identified in this dissertation that could have consequences for both wild and selectively bred populations in the future. The results from this dissertation will support subsequent studies to accurately predict the future success of wild oyster populations (e.g., effects to recruitment) and aquaculture production based on their physiological performances in the face of ongoing climate change.

AUTHOR'S NOTE

Chapter 2 of this dissertation has been prepared as a manuscript for a peer-reviewed scientific journal. As such, the formatting for chapter 2 follows the guidelines of the scientific journal to which the manuscript was submitted.

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Linking physiological effects of environmental stressors from cellular to whole-organismal levels in the early-life history stages of *Crassostrea virginica* (Eastern oyster)

CHAPTER 1

Introduction

Eastern oysters (*Crassostrea virginica*)

The Eastern oyster, *Crassostrea virginica* (Gemlin, 1791), is an ecologically and economically important species to the East and Gulf coasts of the United States with a large biogeographical range from the Gulf of St. Lawrence to the Gulf of Mexico. Oysters are considered foundation species, organisms that affect the biodiversity within an ecosystem through non-trophic interactions and modulations of nutrient and energy fluxes (Ellison, 2019), because of their role as an ecosystem engineer, creating three-dimensional reef-like structures in otherwise flat, soft-sediment environments (Jones et al., 1994; Lemasson et al., 2017). Oyster reefs provide habitat to a number of species like polychaetes, bryozoans, and crustacea (Wells, 1961). Oyster reefs contain a variety of interstitial spaces that also make the structures valuable as a nursery habitat for many fish and larger crustacean species, including economically important ones, like blue crabs and striped bass (Nestlerode et al., 2007; Wells, 1961). Furthermore, reef structures can attenuate wave energy, which reduces erosion, protects other essential habitats, like sea grass beds, and protects shoreline structures, like houses (Grabowski and Peterson, 2007). Additionally, the attenuation of wave energy includes those from storms (i.e., hurricanes), providing shoreline protection and stabilization (Lemasson et al., 2017).

Beyond services directly related to oyster reefs are those from individual oysters, like water column filtration, nutrient cycling, and food provisioning. One of the most touted attributes of oysters is their ability to filter and ‘clean’ the surrounding waters (Newell, 1988). Oysters are filter feeders that mainly consume phytoplankton, though they can consume dissolved organic matter, especially during the larval stage (Langdon and Newell, 1996; Manahan, 1990; Moran and Manahan, 2004). Because oysters filter particles out of the water column, they can also help remove suspended solids and concentrate them in their pseudofeces, which, in addition to their phytoplankton consumption, can increase light penetration throughout the water column (Grabowski and Peterson, 2007). Fecal production also enhances nutrient cycling and removal, and the use of oysters, both on aquaculture farms and subtidal restored reefs, has been proposed as one type of mitigation for nutrient pollution and eutrophication in coastal and estuarine waters (Kellogg et al., 2014).

Oysters are an important food source for many species, including humans. As oysters are at a low trophic level, they aid in the transfer of energy from phytoplankton up the food web, supporting various gastropods, crustaceans, and fish species (White and Wilson, 1996). Oysters are a good source of protein for human populations as well, with evidence of consumption dating back several centuries to various indigenous populations (Jenkins and Gallivan, 2019; Nell, 2001; Reece et al., 2020). Harvest and additional processing industries grew around Eastern oyster populations along the East and Gulf coasts of the U.S., with the Chesapeake Bay eventually becoming the epicenter around the mid-1800s based on the quantity of oysters harvested (Reece et al., 2020; Rothschild et al., 1994); however, overharvesting, technological advancements causing habitat destruction and loss, environmental degradation, lack of regulation and management, and the rise of two deadly oyster diseases caused wild oyster populations in the Chesapeake Bay to decline to an estimated 0.3% of historic harvest levels (Rothschild et al., 1994, Wilberg et al., 2011). Wild population numbers have never recovered, even with many past and ongoing restoration campaigns, like those conducted by the Chesapeake Bay Program and its partners (see https://www.chesapeakeprogress.com/abundant-life/oysters for more details; Mann and Powell, 2007; Reece et al., 2020). Due to the cultural and economic importance of wild oysters as a food source, oyster aquaculture developed around the 1970s and has become an important and growing component of the Chesapeake Bay economy, valued around $14.5 million in Virginia alone (Hudson, 2019; Reece et al., 2020). Oysters are culturally, economically, and ecologically important, which is why understanding what may affect their viability and performance in the future is necessary.

Early life stages

Oysters have a biphasic life cycle with a separate free-swimming, planktonic larval stage that is morphologically and physiologically distinct from their sessile adult form. Eastern oysters sexually reproduce via broadcast spawning, where male and female gametes are released into the water column for fertilization (Thorson, 1950). After fertilization, larvae develop as non-feeding trochophores, relying on maternally invested endogenous energy reserves to begin building their larval shell (Kennedy et al., 1996). After the trochophore stage (~ 24 – 48 h duration), larvae enter into the prodissoconch I stage (~24 h duration) when the larval shell is fully formed with a flat hinge (i.e., where the two shell valves meet) that resembles the letter 'D,' and larvae are aptly referred to as D-shaped veligers. After the D-stage, veligers transition into the prodissoconch II (PDII) stage (~14 days duration), which lasts until settlement. Towards the end of PDII, veligers transition into pediveligers by developing eyespots and foot muscles, signs that larvae are competent and ready to settle onto the benthos for metamorphosis (Baker and Mann, 1994a).

On average, the entire larval stage lasts ~18 – 20 days during which larvae are undergoing developmental processes that are energy intensive, making them vulnerable to external stressors. More than 80% of the total estimated energy pool of oyster larvae is utilized for developmental processes inclusive of calcification, protein synthesis, and ion regulation (Pan et al., 2018). Of the remaining energy pool, most is assumed to be utilized for DNA and RNA synthesis, while a small portion is likely utilized to create long-term lipid stores, from triglycerides, for utilization during metamorphosis, specifically when oysters cannot feed due to morphogenic processes (Pan et al., 2018). This leaves little leeway in the larval energy budget to satisfy energy demands due to any unexpected, external stress. Lastly, larval oyster shells are made of the mineral form of calcium carbonate ($CaCO_3$) called aragonite, which is more soluble in seawater than calcite, the form that juvenile and adult oysters use (Stenzel, 1964), further adding to the vulnerability of oyster larvae.

Following the larval stage are settlement and metamorphosis, together referred to as the settlement stage for the remainder of this chapter, processes by which larvae transition into the juvenile stage. The settlement stage is another energy-intensive period, often considered a bottleneck to the successful recruitment of individuals to the reproductive population (Espinel-Velasco et al., 2018). The settlement stage is morphologically, physiologically, and ecologically dramatic, involving shifts in body form and function, energy allocation, and environmental conditions, respectively (Bishop et al., 2006). For Eastern oysters, the settlement stage, on average, takes approximately three to four days, though this timespan differs among individuals, sometimes taking longer to complete (Baker and Mann, 1994b). More detailed information on the settlement stage is provided in chapter 5. Once oysters have metamorphosed into juveniles, they are still vulnerable for the first few months, though not to the same degree as are their previous life stages, because they must grow quickly to escape predation and be more resilient to physical stressors, like waves. The success of vulnerable, early life stages of oysters dictates the future success of oyster populations, so understanding how each respond to external stress is important, as is understanding how the stress experienced from one life stage may affect a later stage given how interdependent these early life stages are (e.g., larval long-term energy stores are necessary for metamorphosis).

Oyster aquaculture and the triploid oyster

Aquaculture of the Eastern oyster began out of necessity due to a sharp decline in the wild populations from habitat degradation and loss, overharvesting, mismanagement of the fishery, lack of regulations and enforcement, and lastly, increasing disease prevalence. Oyster aquaculture production is either extensive or intensive, but both methods comprise hatcheries and farms. Hatcheries spawn and produce both eyed larvae that are sold to farmers for extensive production (i.e., spat-on-shell) and spat (i.e., 2 – 3 month-old juvenile oysters) for intensive methods (i.e., rearing individual oysters in containers; Reece et al., 2020). Diploid and triploid oysters are spawned for aquaculture, though triploids are generally preferred and becoming more popular. In Virginia, triploids made up 97% and 79% of oyster production for intensive and extensive culture, respectively, as reported by hatcheries in 2018 (Hudson, 2019).

Oyster farmers prefer triploids because of their fast growth and disease resistance (Barber and Mann, 1991; Dégremont et al., 2012; Wadsworth et al., 2019). In Virginia, around the late 1990s, the Aquaculture and Breeding Technology Center (ABC) at the Virginia Institute of Marine Science (VIMS) began developing broodstock lines for disease resistance (Frank-Lawale et al., 2014). Prior to selective breeding for disease resistance, triploid Eastern oysters were developed for their sterility (Stanley et al., 1981). The development of sterility through triploidy meant that oysters could be consumed throughout the year because glycogen stores that are responsible for the flavor and texture in quality oysters would be retained rather than depleted during gametogenesis and spawning (Stanley et al., 1981). Additionally, oyster producers and growers noted an unintended benefit of triploid oysters, their fast growth compared to diploid oysters (Dégremont et al., 2012). The sterility of triploid oysters meant that energy that would have been utilized for reproductive processes was redistributed, manifesting as faster growth rates, which ultimately decreased the time to market (see Chapter 4 for more details; Allen and

Downing, 1986; Dégremont et al., 2012). Energy allocation in oysters produced from these genetically distinct broodstock lines of fast-growing, disease-resistant oysters (Frank-Lawale et al., 2014; Goncalves et al., 2017; Thompson et al., 2015) is likely different than in their wild conspecifics. Understanding how potentially different energy budgets among broodstock and wild oysters may lead to different outcomes within the context of environmental stress is important for the continued success of the growing aquaculture industry, and as breeding programs begin to focus on selecting for environmental stress tolerance (McCarty et al., 2020).

Climate change in Chesapeake Bay and effects on oysters

Climate change has already altered ecosystems and impacted oysters within Chesapeake Bay with no indication of subsiding in the foreseeable future (Najjar et al., 2010; Wuebbles et al., 2017). Climate change affects many chemical and physical properties of water within marine environments (IPCC, 2021), and ocean acidification, ocean warming, and changes in salinity, specifically, are three stressors that can greatly affect Eastern oyster populations. Globally, oceans and coastal waters have absorbed 93% of the excess heat produced by anthropogenic climate change (IPCC, 2021), and the Chesapeake Bay is no exception. Water temperatures within the Chesapeake Bay have increased by ~1 – 2°C since the 1950s and are predicted to increase another 2 – 5°C by the end of this century (Ding and Elmore, 2015; Hinson et al., 2022; Muhling et al., 2018; Najjar et al., 2010; Preston, 2004). Beyond ocean warming, increasing atmospheric temperatures affect climate cycles and weather patterns and ultimately, precipitation (IPCC, 2021; Wuebbles et al., 2017). Heavy precipitation events have become more frequent and intense since the early 20th century for most of the United States, especially in the Northeast, and are expected to continue increasing throughout this century (Wuebbles et al., 2017). As the Chesapeake Bay watershed extends into the Northeast, more frequent and intense precipitation events could lead to greater freshwater flow into the Bay and impact the salinity (Najjar et al., 2010, Wuebbles et al., 2017). Salinity is predicted to decrease by 1 – 3 units in certain tributaries to the Bay by 2100 (Muhling et al., 2018); however, models also predict that evapotranspiration within the Chesapeake Bay watershed will increase with atmospheric temperature, decreasing runoff into the Bay, making predictions for changes in salinity highly variable (Ross et al., 2021). Furthermore, sea level rise is likely more influential on salinity in the lower Chesapeake Bay with models predicting an increase in salinity by 1.5 units by the mid-21st century (Li et al., 2023). Organisms within the Bay will be impacted by future temperature and salinity changes, as well as increasing coastal and ocean acidification.

The main drivers of coastal and ocean acidification (COA) are ocean acidification (OA) and eutrophication, supported by ocean warming, that promotes increased biological activity (Cai et al., 2021). Thirty percent of the anthropogenic atmospheric CO_2 emitted since the industrial revolution has been absorbed by ocean and coastal waters, causing pH to decline by ~0.1 units (Sabine et al., 2004). Absorption of anthropogenic CO_2 is the driver behind this phenomenon, called OA, which occurs through a series of equilibrium reactions resulting in an increase in the thermodynamically favored bicarbonate (HCO_3^-) ion and subsequent decrease in the biologically important carbonate (CO_3^{2-}) ion used by calcifying organisms (Sabine et al., 2004). As climate change continues, OA is predicted to decrease oceanic pH by another ~0.15 – 0.4 units by 2100 (IPCC, 2021); however, coastal zones already experience low pH values and fluctuations equal to or greater than future open ocean predictions due in part to the compounding effect of coastal acidification (Cai et al., 2021, Hofmann et al., 2011). Coastal acidification occurs after increased nutrient inputs from terrestrial runoff stimulate phytoplankton blooms that die and are respired by microbes, releasing CO_2 into the water column that can affect the pH. If the water column is stratified, then the surrounding pH in the bottom layer will decrease with increasing coastal acidification (Arnold et al., 2017). As warming continues, periods of intense biological activity will be prolonged, further acidifying the waters of the Chesapeake Bay (Cai et al., 2021). pH in Chesapeake Bay has already decreased by ~0.2 units during summer months since the 1980's in polyhaline regions (i.e., salinities > 18), resulting in an average summertime pH of 7.8 (Najjar et al., 2010; Waldbusser et al., 2011). Projections for the Bay anticipate further declines in average annual pH by ~0.1 – 0.3 units by the mid-21st century alongside other changes to the entire carbonate chemistry system (reviewed in Cai et al., 2021), like declines in calcium carbonate ($CaCO_3$) saturation state (Li et al., 2023), which is an important indicator of stressful conditions for calcifying organisms (Waldbusser et al., 2015a,b).

Climate change and the cellular stress response

Warmer water temperatures, changing salinities, and the decline in CO_3^{2-} and pH associated with increasing COA can be stressful to marine invertebrates. Generalized species' responses to climate change include changes to survival, growth, reproduction, and metabolic processes (Doney et al., 2012). There are, of course, species-specific responses to environmental stress, and those applicable to Eastern oysters are reviewed below and in the subsequent chapters; however, there are whole-organismal and cellular physiological responses to environmental stress that are conserved across marine invertebrates. As marine invertebrates are ectothermic and lack robust acid-base and ionic regulatory processes, climate change can have profound consequences on their physiologies (Doney et al., 2012). As temperature increases, basal metabolism increases, which can have beneficial effects on growth and development (Solan and Whiteley, 2016). If temperatures rise past an organism's thermal limit, then biochemical reactions, including protein production and the cellular stress response, are impaired, and energy demands are not met, decreasing growth, development, and reproduction before ultimately increasing mortality (Pörtner, 2010). COA and changes in salinity affect the acid-base balance and osmoregulation in marine invertebrates, respectively (Somero et al., 2017). Tolerance of both of these climate change stressors can be energetically demanding, straining energy budgets and resulting in similar negative outcomes as previously described for ocean warming (Solan and Whiteley, 2016). Depending on the species- and ecosystem-specific contexts, however, whole-organismal physiological responses to environmental stress will have different consequences (Doney et al., 2012). Regardless of the context, behind these whole-organismal responses are highly conserved cellular mechanisms counteracting stress effects to cellular functioning and signaling and biochemical molecules (Kültz, 2005; Solan and Whiteley, 2016).

Environmental stress can affect energy budgets and allocation as cells respond to molecular damage, ultimately impacting homeostasis and inducing the cellular stress response (Pörtner, 2008; Sokolova et al., 2012; Somero et al., 2017). Homeostasis refers to the baseline biochemical functioning necessary to maintain higher-level physiological mechanisms for organismal survival (Somero et al., 2017). Cellular stress response (CSR) mechanisms and processes are ubiquitous across all species because they are critical for survival of an organism (Kültz, 2005). The CSR employs a suite of proteins and enzymes to repair or remove damaged biomolecules and cellular structures, maintain metabolism, and increase tolerance to further stress (Kültz, 2005; Solan and Whiteley, 2016). Once cellular conditions have been initially stabilized, the cellular homeostatic response (CHR) is activated to maintain cellular functioning until environmental conditions change again. The CHR can be species, cell, and stressor specific but often relies on osmoregulation for cellular volume and ionic control. Without proper osmoregulation, the cell structure and integrity may be affected and impact protein and cell signaling functions (Solan and Whiteley, 2016). Maintaining homeostasis, and therefore the CSR/CHR, is a necessary priority and can reduce the amount of energy allocated to other important, high-level biological processes (e.g., growth and reproduction) depending on the duration and magnitude of the environmental stressor (Sokolova et al., 2012).

The oxidative stress response is an important component of the CSR that is triggered by most environmental stressors, including COA, ocean warming, and salinity changes because under stressful conditions, organisms increase their metabolism to meet energy demands (Solan and Whiteley, 2016). Specifically, the oxidative stress response is combating and repairing damage caused by reactive oxygen species (ROS; Kültz, 2005). All organisms naturally produce ROS during aerobic metabolism as electrons are transferred sequentially to the final electron acceptor, oxygen, which has two unpaired elections. During the electron transfer, highly dangerous free radical ROS can be created and escape into the cell before the second, neutralizing electron is transferred. Per their name, ROS are highly reactive with other molecules and can cause significant damage to DNA, membrane lipids, and proteins if oxidative stress response molecules (i.e., antioxidants) are not sufficiently abundant, which can occur under environmental stress (Lesser, 2006). Under extreme environmental stress (e.g., long exposures or severe conditions), ROS production can be greater than the pool of antioxidant molecules since ROS production is positively correlated with metabolism (i.e., respiration), resulting in increased cellular damage. Energy allocations then shift to prioritize maintaining homeostasis through increased production of antioxidants and other aspects of the CSR to repair and remove damaged molecules (Kültz, 2005; Sokolova et al., 2012). Organismal responses to stress begin at the cellular level and impact whole-organismal responses, which is why incorporating physiological metrics across both levels is important to provide a more holistic understanding of how marine organisms will respond to future environmental change.

Effects of climate change on early life stages of oysters

Oysters are susceptible to increasing temperatures, COA, and salinity changes because they are ectothermic osmoconformers, meaning that they do not have strong regulation of their internal fluids to buffer tissues and metabolic processes from changes in the surrounding environment (Davenport, 1985; Gazeau et al., 2013). The larval, settlement, and juvenile stages are even more vulnerable to environmental stressors compared to the adult stage because they have limited energy to allocate towards processes unrelated to development and growth (Harvey et al., 2013; Pan et al., 2018; Pandori and Sorte, 2019). Salinity is well-known to affect the physiology of oysters through changes in osmotic control, as previously described above, ultimately dictating the biogeographic ranges of oyster populations (Shumway, 1996). Salinity changes can cause cellular stress and be energetically demanding because oysters close their valves to control and stabilize their osmotic state until they begin utilizing anaerobic processes and need to re-open (Solan and Whiteley, 2016). Furthermore, oysters cannot feed to meet energy demands when their valves are closed, another reason why low salinities are detrimental (McFarland et al., 2022). Overall, changes in salinity, in addition to COA and elevated temperatures, individually impair development, growth, and calcification of larval and juvenile oysters (Byrne, 2011; Gazeau et al., 2013; Przeslawski et al., 2015).

Ocean warming will likely have a profound effect on Eastern oysters even though oysters reside in areas that annually, and even daily, experience a wide range of temperatures. Although Eastern oysters cannot regulate their own internal temperatures as ectotherms, oysters can tolerate temperatures up to almost 50°C (Shumway, 1996); however, the early life stages of marine bivalves, larvae especially, are not as tolerant to warming as later stages (Byrne, 2011; Byrne and Przeslawski, 2013). Larval development can benefit from slight increases in temperature because temperature directly influences the rate of biochemical, and ultimately metabolic, reactions (Hochachka and Somero, 2002). As temperatures approach upper tolerance limits, which can shift with each life stage (Byrne, 2011), physiological processes, in general, begin to slow as oxygen supplies are unable to keep pace with overall energy demand and energy is prioritized for processes necessary for survival (Pörtner, 2010; Sokolova et al., 2012). In the early life stages of Eastern oysters and other bivalves, ocean warming can negatively affect survival, shell growth, development, lipid stores, and water filtration (McFarland et al., 2022; Rayssac et al., 2010; Rybovich et al., 2016; Speights et al., 2017; Talmage and Gobler, 2011). Ocean warming, therefore, may have negative consequences on future Eastern oyster populations.

Similar to temperature, COA can negatively affect oysters (Byrne and Przeslawski, 2013; Gazeau et al., 2013; Przeslawski et al., 2015). Early OA research on *Crassostrea gigas* focused on direct effects to shell growth and the process of calcification because increasing CO_2 concentrations decrease the carbonate ion concentration, which makes calcification less thermodynamically favorable and more energetically intensive (Kurihara et al., 2007; Miller et al., 2009). Studies specifically focused on effects to larval shell growth because the aragonite form of $CaCO_3$ that larval oysters use is more soluble in seawater and, therefore, more sensitive to acidic conditions, compared to the calcite form utilized by juvenile and adult oysters (Brunner et al., 2016; Waldbusser et al., 2015a, 2015b, 2013). Under conditions of OA, multiple species of oyster larvae experience abnormal or weaker shell development, decreased growth rates, and delays in overall development (Gobler and Talmage, 2014; Hettinger et al., 2012; Talmage and

Gobler, 2010; Waldbusser et al., 2011). Though juvenile oysters, in general, are more robust to COA compared to larvae because they utilize a more stable form of $CaCO_3$, calcite, they still exhibit similar results found during the larval stage (Talmage and Gobler, 2011, 2009). Because oysters broadly have limited control of their internal pH, Eastern oysters and other bivalves utilize active and passive buffering mechanisms, like ion regulation and shell dissolution, respectively, to combat conditions of COA (Michaelidis et al., 2005). If active buffering mechanisms are prolonged, however, increases in metabolism cannot be supported through increased respiration rates alone (Frieder et al., 2017; Lannig et al., 2010; Pan et al., 2015), which eventually leads to reduced ATP-levels, tissue growth, and shell growth, and ultimately metabolic depression (Beniash et al., 2010; Frieder et al., 2018; Melzner et al., 2020). Though the individual effects of OA, ocean warming, and salinity have been extensively researched, studies observing the interactive effects of environmental stressors on the early life stages of oysters across different physiological processes would greatly increase knowledge of potential tolerances within Eastern oysters to ongoing climate change.

Dissertation objectives and structure

The goal of this dissertation is to understand how the early life stages of *C. virginica* are physiologically affected by multiple global climate change stressors from a holistic perspective by incorporating local environmental data, observations across three life stages (i.e., carryover effects), responses from two important types of Eastern oysters, and physiological metrics from the cellular to whole-organism levels. This dissertation aims to fill essential knowledge gaps in our understanding about continuous, sub-lethal impacts of climate change stressors across multiple life stages of Eastern oysters. Effects of multiple environmental stressors – COA, warming, and salinity – from the larval to juvenile stages, as well as from the settlement to juvenile stages, were observed. Experiments were designed based on local environmental data since coastal systems regularly experience projected conditions for 2100 in the open ocean as reported by the IPCC (Cai et al., 2021, Hofmann et al., 2011; IPCC, 2021). Furthermore, responses of both wild and selectively bred *C. virginica* were evaluated to understand how climate change may impact Eastern oysters as a species across their various socio-ecological roles. Lastly, a suite of physiological responses from the cellular to whole-organismal levels were measured for an integrated, holistic approach.

Chapter two investigates the relative influence of three environmental-tolerance mechanisms (selective mortality, carryover effects, and phenotypic plasticity) on juvenile oyster physiology. Oysters were exposed to typical salinity differences observed in the Rappahannock River as larvae through the settlement stage (i.e., settlement and metamorphosis). Juvenile oysters were then placed into two Chesapeake Bay tributaries of differing salinity to observe potential shifts in the relative influence of the environmental-tolerance mechanisms under different environmental conditions. Results from this study help identify which mechanism is likely driving the performance of oysters at their most vulnerable life and transitional stages as well as during an important aquaculture industry transition from the hatchery to farm.

Chapter three examines potential carryover effects produced when wild Eastern oyster larvae experience conditions of acidification and ocean warming, either individually or combined. Carryover effects, effects that occur during one life stage and impact a later stage, were generally assessed in juvenile oysters as well as under different environmental conditions to understand potential changes in the relative influence of carryover effects. This study adds to the few conducted on carryover effects in *C. virginica* and multiple stressors. These results help elucidate physiological mechanisms of tolerance or bottlenecks that may impact this species' long-term success in a changing environment.

Chapter four compares responses of larvae from two groups of Eastern oysters important to Chesapeake Bay, wild and selectively bred, to multiple environmental stressors. Findings from this study identify potential vulnerabilities and strengths of each type of Eastern oyster when faced with multiple environmental stressors. This study is one of the first comparing wild and selectively bred Eastern oysters under multiple environmental stressors.

Chapter five explores how the experience of acidic conditions during the settlement stage carries over to produce physiological effects in the juvenile stage. Alongside results from chapter three, this study informs the emergence of and physiological mechanisms of carryover effects since most larval carryover effects studies to date include the settlement stage with the larval experimental exposure period before carryover effects are assessed during the juvenile stage.

Chapter six summarizes all findings within the broader context of the field of marine invertebrate ecophysiology and applied aquaculture research.

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CHAPTER 2

Relative importance of environmental-tolerance mechanisms in response to typical salinity shifts during oyster aquaculture production

Abstract

Variability of water conditions in coastal environments could affect oyster aquaculture production through three main environmental-tolerance mechanisms: carryover effects, selective mortality, and phenotypic plasticity. Aquaculture production of Eastern oyster, *Crassostrea virginica*, larvae occurs on weekly timescales in Virginia, with variations in salinity experienced by subsequent larval cohorts. The present study examined the relative importance of carryover effects – phenotypic changes during a previous life stage that impact a later stage – and phenotypic plasticity in shaping the performance of juvenile oysters after an experience of typical differences in salinity during the larval stage. Genetic diversity was also assessed to rule out large shifts in allele frequencies, or loss of diversity, that would suggest observed effects were the result of selective mortality rather than carryover effects or phenotypic plasticity. Larval oysters were reared through settlement and metamorphosis under two salinity conditions that represent typical differences between consecutive spawns in a hatchery. Juveniles were then raised *in situ* in two Virginia tributaries of the lower Chesapeake Bay with distinct salinity conditions, the Rappahannock and York rivers. Metrics of survival, growth, oxidative stress, and condition index were compared to assess carryover effects and phenotypic plasticity. Juvenile oyster survival and physiology correlated with *in situ* environmental conditions rather than previous larval salinity experience. Specifically, juvenile oysters raised in the Rappahannock River had greater survival (13%), shell length (14%), condition index (38%), and dry tissue weight (78%) than those raised in the York River, regardless of larval salinity. Rappahannock River oysters also had 20% lower total antioxidant capacity than York River oysters. Genetic diversity remained high with no large shifts in allelic frequencies that would suggest non-random loss of alleles attributable to selection. Our results suggest that typical salinity differences

experienced in shellfish hatcheries 48 hours after fertilization likely do not impact juvenile oyster performance during grow-out; rather, phenotypic plasticity likely underpins juvenile oyster performance during the transition from hatchery to farms. The importance of phenotypic plasticity presents another reason why farm site selection is critical to the performance and success of aquaculture product. Future studies are needed to further identify whether larval responses to salinity conditions are dependent on additional environmentally relevant conditions like temperature or the timing of exposure post-fertilization to better understand the relative importance of phenotypic plasticity, carryover effects, and selective mortality within oyster aquaculture.

1. Introduction

Aquaculture of the Eastern oyster, *Crassostrea virginica* (Gmelin, 1791), occurs in highly variable coastal environments along the East and Gulf coasts of the United States where oysters routinely encounter challenging environmental conditions (Proestou et al., 2016; Shumway, 1996). During hatchery production where spawning occurs weekly, temporal changes in water conditions may produce physiological and genetic differences between cohorts of larvae that could impact production on farms. Depending on magnitude and duration, changes in environmental conditions can have cascading impacts from the molecular to whole-organism levels that can last beyond a single life stage due to carryover effects – phenotypic changes from a previous life stage experience that impact a later stage – or selective mortality (Petitjean et al., 2019; Sokolova et al., 2012). Alternatively, phenotypic plasticity may enable larvae to maintain performance under a range of environmental conditions due to the production of multiple phenotypes from one genotype (Pigliucci, 2001). As mechanisms of environmental tolerance, carryover effects and selective mortality could negatively impact aquaculture production of the Eastern oyster through declines in organismal performance (e.g., slower growth rates and therefore time to market) and genetic diversity, respectively. Alternatively, phenotypic plasticity may help sustain production through continued performance of oysters regardless of environmental conditions. The relative influence of these mechanisms on oyster performance within the context of shellfish aquaculture and present-day variations in environmental conditions is currently unknown.

Experience of varied environmental conditions during an oyster's early life history can have latent phenotypic consequences impacting performance, referred to as within-generation carryover effects (O'connor et al., 2014). Carryover effects have been identified in mammals

(Skibiel et al., 2022), birds (Steenweg et al., 2022), amphibians (Le Sage et al., 2022), fish (Jonsson and Jonsson, 2014), insects (Ezeakacha and Yee, 2019), and marine invertebrates (Hettinger et al., 2012; Richardson and Allen, 2023; Shen et al., 2022). In marine bivalves specifically, larval experience of ocean acidification conditions can carry over and negatively affect shell and tissue growth (Hettinger et al., 2013, 2012) and metabolism (Zhao et al., 2019) of juveniles, while early juvenile exposure to ocean warming and hypoxia carried over to negatively impact early tissue and shell growth of adults (Donelan et al., 2021). At present, carryover effect studies on bivalves have mainly focused on global climate change stressors, like ocean acidification and temperature (Goncalves et al., 2016; Parker et al., 2015; Ross et al., 2016; Spencer et al., 2020), with few focused on local stressors, like salinity (Manuel et al., 2023).

The underlying mechanisms responsible for carryover effects, while not well understood, may be linked to shifts in energy allocation (Hettinger et al., 2013, 2012; Pechenik et al., 1998). When organisms experience moderate environmental stress, they begin to allocate more energy towards cellular processes necessary for survival, negatively impacting other aspects of their biology, like growth and reproduction (Petitjean et al., 2019; Sokolova et al., 2012). Carryover effects likely stem from these costly metabolic shifts, especially when they occur prior to an energetically intensive life-stage transition, like metamorphosis, resulting in the next life stage beginning with an energy deficit (Pechenik et al., 1998). A developing alternative hypothesis for mechanisms of carryover effects that are not directly tied to energy deficits is epigenetics. While there is limited evidence with respect to marine organisms, epigenetic changes due to environmental experience could underpin within-generation carryover effects (Eirin-Lopez and Putnam, 2019).

Environmental stress can lead to non-random, or selective, mortality, selecting for phenotypes and the underlying genotypes able to survive that stress. Selective mortality is generally beneficial for the persistence of wild populations (Darwin, 1859; Gardner and Skibinski, 1991). Oysters are r-selected species, organisms that have high fecundity and little investment per offspring to ensure the continuance of the population regardless of high mortality, which could occur because of unpredictable environmental conditions. While survival in hatcheries is likely higher than in the wild, genetic diversity is already lower in selectively bred broodstock populations compared to wild populations, increasing the vulnerability of aquaculture production to further decreases in genetic diversity from selective mortality (Araki and Schmid, 2010; Lind et al., 2008; Varney and Wilbur, 2020; Zhang et al., 2017). Lower genetic diversity and smaller effective population sizes in selectively bred populations can also lead to higher levels of lethal mutations, or inbreeding depressions, further decreasing genetic diversity and effective population size (Gamfeldt and Källström, 2007; Plough, 2018; Plough et al., 2016). Although oysters produced for aquaculture are often selectively bred to thrive in certain environmental conditions, inbreeding depression may become exacerbated in the presence of additional environmental stressors, leading to a lower likelihood of survival in later life stages (Gamfeldt and Källström, 2007; Plough, 2012). Within oyster aquaculture, higher mortality could leave hatcheries and farmers with not only decreased production, but also the possibility of complete production loss from unexpected environmental stress.

Of the three environmental-tolerance mechanisms listed earlier, phenotypic plasticity is likely more beneficial to oyster aquaculture production as a mechanism of resilience to present-day environmental variability and future climate change via rapid acclimatization (Hofmann and Todgham, 2010; Putnam et al., 2016; Seebacher et al., 2015). Phenotypic plasticity, broadly, is the production of multiple phenotypes by one genotype under differing environmental conditions (Pigliucci, 2001). As a means of interacting with and responding to surrounding environments, phenotypic plasticity is ubiquitous among taxa and is expressed at almost all biological scales (Pigliucci, 2001). Plasticity allows organisms to maintain baseline performance under stress and therefore resist loss of basic function during rapid changes in their environment (Putnam et al., 2016; Smith et al., 2013). Oysters have a high degree of phenotypic plasticity, likely evolved from living in highly variable environments as sessile organisms (Robinson et al., 2014; Shumway, 1996). For example, oysters collected from different environments (i.e., reef locations) maintained osmotic performance when exposed to various salinity conditions, and while all oysters expressed some level of plasticity, the magnitude was dependent on the previous-environment to current-environment combinations (Eierman and Hare, 2016). The ability for oysters to effectively respond to an array of environmental conditions via phenotypic plasticity lies within their abundant stress-response genes that have overlapping functions and genetic duplications (Guo et al., 2015; Modak et al., 2021; Wang et al., 2012), which is likely beneficial to aquaculture production.

Throughout the hatchery process and the transition of oysters from hatchery to farm, there are multiple points when oysters may experience stress due to a rapid change in their environment. Salinity varies spatially and temporally in coastal environments where oyster aquaculture takes place (Muhling et al., 2018), often differing weekly between successive larval cohorts throughout a production season (Figure 1), as well as between larval rearing conditions and farm environments. Salinity is also a well-known environmental stressor to oysters, affecting their development, physiology, and genetics (Casas et al., 2018a; Davis, 1958; Hand and Stickle, 1977; Méthé et al., 2015a; Shumway, 1996), making it a suitable factor for examining the relative influence of carryover effects, selective mortality, and phenotypic plasticity on larval and juvenile oyster performance. To study the role of these environmental-tolerance mechanisms on oyster aquaculture production, it is necessary to utilize typical differences in salinity conditions at the hatchery stage. In the mesohaline region of the Chesapeake Bay where oyster aquaculture and hatchery production are common, salinity frequently differs by less than one unit per week on average (Figure 1). Though Eastern oysters reside over a wide range of salinities due to their vast biogeographic range (5 – 40; Shumway, 1996), individual populations have more limited salinity tolerances that affect their ability to withstand shifts in salinity (Eierman and Hare, 2013; Scharping et al., 2019; Swam et al., 2022). Larval oysters are more detrimentally sensitive to small changes in salinity (~2) at the low end of their tolerance range, while more capable of withstanding larger differences within their optimal salinity range (Scharping et al., 2019). Since salinity varies on a fine scale between weekly larval cohort production in hatcheries within mesohaline regions (Figure 1), selective mortality in response to these small differences is unlikely if average salinity conditions are within the optimal range of 10 – 17 (McFarland et al., 2022; Scharping et al., 2019).

Therefore, the aim of this study was to examine the relative importance of carryover effects and phenotypic plasticity in shaping the performance of juvenile Eastern oysters, *C. virginica*, through the hatchery-to-farm transition in oyster aquaculture. To assess the relative influence of these environmental-tolerance mechanisms on oyster aquaculture production, metrics of survival, growth, oxidative stress, and condition index were measured. Simultaneously, genetic diversity was monitored for the occurrence of large shifts in allele frequencies to confirm if selective mortality had occurred. If carryover effects from the larval oysters' environmental experience were driving juvenile performance under ambient common-garden conditions (i.e., the same environment), the physiological response of juvenile oysters would differ based on their larval experience, and there would be random loss of genetic diversity in larval and juvenile oysters (Figure 2a). If selective mortality were driving juvenile performance, non-random loss of genetic diversity would be expected in juvenile oysters based on their larval experience. Furthermore, the physiological responses of juvenile oysters would be similar regardless of larval environmental experience (Figure 2b). Finally, if phenotypic plasticity were driving juvenile performance, there would be random loss of genetic diversity associated with previous larval environmental conditions, while physiological responses would be correlated with the juvenile environment rather than the larval environmental conditions (Figure 2c).

2. Materials and methods

2.1. Experimental design

To examine environmental-tolerance mechanisms associated with salinity exposure of *Crassostrea virginica* larvae, oysters were reared from fertilization through settlement and metamorphosis (i.e., the settlement phase) under different ecologically relevant salinities and assessed for survival, growth, oxidative stress, and genetic diversity. Juvenile oysters were then deployed to two experimental field sites in the York and Rappahannock rivers, two tributaries of Chesapeake Bay of known differing salinities (York: 19.57 $\pm$ 1.70 [CBNERR-VA VIMS, 2018]; Rappahannock: 15.85 $\pm$ 1.99 [M. Congrove, unpublished data]; mean $\pm$ standard deviation), to mimic the grow-out phase of oyster aquaculture on farms. After 4 and 16 weeks, oysters were subsampled for physiological and genetic parameters to test for the relative influence of carryover effects and phenotypic plasticity from the larval salinity treatments. Alongside survival, genetic diversity was assessed at each experimental stage to rule out the occurrence of large shifts in allele frequencies, or loss of diversity, in comparisons among treatments and stages that would suggest observed effects were the result of non-random survival rather than carryover effects or phenotypic plasticity.

Two salinity treatments were used – thirteen (S_{13}) and fifteen (S_{15}) – whose levels were based on environmental data provided by our commercial hatchery partner, Oyster Seed Holdings, Inc. (OSH), located on the Rappahannock River in Grimstead, VA, and retrieved from the NOAA CBIB Stingray Point buoy (Figure 1). Average ambient and frequent minimum salinity values that occurred during the hatchery's 2016 and 2017 operating seasons were chosen; due to an anomalously wet spring season in 2018 in Virginia, S_{13} served as the control treatment representative of incoming water salinity at the hatchery. The two treatment groups were cultured at the Virginia Institute of Marine Science (VIMS) in the Aquaculture Breeding and Genetics Technology Center (ABC), receiving manipulated water from the York River (see section *2.1.1 Treatment Water Generation*). To evaluate whether larval culturing at VIMS produced representative results for hatchery production, a third treatment group, referred to as hatchery (S_H), was cultured at OSH, receiving unmanipulated water from the Rappahannock River. To ensure continuity among all treatments, larval culturing practices at OSH were adopted such that all cultures received water changes, food, and sampling on the same days.

2.1.1. Treatment water generation

Water from the York River was mechanically filtered through sand, cartridge, and ultraviolet filtration systems, and finally through 1 µm mesh. After filtration, the water was chemically manipulated to create the S_{13} and S_{15} treatments at VIMS ABC while controlling for pH, alkalinity, temperature, and dissolved oxygen. Briefly, salinity was manipulated using deionized water or Instant Ocean®, and then alkalinity was adjusted through additions of 1N

NaOH or 1N HCl, following Lunden et al. (2014; see *S1.1 Treatment Water Generation*). The S_H treatment group at OSH received 1 μm mechanically filtered water from the Rappahannock River at ambient salinity.

2.2. Larval culturing

Triploid *C. virginica* larvae (two days post-fertilization) were obtained from OSH in Summer 2018. Female, diploid oysters from broodstock line LOLA (n = 33; Bushek and Allen, 1996) and male, tetraploid oysters from broodstock line GNL (n=4; Guévélou and Allen, 2016), both produced by VIMS ABC, were strip-spawned (Allen and Bushek, 1992). Mantle and/or gill tissue was subsampled from all adult parents and stored in 95% ethanol for genetic assessment. All eggs were then pooled and rinsed to remove harmful bacteria, then divided into four aliquots prior to fertilization. Each aliquot was then fertilized by sperm from one male. After fertilization was completed (~ 1 hour), embryos were pooled and rinsed to remove any remaining sperm and bacteria. Embryos were then reared at OSH for 48 hours under ambient conditions prior to transport to VIMS and the experimental start.

Beginning at 2 days-post-fertilization (dpf), larvae were cultured through metamorphosis (about 4 weeks) under the three salinity treatments (S_{13}, S_{15}, S_H) using three tanks per treatment to create biological pseudoreplicates. Culturing tanks at VIMS ABC (S_{13} and S_{15}) were 50 L, while those at OSH (S_H) were 100 L. To control for the tank size differences, larval density was standardized across treatments. Larval densities started at 15 larvae mL^{-1} and were gradually reduced to 2 larvae mL^{-1} before metamorphosis following hatchery best practices (M. Congrove, pers. comm.; Table S1). To ensure continuity among the treatments at VIMS ABC and OSH, larvae were fed daily on an *ad libitum* diet of a mixture of microalgal species (*Isochrysis galbana*, *Thalassiosira weissflogii*, and *Tetraselmis* sp.) cultured at OSH. To characterize tank conditions during the larval and settlement stages, temperature, dissolved oxygen, pH, conductivity, and salinity were measured with a YSI Pro Plus twice daily in S_{13} and S_{15} treatments and three times per week in S_H. Water samples were collected for measurements of pH and total alkalinity on water change days (see *S1*.5 *Carbonate Chemistry Analysis*).

A full water change of each larval tank was conducted three times per week to remove any build-up of nitrogenous waste in the culture water. Larval sampling occurred at the water change. Tanks were emptied onto a mesh sieve of a size appropriate for the smallest larvae observed at last measurement to conservatively capture all individuals (Table S1). Larvae were then returned to the tank or concentrated into a small volume of treatment water for sampling. Physiological sample collection occurred at 4, 8, 13, and 18 dpf for larvae in S_{13} and S_{15}. The S_H larvae at OSH were sampled twice, half-way through the larval stage at 8 dpf and before settlement at 18 dpf for comparison with S_{13} control larvae.

To assess survival, larval abundance was determined from a minimum of 3 sequential counts of 20-50 µL of the larval concentrate. Larval abundance was compared to the previous sampling timepoint to calculate percent survival. Larvae were then volumetrically sampled for growth (n ≅ 200 individuals), total protein content (n ≅ 5,000), and total antioxidant capacity (n ≅ 10,000). Once all samples were collected, larvae were returned to the culturing tank at the appropriate density (Table S1), and samples were preserved at -80°C until later analysis.

Once larval shell lengths (measured parallel to the hinge) were larger than 300 µm and showed pre-metamorphic attributes, like eyespots and foot muscle protrusion (termed eyed larvae), approximately 75,000 eyed larvae were harvested per treatment replicate and put into a downwelling set-up with the same larval treatment conditions for the settlement phase (see *S1.3 Settlement*). Prior to settlement, whole oysters from each treatment replicate (n ≅ 500 individuals) were preserved in 95% ethanol for genetic analysis. The settlement phase proceeded under treatment conditions for about a week and a half. Once adult morphological features (e.g., gills) were observed, little to no signs of pre-metamorphic features remained, and shell lengths of individuals were >700 µm, oysters were placed in common garden conditions in the ABC nursery. During common garden, oysters received unfiltered York River water in an upwelling system for about 1.5 months until they were large enough for field deployment (>5 mm; see *S1.4 Common garden)*.

2.3. Juvenile field deployment

Oysters from all treatment replicates (n = 2,000 individuals) were deployed to two experimental field sites in the Rappahannock (RR) and York (YR) rivers, tributaries of Chesapeake Bay. Prior to the deployment of oysters, the initial shell length of 50 randomly selected individuals from each treatment replicate was measured. These individuals were then sampled for total antioxidant capacity (n = 3, whole-body tissue), dry tissue weight (n = 10, whole-body tissue), and condition index (n = 10, whole individual; see *2.4 Physiological Assays*). All physiological samples were preserved at -80^{o}C until analysis.

Oysters were deployed in mesh bags inside single-tiered cages placed at the sediment surface in the intertidal zone with similar estimated levels of tidal exposure. Environmental data was collected at each site using a SeapHOx™ (V1 and V2, Sea-Bird Scientific) that recorded pH, temperature, salinity, and dissolved oxygen every 10 minutes. Carbonate chemistry samples were collected every two weeks for field validation of the SeapHOx™ (see *S1.5 Carbonate Chemistry Analysis*). Data from the Virginia Estuarine and Coastal Observing System were used to fill gaps in the SeapHOx™ time series from the YR location (CBNERR-VA VIMS, 2018). The offset between discrete water samples and SeapHOx pH was calculated for each instrument (RR: 0.14 ± 0.05, YR: 0.13 ± 0.07; mean ± standard error).

At 4 and 16 weeks, oysters were sampled at each site for shell length, survival, total antioxidant capacity, dry tissue weight, and condition index. Cumulative survival was measured using a random sub-sample of 300 individuals from each replicate. Live oysters were measured for growth and sampled for physiological measurements, as described above. Physiological samples were immediately put on dry ice, and later stored at -80^{o}C until analysis. Genetic samples were taken after 16 weeks. Fifty randomly selected oysters from each bag were transported on ice to VIMS and measured for size, and a subsample of soft tissue was preserved in 95% ethanol for later genetic workup.

2.4. Physiological assays

Total protein content of larval oysters was measured spectrophotometrically using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, 23225; Smith et al., 1985) and a SpectraMax® iD3 (Molecular Devices) microplate plate reader. Briefly, oyster samples were homogenized in Milli-Q ultrapure water (Millipore Sigma) and incubated with the working reagent at 37^{o}C for 30 minutes before the absorbance at 562 nm was measured at room temperature. Each sample from the treatment replicates was measured in triplicate. Total protein content in the oyster samples were calculated using a standard curve of known concentrations of bovine serum albumin (0-2,000 μg mL^{-1}).

Total antioxidant capacity was measured using the ferric reducing/antioxidant potential (FRAP) assay from (Griffin and Bhagooli, 2004). First, tissue samples were homogenized in 10mM Tris-HCL buffer. Homogenized samples were then mixed with a working reagent (300mM acetate buffer, 10mM 2,4,6-tripyridyl-s-triazine, and 20mM $FeCl_3 \cdot 6H_2O$) followed by a

6-minute incubation at 37°C. Each sample from the treatment replicates was measured in triplicate. The oxidizing reaction produces a colored compound whose absorbance can then be measured at 595 nm using the SpectraMax® iD3 microplate plate reader at 37°C. FRAP in oyster tissues was calculated using a standard curve of known oxidized Fe^{II-} concentrations using 20mM $FeCl_3 \cdot 6H_2O$ (0-1,000μM), and then normalized to total protein content to reduce bias from potential size differences across treatments.

To measure the shell length of larval and early juvenile oysters, a minimum of 100 randomly selected individuals were photographed using a compound microscope (Olympus BX51) or dissection microscope (Nikon SMZ1500), respectively. ImageJ (Schneider et al., 2012) was then used to measure area and longest length (hinge to bill) of 50 randomly selected oysters. Calipers were used to measure the shell length of juvenile oysters during the field deployment.

Juvenile dry tissue weight, indicative of tissue growth during field deployment, was measured in conjunction with condition index. Intact oysters were gently cleaned with a toothbrush under DI water to remove any sediment or other material and then shucked. Tissue and shells were dried individually at 60°C until a constant weight was achieved (± 0.001 g). Condition index was calculated as a ratio of the dry weight of whole-body tissue to dry weight of the shell (Rainer and Mann, 1992).

2.5. Genetic Analysis

2.5.1. DNA Isolation

It was empirically determined that DNA could be consistently isolated and amplified from individual larval oysters of 300 μm size and larger (data not included). Thus, only eyed larvae and juvenile oysters were used for genetic analysis, in addition to broodstock samples which were used to establish baseline genetic diversity. Oysters were randomly selected at the beginning of the settlement phase as eyed larvae (hereafter referred to S_{eyed}) and at the end of the field deployment as juveniles from all treatment replicates. All tissue samples from broodstock, S_{eyed}, and juvenile oysters underwent a DNA extraction protocol using Puramag™ carboxylated magnetic beads (McLab, San Francisco, CA; See *S1.6 Genetics*). DNA quantity was assessed with a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific), and isolates were diluted, if necessary, to 50 ng/µL or less prior to amplification.

2.5.2. Microsatellite Data Acquisition

To evaluate loss of genetic diversity, *C. virginica* primers targeting tri- and tetranucleotide microsatellite loci were tested and optimized for oysters from Virginia (Brown et al., 2000; Carlsson and Reece, 2007; Reece et al., 2004; Wang et al., 2009; Wang and Guo, 2007) see *S1.6 Genetics* for optimization methods). A total of 18 microsatellite loci were optimized into five multiplex groups (Table S2). Broodstock, S_{eyed}, and juvenile DNA isolates underwent multiplex PCR amplification using the Qiagen Type-It Microsatellite PCR Kit (Qiagen) with the following components: 3.25 µL Type-It Mastermix, 0.5 µL 1mg/mL BSA, 0.625 µL primer mix (consisting of 1 part locus specific forward primer modified to include a universal tail sequence, e.g., T3, to 4 parts locus specific reverse primer to 4 parts of the fluorescently labeled universal primer), 1.125 µL PCR water, and 0.5 µL template DNA. The PCR cycling protocol was as follows: 95°C initial denaturation for 5 minutes, 28 cycles of 95°C denaturation for 30 seconds, 90 seconds at the annealing temperature, 72°C extension for 30 seconds, and a final extension at 60°C for 30 minutes. Alleles were size separated on either an ABI Genetic Analyzer 3130XL (broodstock and juveniles) or 3,500 (S_{eyed}) with a GeneScan™ 600 LIZ® Size Standard v2.0 (ThermoFisher Scientific). Broodstock chromatographs were used to size alleles in GeneMarker V2.6.0 (Softgenetics, State College, PA) and establish allele panels for each locus. S_{eyed} and juvenile alleles were then sized based on the broodstock panels. All alleles were sized twice, and where a consensus could not be reached data was coded as missing.

2.6. Statistical Analyses

2.6.1. Physiological Analyses

Water conditions (temperature, salinity, dissolved oxygen, pH, total alkalinity, dissolved inorganic carbon, and aragonite saturation state) of treatments during larval and settlement stages were analyzed with *nlme* in the statistical program R (version 3.1-155; Pinheiro et al., 2020; Pinheiro and Bates, 2000) using separate linear mixed effects models with larval salinity treatment as a fixed factor and tank replicate as a random factor. All treatment water parameters satisfied the assumptions for parametric testing.

To detect direct effects of salinity conditions on larval oysters, larval oyster traits (shell growth, total protein content, total antioxidant capacity, and survival) were analyzed with *nlme* using separate ANCOVAs with larval treatment as the fixed effect and age as a continuous cofactor. The shell length of eyed larvae was analyzed with an ANOVA because age was not needed as a cofactor. Total protein content was log transformed and shell growth was square root transformed to meet assumptions of normality and homogeneity. All other larval oyster traits met the necessary statistical assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity. The Shapiro-Wilkes test was analyzed in *stats* (R Core Team, 2022), and the Levine's test was conducted with *car* (Fox and Weisberg, 2019).

Water conditions (temperature, salinity, dissolved oxygen, pH, total alkalinity, dissolved inorganic carbon, and calcite saturation state) from the juvenile field deployment were compared between field locations using a Kruskal-Wallis test (*stats*; R Core Team, 2022) because parameters did not meet the assumptions of normality and homogeneity of the Shapiro-Wilkes and Levine's tests, respectively.

To assess initial signs of carryover effects from the larval salinity experience or phenotypic plasticity, oyster traits measured after the completion of the settlement phase (shell length, total protein content, and total antioxidant capacity) were analyzed using an independent two-sample student's t-test between S_{13} and S_{15} (*stats*; R Core Team, 2022). Normality and homogeneity assumptions of the Shapiro-Wilkes and Levine's tests, respectively, were met (Fox and Weisberg, 2019; R Core Team, 2022).

To further observe potential carryover effects of larval salinity experience or phenotypic plasticity, as well as shifts in relative influence of carryover effects in different environmental conditions, juvenile oyster traits (shell growth, dry tissue weight, total antioxidant capacity, condition index and survival) were analyzed with *nlme* using separate ANCOVAs with larval salinity treatments and experimental field sites as fixed effects and time as a continuous cofactor. Dry tissue weight was natural log transformed to meet assumptions of normality and homogeneity. All other juvenile oyster traits met the necessary statistical assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity (Fox and Weisberg, 2019; R Core Team, 2022). All statistical analyses were completed in R (version 4.1.3; R Core Team, 2022).

2.6.2. Genetic Analysis

Alleles at microsatellite loci that were not able to be scored consistently, or that were present in offspring but not detected in the broodstock genotypes, were excluded from further analyses. Any individual oyster or microsatellite locus with more than 25% missing data was excluded from further analyses. The remaining data was converted to a Genepop file format

(Raymond and Rousset, 1995; Rousset, 2008). Oysters were grouped by sampling event (broodstock, S_{eyed}, juveniles), treatment (S_{13}, S_{15}), and field deployment location (YR, RR), with each unique combination of these three factors constituting a sample. Data from the three replicates for each of these samples was merged. To correct for unknown dosage in the triploid and tetraploid oysters (e.g., AAB vs. ABB for triploids), which makes accurate estimation of allele frequencies difficult, the dosage correction tool implemented in GenoDive v3.0 (Meirmans, 2020) was used to impute all missing data based on sample-by-sample allele frequencies. Genetic diversity indices including number of alleles, allele frequencies, observed (H_O) and expected heterozygosity (H_S), and inbreeding coefficients (G_{IS}) were calculated for each sample in GenoDive (Meirmans, 2020). GenoDive was also used to compute pairwise F_{ST} values and significance was assessed based on 9,999 iterations of the data using an initial α = 0.05. P-values were corrected for the false-discovery rate (FDR; Benjamini and Hochberg, 1995). The Genepop software (Raymond and Rousset, 1995; Rousset, 2008) was used to identify significant differences in genic (allelic) frequencies among samples on a locus-by-locus basis using the exact G test, and significance was assessed with 100 batches of 1,000 Markov Chain Monte Carlo (MCMC) iterations each following a burn-in of 1,000 iterations. Genepop takes only diploid data, so allele frequencies were used to convert triploid to diploid data prior to calculations. Significant locus-by-locus comparisons (<0.05) were summed for each pairwise comparison. To identify samples with rare alleles, a private alleles analysis was completed in Genepop.

3. Results

3.1. Water conditions

3.1.1. Larval, settlement, and nursery phases

Average larval culture water conditions were significantly different between laboratory treatment groups for many parameters measured (Tables 1, S3). Salinity differed by 1.77 units, dissolved oxygen differed by 0.2 mg L^{-1}, total alkalinity differed by 29 µmol kg^{-1}, and dissolved inorganic carbon differed by 35 µmol kg^{-1} (Table 1). All other water characteristics were not statistically different between treatments. Due to the anomalously wet spring and lack of control over culture conditions at OSH, water conditions tended to differ between S_{13} and S_H (Table 1). Specifically, salinity was lower, while temperature, pH, and aragonite saturation state were all higher in the S_H treatment than S_{13} (Table 1).

During the settlement phase, except for salinity (S_{13} = 13.20 ± 0.02, S_{15} = 15.00 ± 0.02; mean ± standard error) and dissolved oxygen (S_{13} = 7.28 ± 0.07, S_{15} = 7.09 ± 0.05; mean ± standard error), average water conditions were not significantly different between laboratory treatment groups (Tables S4, S5). Oysters experienced temperatures of ~ 25°C, pH values of ~ 7.8, total alkalinities of ~1510 µmol kg^{-1}, and calcite saturation states of ~1.25 during the settlement phase (Table S4). After the settlement phase was completed, oysters experienced temperatures of ~ 28°C, salinities of ~15.2, pH values of ~ 7.6, total alkalinities of ~1410 µmol kg^{-1}, and calcite saturation states of ~1.07 in common garden during the nursery phase (Table S6).

3.1.2. Juvenile field deployment

Oysters experienced significantly different water conditions between the two field sites over the deployment period (Table 2, S7). On average, salinity was higher by 5.29 units, pH was lower by 0.17 units, total alkalinity was greater by 184 µmol kg^{-1}, and dissolved inorganic carbon was greater by 188 µmol kg^{-1} in the YR than RR. Though not significantly different, temperature was greater by 0.95°C in the YR. Calcite saturation state and dissolved oxygen were similar between the two locations.

3.2. Comparisons of allele frequencies based on the larval environment

Three loci and 35 individuals were dropped from the final dataset due to missing data, resulting in a final dataset of 15 loci, 37 broodstock individuals, 112 eyed larvae, and 183 juveniles. Broodstock genotypes consisted of 102 alleles across the 15 microsatellite loci (Table S8). Alleles per locus ranged from three (Cvi2k14, RUCV80, RUCV109, RUCV159) to 15 (Cvi13). A private alleles analysis revealed five private alleles across five different loci, all of which were unique to the broodstock. Overall, 21 and 18 broodstock alleles were not recovered in the first post-fertilization analysis of the offspring ($S_{13\text{-eyed}}$ and $S_{15\text{-eyed}}$, respectively), though many of these alleles were detected in juvenile samples at the end of the experiment at a lower frequency relative to the broodstock (Table 3). The number of alleles recovered when samples were separated by life stage and salinity treatment ranged from 81-88 and when salinity treatments were combined across sampling events, the number of alleles ranged from 90-94 (Table 3).

Observed and expected heterozygosity (H_O and H_S, respectively) for the microsatellite data for all samples after dosage correction are displayed in Table 3. Observed heterozygosity (H_o) values were lower than expected heterozygosity (H_s) values in all samples. Values of G_{IS} were positive in all samples and values in sampling event by treatment groups ranged from 0.203 in $S_{13\text{-eyed}}$ to 0.144 in RR_{15}. The highest number of significant locus-by-locus genic differences (9 of 11 significant differences) and the highest F_{ST} values (0.033-0.047; Table S9) involved comparisons between the broodstock and all offspring samples. Genic differences across all loci were significant between all sample pairs except RR_{13} and YR_{15}, with a p-value of 0.070 (results not presented).

Pairwise F_{ST} values between S_{eyed} treatments and the corresponding juvenile samples were all statistically significant with F_{ST} values around 0.01 and 4-6 significant genic (locus) differences (Table 4, S10). Pairwise F_{ST} comparisons of juvenile field deployment location by salinity treatment groups resulted in estimates that were extremely small, but were in most cases statistically significant, except for the comparisons RR_{15} vs. YR_{13} and RR_{13} vs. YR_{15} (Table 5). All cross-treatment comparisons produced F_{ST} values of <0.01 and between 2 and 4 significant genic differences.

Within salinity treatments, juvenile samples placed in the two field locations (RR and YR) retained similar numbers of alleles, with both S_{13} juvenile samples having 88 alleles at the end of the experiment, and with the S_{15} samples having 81 (RR) and 85 (YR) alleles (Tables 3, S11). The pairwise comparison of RR_{13} and YR_{13} was statistically significant, as was the comparison of RR_{15} and YR_{15} and the number of significant genic differences were 3 and 5, respectively (Table 5). F_{ST} values and counts of significant genic differences were on a similar level to cross-treatment comparisons within the same field sites.

3.3. Carryover and direct effects of larval environment on oyster physiology

Throughout the juvenile field deployment when carryover effects were assessed, juvenile physiology was similar among larval treatment groups (Table 6). Specifically, survival, shell length, dry tissue weight, total antioxidant capacity, and condition index did not significantly differ based on what salinity oysters experienced as larvae (Table 6).

In general, larval cultures raised under ecologically relevant salinities (Figure 1) were physiologically similar, with no direct effects of salinity detected; however, the hatchery comparison treatment (S_H) tended to differ physiologically from S_{13} (Figure S1). Shell length, total protein content, and total antioxidant capacity did not differ between larvae reared in the S_{13} and S_{15} treatments (Figure 3, Table 6). Survival remained above 75% throughout larval development and did not vary significantly by salinity treatment or larval age (Table 6). Larval age had a significant effect on shell length, total protein content, and total antioxidant capacity (Table 6). Both shell length and total protein content increased from four to eighteen dpf by 220% and 561%, respectively (Figure 3a-b). Total antioxidant capacity decreased 62% in larval oysters between 4 and 18 dpf (Figure 3c). Prior to settlement, the shell length of eyed larvae was also similar between treatments (Figure S2, Table 6).

Salinity did not directly affect oysters during the settlement phase. After the settlement phase was completed, there were no significant differences in shell length, total protein content, and total antioxidant capacity between treatment groups (Figure S3, Table S12).

3.4. Phenotypic plasticity in response to juvenile environments

During the juvenile stage when oysters were deployed in the York and Rappahannock Rivers, physiology of the oysters differed significantly by site and time (Table 6). Specifically, condition index, dry tissue weight, and shell length all changed significantly over time, increasing by 11%, 102%, and 20%, respectively, over four months while oysters were deployed in the York and Rappahannock Rivers (Figure 4b,c,e). RR oysters had greater survival (13% difference), shell length (14% difference), condition index (38% difference), and dry tissue weight (78% difference) than the YR oysters at the end of deployment (Figure 4a-d). RR oysters also had lower total antioxidant capacity than YR oysters by 20% (Figure 4e).

4. Discussion

Oysters encounter different water conditions during hatchery and grow-out phases of aquaculture that could impact performance. Phenotypic plasticity likely played a more prominent role than carryover effects in shaping juvenile *C. virginica* performance after individuals experienced typical differences in hatchery salinity conditions (<=2 units). Oysters are also likely to encounter different environmental conditions on farms compared to those experienced in the hatchery, potentially changing the relative influence of the two mechanisms and impacting performance. Juvenile oyster physiology varied with field deployment location (i.e., varied water conditions), not with previous larval experience based on a typical salinity difference of two (Figure 4, Table 6). Genetic diversity also remained high throughout the study, with no large shifts in allelic frequencies (Tables 3 – 5). Conserving physiological function along with genetic diversity across various life stages is likely important to the success of oyster performance in dynamic coastal environments, a finding supported by the results of previous studies (Jones et al., 2019; Modak et al., 2021; Sirovy et al., 2021).

Phenotypic plasticity likely sustained oyster performance during the larval stage in the hatchery and in juveniles during field deployment. Oyster physiology was similar between treatment groups (S_{13}, S_{15}) during the larval stage and within sites at deployment where oysters experienced different water conditions to those in the hatchery (Table 1, 2). Allelic differences were also highly random within each treatment (Figure 2). Plasticity is a known mechanism of environmental tolerance utilized by organisms living within highly variable environments, such as Eastern oysters (Hofmann and Todgham, 2010; Seebacher et al., 2015). Other studies confirm Eastern oysters maintain high plasticity in response to various environments and conditions (Chapman et al., 2011; Eierman and Hare, 2016). One characteristic of highly plastic organisms is their ability to respond relatively quickly to changes in environmental conditions, which was observed in juvenile oysters within weeks of field deployment under different environments (present study) and in transplant experiments with adult oysters (Eierman and Hare, 2016; Sirovy et al., 2021). Furthermore, changes in the gene expression of Eastern oysters, a mechanism of rapid physiological response, are mainly influenced by the current environmental conditions, not parentage or previous life experiences (Barbosa et al., 2022; Sirovy et al., 2021).

Typical salinity differences between larval cohorts likely do not impact oyster performance after the larval stage, as there was no evidence of carryover effects after the settlement phase (i.e., time inclusive of both settlement and metamorphic processes) or during field deployment. Within experimental field deployment locations in the Rappahannock and York Rivers, juvenile oysters performed similarly with respect to physiological measurements of survival, shell length, condition index, dry tissue weight, and total antioxidant capacity regardless of their larval experience (Figure 4, Table 6). The small difference in salinity between treatments (2 units) may not have directly produced any physiological differences in the larvae; alternatively, the larvae may have acclimatized to the salinity treatments, removing any initial physiological divergence. Juvenile oysters whose larvae were reared at the commercial hatchery (S_H) were physiologically similar to individuals in the S_{13} treatment during field deployment, despite differences at the larval stage where salinity differed by 1 unit. S_H larvae had less total protein content and total antioxidant capacity than those in the S_{13} treatment prior to the settlement phase (Figure S1), likely due to issues with feeding and other uncontrollable environmental differences with hatchery conditions. By the start of the field deployment within each location, the individuals raised in the S_H treatment were physiologically similar to individuals reared in not only S_{13}, but also S_{15} (unpublished data). The lack of physiological differences among larval treatments at the juvenile stage likely indicates that carryover effects are not a main driver of juvenile performance when larval oysters experience typical differences in salinity in the hatchery.

Hatchery culturing conditions likely did not affect the genetic makeup of the animals used in this study beyond the initial spawning event. Allelic diversity declined significantly from the broodstock to offspring (Tables S9 and S11), which has been documented previously in both *C. gigas* and *C. virginica* (Appleyard and Ward, 2006; Hughes et al., 2019; Varney and Wilbur, 2020). On average, survival in oyster culturing after the settlement period is around 10-20% (VIMS ABC, pers. comm.; Ernande et al., 2003; Plough and Hedgecock, 2011), and selective mortality occurring prior to, or during, metamorphosis has been linked to deleterious alleles (Plough, 2018; Plough and Hedgecock, 2011). There was no evidence of differential survival or large shifts in allele frequencies among juvenile oysters after the settlement or field deployment phases; therefore, it is unlikely that selection events related to previous environmental experience occurred (Tables 3 – 6). Percent survival after the settlement phase in the current study was consistent with survival in previous studies (~ 30%) with no obvious change in allelic diversity that would support the occurrence of selective mortality since allelic differentiation values remained similar to those prior to settlement (Table S9). In addition, survival was similar between larval salinity treatments throughout the study (Table 6), including individuals within the S_H treatment, further suggesting that survival was likely not genotype-dependent (Tables 3-5, S10, S11).

The relatively low number of samples and loci assayed in this study likely made it difficult to assess non-random genetic selection. Several rare alleles were found within the broodstock used for this study at frequencies below 3.7% and were not detected during the larval stage. The absence of detection was possibly due to a lack of inheritance or equal genetic contribution from the parents (Table S8), which was supported by the lower observed heterozygosity (H_o) values compared to expected values (H_s) in all samples (Table 3), as is common in oysters (Bernatchez et al., 2019, Reece et al., 2004). Alternatively, rare alleles are more likely to be lost in bottlenecking events (Maruyama and Fuerst, 1985), such as the population reductions that occur during the culturing process (e.g., culture density decreases), or they may remain in the population but not be observed due to the limited random sampling of individuals for genetic analysis. In some cases, broodstock alleles that were not observed during the larval stage were recovered in the juvenile samples, supporting the contention of sampling error. Furthermore, the aquaculture oysters produced in Virginia are mostly triploids, making genetic analysis more complex and the results more difficult to interpret as unknown dosage of biallelic heterozygotes may bias results and many analyses assume haploid or diploid data and are not optimized for triploids.

Carryover effects and selective mortality may play a larger role in shaping oyster performance when environmental differences among larval cohorts or between hatchery and grow-out environments is greater in magnitude than tested in the present study. In previous work on carryover effects, the differences between control and treatment conditions were greater than those used in this study, like a difference of 0.4 pH units on the logarithmic scale (Donelan et al., 2021; Hettinger et al., 2013, 2012; Talmage and Gobler, 2011; Torres et al., 2016). Carryover effects of low salinity experience have been found to negatively affect post-metamorphic survival and growth in polychaetes, *Capitella* sp. I (Pechenik, 2006, Pechenik et al., 2001). Though several treatment conditions were separated by two to three salinity units, the greatest change from spawning salinity to treatment condition was twenty salinity units (Pechenik et al., 2001). Furthermore, pronounced genetic selection events have been found to occur in oysters under more stressful conditions than implemented in this study (Plough, 2012). With increasing springtime precipitation projected for the mid-Atlantic region under climate change scenarios (Muhling et al., 2018; Walsh et al., 2014), weekly salinity differences may increase in magnitude in the future, potentially impacting larval physiology and genetics between hatchery-reared larval cohorts through carryover effects and selective mortality, an area for future investigation.

Carryover effects and selective mortality may emerge as more important drivers of performance when stressful conditions align with sensitivity periods in organismal development. In non-brooding oysters and other bivalve species, the most vulnerable life stage is the larval period, and within that stage, the first 48 hours after fertilization is considered the most sensitive and susceptible portion (Barton et al., 2012; Gray et al., 2022). During those first 48 hours, larvae are actively precipitating their initial shell, which is energetically expensive and leaves them more exposed to environmental conditions due to its greater surface area (Waldbusser et al., 2013). Furthermore, larvae are utilizing finite, endogenous energy reserves during this time when they are developing their feeding structures (Waldbusser et al., 2015). Our larval exposures started at 48 hours post fertilization based on logistics with the commercial hatchery partner, which may have precluded observations of carryover effects. Many studies aiming to observe carryover effects in broadcast spawning oysters and other mollusk species began treatment exposure within hours of fertilization (Clark and Gobler, 2016; Mackenzie et al., 2022; Parker et al., 2015; Shen et al., 2022; Talmage and Gobler, 2011, 2009). Experience of stressful environmental conditions during the first 48 hours after fertilization can have negative effects on the rest of the larval stage (Ragg et al., 2019; White et al., 2013). Larvae during the first 48 hours post-fertilization may be more sensitive to minute changes in environmental salinities, and therefore typical salinity differences like those employed in the current study may induce physiological or genetic differences between larval cohorts if experienced within the first 48 hours post-fertilization. An important consideration in future studies is the alignment between the timing of organismal development and exposure to various environmental conditions to better understand potential impacts to marine bivalve populations and aquaculture production.

As no carryover effects were observed, the environmental conditions experienced by juvenile oysters played an important role in their survival and growth. Environmental conditions differed between field deployment locations, potentially influencing juvenile oyster responses, though the present study was not designed to attribute differences in oyster performance to particular *in situ* water parameters. Salinity, total alkalinity, and dissolved inorganic carbon were all lower at the Rappahannock River site compared to the York River site and correlated with better oyster performance (Figure 4, Tables 2). Oysters commonly exhibit lower filtration rates with exposure to lower salinities (Casas et al., 2018b; McFarland et al., 2022; Méthé et al., 2015b); however, there is evidence that oysters grown in the Rappahannock River have higher filtration rates compared to those in the York River despite lower average salinities (Mizuta et al., 2021). Though increased filtration rates can be a sign of stress under certain conditions, they can also lead to greater consumption of phytoplankton and particulate organic matter. While chlorophyll-a concentrations and phytoplankton communities were not assessed here, the field site in the Rappahannock River may have had greater algal concentrations or algal diversity than that of the York River during our study period, leading to greater performance of oysters in the Rappahannock River. Similarly, other water conditions that were not measured in the present study, like residence time and currents, which both impact algal concentrations for feeding

(Campbell and Hall, 2019), may also have contributed to differences in oyster performance during the juvenile phase.

In the context of shellfish aquaculture operating in mesohaline environments, typical salinity differences experienced between larval cohorts after 48 hours of fertilization will not likely hinder the production of oyster seed or the performance of the oysters once transitioned to more saline farm environments. The consensus between this study and previous work that oysters maintain their physiology after experiencing shifts in environmental conditions highlights the importance of phenotypic plasticity to the performance of these sessile, ectothermic organisms in variable environments, with implications for aquaculture production both in hatcheries with weekly larval cohorts and in the transition from hatchery to farms for grow-out. The important role of phenotypic plasticity is another reason why farm site selection is essential for the performance and success of oyster product since the physiology of juvenile oysters was responsive to their *in situ* environment and not influenced by larval experiences. Neither carryover effects nor large shifts in allelic frequencies were detected. Other interactions between impacts of *in situ* environmental conditions on juvenile oyster performance and current hatchery practices for larval rearing could be important for aquaculture production. To further understand the relative importance of phenotypic plasticity, carryover effects, and selective mortality within oyster aquaculture, investigations should focus on identifying whether larval responses to salinity conditions are dependent on other water conditions, like temperature, or exposure to different water conditions within the first 48 hours after fertilization.

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Tables

Table 1. *Average water chemistry conditions experienced by larvae in salinity treatment groups. Values represent mean ± standard error calculated from treatment replicates over the entire larval period. Temperature, salinity, dissolved oxygen, pH_{tot} (pH, total scale), and total alkalinity are measured values. DIC (dissolved inorganic carbon), $\Omega_{calcite}$ (saturation state of calcite), and $\Omega_{aragonite}$ (saturation state of aragonite) are calculated values. Asterisks (*) indicate significant differences between water properties of laboratory treatments ($p<0.05$; linear mixed effects model; Table S2).*

Water Properties	Treatments		
	Laboratory		
	Thirteen (S_{13})	Fifteen (S_{15})	Hatchery (S_H)
<u>Larval Culture</u>			
Temperature (°C)	25.20 ± 0.08	25.07 ± 0.09	27.41 ± 0.17
Salinity	13.06 ± 0.01*	14.83 ± 0.02*	11.80 ± 0.05
Dissolved Oxygen (mg L^{-1})	7.03 ± 0.05*	6.83 ± 0.05*	6.70 ± 0.07
pH_{tot}	7.56 ± 0.01	7.51 ± 0.02	7.74 ± 0.01
Total Alkalinity (µmol kg^{-1})	1418 ± 4*	1447 ± <1*	1446 ± <1
DIC (µmol kg^{-1})	1413 ± 5*	1448 ± 3*	1412 ± 1
$\Omega_{aragonite}$	0.51 ± 0.01	0.49 ± 0.02	0.76 ± 0.01

Table 2. *Average water chemistry conditions during the field deployment period for juvenile oysters. Field deployment conditions are represented by mean ± standard error calculated over the entire field deployment period (four months) in the York and Rappahannock Rivers. Temperature, salinity, dissolved oxygen, pH_{tot} (pH, total scale), and total alkalinity are measured values. DIC (dissolved inorganic carbon), $\Omega_{calcite}$ (saturation state of calcite), and $\Omega_{aragonite}$ (saturation state of aragonite) are calculated values. Asterisks (*) indicate significant differences between water properties of field sites ($p<0.05$; Kruskal-Wallis; Table S2).*

	Field deployment	
	York River (YR)	Rappahannock River (RR)
Temperature (°C)	18.48 ± 0.07	17.53 ± 0.08
Salinity	14.43 ± 0.01*	9.14 ± 0.01*
Dissolved Oxygen (mg L^{-1})	6.52 ± 0.01	6.31 ±0.01
pH_{tot}	7.74 ± 0.01*	7.91 ± 0.01*
Total Alkalinity (µmol kg^{-1})	1491 ± 1*	1307 ± 1*
DIC (µmol kg^{-1})	1464 ± 1*	1276 ± 1*
$\Omega_{calcite}$	1.11 ± 0.01	1.12 ± 0.01

Table 3. *Indices of genetic diversity, including sample size (n), total alleles, observed heterozygosity (H_O), expected heterozygosity (H_S), and inbreeding coefficient (G_{IS}) calculated from 15 microsatellite loci with dosage correction. Results are listed for each individual sample (sampling event by treatment), each sampling event (pre-metamorphosis (S_{eyed}), and Rappahannock River (RR) and York River (YR) juvenile outplant locations), each salinity treatment (salinity of 13 (S_{13}) and salinity of 15 (S_{15})), and for all samples together (Total; with 95% confidence intervals).*

Population	n	Total Alleles	H_O	H_S	G_{IS}
$S_{13\text{-eyed}}$	53	81	0.445	0.558	0.203
$S_{15\text{-eyed}}$	59	84	0.464	0.556	0.164
RR_{13}	45	88	0.467	0.557	0.162
RR_{15}	47	81	0.475	0.555	0.144
YR_{13}	48	88	0.458	0.552	0.170
YR_{15}	43	85	0.453	0.540	0.162
S_{eyed}	112	90	0.431	0.549	0.215
RR	92	91	0.454	0.573	0.207
YR	91	94	0.440	0.561	0.216
S_{13}	146	94	0.456	0.559	0.185
S_{15}	149	95	0.464	0.554	0.162
Total	295	102	0.442	0.561	0.212
CI 2.5%	-	-	0.353	0.460	0.099
CI 97.5%	-	-	0.523	0.659	0.327

Table 4. *Pairwise comparisons between larvae and juveniles within the same salinity treatments, including F_{ST}'s calculated from 15 microsatellite loci, with comparisons with significant FDR-corrected p-values bolded, and the sum of significant locus-by-locus genic (allelic) differentiation tests out of 15 total loci. Samples are juveniles deployed in either the Rappahannock River (RR) or York River (YR), and pre-metamorphic larvae (S_{eyed}), with the subscript indicating the salinity treatment (13 or 15).*

	F_{ST}	Genic Differentiation
$S_{13\text{-eyed}}$ - RR_{13}	**0.008**	5
$S_{13\text{-eyed}}$ - YR_{13}	**0.012**	6
$S_{15\text{-eyed}}$ – RR_{15}	**0.010**	6
$S_{15\text{-eyed}}$ - YR_{15}	**0.007**	4

Table 5. *Pairwise comparisons between salinity treatments in juvenile samples, with F_{ST}'s calculated from 15 microsatellite loci on the upper diagonal, with comparisons with significant FDR-corrected p-values bolded, and the sum of significant locus-by-locus genic (allelic) differentiation tests out of 15 total loci on the lower diagonal. Samples are juveniles deployed in either the Rappahannock River (RR) or York River (YR), with the subscript indicating the salinity treatment (13 or 15).*

	RR_{13}	YR_{13}	RR_{15}	YR_{15}
RR_{13}	--	**0.008**	**0.008**	0.002
YR_{13}	3	--	0.001	**0.008**
RR_{15}	2	3	--	**0.007**
YR_{15}	4	4	5	--

Table 6. *Mixed effects linear model results on the comparison of larval and juvenile physiological metrics, with age and time as covariates, respectively. Tank was a random factor for larval comparisons. Asterisks indicate statistical significance below an alpha threshold of 0.05. Under each response variable are the independent variables of interest in italics.*

	Statistical Value	P value
Larval Stage		
Survival		
Age	$F_{(1,39)} = 1.057$	0.310
Treatment	$F_{(1,39)} = 0.764$	0.388
Shell Length		
Age	$F_{(1,39)} = 4945$	<0.001*
Treatment	$F_{(1,39)} = 2.232$	0.143
Eyed Larval Shell		
Eyed Stage	$F_{(1,8)} = 266$	<0.001*
Treatment	$F_{(1,8)} = 1.457$	0.262
Total Protein Content		
Age	$F_{(1,21)} = 826$	<0.001*
Treatment	$F_{(1,21)} = 0.0002$	0.988
Total Antioxidant Capacity		
Age	$F_{(1,21)} = 19.80$	<0.001*
Treatment	$F_{(1,21)} = 1.199$	0.287
Juvenile Stage		
Survival		
Time	$F_{(1,20)} = 0.631$	0.436
Site	$F_{(1,20)} = 51.76$	<0.001*
Treatment	$F_{(1,20)} = 0.046$	0.833
Condition Index		
Time	$F_{(1,20)} = 4.908$	0.039*
Site	$F_{(1,20)} = 53.30$	<0.001*
Treatment	$F_{(1,20)} = 0.680$	0.419
Shell Length		
Time	$F_{(1,20)} = 29.02$	<0.001*
Site	$F_{(1,20)} = 17.94$	<0.001*
Treatment	$F_{(1,20)} = 3.606$	0.072
Dry Tissue Weight		
Time	$F_{(1,20)} = 29.78$	<0.001*
Site	$F_{(1,20)} = 22.51$	0.001*
Treatment	$F_{(1,20)} = 3.856$	0.064
Total Antioxidant Capacity		
Time	$F_{(1,20)} = 0.482$	0.495
Site	$F_{(1,20)} = 8.134$	0.009*
Treatment	$F_{(1,20)} = 0.439$	0.515

Figures

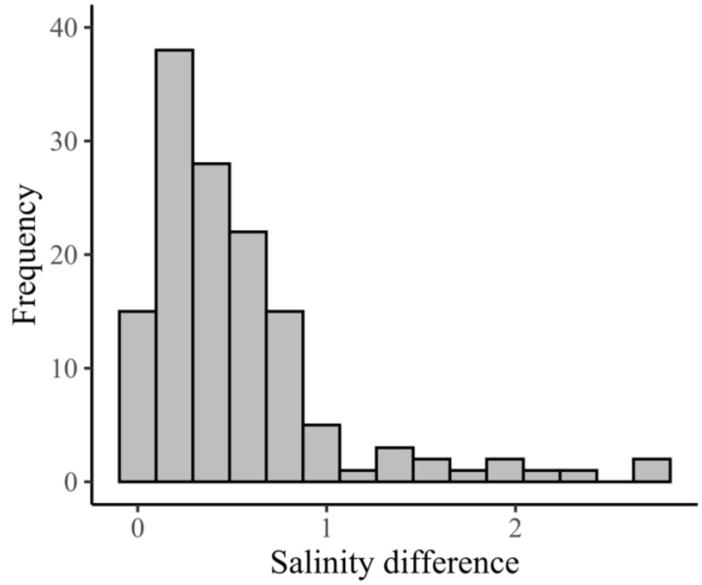

Figure 1. *Frequency of differences in average salinity (7 days) between consecutive weeks during the hatchery season (March - September). Salinity data are from National Oceanic and Atmospheric Administration (NOAA)'s Chesapeake Bay Interpretive Buoy System (CBIB)'s Stingray Point buoy at the mouth of the Rappahannock River, Virginia from 2015 – 2018 because of its proximity to our commercial hatchery partner (https://buoybay.noaa.gov/locations/stingray-point).*

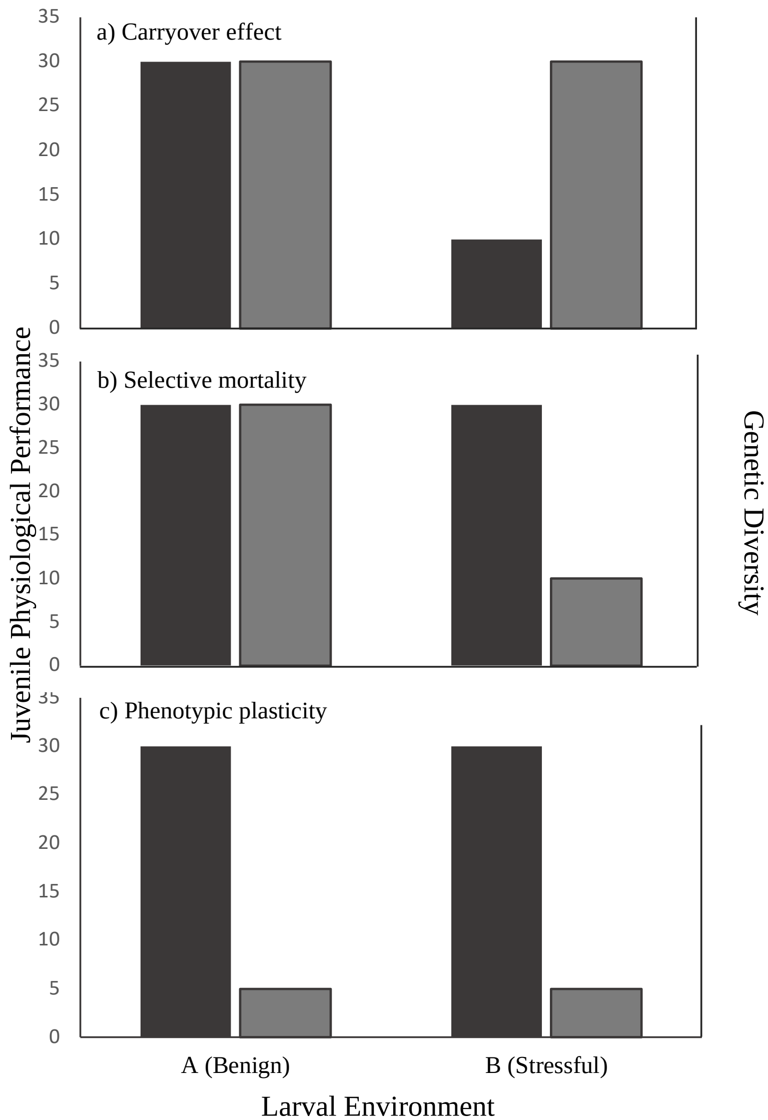

Figure 2. *Hypothesized patterns of physiological performance (black) and non-random loss of genetic diversity (gray) of juvenile oysters in common-garden environmental conditions as a function of their larval environment (Environment A, Environment B) and environmental-tolerance mechanism. Carryover effects (a) would not affect the genetic diversity but would decrease physiological responses in juvenile oysters that experienced stressful environmental conditions as larvae. Selective mortality (b) would increase the non-random loss of genetic diversity in oyster larvae experiencing stress and remain low into the juvenile stage with no effect on juvenile physiology. Lastly, phenotypic plasticity (c) would cause juvenile genetic diversity and physiology to be similar regardless of larval experience. While binary responses were shown in the graphs above for clarity, it is likely that more than one mechanism may be simultaneously driving organismal performance. In this theoretical example, high physiological performance represents a positive indicator of oyster health.*

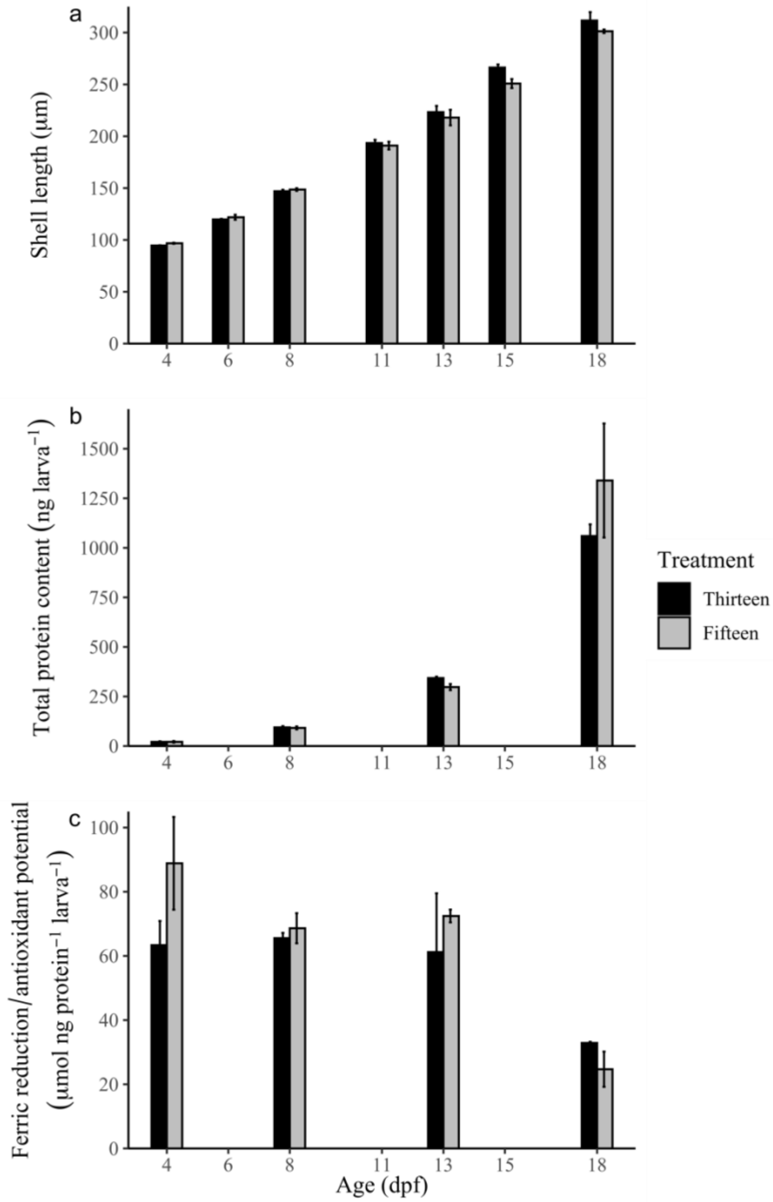

Figure 3. *Physiology of oyster larvae cultured under salinities of thirteen (black) and fifteen (gray). Larvae began to show signs of metamorphosis at 18 days post fertilization. Shell length (a), measured perpendicular from hinge to edge of larval shell, total protein content (b), and total antioxidant concentration (c) were similar among larvae raised at the different salinities. Bars represent mean ± standard error.*

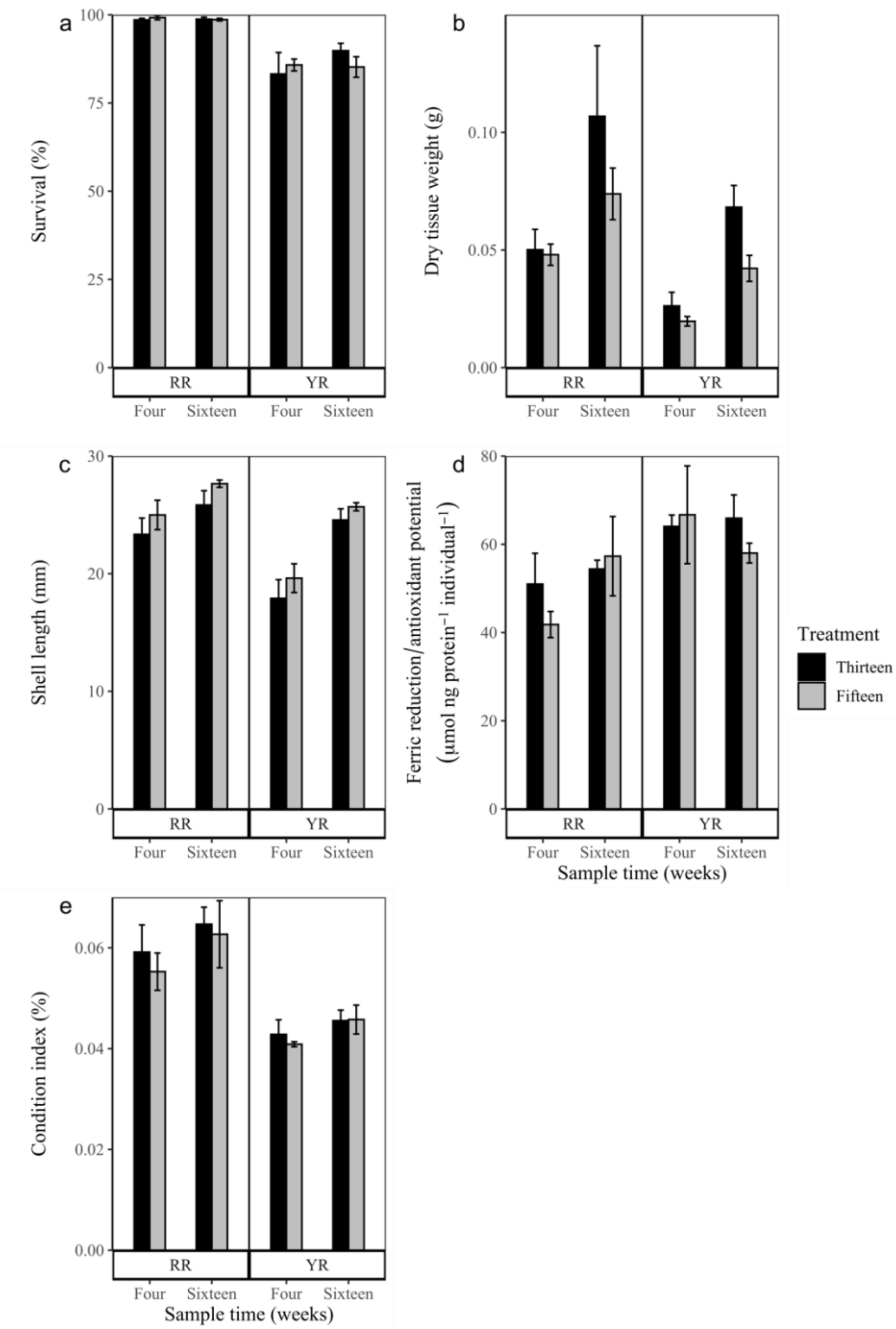

Figure 4. *Effect of* in situ *environmental conditions and previous larval salinity experience on the physiology of juvenile oysters. Oysters were deployed in the York (YR) and Rappahannock Rivers (RR) for four months after the common garden period. Overall, the juvenile environment had a significant effect on oyster physiology compared to larval salinity experience. Specifically, RR oysters had increased survival (a), greater dry tissue weight (b), larger shell length (c), measured from hinge to edge of shell, lower total antioxidant capacity (d), and greater condition index (e), measured as the weight of whole-body tissue over the shell, than YR oysters. Oysters that experienced a salinity of thirteen (black) during the larval stage were similar to those raised in a salinity of fifteen (gray) across all physiological metrics and juvenile environmental conditions. Bars represent mean ± standard error.*

Supplemental Material

S1. Material and methods

S1.1 Treatment water generation

Treatment water preparation started 36 hours prior to utilization. Salinity of the incoming water at VIMS ABC was measured using a YSI Pro Plus to calculate the amount of deionized water (DI) or Instant Ocean® (IO) needed. Dilution was calculated using equations (1) and (2), where V_{IN} refers to the volume of the incoming water, and V_{DI} is the volume of deionized water needed to achieve the treatment salinity value.

$$V_{IN} = \frac{(200\ L)(treatment\ salinity)}{(incoming\ salinity)} \qquad (1)$$

$$V_{DI} = 200\ L - V_{IN} \qquad (2)$$

To calculate the grams of IO needed to increase the salinity of the incoming water, equation (3) was used where S_{IN} is the salinity of the incoming water and S_T is the salinity of the treatment. After the salinity was manipulated, the water was bubbled with atmospheric air overnight.

$$IO\ g = 200\ L\ (S_T - S_{IN}) \qquad (3)$$

The following morning, the salinity of each batch of treatment water was measured with the YSI Pro Plus, and water samples were taken for same-day measurements of total alkalinity (see *S1.4 Carbonate Chemistry Analysis*). Because total alkalinity increased from the addition of IO or decreased with addition of DI, chemical manipulations were used to bring the manipulated treatments at VIMS ABC to that of S_H (Lunden et al. 2014). Equation (4) was used to calculate the volumes of NaOH or HCl needed to increase or decrease total alkalinity, respectively, based on the molarity (N) of each solution. TA_I and TA_f refer to the total alkalinity of the incoming water and salinity-adjusted water.

$$V_{HCl/NaOH} = \frac{m_{sw}\left(TA_i - TA_f\right)}{N_{HCl/NaOH}} \qquad (4)$$

Mass of the seawater (m_{sw}) was determined using a temperature-salinity (T-S) diagram to calculate the density of the treatment water, which was then multiplied by total volume of the treatment water, 200 L, to get the mass. The specified volumes of HCl and NaOH were then added to the respective treatments and bubbled overnight to equilibrate the pH of the treatment water with that of the atmosphere.

S1.2 Larval Culturing

Table S1. *Larval density and sieve size schedule utilized by OSH during the culturing process.*

Larval Age (days post fertilization)	Larval Density (larvae mL^{-1})	Sieve Screen Size (μm)
0	30	48
2	15	48
4	15	60
6	10	80
8	10	100
10	8	125
12	6	150
14	4	175
16	2	190

S1.3 Settlement

When 10% or more of the larvae were larger than 300 μm and showed pre-metamorphic attributes, like eyespots and foot muscle protrusion (termed eyed larvae, S_{eyed}), each culture was gently poured through two sieves: one designed to harvest larvae ready for settlement (225 μm), and one to catch the rest. Larval density was determined as above for concentrates from both sieves. Afterwards, larvae that did not catch on the harvesting screen were rinsed back into the larval tank. S_{eyed} were sampled for growth, as above, total protein content (n = 1,000 individuals), and total antioxidant capacity (n = 5,000). S_{eyed} were also sampled for genetics (n = 250 per sample), preserved in 95% ethanol, and then stored at 4°C until DNA isolation. Remaining S_{eyed} were held in a moist cloth at 4 °C. This procedure was repeated over the next three days or until the remaining culture had eyespots. Once all S_{eyed} were harvested, they were pooled within each treatment replicate. Approximately 75,000 S_{eyed} from the pool were placed in a downwelling system in their respective culture tank.

The downwelling system consisted of a 150 μm sieve containing cultch (finely ground oyster shell) that was suspended at the surface of the water. Attached to the outside of the sieve was an air lift (PVC pipe plumbed with lab air) that gently pumped water onto the sieve. Settlement proceeded under treatment conditions for about a week and a half, when adult morphological features, like gills, with little to no signs of eyespots or foot-muscles, were observed, and shell lengths were mostly greater than 700 μm. Water changes, tank cleaning, feeding, and monitoring of tank conditions were conducted as previously described (see *2.2. Larval Culturing*). After the settlement phase was completed, S_H oysters at OSH were brought back to VIMS ABC. Settlement success, or the percent of the individuals initially put into the downwelling system that survived, was measured in all treatment groups. Physiological and genetic samples were collected.

S1.4 Common garden

Oysters were then placed in an upwelling system to reach a size appropriate for field deployments. In the upwelling system, each treatment replicate was contained within a PVC cylinder with 500 µm mesh on the bottom, and incoming water flowed up past the oysters to the drain. All replicates were held in one tank of flow-through, unfiltered York River water, allowing juvenile oysters to feed *ad libidum* on available phytoplankton. One HOBO® U24 series logger and a SeapHOx™ V1 (Sea-Bird Scientific) were used to document water conditions in the flow-through tank. The HOBO® logger recorded measurements every 30 minutes, while the SeapHOx™ measured temperature, salinity, dissolved oxygen, and pH every 10 minutes. To provide check samples for the SeapHOx™, once a week, water conditions were measured with the YSI Pro Plus, and water samples were collected for measurements of pH and total alkalinity (see S1*.5 Carbonate Chemistry Analysis*). Average difference between water samples and seapHOx pH was calculated (offset = 0.043 ± 0.017 standard error). Three times a week, the nursery tank and upwellers were drained, cleaned, and refilled.

S1.5 Carbonate Chemistry Analysis

Some of the water samples collected to characterize the carbonate chemistry conditions throughout the experiment required preservation. For measurements of total alkalinity, water was either frozen or collected in acid-washed 500 mL glass bottles, poisoned with saturated mercuric chloride ($HgCl_2$), and stored at 4°C until analysis. Spectrophotometric pH was analyzed within an hour of collection or from the stored, poisoned samples.

Total alkalinity was measured following SOP 3b (Dickson, 2007) and using a Metrohm 855 Robotic Titrosampler with ~0.1M HCl in 0.6M NaCl (A. Dickson Laboratory, Scripps Institution of Oceanography). The accuracy of the Titrosampler was ± 10 µmol kg SW^{-1}, assessed using a certified reference material (A. Dickson Laboratory, Scripps Institution of Oceanography), and water samples were run in technical duplicates. The calculated total alkalinity was adjusted for the dilution of saturated $HgCl_2$ addition for poisoned samples.

Water pH (total scale) was measured in technical triplicates following SOP 6b (Dickson, 2007) and using a temperature-controlled Thermo Scientific™ Evolution™ 201 UV-Visible spectrophotometer. Absorbance was measured with and without m-cresol purple dye at 730, 578, and 434 nm at 25°C. Water pH at *in situ* temperature was calculated using the Seacarb package in R (Gattuso et al., 2021), using the temperature at the time of sample collection. Total alkalinity and pH were used to calculate other carbonate chemistry parameters using the Seacarb package in R, using complementary temperature and salinity data.

S1.6 Genetics

All DNA was isolated with Puramag™ carboxylated magnetic beads (McLab, San Francisco, CA). Oyster tissues were lysed in a digestion buffer (25nM Tris, 25nM EDTA, 2M guanidine hydrochloride, 5mM $CACl_2$, 0.5% Triton X-100, and 1% N-Lauroyl-Sarcosine) with a final concentration of 0.2mg/mL proteinase K. For juvenile and adult samples, a small piece of tissue was lysed overnight at 55°C, while whole larval oysters were crushed and lysed for one hour. Magnetic beads were cleaned three times with 0.5M EDTA and diluted 10X with a 2.5 M NaCl and 20% PEG solution. Equal parts cleaned beads and oyster lysate were mixed, and DNA was allowed to bind to beads before being placed on a magnetic stand, washed twice with 70% ethanol, and eluted with 0.1X TE buffer.

A panel of microsatellite loci were tested and optimized for the evaluation of loss of alleles. In total, 62 primer pairs were obtained from several publications (Brown et al. 2000; Reece et al. 2004; Carlsson & Reece 2007; Wang & Guo 2007; Wang et al. 2009) and synthesized with T3 tails on the 3' end of the forward primers. Primers were first tested on tetraploid male oysters obtained from VIMS ABC to ascertain the ideal annealing temperature using gradient PCR. For each primer pair, initial gradients ranged from 5°C below to 5°C above the published annealing temperature. PCR reactions using the Qiagen *Taq* PCR Core Kit (Qiagen, Germantown, MD) consisted of 1 μL 1mg/mL BSA, 0.5 μL 10X PCR Buffer, 0.1 μL 10mM dNTPs, 0.05 μL of each primer (10μM), 0.025 μL 50U/μL *Taq*, 2.963 μL PCR water, and 0.5 μL template DNA. PCR cycling conditions were as follows: 94°C initial denaturation for 3 minutes, 35 cycles of 94°C denaturation for 1 minute, 1 minute at the gradient annealing temperatures, 72°C extension for 1 minute, and a final extension at 72°C for 7 minutes. PCR products were visualized with gel electrophoresis to evaluate amplification success and the presence of any nontarget amplification products. Gradients were adjusted, if necessary, for a second round of PCR optimization including a T3 fluorescent probe (FAM, VIC, PET, or NED; 0.05 μL of a 10 μM probe added to above recipe), and alleles were separated on an ABI Genetic Analyzer 3130XL (ThermoFisher Scientific, Waltham, MA). Chromatograms were evaluated in GeneMarker (SoftGenetics, State College, PA) for amplitude of fluorescent signal and nonspecific amplification, and the final annealing temperature was chosen for each primer pair.

All primers with successful amplification in the tetraploid oysters were further optimized on 16 of the broodstock oysters spawned for this experiment to assess the level of polymorphism. T3 PCRs and sequencing were repeated using the same protocols described above, and alleles were sized in GeneMarker. Primer pairs meeting the quality criteria established above and also having three or more alleles present in the 16 broodstock oysters were chosen as genetic markers for the carryover effects study. These were grouped into multiplex groups based on similar or equal annealing temperatures, and assigned a fluorescent dye (FAM, VIC, PET, or NED) and tail (T3, T7, m13f, A, B, C, or D; see Table S2 for tail sequences). All tails are commercially available with the exception of A-D, which are from (Blacket et al., 2012). Forward primers were re-ordered with the assigned tail sequences attached to the 3' end.

Table S2. *Crassostrea virginica microsatellite loci, their sources, multiplex group for this study, tail sequence and corresponding fluorescent dye, annealing temperature, range of base pair lengths for alleles, and number of alleles.*

Primer	Source	Multiplex Group	Tail/Dye	Annealing Temperature (°C)	Base Pair Range	Number of Alleles
RUCV89	Wang & Guo 2007	Cvi1	tail A FAM	50	221-237	5
Cvi2i4	Reece *et al.*, 2004	Cvi1	tail B VIC	50	338-411	14
Cvi13	Brown *et al.*, 2000	Cvi1	m13f NED	50	150-254	15
Cvi2k14	Reece *et al.*, 2004	Cvi1	T3 PET	50	225-331	3
RUCV51	Wang & Guo 2007	Cvi2	T3 FAM	55	169-196	8
RUCV114	Wang *et al.*, 2009	Cvi2	tail D PET	55	227-288	8
RUCV66	Wang & Guo 2007	Cvi2	tail C NED	55	308-352	7
Cvi6	Brown *et al.*, 2000	Cvi3	tail A FAM	55	179-245	10
Cvi9	Brown *et al.*, 2000	Cvi3	tail B VIC	55	115-148	11
RUCV109	Wang *et al.*, 2009	Cvi3	m13f NED	55	221-227	3
RUCV159	Wang *et al.*, 2009	Cvi3	T3 PET	55	265-283	3
RUCV68	Wang & Guo 2007	Cvi4	tail A FAM	61.4	336-356	9
RUCV274	Wang *et al.*, 2009	Cvi4	T3 PET	61.4	242-258	6
RUCV80	Wang & Guo 2007	Cvi4	T3 PET	61.4	163-169	3
RUCV148	Wang *et al.*, 2009	Cvi4	tail B VIC	61.4	226-263	5
Cvi7	Brown *et al.*, 2000	Cvi5	T3 PET	55	210-221	7
RUCV74	Wang & Guo 2007	Cvi5	T7 VIC	55	171-180	5
Cvi1264E	Carlsson & Reece, 2007	Cvi5	T3 FAM	45.8	170-205	7

S2. Results

S2.1 Water Conditions

Table S3. *Mixed effects linear model results for the comparison of larval treatment water characteristics between S_{13} and S_{15} with tank as a random factor. Asterisks indicate statistical significance below an alpha threshold of 0.05. Under each response variable are the independent variables of interest in italics.*

	Statistical Value	**P value**
Temperature	$F_{(4,201)} = 0.001$	0.979
Salinity	$F_{(4,201)} = 7203.000$	<0.001*
Dissolved Oxygen	$F_{(4,201)} = 11.236$	<0.001*
pH	$F_{(4,36)} = 2.417$	0.120
Total Alkalinity	$F_{(4,36)} = 12.474$	<0.001*
DIC	$F_{(4,36)} = 7.966$	0.005*
$\Omega_{calcite}$	$F_{(4,36)} = 0.472$	0.492
$\Omega_{aragonite}$	$F_{(4,36)} = 0.277$	0.599

Table S4. *Average water chemistry conditions for all treatments during the settlement phase. Values represent mean ± standard error calculated from all treatment replicates over the entire larval period. Temperature, salinity, dissolved oxygen, pH_{tot} (pH, total scale), and total alkalinity are measured values. DIC (dissolved inorganic carbon), $\Omega_{calcite}$ (saturation state of calcite), and $\Omega_{aragonite}$ (saturation state of aragonite) are calculated values.*

	Treatments		
	Laboratory		
Water Properties	Thirteen (S_{13})	Fifteen (S_{15})	Hatchery (S_H)
Temperature (°C)	25.51 ± 0.27	25.62 ± 0.27	27.17 ± 0.63
Salinity	13.20 ± 0.02*	15.00 ± 0.02*	12.67 ± 0.07
Dissolved Oxygen (mg L^{-1})	7.28 ± 0.07*	7.09 ± 0.05*	6.07 ± 0.098
pH_{tot}	7.71 ± 0.01	7.68 ± 0.01	7.92 ± 0.01
Total Alkalinity (umol kg^{-1})	1513 ± 29	1528 ± 1	1492 ± <1
DIC (umol kg^{-1})	1481 ± 27	1494 ± 1	1418 ± 1
$\Omega_{calcite}$	1.26 ± 0.05	1.27 ± 0.03	1.99 ± 0.05
$\Omega_{aragonite}$	0.75 ± 0.03	0.77 ± 0.02	1.18 ± 0.01

Table S5. *Mixed effects linear model results for the comparison of treatment water characteristics during the settlement phase between S_{13} and S_{15}* with tank as a random factor. *Asterisks indicate statistical significance below an alpha threshold of 0.05. Under each response variable are the independent variables of interest in italics.*

	Statistical Value	**P value**
Temperature	$F_{(4,108)} = 0.083$	0.773
Salinity	$F_{(4,108)} = 3{,}581.000$	<0.001*
Dissolved Oxygen	$F_{(4,108)} = 5.157$	0.023*
pH	$F_{(4,23)} = 0.808$	0.369
Total Alkalinity	$F_{(4,23)} = 0.218$	0.641
DIC	$F_{(4,23)} = 0.197$	0.657
$\Omega_{calcite}$	$F_{(4,23)} = 0.011$	0.917
$\Omega_{aragonite}$	$F_{(4,23)} = 0.081$	0.777

Table S6*. Average water chemistry conditions (mean ± standard error) in common garden during the nursery period. Temperature, salinity, dissolved oxygen, pH_{tot} (pH, total scale), and total alkalinity are measured values. DIC (dissolved inorganic carbon), $\Omega_{calcite}$ (saturation state of calcite), and $\Omega_{aragonite}$ (saturation state of aragonite) were all calculated.*

	Nursery
Temperature (°C)	28.04 ± 0.02
Salinity	15.20 ± 0.01
Dissolved Oxygen (mg L^{-1})	3.05 ±0.03
pH_{tot}	7.56 ± 0.01
Total Alkalinity (umol kg^{-1})	1416 ± 2.42
DIC (umol kg^{-1})	1396 ± 2.34
$\Omega_{calcite}$	1.07 ± 0.01
$\Omega_{aragonite}$	0.65 ± 0.01

Table S7. *Kruskal-Wallis test results for the comparison of juvenile water characteristics between field sites (Rappahannock and York Rivers). Asterisks indicate statistical significance below an alpha threshold of 0.05. Under each response variable are the independent variables of interest in italics.*

	Statistical Value	**P value**
Temperature	$\chi^2_{(1)} = 3.75$	0.053
Salinity	$\chi^2_{(1)} = 137.32$	<0.001*
Dissolved Oxygen	$\chi^2_{(1)} = 3.36$	0.067
pH	$\chi^2_{(1)} = 70.26$	<0.001*
Total Alkalinity	$\chi^2_{(1)} = 76.94$	<0.001*
DIC	$\chi^2_{(1)} = 86.26$	<0.001*
$\Omega_{calcite}$	$\chi^2_{(1)} = 0.28$	0.597

S2.2 Genetics

Table S8. Allelic richness for 15 microsatellite loci. Results are listed for broodstock, each individual sample (sampling event X treatment), each sampling event, and each salinity treatment.

	RUCV89	Cvi13	Cvi2k14	RUCV51	RUCV114	RUCV66	Cvi6	Cvi9	RUCV109	RUCV159	RUCV68	RUCV80	RUCV148	RUCV74	Cvi1264E	Totals
BDS	5	15	3	8	8	7	10	11	3	3	9	3	5	5	7	102
$S_{13\text{-eyed}}$	4	10	3	8	5	6	10	10	3	3	6	1	5	3	4	81
$S_{15\text{-eyed}}$	4	11	3	5	7	7	10	9	2	3	7	2	4	4	6	84
RR_{13}	3	10	3	7	7	7	10	10	3	3	7	3	5	5	5	88
RR_{15}	4	10	3	5	6	6	7	10	3	3	7	2	5	5	5	81
YR_{13}	3	11	3	8	6	6	10	10	3	3	7	3	5	4	6	88
YR_{15}	4	12	3	6	8	7	9	10	3	3	5	3	4	4	4	85
S_{eyed}	4	11	3	8	7	7	10	10	3	3	7	2	5	4	6	90
RR	4	12	3	7	7	7	10	10	3	3	7	3	5	5	5	91
YR	4	13	3	8	8	7	10	10	3	3	7	3	5	4	6	94
13	4	12	3	8	8	7	10	10	3	3	7	3	5	5	6	94
15	5	13	3	7	8	7	10	10	3	3	7	3	5	5	6	95

Table S9. Pairwise comparisons between sampling event by salinity treatment groups presented in a heatmap, where F_{ST}'s calculated from 15 microsatellite loci are on the upper diagonal, with comparisons with significant FDR-corrected p-values bolded, and the sum of significant locus-by-locus genic (allelic) differentiation tests out of 15 total loci on the lower diagonal. Red cells contain larger and more significant differences while beige cells contain smaller F_{ST}'s and less significant genic differences.

	BDS	RR_{13}	RR_{15}	$S_{13\text{-eyed}}$	$S_{15\text{-eyed}}$	YR_{13}	YR_{15}
BDS	--	**0.035**	**0.039**	**0.033**	**0.039**	**0.042**	**0.047**
RR_{13}	11	--	**0.006**	**0.008**	**0.008**	**0.010**	0.002
RR_{15}	11	4	--	**0.010**	**0.010**	0.001	**0.010**
$S_{13\text{-eyed}}$	10	5	7	--	**0.006**	**0.012**	**0.006**
$S_{15\text{-eyed}}$	11	2	6	4	--	**0.010**	**0.007**
YR_{13}	9	4	3	6	4	--	**0.013**
YR_{15}	11	2	5	3	4	4	--

S2.3 Hatchery comparison

There were physiological differences between larval oysters in the S_{13} and S_H treatments (Figure S1). While shell length showed little difference between the two treatments at both sampling time points, total protein content differed by the end of the larval period at 18 dpf (Figure S1b) and total antioxidant capacity were different at both sampling time points (Figure S1c). Between the two treatments, S_{13} larvae had almost twice the total protein content and total antioxidant capacity that S_H larvae had by the start of metamorphosis. Larval and juvenile S_H samples were genetically distinct from the corresponding S_{13} samples based on allelic identity (F_{ST} and genic differentiation; Table S10).The overall genetic diversity in the larval S_H and S_{13} samples were comparable, and the numbers of alleles in the juvenile samples (RR_H and YR_H) were 8-9 alleles less than those of the corresponding S_{13} juvenile samples, despite the S_H juvenile samples being larger than the S_{13} juvenile samples (Table S11). The heterozygosities and inbreeding coefficients were comparable between corresponding S_H and S_{13} samples.

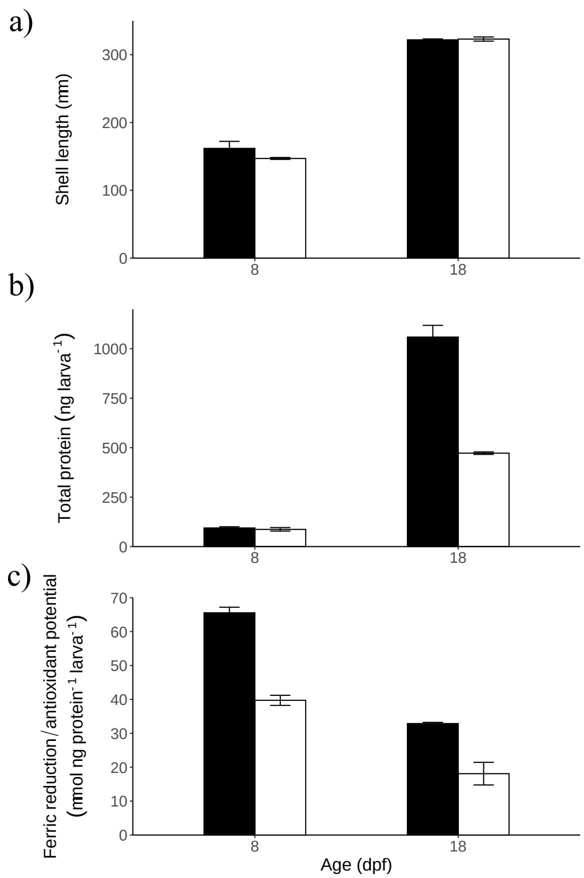

Figure S1. *Comparison of physiology between larval oysters cultured in a local, commercial hatchery (S_H; white) to those in the thirteen-salinity treatment (S_{13}; black) at VIMS. Larvae in the hatchery were cultured from fertilization under ambient water conditions from the Rappahannock River, while those at VIMS were exposed to their salinity treatments starting at 2 dpf. Larvae remained in their treatments until 18 dpf. Larvae from the hatchery were sampled at days 8 and 18. Shell length (a), measured perpendicular from hinge to edge of larval shell, did not differ between the two cultures at either time point. Total protein content (b) was similar between the two cultures at 8 dpf but was greater in S_{13} treatment larvae by 18 dpf. Lastly, total antioxidant capacity (c) was greater in S_{13} treatment larvae at both sampling time points.*

Table S10. Pairwise comparisons between hatchery samples and the comparable salinity treatment (S_{13}), including F_{ST}'s calculated from 15 microsatellite loci, with comparisons with significant FDR-corrected p-values bolded, and the sum of significant locus-by-locus genic (allelic) differentiation tests out of 15 total loci. Samples are Rappahannock River (RR), York River (YR), and pre-metamorphic larvae (S_{eyed}), with the subscript indicating the hatchery (H) or salinity treatment (13).

	F_{ST}	Genic Differentiation
$S_{H\text{-eyed}}$ – $S_{13\text{-eyed}}$	**0.006**	6
RR_H - RR_{13}	**0.006**	4
YR_H - YR_{13}	**0.014**	3

__Table S11.__ Indices of genetic diversity for the hatchery treatment (H), including observed heterozygosity (HO), expected heterozygosity (HS), and inbreeding coefficient (GIS), calculated from 15 microsatellite loci with dosage correction.

Population	n	Total Alleles	H_O	H_S	G_{IS}
S_H	89	88	0.466	0.552	0.156
RR_H	63	80	0.480	0.561	0.144
YR_H	65	79	0.442	0.545	0.189

S2.4 Settlement Phase

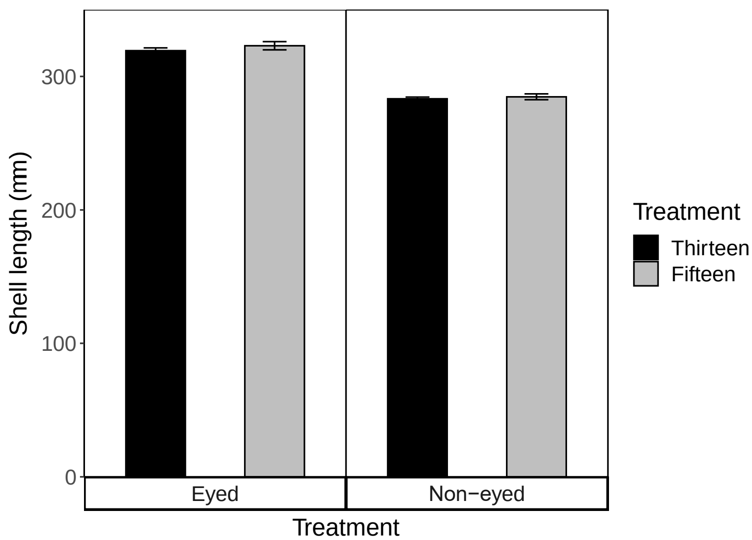

Figure S2. *Shell length at settlement for oyster larvae raised under two salinities. Larvae exposed to a salinity of thirteen (black) were of similar size at metamorphosis (eyed larvae, left panel) to those experiencing a salinity of fifteen (gray). Shell length of larvae without visible signs of metamorphosis (right panel) was also similar across the environmentally relevant salinity difference. Bars represent mean ± standard error.*

Table S12. *T-test model results for the post settlement period. Asterisks indicate statistical significance below an alpha threshold of 0.05. Under each response variable are the independent variables of interest in italics.*

	Statistical Value	**P value**
Post Settlement		
Shell Length	$T_{(4)} = -0.480$	0.664
Total Protein Content	$T_{(4)} = -0.997$	0.375
Total Antioxidant Capacity	$T_{(4)} = -1.680$	0.1682

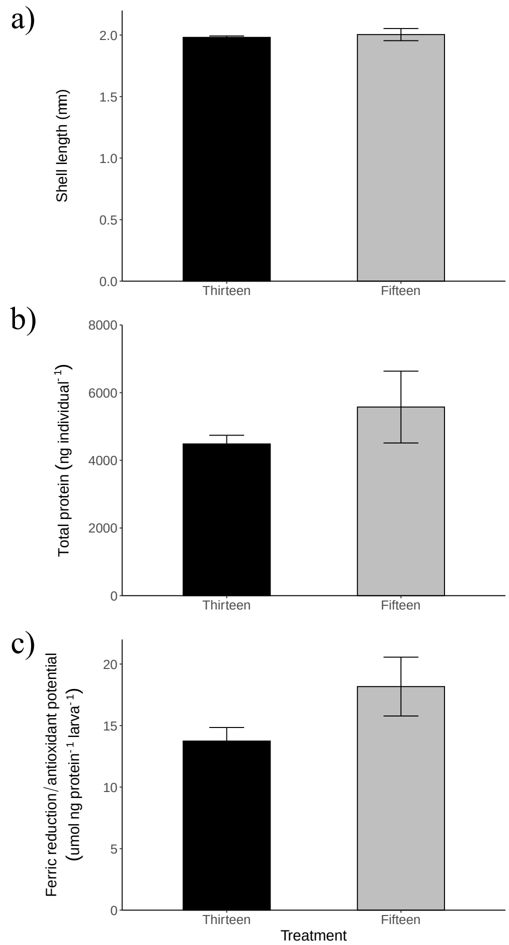

Figure S3. *Effect of larval salinity experience on the physiology of juvenile oysters after completion of the settlement phase. Larval salinity experience had no significant effect on oyster physiology after the settlement phase was complete. Specifically, oysters that experienced a salinity of thirteen (black) through the larval and settlement stages had similar shell lengths (a), measured perpendicular from hinge to edge of shell, total protein content (b), and total antioxidant capacity (c) to those raised in a salinity of fifteen (gray).*

CHAPTER 3

Carryover effects of temperature and pH on juvenile, wild Eastern Oysters, *Crassostrea virginica*

Abstract

As ocean acidification and warming simultaneously change oyster habitat in coastal environments, it is important to understand how these two global climate change stressors may affect the physiology of larval oysters, the most vulnerable life stage. Furthermore, impacts to the larval stage may affect oysters during and after the settlement phase (i.e., settlement and metamorphosis), another highly energetically demanding life stage. Effects that occur during one life stage and impact a later stage are referred to as carryover effects. The purpose of this study was to understand how conditions of ocean acidification and ocean warming, individually and simultaneously, would affect larval oysters and if those effects would carry over to influence the physiology of juvenile oysters in different environmental conditions. Larval Eastern oysters, *Crassostrea virginica*, were spawned using wild adults from a reef in the Rappahannock River, a tributary of the Chesapeake Bay. Larvae were exposed to full-factorial combinations of two temperature (25°C, 29°C) and carbonate chemistry (pH 7.2, pH 7.8) levels that represent either average present-day ambient (25°C, pH 7.8) or extreme summer conditions anticipated to become more frequent in the future. Then, to observe potential carryover effects from the larval experience, early juvenile oysters from each larval treatment were re-distributed across all four treatment conditions factorially for 16 larval-to-juvenile treatment combinations. Carryover effects from the individual stressors were identified from measurements of oxidative stress defense and damage molecules, biomass accumulation, changes in long-term energy stores, rates of oxygen consumption, and shell growth. Carryover effects associated with exposure to multiple stressors were unable to be evaluated due to 100% mortality of larval oysters in the Multiple Stressor treatment (29°C, pH 7.2) ~11 days-post-fertilization (dpf). Carryover effects from conditions of acidification were more persistent and mostly negative, whereas warming had

shorter, but positive, carryover effects. Warming also had a cross-tolerant effect when stressors were experienced sequentially throughout the study. Some carryover effects did not persist past the first week of the juvenile stage, which may have been masked by potential selective mortality, as juvenile oyster survival was greatly affected by acidified conditions. Overall, larval and juvenile responses to multiple environmental stressors are intricate and complex. While juvenile Eastern oysters did experience carryover effects from conditions of ocean acidification and ocean warming, they were ultimately able to compensate for those that were negative, indicating potential tolerances to future environmental conditions.

Introduction

Estuarine organisms, such as the Eastern oyster (*Crassostrea virginica*), experience highly dynamic environmental conditions due to tidal forces, riverine inputs, and biological activity. Estuarine environments are becoming more stressful as ongoing shifts in abiotic conditions, due to global climate change in addition to the underlying dynamic processes already occurring, result in more frequent extreme conditions (Hofmann et al., 2011). Because environmental conditions undergo diurnal fluctuations within estuaries, organisms can experience more than one environmental stressor simultaneously or sequentially (Cheng et al., 2015), which is why it is important to understand how physiological mechanisms may shift in response to multiple environmental stressors (Gunderson et al., 2016). While single-stressor studies are more feasible, their results may prove misleading when interpreted in a multiple stressor context because of potential nonlinear (i.e., synergistic) interactions between concurrent stressors (Côté et al., 2016; Crain et al., 2008; Gunderson et al., 2016; Todgham and Stillman, 2013). Though studying organismal responses to all possible combinations of environmental stressors is not feasible (Côté et al., 2016), targeted studies have begun to identify patterns and overlapping physiological mechanisms in response to multiple climate change stressors. Identifying common mechanisms of organismal responses to multiple environmental stressors will increase the accuracy of future predictions and models, and ultimately, improve conservation and fisheries management plans (Breitburg et al., 2015).

Eastern oyster populations in Chesapeake Bay, and other areas within their geographic range, will likely be affected simultaneously by ocean acidification and ocean warming. Individually, these two stressors impair development, growth, reproduction, and calcification in oysters (See Chapter 1 for more details; Byrne, 2011; Byrne and Przeslawski, 2013; Gazeau et al., 2013; Mcafee et al., 2018). Furthermore, the early life stages of oysters, larvae and juveniles, are particularly vulnerable due to the energy demands of rapid growth and development (Pandori and Sorte, 2019; Przeslawski et al., 2015). As a result, success of these vulnerable early life stages may be compromised by additional stress from environmental change (Kurihara et al., 2007; Waldbusser et al., 2011). While warming and acidification can individually be harmful, prior work reports inconsistent simultaneous effects of these two stressors to larval and juvenile oyster biology and physiology (Przeslawski et al., 2015; Speights et al., 2017). In some instances, warming ameliorated negative effects of acidification on shell and tissue growth, for example (Cole et al., 2016; Ko et al., 2014; Parker et al., 2010), while in other studies the simultaneous experience of multiple stressors amplified the negative, single stressor effects (Talmage and Gobler, 2011). Even within an experiment, the combined effects of warming and acidification can be complex and vary considerably between the physiological metrics measured (Ko et al., 2014; Parker et al., 2010).

To holistically understand the effects of multiple environmental stressors on Eastern oysters, it is necessary to consider how those effects may persist beyond a single life stage. Phenotypic changes that occur during one life stage and impact a later one are referred to as within-generation carryover effects (O'connor et al., 2014), and environmental stressors are one known cause, likely due to bioenergetic shifts (See Chapter 2 for more details; Pechenik et al., 1998; Sokolova et al., 2012). Carryover effects have been identified in oysters after exposing the larval or juvenile stages to various stressful environmental conditions, though most were single stressor exposures (Hettinger et al., 2013, 2012; Mackenzie et al., 2022; Manuel et al., 2023). Studies observing carryover effects from single environmental stressors have found mixed results. Specifically, negative effects to larval shell growth from ocean acidification conditions carried over into the juvenile stage, decreasing growth by 41% in laboratory studies (Hettinger et al., 2012), which also held true when tested *in situ* (Hettinger et al., 2013). In other studies, oyster larvae exposed to acidification had better juvenile and adult health when compared to individuals exposed to artificially buffered water with higher pH (Mackenzie et al., 2022). Though results are mixed from carryover-effect studies employing single stressors, observing carryover effects within the context of multiple environmental stressors is more ecologically relevant for reasons mentioned above; however, few studies have observed carryover effects of multiple stressors (Donelan et al., 2023, 2021), and even fewer specifically observed the effects of ocean acidification and warming. Most of the multiple stressor studies observing the effects of previous environmental experience on later life stages are technically cross- or trans-generational studies observing how parental exposure impacts offspring responses (Parker et al., 2017; Spencer et al., 2020) and are not directly relevant to the purpose of this chapter. From the few carryover effect studies conducted with multiple stressors, carryover effects may be context-dependent, making their interpretation highly complex (Donelan et al., 2021).

Exposure to one stressor can make organisms more robust or vulnerable to additional stressors, which is referred to as cross-tolerance and cross-susceptibility, respectively (Todgham and Stillman, 2013). Carryover effects may manifest differently under various environmental conditions due to potential cross-tolerant or cross-susceptible effects from previous environmental stress experience. Cross-tolerance occurs when exposure to one environmental stressor activates similar cellular response pathways used with additional stressors, or separate pathways that converge on the same physiological function. Meanwhile, cross-susceptibility transpires when the response pathway is impaired, or energy has been mostly depleted by a previous stressor (Kroeker et al., 2017; Todgham and Stillman, 2013). An environmental stressor can be both beneficial or disadvantageous in a multiple-stressor scenario depending on the subsequent stressor, environmental context, or species. For example, ocean warming can make organisms cross-tolerant to hypoxic conditions (Cheng et al., 2015; Collins et al., 2021; Todgham et al., 2005) but can make them cross-susceptible under ocean acidification (Parker et al., 2017; Stevens and Gobler, 2018). To best understand the complex impacts of climate change on oysters, it is important to observe carryover effects within the context of multiple environmental scenarios, as carryover effects from previous exposure to one environmental stressor may differ depending on the environmental context later (Donelan et al., 2021).

Therefore, the purpose of this study was to understand how conditions of ocean acidification and ocean warming might affect the physiology of larval *C. virginica*, if those effects would carry over to impact the performance of juvenile oysters and lastly, if those carryover effects would change under different future environmental scenarios in the juvenile phase. I hypothesized that temperature and low pH, individually, would decrease survival, growth, and long-term energy storage, and increase oxidative stress levels and metabolic rates in larval oysters. Furthermore, I predicted that the interaction of the two stressors would be negative and synergistic in larvae. Lastly, I hypothesized that the negative impacts of the environmental stressors during the larval stage would carry over into the juvenile stage regardless of the water conditions that the juveniles experience, impacting their overall performance.

Methods

1. Experimental Design

To examine how the larval experience of multiple environmental stressors, ocean acidification and ocean warming, carries over to affect the physiology of juvenile *Crassostrea virginica*, larvae were reared from fertilization through settlement and metamorphosis (i.e., the settlement stage) under four ecologically relevant combinations of water temperature and pH in the Environmental Change Aquarium System (see *section 1.1 Environmental Change Aquarium System*) at the Virginia Institute of Marine Science (VIMS). Juvenile oysters from each larval treatment were then equally divided and randomly re-distributed among all four treatment conditions for a total of sixteen larval-exposure-to-juvenile-treatment combinations (Figure 1). Oysters were subsampled for survival, growth, oxidative stress, long-term energy storage, and whole-organism metabolism during both the larval and juvenile stages to observe the potential carryover effects from acidified conditions and warming. Specifically, oysters were subsampled at 2, 4, 6, and 8 dpf during the larval phase of the experiment, at 20 dpf and 32 dpf during the settlement phase, and then at 1 week and 4 weeks during the first month of the juvenile phase.

Due to unforeseen issues, larvae in two replicate aquaria for the Multiple Stressor treatment experienced 100% mortality by 11 dpf. The third replicate aquaria only had ~8000 individual larvae by 15 dpf following a sharp decline in surviving individuals from ~500,000 at 11 dpf to ~170,000 larvae at 13 dpf. A sign of either contamination of treatment water or poor larval health is increasing bacterial growth, which was noted across all three replicate larval cultures for the Multiple Stressor treatment by 11 dpf; however, if there was a contamination event, then it would have been likely that all larval cultures across all treatments and replicates would have been impacted, which was not the case. Because there was one replicate tank within the Temperature treatment that also experienced 100% mortality by 13 dpf, and there were sharp declines in larval abundance within all three Low pH treatment replicate cultures around 11 dpf, it is likely that the simultaneous experience of pH and warming caused the morality of oyster larvae by 11 dpf. With one replicate aquaria remaining by the eyed larval phase with too few individuals, the Multiple Stressor larval treatment was not included in the settlement phase of the experiment. Larval data from the Multiple Stressor treatment are therefore presented for 2, 4, 6, and 8 dpf only.

The four treatment conditions were created from a full factorial combination of two temperature and two pH levels based on summertime average ambient and extreme values observed in the lower Rappahannock River for the month of June, which aligned with the larval phase of the present study (Figure 2a, b): Control (CN: 25°C, pH 7.8), High Temperature (HT: 29°C, pH 7.8), Low pH (LP: 25°C, pH 7.2), and Multiple Stressors (MS: 29°C, pH 7.2). The environmental data used to identify treatment levels were collected by a commercial hatchery partner, Oyster Seed Holdings, Inc. (OSH), located on the Rappahannock River in Grimstead, VA. Oysters in all treatment groups were cultured at a salinity of 12, also reflective of average summertime conditions in the lower Rappahannock River.

1.1. Environmental Change Aquarium System

Experimental treatments were executed in 100 L aquaria where both water temperature and pH were independently controlled. All aquaria were equipped with a GF Signet, LLC DryLoc pH 2724-2726 series glass electrode (Irwindale, CA), recirculation pump for water mixing, and two air stones through which CO_2 or CO_2-free air was delivered. Surrounding each aquarium was a recirculating water jacket containing a Signet, LLC 2350 thermal probe. Heat exchangers connected to external closed loops of recirculating hot and cold water were used to control the temperature of the water jacket. Water temperature and pH in each aquarium were controlled and maintained at setpoint conditions using a software control system that received data every minute from the pH electrodes and thermal probes in each aquarium. When aquarium temperature deviated from its setpoint, a four-way valve was autonomously opened to send hot or cold water through the heat exchanger in the aquarium’s water jacket, as needed. When aquarium pH deviated from its setpoint, a solenoid was opened to deliver CO_2 or CO_2-free air to the aquarium. Pure CO_2 was bubbled directly into the aquarium from a compressed gas cylinder, while the CO_2-free air was generated by a CAS Series CO_2 Adsorber.

All aquaria received 1µm-filtered York River water. River water was first filtered to 5 µm using a series of mixed media beds and cartridge filters. The 5 µm-filtered water was then pumped into a mixing tank where the water was aerated, and its salinity was adjusted to the desired level for the experiment (12) using additions of either deionized (DI) water or a brine solution made with Instant Ocean® (salinity of 80). Salinity conditions were maintained and monitored in the mixing tank using a Signet, LLC 2842-1V(D) PVDF conductivity electrode. Water from the mixing tank was passed through two, 1 µm filters before being pumped into the individual aquaria.

2. Larval Phase

Adult, diploid *C. virginica* were collected via dredge in March 2021 from Parrots Rock Reef West (37.605837°N, 76.421667°W), a sanctuary reef located near the mouth of the Rappahannock River. Adult oysters were held in floating cages at the VIMS Kauffman Aquaculture Center (KAC), located on the Rappahannock River near the original reef location. Gonad development in the oysters was checked every two weeks. When oysters were almost fully ripe at the beginning of June 2021, they were held in a temperature-controlled tank in KAC to delay the onset of spawning until the start of the experiment in mid-June.

Diploid larvae were produced from 10 female and 5 male oysters via strip-spawning (Allen and Bushek, 1992). Briefly, after oysters with high-quality gametes were identified and selected, eggs were stripped from each female oyster and pooled together. The pooled eggs were rinsed to remove harmful bacteria, then divided equally into 5 aliquots, as determined by the number of male oysters utilized. Each aliquot was then fertilized by the sperm from one male. After fertilization was completed (~1 hour), embryos were re-pooled and rinsed to remove any remaining sperm and bacteria. About two hours after fertilization, embryos were randomly divided and placed into three research aquaria (n ≅ 3,000,000 per aquarium) maintained at each of the four treatment conditions to create biological pseudo replicates. To control for density-dependent growth patterns, larval density was standardized across aquaria throughout the experiment. Densities started at 30 embryos mL^{-1} and were gradually reduced to 2 larvae mL^{-1} prior to the settlement phase (M. Congrove, pers. comm.; *Chapter 2, Table S1*). Larvae were fed daily *ad libitum* with a diet of a mixture of microalgal species (*Isochrysis galbana*, *Thalassiosira weissflogii*, and *Tetraselmis* sp.) cultured at VIMS Aquaculture and Breeding Technology Center (ABC).

Three times per week, a full water change and aquarium cleaning were conducted to remove any build-up of nitrogenous waste in the culture water and to subsample the larvae (days 2, 4, 6, and 8 postfertilization). Aquaria were emptied onto a mesh sieve of a size appropriate for the smallest larvae observed at last measurement to conservatively capture all individuals (*Chapter 2, Table S1*). Larvae were then returned to the aquarium or concentrated into a small volume of treatment water for sampling. To assess survival, and collect samples for physiological assessments, larval abundance in each aquarium was calculated using the density and total volume of the concentrated larvae. The density of larvae was calculated from a minimum of three sequential counts of 50 µL of the concentrated oyster larvae, during which images of individual larvae (n ≅ 100) were also captured for later shell growth measurements using a Leica DM compound microscope (Deerfield, IL) with a Leica MC170 HD camera. Larvae were then volumetrically sub-sampled by homogenizing the concentrated larvae and taking aliquots calculated using the above measured larval density for measurements of total antioxidant capacity and total protein (n ≅ 20,000), lipid peroxidation (n ≅ 20,000), triglyceride content (n ≅ 10,000), and respirometry (day 4 only; n ≅ 1,000). Once all samples were collected, remaining larvae were returned to their respective aquarium at the appropriate density (*Chapter 2, Table S1*), and samples were preserved at -80^{o}C until later analysis.

Both water samples and the continuous measurements by aquarium probes were used to characterize tank conditions during the experiment (see *Section 1.1. Environmental Change Aquarium System*). Prior to each water change, temperature, dissolved oxygen, pH, conductivity, and salinity in each aquarium were measured using a YSI Pro Plus (YSI Inc., Yellow Springs, OH). At the same time, water samples were collected from each aquarium for independent measurements of pH_{total} and from the mixing tank for measurements of total alkalinity (see *Section 5. Carbonate Chemistry Analysis*).

3. Settlement Phase

When 10% or more of the larvae showed pre-metamorphic attributes, like eyespots and foot muscle protrusion (termed eyed larvae) and shell lengths were larger than 300 μm, each culture was gently poured through two sieves: one designed to harvest larvae ready for settlement (225 μm), and one to catch the rest. Larval density was determined as above for concentrates from both sieves. Afterwards, larvae that did not catch on the harvesting screen were rinsed back into the aquarium. Eyed larvae were held in a moist cloth at 4°C. This procedure was repeated at the next water change until all larvae were harvested. Then, all larvae held at 4°C from the same treatment replicate were combined and then sub-sampled for abundance and growth (20 dpf), as above. Sub-samples of eyed larvae were also collected as described above for the larval samples for measurements of total antioxidant capacity, total protein, lipid peroxidation, and triglyceride content (n ≅ 5,000). Once sampling was complete, approximately 75,000 eyed larvae from each treatment replicate were placed in a downwelling system in their respective culture aquarium.

Experimental aquaria were set up as static downwelling systems. A 150 μm sieve containing cultch (finely ground oyster shell) was suspended about 2 cm below the surface of the water. The water in each aquarium was recirculated downward onto the sieve using the recirculation pump, which maintained homogenous treatment conditions, delivered and suspended algal food, and promoted settlement onto individual pieces of cultch. Water changes, feeding, and monitoring of aquaria water conditions were conducted as previously described (see *Section 2. Larval Culture*).

Settlement proceeded under treatment conditions for about a week and a half. Once adult morphological features (e.g., gills) were observed, there were little to no signs of eyespots or foot-muscles, and shell lengths were mostly greater than 700 μm, oysters were transitioned to the juvenile portion of the experiment. Oyster abundance was assessed (32 dpf) for survival and sample collection prior to transition into the juvenile phase of the experiment. Abundance of settled oysters in each aquarium was determined volumetrically from a random, concentrated sub-sample after measuring the total volume of oysters per treatment replicate. The density was calculated from a minimum of three sequential counts of 100 μL of the concentrated oyster sub-sample, during which images of individual oysters (n ≅ 60) were also captured for later shell growth measurements using a Leica S9i dissection microscope (Deerfield, IL) with integrated camera. Physiological samples (n ≅ 500) were collected for total antioxidant capacity, total protein content, lipid peroxidation, and triglyceride content.

4. Juvenile Phase

Juvenile oysters from each treatment replicate were randomly divided (n ≅ 5,000) and redistributed amongst one replicate aquarium of each of the four treatment conditions. Each replicate aquarium contained oysters from three larval treatments, each in an individual container with 500 μm mesh sides, mimicking upwelling containers used in the nursery phase at oyster hatcheries. Oysters were fed Shellfish Diet 1800® every day according to instructions provided by Reed Mariculture Inc. (Campbell, CA). Aquaria operated in flow through mode during the day at a rate of 250 mL min^{-1} to turn over aquaria water equivalent to a little more than one full volume of each aquarium per 24 h to avoid build-up of nitrogenous waste and waste from unconsumed food in between aquarium cleanings. To ensure oysters had enough time to feed, aquaria were operated in static mode overnight; the recirculation pump in each aquarium maintained suspension and circulation of treatment water and food. Water changes, aquaria cleaning, and monitoring of aquaria water conditions were conducted as previously described (see *Section 2. Larval Culture*) but at a lower frequency of once per week.

Whole oysters were collected from each aquarium at one and four week(s) after the start of the juvenile stage of the experiment to assess any carryover effects from the larval environmental experience or potential acclimatization. Juvenile oyster abundance was measured volumetrically, and shell growth images were captured using the same dissection microscope and integrated camera, as described above. The same suite of physiological samples was collected as described above (n_{week1} = 200, n_{week4} = 50). All samples were preserved at -80°C until analysis.

5. Carbonate Chemistry

Carbonate chemistry conditions were characterized from water samples collected throughout the experiment. Total alkalinity was measured using open-cell potentiometric titrations following best practices (SOP 3b; Dickson et al., 2007). pH_{total} was measured spectrophotometrically using m-cresol purple dye, following best practices (SOP6b; Dickson et al., 2007). All pH samples were run within an hour of collection; water samples used for total alkalinity measurements were held at -20°C until later analysis. Total alkalinity, pH and complementary temperature and salinity data were used to calculate other carbonate chemistry parameters using the Seacarb package in R (Gattuso et al., 2021) and the following constants: K_1 and K_2 for carbonic acid (Lueker et al. 2000), K_F (Perez and Fraga 1987), total boron (Lee et al. 2010), and K_{SO4} (Dickson et al. 1990). Refer to *Chapter 2, Section S1.5* for more detailed methods of the carbonate chemistry measurements.

6. Physiological Assays

Measurements of total protein content, total antioxidant capacity, and shell length were conducted as described in detail in *Chapter 2, Section 2.4* and are summarized here. Total protein content was measured using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Smith et al., 1985). Total antioxidant capacity was measured using the ferric reducing/antioxidant potential (FRAP) assay from (Griffin and Bhagooli, 2004). All spectrophotometric assays were conducted using a Molecular Devices SpectraMax® iD3 (San Jose, CA) microplate plate reader. ImageJ was used to measure shell size from photos captured at the time of the larval, settlement, and juvenile oyster density sub-samples described above (Schneider et al., 2012).

6.1. Lipid Peroxidation

Lipid peroxidation was measured as malondialdehyde (MDA) content using the Lipid Peroxidation Malondialdehyde Assay kit (Colorimetric/Fluorometric) from Abcam (118970). Samples of oysters from the larval, settlement, and juvenile stages were homogenized in the lysis buffer provided in the assay kit (1:100 2,6-Di-tert-butyl-4-methylphenol [BHT]). Oysters from the larval and settlement stages were homogenized using sonication, while juvenile oyster samples were homogenized with bead beating. Sample homogenates were then mixed with the working reagent (thiobarbituric acid and glacial acetic acid) and incubated at 95°C for an hour. After cooling to room temperature, sample reactions of larvae 6 dpf and older were read at 532 nm. Samples of younger larvae (2 and 4 dpf) were read fluorometrically to increase the detection of their smaller MDA concentrations, with an excitation at 532 nm and emission reading at 553 nm using the SpectraMax® iD3 microplate plate reader. Samples from each treatment replicate were measured in technical triplicates. Duplicate standard curves of known MDA concentrations (colorimetric: 0 – 5 nmol, fluorometric: 0 – 0.5 nmol) created from the provided MDA standard were included on each assay plate and used to calculate MDA content in oyster samples. MDA content was then normalized to total protein content to reduce bias from potential biomass differences among individuals.

6.2. Triglyceride Content

Triglyceride (TG) content was measured using the Triglyceride Assay Kit from Abcam (65336). Samples of oysters from the larval, settlement, and juvenile stages were homogenized in 5% NP-40 and analyzed in technical duplicate pairs. Oysters from the larval and settlement stages were homogenized using sonication, while juvenile oyster samples were homogenized with bead beating. One member of each pair received an aliquot of lipase, which converts TG into free fatty acids and glycerol, the end products quantified by the assay. The other member of each pair did not receive lipase and served as a blank to control for background glycerol levels. All reactions incubated at room temperature for 20 minutes. The working reagent (assay buffer, enzyme mix, and probe all provided in the kit) was added, followed by a second incubation for an hour at room temperature. Absorbance at 570 nm was measured using the SpectraMax® iD3 microplate plate reader. Duplicate standard curves of known TG concentrations (0 – 10 nmol) created from the provided TG standard were included on each assay plate and used to calculate TG concentrations in oyster samples. TG content was then normalized to total protein content to reduce bias from potential size differences across treatments.

6.3. Respirometry

Based on methods developed by Marsh and Manahan (1999), oxygen consumption of oyster larvae and juveniles was measured using endpoint respirometry. Twenty-four hours prior to measurement, sub-sampled oysters were isolated in separate containers under treatment conditions without food to ensure complete digestion. Oysters were then placed into micro biological oxygen demand (BOD) vials filled with their respective, oxygen-saturated treatment water after being filtered to 0.2 µm (FSW) to remove any algal and microbial cells that might confound the oxygen measurements. The BOD vials containing oysters were then incubated, alongside BOD vials with treatment FSW only, at their respective treatment temperatures for several hours (larvae: ~7 hrs, juveniles: ~2 hrs) before end-point measurements of the oxygen concentration were made using a PreSens Microx 4 Oxygen Microsensor (Regensburg, Germany). The oxygen microsensor was calibrated using a 0% O_2 in saturated $NaSO_4$ and a 100% O_2 in FSW. An initial measurement of the 100% O_2 solution was captured before beginning the measurements of the BOD vials. After uncapping each BOD vial, an airtight syringe was used to carefully remove the sample water to decrease the possibility of atmospheric O_2 contaminating the sample. The sample was then carefully dispensed into the airtight measurement chamber where the oxygen microsensor was located. Three consecutive measurements of dissolved oxygen concentrations were recorded after two minutes, allowing the microsensor to equilibrate and stabilize. The chamber and syringe were rinsed with FSW between samples to ensure there was no cross-contamination. Oxygen consumption rates were calculated by removing background microbial oxygen consumption (from blank BOD vial measurements), correcting for sensor drift, and accounting for vial volume, incubation time, and biomass.

Larvae from each treatment replicate were measured in triplicate BOD vials, with about 300 larvae per vial. Larvae from each vial were counted after the respirometry measurement for an exact count for measurement standardization; however, respirometry data from oyster larvae could not be analyzed due to measurement error. Oxygen consumption of juvenile oysters was measured after five days in juvenile treatment conditions to capture initial responses or carryover effects in metabolism. Oxygen consumption was then measured again after three and half weeks in juvenile treatments to observe any potential sustained carryover effects or acclimatization. Due to large number of treatment replicate combinations, juvenile measurements were conducted over three consecutive days, with one replicate from each larval-treatment-to-juvenile-treatment combination analyzed each day. Juvenile oysters were then preserved at -80°C for later analysis of total protein (see *Chapter 2, Section 2.4*). After total protein was analyzed, oxygen consumption rates were normalized to protein content to reduce bias from potential biomass differences among individuals.

7. Statistics

Water conditions among the four treatments were analyzed over the larval and juvenile phases of the experiment, separately. Water conditions in the settlement period were analyzed similarly but only among three treatment conditions. All water conditions, except for total alkalinity, were analyzed using a linear mixed effects model with the *nlme* package (version 3.1-160; Pinheiro et al., 2020; Pinheiro and Bates, 2000) in R (version 4.2.2; R Core Team, 2022). Posthoc testing was done using the *multcomp* package (version 1.4-20; Hothorn et al., 2008) in

R. Treatment was coded as four factors and treated as a fixed effect, while aquarium replicate was included as a random factor to account for any aquaria effects. Several response variables were transformed to satisfy the assumptions of normality for the Shapiro-Wilkes test and homogeneity for the Levine's test. For the larval phase, aragonite saturation state received a reciprocal transformation. For the settlement phase, water temperature, pH, and aragonite saturation state received a reciprocal transformation. Lastly, for the juvenile phase, dissolved inorganic carbon received a reciprocal transformation, and aragonite saturation state was log transformed. The Shapiro-Wilkes test was analyzed in *stats* (R Core Team, 2022), and the Levine's test was conducted with *car* (Fox and Weisberg, 2019).

To detect differences in the physiological responses of larvae to multiple environmental stressors, linear mixed effects models and posthoc tests were used with the same R packages described above. pH and temperature were treated as separate fixed effects, with two levels each, and larval age was included as a fixed effect. Aquarium replicate was included as a random factor to account for any aquaria effects. Post-hoc tests were not performed when 3-way interactions were detected. Instead, effects were interpreted from plots of the data. Model selection was performed incrementally, following Burnham and Anderson (2002). Two response variables were transformed to satisfy the assumption of normality for the Shapiro-Wilkes test: lipid peroxidation was square root transformed, and cumulative survival was log transformed. After the completion of all transformations, all response variables satisfied the assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity. The Shapiro-Wilkes test was analyzed in *stats* (R Core Team, 2022), and the Levine's test was conducted with *car* (Fox and Weisberg, 2019).

Similar to the statistical tests used to detect physiological differences during the larval stage, a linear mixed effects model was used to detect physiological differences between the three remaining treatments (i.e., the Multiple Stressors treatment was absent) in the settlement stage. The three treatment conditions were coded as factors as well as oyster age because sampling occurred at the beginning and end of the settlement phase. Aquarium replicates were included as a random factor to account for any aquaria effects. Model selection was performed incrementally, following Burnham and Anderson (2002). Several response variables were transformed to satisfy the assumption of normality for the Shapiro-Wilkes test: total antioxidant capacity and lipid peroxidation were log transformed, and shell length was reciprocal transformed. After the completion of all transformations, all response variables satisfied the assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity. The Shapiro-Wilkes test was analyzed in *stats* (R Core Team, 2022), and the Levine's test was conducted with *car* (Fox and Weisberg, 2019).

A linear mixed effects model and post hoc tests, using the same R packages as described above, were used to detect potential carryover effects from the larval experience on juvenile oyster physiology. Larval and juvenile treatment conditions were included as separate fixed effects, with three and four levels each, respectively. Sampling week was also included as a factor. Juvenile aquarium replicate was included as a random factor to account for any aquaria effects. Post-hoc tests were not performed when 3-way interactions were detected. Instead, effects were interpreted from plots of the data. Furthermore, contrasts did not include the Multiple Stressors juvenile treatment because the treatment was not carried all the way through the experiment. Model selection was performed incrementally, following Burnham and Anderson (2002). All but one response variable, oxygen consumption standardized by total protein, were transformed to satisfy the assumption of normality for the Shapiro-Wilkes test: lipid peroxidation, total protein content, triglyceride content, and shell length were log transformed, oxygen consumption standardized per individual and percent survival were square root transformed, and total antioxidant capacity was reciprocal transformed. After the completion of all transformations, all response variables satisfied the assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity. The Shapiro-Wilkes test was analyzed in *stats* (R Core Team, 2022), and the Levine's test was conducted with *car* (Fox and Weisberg, 2019).

Results

1. Treatment Water Conditions

    a. Larval phase

Expected differences in temperature, pH, aragonite saturation state (Ω_{ar}), and dissolved inorganic carbon (DIC) among the four larval treatments were confirmed (Table 1). Temperature was significantly greater in the high temperature treatments (i.e., High Temperature and Multiple Stressors) compared to the ambient temperature treatments (i.e., Control and Low pH) by more than 3.62°C (Tables 1, 2). pH was significantly higher in the ambient pH treatments (i.e., Control and High Temperature) compared to the acidic treatments (i.e., Low pH and Multiple Stressors) by more than 0.5 units (Tables 1, 2). Due to the differences in pH, DIC was greater and Ω_{ar} was lower in the low-pH treatments compared to the ambient pH treatments by more than 73 µmol kg^{-1} and 0.23 units, respectively (Tables 1, 2). Salinity was similar among treatments (Tables 1, 2). Beyond expected differences among treatment conditions, dissolved oxygen was significantly lower in the high temperature treatment compared to ambient by more than 0.81 mg L^{-1}, but average conditions for each treatment were ≥6.90 mg L^{-1} (Tables 1, 2). Total alkalinity was 1146 ± 62 µmol kg^{-1} (mean ± standard error) during the larval stage.

b. Settlement phase

Anticipated differences in treatment conditions during the settlement phase were confirmed among treatments for temperature, pH, DIC, and Ω_{ar} (Table 1). Temperature was greater by more than 3.5°C in the High Temperature treatment compared to the other two treatments (Tables 1, 2). pH was significantly different between pairs of all three treatments with the greatest difference of 0.58 units between Control and Low pH, and the smallest difference of 0.12 units between the Control and High Temperature treatments (Tables 1, 2). DIC and Ω_{ar} were significantly different between the low-pH treatment and the two ambient-pH treatments, differing by more than 44 μmol kg^{-1} and 0.23 units, respectively (Tables 1, 2). Aside from anticipated differences, salinity and dissolved oxygen differed between the High Temperature treatment and each of the two ambient-temperature treatments by 0.23 units and 1.24 mg L^{-1}, respectively, but average conditions for each treatment were greater than 6.50 mg L^{-1} (Tables 1, 2). Total alkalinity was 1004 ± 2.59 μmol kg^{-1} (mean ± standard error) during the settlement phase.

c. Juvenile phase

As expected, differences in temperature, pH, DIC, and Ω_{ar} among treatments were confirmed (Table 1). Temperature was greater by more than 3.5°C in the High Temperature treatments compared to ambient (Tables 1,2). pH was lower in the low-pH treatments compared to ambient treatments by more than 0.49 units (Tables 1,2). DIC and Ω_{ar} similarly differed among the low- and ambient-pH treatments by 59 μmol kg^{-1} and 0.28 units, respectively (Tables 1,2). Aside from anticipated differences, salinity differed between the High Temperature treatment and the three other treatments by less than 0.12 units, and dissolved oxygen differed between the Control treatment and the two low-pH treatments by more than 0.98 mg L^{-1}, but average conditions for each treatment were greater than 6.60 mg L^{-1} (Tables 1,2). Total alkalinity was 949 ± 1 μmol kg^{-1} (mean ± standard error) during the juvenile phase.

2. Effects of multiple environmental stressors on oyster physiology during the larval and settlement phases

Shell length and total protein content were analyzed as proxies of shell and tissue growth, respectively, to observe changes under multiple environmental stressors. Larval age significantly interacted with both temperature and pH, independently (Table 3). Larval shells grew faster under warmer water temperatures compared to ambient conditions, resulting in 15% larger shell lengths by 8 dpf, while acidified conditions had a slightly negative impact around day 2 but was diminished by 4 dpf (Figure 3a). A significant interaction between treatment conditions and oyster age was also observed during the settlement phase (Table 4). Initially, the shell growth of eyed larvae (20 dpf) continued to be negatively affected by acidified conditions compared to ambient conditions but by the end of the settlement period, oysters in the Low pH treatment had larger shell lengths than the other two treatment conditions (Table 4, Figure 4a). During the larval phase, variation in total protein content was explained by a significant interaction between temperature and larval age, while pH had no significant effect (Table 3). Early in the larval phase, temperature likely did not impact the total protein of larvae, but by 8 dpf, those in the high-temperature treatments had almost double the total protein content compared to those in ambient conditions, on average (Figure 3b). By the settlement phase, only oyster age had a significant effect on total protein content, with oysters quadrupling their protein content by 32 dpf. Treatment conditions had no significant effect on protein content during the settlement phase (Table 4, Figure 4b).

Total antioxidant capacity and lipid peroxidation were measured to observe how multiple environmental stressors during the larval and settlement stages may induce oxidative stress and the corresponding cellular response. During the larval phase, total antioxidant capacity in larvae at warmer temperatures was significantly lower by 15% compared to that of larvae in ambient temperatures, and total antioxidant capacity, regardless of treatment conditions, decreased over time (Table 3, Figure 3c). pH did not significantly affect larval total antioxidant capacity (Table 3). By the settlement phase, there was a significant interaction between treatment conditions and oyster age (Table 4), likely from the increase in total antioxidant capacity in oysters exposed to the Low pH treatment at the end of the settlement phase (Figure 4c). This interaction, however, was weak as indicated by the lack of significance of contrasts during post hoc testing (Table 4). Lipid peroxidation was not significantly affected by environmental conditions (i.e., temperature and pH) during the larval stage but significantly changed with age (Table 3). Regardless of treatment conditions, lipid peroxidation began to plateau around 4 dpf after a sharp increase in levels (Figure 3d). There was a weak interaction between treatment and oyster age on lipid peroxidation levels during the settlement phase (Table 4), again, likely driven by an increase in peroxidation levels in individuals experiencing the Low pH treatment (Figure 4d).

To understand how long-term energy stores were affected during the larval and settlement stages by multiple environmental stressors, triglyceride content was measured. Temperature and pH had an interactive effect on triglyceride content during the larval stage (Table 3). Specifically, triglyceride content was negatively affected by warmer temperatures when larvae were already experiencing acidified conditions (Table 3, Figure 3e), but this effect was not detected under control pH conditions. pH also significantly interacted with larval age (Table 3); triglyceride content of larvae under ambient conditions decreased over time, while that in larvae in low-pH conditions increased (Figure 3e). By the settlement phase, treatment effects were no longer detectable (Table 4). The only noticeable affect was the significant decline in triglyceride content over the duration of settlement (Table 4, Figure 4e).

Cumulative survival through 8 days-post-fertilization (8 dpf) during the larval phase was significantly affected by both temperature and pH, individually (Table 3, Figure 3f). High temperature and Low pH decreased cumulative survival by 66% and 71%, respectively. During the settlement phase, there was a significant interaction between treatment condition and oyster age (Table 4). At 20 dpf, percent survival was similar among the treatment conditions, but by the end of the settlement phase, survival in the Low pH treatment had decreased by 44% from the start of settlement and was significantly lower than in the other two treatments (Table 4, Figure 4f).

3. Carryover effects of multiple environmental stressors on juvenile oyster physiology

Juvenile oyster physiology was affected by both previous larval treatment experience and direct juvenile exposures, and some of these effects varied between the two sampling timepoints during the juvenile phase (Table 5). Specifically, a three-way interaction between juvenile treatment, larval treatment, and sampling timepoint explained a significant amount of variation in shell length (Table 5). After a week in juvenile treatment conditions, oysters that remained in the High Temperature and Low pH treatments for the larval, settlement, and juvenile phases had longer shell lengths than those in all other combinations of larval and juvenile treatment conditions; however, after four weeks in the juvenile treatment conditions, there were no obvious effects of either juvenile or previous larval treatment conditions (Figures 5a,b). There was a weak interactive effect between larval treatment and juvenile treatment on total protein content in juvenile oysters (Table 5). Similar to shell growth, juvenile oysters that had experienced the High Temperature treatment from fertilization through the juvenile phase of the experiment had significantly more total protein than oysters that had previously experienced the Control treatment as larvae and then the High Temperature treatment as juveniles (Table 5, Figures 5c,d). Variation in total protein content was explained by a significant interaction between larval treatment and sampling timepoint (Table 5). While juveniles that experienced the Control treatment conditions as larvae increased their total protein content between the two sampling points, those that had been exposed to the Low pH treatment as larvae only increased their total protein content slightly (Table 5, Figures c,d).

The oxidative stress response defense and damage molecules increased significantly over the juvenile phase (Table 5). Total antioxidant capacity increased by 89%, and lipid peroxidation increased by 69% (Figures 5e,f). While juvenile total antioxidant capacity did not vary significantly among larval or juvenile treatments, lipid peroxidation in juvenile oysters was affected by an interaction between previous larval treatment and present juvenile treatment (Table 5). Specifically, for juvenile oysters in the High Temperature juvenile treatment, those that experienced the Control and Low pH treatments as larvae had significantly more lipid peroxidation than those in the High Temperature treatment as larvae (Table 5, Figure 5g,h).

Triglyceride content in juvenile oysters was significantly affected by a three-way interaction between larval treatment, juvenile treatment, and sampling timepoint (Table 5). After one week of residing in their assigned juvenile treatment conditions, juveniles in the Control treatment, regardless of previous experience, had increased triglyceride content compared to those in the other three juvenile treatments (Figure 5i). This pattern continued after four weeks only for juveniles that had experienced the Low pH treatment during the larval and settlement stages (Figure 5j). Furthermore, juveniles that previously experienced the Control treatment during the larval and settlement stages had more triglyceride across the juvenile treatments after one week in treatments, except for those in the High Temperature treatment, compared to oysters that experienced the two other larval treatments (Figure 5i). This pattern switched after four weeks, with juvenile oysters in the High Temperature treatment that had experienced the Control treatment as larvae having elevated triglyceride content (Figure 5j).

Oxygen consumption rates, standardized either to total protein or per individual oyster, were not affected by larval treatment but were affected by a significant interaction between juvenile treatment conditions and sampling time point (Table 5). Though significant contrasts were not detected during posthoc testing, there were some patterns that emerged (Table 5). Initially, oxygen consumption was similar across all juvenile treatment conditions but after four weeks, oysters in the acidic treatments (i.e., Low pH and Multiple Stressor) had lower rates of oxygen consumption compared to the Control treatment (Figures 5k,l).

Lastly, cumulative percent survival after four weeks in treatment conditions was only affected by the juvenile treatment, not previous larval experience (Table 5). Specifically, oysters in the acidified treatments (i.e., Low pH and Multiple Stressor) had significantly lower survival compared to those at ambient pH (i.e., Control and High Temperature; Table 5, Figure 6).

Discussion

Conditions of ocean acidification and ocean warming did affect the larval stage of the Eastern oyster and carry over into the early juvenile stage, though these effects were nuanced and context dependent. Overall, acidified conditions had more pronounced effects on oyster physiology compared to ocean warming. The effects from conditions of acidification were more persistent and detrimental than those of ocean warming, which were generally more positive and transient. Specifically, oysters were lethally affected by conditions of acidification across all three life stages, while warming only negatively affected survival during the larval stage (Tables

3 – 5, Figures 3f, 4f, 6). Juvenile oysters experienced carryover effects associated with both warming and acidification; however, carryover effects associated with the simultaneous experience of both stressors during the larval stage were unable to be evaluated due to the complete mortality of larval oysters in the Multiple Stressor treatment around 11 days-post-fertilization (dpf). The remainder of the study focused on potential carryover and direct effects from each stressor individually. Negative carryover effects were more likely when larvae experienced acidified conditions, most notably demonstrated by the total protein and lipid peroxidation data (Table 5, Figures 3c-d, g-h). Carryover effects were also evident in two other physiological metrics - shell growth and triglyceride content. Juvenile shell growth was positively affected by the larval experience of both environmental stressors, while triglyceride content in juvenile oysters was negatively affected by larval experience of warming (Table 5, Figures 5a-b, i-j). Though several carryover effects were found, most of them were minimal, except for total protein, and some were not detectable by the end of the experiment whether due to acclimatization, or potentially, selective mortality.

Shell growth was minimally impacted by the larval environmental experience of both acidified and warming conditions likely because of context-dependent carryover effects. Within the first week of the juvenile stage, oyster shell growth was positively affected by prior acidification experience, as well as warming, in contrast with other carryover effect studies where the shell growth of juvenile oysters was negatively affected by early acidification exposure (Hettinger et al., 2013, 2012). The positive effect seen here was specifically for oysters whose juvenile treatment matched their larval treatment, for both acidification and warming conditions (Table 5, Figure 5a). This instance of carryover effects in shell growth could be a case of context-dependent carryover effects, effects that only manifest when organisms are placed into the right combination of environmental stressors after their early stress experience (Donelan et al., 2021). Context-dependent carryover effects were observed in juvenile Eastern oysters when exposed to specific combinations of hypoxia and warming two months after their initial exposure to environmental stress (Donelan et al., 2021). In the present study, the context-dependent carryover effects for shell growth did not persist through the first four weeks of the juvenile stage. For juvenile oysters that experienced different treatment conditions, and by the end of the experiment (4 weeks), shell length was similar among juvenile oysters in the various combinations of larval and juvenile treatments (Table 5, Figure 5a,b). Although the positive carryover effects to shell growth were transient, other carryover effects were more persistent.

Conditions of ocean acidification caused persistent, sub-lethal effects to the larval oysters' cellular stress response and energy allocation that affected juvenile oyster physiology. Specifically, total protein content was lower and lipid peroxidation was higher for juvenile oysters that experienced acidification conditions as larvae, regardless of their current environmental conditions (Table 5, Figures 5c,d,g,h). Oysters can be susceptible to increased lipid peroxidation from oxidative stress under ocean acidification at ambient temperatures (Matoo et al., 2013; Tomanek et al., 2011), as well as decreased protein accretion due to increased protein degradation (Frieder et al., 2018). Lower total protein content and higher lipid peroxidation levels under acidified conditions were both likely initiated during the larval stage, though they were not statistically significant among pH levels (Tables 3, Figures 3b,d). These negative physiological effects were also observed in the juvenile oysters (Figures 3c-d, g-h). Sustained negative effects from larval acidification experience have been reported in previous studies (Hettinger et al., 2013, 2012, Shen et al., 2022). In Pacific abalone (*Haliotis discus hannai*), larval exposure to hypoxia did not impact juvenile shell growth but did decrease juvenile weight and survival, similar to the present study, and these effects were attributed to bioenergetic shifts between stress tolerance and growth (Shen et al., 2022), which could also explain the results found here.

Persistent carryover effects may be due to a bioenergetic shift to prioritize and maintain shell growth at the continued expense of other physiological processes (Table 5, Figure 5). When organisms experience stressful conditions, abiotic or biotic, they generally prioritize basal metabolic processes that are necessary for survival, at the expense of other important biological processes like growth and reproduction (Sokolova et al., 2012). Within this framework, shell growth is not considered a process necessary for survival; however, this framework was meant as a generalization for marine invertebrates to explore potential effects of multiple environmental stressors at the organismal level (Sokolova et al., 2012). As the majority of the organismal life cycle is dedicated to the adult stage, the bioenergetic framework provided above likely pertains mostly to adult invertebrates rather than trying to accommodate the complex nuances of the shorter, early life history stages. As this framework pertains to the present study, processes necessary to survive during the early life history stages of the Eastern oyster may have evolved to prioritize shell growth since that is a larva's main means of protection and survival against predation as well as poor environmental conditions (Pruett and Weissburg, 2019; Shumway, 1996). Here, juvenile oysters transitioning out of the settlement stage maintained shell growth, regardless of environmental conditions, which may have negatively affected other aspects of their physiology, like tissue growth (Figures 4a-b, 5a-d), because maintaining shell growth under acidified conditions requires more energy (Frieder et al., 2017; Zhao et al., 2017).

There were several indications that energy demands were higher in oysters with previous experience of acidified conditions. First, at the end of settlement, oysters in the Low pH treatment had significantly higher levels of lipid peroxidation from oxidative stress (Table 4, Figure 4d), which can be caused by an increase in metabolic processes (Abele et al., 2007). This pattern carried over into the juvenile stage (Table 5), lasting through the end of the experiment (Figures 5g-h). Second, triglyceride content, after one week in juvenile treatment conditions, was lower in oysters that had previous environmental stress experience, including conditions of acidification (Table 5, Figure 5i). Triglycerides are one of the main classes of lipids that oysters and other marine invertebrates use to store energy (Giese, 1966). Declines in triglyceride content have been linked to increased metabolism in marine bivalves, including adult *C. virginica*, when experiencing environmental stressors (Ivanina et al., 2013; Laudicella et al., 2020; Pernet et al., 2007; Rayssac et al., 2010). The higher energy demands of oysters with previous experience of acidified conditions did not persist throughout the juvenile stage, however; the negative carryover effects on triglyceride content found in the present study were undetectable after four weeks of the juvenile stage, suggesting that oysters acclimatized to juvenile treatment conditions (Figure 5j). Because the oysters in this study were fed *ad libitum*, these results suggest that juvenile oysters may be able to compensate for negative carryover effects during summer months when food sources are abundant. Under a food-limited situation, however, the carryover effects in this study may have different outcomes, possibly detrimental, as shown in other oyster species and bivalves (Cole et al., 2016; Ramajo et al., 2016; Shen et al., 2022; Thomsen et al., 2013). If low food availability overlaps with a period of energetically demanding developmental processes (i.e., early juvenile shell growth), in addition to carryover effects from environmental stress that deplete energy stores and increase cellular stress and damage, then mortality may increase from an inability to meet energy demands (Pan et al., 2015).

Warming had a cross-tolerance effect during the larval stage that carried over into the juvenile stage. In general, increased temperatures were more influential on larval physiology than acidified conditions, but the effects diminished by the juvenile stage. Temperature positively affected oyster physiology during the larval stage, as shown through increased shell and tissue growth rates and decreased total antioxidant capacity, which likely indicates lower oxidative stress experience (Table 3, Figures 3a-c). Furthermore, when comparing the effects of warming in addition to acidification (i.e., Low pH and Multiple Stressor), warming ameliorated the negative effects caused by acidified conditions under ambient temperatures (Figures 3a-c). The ameliorative impact of warming under ocean acidification has been identified in oysters and other marine invertebrates and is likely due to the presence and abundance of heat shock proteins (Czaja et al., 2023; Ko et al., 2014; Matoo et al., 2013). Sessile, marine invertebrates living in highly thermally variable or intertidal environments have an abundance of heat shock proteins that aid in protection from thermal stress caused by the increase in metabolism (Dutton and Hofmann, 2009; Hofmann and Todgham, 2010). Heat shock proteins are also one of the larger components of the general cellular stress response (Kültz, 2005), which is why heat exposure has been observed to make organisms more tolerant to additional or subsequent stressors (Agrawal and Jurgens, 2023; Matoo et al., 2013; Todgham et al., 2005). As part of the cellular stress response, heat shock proteins can respond to oxidative stress damage (Feder and Hofmann, 2002), which is potentially why larvae exposed to warmer temperatures, Multiple Stressor treatment included, in the present study had less lipid damage and less of a need to produce antioxidant molecules (Figures 3c-d). The cross-tolerance protection from lipid damage did carry over, lasting through the first four weeks of the juvenile stage (Table 5, Figures 5g-h), unlike shell growth.

Several carryover effects observed within the first week of the juvenile stage were lost after four weeks in juvenile treatment conditions, which could have resulted from compensation through acclimatization; however, an alternative explanation is that selective mortality, rather than compensatory mechanisms, may be underpinning the lack of detection, or loss, of carryover effects from both conditions of acidification and warming. Selective mortality occurs when certain genotypes are non-advantageous under specific external stressors, including environmental stress, and are then non-randomly selected against through mortality, leaving genotypes better suited for the present conditions (Chapter 2; Gardner and Skibinski, 1991). Juvenile survival after four weeks in treatment conditions was negatively affected by conditions of acidification, and though not significant, survival was 28% lower, on average, in the High Temperature treatment compared to the Control treatment (Table 5, Figure 6). Selective mortality and low survival of juveniles in the environmental stress treatments after four weeks may have resulted in the disappearance of the positive carryover effects on juvenile shell growth, especially if shell growth was too energetically taxing under the continued exposure to environmental stress (Figures 5a-b). Similarly, the loss of carryover effects on juvenile triglyceride at the end of the experiment could potentially be a product of selective mortality since the carryover effects were likely related to high energy demands potentially depleting energy stores (Table 5, Figures i-j). Additionally, the shift to lower respiration rates in juvenile oysters experiencing acidic conditions by the last week of the experiment (Table 5, Figures 5k-l) could be explained by selective mortality of individuals with unsustainable energy demands under environmental stress. Individuals that were able to maintain physiological processes under lower metabolic costs when exposed to stressful environmental conditions may have been preferentially selected for over those unable to meet energy demands, especially when long-term energy stores were already depleted during the larval phase.

Eastern oysters from the mesohaline region of the Chesapeake Bay are robust to conditions of acidification and warming (Figure 2) even though both environmental stressors do affect the physiology of young Eastern oysters, and these effects carry over into the early juvenile stage. Conditions of acidification were more lethal to oysters than warming, though this may differ at other temperature and acidification levels, especially as oysters reach their thermal limit (Pörtner, 2010). Carryover effects from acidified conditions compared to warming were more persistent and mostly negative, likely due to the initiation of negative effects during the larval stage. Warming also has a cross-tolerant effect during the larval stage to the addition of acidification, as well as during the juvenile stage to any sequential environmental stressor. The ability for heat exposure to increase an organism's tolerance to additional stressors may be due to increased metabolic rates and pools of heat shock proteins that can double as oxidative stress protection (Agrawal and Jurgens, 2023). Overall, whether from selective mortality, compensation, or acclimatization, juvenile Eastern oysters were able to recover from negative larval carryover effects of warming and acidification within the first four weeks of the juvenile stage, which may indicate they could tolerate similar scenarios of long-term acidification and warming *in situ*. As this is one of the first attempts to observe within-generation carryover effects in juvenile oysters from the larval experience of multiple environmental stressors, future studies should focus on potential cross-tolerant carryover effects of warming, duration of carryover effects from acidified conditions beyond the first month of the juvenile stage, and how these results change under different temperature and pH values. To understand the implications of carryover effects associated with multiple environmental stressors on future oyster populations, future studies should observe how the simultaneous larval experience of warming and acidification may carry over, as well as how the relative influence of carryover effects shifts under different ecologically relevant temporal exposures like diel and seasonal cycles and asynchronous versus concurrent stressor experiences.

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Tables

Table 1. *Analysis of water conditions for the larval, settlement, and juvenile phases of the experiment. Comparisons were made using mixed effects linear models, with treatment as a fixed effect and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) from final reduced models are reported. Tukey post-hoc tests were performed where appropriate, and test statistics z and p-values (p) are reported. Asterisks indicate statistical significance below an alpha threshold of 0.05. Treatment conditions: CN – Control, HT – High Temperature, LP – Low pH, and MS – Multiple Stressor.*

Water condition	Chi-square	p	Post hoc comparison	Z value	p
Larval Phase					
Temperature	$X^2_{(3,12)}$ = 2263.30	<0001*	CN – HT	32.549	<0.001*
			CN – LP	-1.326	1.000
			CN – MS	33.369	<0.001*
			HT – LP	33.875	<0.001*
			HT – MS	-0.819	1.000
			LP – MS	-34.694	<0.001*
Salinity	$X^2_{(3,12)}$ = 3.162	0.367			
Dissolved Oxygen	$X^2_{(3,12)}$ = 54.058	<0.001*	CN – HT	-4.976	<0.001*
			CN – LP	-0.120	1.000
			CN – MS	-5.512	<0.001*
			HT – LP	-4.856	<0.001*
			HT – MS	0.537	1.000
			LP – MS	5.393	<0.001*
pH_{tot}	$X^2_{(3,12)}$ = 436.57	<0.001*	CN – HT	1.362	1.000
			CN – LP	-13.984	<0.001*
			CN – MS	-14.139	<0.001*
			HT – LP	15.346	<0.001*
			HT – MS	15.501	<0.001*
			LP – MS	0.155	1.000
DIC	$X^2_{(3,12)}$ = 789.50	<0.001*	CN – HT	-1.119	1.000
			CN – LP	19.877	<0.001*
			CN – MS	18.672	<0.001*
			HT – LP	-20.997	<0.001*
			HT – MS	-19.791	<0.001*
			LP – MS	1.206	1.000
$\Omega_{aragonite}$	$X^2_{(3,12)}$ = 424.19	<0.001*	CN – HT	-1.352	1.000
			CN – LP	23.640	<0.001*
			CN – MS	19.404	<0.001*

			HT – LP	-24.992	<0.001*
			HT – MS	-20.755	<0.001*
			LP – MS	4.237	<0.001*
Settlement Phase					
Temperature	$X^2_{(2,9)} = 876.92$	<0.001*	CN – LP	0.469	1.000
			CN – HT	-25.408	<0.001*
			HT – LP	-25.877	<0.001*
Salinity	$X^2_{(2,9)} = 45.61$	<0.001*	CN – LP	-0.043	1.000
			CN – HT	5.827	<0.001*
			HT – LP	5.870	<0.001*
Dissolved Oxygen	$X^2_{(2,9)} = 25.018$	<0.001*	CN – LP	-0.737	1.000
			CN – HT	-4.653	<0.001*
			HT – LP	-3.916	<0.001*
pH_{tot}	$X^2_{(2,9)} = 311.59$	<0.001*	CN – LP	16.625	<0.001*
			CN – HT	3.175	0.004*
			HT – LP	-13.450	<0.001*
DIC	$X^2_{(2,9)} = 18.15$	<0.001*	CN – LP	4.115	<0.001*
			CN – HT	1.130	0.811
			HT – LP	-3.012	0.008*
$\Omega_{aragonite}$	$X^2_{(2,9)} = 243.31$	<0.001*	CN – LP	13.249	<0.001*
			CN – HT	-0.506	1.000
			HT – LP	-13.754	<0.001*
Juvenile Phase					
Temperature	$X^2_{(3,12)} = 1414.20$	<0.001*	CN – HT	25.493	<0.001*
			CN – LP	-0.597	1.000
			CN – MS	27.040	<0.001*
			HT – LP	26.090	<0.001*
			HT – MS	-1.547	0.731
			LP – MS	-27.638	<0.001*
Salinity	$X^2_{(3,12)} = 1414.20$	<0.001*	CN – HT	4.502	<0.001*
			CN – LP	-2.168	0.181
			CN – MS	0.204	1.000
			HT – LP	6.670	<0.001*
			HT – MS	4.298	<0.001*
			LP – MS	-2.372	0.106
Dissolved Oxygen	$X^2_{(3,12)} = 14.39$	0.002*	CN – HT	-1.812	0.420
			CN – LP	-2.642	0.049*
			CN – MS	-3.658	0.002*

			HT – LP	0.830	1.000
			HT – MS	1.847	0.389
			LP – MS	1.016	1.000
pH_{tot}	$X^2_{(3,12)} = 1090.00$	<0.001*	CN – HT	-1.932	0.320
			CN – LP	-24.180	<0.001*
			CN – MS	-24.361	<0.001*
			HT – LP	22.248	<0.001*
			HT – MS	22.429	<0.001*
			LP – MS	0.180	1.000
DIC	$X^2_{(3,12)} = 447.04$	<0.001*	CN – HT	0.763	1.000
			CN – LP	-14.921	<0.001*
			CN – MS	-14.179	<0.001*
			HT – LP	15.684	<0.001*
			HT – MS	14.942	<0.001*
			LP – MS	-0.742	1.000
$\Omega_{aragonite}$	$X^2_{(3,12)} = 802.47$	<0.001*	CN – HT	2.002	0.272
			CN – LP	-19.743	<0.001*
			CN – MS	-18.153	<0.001*
			HT – LP	21.745	<0.001*
			HT – MS	20.155	<0.001*
			LP – MS	-1.591	0.670

Table 2. *Water conditions of the larval, settlement, and juvenile phases of the experiment. Values represent mean ± standard error calculated across all treatment replicates over the duration of each phase. Temperature, salinity, dissolved oxygen, and* pH_{tot} *(pH, total scale) are measured values. DIC (dissolved inorganic carbon),* $\Omega_{calcite}$ *(saturation state of calcite), and* $\Omega_{aragonite}$ *(saturation state of aragonite) are calculated values.*

	Treatment			
Water Conditions	Control	Low pH	High Temperature	Multiple Stressors
Larval Phase				
Temperature (°C)	25.07 ± 0.35	24.93 ± 0.30	28.69 ± 0.61	28.78 ± 0.57
Salinity	12.94 ± 0.10	12.98 ± 0.12	12.89 ± 0.02	13.14 ± 0.20
Dissolved Oxygen (mg L^{-1})	7.88 ± 0.59	7.87 ± 0.64	7.06 ± 0.53	6.97 ± 0.75
pH_{tot}	7.54 ± 0.15	7.03 ± 0.10	7.59 ± 0.14	7.02 ± 0.10
DIC (µmol kg^{-1})	1056 ± 47	1133 ± 53	1051 ± 45	1129 ± 50
$\Omega_{aragonite}$	0.37 ± 0.03	0.13 ± 0.01	0.42 ± 0.03	0.14 ± 0.13
Settlement Phase				
Temperature (°C)	24.92 ± 0.29	24.86 ± 0.26	28.44 ± 0.51	-
Salinity	13.12 ± 0.19	13.11 ± 0.17	13.35 ± 0.20	-
Dissolved Oxygen (mg L^{-1})	7.76 ± 0.30	7.56 ± 0.38	6.52 ± 0.60	-
pH_{tot}	7.69 ± 0.10	7.11 ± 0.09	7.57 ± 0.15	-
DIC (µmol kg^{-1})	989 ± 18	1045 ± 19	1001 ± 23	-
$\Omega_{aragonite}$	0.39 ± 0.02	0.16 ± 0.01	0.42 ± 0.07	-
Juvenile Phase				
Temperature (°C)	24.97 ± 0.42	24.89 ± 0.29	28.49 ± 0.64	28.70 ± 0.51
Salinity	12.91 ± 0.08	12.87 ± 0.08	12.99 ± 0.10	12.91 ± 0.06
Dissolved Oxygen (mg L^{-1})	8.13 ± 0.22	7.08 ± 1.10	7.41 ± 0.30	6.68 ± 0.83
pH_{tot}	7.68 ± 0.12	7.15 ± 0.10	7.64 ± 0.14	7.14 ± 0.10
DIC (µmol kg^{-1})	926 ± 54	987 ± 57	923 ± 54	985 ± 58
$\Omega_{aragonite}$	0.45 ± 0.03	0.16 ± 0.01	0.50 ± 0.04	0.17 ± 0.02

Table 3. *Analysis of larval physiology. Comparisons were made using mixed effects linear models with pH, temperature, and age as a fixed effects and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) are reported from final reduced models. Tukey post-hoc tests were performed where appropriate, and test statistics z and p-values (p) are reported. Asterisks indicate statistical significance below an alpha threshold of 0.05.*

Physiological metric	Chi-square	p	Post hoc comparison	Z value	p
Shell Length					
*Temperature*Age*	$X^2_{(1,48)} = 10.617$	0.001*			
*pH*Age*	$X^2_{(1,48)} = 8.215$	0.004*			
pH	$X^2_{(1,48)} = 12.151$	<0.001*			
Temperature	$X^2_{(1,48)} = 0.117$	0.732			
Age	$X^2_{(1,48)} = 173.836$	<0.001*			
Total Protein Content					
*Temperature*Age*	$X^2_{(1,48)} = 10.982$	<0.001*			
pH	$X^2_{(1,48)} = 1.069$	0.301			
Temperature	$X^2_{(1,48)} = 0.502$	0.479			
Age	$X^2_{(1,48)} = 45.366$	<0.001*			
Total Antioxidant Capacity					
pH	$X^2_{(1,48)} = 1.046$	0.306			
Temperature	$X^2_{(1,48)} = 9.467$	0.002*			
Age	$X^2_{(1,48)} = 5.346$	0.021*			
Lipid Peroxidation					
pH	$X^2_{(1,48)} = 0.006$	0.939			
Temperature	$X^2_{(1,48)} = 3.191$	0.074			
Age	$X^2_{(1,48)} = 51.436$	<0.001*			
Triglyceride Content					
*pH*Temperature*	$X^2_{(1,48)} = 9.703$	0.002*	pH 7.8: 25°C vs. 29°C	2.011	0.177

			pH 7.2: 25°C vs. 29°C	6.416	<0.001*
			25°C: pH 7.8 vs pH 7.2	-0.742	1.000
			29°C: pH 7.8 vs pH 7.2	1.613	0.427
*pH*Age*	$X^2_{(1,48)} = 12.959$	<0.001*			
pH	$X^2_{(1,48)} = 0.550$	0.458			
Temperature	$X^2_{(1,48)} = 4.045$	0.044*			
Age	$X^2_{(1,48)} = 6.712$	0.010*			
Survival					
pH	$X^2_{(1,12)} = 5.811$	0.016*			
Temperature	$X^2_{(1,12)} = 3.999$	0.046*			

Table 4. *Analysis of oyster physiology during the settlement period. Comparisons were made using mixed effects linear models with treatment as a fixed effect and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) are reported. Tukey post-hoc tests were performed where appropriate, and test statistics z and p-values (p) are reported. Asterisks indicate statistical significance below an alpha threshold of 0.05. Treatment conditions: CN – Control, HT – High Temperature, and LP – Low pH. Oysters ages: 20 and 32 days-post-fertilization*

Physiological metric	Chi-square	p	Post hoc comparison	Z value	p
Shell Size					
*Larval Treatment*Age*	$X^2_{(2,18)} = 15.439$	<0.001*	CN: 20 vs 32	36.190	<0.001*
			HT: 20 vs 32	36.899	<0.001*
			LP: 20 vs 32	37.446	<0.001*
			20: CN vs HT	-1.588	1.000
			20: CN vs LP	-4.274	<0.001*
			20: HT vs LP	-3.384	0.006*
			32: CN vs HT	36.899	<0.001*
			32: CN vs LP	37.446	<0.001*
			32: HT vs LP	26.980	<0.001*
Larval Treatment	$X^2_{(2,18)} = 18.666$	<0.001*			
Age	$X^2_{(1,18)} = 1309.693$	<0.001*			
Total Protein Content					
Larval Treatment	$X^2_{(2,18)} = 2.719$	0.257			
Age	$X^2_{(1,18)} = 22.068$	<0.001*			
Total Antioxidant Capacity					
*Larval Treatment*Age*	$X^2_{(2,18)} = 7.907$	0.019*	CN: 20 vs 32	0.026	1.000
			HT: 20 vs 32	0.031	1.000
			LP: 20 vs 32	-1.666	0.862
			20: CN vs HT	-0.363	1.000
			20: CN vs LP	0.597	1.000
			20: HT vs LP	0.135	1.000
			32: CN vs HT	0.031	1.000
			32: CN vs LP	-1.666	0.862
			32: HT vs LP	-1.051	1.000
Larval Treatment	$X^2_{(2,18)} = 0.940$	0.625			
Age	$X^2_{(1,18)} = 0.001$	0.979			
Lipid Peroxidation					
*Larval Treatment*Age*	$X^2_{(2,18)} = 11.060$	0.004*	CN: 20 vs 32	1.910	0.505
			HT: 20 vs 32	1.205	1.000
			LP: 20 vs 32	-0.891	1.000
			20: CN vs HT	-0.456	1.000
			20: CN vs LP	0.641	1.000
			20: HT vs LP	0.107	1.000
			32: CN vs HT	1.205	1.000

			32: CN vs LP	-0.891	1.000
			32: HT vs LP	-1.129	1.000
Larval Treatment	$X^2_{(2,18)} = 1.823$	0.402			
Age	$X^2_{(1,18)} = 5.472$	0.019*			
Triglyceride Content					
Larval Treatment	$X^2_{(2,18)} = 2.210$	0.331			
Age	$X^2_{(1,18)} = 61.624$	<0.001*			
Survival					
*Larval Treatment*Age*	$X^2_{(2,18)} = 11.664$	<0.001*	CN: 20 vs 32	-1.379	1.000
			HT: 20 vs 32	0.546	1.000
			LP: 20 vs 32	3.166	0.014*
			20: CN vs HT	-0.482	1.000
			20: CN vs LP	0.757	1.000
			20: HT vs LP	0.159	1.000
			32: CN vs HT	0.546	1.000
			32: CN vs LP	3.166	0.014*
			32: HT vs LP	2.958	0.028*
Larval Treatment	$X^2_{(2,18)} = 1.561$	0.458			
Age	$X^2_{(1,18)} = 1.902$	0.168			

Table 5. *Analysis of juvenile physiology. Comparisons were made using mixed effects linear models with larval treatment, juvenile treatment, and juvenile sampling week as categorical variables and fixed effects and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) are reported from final reduced models. Tukey post-hoc tests were performed where appropriate, and test statistics z and p-values (p) are reported. Asterisks indicate statistical significance below an alpha threshold of 0.05. Larval Treatments: CN – Control, HT – High Temperature, and LP – Low pH.*

Physiological metric	Chi-square	p	Post hoc comparison	Z value	p
Shell Length					
*Juvenile Treatment *Larval Treatment*Week*	$X^2_{(6,72)} = 43.617$	<0.001*			
*Juvenile Treatment *Larval Treatment*	$X^2_{(6,72)} = 1.708$	0.944			
*Juvenile Treatment*Week*	$X^2_{(3,72)} = 9.025$	0.029*			
*Larval Treatment*Week*	$X^2_{(2,72)} = 2.386$	0.303			
Juvenile Treatment	$X^2_{(3,72)} = 4.568$	0.206			
Larval Treatment	$X^2_{(2,72)} = 3.235$	0.198			
Week	$X^2_{(1,72)} = 0.696$	0.404			
Total Protein Content					
*Juvenile Treatment*Larval Treatment*	$X^2_{(6,72)} = 13.340$	0.038*	Juvenile Control:		
			Larval CN – Larval HT	-1.825	0.611
			Larval CN – Larval LP	2.711	0.064
			Larval HT – Larval LP	0.511	1.000
			Juvenile High Temperature:		
			Larval CN – Larval HT	-3.558	0.003*
			Larval CN – Larval LP	2.033	0.378
			Larval HT – Larval LP	-1.429	1.000
			Juvenile Low pH:		
			Larval CN – Larval HT	-1.932	0.480
			Larval CN – Larval LP	1.529	1.000

			Larval HT – Larval LP	0.175	1.000
*Larval Treatment*Week*	$X^2_{(2,72)} = 7.800$	0.020*	CN: Week 1 vs Week 4	3.137	0.005*
			HT: Week 1 vs Week 4	0.483	1.000
			LP: Week 1 vs Week 4	2.713	0.020*
Juvenile Treatment	$X^2_{(3,72)} = 7.984$	0.046*			
Larval Treatment	$X^2_{(2,72)} = 20.837$	<0.001*			
Week	$X^2_{(1,72)} = 9.84$	0.002*			
Total Antioxidant Capacity					
Juvenile Treatment	$X^2_{(3,72)} = 4.112$	0.250			
Larval Treatment	$X^2_{(2,72)} = 5.584$	0.061			
Week	$X^2_{(1,72)} = 45.903$	<0.001*			
Lipid Peroxidation					
*Juvenile Treatment*Larval Treatment*	$X^2_{(6,72)} = 20.141$	0.003*	Juvenile Control:		
			Larval CN – Larval HT	1.943	0.468
			Larval CN – Larval LP	-1.365	1.000
			Larval HT – Larval LP	0.334	1.000
			Juvenile High Temperature:		
			Larval CN – Larval HT	5.528	<0.001*
			Larval CN – Larval LP	-1.574	1.000
			Larval HT – Larval LP	4.563	<0.001*
			Juvenile Low pH:		
			Larval CN – Larval HT	1.656	0.879
			Larval CN – Larval LP	-0.847	1.000
			Larval HT – Larval LP	0.527	1.000
Juvenile Treatment	$X^2_{(3,72)} = 7.220$	0.065			
Larval Treatment	$X^2_{(2,72)} = 11.055$	0.004*			
Week	$X^2_{(1,72)} =6.808$	0.009*			

Triglyceride Content					
*Juvenile Treatment*Larval Treatment*Week*	$X^2_{(6,72)} = 14.465$	0.025*			
*Juvenile Treatment*Larval Treatment*	$X^2_{(6,72)} = 18.711$	0.005*			
*Juvenile Treatment*Week*	$X^2_{(3,72)} = 9.797$	0.020*			
*Larval Treatment*Week*	$X^2_{(2,72)} = 11.301$	0.004*			
Juvenile Treatment	$X^2_{(3,72)} = 6.199$	0.102			
Larval Treatment	$X^2_{(2,72)} = 17.949$	<0.001*			
Week	$X^2_{(1,72)} = 2.625$	0.105			
Respiration (per ng Protein)					
*Juvenile Treatment*Week*	$X^2_{(3,72)} = 7.991$	0.046*	Week One:		
			Control – High Temperature	1.263	1.000
			Control – Low pH	-0.120	1.000
			High Temperature – Low pH	0.999	1.000
			Week Four:		
			Control – High Temperature	1.192	1.000
			Control – Low pH	2.267	0.140
			High Temperature – Low pH	1.997	0.275
Juvenile Treatment	$X^2_{(3,72)} = 5.472$	0.140			
Larval Treatment	$X^2_{(2,72)} = 2.381$	0.304			
Week	$X^2_{(1,72)} = 0.186$	0.667			
Respiration (per Individual)					
*Juvenile Treatment*Week*	$X^2_{(3,72)} = 18.228$	<0.001*	Week One:		
			Control – High Temperature	6.820	<0.001*
			Control – Low pH	5.200	<0.001*
			High Temperature – Low pH	3.222	0.008*

			Week Four:		
			Control – High Temperature	1.590	0.671
			Control – Low pH	1.605	0.651
			High Temperature – Low pH	1.845	0.391
Juvenile Treatment	$X^2_{(3,72)} = 3.442$	0.328			
Larval Treatment	$X^2_{(2,72)} = 3.398$	0.183			
Week	$X^2_{(1,72)} = 110.611$	<0.001*			
Survival					
Juvenile Treatment	$X^2_{(3,36)} = 68.744$	<0.001*	Control – High Temperature	-1.276	0.579
			Control – Low pH	-4.767	<0.001*
			Control – Multiple Stressors	-6.529	<0.001*
			High Temperature – Low pH	-3.492	0.003*
			High Temperature – Multiple stressors	-5.254	<0.001*
			Low pH – Multiple Stressors	-1.762	0.262
Larval Treatment	$X^2_{(2,36)} = 3.382$	0.184			

Figures

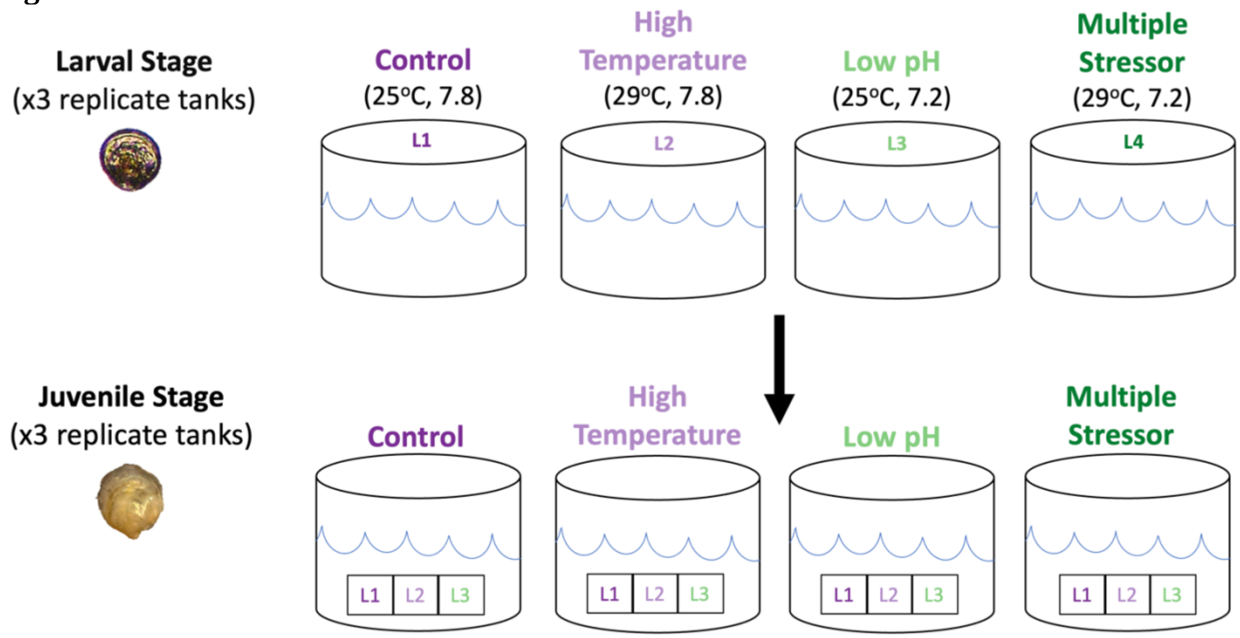


Figure 1. *The experimental design used to observe how exposure to conditions of ocean acidification and ocean warming may carry over to affect the physiology of juvenile oysters. Larvae were exposed to treatment conditions from fertilization through settlement and metamorphosis, after which oysters from each larval treatment were evenly divided and re-distributed amongst all four juvenile treatment conditions. Due to unforeseen culturing issues, larvae in the Multiple Stressor treatment died prior to settlement, and this treatment group was not represented in the juvenile stage of the experiment.*

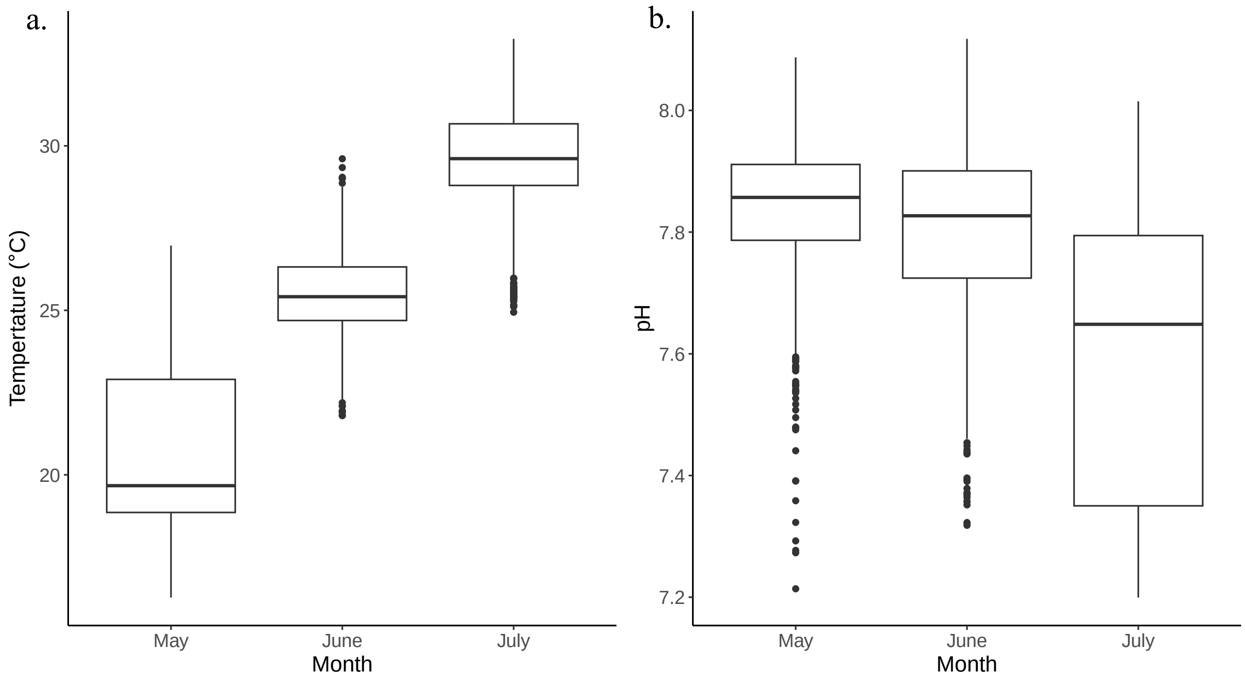


Figure 2. *Environmental data from the Rappahannock River that were used to design treatment conditions for the present study, specifically temperature (a) and pH (b). The box represents the interquartile range with the median black line indicating median monthly values. Outliers are indicated by black points. Data were collected by a local commercial hatchery partner in 2016 and 2017 during peak hatchery production months, which overlapped with peak spawning months for wild oyster populations in Chesapeake Bay. Treatment conditions were based on average and extreme conditions observed during the month of June to best match environmental conditions at the start of the experiment in June 2021, with the exception of extreme low pH, which was based on a pH of 7.2 seen in May and July.*

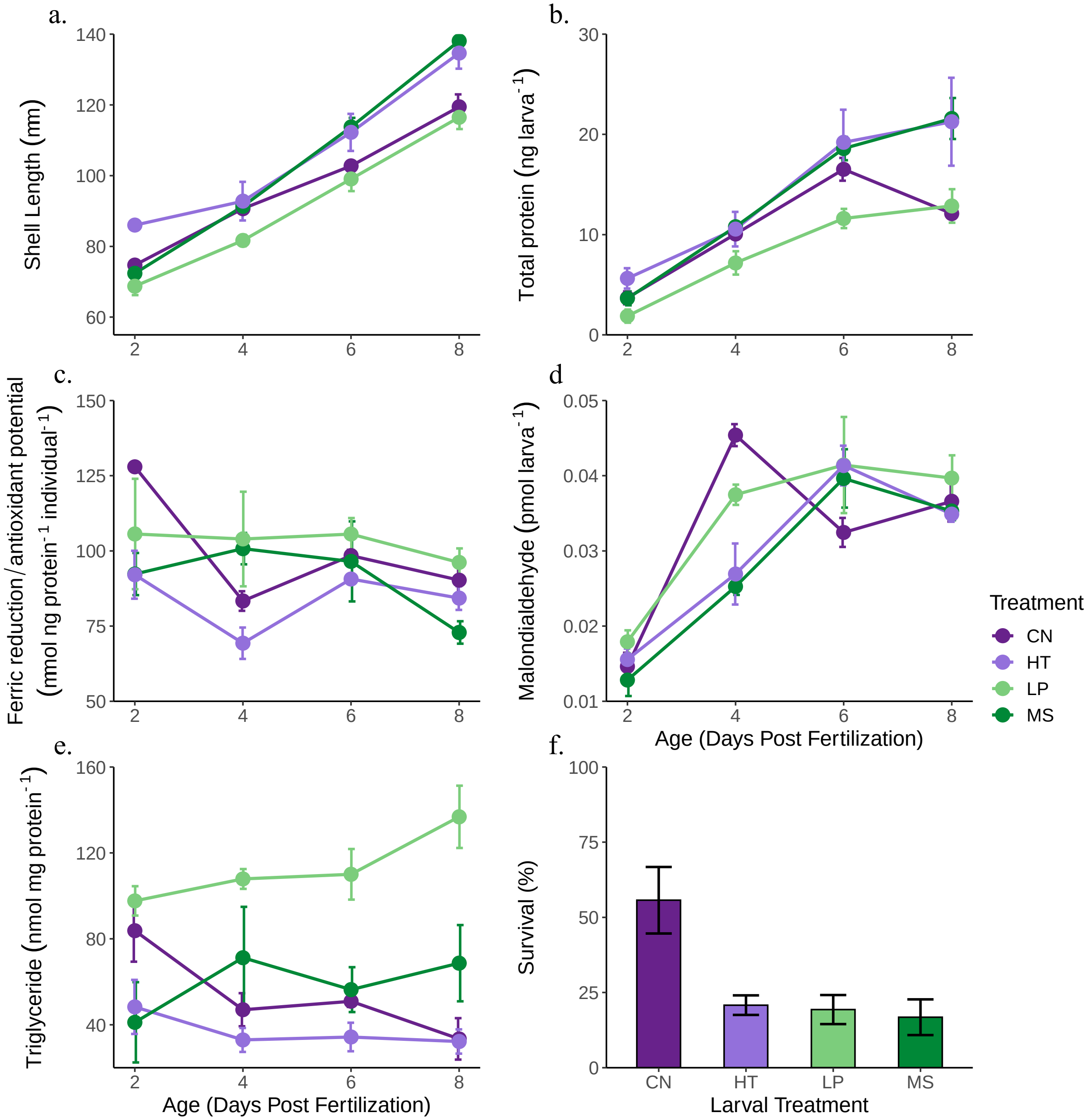


Figure 3. *Effects of multiple environmental stressors on larval Eastern oyster physiology. Larval shell length (a), total protein content (b), total antioxidant capacity (c), lipid peroxidation (d), triglyceride content (e), and cumulative survival (f) were measured through 8 dpf. Survival was 0% by 11 dpf for oysters in the Multiple Stressors treatment. Larvae were exposed to four treatment combinations of two pH and two temperature values representative of present-day average summertime ambient and future extreme conditions: Control (CN: 25°C, pH 7.8; dark purple), High Temperature (HT: 29°C, pH 7.8; light purple), Low pH (LP: 25°C, pH 7.2; light green), and Multiple Stressor (MS: 29°C, pH 7.2; dark green). Error bars represent standard error.*

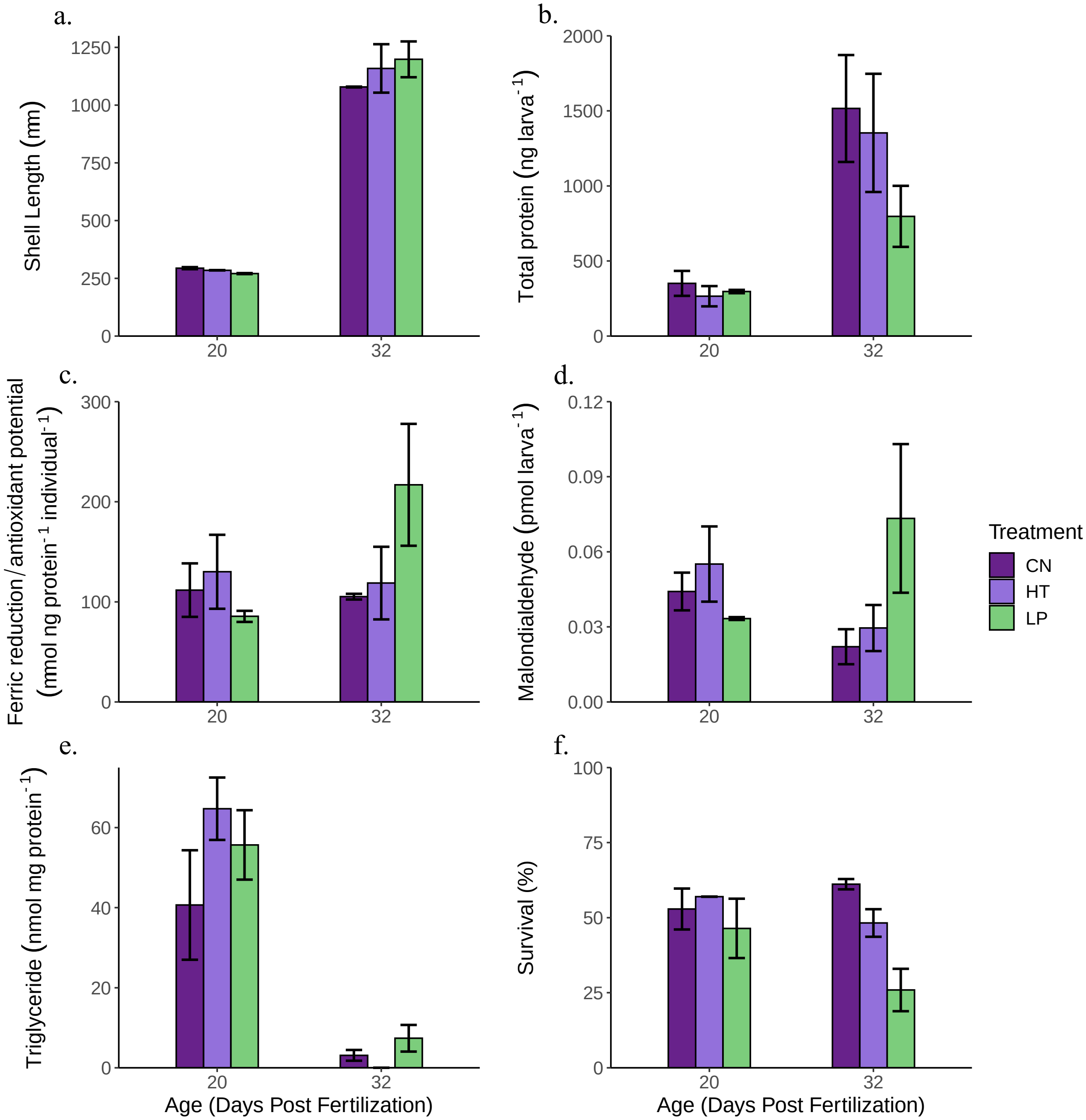
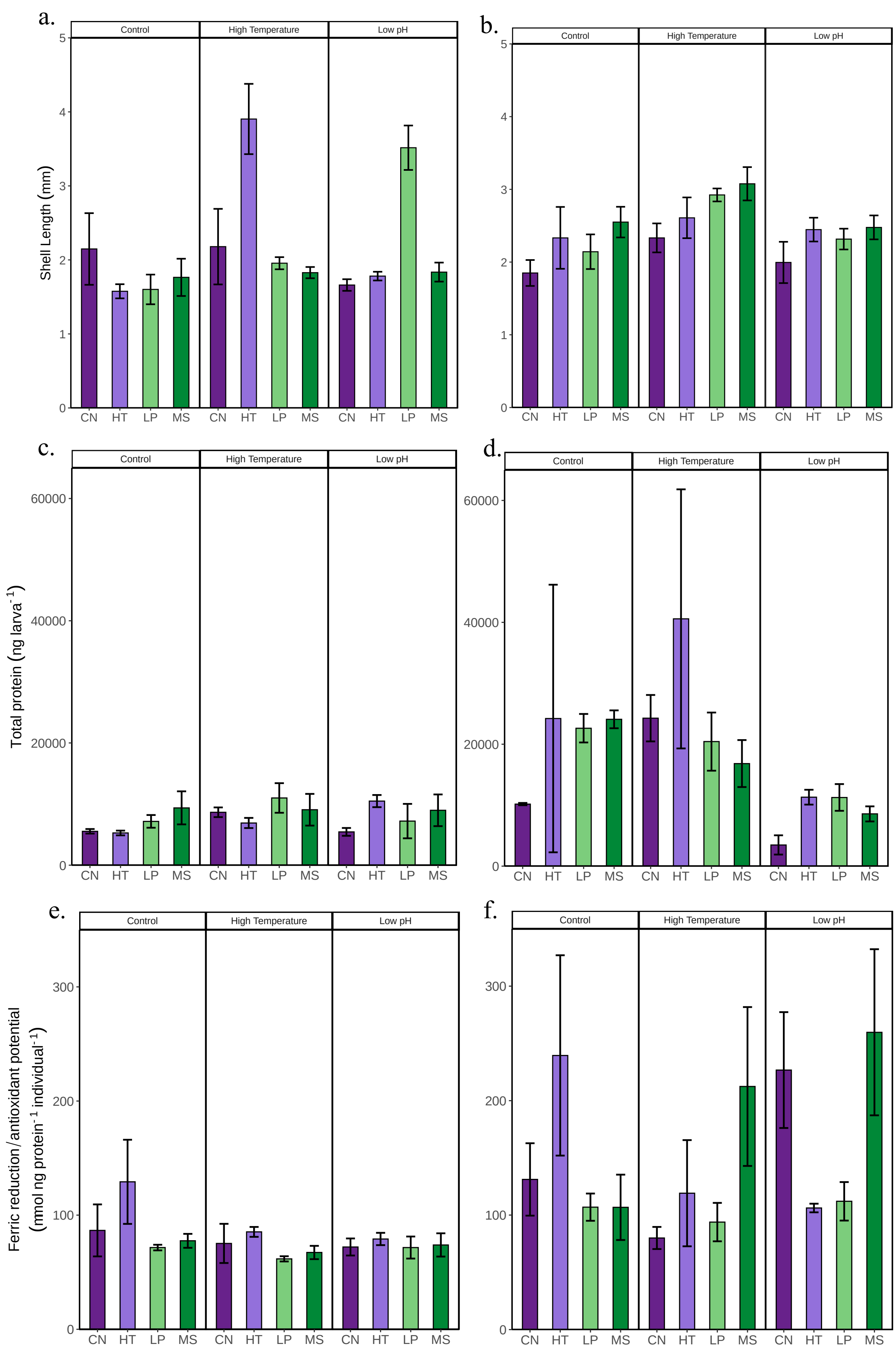
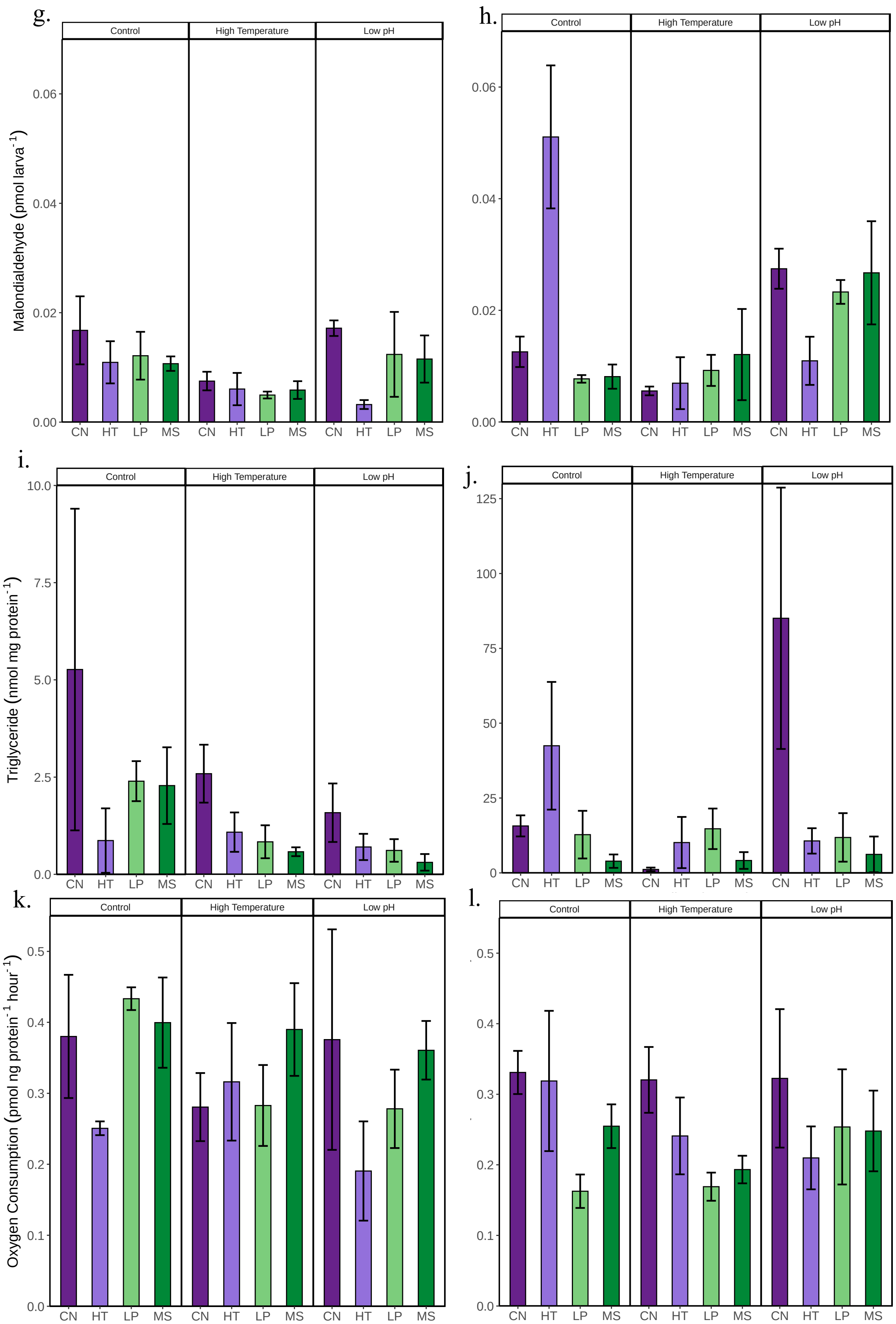


Figure 4. *Effects of multiple environmental stressors on Eastern oyster physiology during the settlement phase (i.e., settlement and metamorphosis). Shell length (a), total protein content (b), total antioxidant capacity (c), lipid peroxidation (d), triglyceride content (e), and percent survival (f) were measured through at the beginning (20 dpf) and end (32 dpf) of the settlement phase. Oysters were exposed from fertilization to four treatment combinations of two pH and two temperature values representative of present-day average summertime ambient and future extreme conditions: Control (CN: 25°C, pH 7.8; dark purple), High Temperature (HT: 29°C, pH 7.8; light purple), Low pH (LP: 25°C, pH 7.2; light green), but larvae in the Multiple Stressor (29°C, pH 7.2) treatment reached 100% mortality around 11 dpf. Error bars represent standard error.*


a.
b.
c.
d.
e.
f.
Control
High Temperature
Low pH
Shell Length (mm)
Total protein (ng larva^{-1})
Ferric reduction/antioxidant potential
(mmol ng protein^{-1} individual^{-1})
CN
HT
LP
MS



g.
h.
i.
j.
k.
l.
Control
High Temperature
Low pH
Malondialdehyde (pmol larva-1)
Triglyceride (nmol mg protein-1)
Oxygen Consumption (pmol ng protein-1 hour-1)
CN
HT
LP
MS

Figure 5. *Potential carryover effects of multiple environmental stressors from the larval and settlement stage experiences on Eastern oyster physiology during the juvenile stage. Shell length (a,b), total protein content (c,d), total antioxidant capacity (e,f), lipid peroxidation (g,h), triglyceride content (i,j), and percent survival (k,l) were measured after one (a,c,e,g,i,k) and four (b,d,f,h,j,l) weeks in treatment conditions. Oysters were exposed from fertilization through the settlement stage to four treatment conditions representative of present-day average summertime ambient and future extreme conditions, though the Multiple Stressor treatment failed around 11 dpf. After completion of the settlement stage, oysters from each remaining treatment were evenly divided and exposed to all four treatment conditions again to understand how the larval and settlement experience may affect how juvenile oysters respond to different environmental conditions. The juvenile treatments are expressed as colored bars: Control (CN: 25°C, pH 7.8; dark purple), High Temperature (HT: 29°C, pH 7.8; light purple), Low pH (LP: 25°C, pH 7.2; light green), and Multiple Stressor (29°C, pH 7.2). The juvenile responses are grouped into three panels labeled with the previous larval-through-settlement stage experience. Error bars represent standard error.*

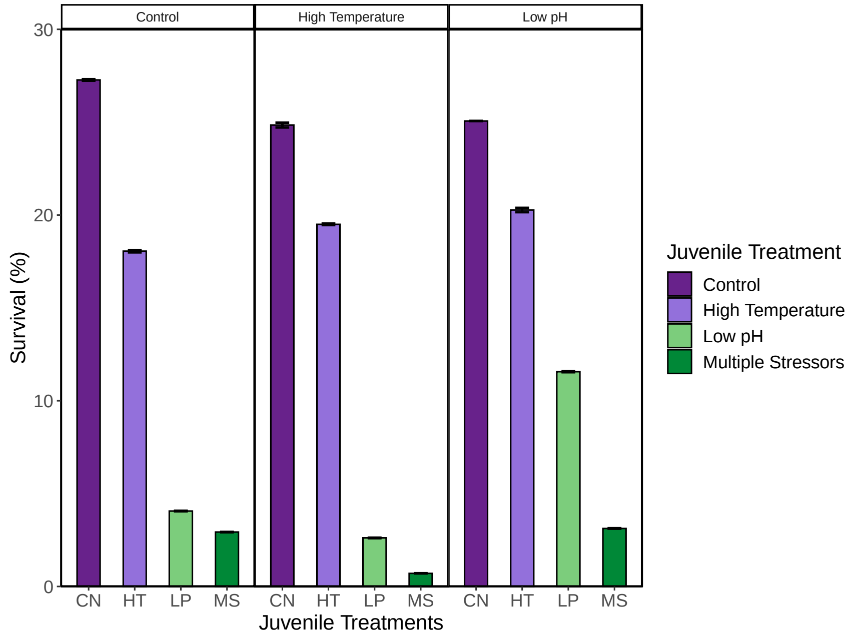


Figure 6. *Effect of previous larval and current juvenile experience of multiple environmental stressors on percent survival of juvenile Eastern oysters after four weeks in treatment conditions. Oysters were exposed from fertilization through the settlement stage to four treatment conditions representative of present-day average summertime ambient and future extreme conditions, though the Multiple Stressor treatment failed around 11 dpf. After completion of the settlement stage, oysters from each remaining treatment were evenly divided and exposed to all four treatment conditions again to understand how the larval and settlement experience may affect how juvenile oysters respond to different environmental conditions. The juvenile treatments are expressed as colored bars: Control (CN: 25°C, pH 7.8; dark purple), High Temperature (HT: 29°C, pH 7.8; light purple), Low pH (LP: 25°C, pH 7.2; light green), and Multiple Stressor (29°C, pH 7.2). The juvenile responses are grouped into three panels labeled with the previous larval-through-settlement stage experience. Error bars represent standard error.*

CHAPTER 4

Comparison of the physiological responses of larvae from selectively bred and wild *Crassostrea virginica* when exposed to multiple environmental stressors

Abstract

The Eastern oyster maintains two important roles: one as a reef-building, ecosystem engineer and the other as selectively bred broodstock for aquaculture. As oysters bred for aquaculture have been selected over several generations for traits like fast growth and disease resistance, their underlying physiology and allocation of energy is likely different from wild oysters. These potential differences in energy allocation could affect the ability of each oyster type to respond to external stress. As climate change continues to threaten marine ecosystems, understanding how these two types of Eastern oysters perform under multiple environmental stressors is important. The purpose of this study was to compare how the physiology of larvae from wild and selectively bred oysters was affected when exposed to both individual and simultaneous conditions of ocean acidification and ocean warming. Experiments were conducted in two separate years with larvae spawned from a well-established aquaculture broodstock line in Virginia (LILY) and from wild adults from a reef in the Rappahannock River, a tributary of the Chesapeake Bay. In experiments with wild and selectively bred oysters, larvae were exposed to full-factorial combinations of two temperature (25°C, 29°C) and carbonate chemistry (pH 7.2, pH 7.8) levels that represent average present-day ambient (25°C, pH 7.8) and extreme summer conditions anticipated to become more frequent in the future. Oxidative stress defense and damage molecules, biomass accumulation, shell growth, changes in long-term energy stores, and cumulative survival were measured through 8 days-post-fertilization (dpf) in both experiments. Wild oyster larvae experienced stronger sub-lethal and lethal effects of the stressors within the first few days of the larval stage compared to selectively bred larvae. Specifically, tissue and shell growth decreased under conditions of acidification, and lipid damage from oxidative stress in general was greater across all treatment conditions in wild oyster larvae. By the end of the

experiment (8 dpf), surviving wild oyster larvae had compensated for earlier negative physiological effects from acidified conditions, while selectively bred larvae were beginning to show signs of sub-lethal stress through decreased tissue growth and increased oxidative stress (i.e., greater concentrations of antioxidants and lipid damage). Overall, larvae from wild and selectively bred Eastern oysters have differing physiological responses to individual and simultaneous conditions of ocean acidification and ocean warming, and this study provides evidence to suggest that wild oyster larvae may be more successful in the face of ongoing climate change compared to selectively bred oyster larvae. This study is a good starting point for further exploration into the physiological tolerances of various broodstock lines and wild oyster populations to environmental stress to better predict the future of the Eastern oyster as a species.

Introduction

Oysters are economically and environmentally important organisms because of the various direct (e.g., shoreline stabilization) and indirect (e.g., nursery habitat for economically important species) ecosystem services they provide (Chapter 1; Gazeau et al., 2013; Grabowski et al., 2012; Lemasson et al., 2017). Over recent decades, the economic importance of oysters as an aquaculture species has grown, and with that, the development of selective breeding to enhance production (Botta et al., 2020). Along the East and Gulf coasts of the United States, aquaculture of the Eastern oyster, *Crassostrea virginica*, dominates production and overlaps in geographical extent with the remaining wild populations. Overharvesting of wild Eastern oysters along with increased disease prevalence and habitat loss, specifically in the Chesapeake Bay, reduced wild populations to an estimated 0.3% of historic levels (Rothschild et al., 1994, Wilberg et al., 2011). The decline of the wild oyster fishery is partially responsible for spurring the development of selective-breeding programs and the rise of oyster aquaculture production (Reece et al., 2020). With the rise of selective breeding for aquaculture alongside restoration and conservation efforts for wild populations, the Eastern oyster maintains two important roles: one as a reef-building, ecosystem engineer and the other as selectively bred broodstock supporting a $14.5 million aquaculture industry in Virginia alone (Hudson, 2019).

As with many organisms bred for human consumption, disease resistance and fast growth are commonly prioritized traits (Gjedrem et al., 2012). Two parasitic diseases, *Haplosporidium nelson* (MSX) and *Perkinsus marinus* (Dermo), were partially responsible for the decline of wild Eastern oyster populations and the associated harvest fishery, initiating selective breeding efforts to enhance disease-resistance in oysters (Frank-Lawale et al., 2014; Ragone Calvo et al., 2003; Reece et al., 2020). Additionally, triploid Eastern oysters were developed to improve the growth rate of disease-resistant broodstock lines through sterility since gamete production is energetically demanding (Dégremont et al., 2012; Guo et al., 1996). Triploid oysters are commonly used across most cultured species (Wadsworth et al., 2019), and in Virginia specifically, triploid oysters made up 97% of production in 2018 (Hudson, 2019). As selective breeding over multiple generations inherently changes the underlying physiology and allocation of energy within organisms (Andersson and Georges, 2004), and given the known differences in growth, reproduction, and disease-resistance between wild and selectively bred Eastern oysters (Barber and Mann, 1991; Dégremont et al., 2012), important biological processes like baseline metabolism, development, and long-term energy storage could be different (Sokolova et al., 2012).

Differences in energy allocation could have implications for an organism's ability to physiologically respond to external stress. Ocean acidification and ocean warming are known global climate change stressors, affecting whole-organismal and cellular physiological responses in oysters independently and concurrently (Byrne and Przeslawski, 2013; Gazeau et al., 2013; Przeslawski et al., 2015). In general, when organisms experience stress, whether from abiotic or biotic environmental factors, basal physiological processes are prioritized over other biological functions, such as growth, to conserve energy and increase the likelihood of survival (Sokolova et al., 2012). If stressful conditions are more extreme or prolonged, organisms cease biological activities beyond what is necessary for survival, negatively affecting important processes, like long-term energy storage, which could have consequences for the organism well after conditions improve (i.e., latent or carryover effects; Pechenik, 2006; Sokolova et al., 2012). In this way, trait selection for aquaculture production may indirectly benefit oysters with respect to global climate change (Tan et al., 2020). Oysters used for aquaculture that are bred for resilience to multiple diseases may have a more robust cellular stress-response system due to their enhanced immune systems, which are often linked to, and overlap with, the cellular stress response system (Guo et al., 2015). Therefore, alongside their optimized growth rates, selectively bred oysters may have more energy to maintain basal metabolic processes before resorting to bioenergetic shifts at the expense of other important biological processes. While selectively bred oysters may be more tolerant of environmental stress from an energetics perspective, genetic diversity should be taken into consideration. Selection can decrease genetic diversity and result in narrowed environmental tolerance ranges, potentially affecting the ability of selected oyster broodstock lines to withstand environmental stress (Appleyard and Ward, 2006).

Wild and selectively bred *C. virginica* may respond differently to environmental stress, especially during the highly vulnerable larval stage. Studies comparing the physiological performance of wild and selectively bread Sydney Rock, *Saccostrea glomerata*, and Pacific, *C. gigas*, oysters have found that overall, selectively bred oysters may be better equipped to withstand acidification or warming (Durland et al., 2021, 2019; Fitzer et al., 2019; Goncalves et al., 2016; Hall et al., 2020; Parker et al., 2012, 2011; Stapp et al., 2018), though no studies have observed whether that resilience is maintained under multiple environmental stressors. Increased resilience in selectively bred oysters has been linked to increased metabolic rates in oyster larvae (Parker et al., 2012), adaptive energy allocation (Goncalves et al., 2018, 2016), upregulation of genes related to the cellular stress response (Goncalves et al., 2016) but overall fewer genetic changes (Durland et al., 2021), and downregulation of the proteome (i.e., conserving energy; Thompson et al., 2015). With respect to *C. virginica*, few studies have compared how wild and selectively bred oysters respond to global climate change stressors. In one study, selectively bred, adult *C. virginica* had lower oxygen demand and basal metabolic rates compared to wild oysters, irrespective of the acute thermal stress they were exposed to, which may make selectively bred Eastern oysters more resilient to environmental stress (Hall et al., 2020). Holistically understanding how global climate change will affect *C. virginica* is crucial for the development and identification of resilient broodstock lines and wild reef populations in support of the longevity and success of aquaculture production and restoration efforts, respectively.

The purpose of this study was to compare the physiological responses of larvae from selectively bred and wild *C. virginica* when exposed to multiple environmental stressors, using cellular to whole-organism physiological metrics. To compare physiological responses under environmental stress, larvae from wild and selectively bred oysters were exposed to conditions of ocean acidification and ocean warming individually and concurrently. Survival, growth, oxidative stress defense and damage molecules, long-term energy storage, and metabolism were assessed for a more complete understanding of the differences between the two larval types. It was hypothesized that the larvae spawned from selectively bred broodstock, LILY, would be more resilient to environmental stress because of their selection for low-salinity tolerance and fast growth. The resistance in the selectively bred larvae to environmental stress would be evidenced by increased survival, growth, and long-term energy storage, less oxidative stress indicated by lower defense and damage molecule concentrations, and higher metabolic rates that are indicative of greater energy production compared to larvae from wild adults.

Methods

1. Experimental Design

To compare the cellular and whole-organism physiology of selectively bred and wild oyster larvae in response to multiple environmental stressors, ocean acidification and ocean warming, the experimental design and treatment conditions from the larval portion of *Chapter 3* were used. Additionally, the data used in this chapter for wild oyster larvae were generated in the experiment described in *Chapter 3*. For details about the experiment with wild oyster larvae, refer to *Chapter 3, Section 2*. Details about the complementary experiment with selectively bred oyster larvae will be described below.

In brief, larvae were cultured under a full factorial combination of two temperature and two pH conditions based on summertime average ambient and extreme values observed in the Rappahannock River (see *Chapter 3, Section 1* and *Chapter 3, Figure 2* for more details) for a total of four treatment conditions: Control (CN: 25°C, pH 7.8), High Temperature (HT: 29°C, pH 7.8), Low pH (LP: 25°C, pH 7.2), and Multiple Stressors (MS: 29°C, pH 7.2). Larvae were reared under these conditions at a salinity of 12 from fertilization through 11 days-post-fertilization (dpf) at VIMS in the Environmental Change Aquarium System (see *Chapter 3, Section 1.1*). Experiments were conducted in July 2019 and July 2021 with larvae spawned from selectively bred and wild oysters, respectively. Larvae were sampled for metrics of survival, growth, oxidative stress, long-term energy storage at 2, 4, 6, and 8 dpf, and whole-organism metabolism was measured once at 4 dpf. The experiment was originally planned to extend through the entire larval period until signs of metamorphic competency were seen, but in 2019, many of the larval cultures across all treatments began to decline after 11 dpf for reasons that are unknown. Also, the experiment with larvae from wild oysters was planned for Summer 2020 but was rescheduled to the Summer of 2021 due to the COVID-19 pandemic.

2. Larval Culturing

Triploid *C. virginica* larvae were produced from selectively bred broodstock at VIMS Aquaculture and Breeding Technology Center (ABC) in June 2019, and diploid larvae were produced from wild oysters collected from Parrot’s Rock Reef at VIMS in June 2021. For the

2019 spawn, female, diploid oysters (n = 25) and male, tetraploid oysters (n = 10) from broodstock line LILY (Frank-Lawale et al., 2014), were produced by and obtained from VIMS ABC, then conditioned naturally in Sarah's Creek in the York River, VA prior to spawning day. For details about the adult oysters utilized in the 2021 experimental run, refer to *Chapter 3, section 2*. All larvae were produced using strip-spawning (Allen and Bushek, 1992). Briefly, gametes were stripped from adults, and eggs were pooled together. The pooled eggs were rinsed to remove harmful bacteria, then divided equally into a number of aliquots that was determined by the number of male oysters utilized. Each aliquot was then fertilized by sperm from one male. After fertilization was completed (~ 1 hour), embryos were re-pooled and rinsed to remove any remaining sperm and bacteria. Embryos (n ≅ 3,000,000 per replicate aquarium) were randomly divided and placed into each treatment replicate about two hours after fertilization.

Larval culture conditions were maintained following best practices and were monitored regularly. Larval density was standardized across treatments throughout the experiment to control for density-dependent growth patterns. Densities started at 30 embryos mL^{-1} and were gradually reduced to 2 larvae mL^{-1} prior to the settlement phase (M. Congrove, pers. comm.; *Chapter 2, Table S1*). Larvae were fed daily on an *ad libitum* diet of a mixture of microalgal species (*Isochrysis galbana*, *Thalassiosira weissflogii*, and *Tetraselmis* sp.) cultured at VIMS ABC. Every two days, a 100% water change was conducted to remove any build-up of nitrogenous waste in the culture water. Water samples were collected from each aquarium for independent measurements of pH_{total} and from the mixing tank for measurements of total alkalinity prior to each water change (see *Section 3. Carbonate Chemistry Analysis*). Temperature, dissolved oxygen, pH, conductivity, and salinity were measured using a YSI Pro Plus (YSI Inc., Yellow Springs, OH) alongside all water samples. Aquarium conditions were continuously monitored by the aquarium system glass electrodes (refer to *Chapter 3, Section 1.1*).

Larvae were sub-sampled on days 2, 4, 6, and 8. To assess survival, and collect samples for physiological assessments, larval abundance in each aquarium was determined and compared to the abundance at the previous sampling timepoint to calculate percent survival. Photographs were taken at the same time of the larval abundance assessment for later measurements of shell growth. Larvae were then volumetrically sub-sampled by homogenizing the concentrated larvae and taking aliquots calculated from above measured larval density for total antioxidant capacity and total protein (n ≅ 20,000), lipid peroxidation (n ≅ 20,000), triglyceride content (n ≅ 10,000), and respirometry (day 4 only; n ≅ 1,000). Once all samples were collected, remaining larvae were returned to their respective aquarium at the appropriate density, and samples were preserved at -80^{o}C until later analysis. Details about larval production, culturing, and experimental design can be found in *Chapter 3, Section 2* and pertain to experiments with each type of larvae.

3. Carbonate Chemistry Analysis

Carbonate chemistry conditions were characterized throughout the experiment using water samples. Total alkalinity was measured through open-cell potentiometric titration method following best practices (SOP3b; Dickson et al., 2007). pH_{total} was measured spectrophotometrically using m-cresol purple dye, following best practices (SOP6b; Dickson et al., 2007). All pH samples were run within an hour of collection; water samples used for total alkalinity measurements were held at -20°C until later analysis. Total alkalinity and pH were used to calculate other carbonate chemistry parameters using the Seacarb package in R (Gattuso et al., 2021), complementary temperature and salinity data, and the following constants: K_1 and

K_2 for carbonic acid (Lueker et al. 2000), K_F (Perez and Fraga 1987), total boron (Lee et al. 2010), and K_{SO4} (Dickson et al. 1990). Refer to *Chapter 2, Section S1.5* for more detailed methods of the carbonate chemistry measurements.

4. Physiological Assays

Total protein content, triglyceride content, total antioxidant capacity, lipid peroxidation, shell size, and respiration (day 4 only) were measured from samples of larvae collected throughout the experiment. Total protein content was measured using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Smith et al., 1985). Total antioxidant capacity was measured using the ferric reducing/antioxidant potential (FRAP) assay from (Griffin and Bhagooli, 2004). Lipid peroxidation was measured with the Lipid Peroxidation (MDA) Assay Kit (Cat. No. ab118970, Abcam). Triglyceride content was measured using the Triglyceride Quantification Kit (Cat. No. ab65336, Abcam). All spectrophotometric assays were conducted using a Molecular Devices SpectraMax® iD3 (San Jose, CA) microplate plate reader. ImageJ was used to measure shell size from photos captured using a Leica compound microscope mounted with a Leica MC170 HD camera at the time of the larval density sub-samples described above (Schneider et al., 2012). Lastly, rates of larval respiration were measured using endpoint respirometry. Larvae from each treatment replicate were measured in triplicate, with about 300 larvae per triplicate. Larvae were incubated in micro biological oxygen demand vials for several hours under treatment conditions. Oxygen consumption was measured using a PreSens Microx 4 Oxygen Microsensor (Regensburg, Germany; Marsh and Manahan, 1999). Due to measurement error during respirometry with the wild oyster larvae, only data from the selectively bred oyster larvae are reported here. Refer to *Chapter 2, Section 2.4* for more detailed methods of total antioxidant capacity, total protein content, and shell size. Refer to *Chapter 3, Section 6* for more detailed methods of lipid peroxidation, triglyceride content, and respiration.

5. Statistical Analyses

Water conditions between the two larval oyster types, wild and selectively bred, were analyzed over the entire experimental period. All water conditions, with the exception of total alkalinity, were analyzed using a linear mixed effects model with the *nlme* package (version 3.1-160; Pinheiro et al., 2020; Pinheiro and Bates, 2000) in R (version 4.2.2; R Core Team, 2022). Posthoc testing was done using the *multcomp* package (version 1.4-20; Hothorn et al., 2008) in R. The four treatments were coded as factors and treated as a fixed effect along with larval type (wild or selectively bred), while aquarium replicate was included as a random factor to account for any aquaria effects. A Welch's Two Sample T-test was used to analyze differences in total alkalinity between the two experiments since total alkalinity of water used to fill the aquaria was measured (see *Environmental Change Aquarium System* in *Chapter 3, Section 1.1*). Data of all water parameters satisfied the assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity. The Shapiro-Wilkes test was analyzed in *stats* (R Core Team, 2022), and the Levine's test was conducted with *car* (Fox and Weisberg, 2019).

To detect differences in the physiological responses of larvae from wild and selectively bred Eastern oysters to multiple environmental stressors, linear mixed effects models and posthoc tests were used as described above. The pH and temperature conditions that made up the four environmental treatments were treated as separate fixed effects, with two levels each, alongside larval type (wild or selectively bred) and age. Aquarium replicate was included as a random factor to account for any aquaria effects. Model selection was performed incrementally, following Burnham and Anderson (2002). Post-hoc tests were not performed when 3- and 4-way interactions were detected. Instead, effects were interpreted from plots of the data. All response variables were transformed to satisfy the assumption of the Shaprio-Wilkes test for normalcy: cumulative survival, shell length, and lipid peroxidation were natural log transformed, while total protein content and triglyceride content were square-root transformed, and total antioxidant capacity was reciprocal transformed. After the completion of all transformations, all response variables satisfied the assumptions of the Shapiro-Wilkes test for normalcy and Levine's test for homogeneity. The Shapiro-Wilkes test was analyzed in *stats* (R Core Team, 2022), and the Levine's test was conducted with *car* (Fox and Weisberg, 2019).

Results

1. Water conditions

Environmental conditions were similar between the two experimental runs within each treatment, with the exception of dissolved inorganic carbon (DIC); no significant interactions were found among water parameters between the two types of larvae and four treatment conditions (Table 1). The difference in DIC between the same treatment in both experiments was generally smaller than the difference between acidified and non-acidified treatments (Table 2). When averaged across treatment conditions, there were some significant differences in certain water parameters between the two experiments. In the wild oyster experiment compared to the selectively bred oyster experiment, average dissolved oxygen was significantly lower by 0.430 mg L^{-1}, while average pH, Ω_{ag}, salinity, and total alkalinity were greater by 0.120 pH units, 0.063 Ω_{ag} units, 1.2 salinity units, and 228 µmol kg^{-1}, respectively (Tables 1, 2).

For both experiments, expected differences in pH and temperature among treatments were confirmed. pH was significantly greater in the high pH treatments (i.e., Control and High Temperature) compared to the acidified treatments (i.e., Low pH and Multiple Stressor) by more than 0.5 units (Tables 1, 2). Similarly, temperature was significantly greater in the high temperature treatments (i.e., High Temperature and Multiple Stressor) compared to the ambient temperature treatments (i.e., Control and Low pH) by 3.66°C (Tables 1, 2). Due to differences in pH, differences in Ω_{ag} subsequently varied between treatments. Beyond expected differences among treatment conditions, there were other water parameters that differed. Dissolved oxygen was significantly greater in the low temperature treatments (i.e., Control and Low pH) compared to the warmer treatments (i.e., High Temperature and Multiple Stressor) by 0.75 mg L^{-1} (Tables 1, 2).

2. Effects of multiple environmental stressors on the physiology of larvae from wild and selectively bred oysters

    a. Survival

Temperature significantly impacted cumulative survival in oyster larvae, regardless of type (Table 3). Larvae exposed to warmer temperatures (i.e., High Temperature and Multiple Stressor treatments) had lower percent survival over the course of the experiment compared to those that were reared under ambient conditions (i.e., Control and Low pH treatments; Figure 1), likely driven by high survival of wild larvae in the Control treatment.

pH conditions affected survival differently between the larval types (Table 3). In wild oyster larvae, survival was negatively affected by conditions of acidification compared to ambient pH likely driven by the high survival in the Control treatment, but in selectively bred larvae, survival did not significantly differ between pH conditions (Table 3, Figure 1).

    b. Shell and Tissue Growth

To understand how multiple environmental stressors would affect growth in both larval wild and selectively bred oysters, shell length and total protein were measured as proxies for shell and tissue growth, respectively. The four-way interaction of temperature, pH, larval type, and age explained a significant portion of the variation in both shell length and total protein content (Table 3). Larvae raised at increased temperatures and ambient pH (i.e., High Temperature treatment) had larger shell lengths compared to those raised in control conditions, regardless of larval type, though wild oyster larvae, in general, had larger shell lengths compared with larvae from selectively bred oysters (Figure 2a,b). Acidified conditions at ambient temperatures (i.e., Low pH treatment) had a slight negative effect on shell length within the first few days post-fertilization for both larval types, most notably at 2 dpf for selectively bred oyster larvae and 4 dpf for wild oyster larvae (Figure 2 a,b). The combination of the two environmental stressors (i.e., Multiple Stressor treatment) positively affected shell growth compared to larvae in the control treatment over the course of the experiment in wild oyster larvae, but this trend was not observed in selectively bred larvae (Figure 2a,b).

Total protein content was generally similar between larval types and environmental conditions, especially early in the larval stage (Figure 2c,d); however, wild oyster larvae in Low pH started to experience slower accumulation of total protein by day 4, continuing through day 6 (Figure 2c). By 8 dpf, wild oyster larvae exposed to the Low pH treatment had similar total protein content to those in the Control treatment (Figure 2c). This effect of acidification at ambient temperature was not observed in the selectively bred larvae. Regardless of larval type, warmer temperatures under ambient pH (i.e., High Temperature) did result in higher total protein content (by approximately 41%) compared to Control conditions, especially by 8 dpf (Figure 2c,d). While accumulation of total protein tended to be faster in larvae from selectively bred oysters compared to wild oysters in general, under the Multiple Stressor treatment, wild oysters had greater total protein content by 6 dpf than did selectively bred oysters (Figure 2c,d).

c. Oxidative Stress Defense and Damage

To understand differences in the cellular stress response between larval types when exposed to conditions of warming and acidification, total antioxidant capacity and lipid peroxidation were measured to assess oxidative stress and damage, respectively. Temperature significantly affected total antioxidant capacity, regardless of larval type, larval age, and pH conditions (Table 3). Larvae exposed to warmer temperatures (i.e., High Temperature and Multiple Stressors) had lower total antioxidant capacity, on average, compared to those under ambient temperatures (i.e., Control and Low pH treatments; Figure 3a,b). Total antioxidant capacity was also significantly affected by pH, but this effect differed through time and by larval type (Table 3). As larvae from selectively bred oysters progressed through the larval stage under conditions of acidification (i.e., Low pH and Multiple Stressor treatments), total antioxidant capacity became relatively elevated while the effects of pH were minimal through the experimental period for wild oyster larvae (Figure 3a,b). By 8 dpf, the total antioxidant capacity of larvae from selectively bread oysters in acidified conditions was 66% greater than that of wild oyster larvae.

There was a significant interaction of pH and temperature on lipid peroxidation levels (Table 3). Specifically, under conditions of acidification, larvae had less lipid peroxidation at warmer temperatures (i.e., Multiple Stressor treatment) compared to ambient temperatures (i.e., Low pH treatment), but under ambient pH, lipid peroxidation levels were similar across temperature conditions (i.e., Control and High Temperature treatments; Figure 3c,d). The effect of temperature on lipid peroxidation levels differed with larval age (Table 3). Initially, larvae exposed to ambient temperatures (i.e., Control and Low pH treatments) experienced greater lipid peroxidation than those at warmer temperatures but towards the last sampling timepoint, lipid peroxidation levels were lower overall. There was also a significant interactive effect of larval type and age on lipid peroxidation levels (Table 3). Lipid peroxidation was initially lower in selectively bred oysters compared to wild (4 and 6 dpf), but by the end of the experiment, selectively bred oyster larvae had 30% more lipid peroxidation than wild oyster larvae (Figure c,d).

d. Long-term energy stores and metabolism

Triglyceride content and respiration rate were measured as proxies for energy reserves and aerobic metabolism, respectively, to identify potential differences in energy allocation and integrated effects of multiple environmental stressors. The four-way interaction between temperature and pH conditions, as well as larval type and age, explained a significant portion of the variation in triglyceride content (Table 3). Triglyceride content tended to be similar throughout the experimental period across the two larval oyster types, wild and selectively bred (Figure 3e,f), with the exception of those in the Low pH treatment. Larvae from wild oysters increased in triglyceride content over time when they were exposed to low pH and ambient temperatures (i.e., Low pH treatment; Figure 3e), while triglyceride content in larvae from selectively bred oysters under the same conditions remained similar to those experiencing all other treatment conditions (Figure 3f).

Temperature and pH had an interactive effect on the respiration rates of larvae from selectively bred oysters (Table 3). Specifically, under ambient pH conditions, oysters exposed to warmer temperatures (i.e., High Temperature treatment) respired less than those at ambient temperatures (i.e., Control treatment) regardless of whether oxygen consumption rates are standardized by total protein of the sample or per individual (Figure 4a,b; Table 3). At warmer temperatures, larvae that experience low pH (i.e., Multiple Stressor treatment) compared to ambient pH (i.e., High Temperature treatment) have greater oxygen consumption regardless of standardization by total protein of the sample or per individual (Figure 4a,b; Table 3). For the larvae subsampled for respirometry, the average total protein per larva at the time of measurement tended to be greatest in those experiencing warmer temperatures (Figure 4c).

Discussion

Though selectively bred Eastern oysters have been developed for disease resistance and fast growth over several generations, larval wild oysters may be more resilient to multiple environmental stressors. For selectively bred and wild *C. virginica* larvae exposed from fertilization through the first half of their larval stage (8 dpf) to conditions of ocean acidification and ocean warming, the effects were complex, with differences observed depending on larval type and age. Though wild oyster larvae experienced more lethal effects of the environmental stressors (Figure 1; Table 3), by the end of the experiment (8dpf), surviving wild oyster larvae had compensated for earlier negative physiological effects from acidified conditions, while selectively bred larvae were beginning to show signs of sub-lethal stress when exposed to multiple environmental stressors (Figures 2, 3; Table 3). Thus, larvae from wild oysters were more sensitive to environmental stress in the first few days of their lives, but they likely acclimatized to stressful conditions and benefitted from the addition of warming under conditions of acidification. For larvae from selectively bred oysters, the development of negative effects from conditions of acidification and warming later in the larval stage could have consequences during metamorphosis, potentially affecting aquaculture production.

Within the first few days of the larval stage, larval growth under conditions of ocean acidification was negatively affected, unlike for selectively bred larvae after the first two days of exposure. Larval shell lengths of selectively bred and wild oysters were initially impacted by acidified conditions within the first two days of exposure compared to those in other environmental conditions (Figure 2a,b), but selectively bred oyster larvae were able to recover faster than those from wild oysters. The first 48 hours of the larval stage are vulnerable because the energetically demanding initial formation of the larval shell, prodissoconch I, coincides with reliance on maternal endogenous energy reserves due to lack of robust feeding structures to capture food (Gallager and Mann, 1986; Waldbusser et al., 2013). Furthermore, during the period of initial shell formation, calcification sites are in greater contact with seawater, and larvae have not fully developed physiological mechanisms (i.e., acid base regulation) to help control environmental conditions at the site of calcification. Therefore, exposure to acidification greatly affects calcification, and ultimately shell growth, due to reduced calcium carbonate saturation states (Waldbusser et al., 2013, 2015). Though initial larval shell growth in selectively bred oysters was likely affected by acidic conditions, total protein content (i.e., tissue growth) was unaffected (Figure 2d), unlike in wild oyster larvae (Figure 2c). Larval shell and total protein content in wild oysters remained negatively affected by acidified conditions through 6 dpf (Figure 2; Table 3), as seen in other studies (Frieder et al., 2018), though simultaneous experience of warmer temperatures ameliorated the effect (Figure 2a,c). Previous studies have found similar growth results with respect to the comparison of wild oyster larvae to those from selectively bred oysters under environmental stress, attributing the success of larvae from selectively bred oysters to bioenergetics (Durland et al., 2019; Parker et al., 2011).

The sensitivity of wild oyster larvae to conditions of ocean acidification early in the larval stage may have been linked to the cellular stress response. Through 6 dpf, while wild oyster larvae were exhibiting slower growth under acidified conditions, lipid peroxidation, damage to lipid molecules from oxidative stress, was higher in wild oyster larvae, likely due to increased levels of oxidative stress (Figure 3c,d; Table 3). In support of greater oxidative stress experience, wild oyster larvae did have slightly lower total antioxidant capacities when exposed to low pH compared to larvae from selectively bred oysters (i.e., Low pH and Multiple Stressor treatments), suggestive of an increased reaction rate with reactive oxygen species, which are the agents of oxidative stress (Figure 3a,b; Table 3). While total antioxidant capacity alone can be difficult to interpret (Griffin and Bhagooli, 2004), lower total antioxidant capacity in combination with high lipid peroxidation likely indicates an inability of wild oyster larvae to produce enough antioxidants to combat oxidative stress before cellular damage was incurred under conditions of ocean acidification. Oysters exposed to ocean acidification generally upregulate genes and proteins related to oxidative stress defenses (Dineshram et al., 2015; Tomanek et al., 2011; Wang et al., 2012; Zhang et al., 2016), and there is evidence showing greater upregulation in selectively bred oysters compared to wild populations (Goncalves et al., 2017, 2016), supporting results found in the present study.

More readily available energy in selectively bred oysters may explain the increased total antioxidant capacity and lower oxidative stress damage observed early in the larval stage in the present study (Figure 3b,d; Table 3). In selectively bred *Saccostrea glomerata* adults, metabolism and energy metabolism-related proteins (i.e., phosphorylases) increased when exposed to ocean acidification, supplying excess energy to physiological processes under stress (Goncalves et al., 2018; Parker et al., 2012; Thompson et al., 2015). Respiration rates in larvae from the present study provide evidence to support that selectively bred oysters were not metabolically stressed early in the larval stage. Respiration rates of larvae that were cultured under conditions of acidification through 4 dpf were not significantly different, or were slightly lower than, those of larvae raised under the Control treatment conditions with ambient pH (Figure 4a,b; Table 3). This pattern was also observed in Pacific oyster (*C. gigas*) larvae under mild acidification levels and attributed to re-allocation of existing energy pools to continue supporting protein growth (Frieder et al., 2018). Another potential source of available energy within the first few days of the larval life stage is maternally provisioned lipids (Gallager et al., 1986; Gallager and Mann, 1986). Selectively bred larvae may be more efficient at utilizing maternally invested triglycerides under acidified conditions within the first few days of the larval stage. Triglyceride content in selectively bred larvae declined from day 2 to 4 under exposure to acidified conditions and may explain the growth recovery that occurred during the same timeframe (Figure 3f, Table 3). After that decline, triglyceride levels in selectively bred larvae remained similar among treatment conditions and to those in wild oyster larvae (Figure 3e,f). Larvae from selectively bred oysters are more resilient than wild oyster larvae to conditions of ocean acidification during the first few days of the larval stage, likely due to their ability to meet the energy demands of the oxidative stress response.

The relative sensitivities of wild and selectively bred oyster larvae to multiple stressors changed by the end of the experiment (i.e., halfway through the larval stage). Selectively bred Eastern oysters began exhibiting signs of sub-lethal stress, while wild oysters may have acclimatized to stressful environmental conditions. By 8 dpf, both shell and tissue growth in wild oyster larvae exposed to acidified conditions recovered (Figure 2a,c), lipid peroxidation leveled off (Figure 3c), and total antioxidant capacity remained the same (Figure 3a), indicating that energy demands had either decreased or that wild oysters had acclimatized to increased demands. Selectively bred larvae, however, continued to accumulate lipid damage via increased lipid peroxidation levels alongside a subsequent increase in antioxidant molecules (Figure 3b,d), which likely indicates an increase in oxidative stress. Furthermore, while shell growth remained unaffected by conditions of acidification, signs of stress appeared through stable, depressed total protein content in selectively bred oyster larvae that were exposed to simultaneous conditions of acidification and warming relative to individuals in the other environmental conditions (Figure 2d). In addition to the negative effect on total protein at day 8, there was a subsequent slight increase in lipid peroxidation, and large increase in total antioxidant capacity in selectively bred larvae. Furthermore, lipid peroxidation levels in selectively bred oysters, regardless of environmental condition, were much higher than in wild oysters, indicative of greater oxidative stress overall. These results differ from other studies where selectively bred oysters were more robust to environmental stress through increased survival, growth, efficient filtration rates, and completion of metamorphosis, but it is important to note that those previous studies were conducted using a single environmental stressor (Durland et al., 2021, 2019; Fitzer et al., 2019; Goncalves et al., 2016; Hall et al., 2020; Lee et al., 2016; Mcafee et al., 2018; Parker et al., 2012, 2011; Stapp et al., 2018). As the literature presently stands, selectively bred oysters, when compared to wild oysters, are likely more resilient to individual environmental stressors and may be more susceptible to combined effects of multiple stressors, as shown in the present study.

The effects of warming transcend any potential physiological or genetic differences between oyster type, unlike conditions of ocean acidification. Survival, total antioxidant capacity, lipid peroxidation, and respiration rate (selectively bred oyster larvae) all differed with respect to temperature independently of oyster type (Table 3). All four metrics declined under warmer temperatures. Although the interactive effects of acidification and warming on both growth metrics, shell length and total protein, were complex (Table 3), there was a general, positive effect of warmer temperatures on larval growth across both wild and selectively bred oysters (Figure 2). In general, the warmer temperature likely enhanced larval growth, regardless of acidified conditions, and without the expense of an increase in metabolic demand or oxidative stress. A study on wild and selectively bred *C. gigas* larvae similarly found that, regardless of oyster type, the cost of protein synthesis was lower at warmer temperatures (Lee et al., 2016), meaning that protein accretion (i.e., tissue growth) under warmer conditions likely does not require an increase in metabolism to meet energy demands. If metabolism does not increase, as seen with the lower respiration rates in the selectively bred larvae, then oxidative stress would remain low because reactive oxygen species production increases with metabolism (Lesser, 2006). Alternatively, at warmer temperatures, it is possible that enzymatic, compared with non-enzymatic, antioxidants play a larger role in oxidative stress defenses, as studies on *C. virginica* both in the lab and field have identified an increase in specific enzymatic antioxidants, like superoxide dismutase, with increasing temperatures (Rahman and Rahman, 2021a, 2021b; Soldatov et al., 2007). An increased role of enzymatic antioxidants at higher temperatures would explain the low non-enzymatic antioxidant concentrations and subsequent low lipid damage identified in the present study. Under more extreme scenarios, the effects of warming will become detrimental when temperatures reach beyond the upper thermal limit for larval oysters (Pörtner, 2010; Sokolova et al., 2012, Rahman and Rahman, 2021a), which was not the goal of this study and should be observed in future studies to understand how lethal limits may shift in the presence of additional environmental stressors.

In the long run, genetic diversity and selective mortality may impact the ability of selectively bred and wild oysters to withstand environmental stress. Oysters are highly genetically diverse with ~3.8 million single-nucleotide-polymorphisms in their genome and can differentially express 21% of genes in response to at least one environmental stressor (Wang et al., 2012). The high genetic diversity in oysters may be partially explained by the high proportion of expressed distorted genetic markers (27-49%), a measure of genetic load or deleterious alleles (Plough, 2016). High genetic load causes selective mortality throughout development, especially in the larval and settlement stages (Plough and Hedgecock, 2011), environmental stress aside (Plough, 2016; Plough et al., 2016). Selective pressures increase under environmental stress in favor of genotypes that can perform best in those conditions (Plough, 2012). Though this study did not measure changes in genetic diversity, it is possible that some amount of selective mortality of unsuitable genotypes occurred in wild oyster larvae, since cumulative survival was negatively impacted under conditions of acidification and warming (Figure 1a; Table 3). Selective mortality due to treatment conditions does not explain the physiological decline of selectively bred oysters by 8 dpf though. If there was selective mortality in selectively bred oysters in the present study, it was likely due only to genetic load, not environmental stressors since no differential effect on cumulative survival was observed (Figure 1b; Table 3), which is in agreement with other studies (Durland et al., 2021); however, as signs of sub-lethal stress in selectively bred oysters were emerging at the end of the study period, it begs the question of whether selective mortality from environmental stress would occur as larvae begin to approach their second developmental bottleneck, metamorphosis. It is well known that selectively bred oysters are not genetically diverse compared to their wild conspecifics (Carlsson et al., 2006; Durland et al., 2021). Less genetic diversity could pose a problem to aquaculture production if genetic diversity of selectively bred oysters is further constrained by simultaneous environmental stressors, as evidenced in this study, and if the occurrence of selective mortality increases as oysters approach metamorphosis, an area for future studies.

Based on the results of this chapter, the Eastern oyster aquaculture industry may be relatively vulnerable to multiple environmental stressors, while wild populations, being more genetically diverse at present with rich environmental histories, may have greater success. It is important to note that only one wild population and one selectively bred broodstock line were utilized in the present study, and there can be substantial genetic variation among wild and selectively bred oyster populations (Frieder et al., 2017; Pan et al., 2018, 2016). Furthermore, this study only observed the first half of the larval stage; however, results from this study have highlighted potential susceptibility in larvae from a well-established broodstock line, which may provide an explanation for recent failure of hatchery cultures that have been relatively unexplainable to date (Gray et al., 2022). Resilience to specific environmental stressors is heritable (McCarty et al., 2020; McFarland et al., 2020) but is often overlooked in deference to more immediate and pressing issues, like disease resistance (Frank-Lawale et al., 2014). Though, as seen in this study, responses to multiple environmental stressors are complex, and incorporating selection for resilience to environmental stressors in commercial broodstock will become easier through the future identification of heritable traits and with the development and utilization of more advanced genetic tools (Tan et al., 2020; Tan and Zheng, 2020).

C. virginica from wild and selectively bred oysters in the mesohaline region of the Chesapeake Bay responded differently to conditions of acidification, but not warming, throughout the first half of the larval stage. In the first few days of exposure to acidified conditions during the larval period, wild oysters had smaller shells, less tissue biomass, and more lipid damage alongside a low total antioxidant capacity, likely indicating that their cellular stress response was unable to keep pace with oxidative stress levels. Selectively bred oyster larvae during this early larval period were able to maintain their growth, metabolic rates, and cellular stress responses that aided in keeping damage from cellular stress low. During these first few days, wild oyster larvae benefited from the addition of warming, which improved growth and decreased damage from oxidative stress, unlike selectively bred oysters. Though exposure to simultaneous multiple environmental stressors initially did not affect selectively bred oyster larvae, by 8 dpf, evidence of stress was beginning to emerge through depressed tissue growth. Furthermore, larvae from selectively bred oysters continued accumulating lipid damage from oxidative stress, which was further supported with increased total antioxidant capacity. Wild oyster larvae at this point in development may have acclimatized to conditions of acidification, as evidenced by recovered shell and tissue growth and plateaued lipid peroxidation levels. Because of acclimatization of their larvae to conditions of acidification and positive response to the addition of warming, wild oyster larvae may be more successful in the face of ongoing climate change compared to selectively bred oyster larvae, though future studies should compare the physiological responses of different broodstock lines and reef populations under multiple environmental stressor conditions through the entirety of the larval stage and into later life stages to better understand the potential variations in resilience.

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Tables

Table 1. *Analysis of water conditions of the larval experiments of wild and selectively bred oysters for all treatment groups. Comparisons were made using mixed effects linear models, with treatment and larval type as fixed effects and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) from final reduced models are reported. Tukey post-hoc tests were performed where appropriate, and test statistics z and p-values (p) are reported. Asterisks indicate statistical significance below an alpha threshold of 0.05. Treatment conditions: CN – Control, HT – High Temperature, LP – Low pH, and MS – Multiple Stressor. Larval type: W – wild, SB – Selectively bred.*

Water condition	Chi-square	p	Post hoc comparison	Z value	p
Temperature					
Type	$X^2_{(1,24)} = 0.52$	0.470			
Treatment	$X^2_{(1,24)} = 1371$	<0.001*	CN – MS	26.653	<0.001*
			CN – LP	-0.708	1.000
			CN – HT	24.941	<0.001*
			HT – MS	-1.712	0.521
			HT – LP	25.650	<0.001*
			LP – MS	-27.362	<0.001*
Salinity					
Type	$X^2_{(1,24)} = 5.45$	<0.001*			
Treatment	$X^2_{(1,24)} = 489$	0.142			
Dissolved Oxygen					
Type	$X^2_{(1,24)} = 37.21$	<0.001*			
Treatment	$X^2_{(1,24)} = 113$	<0.001*	CN – MS	-7.634	<0.001*
			CN – LP	-0.067	1.000
			CN – HT	-7.492	<0.001*
			HT – MS	0.142	1.000
			HT – LP	-7.425	<0.001*
			LP – MS	7.567	<0.001*
pH_{tot}					
Type	$X^2_{(1,24)} = 60.04$	<0.001*			
Treatment	$X^2_{(1,24)} = 1346$	<0.001*	CN – MS	-25.006	<0.001*
			CN – LP	-24.871	<0.001*
			CN – HT	1.940	0.314
			HT – MS	26.946	<0.001*
			HT – LP	26.811	<0.001*
			LP – MS	0.135	1.000
Total Alkalinity	$t_{(60)} = 2.640$	0.010*			

DIC					
*Treatment*Type*	$X^2_{(1,24)} = 11.63$	0.009*	CN: W – SB	-7.943	<0.001*
			LP: W – SB	-12.615	<0.001*
			HT: W – SB	-9.288	<0.001*
			MS: W – SB	-10.226	<0.001*
Treatment	$X^2_{(3,24)} = 420.421$	<0.001*			
Type	$X^2_{(1,24)} = 63.086$	<0.001*			
$\Omega_{aragonite}$					
Type	$X^2_{(1,24)} = 18.91$	<0.001*			
Treatment	$X^2_{(1,24)} = 385$	<0.001*	CN – MS	-11.724	<0.001*
			CN – LP	-12.120	<0.001*
			CN – HT	3.476	0.003*
			HT – MS	15.199	<0.001*
			HT – LP	15.596	<0.001*
			LP – MS	-0.396	1.000

Table 2. *Water conditions of the experiments with larvae from wild and selectively bred oysters for all treatment groups. Values represent mean ± standard error calculated across all treatment replicates over the duration of the experiment. Temperature, salinity, dissolved oxygen, and* pH_{tot} *(pH, total scale) are measured values. DIC (dissolved inorganic carbon),* $\Omega_{calcite}$ *(saturation state of calcite), and* $\Omega_{aragonite}$ *(saturation state of aragonite) are calculated values.*

	Larval Type	
Water Conditions	Wild	Selectively bred
Temperature (°C)		
Control	25.07 ± 0.35	25.02 ± 0.30
Low pH	24.93 ± 0.30	24.97 ± 0.34
High Temperature	28.69 ± 0.61	28.38 ± 0.90
Multiple Stressors	28.78 ± 0.57	28.79 ± 0.49
Salinity		
Control	12.94 ± 0.10	11.74 ± 0.01
Low pH	12.98 ± 0.12	11.74 ± 0.02
High Temperature	12.89 ± 0.02	11.77 ± 0.01
Multiple Stressors	13.14 ± 0.20	11.84 ± 0.10
Dissolved Oxygen (mg L^{-1})		
Control	7.88 ± 0.59	7.33 ± 0.10
Low pH	7.87 ± 0.64	7.34 ± 0.11
High Temperature	7.06 ± 0.53	6.68 ± 0.14
Multiple Stressors	6.97 ± 0.75	6.74 ± 0.17
pH_{tot}		
Control	7.54 ± 0.15	7.69 ± 0.10
Low pH	7.03 ± 0.10	7.13 ± 0.07
High Temperature	7.59 ± 0.14	7.72 ± 0.08
Multiple Stressors	7.02 ± 0.10	7.12 ± 0.08
Total Alkalinity (µmol kg^{-1})	1146 ± 62	917 ± 61
DIC (µmol kg^{-1})		
Control	1056 ± 47	1020 ± 69
Low pH	1133 ± 53	1077 ± 77
High Temperature	1051 ± 45	1010 ± 71
Multiple Stressors	1129 ± 50	1082 ± 77
$\Omega_{aragonite}$		
Control	0.37 ± 0.03	0.43 ± 0.03
Low pH	0.13 ± 0.01	0.18 ± 0.03

High Temperature	0.42 ± 0.03	0.52 ± 0.06
Multiple Stressors	0.14 ± 0.13	0.18 ± 0.02

Table 3. *Analysis of larval physiology of wild and selectively bred oysters. Comparisons were made using mixed effects linear models, with pH, temperature, larval type, and age as a fixed effects and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) are reported from final reduced models. Tukey post-hoc tests were performed where appropriate, and test statistics z and p-values (p) are reported. Asterisks indicate statistical significance below an alpha threshold of 0.05. Larval type: W – wild, SB – Selectively bred.*

Physiological metric	Chi-square	p	Post hoc comparison	Z value	p
Survival					
*pH*Type*	$X^2_{(1,24)} = 3.791$	0.052*	pH 7.8: W vs. SB	1.558	0.477
			pH 7.2: W vs. SB	-1.196	0.928
			W: pH 7.8 vs. pH 7.2	3.102	0.008*
			SB: pH 7.8 vs. pH 7.2	0.349	1.000
pH	$X^2_{(1,24)} = 9.625$	0.002			
Temperature	$X^2_{(1,24)} = 7.347$	0.007*			
Type	$X^2_{(1,24)} = 2.427$	0.119			
Shell Length					
*pH*Temperature*Type*Age*	$X^2_{(1,95)} = 14.761$	<0.001*			
*pH*Temperature*Type*	$X^2_{(1,95)} = 7.575$	0.006*			
*pH*Temperature*Age*	$X^2_{(1,95)} = 3.069$	0.080			
*pH*Type*Age*	$X^2_{(1,95)} = 2.108$	0.146			
*Temperature*Type*Age*	$X^2_{(1,95)} = 2.908$	0.088			
*pH*Temperature*	$X^2_{(1,95)} = 1.478$	0.224			
*pH*Type*	$X^2_{(1,95)} = 1.048$	0.306			
*pH*Age*	$X^2_{(1,95)} = 0.975$	0.324			
*Temperature*Type*	$X^2_{(1,95)} = 1.390$	0.238			
*Temperature*Age*	$X^2_{(1,95)} = 0.002$	0.988			
*Type*Age*	$X^2_{(1,95)} = 1.634$	0.201			
pH	$X^2_{(1,95)} = 3.689$	0.055*			

Temperature	$X^2_{(1,95)} = 3.291$	0.070
Type	$X^2_{(1,95)} = 3.961$	0.047*
Age	$X^2_{(1,95)} = 154.220$	<0.001*
Total Protein Content		
*pH*Temperature*Type*Age*	$X^2_{(1,95)} = 3.724$	0.054*
*pH*Temperature*Type*	$X^2_{(1,95)} = 0.394$	0.530
*pH*Temperature*Age*	$X^2_{(1,95)} = 0.099$	0.753
*pH*Type*Age*	$X^2_{(1,95)} = 0.742$	0.389
*Temperature*Type*Age*	$X^2_{(1,95)} = 0.360$	0.549
*pH*Temperature*	$X^2_{(1,95)} = 0.007$	0.933
*pH*Type*	$X^2_{(1,95)} = 1.273$	0.259
*pH*Age*	$X^2_{(1,95)} = 0.243$	0.622
*Temperature*Type*	$X^2_{(1,95)} = 0.032$	0.857
*Temperature*Age*	$X^2_{(1,95)} = 1.891$	0.169
*Type*Age*	$X^2_{(1,95)} = 5.134$	0.023*
pH	$X^2_{(1,95)} = 1.316$	0.251
Temperature	$X^2_{(1,95)} = 0.023$	0.879
Type	$X^2_{(1,95)} = 4.742$	0.029*
Age	$X^2_{(1,95)} = 25.826$	<0.001*
Total Antioxidant Capacity		
*pH*Type*Age*	$X^2_{(1,95)} = 8.323$	0.004*
*pH*Temperature*	$X^2_{(1,95)} = 1.547$	0.214
*pH*Type*	$X^2_{(1,95)} = 3.300$	0.069
*pH*Age*	$X^2_{(1,95)} = 0.586$	0.444
*Temperature*Type*	$X^2_{(1,95)} = 0.587$	0.443
*Temperature*Age*	$X^2_{(1,95)} = 1.731$	0.188

*Type*Age*	$X^2_{(1,95)} = 2.760$	0.097			
pH	$X^2_{(1,95)} = 0.696$	0.404			
Temperature	$X^2_{(1,95)} = 9.547$	0.002*			
Type	$X^2_{(1,95)} = 1.512$	0.219			
Age	$X^2_{(1,95)} = 2.295$	0.130			
Lipid Peroxidation					
*pH*Temperature*	$X^2_{(1,95)} = 3.994$	0.046*	pH 7.8: 25°C vs. 29°C	1.931	0.214
			pH 7.2: 25°C vs. 29°C	3.395	0.003*
			25°C: pH 7.8 vs pH 7.2	-1.905	0.227
			29°C: pH 7.8 vs pH 7.2	0.915	1.000
*Temperature*Age*	$X^2_{(1,95)} = 4.366$	0.037*			
*Type*Age*	$X^2_{(1,95)} = 5.807$	0.016*			
pH	$X^2_{(1,95)} = 3.630$	0.057*			
Temperature	$X^2_{(1,95)} = 3.728$	0.054*			
Type	$X^2_{(1,95)} = 10.182$	0.001*			
Age	$X^2_{(1,95)} = 30.082$	<0.001*			
Triglyceride Content					
*pH*Temperature*Type*Age*	$X^2_{(1,95)} = 3.663$	0.056*			
*pH*Temperature*Type*	$X^2_{(1,95)} = 2.977$	0.084			
*pH*Temperature*Age*	$X^2_{(1,95)} = 0.594$	0.441			
*pH*Type*Age*	$X^2_{(1,95)} = 7.221$	0.007*			
*Temperature*Type*Age*	$X^2_{(1,95)} = 0.447$	0.504			
*pH*Temperature*	$X^2_{(1,95)} = 0.003$	0.956			
*pH*Type*	$X^2_{(1,95)} = 3.022$	0.082			
*pH*Age*	$X^2_{(1,95)} = 5.990$	0.014*			
*Temperature*Type*	$X^2_{(1,95)} = 1.761$	0.185			

*Temperature*Age*	$X^2_{(1,95)} = 1.046$	0.306			
*Type*Age*	$X^2_{(1,95)} = 0.542$	0.462			
pH	$X^2_{(1,95)} = 0.145$	0.704			
Temperature	$X^2_{(1,95)} = 2.597$	0.107			
Type	$X^2_{(1,95)} = 1.022$	0.312			
Age	$X^2_{(1,95)} = 4.995$	0.025*			
Respiration per Protein (SB only)					
*pH*Temperature*	$X^2_{(1,12)} = 10.587$	0.001*	pH 7.8: 25°C vs. 29°C	4.904	<0.001*
			pH 7.2: 25°C vs. 29°C	0.302	1.000
			25°C: pH 7.8 vs pH 7.2	1.636	0.407
			29°C: pH 7.8 vs pH 7.2	-2.965	0.012*
pH	$X^2_{(1,12)} = 2.677$	0.102			
Temperature	$X^2_{(1,12)} = 24.048$	<0.001*			
Respiration per Larva (SB only)					
*pH*Temperature*	$X^2_{(1,12)} = 23.749$	<0.001*	pH 7.8: 25°C vs. 29°C	4.424	<0.001*
			pH 7.2: 25°C vs. 29°C	-2.468	0.054*
			25°C: pH 7.8 vs pH 7.2	3.696	0.001*
			29°C: pH 7.8 vs pH 7.2	-3.195	0.006*
pH	$X^2_{(1,12)} = 13.664$	<0.001*			
Temperature	$X^2_{(1,12)} = 19.570$	<0.001*			

Figures

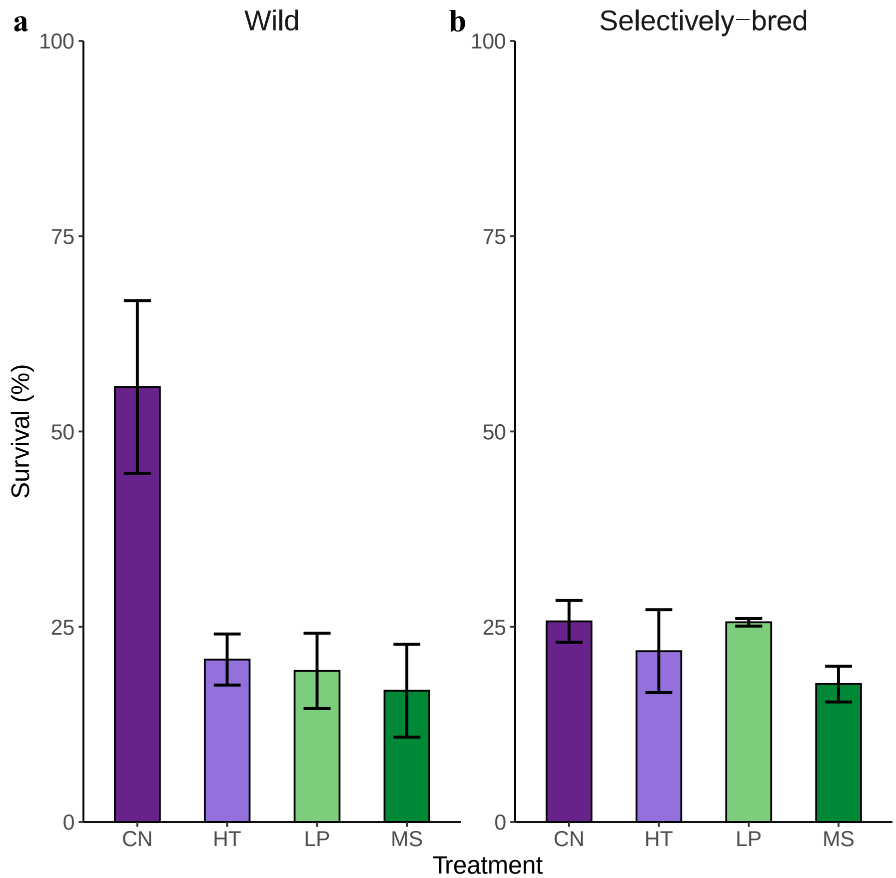

Figure 1. *Effects of multiple environmental stressors on cumulative percent survival through 8 days-post-fertilization of larvae from wild (a) and selectively bred (b) Eastern oysters. Larvae were exposed to four treatment combinations of two pH and two temperature values representative of present-day average summertime ambient and future extreme conditions: Control (CN: 25°C, pH 7.8; dark purple), High Temperature (HT: 29°C, pH 7.8; light purple), Low pH (LP: 25°C, pH 7.2; light green), and Multiple Stressor (MS: 29°C, pH 7.2; dark green). Error bars represent standard error.*

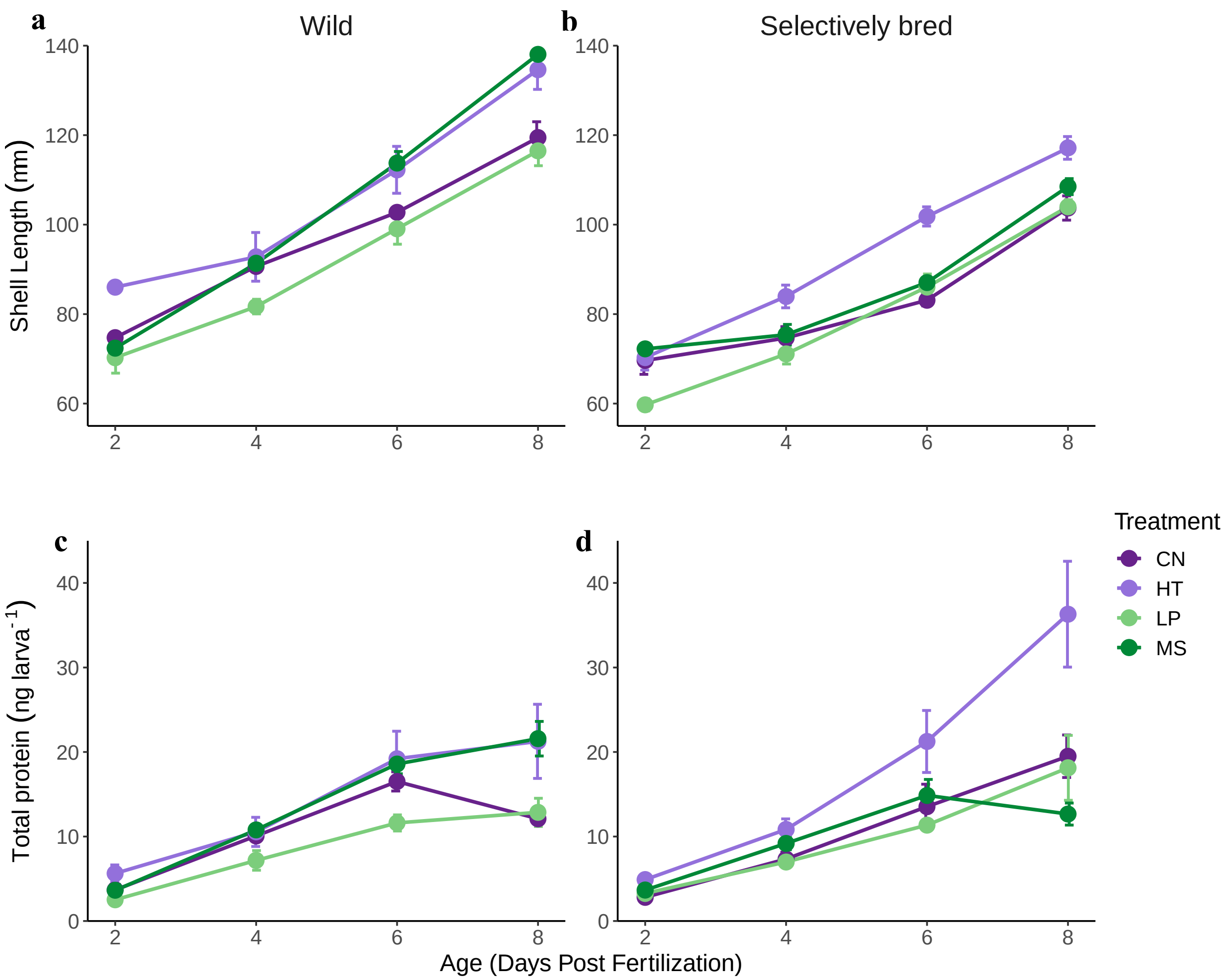

Figure 2. *Effects of multiple environmental stressors on shell length (a and b) and total protein content (c and d), proxies for shell and tissue growth, of larvae from wild (left panels) and selectively bred (right panels) Eastern oysters. Larvae were exposed to four treatment combinations of two pH and two temperature values representative of present-day average summertime ambient and future extreme conditions: Control (CN: 25°C, pH 7.8; dark purple), High Temperature (HT: 29°C, pH 7.8; light purple), Low pH (LP: 25°C, pH 7.2; light green), and Multiple Stressor (MS: 29°C, pH 7.2; dark green). Error bars represent standard error.*

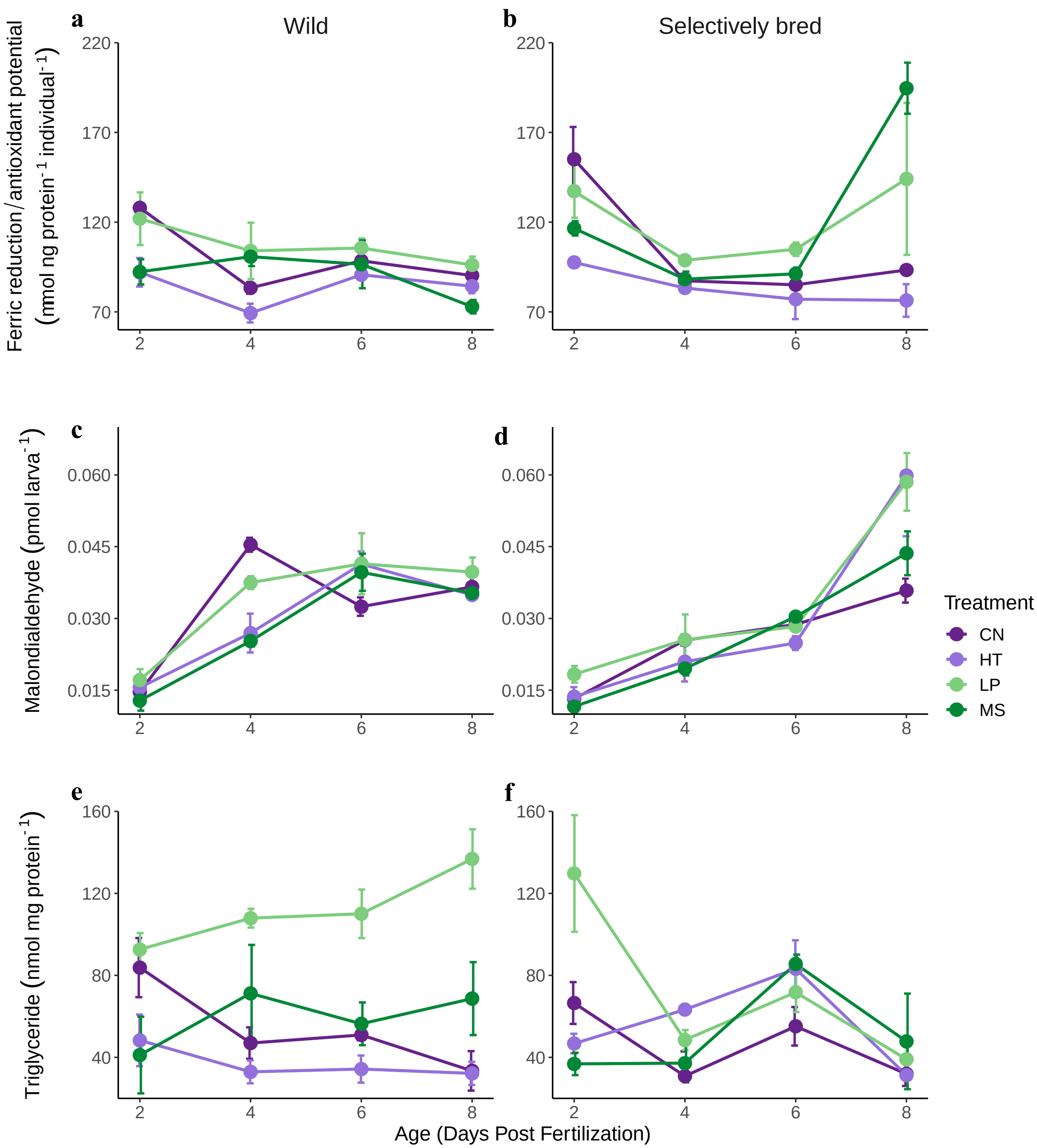

Figure 3. *Effects of multiple environmental stressors on total antioxidant capacity (a and b), lipid peroxidation (c and d), and triglyceride content (e and f) of larvae from wild (left panels) and selectively bred (right panels) Eastern oysters. Larvae were exposed to four treatment combinations of two pH and two temperature values representative of present-day average summertime ambient and future extreme conditions: Control (CN: 25°C, pH 7.8; dark purple), High Temperature (HT: 29°C, pH 7.8; light purple), Low pH (LP: 25°C, pH 7.2; light green), and Multiple Stressor (MS: 29°C, pH 7.2; dark green). Error bars represent standard error.*

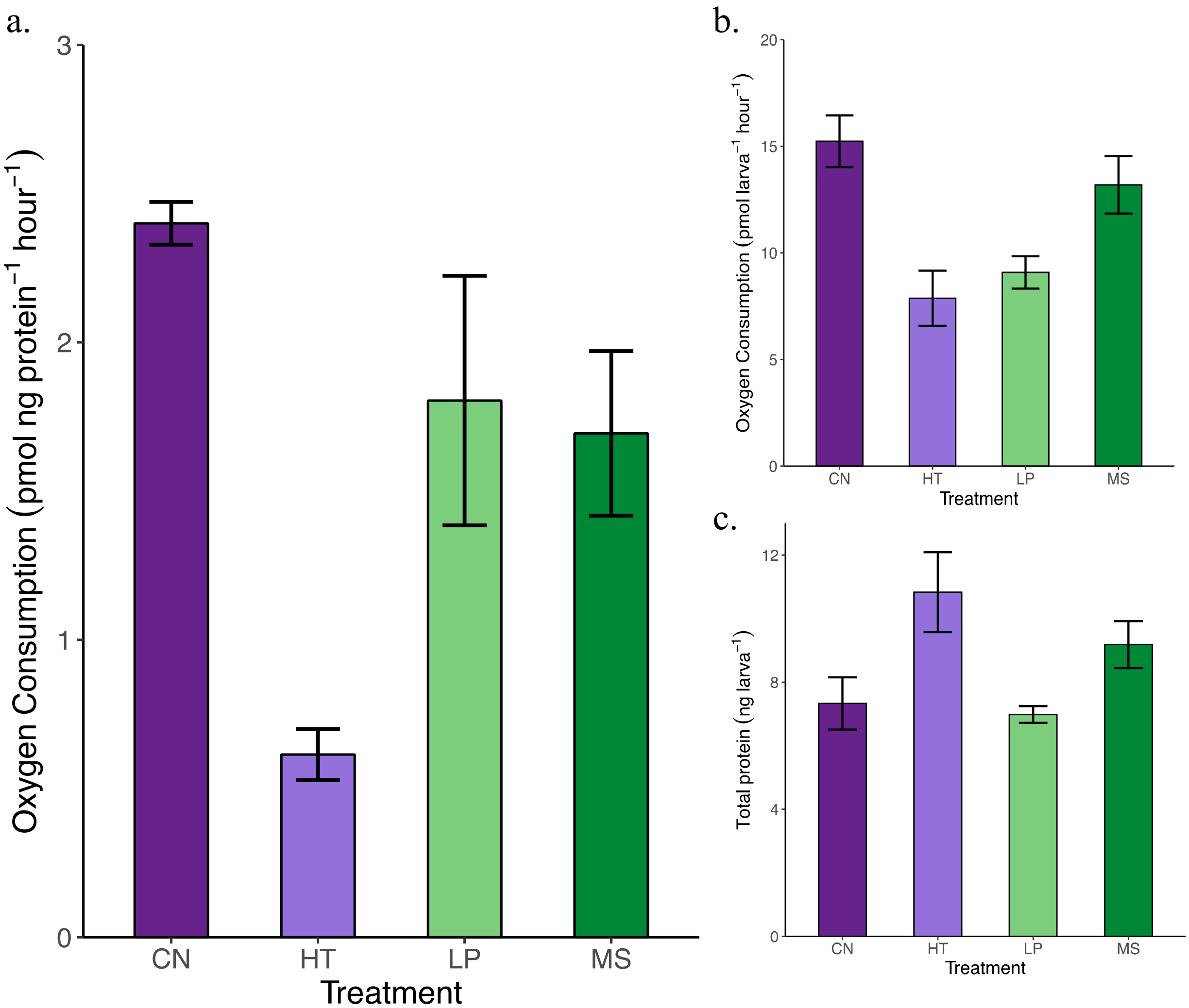

Figure 4. *Effects of multiple environmental stressors on rates of oxygen consumption standardized by total protein (a) and per individual (b), as well as total protein per individual (c) of larvae at 4 days-post-fertilization from selectively bred Eastern oysters. Larvae were exposed to four treatment combinations of two pH and two temperature values representative of present-day average summertime ambient and future extreme conditions: Control (CN: 25°C, pH 7.8; dark purple), High Temperature (HT: 29°C, pH 7.8; light purple), Low pH (LP: 25°C, pH 7.2; light green), and Multiple Stressor (MS: 29°C, pH 7.2; dark green). Asterisks and brackets indicate significant differences in oxygen consumption standardized by total protein between treatment pairs ($p < 0.05$; mixed effects linear model with a Bonferroni post-hoc test). Error bars represent standard error.*

CHAPTER 5

Carryover effects on juvenile Eastern oysters, *Crassostrea virginica*, from exposure to conditions of ocean acidification during the settlement stage

Abstract

In bivalves, exposure to conditions of coastal and ocean acidification during the larval stage can cause carryover effects, phenotypic changes from one life stage that affect a later stage; however, studies that have identified carryover effects in bivalves have often combined the larval and settlement periods in the initial exposure to acidification, making it difficult to directly attribute the origins of carryover effects from environmental stress to either the larval or settlement stages. As the settlement period, defined as the time inclusive of settlement and metamorphic processes, is a well-known bottleneck point within the lifecycle of oysters, understanding the relative influence of the settlement phase in the development of carryover effects may help focus future climate change studies as well as restoration and aquaculture efforts. The present study observed how conditions of coastal and ocean acidification experienced during settlement affected the physiology of Eastern oysters, *Crassostrea virginica*, and whether those effects carried over to influence physiology of the juvenile stage. Pre-metamorphic oyster larvae (i.e., eyed larvae) were exposed to three pH conditions through the end of the settlement stage, after which juvenile oysters were placed into common garden conditions to assess potential carryover effects. Survival, growth, oxidative stress defense and damage molecules, long-term energy storage, and aerobic metabolism were assessed during and after the settlement phase to understand direct and carryover effects of acidified conditions. No detectable carryover effects from acidified conditions were found in juvenile oysters, as no differences among the three settlement stage treatments were detected with respect to juvenile physiology, even though direct effects during settlement were identified. After three days of treatment exposure, which likely overlapped with peak metamorphic changes as evidenced by increased respiration rates, total protein content was significantly lower in oysters at the

beginning of the settlement stage under exposure to conditions of extreme acidification (i.e., pH 7.0) compared to those under ambient conditions (i.e., pH 7.8). Furthermore, respiration rates, a proxy for overall energy demand, tended to be higher in oysters experiencing some level of acidification within the first few days of the settlement period. Survival, shell length, oxidative stress defense and damage, and long-term energy storage metrics in oysters throughout the settlement stage were not significantly affected by conditions of acidification. Eastern oysters likely acclimatized to near-future acidic conditions during settlement, supported by evidence of increased utilization of endogenous energy sources alongside higher energetic demands under increasing amounts of environmental stress in these treatment groups. Due to the relatively short period of intense energetic demands associated with developmental changes during the settlement stage compared with other sensitive life stages (i.e., larval), oysters undergoing settlement may be able to begin recovery from environmental stress sooner, which could potentially ameliorate any lasting carryover effects. Future studies should simulate other environmental stressors (e.g., ocean warming) individually and simultaneously with coastal and ocean acidification to better understand the likelihood of oysters developing carryover effects after exposure during the settlement period.

Introduction

The behavioral, structural, and physiological changes that oysters undergo throughout metamorphosis and settlement are energetically intensive, likely diminishing excess energy for dealing with external stressors. Metamorphosis, or the transition from the larval to juvenile stage, is morphologically, physiologically, and ecologically dramatic, involving shifts in body form and function, energy allocation, and environmental conditions, respectively (Bishop et al., 2006). For many marine invertebrates, especially marine molluscs, metamorphosis encompasses all three of these drastic shifts as they transition from a pelagic, free-swimming larva to a benthic, sessile juvenile (Heyland and Moroz, 2006; Joyce and Vogeler, 2018). In oysters, metamorphosis involves the loss of larval structures like the ciliated velum used for feeding and swimming as well as the loss of settlement features like the eyespot and foot (Baker and Mann, 1994a; Kennedy et al., 1996). Furthermore, shell composition changes with respect to the molecular structure of calcium carbonate precipitated, from aragonite to calcite (Stenzel, 1964). Because the metamorphic stage involves a change from pelagic to benthic ecosystems, the terms metamorphosis and settlement have often been used interchangeably. While they do occur around the same period in an organism's life cycle, they are two distinct processes that happen in tandem with one another. Settlement refers to the exploratory, reversible period when larvae leave their planktonic phase and move towards the benthos to find a suitable habitat, whereas metamorphosis refers to the permanent cementation to a substrate as well as the morphological and physiological changes that occur between the larval and juvenile stages to make the organism more successful in their new environment (Burke, 1983; Hadfield and Paul, 2001; Joyce and Vogeler, 2018). For this chapter, the use of the term settlement refers to the developmental period inclusive of both processes: metamorphosis and settlement.

Oysters undergoing settlement may be more vulnerable to environmental stress due to the high demand of energy for metamorphosis. A decline in feeding rates has been observed prior to, and during, settlement, caused by a decrease in swimming activity and morphologic changes in feeding structures (Espinel-Velasco et al., 2018; Sangare et al., 2019). While oysters can ingest algal cells throughout most of the settlement period (Baker and Mann, 1994b), they rely heavily on the catabolism of endogenous energy reserves, like proteins and lipids, to provide the energy needed for the morphological and physiological changes during metamorphosis (Brunner et al., 2016; Garcia-Esquivel et al., 2001). External stress from the surrounding environment may prove detrimental to oysters going through settlement if physiological responses to environmental stress require additional energy expenditures (Sokolova et al., 2012). All organisms maintain a pool of stress-response molecules that can be used almost instantaneously in response to cellular stress, termed constitutive expression, while others are induced in response to a certain threshold of cellular damage, ultimately requiring energy (Kültz, 2005). If oysters experience extreme, or prolonged, environmental stress necessitating the production of more stress response molecules during settlement when energy is already a limited resource, basal metabolic processes (e.g., protein turnover) may be affected (Frieder et al., 2018), ultimately impacting the successful completion of metamorphosis.

Though settlement is an important bottleneck for successful development in wild oyster populations and aquaculture production, there have been relatively few studies explicitly observing potential impacts of environmental stress on oysters during settlement (Espinel-Velasco et al., 2018). Baker and Mann (1992; 1994a, 1994b) found generally negative effects of hypoxia and anoxia on oysters going through settlement and metamorphosis. Specifically, hypoxia and anoxia delayed development and growth rate, increased mortality, and decreased feeding rates in oysters during settlement. Similar biological effects have been found in pre-metamorphic and metamorphosing bivalves exposed to conditions of ocean acidification (Espinel-Velasco et al., 2018; Gobler and Talmage, 2014; Talmage and Gobler, 2009). In addition, the paucity of studies on metamorphosis under environmental stress conditions have measured general, whole-organismal physiological responses, like growth and developmental timing, and few have examined changes to underlying physiological mechanisms, like energy stores or the cellular stress response. One study examined potential underlying physiological explanations for known whole-organismal responses to acidification, like reduced shell formation, using the proteomic response of metamorphosing tubeworm larvae, *Hydroides elegans* (Mukherjee et al., 2013). Larvae exposed to acidification had significantly lower amounts of total protein than larvae raised in the other treatments. Specifically, larvae under acidification had greater downregulation of proteins used in calcification and metabolism compared to the control group, potentially explaining patterns of reduced growth and metamorphosis, respectively. Utilizing an integrated, holistic approach to climate change research by linking physiological changes in cellular processes to whole-organismal responses can illuminate the severity of future environmental stress.

While environmental stress directly impacts juvenile stages of bivalves and other marine invertebrates (Gobler and Talmage, 2013; Hettinger et al., 2013, 2012; Parker et al., 2015; Talmage and Gobler, 2011), the impacts of environmental stress during settlement and whether these impacts can affect juvenile performance are still unknown. Specifically, exposure to conditions of acidification, whether coastal or ocean acidification, may negatively affect bivalves undergoing settlement through cellular stress and less favorable calcification conditions that would require further draws on limited endogenous energy reserves and ultimately affect tissue growth. If environmental stress experienced during settlement does affect overall energy reserves and allocation, then that deficit may carry over and negatively impact the performance of later life stages (Pechenik et al., 1998). Carryover effects, phenotypic changes during an earlier life stage that impact a later stage, have been identified in bivalves after the larval or juvenile stages are exposed to an environmental stressor, like ocean acidification (Goncalves et al., 2016; Hettinger et al., 2012; Parker et al., 2015; Ross et al., 2016; Spencer et al., 2020). To date, larval carryover effect studies do not isolate the settlement phase from the larval experimental exposure (e.g., Ch. 2; Hettinger et al., 2012, 2013), making it difficult to tease apart the relative sensitivity of these two sensitive life stages in the generation of latent effects. Isolating effects of acidification stress during settlement will allow the identification of potential bottlenecks to successful juvenile recruitment and performance during this highly vulnerable time.

The purpose of this study was to understand how conditions of coastal and ocean acidification experienced during settlement would affect the physiology of *Crassostrea virginica*, and if those effects would carry over and influence oyster performance during the first month of the juvenile stage. To observe potential impacts of acidified conditions on oysters undergoing settlement, survival, growth, oxidative stress defense and damage molecules, long-term energy storage, and metabolism were assessed. The same metrics were re-measured for oyster juveniles to detect potential presence and persistence of carryover effects. It was hypothesized that experience of acidic conditions would decrease survival, growth, and long-term energy storage, while increasing the abundance of oxidative stress defense and damage molecules and metabolic rates during the settlement stage. Furthermore, oysters exposed to conditions of ocean acidification during settlement would have reduced performance as juveniles compared to individuals exposed to control conditions, regardless of the present environmental experience.

Methods

1. Experimental Design

To observe carryover effects associated with exposure to conditions of ocean acidification during settlement, *Crassostrea virginica* were cultured under three ecologically relevant pH treatments from metamorphically competent eyed larvae through the end of settlement, a period of approximately thirteen days, in the Environmental Change Aquarium System at the Virginia Institute of Marine Science (VIMS). Oysters were assessed at three and eleven days of treatment exposure (18- and 26-days post-fertilization [dpf], respectively) during the settlement stage for growth, oxidative stress, long-term energy storage, and whole-organism metabolism. Juveniles from all three treatment groups were then held under common garden conditions of unfiltered York River water, referred to as the nursery phase. The physiological parameters, except for metabolism and long-term energy storage, were re-assessed after one and five weeks in the nursery to test for the presence and persistence of carryover effects from the carbonate chemistry conditions experienced during settlement, or for potential acclimatization to the new environment (Figure 1).

The three experimental treatments were designed to be relevant to oysters growing in the Rappahannock River in Virginia, an area of active oyster aquaculture production. Treatment levels represent average pH conditions (pH 7.8; Control), low pH conditions that are frequently observed (pH 7.4; Low pH), and lower pH conditions that are less frequently observed (pH 7.0; Extreme Low pH), based on pH data collected during July in 2016 and 2017 by a commercial hatchery partner, Oyster Seed Holdings, Inc. (OSH) located in the Rappahannock River in Grimstead, VA (see *Chapter 3, Figure 2)*. Eyed larvae were cultured under the three pH treatments at a salinity of 15 and temperature of 25°C that mimic summertime average conditions for the lower portion of the Rappahannock River based on environmental data provided by OSH and retrieved from the NOAA Chesapeake Bay Integrated Buoy System (CBIB) Stingray Point buoy (see *Section 2 settlement phase*).

2. Settlement Phase

Triploid *C. virginica* eyed larvae (15 dpf) were purchased from Ward Oyster Company (DEBY broodstock line, [Ragone Calvo et al., 2003], batch C54, Mobjack Bay, Gloucester, VA) in July 2019. Adult diploid females (n = 16) and tetraploid males (n = 3) were strip-spawned to produce gametes that were then pooled across all males and females prior to fertilization. The larvae were cultured under ambient environmental conditions at Ward Oyster Co. for 15 days prior to the start of the experiment where salinity was on average ~15 (unpublished data; Ward Oyster Company), matching experimental conditions. The eyed larvae were randomly divided amongst the three pH treatments described above.

Eyed larvae (n ≅ 75,000 per aquarium) were cultured through settlement (13 days total) in July 2019. pH treatments were generated in three aquaria each in the Environmental Change Aquarium System at VIMS, to create biological pseudoreplicates. Briefly, temperature and pH conditions were individually controlled in nine, 100 L aquaria using a software control system. Tank conditions were continuously measured by pH electrodes and thermistors, and water conditions were autonomously adjusted to meet setpoint conditions using heat exchangers connected to hot- and cold-water loops and controlled delivery of CO_2 or CO_2-free air. In 2019, generation of CO_2-free air was achieved by passing compressed atmospheric air through Drierite to remove any moisture and then soda ash to strip out CO_2. For more details about the aquarium system, refer to *Chapter 3, Section 1.1*.

Experimental aquaria were set up as static downwelling systems with a 150 µm-sieve that was suspended about 2 cm below the surface of the water containing finely ground oyster shell, or cultch. The water in each aquarium was recirculated downward onto the sieve using the recirculation pump, which maintained homogenous treatment conditions, delivered and suspended larval food, and promoted settlement onto individual pieces of cultch. Larvae were fed Shellfish Diet LPB® every day according to instructions provided by Reed Mariculture Inc. (Campbell, CA). A 100% water change was conducted three times per week to remove any build-up of nitrogenous waste in the culture water.

Prior to each water change, water samples were collected from each aquarium for independent measurements of pH_{total} and from the mixing tank for measurements of total alkalinity (see *Section 4 carbonate chemistry analysis*). Complementing each water sample were measurements of temperature, dissolved oxygen, pH, conductivity, and salinity using a YSI Pro Plus (YSI Inc., Yellow Springs, OH). Individual aquarium conditions were also continuously monitored by glass electrodes (refer to *Chapter 3, Section 1.1*).

Sample collection for physiological analyses occurred at three and eleven days of treatment exposure (18 and 26 dpf, respectively) to test for acute and sustained stress, respectively. First, oyster density was determined volumetrically from a random, concentrated sub-sample after measuring the total volume of oysters per treatment replicate. The density was calculated from a minimum of three sequential counts of 50 µL of the concentrated, homogenized oyster sub-sample, during which images of individual oysters ($n \cong 100$) were also captured using a Leica DM compound microscope (Deerfield, IL) with a Leica MC170 HD camera. Then whole oysters were sampled volumetrically for triglyceride content, total antioxidant capacity, lipid peroxidation, and total protein content based on desired counts (n_{day3}

≅ 1000, n_{day11} ≅ 500; see *Section 5 physiological assays* for more details); samples were preserved at -80°C until later analysis. Oysters were also collected for respirometry on days 4 (n ≅ 1000) and 11 (n ≅ 100) of exposure after being held under treatment conditions for 24 hours without any food prior to measurement. Once all physiological samples were collected, the remaining oysters were returned to the aquarium.

3. Juvenile Phase

Once the oysters began to develop adult morphological features (i.e., gills), relatively no eye spots or foot muscles remained, and shell sizes were generally greater than 700 µm, oysters from all treatment replicates were held under common garden conditions in an upwelling system at VIMS Aquaculture and Breeding Technology Center (ABC) for five weeks. In the upwelling system, each treatment replicate group was individually contained within a PVC cylinder with 500 µm mesh on the bottom in one flow-through tank (i.e., raceway). The incoming, unfiltered York River water flowed up past the oysters to the drain, allowing juvenile oysters to feed *ad libidum* on available phytoplankton. Nursery tanks were drained, cleaned, and refilled three times a week to abate sediment build-up or clogging of the mesh. To document nursery tank water conditions, a HOBO® U24 series logger (Onset®) recorded water temperature and conductivity every 30 minutes. A SeapHOx™ V2 (Sea-Bird Scientific) measured water temperature, salinity, dissolved oxygen, and pH every 10 minutes. Water samples were collected once a week for independent measurements of carbonate chemistry as *in situ* validation for the SeapHOx™ (See *Section 4 carbonate chemistry analysis*; Rivest et al., 2016).

Whole oysters were collected after one and five week(s) under common garden conditions from all treatment replicates to assess any carryover effects from experience of low pH during settlement or potential acclimatization. Oysters were preserved at -80°C until analysis of shell growth (n = 50), total antioxidant capacity (n_{week1} = 200, n_{week5} = 5), total protein content (from total antioxidant capacity samples), lipid peroxidation (n_{week1} = 200, n_{week5} = 5), and triglyceride content (n_{week1} = 200, n_{week5} = 5). Shell growth (i.e., shell length, hinge to bill) for the first sampling time point was measured from images captured with a Leica S9i dissection microscope with integrated camera after preservation in 95% ethanol. Juvenile shell length at the end of the experiment was measured using calipers.

4. Carbonate Chemistry Analysis

Carbonate chemistry conditions were characterized from water samples collected throughout the experiment. Total alkalinity was measured using open-cell potentiometric titrations following best practices (SOP 3b; Dickson et al., 2007). pH_{total} was measured spectrophotometrically using m-cresol purple dye, following best practices (SOP6b; Dickson et al., 2007). All pH samples were run within an hour of collection; water samples used for total alkalinity measurements were held at -20°C until later analysis. Total alkalinity and pH were used to calculate other carbonate chemistry parameters using the Seacarb package in R (Gattuso et al., 2021), complementary temperature and salinity data, and the following constants: K_1 and K_2 for carbonic acid (Lueker et al. 2000), K_F (Perez and Fraga 1987), total boron (Lee et al. 2010), and K_{SO4} (Dickson et al. 1990). Refer to *Chapter 2, Section S1.5* for more detailed methods of the carbonate chemistry measurements.

5. Physiological Assays

Total protein content was measured using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, 23225; Smith et al., 1985). Total antioxidant capacity was measured using the ferric reducing/antioxidant potential (FRAP) assay following (Griffin and Bhagooli, 2004). Lipid peroxidation was measured with the Lipid Peroxidation (MDA) Assay

Kit (Cat. No. ab118970, Abcam). Triglyceride content was measured using the Triglyceride Quantification Kit (Cat. No. ab65336, Abcam). All spectrophotometric assays were conducted using a Molecular Devices SpectraMax® iD3 microplate plate reader. Refer to *Chapter 2, Section 2.4* and *Chapter 3, Section 6* for more detailed methods of the individual physiological assays. To measure the shell length of metamorphosing and juvenile oysters, ImageJ (Schneider et al., 2012) was used to measure the longest length (hinge to bill) of 50 randomly selected oysters from images captured during sampling, as described above. Lastly, rates of oyster respiration were measured at the beginning and end of settlement using endpoint respirometry. For each treatment replicate, three sub-samples of 10 oysters each were incubated in micro biological oxygen demand vials for several hours at 25°C. Oxygen consumption was measured using a PreSens Microx 4 Oxygen Microsensor (Regensburg, Germany; Marsh and Manahan, 1999). For respirometry methods, refer to *Chapter 3, Section 6.3*.

6. Statistical Analyses

To detect effects of ocean acidification on the physiology of Eastern oysters undergoing settlement and any potential carryover effects from that exposure on juvenile physiology, a linear mixed effects model was used with the *nlme* package (version 3.1-160; Pinheiro et al., 2020; Pinheiro and Bates, 2000) in R (version 4.2.2; R Core Team, 2022). Effects of experimental treatment on each physiological response variable at each sampling timepoint were analyzed independently. Water conditions were analyzed over the entire settlement period using the same linear mixed effects model as previously described. Posthoc testing was done using the *multcomp* package (version 1.4-20; Hothorn et al., 2008) in R. The pH treatment was treated as a fixed effect while tank replicate was included as a random factor to account for any tank effects. All water parameters and physiological response variables satisfied the assumptions of the Shapiro-

Wilkes test for normalcy and Levine's test for homogeneity. The Shapiro-Wilkes test was analyzed in *stats* (R Core Team, 2022), and the Levine's test was conducted with *car* (Fox and Weisberg, 2019).

Results

1. Experimental Water Conditions

As expected, several water condition parameters differed significantly among the three treatments over the entire settlement period, specifically pH, aragonite and calcite saturation states, and dissolved oxygen (Tables 1, 2). The greatest differences in aragonite and calcite saturation states, as well as in dissolved oxygen, were 0.28, 0.45, and 0.78 mg L^{-1}, respectively, between the Control and Extreme Low treatments (Table 1). Dissolved inorganic carbon (DIC) was significantly lower in the Control treatment than in the other two treatments (Control – Low pH: 63 μmol kg^{-1}, Control – Extreme Low pH: 108 μmol kg^{-1}; Table 1) and did not significantly differ between the two low pH treatments (Tables 1, 2). Temperature and salinity were similar across all treatment conditions (Tables 1, 2). Total alkalinity in the aquaria was 907 ± 179 μmol/kg (mean ± SE). Water conditions experienced by juvenile oysters are summarized in Table 3.

2. Effects of acidification conditions on oyster physiology during settlement

Oyster physiology during settlement generally did not differ among treatment groups (Table 4). After three days of treatment exposure at the beginning of the settlement process, exposure to either low pH treatment did not significantly affect shell size, total antioxidant capacity, lipid peroxidation, triglyceride content, or respiration (Figure 2c, Table 4). Though triglyceride content did not differ among pH treatments at the first sampling time point (18 dpf), triglyceride content did decrease by more than 84% from the triglyceride content of larvae subsampled at 15 dpf prior to the start of the experiment (triglyceride content = 372 nmol mg protein^{-1}). Acidification exposure, however, negatively affected total protein content in oysters at the beginning of settlement (Table 4). Specifically, oysters in the Control treatment had 61.1% more total protein content compared to those in the Extreme Low pH treatment, though total protein content was not different between oysters in either low pH treatment or between those in the Control and Low pH treatments (Figure 1a, Table 4).

By the end of settlement, after eleven days of treatment exposure, total protein content no longer differed between treatment groups (Figure 2b, Table 4). Shell size, total antioxidant capacity, triglyceride content, and lipid peroxidation were also similar among oysters in all treatment groups (Table 4). Additionally, survival, measured at the end of the settlement stage was also not significantly affected by treatment conditions (Table 4). Acidification exposure did have a marginally significant effect on oyster respiration at the end of settlement (Table 4). Oysters in the Extreme Low pH consumed oxygen more slowly compared to those in the Low pH treatment (Figure 2d, Table 4). Oysters in the control treatment had similar respiration rates to oysters in the other two treatment conditions (Figure 2d, Table 4).

3. Carryover effects of acidification experience during settlement on juvenile oyster physiology

Physiology of juvenile oysters throughout the nursery phase was similar among settlement treatment groups (Table 5). Shell size, total protein content, total antioxidant capacity, and lipid peroxidation were not statistically different among the three settlement conditions after one or five weeks in common garden (Table 5).

Discussion

The settlement phase, which, as defined here, encompasses both settlement and metamorphic processes, is an energy-intensive period during which oysters are potentially vulnerable to external stressors. The goal of this chapter was to understand how oysters going through settlement respond physiologically to conditions of ocean acidification, and if those effects may carry over and impact the juvenile stage. While there was evidence that conditions of ocean acidification did affect certain aspects of oyster physiology during settlement (Figure 2, Table 3), there were no detectable carryover effects, positive or negative, from acidification experience during settlement on juvenile performance (Table 4). Eastern oysters can likely acclimatize to near-future acidic conditions during settlement, one of the most energetically demanding periods in their life cycle.

Conditions of ocean acidification negatively affected oysters within the first few days of settlement, coinciding with the most intense period of morphogenic processes (Baker and Mann, 1994a). While the timing of metamorphosis varies considerably among individuals, between several hours to two weeks, Eastern oysters, on average, complete metamorphosis in three days, matching the first sampling timepoint in this study (Baker and Mann 1994a). Respiration rates, a proxy for aerobic metabolism and overall energy demands, were elevated at the first sampling timepoint compared to the end of the settlement period by 1.14, 2.13, and 3.5 times in the Control, Low pH, and Extreme Low pH treatments, respectively (Figure 2c,d; Table 3), providing evidence for peak metamorphosis. Furthermore, Baker and Mann (1994b) found that during this energetically intensive time, there is a period after cementation (~24 hrs) where oysters do not consume food and noted that evidence of digestion, in the appearance of pseudofeces, did not occur until near the end of the metamorphic process (~48 hrs). Oysters therefore rely on endogenous energy sources (i.e., lipids and proteins) to carry them through morphogenic transitions during the first few days of settlement (Garcia-Esquivel et al., 2001). In the present study, evidence supports the use of endogenous energy reserves: first, triglyceride content declined by more than 84% from the start of the experiment to the first sampling timepoint, and second, oysters that experienced the Extreme Low pH treatment (i.e., pH 7.0) had lower total protein content compared to those in the Control treatment after three days of treatment exposure (Figure 2a, Table 3). Individuals experiencing the Low pH treatment (i.e., pH 7.4) did have lower total protein content but not significantly different from either of the other two treatments (Figure 2a, Table 3).

Interestingly, conditions of ocean acidification did not affect shell growth during the settlement period, as shell size did not differ between individuals in the three treatments (Table 3). In previous ocean acidification studies, shell size has frequently been found to be negatively impacted (Beniash et al., 2010; Frieder et al., 2018; Talmage and Gobler, 2009) more often than not impacted (Lim et al., 2021), though these studies focus on the larval phase that utilizes the more soluble form of calcium carbonate (i.e., aragonite versus calcite which juveniles precipitate) to build their shells. One possible explanation supported by the observed decrease in tissue biomass after three days of exposure to acidification is the prioritization of calcification to maintain shell growth, which would in turn leave less energy remaining for protein synthesis and somatic growth creation, another energy intensive process (Frieder et al., 2018; Pan et al., 2018).

Maintaining elevated calcification rates may have evolved as a strategy to combat high post-settlement mortality, but at a price. Prioritization of shell growth and calcification in newly settled marine invertebrates likely reduces the risk of predation and mitigates physical properties of their surrounding habitat, like preventing smothering from sedimentation (Hunt and Scheibling, 1997; Pruett and Weissburg, 2019); however, calcification under acidic conditions can require more energy at the expense of other important biological and physiological processes, like tissue biomass accumulation (Frieder et al., 2017; Hettinger et al., 2012; Liu et al., 2020; Sokolova et al., 2012; Waldbusser et al., 2013; Zhao et al., 2017). When experiencing ocean acidification, oysters need to modify their internal calcifying fluid to be thermodynamically favorable to convert bicarbonate, which is more prevalent in the water column under acidified conditions, into carbonate before secreting the final product, calcium carbonate (Ramesh et al., 2017; Zhao et al., 2018). Though no statistical significance was detected, there was a pattern of higher respiration rates from individuals in the Low pH and Extreme Low pH treatments compared those in the Control treatment (~53% higher) at the first sampling point, signaling a potential increase in energy demand under acidic conditions. Increased metabolism in oysters experiencing ocean acidification has been found in juvenile Eastern oysters (Beniash et al., 2010), providing some support that further testing of oysters during the settlement period using higher replication or different treatment conditions may detect significant differences in respiration rate. Although oysters in the Extreme Low pH treatment were able to maintain shell growth, there was a trade-off for their protein content and ultimately, tissue biomass accumulation, likely due to sub-optimal calcification conditions and lack of additional energy sources due to the absence of ingestion (Pörtner, 2010; Sokolova et al., 2012).

A decline in total protein content in oysters experiencing acidification conditions could be indicative of an increased utilization of endogenous energy sources. To support the higher costs of maintaining shell growth, oysters under acidified conditions likely relied more heavily on the catabolism of proteins for energy compared to individuals in ambient conditions. As previously mentioned, oysters going through peak metamorphosis essentially cease feeding though energy demand is high, illustrated by higher respiration rates in this study. During peak metamorphosis, oysters have been found to exhibit decreased protein and lipid contents due to catabolism of endogenous energy sources stored in tissues (Garcia-Esquivel et al., 2001). Furthermore, one study discovered that individuals going through peak metamorphosis while experiencing environmental stress caused by exposure to a harmful algal bloom (HAB) catabolized more protein compared to those in ambient, non-HAB conditions (Garcia-Esquivel et al., 2001). Here, acidification exposure did not affect triglyceride levels (Table 3), though there was a large decline (> 84%) in triglyceride content between the start of the experiment and the first sampling timepoint, suggesting that larvae across all three treatments were utilizing endogenous lipid stores to a similar degree; however, it is possible that oysters experiencing extreme ocean acidification (i.e., pH around 7.0) during peak metamorphosis relied instead on the catabolism of proteins to supply the energy needed to sustain shell growth at the expense of tissue biomass accumulation (Lim et al., 2021). Any deficit in total protein did not produce carryover effects, however. Rather, by the end of settlement and treatment exposure, protein content of oysters in the most acidic treatment had recovered to the level of the oysters in the two other treatment groups, though the high variability in protein content among replicates may be masking a true trend.

Experience of acidification conditions during settlement did not carry over into the juvenile phase likely because oysters acclimatized to the environmental stress by the end of the settlement period. Tissue biomass (i.e., total protein content) of oysters exposed to the Extreme Low pH treatment, which differed at three days of exposure, was not different than that in individuals from the Control treatment by the second sampling timepoint at the end of settlement (Figure 2b, Table 3). Furthermore, individuals in the Extreme Low pH treatment had significantly lower respiration rates by the end of settlement compared to those in the Low pH treatment and similar to those in the Control (Figure 2d, Table 3), suggesting that oysters in the Extreme Low pH treatment had acclimatized to their treatment conditions. Selective mortality, when environmentally stressful conditions select for advantageous genotypes through differential survival (Gardner and Skibinski, 1991), was likely not responsible for the perceived recovery of tissue biomass and respiration rates, as survival did not differ among the three treatment groups (Table 3).

Recovery from ocean acidification effects may have been supported by a high-quality food source readily available after the peak metamorphic changes occurred as well as overlapping cellular stress responses with settlement processes. Oysters going through settlement resume algal consumption after 48-72 hours once the major metamorphic changes have occurred, rather than relying on endogenous energy sources for the rest of settlement. Oysters in this study were fed *ad libitum* with an ideal diet for commercial shellfish culture. Though studies have shown that environmental stress can decrease feeding ability in oysters (Baker and Mann, 1994b; Clements and Darrow, 2018), others have noted the importance of food availability in resilience to ocean acidification and other environmental stressors for several bivalve species (Brockington and Clarke, 2001; Leung et al., 2019; Ramajo et al., 2016; Thomsen et al., 2013; Torres et al., 2016). Access to high-quality food not only provides exogenous energy to support active stress responses, but also can aid in oxidative stress defense through the provision of dietary antioxidants and restoration of long-term energy stores (Amaro et al., 2019; Gibbs et al., 2021; Lesser, 2006). While not statistically significant, oysters in the Extreme Low pH treatment did have almost double the total antioxidant concentration during settlement than individuals in the Control treatment on average, which correlated with less lipid peroxidation (i.e., damage due to oxidative stress). Additionally, overlapping cellular stress responses to settlement and acidified conditions may lessen the energetic costs of supporting these processes simultaneously, leaving energy for allocation to other biological processes (e.g., growth) and ultimately aiding in acclimatization. The developmental up-regulation of genes controlling the cellular stress response (e.g., antioxidant production) and membrane function, for easier molecular transport, have been linked to both settlement and environmental stress experience (Dineshram et al., 2016; Durland et al., 2021; Heyland and Moroz, 2006; Tomanek et al., 2011). Additionally, DNA methylation has been identified as a potentially important mechanism in facilitating transcription of genes controlling aspects of the cellular stress response and metabolic pathways, and changes in methylation patterns have been observed in organisms experiencing acidification and other environmental stressors (Downey-Wall et al., 2020; Lim et al., 2021; Putnam et al., 2016; Wang et al., 2021). High-quality food in addition to oxidative stress and associated cellular responses may be important in emergence from the non-feeding settlement period, especially under environmental stress.

Though oysters undergoing peak metamorphosis may be vulnerable to environmental stress, the timescale of this developmental period is short relative to other sensitive life stages (i.e., larval), enabling oysters to begin recovery sooner, potentially diminishing chances of lasting carryover effects. Studies identifying carryover effects from ocean acidification exposed oysters over the entire larval stage through the settlement period, as previously mentioned (Hettinger et al., 2012). While the lengths of the settlement and larval stages can be similar, the highest developmental energy demands are likely condensed to peak metamorphosis (~2-3 days) compared to the larval stage where oysters experience near-constant demand (Pan et al., 2018). In larval oysters, it is estimated that more than 80% of the total energy pool is allocated for development, leaving little energy for handling external stressors (Pan et al., 2018). While energy budget estimates for metamorphosing oysters are sparse, energy allocation for developmental processes likely decreases after the completion of peak metamorphosis when structures have transitioned into their near-final forms (Baker and Mann, 1994). With continued exposure to environmental stress during the larval stage, energy budgets and pools are constantly strained with little ability to allocate reserves towards recovery processes, unlike the settlement phase, likely leading to carryover effects (Frieder et al., 2018; Marshall and Morgan, 2011). In the present study, oysters exposed to conditions of acidification at peak metamorphosis were negatively affected, but ultimately recovered before the end of settlement. Carryover effects from environmental stress experienced during settlement may be less likely to occur given the shorter period of high energy demand compared to other life stages. Documented carryover effects in previous studies likely stem from stress experienced during the larval stage rather than the settlement phase, supported by the findings and rationale from this study. Future studies, however, should observe whether similar results are found when oysters going through settlement are exposed to other environmental stressors (e.g., ocean warming) individually and simultaneously with ocean acidification to better understand the likelihood of developing carryover effects.

Eastern oysters going through settlement while living in mesohaline environments may be resilient to near-future ocean acidification, protecting future life stages from carryover effects that could impact performance. Oysters in this study were tolerant of moderate conditions of ocean acidification (i.e., pH 7.4), with no significant effects to shell and tissue growth, cellular stress, metabolism, or long-term energy stores. Extreme acidified conditions (i.e., pH 7.0) affected tissue biomass accumulation within the first few days of treatment exposure, which overlapped with the average timing for peak metamorphic activity, but those effects were not detectable by the end of settlement, suggesting that oysters had acclimatized to acidic conditions. Effects of ocean acidification on oxidative stress levels, triglycerides, and metabolism, though non-significant, provide preliminary support for mechanisms of acclimatization during settlement, as in the up-regulation of cellular stress response molecules (e.g., antioxidants) as seen in other studies through changes in DNA methylation and gene regulation that then lead to lower burdens on energy demands. However, future studies with larger sample sizes are needed to confirm these trends as they were not statistically supported due to lack of statistical power. Overall, though settlement is a known bottleneck to the success of oyster populations and aquaculture production, the experience of acidified conditions likely will not compound the energetic costs of settlement and metamorphosis alone. Further studies should observe how oysters undergoing settlement and metamorphosis may respond to additional environmental stressors.

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Tables

Table 1. *Water conditions during the settlement period for all treatment groups. Values represent mean ± standard error calculated across all treatment replicates over the settlement period. Temperature, salinity, dissolved oxygen, and pH_{tot} (pH, total scale) are measured values. DIC (dissolved inorganic carbon), $\Omega_{calcite}$ (saturation state of calcite), and $\Omega_{aragonite}$ (saturation state of aragonite) are calculated values.*

	Treatments		
Water Conditions	Control (7.8)	Low pH (7.4)	Extreme Low pH (7.0)
Temperature (°C)	24.99 ± 0.33	24.92 ± 0.37	24.87 ± 0.32
Salinity	16.55 ± <0.01	16.56 ± <0.01	16.56 ± <0.01
Dissolved Oxygen (mg L^{-1})	6.88 ± 0.13	6.53 ± 0.26	6.10 ± 0.38
pH_{tot}	7.64 ± 0.09	7.38 ± 0.07	7.05 ± 0.07
DIC (μmol kg^{-1})	865 ± 302	928 ± 313	973 ± 324
$\Omega_{aragonite}$	0.39 ± 0.14	0.22 ± 0.08	0.11 ± 0.04
$\Omega_{calcite}$	0.64 ± 0.23	0.37 ± 0.13	0.19 ± 0.07

Table 2. *Analysis of water conditions during the settlement period. Comparisons were made using mixed effects linear models, with pH treatment as a fixed effect and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) are reported. Tukey post-hoc tests were performed where appropriate, and test statistics z and p-values (p) are reported. Asterisks indicate statistical significance below an alpha threshold of 0.05.*

Water condition	Chi-square	p	Post hoc comparison	Z value	p
Temperature	$X^2_{(2,9)} = 2.103$	0.257			
Salinity	$X^2_{(2,9)} = 3.196$	0.202			
Dissolved Oxygen	$X^2_{(2,9)} = 33.610$	<0.001*	Control – Low pH	-2.594	0.026*
			Control – Extreme Low pH	-5.787	<0.001*
			Low pH – Extreme Low	-3.193	0.004*
pH_{tot}	$X^2_{(2,9)} = 376.330$	<0.001*	Control – Low pH	-8.449	<0.001*
			Control – Extreme Low pH	-19.348	<0.001*
			Low pH – Extreme Low	-10.839	<0.001*
DIC	$X^2_{(2,9)} = 19.267$	<0.001*	Control – Low pH	2.579	0.027*
			Control – Extreme Low pH	4.366	<0.001*
			Low pH – Extreme Low	1.787	0.174
$\Omega_{aragonite}$	$X^2_{(2,9)} = 173.960$	<0.001*	Control – Low pH	-7.905	<0.001*
			Control – Extreme Low pH	-13.096	<0.001*
			Low pH – Extreme Low	-5.191	<0.001*
$\Omega_{calcite}$	$X^2_{(2,9)} = 173.970$	<0.001*	Control – Low pH	-7.986	<0.001*
			Control – Extreme Low pH	-13.096	<0.001*
			Low pH – Extreme Low	-5.191	<0.001*

Table 3. *Water conditions experienced by juvenile oysters in all treatment groups under common garden conditions. Summary statistics over the entire common garden period are provided. Temperature, salinity, dissolved oxygen, pH_{tot} (pH, total scale), and total alkalinity are measured values. DIC (dissolved inorganic carbon), and $\Omega_{calcite}$ (saturation state of calcite) are calculated values.*

Water Conditions	Mean ± SE	Common Garden Minimum	Common Garden Maximum
Temperature (°C)	27.06 ± 0.37	24.14	30.34
Salinity	20.10 ± 0.18	18.16	21.52
Dissolved Oxygen (mg L^{-1})	3.48 ± 0.29	0.55	5.74
pH_{tot}	7.64 ± 0.05	7.16	8.11
Total alkalinity (µmol kg^{-1})	1766 ± 22	1485	1811
DIC (µmol kg^{-1})	1717 ± 28	1360	1881
$\Omega_{calcite}$	1.40 ± 0.17	0.44	3.50

Table 4. *Analysis of oyster physiology during the settlement period. Comparisons were made using mixed effects linear models, with pH treatment as a fixed effect and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) are reported. Tukey post-hoc tests were performed where appropriate, and test statistics z and p-values (p) are reported.*

Physiological metric	Chi-square	p	Post hoc comparison	Z value	p
18 dpf (3 days of exposure)					
Shell Size	$X^2_{(2,9)} = 0.872$	0.647			
Total Protein Content	$X^2_{(2,9)} = 8.157$	0.017*	Control – Extreme Low	-2.854	0.012*
			Control – Low pH	-1.520	0.282
			Low pH – Extreme Low	-1.334	0.376
Total Antioxidant Capacity	$X^2_{(2,9)} = 3.451$	0.178			
Lipid Peroxidation	$X^2_{(2,9)} = 0.962$	0.618			
Triglyceride Content	$X^2_{(2,9)} = 1.114$	0.573			
Respiration	$X^2_{(2,9)} = 2.251$	0.324			
26 dpf (11 days of exposure)					
Survival	$X^2_{(2,9)} = 0.961$	0.619			
Shell Size	$X^2_{(2,9)} = 3.540$	0.170			
Total Protein Content	$X^2_{(2,9)} = 0.918$	0.632			
Total Antioxidant Capacity	$X^2_{(2,9)} = 1.357$	0.508			
Lipid Peroxidation	$X^2_{(2,9)} = 1.240$	0.538			
Triglyceride Content	$X^2_{(2,9)} = 2.129$	0.345			
Respiration	$X^2_{(2,9)} = 5.959$	0.051*	Control – Extreme Low	-1.342	0.372
			Control – Low pH	1.095	0.517
			Low pH – Extreme Low	-2.437	0.039*

Table 5. *Analysis of oyster physiology during the juvenile stage in the nursery. Comparisons were made using mixed effects linear models, with pH treatment as a fixed effect and tank as a random factor. Test statistic χ^2, degrees of freedom, and p-values (p) are reported. Asterisks indicate statistical significance below an alpha threshold of 0.05.*

Physiological metric	Chi-square	p
One week of common garden		
Shell Size	$X^2_{(2,9)} = 0.759$	0.684
Total Protein Content	$X^2_{(2,9)} = 1.544$	0.462
Total Antioxidant Capacity	$X^2_{(2,9)} = 0.684$	0.711
Lipid Peroxidation	$X^2_{(2,9)} = 2.171$	0.338
Five weeks of common garden		
Shell Size	$X^2_{(2,9)} = 1.176$	0.556
Total Protein Content	$X^2_{(2,9)} = 0.641$	0.726
Total Antioxidant Capacity	$X^2_{(2,9)} = 0.415$	0.813
Lipid Peroxidation	$X^2_{(2,9)} = 5.642$	0.060

Figures

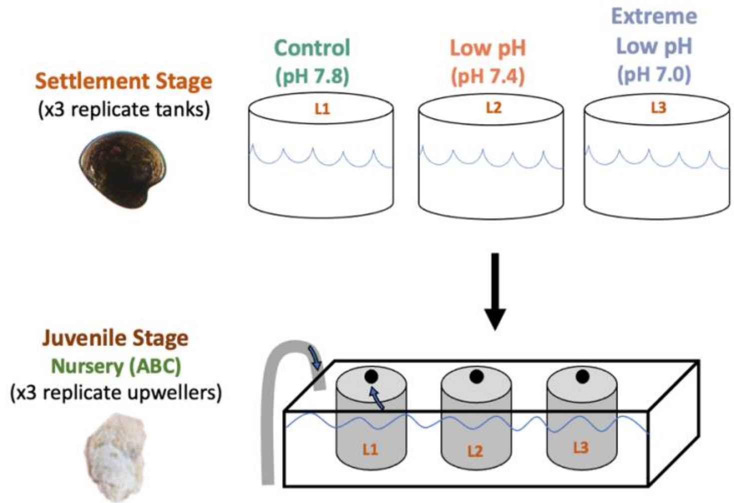

Figure 1. *The experimental design to observe how exposure to ocean acidification conditions during settlement (i.e., settlement and metamorphosis) may carry over to affect the physiology of juvenile oysters using environmentally relevant treatment conditions based on data from the Rappahannock River.*

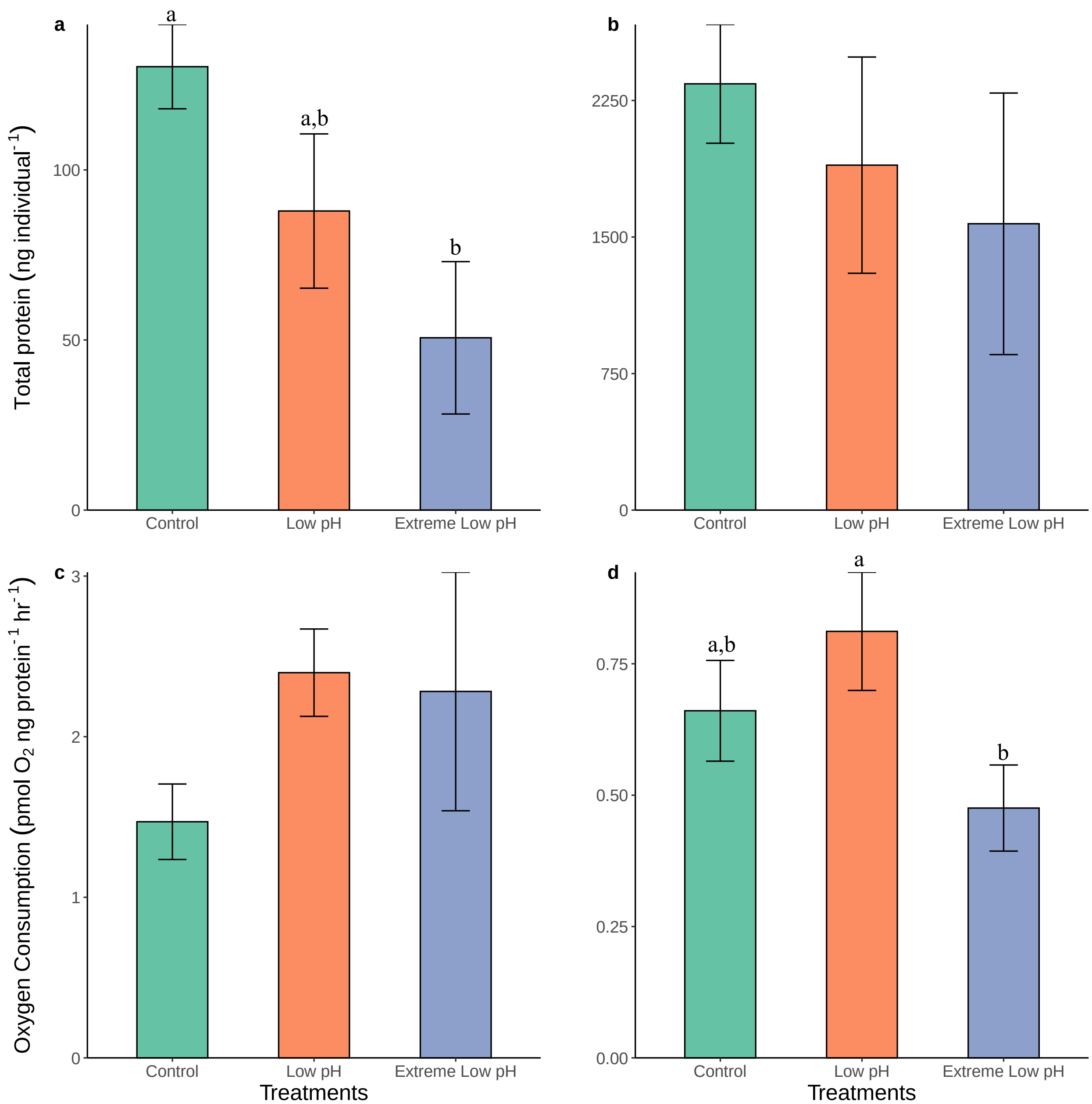

Figure 2. *Effect of acidification conditions on total protein content (a, b), a proxy for tissue biomass accumulation, and oxygen consumption (c,d), a proxy for whole organism metabolism, in Eastern oysters undergoing settlement. Oysters were placed into one of three treatments representative of summertime average ambient (pH 7.8; green), low (7.4; orange), and extreme low (pH 7.0; purple) acidification conditions in the Rappahannock River. Physiological measurements were collected three days after exposure to treatments when oysters were 18 days old (a, c) and after 11 days of treatment exposure when oysters were 26 days old and near the end of their settlement period (b, d). Different letters indicate significant difference between treatments ($p < 0.05$; mixed effects linear model with a Tukey post-hoc test). Error bars represent standard error.*

CHAPTER 6

Conclusion

Climate change is ongoing and will greatly impact marine environments (Doney et al., 2012; IPCC, 2023; Wuebbles et al., 2017). As oysters are an economically important foundation species (Grabowski et al., 2012; Lemasson et al., 2017), continuing to understand how they will perform under future scenarios of environmental stress is necessary to ensure that the benefits of their ecosystem services are maintained. Early life stages of oysters are particularly susceptible to environmental stressors, specifically ocean acidification and ocean warming, and effects from these stressors can carry over into later stages (Byrne, 2011; Byrne and Przeslawski, 2013; Pechenik, 2006; Ross et al., 2016). Eastern oysters may be more robust and tolerant to various climate change stressors compared to other oyster and bivalve species due to their environmental histories in highly dynamic and variable estuarine environments (Ivanina et al., 2018; Stevens and Gobler, 2018; Talmage and Gobler, 2011, 2009); however, their early life stages are still vulnerable to environmental stress, which may have consequences for later life stages.

The goal of this dissertation was to understand how the early life stages of *C. virginica* are physiologically affected by multiple global climate change stressors from a holistic perspective by incorporating local environmental data, observations across three life stages (i.e., carryover effects), responses from two important types of Eastern oysters, and physiological metrics from the cellular to whole-organism levels. Specifically, carryover effects from typical salinity differences experienced by larvae were explored alongside selective mortality and phenotypic plasticity in chapter two. In chapter three, multiple stressor effects from conditions of acidification and ocean warming were observed during the larval stage and into the juvenile stage to study potential carryover effects. Next, larval responses to multiple environmental stressors were compared between selectively bred and wild oysters in chapter four. Lastly in chapter five, oysters were exposed to conditions of acidification during the settlement stage and monitored for carryover effects into the early juvenile stage. For a more holistic understanding of the physiological mechanisms potentially driving the responses of *C. virginica* to various aspects of climate change, a suite of physiological responses from the cellular to whole-organism levels was measured.

Carryover effects of environmental stress in oysters may be more persistent when the stress exposure occurs immediately after fertilization. The first 24 – 48h after fertilization have been identified as the most sensitive period during the larval stage (Brunner et al., 2016; Waldbusser et al., 2013), and events when water conditions reach extreme levels within that short timeframe could have significant consequences for aquaculture production and wild oyster populations. In chapter three, after exposing embryos to four combinations of average and extreme summertime temperature and pH conditions starting at two hours after fertilization, within-generation carryover effects lasting into the first month of the juvenile stage were observed. Similarly, of the studies where within-generation carryover effects were identified, those with lasting effects beyond a week or two into the following life stage began their stress exposure immediately after fertilization (Hettinger et al., 2013, 2012; Richardson and Allen, 2023; Torres et al., 2016). Additionally, no carryover effects were identified in either chapters two or five of this dissertation when oysters were exposed to treatment conditions after the first two days of the larval life stage or during the settlement stage, respectively. Correspondingly, studies that identified minimal carryover effects initiated the stress exposure either towards the end of the larval stage or during the juvenile stage (Donelan et al., 2021; Ragg et al., 2019). Carryover effects may occur from the development of an energy deficit during an energetically demanding period, making it difficult to recover or compensate for those effects (Pechenik, 2006). While the settlement and juvenile stages both require energy for developmental processes, they are followed by less demanding periods that potentially allow for faster compensation of any deficits. The first two days of the larval period, however, are followed by 12 to 16 more days of energy demanding processes related to larval development, making recovery more difficult. The findings in this dissertation can serve as a building block for future studies to explore developmental-timing thresholds for the emergence of carryover effects. Specifically, future studies should delve into the alignment of stress exposures with sensitive and energy-intense periods that are followed by another energetically demanding life stage.

Certain selectively bred broodstock lines of Eastern oysters utilized for aquaculture may be more sensitive to multiple environmental stressors than certain wild populations. Larvae from selectively bred oysters outperformed larvae from wild oysters when initially exposed to individual conditions of acidification and warming based on the results from chapter four, which could be due to better maternal investments (Parker et al., 2012; Waldbusser et al., 2016). After four to six days, however, when mechanisms of the larval stress response (e.g., antioxidant production) were likely more prominent and developed (Song et al., 2016), wild-type oysters performed better than selectively bred oysters under individual and combined scenarios of future acidified and warming conditions. Specifically, the larvae from wild oysters recovered both their tissue and shell growth, and their lipid damage levels plateaued while the larvae from selectively bred oysters continued accumulating lipid damage and their tissue growth began to plateau under the combined multiple stressor treatment. Based on results from the continuation of the experiment on wild oysters to observe carryover effects (chapter three), the negative effects that were beginning to emerge in selectively bred oysters under a scenario of future multiple stressors likely would have continued into the juvenile stage. Though other studies comparing the performance of selectively bred and wild oysters under various environmental stressors have often found that selectively bred oysters perform better (Durland et al., 2019; Hall et al., 2020; Parker et al., 2011), few have specifically been conducted on Eastern oysters. Oyster species have known physiological differences (Dineshram et al., 2015; Jackson et al., 2011; Parker et al., 2010), and furthermore, even within a single species, physiological differences exist among populations (Casas et al., 2017, 2018; Eierman and Hare, 2016, 2013). Additionally, physiological performance can differ among broodstock lines utilized for aquaculture production. Six well-established broodstock lines for the Eastern oyster display disparate responses to varying environmental conditions (Proestou et al., 2016). More studies are needed to assess the performance of established broodstock lines to multiple environmental stressors with the aim of identifying those most tolerant of climate change for further development; however, based on the results of chapter four, larval oysters from the LILY broodstock line (Frank-Lawale et al., 2014) may not be tolerant of acidified and warming conditions, making hatchery production potentially less successful in the future as environmental conditions more frequently reach current extreme values without careful control of larval culture conditions.

Many physiological and cellular stress responses are active under ocean acidification and ocean warming (Pörtner, 2008), which can make teasing apart particular physiological drivers of tolerance or susceptibility difficult. This dissertation research has begun to identify underlying physiological mechanisms responsible for carryover effects and overall performance under future scenarios of multiple environmental stressors by evaluating several metrics spanning cellular to whole-organism levels. One unexpected result based on previous ocean acidification studies was the observation that oysters maintain high rates of shell growth during the settlement and early juvenile stages, regardless of past, present, or poor growing environmental conditions (i.e., calcium carbonate saturation states significantly less than 1), as seen in chapters two, three, and five. Even if a shell growth deficit was generated during the larval stage under future acidification scenarios compared to average ambient conditions, it was compensated for during the settlement stage, as seen in chapters three and five. Maintaining high rates of shell growth under future environmental stress scenarios coincided with decreased rates of tissue growth (measured using total protein) as seen in chapters three and five, and negative effects to tissue growth may have a more persistent carryover effect than other physiological impacts as seen in chapter three. Across all chapters, total protein, as a proxy for tissue mass, was the most sensitive and informative metric through the first month of the juvenile stage. Per the results from this dissertation, future studies involving the early life stages of bivalves through the first month of the juvenile stage should pair observations of shell growth with total protein content or tissue biomass metrics when possible, as shell growth can misrepresent effects of environmental stressors by masking sub-lethal impacts.

Metrics of oxidative stress are another effective category for observing impacts of environmental stress on organisms; however, direct measures of oxidative stress, like lipid peroxidation, are more informative than measures of defense molecules only (i.e., total antioxidant capacity), as defense molecules can be both elevated or depressed under stress depending on whether the study population has adapted to produce or maintain a pool of antioxidants, respectively (Griffin and Bhagooli, 2004). Though total antioxidant capacity was measured across all chapters, the addition of lipid peroxidation in chapter three aided in the interpretation of total antioxidation capacity. In chapters three and four, across life stages and types of oysters, oysters that experienced warmer temperatures had lower total antioxidant capacity, which alone could be interpreted as increased utilization due to greater oxidative stress. The additional observation of lower lipid peroxidation levels under higher temperatures in chapter three indicated a cross-tolerance effect of warming instead. Because the concentrations of oxidative stress molecules can change more rapidly than other metrics, like proteins and lipids, observations are best made over the course of multiple time points during an experiment to understand if the patterns observed at one timepoint represent the general impact of treatment conditions; otherwise, effects may be over or underestimated.

As the field of marine invertebrate ecophysiology progresses, studies need to explore various temporal and interactive effects of multiple environmental stressors. In natural systems, some stressors have diel cycles that vary in magnitude spatially between habitat patches and temporally with the seasons, which can lead to multiple stressors that are experienced asynchronously rather than simultaneously (Cheng et al., 2015; Hofmann et al., 2011; Price et al., 2012). Most multiple stressor studies involving laboratory experiments, chapters three and four included, simulate environmental stressors using constant, average conditions and concurrent stressors. While constant stressor studies are useful for identifying physiological and genetic responses to environmental stress, they could be over- or underestimating the severity of the response due to the low likelihood of constant stressor experience *in situ* (Kroeker et al., 2020). Some studies that incorporated temporal dynamics have produced similar results to studies that used constant, simultaneous stressor exposures (Clark and Gobler, 2016; Keppel et al., 2015), while others have found that results generated under constant, simultaneous exposures have overestimated responses observed under more dynamic conditions (Bible et al., 2017; Cheng et al., 2015; Comeau et al., 2014). As more studies include temporal dynamics, patterns will emerge and aid in modelling efforts to more accurately predict the future success of wild oyster populations (e.g., effects to recruitment) and aquaculture production based on their physiological performances in the face of ongoing climate change.

Overall, the findings in this dissertation have identified potential drivers of carryover effects, responses of young oysters to multiple stressors, useful physiological metrics, and future areas of research. As climate change continues, Eastern oysters will be affected. While this species will persist as it has for millennia, the results from this dissertation will support future work to inform that probability and identify potential solutions to sustain wild populations through conservation and restoration and to support oyster aquaculture.

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