

ABSTRACT

Title of Dissertation: PHYSIOLOGICAL, MOLECULAR, AND
 ECOLOGICAL RESPONSES OF THE
 EASTERN OYSTER, *CRASSOSTREA*
 VIRGINICA, TO HYPOXIA EXPOSURE IN
 THE CHESAPEAKE BAY

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Hypoxia is a naturally occurring phenomenon in coastal waters that is increasing in frequency and extent due to human activities. There is a pressing need to understand how organisms will be able to respond and adapt to future oxygen limitation. The eastern oyster, *Crassostrea virginica*, is an ecologically important bivalve that is threatened by the increasing incidence of low oxygen events. Little is known about the capacity of *C. virginica* to cope with projected deoxygenation or the potential ecological implications of reduced oxygen availability. The primary objectives of this dissertation research were to 1) characterize the intraspecific variability in physiological and molecular responses to hypoxia for oysters from the Chesapeake Bay and 2) develop a model to predict the implications of hypoxia on oyster population ecology. In *Chapter 2* I assessed the survival and heart rate responses under low oxygen stress for oysters sourced from reefs experiencing varying

frequencies of hypoxia exposure. Results indicated that prior hypoxia exposure does not confer increased survival under low oxygen stress but may relate to sublethal physiological differences in tolerance, particularly for oysters with a greater frequency of prior hypoxia exposure. In *Chapter 3*, I used four different analytical approaches, principal components, differential gene expression, co-expression gene network, and transcriptional frontloading analyses, to assess intraspecific differences in oyster transcriptomic response to hypoxia. No statistically significant differences in gene expression response between sites were observed indicating that prior hypoxia exposure may not have affected the regulation of expression under hypoxic stress. However, while not statistically significant, gene expression patterns suggested transcriptional frontloading as a possible mechanism of increased hypoxia tolerance in oysters. Finally, in *Chapter 4*, I developed a Dynamic Energy Budget model integrating dissolved oxygen concentration as a forcing variable to make predictions about oyster growth and reproduction under varying oxygen conditions. Model outputs indicated that low oxygen exposure reduces oyster growth, fecundity, and spawning frequency. Collectively, this dissertation research affirms that low oxygen availability negatively affects oyster physiology and ecology, and emphasizes the importance of continued research into the capacity of oysters to tolerate future increases in coastal hypoxia.

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IN THE CHESAPEAKE BAY

by

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DEDICATION

For my children.

You were my inspiration every step of the way.

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CHAPTER 1: GENERAL INTRODUCTION AND OVERVIEW OF RESEARCH

Environmental Oxygen Availability

Oxygen is one of the most critically important elements as it is essential for aerobic metabolism, sustaining most multicellular life on earth. While early earth's atmosphere was virtually devoid of oxygen, today oxygen comprises approximately 21% of the atmosphere's gaseous content. For most terrestrial organisms, with the exception of those that reside in high altitudes (e.g., Hall et al. 1936) or endosymbiotic bacteria that reside in the gut of other organisms (e.g., Lewis and Rettger 1940), the environmental availability of oxygen rarely varies. In contrast, for organisms that reside in aquatic environments, oxygen availability can vary widely both temporally and spatially (Boutilier 1990).

In aquatic environments, dissolved oxygen comes primarily from two sources. First, diffusion allows for the passage of oxygen across the water surface from the atmosphere, leading to oxygen concentrations in surface waters to be equilibrated with the atmosphere. Physical movement of the water, for example through wind disturbances at the surface, can cause mixing between oxygenated surface waters and deeper waters that do not have contact with the atmosphere (Portela et al. 2020). Second, the production of oxygen by aquatic photosynthetic organisms, such as phytoplankton or aquatic plants, contributes to the concentration of available dissolved oxygen in aquatic environments (e.g., Jenkins and Goldman 1985). Alterations to either of these processes can influence the amount of available oxygen

in water bodies. For example, changes in wind patterns can decrease the available oxygen in coastal estuaries (e.g., Scully 2010). With respect to effects of photosynthetic organisms, shallow coastal waters can become supersaturated with oxygen during the day when photosynthesis predominates over respiration and depleted of oxygen at night when phytoplankton undergo only respiration (e.g., Shen et al. 2008).

Normoxia, hypoxia, and anoxia are three terms used to describe varying levels of oxygen availability in aquatic water bodies. Normoxia corresponds to dissolved oxygen concentrations of approximately 2.0-8.0 mg l⁻¹ and is defined as oxygen concentrations that typically do not significantly affect the physiology of organisms. Hypoxia is typically defined as oxygen concentrations below 2.0 mg l⁻¹ (Diaz and Rosenberg 1995) and is identified as a threshold concentration at which organisms will alter their behavior or physiology in order to maintain cellular homeostasis. Lastly, anoxia is defined as a complete absence of dissolved oxygen. In these conditions, the vast majority of organisms cannot survive for extended periods of time (Diaz and Rosenberg 1995).

It's important to note that while 2.0 mg l⁻¹ typically defines the threshold oxygen concentration considered to be 'hypoxic,' research has indicated that the specific dissolved oxygen concentration that alters organismal behavior or physiology varies widely among species (Vaquer-Sunyer and Duarte 2008). The threshold of 2.0 mg l⁻¹ was established because it corresponded to the concentration at which point mass

mortality of fishes were observed (Renaud 1986). However, it may not necessarily reflect an accurate threshold for many marine organisms. For example, killifish, *Fundulus heteroclitus*, show physiological changes around 4.50 mg l⁻¹ (Voyer and Hennekey 1972) whereas 0.43 mg l⁻¹ denotes a physiological threshold for a species of jellyfish, *Polyorchis penicillatus* (Rutherford and Thuesen 2005). Despite the variability between species, the mean sublethal threshold concentration across a range of marine species is approximately 2.61 mg l⁻¹ (Vaquer-Sunyer and Duarte 2008). Thus, 2.0 mg l⁻¹ may not be a ubiquitous threshold, but it likely represents a stressful concentration for many marine species.

While oxygen concentrations are frequently used by ecologists and oceanographers to define the availability of oxygen in aquatic environments, physiologists often use oxygen partial pressure – also referred to as oxygen tension (Roman et al. 2019). Oxygen concentrations are defined as a quantity of oxygen per unit volume (typically mg l⁻¹ or ml l⁻¹) whereas oxygen tension refers to the pressure exerted by oxygen in a mixture of gases. Oxygen tension may be more physiologically relevant as it relates to the diffusivity of oxygen across organismal respiratory surfaces and thus provides a greater sense of oxygen available for biological processes. Oxygen tension is influenced by the solubility of oxygen in water. Oxygen solubility decreases with increasing temperature and salinity, leading to reductions in the availability of oxygen for organisms (Verberk et al. 2011). However, at similar temperature and salinity, oxygen tension will not vary greatly and oxygen concentration can provide an accurate estimate of oxygen availability.

In addition to the effects of temperature and salinity, oxygen availability in aquatic environments can be altered by changes in biological or physical features of a water body and can vary on daily or seasonal time scales. Natural diel-cycling of oxygen availability occurs in shallow aquatic environments as a result of daily cycles of photosynthetic activity (Kemp and Boynton 1980). Photosynthesis by phytoplankton and other aquatic plants dominates during the day, driving oxygen concentrations up. In the absence of sunlight, photosynthetic activity diminishes and respiration dominates, driving the decrease in dissolved oxygen concentrations. This results in a daily cyclical pattern of dissolved oxygen (Tyler et al. 2009, Breitburg et al. 2015). In the open ocean, Oxygen Minimum Zones (OMZs) are vast areas characterized by reduced dissolved oxygen resulting from natural physical hydrographic features (Sarmiento et al. 1988). OMZs can occur on seasonal time scales as a result of ocean currents that cause an upwelling of nutrients and oxygen-deplete waters from depth (e.g., along the west coast of South America, Helly and Levin 2004) or can occur at ocean depths where the waters are perpetually deoxygenated due to the lack of photosynthetic organisms and isolation from the atmosphere (Sarmiento et al. 1988). Lastly, limited mixing and slow flushing rates can lead to perpetually hypoxic conditions in bottom waters within semi-enclosed coastal water bodies (e.g., the Black Sea, Breitburg et al. 2009).

Organisms living in the above-mentioned environments encounter periods of reduced oxygen as a result of the ambient conditions of their surrounding water, but aspects of

marine organisms' natural history can also increase their likelihood of encountering hypoxia. For example, intertidal organisms experience daily cycles of emersion as a result of tidal fluxes, limiting oxygen availability and their ability to aerobically respire (e.g., Brinkhoff et al. 1983). In addition, burrowing organisms can be subjected to depleted oxygen in their burrows or near the sediment-water interface since oxygenated water only infiltrates the sediment layer a few millimeters (Jørgensen and Revsbech 1985).

Anthropogenically-Induced Hypoxia

While hypoxic conditions can occur as a result of natural processes, anthropogenic activities are resulting in an increase in the frequency and severity of hypoxic events worldwide (Diaz and Rosenberg 2008, Stramma et al. 2008, Keeling et al. 2010). Coastal water bodies are prone to eutrophication resulting from a significant influx of nutrients from agricultural activities and residential runoff. The influx of nutrients leads to phytoplankton blooms, which deplete oxygen in the water through phytoplankton respiration and microbial decomposition of senescent algal cells. The issue is further exacerbated by stratification of the water column, which typically occurs during the summertime when water temperatures are high. High temperatures prevent vertical mixing of oxygenated surface waters and oxygen depleted deep waters, leading to prolonged periods of reduced oxygen at depth (Schmidtko et al. 2017). In the United States, coastal water bodies such as the Chesapeake Bay (Hagy et al. 2004) and the Gulf of Mexico (Rabalais et al. 2002), both with large watershed

areas and significant amounts of nutrient runoff, have experienced increases in the frequency and severity of hypoxic events in recent history.

Global climate change is expected to increase the frequency and severity of hypoxic events (Diaz and Rosenberg 2008, Breitburg et al. 2018). Increased temperatures reduce oxygen solubility in water (Weiss 1970) and have already led to reduced oxygen concentrations throughout the world's oceans (Schmidtko et al. 2017). As global temperatures continue to increase, oxygen availability in ocean waters is expected to continue to decline (Keeling et al. 2010, Long et al. 2016). Hypoxic conditions will be further exacerbated since warmer waters lead to more intensified stratification of the water column, limiting vertical mixing (IPCC 2007). Alterations to coastal boundary currents as a result of climate change will increase the amount of upwelling along coastal margins, bringing deoxygenated waters from depth to the surface (Chan et al. 2008, Sydeman et al. 2014). In addition, increased climate change-induced precipitation has the potential to increase land runoff, leading to a greater influx of nutrients into coastal waters and therefore greater incidence of low oxygen events (Miller and Russell 1992). In comparison to the two other oceanic effects of climate change – ocean warming and ocean acidification – reduced oxygen availability is predicted to have more severe biological consequences for marine organisms (Sampaio et al. 2021).

Consequences of Hypoxia for Marine Organisms

Hypoxia threatens the health of many marine and estuarine organisms (Diaz and Rosenberg 1995). For aerobically respiring organisms, oxygen is a necessary element for their metabolic processes (Grieshaber et al. 1994). Therefore, reduced dissolved oxygen negatively impacts basic metabolic functioning as well as organismal functioning, with implications at the molecular to ecological level. For many species, hypoxia exposure is known to lead to a distinct molecular response intended to preserve homeostatic cellular functioning (Semenza 2009). At an individual level, organisms will alter their behavior, physiology, or even anatomy in order to maintain proper cellular functioning when faced with low oxygen (Grieshaber et al. 1994). In turn, these responses can strongly influence the ecology of marine organisms in the face of reduced oxygen (Diaz and Rosenberg 1995).

Molecular and Cellular Responses to Hypoxia

As the final electron acceptor for aerobic respiration, oxygen availability plays a key role in cellular functioning. The lack of oxygen availability during hypoxia therefore necessitates both the maintenance of energy production to allow for basic cellular functioning as well as reduction in metabolic rates in order to decrease energy demand (Larade and Storey 2002). At a cellular level, the cascade of responses to hypoxia appears to be triggered by the mitochondrial production of reactive oxygen species (ROS) which signal to the cell that oxygen availability has been reduced (Chandel et al. 1998). The accumulation of ROS leads to the activation of a key transcription factor for hypoxia response, hypoxia-inducible factor-1 α (HIF; Chandel

et al. 2000). HIF expression is a highly conserved response to hypoxia in most metazoans (Graham and Barreto 2020) and is important for activating the expression of genes broadly related to promoting oxygen delivery, metabolic depression, initiating aerobic metabolism, and activating immune processes (Greer et al. 2012). In fact, the ubiquitous importance of HIF for signaling hypoxia stress has led to it being proposed as a potential biomarker to indicate the health of some marine invertebrates (e.g., Kodama et al. 2012, Patterson et al. 2014.).

Similar to other environmental stressors, hypoxia also appears to trigger the expression of genes outside of the HIF pathway that are associated with the generalized stress response in marine invertebrates (e.g., Zhang et al. 2016). For example, heat shock proteins (HSP) are molecular chaperones that aid in maintaining protein structure under abiotic stress (Lindquist 1986, Hartl 1996) and their upregulation have been shown to play an important role in generalized organismal stress response (see reviews by Feder and Hofmann 1999, Dahlhoff 2004), including hypoxia (e.g., Patterson et al. 2014, Nie et al. 2018). Similarly, abiotic stressors such as temperature, salinity, and hypoxia can lead to oxidative stress in marine invertebrates as a result of the increased occurrence of ROS (Lushchak 2011). Thus, the increased expression of antioxidant genes, such as Kelch-like ECH associated protein 1 (Keap1), to prevent cellular damage appear to play an important role in hypoxia stress response (Lushchak 2011, Welker et al. 2013).

Behavioral and Physiological Responses to Hypoxia

On an individual level, behavioral responses typically constitute the first line of defense organisms undertake to mitigate against low oxygen conditions. The aim of these behavioral responses is to increase the contact between respiratory surfaces and the surrounding water, increasing the amount of oxygen with which the organism can come in contact. For mobile organisms, movement to waters with greater oxygen availability is the primary method used to maintain proper aerobic functioning (Pihl et al. 1991, Bell et al. 2003). Lacking the ability to move to more favorable areas, organisms can increase ventilation rates in order to increase the amount of water – and oxygen – coming in contact with their respiratory surfaces (e.g., Spitzer et al. 1969). In some organisms, the surface area of respiratory organs can be increased upon exposure to hypoxic waters, allowing for greater oxygen diffusion when oxygen availability is low (e.g., Sollid et al. 2003).

Because they lack the mobility to move to areas with greater oxygen availability, sessile organisms are particularly susceptible to low oxygen conditions. As such, sessile organisms, particularly those with a long evolutionary history of exposure to low oxygen conditions, have evolved unique adaptations to combat low oxygen. Some deep-sea sessile organisms possess respiratory pigments with greater oxygen affinity to allow for the greater mobilization of oxygen within their body, even if environmental concentrations are low (e.g., Sanders and Childress 1990). Many of these organisms also possess larger respiratory surfaces than their close phylogenetic relatives, allowing for greater oxygen diffusion into the body (e.g., Lamont and Gage

2000). Metabolic depression is a commonly observed response of hypoxia tolerant organisms to low oxygen availability, which can be manifested physiologically through reduced respiration rates (e.g., Taylor and Brand 1975) or reductions in heart rate (e.g., deFur and Mangum 1979). Other organisms can switch to anaerobic metabolic pathways upon exposure to hypoxic conditions, allowing for the maintenance of metabolic activity, albeit with lower energetic output (de Zwaan and Wijsman 1976).

Among marine invertebrates, there are three general metabolic responses to declining oxygen tension. First, an oxyconforming response is characterized by the reduction in metabolic rate in tandem with declining oxygen tensions. Second, an oxyregulatory response is characterized by the maintenance of metabolic rate as dissolved oxygen concentrations decline (Prosser and Brown 1961). The third type of response, which is followed by many bivalves (deFur and Mangum 1979, Polhill and Dimock 1996), involves a combination of an oxyregulatory response at higher oxygen concentrations and an oxyconforming response below a critical oxygen concentration, known as P_{crit} (Tang 1933, Fry 1947). The ability to oxyregulate tends to increase tolerance to low dissolved oxygen conditions compared to oxyconformity (Stickle et al. 1989) as oxyregulation allows for maintenance of aerobic respiration during low-oxygen conditions. Therefore, individuals able to maintain oxyregulation over a wider range of dissolved oxygen concentrations (i.e., have a lower P_{crit} value, e.g., Mandic et al. 2013) are more tolerant to low oxygen owing to the greater energetic yield afforded by the maintenance of aerobic pathways (Grieshaber et al. 1994).

Ecological Consequences of Hypoxia

The physiological and behavioral responses of organisms to hypoxia can have broad-reaching ecological consequences. On an individual level, hypoxia has the potential to reduce both growth rates of organisms (e.g., Das and Stickle 1993, McNatt and Rice 2004, Stevens and Gobler 2018) as well as their reproductive potential (e.g., Wiklund and Sundelin 2001, Landry et al. 2007, Long et al. 2014). At the population level, hypoxia can lead to reduced population growth rates (e.g., Marcus et al. 2004, Eby et al. 2005) and alterations to the size structure of populations (e.g., Burd 1985, Pihl et al. 1991, Breitburg 1992). Individual reductions in fecundity – either via reduced numbers of eggs (e.g., Long et al. 2014) or reductions in energy invested in eggs (e.g., Peruzza et al. 2018) – can lead to reductions in recruitment and overall population biomass (Diaz and Rosenberg 1995).

Low dissolved oxygen availability can also influence community interactions within ecosystems. Habitat compression – the reduction in physical area in which organisms can properly function physiologically – as a result of hypoxia can alter the ecological interactions of an area. The movement of mobile organisms from oxygen deplete waters to waters with greater oxygen concentrations during times of habitat compression can lead to changes in inter- and intraspecific interactions, such as greater competition for limited resources (Prince and Goodyear 2006, Campbell and Rice 2014). Similarly, exposure to hypoxia can increase the susceptibility of some organisms to predation. Movement from burrows or changes in behavior in response

to low oxygen conditions can lead organisms to be more easily predated upon, altering trophic dynamics (Nestlerode and Diaz 1998). Lastly, differences in the tolerance of different species to low oxygen can influence the ecological community in areas prone to hypoxic events. For example, habitats that have been severely degraded by long-term exposure to hypoxic or anoxic conditions are primarily dominated by hypoxia-tolerant species and thus suffer reductions in species richness (Diaz and Rosenberg 1995).

Measuring Adaptive Capacity to Hypoxia

Tolerance to hypoxia can differ both inter- and intraspecifically and is a product of the behavioral and physiological mechanisms described above. Understanding intraspecific variability in tolerance to environmental stressors exacerbated by climate change is of particular importance as it can indicate organismal capacity for future adaptation (Somero 2010). One means of assessing the capacity of organisms to adapt to changing conditions is through examination of the responses of conspecifics that reside across a range of environmental conditions (Hoffman and Sgrò 2011). For example, an extensive amount of research has been conducted on thermal tolerance of organisms that span broad latitudinal ranges, as individuals at lower latitudes experience higher average temperatures than those from higher latitudes and therefore exhibit higher tolerances to thermal challenge than their high latitude counterparts (e.g., Kelly et al. 2012, De Frenne et al. 2013). These types of comparisons – termed ‘space-for-time’ substitutions (Blois et al. 2013) – use the natural variability of stress

exposure across space to gain insights into how individuals may respond to environmental conditions expected to change over time.

Organisms can respond to environmental stress through two means, phenotypic plasticity or genetic adaptation (Sanford and Kelly 2011). Phenotypic plasticity (or acclimation) constitutes short-term morphological or physiological responses to environmental stress that increase individual fitness and occur on timescales less than the lifespan of an individual. Genetic adaptation, on the other hand, occurs over multiple generations and results from the differential survival of individuals with greater fitness in local environmental conditions, leading to increases in the frequency of alleles conferring greater fitness within the population. Differences in tolerance to an environmental stressor between conspecifics can result from either phenotypic plasticity or genetic adaptation, although important considerations in experimental design are necessary in order to tease apart the relative contributions of each (Sanford and Kelly 2011).

Assessing the difference in organismal tolerance to environmental stress is typically accomplished through the use of reaction norms (Schlichting and Pigliucci 1998). Reaction norms measure the response of individuals from differing environments across a range of environmental conditions to determine whether individuals from different environments show differing responses to stress that may relate to their tolerance for the stress (Simms 2000). Both physiological responses – such as metabolic rates (e.g., Walsh and Somero 1981) – as well as transcriptomics (Rivera et

al. 2021) – examination of the total mRNA transcripts produced by an individual – can be used as a tool to study variability in tolerance to environmental stress.

Understanding the variability in tolerance to environmental stressors like hypoxia is especially important with respect to ecosystem engineers. Ecosystem engineers or foundation species are species that create or modify a habitat in such a way that they are disproportionately important to the effective functioning of the greater ecosystem (Jones et al. 1994). Given their role in the environment, the persistence of these species is critical for the health of the ecosystems they inhabit (Smale et al. 2019, Thomson et al. 2015). The ability of foundation species to acclimate or adapt to future environmental conditions will be critical for overall ecosystem resilience (Thrush et al. 2009, Prather et al. 2013).

Oyster Natural History and Interactions with Hypoxia

The eastern oyster, *Crassostrea virginica* (Gmelin), is an ecologically and economically important sessile bivalve that has experienced a significant decline in abundance throughout their range (Rothschild et al. 1994). *C. virginica* reside in shallow coastal waters along the east coasts of North and South America where they serve as a foundation species, providing a number of ecosystem services to their local environment (Coen et al. 2007, Grabowski and Peterson 2007). Since oysters have hard external shells and oyster larvae preferentially settle on other oysters, they create large, three-dimensional hard reef structures as they grow. These structures provide habitat and nursery grounds for other marine organisms – including other

economically-important species (Peterson et al. 2003) – help to attenuate waves and reduce coastal erosion (Meyer et al. 1997), and increase water clarity through their filtering abilities (Newell and Koch 2004).

In addition to their ecological importance, *C. virginica* historically has been an important economic commodity throughout most of its range, particularly in the Chesapeake Bay (National Research Council 2004). As a result of its popularity as a culinary delicacy, overharvesting of oysters in the Chesapeake Bay, combined with an increase in disease prevalence, led to a significant decline in oyster abundance throughout the 20th century (Wilberg et al. 2011). Today's oyster population in the Chesapeake Bay is estimated to represent less than 5% of the historic population size (Rothschild et al. 1994). In an effort to restore oyster populations to historic levels, large-scale restoration efforts, focusing initially on shell planting and now focusing primarily on utilizing spat-on-shell, have been undertaken in the Chesapeake Bay since the late 1800s (Mann and Powell 2007).

Despite efforts to breed disease-resistant oyster strains to repopulate the Bay (e.g., Ragone Calvo et al. 2003) and to better regulate commercial oyster harvest (Kirby 2004), other anthropogenically-induced stressors continue to threaten oyster abundance. In the Chesapeake Bay, the summertime reduction in oxygen availability is one such stressor with the potential to affect oyster populations. Since first being documented in the 1930s (Newcombe and Horne 1938), seasonal hypoxia in the mainstem of the Bay has persisted on an annual basis, with an increase in the volume

of hypoxic water over time (Hagy et al. 2004). Hypoxia in the Chesapeake Bay is caused by eutrophication which is driven by the highly-populated and agriculturally-intensive watershed and physical features of the bay that encourage water stratification (Officer et al. 1984). Deepwater areas of the mainstem, as well as deeper areas of highly degraded tributaries, are especially susceptible to hypoxia (Officer et al. 1984, Breitburg et al. 2003, Muller et al. 2016). With increasing water temperatures in the bay associated with climate change, the severity of hypoxic events is expected to increase into the future (Najjar et al. 2010, Ni et al. 2019).

Compared to other marine invertebrates inhabiting the Chesapeake Bay, *C. virginica* is highly tolerant of hypoxic conditions (Stickle et al. 1989) since their sessile life history leads them to be periodically exposed to low oxygen conditions (e.g., Breitburg 1990, Dame 1979). Oysters, like other hypoxia-tolerant organisms, employ a number of physiological mechanisms to increase their tolerance to low oxygen exposure. Typically, the first line of defense against hypoxia that oysters employ is the depression of metabolic activity in order to minimize the amount of energy expended (Storey and Storey 1990). In addition to reducing their energy needs, oysters are highly efficient facultative anaerobes, and can transition easily from aerobic to anaerobic metabolism when oxygen availability is reduced (Grieshaber et al. 1994). Their efficiency with facultative anaerobiosis is further supported through the production of alternative metabolic by-products – specifically alanine, succinate, and propionate (Eberlee et al. 1983) – which affords them greater ATP production than other glycolytic pathways that produce lactate (Larade and Storey 2002). The

large quantity of glycogen stored in their tissues also allows for their efficiency with anaerobiosis as it provides an easily mobilizable fermentable fuel (de Zwaan 1983). Finally, by mobilizing bicarbonate from their shells, oysters can buffer against anaerobic-induced acidosis (Cameron 1990).

Despite the eastern oyster's high tolerance to low oxygen exposure, prolonged exposure to hypoxic conditions, particularly at high temperatures such as those experienced during the seasonal hypoxic events in the bay, can lead to mass mortality events (Lenihan and Peterson 1998), reducing secondary production in the bay and its tributaries (Sturdivant et al. 2014). Hypoxic conditions can occur for up to an estimated 140 days a year in some areas of the bay (Murphy et al. 2011) and documented hypoxic events on oyster beds specifically last 7-14 days (Lenihan and Peterson 1998, Sagasti et al. 2000, Neff et al. 2020). Thus, oyster populations are threatened by these low oxygen episodes. Furthermore, mass mortality events associated with low oxygen conditions can threaten the success of oyster restoration efforts (Johnson et al. 2009).

As the severity of hypoxia continues to increase in coastal oceans and bays, understanding how increased duration and frequency of hypoxia will influence the physiology and ecology of oysters in the Chesapeake Bay is important. The research from this dissertation aims to answer two major questions related to oyster response to hypoxia. First, to what extent do oysters in the Chesapeake Bay vary in their ability to tolerate low oxygen exposure and what mechanisms are used to improve tolerance?

Second, what are some of the implications of low oxygen availability on oyster population ecology?

Differential Tolerance to Hypoxia in Oysters

While large geographic distances don't separate individual oyster reefs in the Chesapeake Bay, oysters distributed throughout the bay still experience spatially heterogeneous oxygen conditions on an annual basis. Deep-water areas are more likely to experience hypoxia during the summer months since they are more susceptible to stratification of the water column and therefore less likely to experience mixing with oxygenated surface waters (Boicourt 1992, Officer et al. 1984). In contrast, oyster reefs located in shallow water areas of the bay do not experience water stratification and therefore generally do not experience seasonal hypoxia. Consequently, oysters in these distinct reefs experience differing oxygen conditions on an annual basis. Since oysters appear to thrive in either of these environments, it's useful to understand how oysters are able to persist in the hypoxic reefs and whether they employ different physiological strategies than normoxic conspecifics when exposed to hypoxic conditions.

When making interspecific comparisons and therefore looking over long evolutionary timescales, it is apparent that oysters differentially adapt to the oxygen conditions they experience in their native environment. For example, the Asian oyster, *Crassostrea ariakensis*, shows a lesser tolerance to hypoxia than its congener *C. virginica* (Lombardi et al. 2013). This difference likely stems from exposure to

different oxygen conditions in their native environments. *C. ariakensis* inhabits coastal waters where they are constantly submerged and therefore unlikely to experience low oxygen whereas *C. virginica* inhabit hypoxia-prone waters and intertidal areas where they periodically experience emersion and a lack of oxygen.

There is evidence that geographically proximal conspecifics from the same estuary can show differential tolerance to abiotic stressors besides hypoxia. In Delaware Bay, for example, oysters from lower salinity areas of the bay show differential gene expression patterns than oysters from higher salinity areas when exposed to salinity conditions in the lab that differ from their native reef (Eierman and Hare 2016). In addition, experiments conducted on the Pacific oyster, *Crassostrea gigas*, indicate that individual oysters from intertidal habitats – where heat stress would be more likely – showed higher tolerance, as measured by higher survival and metabolic rates, under high temperatures compared to subtidal conspecifics (Li et al. 2018).

While these studies indicate the potential for differential tolerance in geographically proximal conspecifics, less is known about whether environmental variations in oxygen availability might similarly lead to differences in tolerance of oysters to low oxygen. It is likely that oysters do differentially tolerate low oxygen as one study from Mobile Bay, Alabama indicated that oysters from a reef that is expected to experience hypoxia had higher survival in low oxygen exposure compared to conspecifics from a normoxic reef (Ford 2005). However, this study lacked water quality monitoring data from the two collection sites to support their assertion that the

more tolerant oysters were actually exposed to low oxygen conditions on their native reef. No additional work has been published examining differences in physiology or gene expression between geographically proximal conspecifics of *C. virginica* acclimatized to differing oxygen regimes. A primary goal of this dissertation research is to address whether variation in hypoxia exposure between distinct oyster reefs in the Chesapeake Bay results in intraspecific differences in tolerance to hypoxia.

Predicting Ecological Effects of Hypoxia on Oysters

As discussed above, hypoxia can have acute physiological and reproductive effects on individuals which can lead to broader ecological consequences. For oysters specifically, low oxygen can reduce ingestion rates (Clark 2014) and respiration rates (Shumway and Koehn 1982), ultimately reducing individual growth rates (Keppel et al. 2016, Stevens and Gobler 2018) or leading to mortality (Lenihan and Peterson 1998). However, how these effects scale more broadly to the population ecology of *C. virginica* is not well understood.

Assessing the long-term effects of hypoxia on oyster population ecology can be logistically or financially prohibitive. However, the use of mechanistic models provides a powerful means to quantify and translate individual effects of hypoxia more broadly to the population. One such model, the Dynamic Energy Budget (DEB) model (Kooijman 2000), uses a broadly applicable framework to model the dynamics of organismal bioenergetics with environmental variables to predict how organismal growth and reproduction may be affected by dynamic environmental conditions. DEB

models have previously been used successfully to predict how hypoxia influences the bioenergetics and ultimately the life history of Atlantic cod (Lavaud et al. 2019) and Peruvian scallop (Aguirre-Velarde et al. 2019). A parameterized DEB model exists for *C. virginica* (Lavaud et al. 2017), but integration of dissolved oxygen as an additional forcing variable has not yet been accomplished. Therefore, a second goal of this dissertation work is to integrate dissolved oxygen into the DEB model for *C. virginica* in order to make predictions about how dissolved oxygen affects oyster growth and reproduction.

Dissertation Research Focus

The primary objectives of this dissertation are to 1) characterize the intraspecific variability in physiological and molecular responses to hypoxia for oysters from the Chesapeake Bay and 2) develop a model for use in predicting the effects of hypoxia on oyster population ecology. *Chapter 2* focuses on whether oysters from reefs with differing oxygen exposure exhibit differential lethal and sublethal tolerance to low oxygen stress. Survival and heart rate responses of oysters from ‘hypoxic’ and ‘normoxic’ sites are compared in order to determine whether prior exposure to low oxygen confers increased tolerance upon subsequent exposure. Results from this chapter provide insights into whether geographically proximal individuals that experience differential exposure to hypoxia show differing tolerance to hypoxia and how these different tolerances relate to the oyster’s capacity for tolerating projected reductions in oxygen availability. *Chapter 3* involves an intraspecific comparison of the transcriptomic response of oysters exposed to low oxygen challenge to assess

whether molecular responses to hypoxia differ between oysters with different histories of exposure to low oxygen. Results from this chapter examine the underlying molecular response of oysters to low oxygen and provide insight into plasticity of gene expression response and mechanisms of variable tolerance to hypoxia. *Chapter 4* focuses on integrating dissolved oxygen into the DEB model for *C. virginica* in order to make predictions about the response of oysters to low oxygen availability. Results from this chapter will provide a mechanistic tool for understanding and predicting how future variation in low oxygen availability may influence the ecology of oysters in the Chesapeake Bay and elsewhere. Overall, results from this dissertation research will advance understanding of both individual as well as ecological implications of hypoxia on *C. virginica*, allowing for a greater ability to predict how future increases in hypoxia frequency and duration will affect an ecologically important marine bivalve.

CHAPTER 2: INTRASPECIFIC COMPARISONS OF LETHAL AND SUBLETHAL HYPOXIA RESPONSE IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

Abstract

Hypoxia is a naturally occurring phenomenon in coastal and oceanic waters that is increasing in frequency and extent as a result of human activities and there is a great need to understand how organisms will adapt to future oxygen limitation. Elucidating how organisms differentially tolerate stressful conditions as a consequence of prior stress exposure can yield important insights into the capacity for future adaptation, but studies addressing these questions with respect to hypoxia are limited. The eastern oyster, *Crassostrea virginica*, is an ecologically important bivalve that resides in areas with variable oxygen conditions for which knowledge on intraspecific variability in tolerance to hypoxia is lacking. To address this knowledge gap, oysters were collected from six different oyster reefs in the Chesapeake Bay – three that experience seasonal hypoxia and three that experience general normoxia throughout the year. Oysters from all sites were exposed to hypoxic and normoxic conditions in the lab and survival and heart rate responses were measured. Regardless of the source site, oysters showed a significant increase in mortality, particularly for oysters of a larger size, and decrease in heart rate upon hypoxic exposure. However, comparisons between sites indicated no significant differences in survival upon experimental hypoxia exposure, although the frequency of oyster's prior hypoxic exposure or the experimental salinity may have contributed to the observed results. In contrast, heart rate measurements showed an overall smaller reduction in heart rate for oysters from

one of the hypoxic sites, and a trend towards smaller reduction in a second site. Results indicate that oysters with prior hypoxia exposure show no difference in lethal responses to hypoxia but may show some degree of sublethal physiological difference in tolerance, particularly for oysters with a greater frequency of prior hypoxia exposure. Overall, these results suggest that low oxygen availability will have negative effects on oyster ecology and restoration, and underscore the importance of continued research into oyster's capacity to tolerate future increases in coastal hypoxia.

Introduction

Hypoxia, often defined as dissolved oxygen concentrations less than 2.0 mg l⁻¹, is a widespread aquatic stressor that can occur as a result of natural phenomena as well as human activity (Diaz and Rosenberg 1995). Over the past century, the frequency of hypoxic events throughout the world's oceans has been increasing (Diaz and Rosenberg 2008, Stramma et al. 2008, Keeling et al. 2010) and oceanic oxygen availability is expected to continue to decline as a result of rising global temperatures associated with climate change and increased eutrophication (Breitburg et al. 2018). This decline in oxygen availability is expected to have detrimental effects on marine organisms and the environments they inhabit, causing mass mortalities (Breitburg 2002), compression of livable habitat (Stramma et al. 2011), and reduced productivity, particularly in anoxic environments (i.e. those lacking oxygen; Rainer and Fitzhardinge 1981). Reduced oxygen availability is predicted to have more severe

biological consequences compared to the effects of other two oceanic effects of climate change, ocean warming and ocean acidification (Sampaio et al. 2021).

There is a great deal of interest exists in understanding how marine organisms will be able to cope with the effects of climate change-associated environmental stressors, such as hypoxia (Somero 2010). Both plasticity – within-generation flexibility in physiological response – as well as adaptive mechanisms, such as natural selection, whereby more fit genotypes survive and increase the frequency of stress tolerance alleles, can play a role in increased tolerance (Sanford and Kelly 2011, Reusch 2014). While the potential physiological and ecological consequences of increasing hypoxic conditions are well-studied (see reviews by Diaz and Rosenberg 1995, Wu 2002, Diaz and Rosenberg 2008), the capacity of organisms to tolerate changing oxygen conditions is not well understood. One way to predict how organisms might respond to future changes to their local environment is through comparisons of physiological responses to environmental stressors in congeners or conspecifics that currently exist across a natural range of environmental stress (Sanford and Kelly 2011). Studies of this type have been effective in advancing knowledge on how marine organisms are expected to respond to rising temperatures (e.g. Kuo and Sanford 2009, Kelly et al. 2012, De Frenne et al. 2013) and ocean acidification (e.g. Crook et al. 2013, Padilla-Gamiño et al. 2015, Thomsen et al. 2017).

The eastern oyster, *Crassostrea virginica*, is an economically and ecologically important bivalve that inhabits coastal areas experiencing a range of dissolved oxygen

availability (Breitburg 1990, Dame 1979). Over the past century, the abundance of *C. virginica* in the Chesapeake Bay and elsewhere has declined significantly as a result of reduced water quality, overharvesting, and disease (Jackson et al. 2001, Kirby 2004, Wilberg et al. 2011, Mackenzie 2007). Efforts are underway to restore oyster populations throughout the Chesapeake Bay, but mass mortality events associated with hypoxia threaten their success (Lenihan and Peterson 1998). In the Chesapeake Bay, hypoxic events have been documented since the middle part of the 20th century (Newcombe and Horne 1938) and are expected to increase in severity into the future (Ni et al. 2019). Hypoxic conditions are spatially heterogeneous as deep-water areas are more prone to stratification that helps to drive seasonal hypoxia. *C. virginica* are distributed throughout the mesohaline portion of the Bay and its tributaries, in depths up to approximately 8 m (MacKenzie 1997), and oyster reefs throughout the Bay are differentially exposed to low oxygen on an annual basis.

In comparison with other estuarine invertebrates, *C. virginica* has a high tolerance to hypoxia (Stickle et al. 1989), an adaptive consequence of their sessile life history that exposes them to periodic low oxygen conditions. Oysters, along with other hypoxia-tolerant marine bivalves, utilize a number of physiological adaptations that allow them to persist during low oxygen exposure. Perhaps the most important defense is the ability of oysters to depress their metabolic activity – a strategy referred to as “turning down the pilot light” (Hochachka et al. 1996) – to minimize energy expenditure (Storey and Storey 1990). Additionally, oysters are highly successful facultative anaerobes, with the ability to efficiently transition from aerobic to

anaerobic respiration when oxygen availability is low (Grieshaber et al. 1994). Oysters also produce alternative metabolic byproducts during anaerobiosis, namely alanine, succinate, and propionate (Eberlee et al. 1983), which allow for greater ATP yield during anaerobic respiration compared to pathways that produce lactate (Larade and Storey 2002). Anaerobiosis is also facilitated in oysters by the storage of large quantities of glycogen in their tissues, which can easily be mobilized for use as a fermentable fuel (de Zwaan 1983). Finally, oysters can buffer against anaerobiosis-induced acidosis by mobilizing bicarbonate from their shells (Cameron 1990). As a result of these physiological adaptations, oysters are highly tolerant to low oxygen availability and are able to persist for more than a month in hypoxic conditions (Stickle et al. 1989).

While there is a great deal of knowledge on the physiological mechanisms of hypoxia tolerance employed by *C. virginica*, much less is understood about whether differences in tolerance or physiological responses to low oxygen exist between conspecifics, particularly for individuals exposed to differing oxygen availability. Inducible increases in hypoxia tolerance as a result of prior exposure to hypoxia have been shown in some species of fish (Gamperl and Farrell 2004, Regan et al. 2017), bivalves (Altieri 2006, Yamada et al. 2016), copepods (Decker et al. 2003) and polychaete worms (Lucey et al. 2019), and, in some cases, the physiological mechanisms underlying these differing tolerances have been identified. For example, a comparison of two populations of three-spine stickleback inhabiting lakes with differing wintertime hypoxia exposure showed that fish from the hypoxia-prone lake

showed higher tolerance to low oxygen, which appeared to be the result of a greater ability to depress their metabolism compared to their counterparts from a lake without wintertime hypoxia exposure (Regan et al. 2017). Some oysters – such as those on seasonally hypoxic reefs (Johnson et al. 2009) or those inhabiting intertidal habitats (Dame 1979) – experience repeated exposure to low oxygen, whereas oysters from shallow, well-ventilated habitats experience hypoxia far less regularly or not at all. Whether more frequent exposure to low oxygen leads to increased tolerance in oysters and what underlying physiological mechanisms allow for this increased tolerance are the foci of this study.

Hypoxia tolerance can be assessed in a number of ways, although survival is one frequently used metric. In general, exposure to hypoxic conditions for prolonged periods of time can lead to mortality (Diaz and Rosenberg 1995), but the amount of time an organism can survive during hypoxic exposure varies among species (Vaquer-Sunyer and Duarte 2008). For example, a comparative study of mortality upon exposure to hypoxia between different species of marine invertebrates showed that crustaceans can survive less than a day when exposed to hypoxia, while bivalves can survive weeks, depending on the ambient temperature (Stickle et al. 1989). However, less is known about intraspecific differences in bivalve mortality in response to hypoxia. One study examined differential mortality of *C. virginica* from two different reefs – one normoxic and one presumed to be hypoxic – in Mobile Bay, Alabama, and found that oysters from the hypoxic reef lived longer in anoxia than those from the normoxic reef (Ford 2005). Similarly, Altieri (2006) found lower

mortality rates in mussels that had previous exposure to daily tidal emersion – used in this study as a proxy for hypoxic conditions – than their subtidal conspecifics upon exposure to hypoxic conditions in the laboratory. Results from these studies indicate that bivalves demonstrate intraspecific differences in tolerance to low oxygen and that these differences can occur in geographically proximal organisms.

Heart rate response is an additional metric that can be used both to identify differing tolerance to hypoxia as well as understand the physiological mechanism of hypoxia tolerance. In bivalves, heart rate is closely linked to metabolic activity (Bayne 1971) and has been shown to vary in response to environmental variables, including temperature, salinity, and dissolved oxygen concentration (Feng 1965, deFur and Mangum 1979). With respect to dissolved oxygen, there are three general heart rate responses to declining oxygen tension among marine invertebrates. Strict oxyconformers reduce their heart rate in tandem with declining oxygen tension, while strict oxyregulators maintain a constant or, in some cases, slightly increasing heart rate as dissolved oxygen concentration declines (Prosser and Brown 1961). The third type of response, which is followed by many bivalves (deFur and Mangum 1979, Polhill and Dimock 1996), is two-phase, where an oxyregulatory response is used at higher oxygen concentrations and an oxyconforming response is used below a critical oxygen concentration, known as P_{crit} (Tang 1933, Fry 1947). In general, although not definitively, the ability to oxyregulate tends to increase tolerance to low dissolved oxygen conditions compared to oxyconformity (Stickle et al. 1989). The increased tolerance is owed to the fact that an oxyregulatory response allows for maintenance of

aerobic respiration during low-oxygen conditions. Thus, organisms that are able to maintain an oxyregulating response over a broader range of oxygen tensions (i.e., have a lower P_{crit} value) are considered to be more tolerant than those that transition to an oxyconforming response at higher oxygen tensions (e.g., Mandic et al. 2013) owing to the greater energetic yield afforded by maintenance of aerobic pathways (Grieshaber et al. 1994). Although hypoxia is often defined by the threshold oxygen concentration of 2.0 mg/l, P_{crit} can vary both inter- (Vaquer-Sunyer and Duarte 2008) and intraspecifically (as a result of ambient conditions; Rogers et al. 2016) and therefore represents a more general threshold for 'stressful' oxygen concentrations.

Heart rates of *C. virginica* across a range of oxygen concentrations have not been measured. However, previous research has shown that they undergo a two-phase response in oxygen consumption and they are typically able to regulate their oxygen consumption rates over a broad range of oxygen tensions (Shumway and Koehn 1982) indicating an overall high level of tolerance to reduced oxygen. Intraspecific comparisons of heart rate response to low oxygen have not been undertaken for *C. virginica*, but these data could prove useful for understanding how individuals with differing prior exposure to hypoxia might show differing physiological response to subsequent low oxygen stress. For example, reduced heart rate response to hypoxic stress has been shown in bay scallops previously acclimated to diel-cycling hypoxia compared to hypoxia-naïve scallops, indicating that heart rate response to hypoxia might be an important indicator of differential hypoxia stress tolerance (Gurr et al. 2021).

The present study intends to determine whether oysters with differing histories of exposure to seasonal hypoxia on their native reef exhibit differing mortality and heart rate response upon experimental laboratory challenge with low oxygen. It is hypothesized that oysters from reefs that experience seasonal hypoxia would show greater tolerance to low oxygen through higher survivorship when exposed to low oxygen conditions than those from normoxic reefs. With respect to heart rate, it is hypothesized that oysters from seasonally hypoxic reefs would be better oxygen regulators than their normoxic counterparts, and therefore would reduce their heart rate to a lesser extent upon exposure to hypoxia. Results from this work will provide insight into how different levels of exposure to hypoxia on a natural oyster reef affects the physiology of oysters which can advance our understanding of oyster capacity for acclimation to future reductions in oxygen availability in coastal waters.

Methods

Site Selection

Six different oyster reefs – three hypoxic reefs and three normoxic reefs – throughout the Maryland portion of the Chesapeake Bay were selected as collection sites for the experiments. Oyster collection sites (Figure 2.1) were identified based on long-term dissolved oxygen data collected monthly from water quality monitoring stations (data from Maryland Department of Natural Resources Eyes on the Bay; <http://eyesonthebay.dnr.maryland.gov> [last accessed 5/21/2021]) located near known

oyster reefs (Tarnowski 2017). Hypoxic sites were characterized by historic annual reductions in bottom water oxygen concentrations below 2.0 mg l^{-1} , while normoxic sites were identified based on data of bottom water oxygen concentrations that infrequently dipped below 2.0 mg l^{-1} (Figure 2.2). Between 2013 and 2017, the five years preceding the first of the experiments, $20.4 \pm 9.0\%$ of the monthly dissolved oxygen measurements (average of approximately 3 monthly measurements per year per site) taken at the hypoxic sites were at or below 2.0 mg l^{-1} , whereas only $1.7 \pm 2.9\%$ of the monthly measurements at the normoxic sites were at or below 2.0 mg l^{-1} (average of <1 monthly measurement per year per site). In addition to their annual dissolved oxygen profiles, temperature and salinity regimes of sites were compared to assess any potential differences in other water quality conditions between sites (Figures 2.3 and 2.4).

Oyster Collection and Acclimation

Oysters used in the experiments were collected during autumn of 2017 (for the heart rate experiment) and 2018 (for the survival experiment) using a combination of hand-collection by divers, dredging (courtesy of the Maryland Department of Natural Resources), and patent tong sampling from the six sites identified for collection. Approximately 100 oysters, all measuring at least 75 mm in length, from each site were collected. Only oysters greater than 75 mm in length (mean of $97.7 \pm 15.1 \text{ mm}$ for the survival experiment and $100.5 \pm 13.7 \text{ mm}$ for the heart rate experiment) were used in the experiments to ensure only adults were used as life stage may influence organismal response to hypoxic exposure (e.g., Leiva et al. 2018). After collection,

oysters were kept for at least one month suspended in the water in 18 mm flexible plastic mesh aquaculture bags at a marina in the South River, Maryland until their use in experimentation. This ensured that oysters were acclimated to the same environmental conditions (survival experiment: temperature $11.77\pm 8.33^{\circ}\text{C}$, salinity 3.82 ± 0.17 ppt, dissolved oxygen 9.13 ± 2.91 mg/l; heart rate experiment: temperature $17.64\pm 9.39^{\circ}\text{C}$, salinity 11.85 ± 2.77 ppt, dissolved oxygen 8.34 ± 1.89 mg/l) prior to their use in the experiments. Twelve (12) oysters per site per treatment were used in the mortality experiment and six oysters per site were used in the heart rate experiment.

Following their acclimation in the South River, oysters were randomly selected from each of the sites for use in experiments. Once in the lab, fouling organisms, such as barnacles and mussels, were removed from the oysters and each oyster was scrubbed each with metal brushes to remove any external debris. Oysters were then cleaned with a 10% bleach solution to aid in removal of bacteria and small fouling organisms, as this was shown to maintain better water quality in the experimental tanks during pilot experiments (*personal observation*). Shell length and whole animal wet weight were measured on all oysters, and each oyster was marked with a unique identifier using fingernail polish. The oysters were held and fed *ad libitum* with commercial shellfish diet (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA) for at least two days in aerated artificial seawater at room temperature (approximately 20°C) and a salinity matching the conditions from the dock at the time of retrieval for

experiments (5 ppt for the survival experiment and 10 ppt for the heart rate experiment) prior to the start of experiments.

Survival Experiment

The survival experiment was conducted January-February 2019. After the two-day lab acclimation, each oyster was randomly placed into one of six 10-gallon aquaria filled with artificial seawater (Instant Ocean®, Spectrum Brands, Blacksburg, VA) at room temperature ($20.5 \pm 1.1^\circ\text{C}$) and 5 ppt salinity. Each tank held a total of 24 oysters, four from each of the six sites. Three of the tanks (hereafter ‘normoxic treatments’) were continuously bubbled with compressed air to maintain normoxic conditions in the tank. The remaining three tanks (hereafter ‘hypoxic treatments’) were briefly bubbled with compressed nitrogen gas until the dissolved oxygen was reduced to less than 2.0 mg l^{-1} . All dissolved oxygen measurements were carried out using a YSI Model 55 dissolved oxygen reader (Yellow Springs Instruments, Yellow Springs, OH). Then, an air stone continuously bubbling compressed air at a very slow rate was added to each of the hypoxic treatment tanks to prevent oxygen concentrations from dropping to anoxic levels while the oysters respired over the course of the experiment. The hypoxic treatment tanks were then sealed with glass lids to prevent oxygen diffusion from the surrounding air. Oysters were not fed throughout the experiment and 50% water changes were undertaken in all tanks every other day.

Tank conditions, including dissolved oxygen concentrations and temperature, were monitored once a day using the oxygen probe. Hypoxic treatment tanks that deviated above or significantly below the 2.0 mg l⁻¹ hypoxia threshold were bubbled again with nitrogen gas or compressed air, respectively, to maintain hypoxic conditions.

Mortality was also assessed daily by tapping on the aquaria and observing oyster responsiveness (Lombardi et al. 2013). Oysters that remained gaping following the tapping and failed to respond to subsequent tactile stimulus were determined to be dead. Dead oysters were removed from the tanks, shucked, and their shell wet weight and dry tissue weight (assessed by drying the tissue in a drying oven at 55°C for one week) were measured. The experiment ran for 46 days, at which time the majority of the oysters in the hypoxic treatments had died.

Heart Rate Experiment

The heart rate experiments were conducted December-February 2017-2018. Following at least two days of acclimation to the laboratory conditions, one oyster at a time was selected for heart rate analysis. Heart rate was measured with an electrocardiogram (iWorx IX-214, iWorx, Dover, NH) using methods developed in our research laboratory (Needham and Paynter, *unpublished data*). Prior to starting the experiment, three holes were drilled into the left valve of each oyster in locations determined to provide the best signal for heart rate measurement (Needham, *personal communication*). iWorx gold needle probes, modified with electrical tape to ensure waterproofing, were then placed through the holes into the tissue of the oyster.

The oyster remained on the benchtop and probes were adjusted until a heart rate signal was detected, at which point the oyster was placed into a 2.5-gallon aerated aquarium filled with oxygenated artificial seawater at room temperature ($20.5\pm 0.8^{\circ}\text{C}$) and 10 ppt salinity. Each oyster was left in the tank to acclimate for 30 minutes prior to beginning measurements. Throughout the acclimation period and the subsequent experiment, the heart rate was continuously monitored to ensure the signal was not lost. Probes were adjusted to reestablish a signal as necessary. After the acclimation period, the oysters heart rate was measured for one hour under normoxic conditions (hereafter termed the ‘baseline treatment’), with oxygen concentrations in the tank being recorded every five minutes. Following the one-hour baseline treatment, the tank was briefly bubbled with compressed nitrogen gas to reduce the oxygen concentrations to $< 2.0 \text{ mg l}^{-1}$. Oysters were left for another 30 minutes of acclimation to the hypoxic conditions and then the heart rate was measured for one hour in hypoxia (hereafter termed the ‘hypoxic treatment’). Oxygen concentrations were again measured using an oxygen probe every five minutes throughout the hypoxic treatment period. Following the completion of the experiment, each oyster was shucked and shell weight and dry tissue weight (determined by drying the tissue at 55°C for one week) were measured.

Survival Experiment Statistical Analysis

The effects of treatment and three metrics of oyster size (wet weight, shell length, and dry tissue weight) on oyster survival was assessed using a Cox proportional hazards model implemented using the SURVIVAL package in R (RStudio Version 1.3.1073 / R

Version 3.6.1). Shell weight and wet weight were strongly correlated ($r=0.99$, $p<0.001$) so shell weight was excluded from the analysis. The Cox model could not be used for comparing the survivorship curves of oysters from the different sites as the survival curves crossed, contradicting the proportional hazards assumption of the model. Therefore, multiple pairwise log-rank comparisons between each pair of sites were conducted, using a Benjamini-Hochberg procedure to correct for multiple comparisons. In addition to assessing the survival curves, LT_{50} values were calculated for the oysters from each site in the hypoxic treatment using the *LT_probit* function in the ECOTOX package in R, treating each tank as a replicate. Differences in LT_{50} values between sites in the hypoxic treatment were analyzed using an ANOVA.

Heart Rate Experiment Statistical Analysis

Heart rate data files were analyzed using LabScribe software (LabScribe2 Version 2.35). Heart rate was manually measured in the baseline and hypoxic treatment periods, taking four one-minute samples for each 10-minute period. Heart rate was then averaged for each oyster across each treatment, baseline and hypoxia. To correct for the natural individual variability in oyster heart rate, the average percent change in heart rate between each oyster's baseline heart rate and their heart rate in hypoxia was calculated.

Heart rate differences between the baseline and hypoxic treatments were assessed using an analysis of variance (ANOVA). Differences in proportional heart rate change were analyzed using an analysis of covariance (ANCOVA) to assess

differences in heart rate response to hypoxia associated with the oyster's site of collection. Covariates in the analysis included oyster wet weight, shell length, dry weight, and site. As with the oyster size data from the survival experiment, wet weight and shell weight were strongly correlated ($r=0.99$, $p<0.001$), thus shell weight was dropped from the model. Where significant differences were observed, a post-hoc Duncan's test was performed to make pairwise comparisons between sites. In addition, linear models were used to determine relationships between metrics of oyster size and heart rates in the each of the two treatments. All analyses were carried out in R (RStudio Version 1.3.1073/R Version 3.6.1).

Results

Survival Experiment

Oxygen conditions in the tanks remained within the target range throughout the duration of the experiment, with average dissolved oxygen concentrations in the normoxic tank of 7.88 ± 0.38 mg/l and average dissolved oxygen concentrations in the hypoxic tank of 1.52 ± 0.66 mg/l (Figure 2.5a). Throughout the experiment, normoxic tanks had a slightly lower temperature compared to the hypoxic tanks ($19.53\pm 0.41^\circ\text{C}$ vs. $21.50\pm 0.44^\circ\text{C}$, $t=34.199$, $df=214.96$, $p<0.001$), likely as a result of greater evaporative cooling from the aeration with compressed air (Figure 2.5b).

At the conclusion of the experiment, 46 days, the mean survival of oysters in the normoxic tanks was $79.2\pm 0.083\%$ and the mean survival of oysters in the hypoxic

tanks was $13.9 \pm 0.13\%$. Overall, oysters in the hypoxic treatment had a statistically higher risk of mortality than oysters in the normoxic treatment (Table 2.1, Figure 2.6, $p < 0.001$), with normoxic treatment oysters having 90% reduced risk of mortality compared to hypoxic treatment oysters. Given that 46 days represents an extreme duration of hypoxia exposure (see *Discussion*), a comparison of survival at 14 days into the experiment was also conducted, which still revealed a significant difference in survival between oysters in hypoxic and normoxic treatments (hazard coefficient = 1.244, SE = 0.572, $p = 0.03$).

While the survival curves of some sites appeared to differ in their mortality risk, particularly in the hypoxic treatment, a comparison of survival curves within each treatment independently did not indicate site-level differences in oyster mortality (Figure 2.7; pairwise adjusted p -values all > 0.05). In the normoxic treatment, LT_{50} values could not be calculated for all tanks as some had no mortality throughout the experiment. In the hypoxic treatment, no significant differences in LT_{50} values between sites were observed (Figure 2.8, $F = 1.261$, $df = 5$, $p = 0.35$).

Oyster body size, as measured through wet weight, had a significant negative effect on oyster survival within the hypoxic treatment (Table 2.1, $p < 0.001$). Other metrics of oyster size did not correlate to differences in survival (shell length, $p = 0.88$; dry weight, $p = 0.24$). To further investigate the effect of oyster wet weight on survival, oysters were grouped into three size classes based on their wet weight (small < 140.2 g, medium 140.2-224.2 g, and large > 224.2 g) using a Jenks analysis to determine

natural breaks in the wet weight data, and pairwise log-rank comparisons were made between the three curves. The survival curves of ‘small’ and ‘large’ oysters differed significantly from one another (Figure 2.9a, $p=0.038$), with large oysters having a 45% greater risk of mortality than small oysters. Within the hypoxic treatment, there was a marginally significant relationship between wet weight and source site, primarily driven by the slightly smaller oysters from Ragged Point (Figure 2.9b; $F=2.304$, $df=5$, $p=0.054$). However, given the similar distribution of small, medium, and large oysters within each site, it’s unlikely that the observed differences in mortality between oyster sizes were driven by differences in tolerances based off of the site from which they were collected.

Heart Rate Experiment

Across all sites, the average heart rate of oysters in the baseline treatment was 20.6 beats per minute (bpm) and 12.6 bpm in the hypoxic treatment, a significant reduction of approximately 40% (Figure 2.10; $F=65.54$, $df=1$, $p<0.001$). The proportional decrease in heart rate was not associated with any metric of body size (ANOVA results: wet weight $p=0.28$, shell length $p=0.70$, dry weight $p=0.64$). While the majority of oysters decreased their heart rate in the hypoxic treatment (two individuals increased their heart rate), the degree to which an individual decreased its heart rate differed among sites ($F=2.94$, $df=5$, $p=0.028$). Ragged Point, one of the hypoxic sites, showed a significantly smaller heart rate reduction (approximately 14% reduction) compared to Cook Point, Harris Creek, Holland Point, and Neal Addition, which all showed approximately 50% reduction in heart rate (Figure 2.11). However,

Ragged Point did not differ from Northwest Middleground, another hypoxic site, which exhibited a decrease in heart rate of approximately 28%.

Oyster body size, as measured through wet weight, correlated negatively with oyster heart rate in the baseline treatment (slope=-0.032, $R^2=0.204$, $p=0.0057$), but showed no association in the hypoxic treatment (linear regression $p=0.34$; Figure 2.12).

Among the sites, wet weights were marginally significantly related to the site from which the oyster was collected ($F=2.67$, $df=5$, $p=0.04$), driven primarily by larger oysters from Cook Point. While the size difference may reflect true differences between sites, given the small sample sizes (six oysters/site), broader generalizations about the population characteristics can't be made.

Discussion

Coastal and oceanic hypoxia is increasing in frequency and severity (Breitburg et al. 2018), particularly in the Chesapeake Bay (Hagy et al. 2004, Ni et al. 2019) and the ability of marine organisms to adapt to low oxygen availability will be key to their resilience in the face of future environmental change. Sessile, reef-building bivalves in particular have a strong influence on the health of coastal ecosystems (Vaughn and Hoellein 2018), thus their ability to persist despite projected future environmental change will be critical for sustaining coastal ecosystem health (e.g. Isdell et al. 2020). Studying how conspecific bivalves currently vary in their response to low oxygen stress provides insight into their capacity for future acclimation. The present study examined the survival and heart rate responses of oysters from reefs that experience

differing exposure to seasonal hypoxia to understand how prior exposure to low oxygen stress may confer differing tolerance upon subsequent exposure. Results indicated a strong treatment effect, with a significant overall increase in mortality and a significant decrease in heart rate for oysters exposed to hypoxia compared to the normoxic treatments. However, oysters from the different sites did not show differences in survival during experimental hypoxic challenge, indicating that prior exposure to hypoxia in these hypoxic site oysters did not lead to increased survival in low oxygen, at least under these experimental conditions. The observed difference between some sites in the oyster's heart rate response to low oxygen – a significantly smaller reduction in heart rate for oysters from one hypoxic site – may indicate increased sublethal tolerance in the hypoxic site oysters. While not a novel observation with respect to acute environmental stressors, the additional finding of increased mortality experienced by large oysters in hypoxia presents an important intraspecific difference in tolerance with broad ecological implications.

Hypoxia exposure increases oyster mortality

One of the key findings from this study is that hypoxia significantly increased the risk of mortality for oysters, regardless of their previous exposure to hypoxic conditions. After 46 days of exposure to persistent hypoxic conditions, approximately 14% of oysters survived, whereas approximately 80% of oysters in normoxic conditions survived. Despite the ability of some oysters to survive prolonged exposure to hypoxia in this experiment, a duration of 46 days likely represents an extreme condition that oysters would rarely experience in their natural habitat. In the

Chesapeake Bay, persistent seasonal hypoxia predominantly affects bottom waters of the main channel with depths >10 m and can last for up to 140 days per year (Murphy et al. 2011). However, oysters are typically not found at these depths, thus they would rarely experience such prolonged exposure to hypoxia in their natural environment. Instead, oysters primarily reside in mainstem and tributary waters up to a depth of 10 m (Kennedy 1991) where documented hypoxic events have been shown to last for a significantly shorter period of time, approximately 7-14 days (Lenihan and Peterson 1998, Sagasti et al. 2000, Neff et al. 2020). The duration of exposure to hypoxia in the present study was significantly longer than the one-to-two-week exposure experienced on natural oyster reefs. However, an analysis of oyster survival at two weeks of experimental exposure to hypoxia indicates that shorter term hypoxic events on oyster reefs would also lead to increased mortality. At two weeks, oysters still showed a difference in survival across treatments, with 82% surviving in hypoxic conditions compared to 96% in the normoxic treatment. Thus, prolonged exposure to hypoxia does increase oyster's risk of mortality, even for durations consistent with documented events on oyster reefs.

Reef-specific effects on lethal and sublethal hypoxia tolerance

Contrary to one of the study's main hypotheses, results indicated that the site from which the oysters originate does not influence survival in hypoxia as oysters from hypoxic sites did not have a lower risk of mortality in the hypoxic treatment than oysters from normoxic sites. These results contrast with findings from previous research in oysters and other bivalves indicating the inducibility of increased bivalve

survivorship in low oxygen as a result of prior exposure to hypoxic conditions. For example, Ford (2005) showed that *C. virginica* from a reef believed to experience hypoxia in Mobile Bay, Alabama had higher LT₅₀ and LT₉₀ values in anoxic conditions than individual oysters from a nearby normoxic reef. Similarly, two independent studies have shown inducible increased hypoxia survival in *Crassostrea gigas* (Meng et al. 2018) and *Mytilus edulis* (Altieri 2006) based off prior exposure to reduced oxygen availability via aerial exposure. In both studies, prior exposure to tidal emersion – aerial exposure being a proxy for hypoxic conditions – led to greater survivorship upon subsequent exposure to reduced oxygen availability compared to individuals acclimated to well-oxygenated habitats.

There are at least two possible explanations for why increased inducible tolerance was not detected in oysters sourced from low-oxygen reefs. One possible reason for the differing results is that, in contrast to the studies described above, the frequency or duration of hypoxic exposure experienced by the oysters in the hypoxic sites from this study was not sufficient to induce differing responses upon experimental exposure to hypoxia. Water quality monitoring data indicates that the oysters from hypoxic sites used in these experiments experienced a decrease in oxygen availability at two or three measured time points during each year, but these sites lack continuous monitoring data to ascertain the duration of the exposure at the timepoint measured and the potential for other exposures between the sampled time points. The dissolved oxygen profile of the hypoxic reef from Ford's study (2005) was not reported, but presumably could have been more prolonged in duration than the oysters in this

study. Additionally, the bivalves from the studies involving intertidal exposure (Meng et al. 2018, Altieri 2006) were exposed to anoxic conditions twice daily during emersion, a much higher frequency of exposure than experienced by the oysters in the present study. In general, theory on phenotypic plasticity in response to environmental stressors indicates that plasticity tends to be more pronounced in environments where the frequency of exposure to the stressor is predictable (Gabriel 2005), but the amount of time between the exposures also tends to play a role. For example, more frequent exposure to thermal stress increases mussel tolerance to subsequent thermal challenge (Prince 2017). Similarly, differential heart rate response to low oxygen stress in bay scallops appears to be related to the frequency of prior exposure to hypoxia (Gurr et al. 2021). Both of these studies looked at exposure frequencies over much shorter timescales than the present study (hours to days), thus how these findings relate to the exposures over the annual timescale of this study is unknown. If multiple annual exposures do not confer differing lethal tolerance to hypoxia in oysters, future work on understanding how varying timescales of exposure to hypoxia stress might influence future organismal response is worth pursuing.

It is also plausible that the experimental salinity as well as the ambient salinity conditions of Chesapeake Bay leading up to the experiment introduced an additional stress that may have influenced survival results. The experimental salinity of 5 ppt was chosen in order to maintain experimental conditions at a level consistent with what the oysters were experiencing during their pre-experiment acclimation.

However, the oysters used for the survival experiment were collected and underwent

their acclimation in 2018, a year in which the Chesapeake Bay area experienced record precipitation (National Weather Service 2019) which led to substantially decreased salinity throughout the Bay and significant oyster mortality in some of the tributaries (Tarnowski 2019). The oysters used in the survival experiment experienced abnormally low salinities on their native reefs and the low salinity exposure continued throughout their acclimation. When the oysters were collected in 2018, the salinities at the collection sites ranged between 5-8 ppt. In comparison, oysters that had been collected for the heart rate experiment in the year prior – with typical precipitation amounts – were collected at salinities of 10-15 ppt. During the month-long pre-experiment acclimation, oysters experienced salinities of 3.82 ± 0.17 ppt. Thus, oysters at the onset of the experiment were likely experiencing some degree of osmotic stress, which continued throughout the experiment.

C. virginica is an osmoconformer, able to tolerate a wide range of salinity conditions, but the experimental salinity of 5 ppt corresponds to a lower threshold of *C. virginica*'s salinity tolerance (Kennedy 1991). At salinities at 5 ppt or lower, oysters will close their valves, reducing their feeding, respiratory, and reproductive activities (Galtsoff 1964). Furthermore, the synergistic effects of low salinity and reduced oxygen availability would potentially add additional complexity to the oyster response to hypoxia, since low salinities tend to lessen the ability of bivalves to regulate their oxygen consumption in declining oxygen tensions (Bayne 1973, Shumway and Koehn 1982). A significant decline in oyster's oxyregulatory ability has been shown at salinities as low as 7 ppt (Shumway and Koehn 1982). The

inability to maintain aerobic pathways under salinity and oxygen stress would lessen energy production, increasing overall oyster mortality in hypoxia and potentially masking any site-specific differences in tolerance to hypoxic stress. Increases in mortality rates under both hypoxic and low-salinity stress have been shown for the Olympic oyster, *Ostrea lurida* (Cheng et al. 2015), and some species of crustaceans (Legeay and Massabuau 2000, McGaw and McMahon 2003). Therefore, the reduced salinities leading up to and during the experiment may have imposed additional osmotic stress on the oysters that could have contributed to the observed results.

Adding additional complexity, differences in low salinity tolerance between sites could have influenced the mortality patterns observed. It is well understood that oyster's prior low salinity exposure does confer some degree of increased tolerance to future low salinity stress (Eierman and Hare 2013). The observed pattern of survival for oysters from Northwest Middleground indicates that differential tolerance to salinity may have played a role in the survival results. Oysters from the hypoxic site Northwest Middleground trended towards the lowest survival (Figures 2.7B and 2.8) of the examined sites, and also experienced the highest range (9-20 ppt) and average (15 ppt) salinity (Figure 2.4) on their native reef. The other reef sites historically had comparatively lower ranges in salinity (Cook Point=7-17 ppt, Holland Point=5-15 ppt, Harris Creek=9-17, Neal Addition=8-18, Ragged Point=7-18). Given the higher range of salinities and higher average salinity experienced at Northwest Middleground, it is plausible that the experimental salinity of 5 ppt imposed more

stress on the oysters from Northwest Middleground compared to oysters from the other sites leading to their trend towards higher mortality rates.

In contrast to results from the survival experiment, differences in heart rate response to hypoxia between sites were observed, indicating that there are intraspecific differences in sublethal physiological response to hypoxia which may relate to the oyster's prior exposure to low oxygen. The smallest proportional decrease in heart rate was observed in one of the hypoxic sites (Ragged Point) and a trend towards a similar small proportional decrease was observed in a second hypoxic site (Northwest Middleground). The proportional change in heart rate for oysters from Northwest Middleground was not statistically different from the normoxic site oysters. However, the sample size for this experiment was small (six individuals/site) and the lack of detectable difference was driven by one oyster from Northwest Middleground that reduced their heart rate by nearly 80% compared to the other five that showed reductions of less than 50%. Removal of this potential outlier individual from the analysis yields a strong effect of site ($p=0.004$), with Ragged Point and Northwest Middleground grouping separately from the rest of the examined sites. For Ragged Point and Northwest Middleground, the trend towards a smaller proportional heart rate response compared to the other sites appears to be related to the frequency of prior hypoxia exposure. Of the six sites used in the study, Ragged Point and Northwest Middleground had the highest frequency of hypoxic measurements (average monthly measurements per year: Northwest Middleground = 3.8, Ragged Point = 3.2, Neal Addition = 1.2, Holland Point = <1, Cook Point = 0, Harris Creek =

0). These findings lend further support to the hypothesis that a higher frequency of hypoxic exposure is important in inducing differential responses. Additionally, the trend towards a reduced cardiac response in low oxygen for oysters with a history of frequent hypoxia exposure supports recent findings that hypoxia-acclimated bivalves exhibit a smaller change in heart rate response compared to hypoxia-naïve bivalves (Gurr et al. 2021).

It is plausible that the smaller reduction in heart rate in oysters from Ragged Point and Northwest Middleground compared to the other sites indicates a potential mechanism of increased tolerance to low oxygen. The lesser decrease in heart rate could be indicative of a lower critical oxygen concentration (P_{crit}) for the oysters from the hypoxic sites (Figure 2.14). P_{crit} denotes the concentration at which an organism transitions from oxyregulation to oxyconformation (Beamish 1964, Ultsch et al. 1974) and compared across species, lower P_{crit} values are shared by organisms with greater hypoxia tolerance (Vaquer-Sunyer and Duarte 2008) as it allows them to maintain aerobic pathways under declining oxygen tensions (Grieshaber et al. 1994). Thus, if the reason for the observed difference in heart rate response is due to a lower P_{crit} for oysters from Ragged Point and Northwest Middleground, this could indicate a greater tolerance to low oxygen for oysters with higher frequency of prior low oxygen exposure. Furthermore, organisms that undergo a more oxyregulatory response, such as some species of cnidarians (Rutherford and Thuesen 2005) and bivalves (Artigaud et al. 2014), have also been shown to be more tolerant to hypoxia compared to those with a greater oxyconforming response as measured by higher survival rates. A

limitation of the present study is that it only provides heart rate responses at two oxygen concentrations, so it's not possible to conclude how oysters from the different sites would alter their heart rate across a range of dissolved oxygen concentrations. More research is needed to elucidate whether the observed difference in heart rate response between sites actually reflects a lower P_{crit} for hypoxic site oysters.

Overall, the results of the survival and heart rate experiments provide variable results regarding inducible oyster tolerance to low oxygen stress. Oysters with differing histories of hypoxia exposure appear to show no difference in gross survival tolerance upon hypoxic exposure, but prior exposure to higher frequencies of hypoxia does appear to confer some increased physiological tolerance, as evidenced by the varying heart rates among oysters from hypoxic versus normoxic sites. Considering the results of both experiments, the possibility that low salinity stress imposed on oysters during the survival experiment played a key role in the lack of observed difference in oyster survival between sites cannot be disregarded. As previously discussed, low salinity stress in concert with oxygen stress would synergistically affect oyster energetics, leading to higher overall mortality and potentially masking site-specific differences in tolerance to low oxygen. Additionally, greater sensitivity to low salinity stress for the hypoxia-acclimated Northwest Middleground oysters could have driven the trend towards higher mortality for oysters from this site. Furthermore, physiological differences in hypoxia response between sites were observed in the heart rate experiment which was conducted at a higher salinity (10 ppt). While the differences in heart rate may present a short-term response to low oxygen that doesn't confer

greater survival, prior research indicates that the hypothesized greater oxyregulatory response/lower P_{crit} exhibited by some oysters with prior hypoxia exposure correlates with greater hypoxic survival (Rutherford and Thuesen 2005). Thus, it remains possible that the synergistic effects of osmotic and respiratory stress may have played a key role in the observed survival patterns. Further research should address the synergistic effects of hypoxia and low-salinity stress, particularly as they may be important co-occurring stressors resulting from climate change, particularly in the Chesapeake Bay (Najjar et al. 2010).

Frequency of exposure could also have played a key role in driving differential tolerances. The two sites that trended towards the lowest reduction in heart rate, Ragged Point and Northwest Middleground, also had the highest frequency of hypoxic exposure (approximately 3 monthly measurements per year). Neal Addition, the third site identified as 'hypoxic', averaged approximately one monthly hypoxic measurement per year and showed a heart rate response that was more similar to the normoxic site oysters. Thus, a threshold duration or frequency of exposure may be necessary to induce a differential physiological response to hypoxia. One clear limitation of the current study is the collection of oysters from sites lacking daily dissolved oxygen data and the inability to fully quantify the extent of hypoxia exposure at each site. However, the results do indicate differential sublethal response to hypoxia driven by prior exposure, which should motivate further research examining how the frequency of hypoxia exposure affects the inducibility of oyster tolerance to low oxygen.

Oyster body size influences mortality and heart rate in hypoxia

Results from both experiments indicate that oyster body size influences the response of oysters to hypoxic conditions. In the mortality experiment, large oysters (>224.2 g) died at a significantly higher rate than small oysters (<140.2 g) (Figure 2.9a). In the heart rate experiment, there was a negative correlation between oyster heart rate and oyster wet weight in normoxic conditions that was not present in hypoxic conditions (Figure 2.12). Furthermore, large oysters (characterized as above) appeared to reduce their heart rate in hypoxia to a lesser extent than would be expected based of the relationship between wet weight and proportional heart rate change shown by small and medium oysters (Figure 2.13).

Reduced tolerance of larger individuals to stressors has been shown across a range of environmental conditions, such as temperature (reviewed by Peck et al. 2009), salinity (Cheng et al. 2016), and dissolved oxygen (Clark et al. 2013). Reduced tolerance to low oxygen in large individuals can be explained by the decrease in mass-specific metabolic rate with increasing size. That is, as bivalve size increases, the respiratory surface-area to body-volume ratio also decreases, leading to a reduction in the amount of oxygen uptake per unit body mass (Nilsson and Östlund-Nilsson 2008). Thus, as oxygen availability decreases, larger individuals are less able to efficiently extract oxygen from the surrounding environment than their smaller conspecifics.

Given that large oysters are less tolerant to hypoxia, reduced oxygen availability in the Chesapeake Bay and other areas abundant in oysters could have profound effects on the oyster size distributions on hypoxia-prone reefs. Hypoxic events could act as acute demographic or natural selection events, causing mass mortalities of large individuals, which could have implications for short-term changes in oyster population or community dynamics as well as longer-term microevolutionary changes. Since *C. virginica* is a protandric hermaphrodite, loss of large individuals in areas prone to low oxygen could reduce female abundance and thus reduce reproductive output, as has been observed in populations subject to size-selective mortality due to disease or overfishing (Rothschild et al. 1994, Powell et al. 2013). This reduction in fecundity of oysters ultimately affects the ability of oyster populations to rebound from significant declines over the past century (Mann and Powell 2007). In addition, the loss of larger individuals during hypoxic events reduces the biomass and secondary production on oyster reefs (Sturdivant et al. 2014) and can have cascading effects for the community assemblage dependent on the reef structure (Luckenbach et al. 2005). Therefore, the continued increase in hypoxia severity predicted in the Chesapeake Bay (Ni et al. 2019) and other key habitats of *C. virginica*, such as the Gulf of Mexico (Laurent et al. 2018), may have broader ecological implications for oysters, as well as oyster-dependent species, in the Chesapeake Bay and elsewhere.

Furthermore, the predicted widespread deoxygenation in the world's oceans may select for a reduced final body size in oysters. In *Drosophila*, flies exposed to low

oxygen over the course of 12 generations had significantly reduced body sizes compared to those raised at normoxic levels (Klok and Harrison 2009). Similar patterns have been observed in reptiles (Owerkowicz et al. 2009) and crustaceans (Seidl et al. 2005). Certainly, over a long-term scale, the reduction in body size would have important implications for the population dynamics and community ecology as on the shorter-term scale described above.

Conclusions

The present study provides novel insights into intraspecific variability in hypoxia response in a marine bivalve. While no site-specific patterns in mortality were observed, the results were possibly obscured by interacting effects of low salinity experienced by oysters during and preceding the experiment. In contrast, a comparison of sublethal cardiac response indicated that oysters from at least one hypoxic reef show a lesser reduction of heart rate than their normoxic reef counterparts, perhaps indicative of a greater oxyregulatory response for oysters with prior hypoxia exposure. Frequency of prior hypoxic exposure also may play an important part in the development of differential physiological tolerance since oysters that trended towards the lowest reductions in heart rate response also experienced the highest frequency of low oxygen exposure at their native reef. Additionally, the differential effect of hypoxia on oysters depending on their body size, while not a novel finding, is still important on a broader ecological and evolutionary scale, with serious implications for oyster restoration efforts in the Chesapeake Bay and elsewhere. The finding of a significant overall effect of hypoxia on both survival and

physiological response reaffirms the importance of continued study into the mechanisms by which oysters will be able to mitigate against the effects of future reductions in oxygen availability.

Table 2.1: Results of Cox proportional hazards model on oyster survival dependent on treatment, wet weight, shell length, and dry weight.

Term	Coefficient	SE	p-value
Treatment	-2.59	0.34	<0.001
Wet weight	0.0083	0.0025	<0.001
Shell length	-0.0017	0.011	0.88
Dry weight	-0.26	0.22	0.24

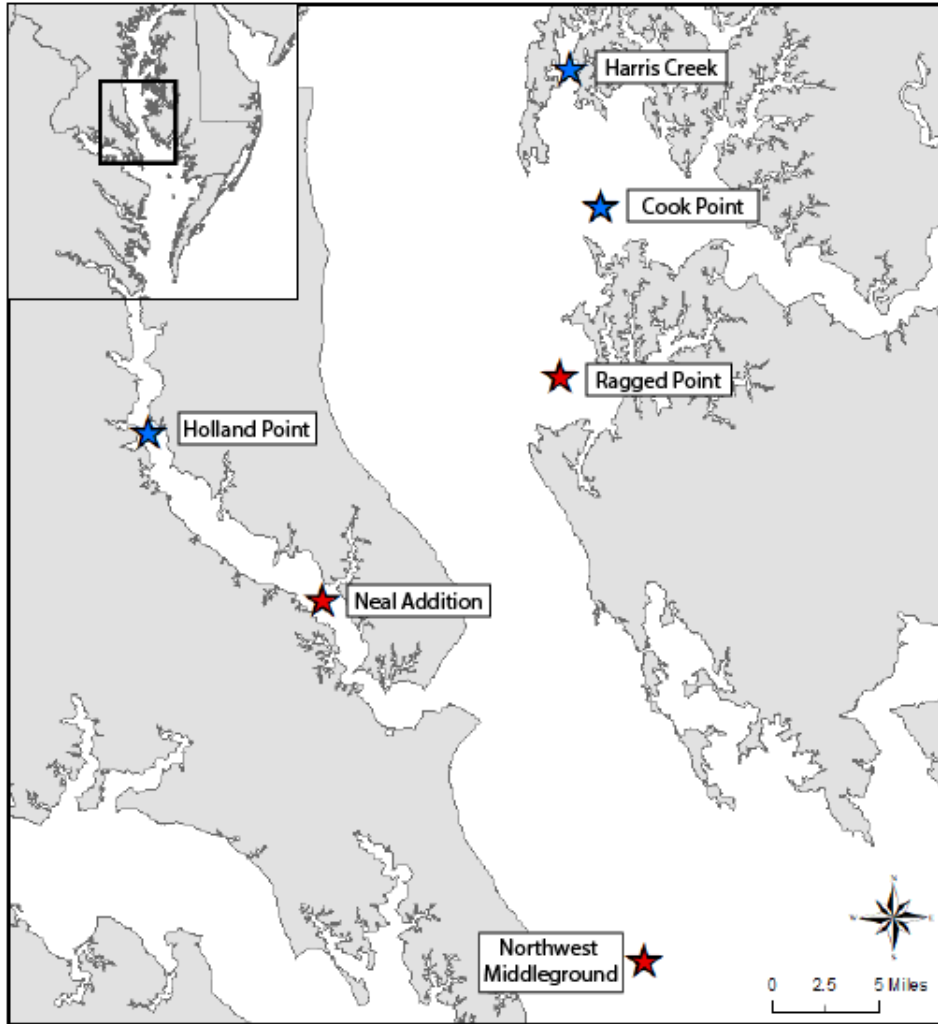


Figure 2.1: Map of oyster collection sites. Red stars indicate sites with summertime hypoxic conditions. Blue stars indicate sites with generally normoxic conditions.

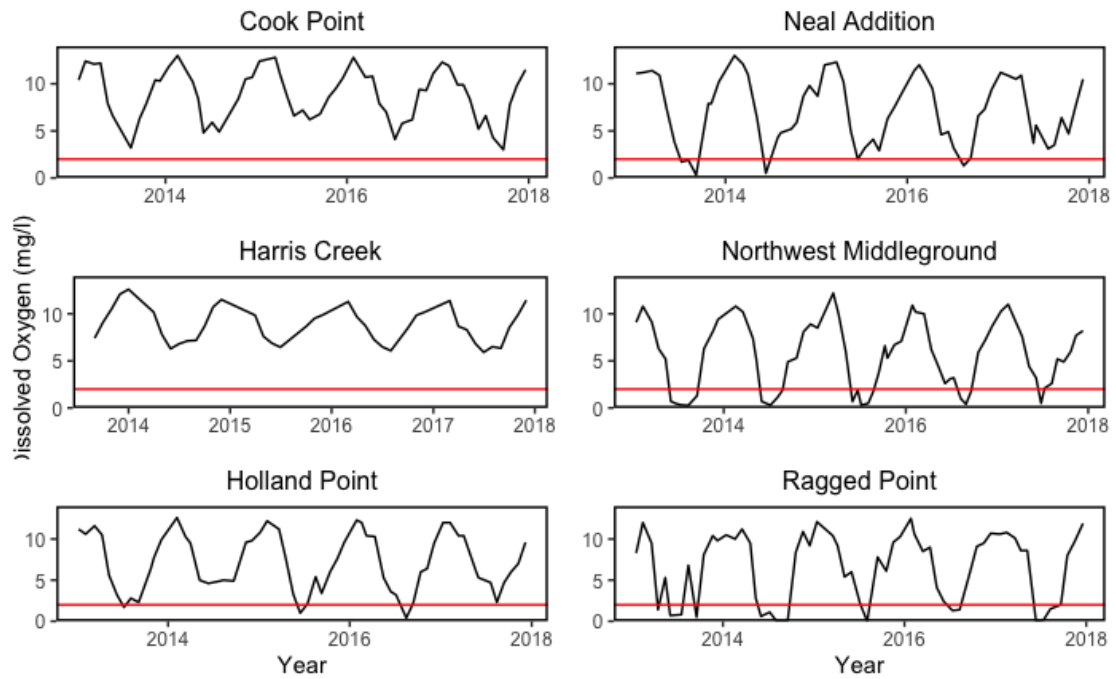


Figure 2.2: Historic dissolved oxygen concentrations (mg l^{-1}) for the six oyster collection sites from 2013-2018. Left-hand panels correspond to ‘normoxic’ sites and right-hand panels correspond to ‘hypoxic’ sites. The red lines on each panel mark 2.0 mg l^{-1} , the threshold concentration for hypoxic conditions.

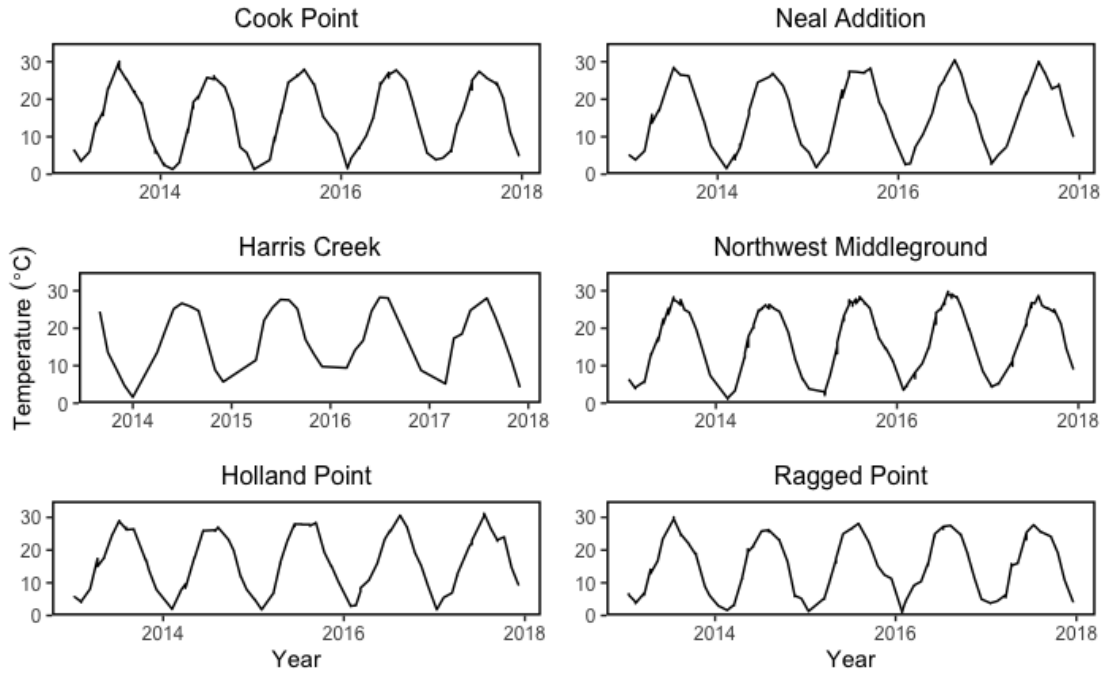


Figure 2.3: Historic temperature (°C) for the six oyster collection sites from 2013-2018. Left-hand panels correspond to ‘normoxic’ sites and right-hand panels correspond to ‘hypoxic’ sites. Temperature profiles between sites were very similar for the years compared.

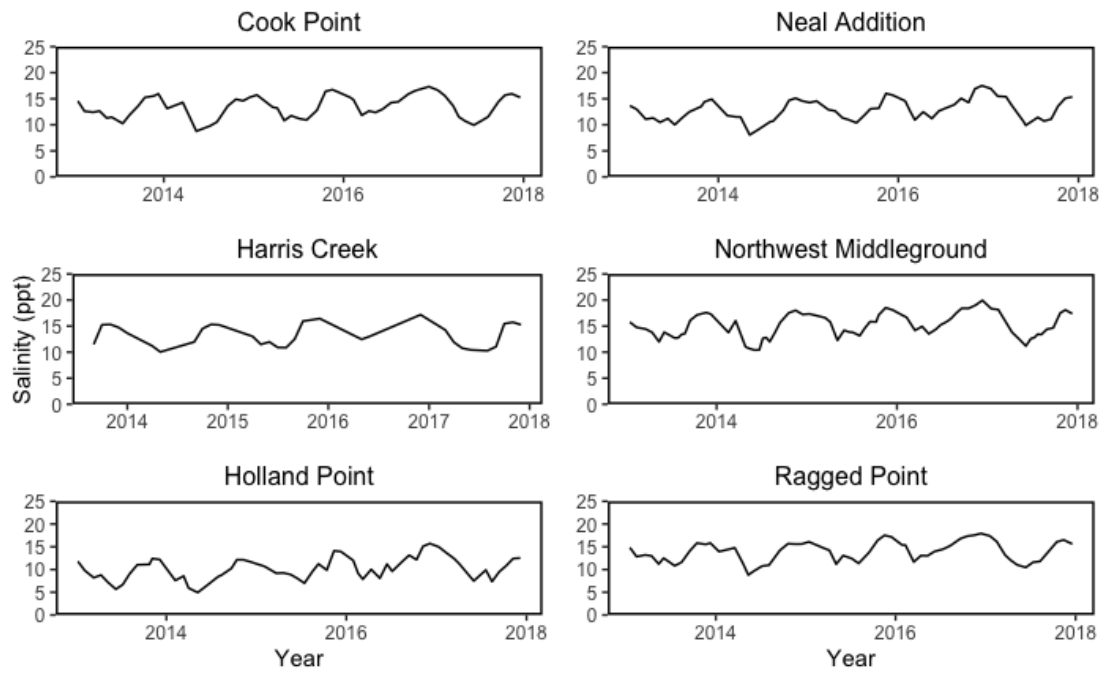


Figure 2.4: Historic salinity (ppt) for the six oyster collection sites from 2013-2018. Left-hand panels correspond to ‘normoxic’ sites and right-hand panels correspond to ‘hypoxic’ sites. Salinity profiles varied slightly between sites but were overall similar for the years compared.

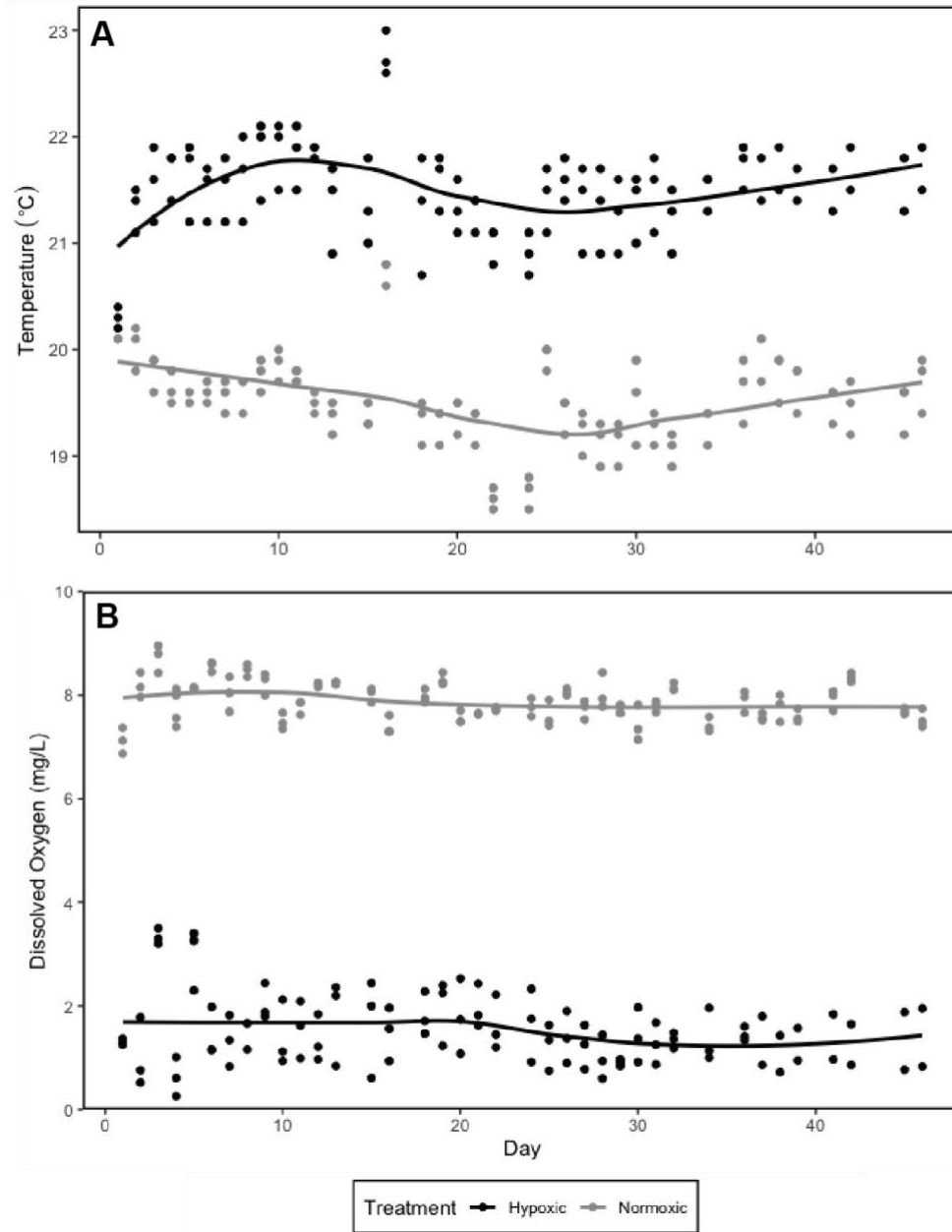


Figure 2.5: Daily temperature (A) and dissolved oxygen concentrations (B) in each of the experimental tanks throughout the duration of the survival experiment. Trend lines in each correspond to a rolling average. Black symbols correspond to hypoxic tank conditions and grey symbols correspond to normoxic tank conditions.

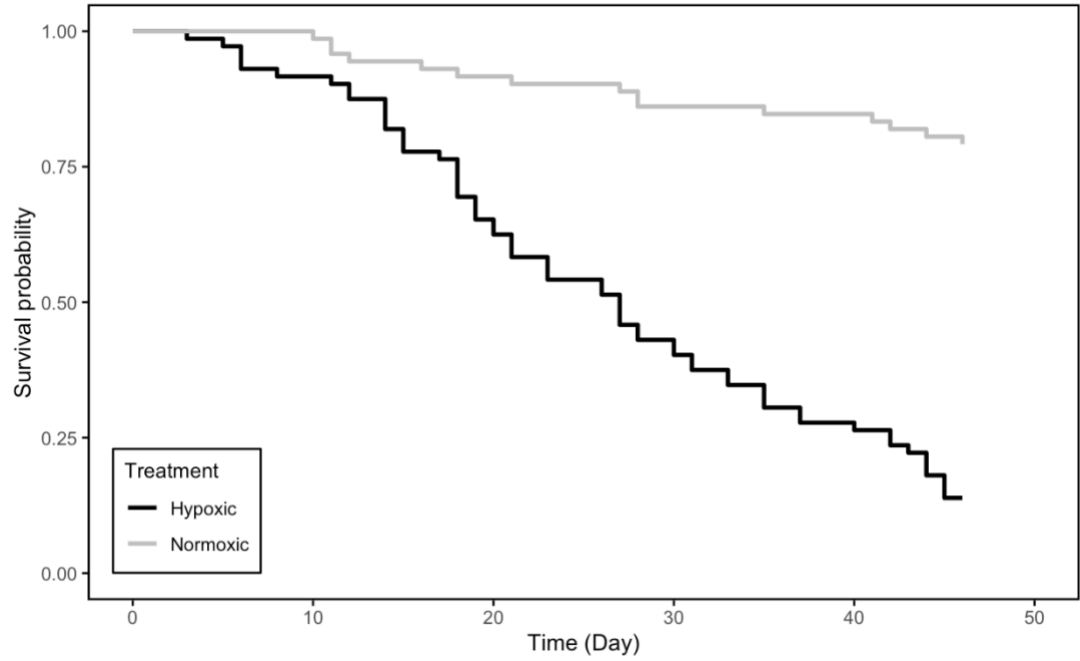


Figure 2.6: Survival of oysters in hypoxic (black line) and normoxic (grey line) treatments. Oysters died at higher rate in hypoxic treatment ($p < 0.001$).

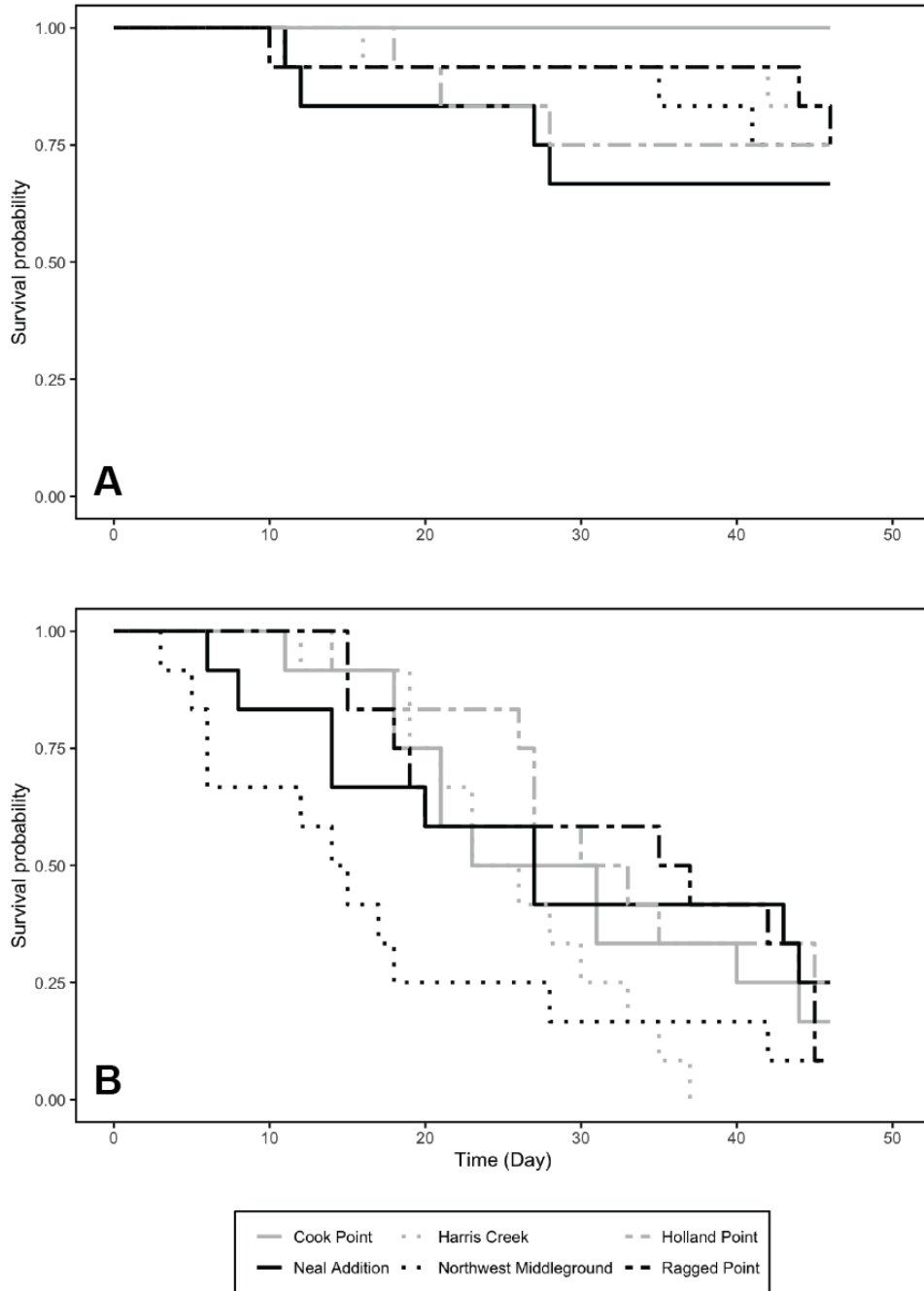


Figure 2.7: Survival curves of oysters from six collection sites in A) normoxic and B) hypoxic treatments. Grey lines correspond survival curves of oysters from normoxic sites and black lines correspond to oysters from hypoxic sites. No significant difference was observed between sites in either treatment (all analyses $p > 0.05$).

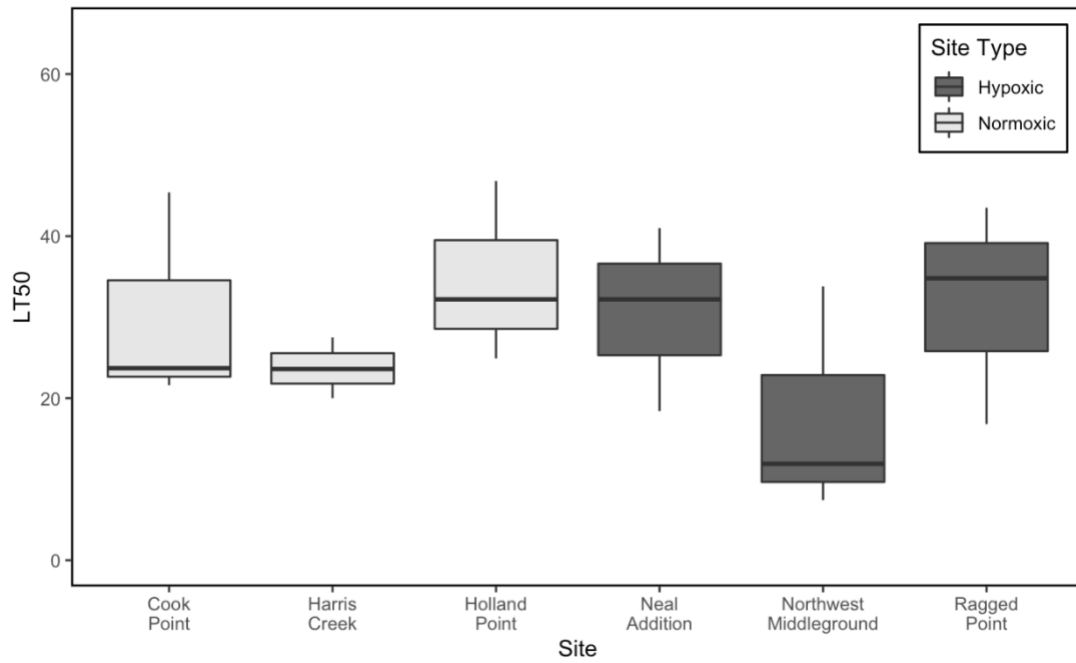


Figure 2.8: Boxplot of LT_{50} values for oysters from the six sites in the hypoxic treatment. No significant differences existed between the different sites ($p=0.35$).

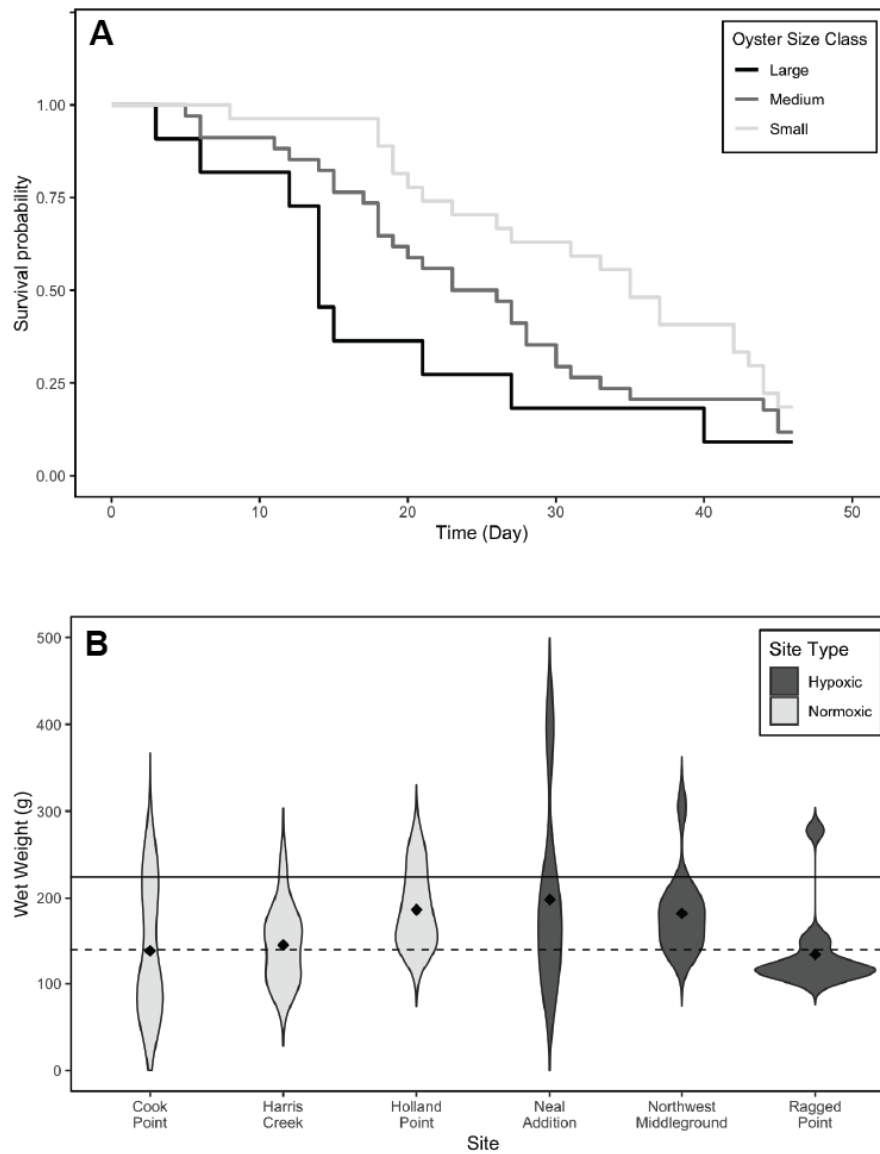


Figure 2.9: A) Survival probability of oysters by wet weight from all sites across the duration of the experiment. The large oysters (>224.2 g wet weight) died at a significantly higher rate than small oysters (<140.2 g wet weight; $p = 0.038$). B) Violin plot of oyster wet weights by site for oysters in the survival experiment. Point inside violins corresponds to mean wet weight. Solid line denotes threshold for ‘large’ oysters and dashed line denotes threshold for ‘small’ oysters. A marginally significant difference existed between sites, driven primarily by Ragged Point ($p=0.054$).

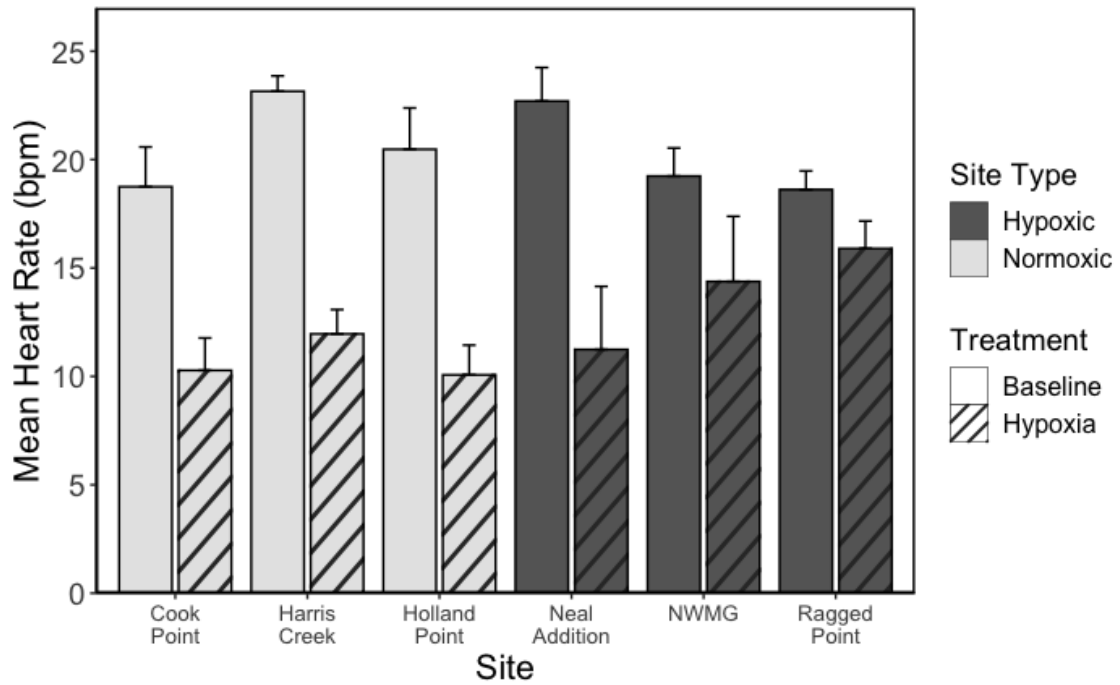


Figure 2.10: Heart rate in baseline (solid) and hypoxic (striped) treatments for oysters from each of the six collection sites. Light grey bars correspond to ‘normoxic’ sites and dark grey bars correspond to ‘hypoxic’ sites. Hypoxia heart rate in all sites was significantly reduced from baseline heart rate ($p < 0.001$). Error bars denote SEM.

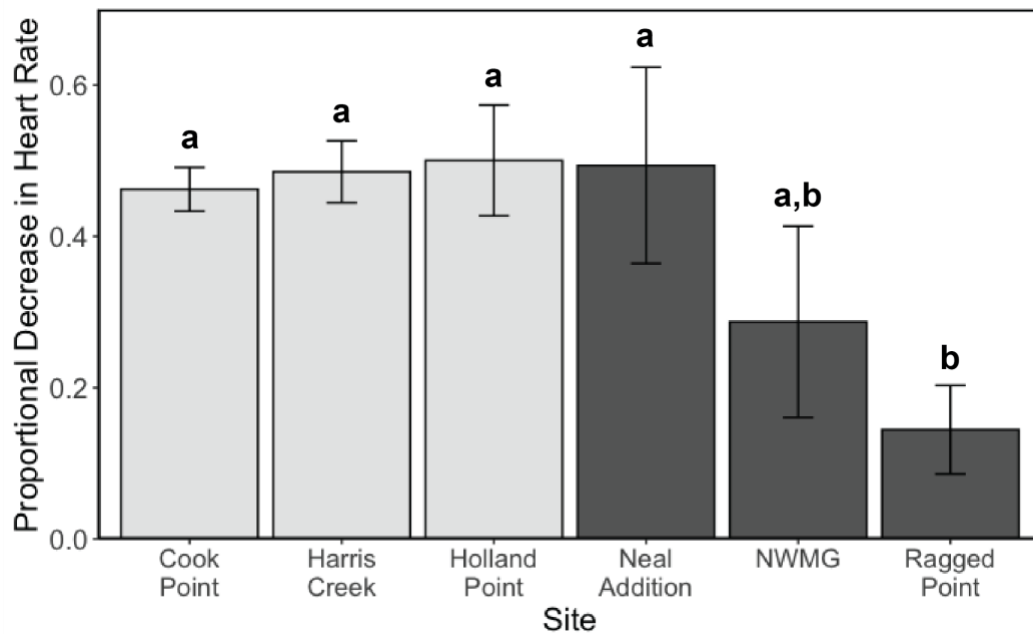


Figure 2.11: Proportional decrease in heart rate as a function of oyster collection site. Light grey bars correspond to normoxic sites and dark grey bars correspond to hypoxic sites. The proportional decrease in heart rate differed between the sites examined ($p=0.0283$). Letters denote statistically significantly different means. Error bars represent SEM.

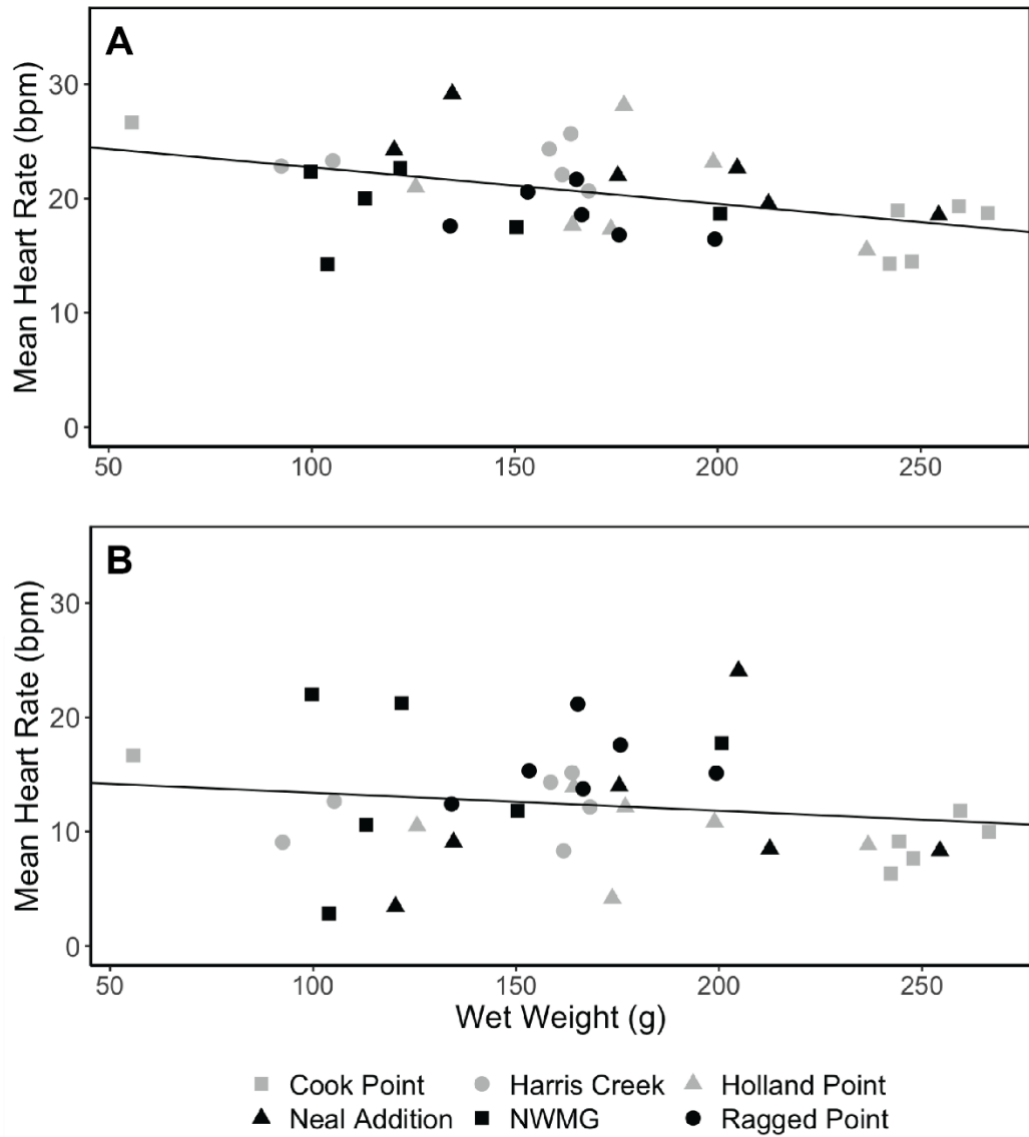


Figure 2.12: Relationship between wet weight and heart rate in the baseline (A) and hypoxic (B) treatments. Lines in each show the best fit line from a linear model. Significant negative relationship between wet weight and mean heart rate ($p = 0.0057$) in the baseline treatment, but not in the normoxic treatment ($p=0.34$).

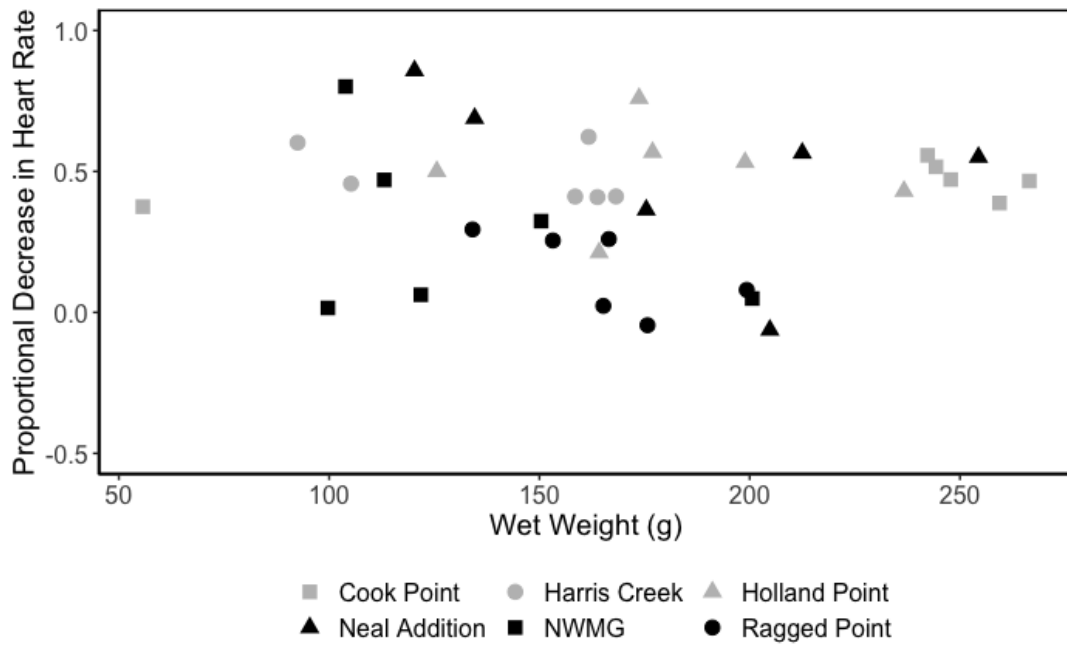


Figure 2.13: Wet weight of oysters in relation to the proportional change in heart rate between hypoxic and baseline treatments. Grey symbols correspond to normoxic sites, black symbols correspond to hypoxic sites.

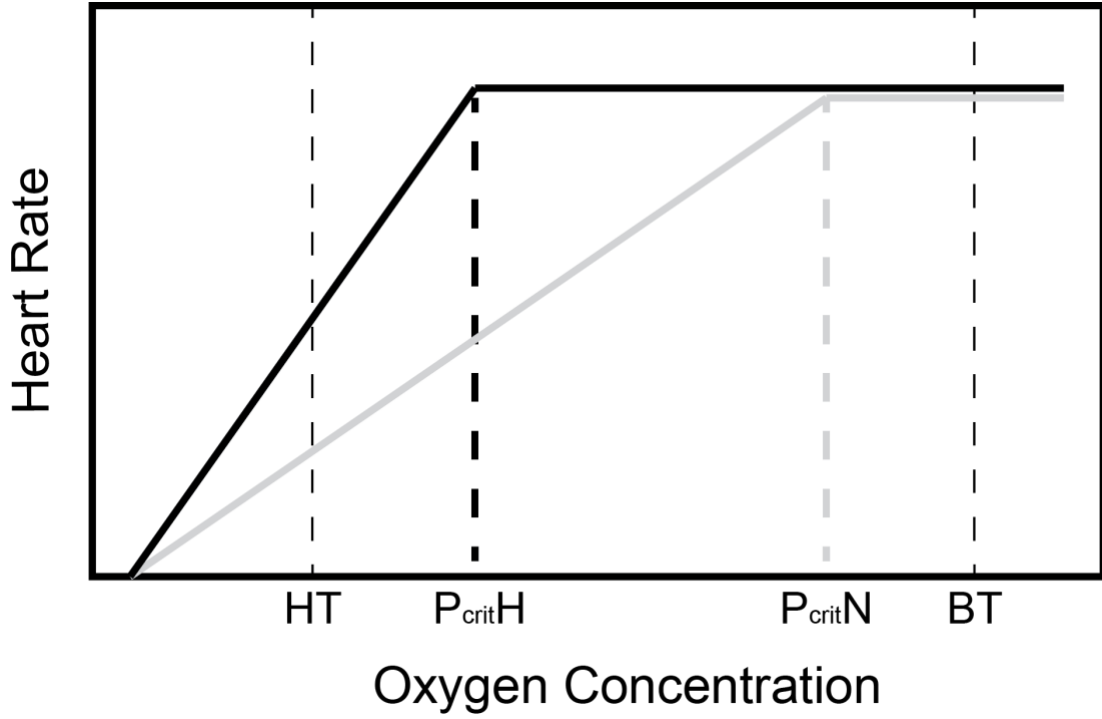


Figure 2.14: Schematic of hypothesized heart rate response curves across a range of dissolved oxygen concentrations for the hypoxic (black line) and normoxic (grey line) site oysters. Oysters from hypoxic sites are theorized to have a lower P_{crit} value (P_{critH} , black dashed line) in comparison to oysters from normoxic sites (P_{critN} , grey dashed line), leading to a smaller decrease in heart rate for hypoxic site oysters in the hypoxic treatment (HT) compared to baseline treatment (BT).

CHAPTER 3: TRANSCRIPTOMIC RESPONSE OF *CRASSOSTREA VIRGINICA* TO LOW OXYGEN EXPOSURE

Abstract

The frequency or severity of abiotic stressors in coastal environments as a result of human activities are predicted to increase into the future and will necessitate the acclimation or adaptation of organisms to increased stress. Hypoxia is a major environmental stressor that is increasing in frequency in coastal and open ocean environments. However, little is known about the capacity of organisms to cope with lower dissolved oxygen levels forecast to occur in estuarine environments in the future. Comparative transcriptomic analyses provide a powerful approach to assess differences in organismal response to environmental stress and, when used to compare responses between conspecifics with different histories of exposure to a stress, can provide insights into the mechanisms underlying differential tolerance. In the present study, gene expression response to low oxygen exposure was compared between eastern oysters, *Crassostrea virginica*, differentially acclimatized to reduced oxygen in order to find evidence for molecular pathways important for increased tolerance to hypoxia. Three different analytical approaches, differential gene expression, co-expression gene network, and transcriptional frontloading analyses, were used to assess intraspecific differences in hypoxic response. Global gene expression analyses indicated that oysters undergo a distinct gene expression response to low oxygen availability, with enrichment for genes that relate to metabolism, stress response, and immune processes. Statistically significant differences in gene

expression response between sites were not detected, although trends in expression of co-expressed genes for two significant modules identified through the gene network analysis indicated the potential for differing expression patterns between sites. Finally, while not found to be statistically significant, gene expression patterns suggested transcriptional frontloading as a possible mechanism of increased hypoxia tolerance in oysters. Overall, the lack of significant pattern of expression supportive of gene frontloading may have resulted from a reduced power from a low sample size or an insufficient magnitude or duration of prior hypoxia exposure to elicit a lasting gene expression response in hypoxic reef oysters. The ability to make predictions about the potential for oysters to exhibit adaptive plasticity in response to future changes in oxygen availability is limited given the results of the present study. Still, results confirm and highlight many of the major physiological pathways associated with hypoxia response in oysters and reinforce the importance of understanding how the duration and frequency of hypoxia exposure influences physiological response and differential tolerance in oysters.

Introduction

Coastal waters, such as estuaries, are dynamic environments where abiotic conditions can fluctuate on hourly, daily, and seasonal timescales. Coastal environments are also changing at a rapid rate as a result of human influence (Scavia et al. 2002) and there is a great need to understand how inhabitants of these environments will be able to tolerate future environmental stress. Hypoxia, broadly defined as oxygen concentrations less than 2.0 mg l^{-1} (Diaz and Rosenberg 1995), is one such abiotic

stressor that is increasing in frequency throughout the world's oceans and coastal waters (Diaz and Rosenberg 2008). Ocean deoxygenation is driven primarily by the increased flux of nutrients to coastal waters and global increases in water temperature which reduce oxygen solubility and intensify water column stratification (Breitburg et al. 2018). There is increasing appreciation for the importance of understanding organismal response to reduced oxygen availability, especially as it has been recognized as having the most acute physiological consequences of the three major consequences of global climate change in the oceans (warming, acidification, and deoxygenation; Sampaio et al. 2021). However, uncertainty remains in the capacity of organisms to tolerate future low oxygen conditions.

The key to organismal resilience in the face of environmental change will lie in the ability of organisms to match their phenotype to their environment, which can be accomplished through phenotypic plasticity and/or genetic adaptation (Visser 2008). Phenotypic plasticity or acclimation involves within-generation adjustments to organismal physiology or morphology, whereas genetic adaptation occurs across generations, as a result of selection acting against deleterious or mal-adapted phenotypes, resulting in increased organismal fitness. Differential exposure to abiotic stress between conspecifics can result in intraspecific differences in tolerance, either resulting from plastic responses or genetic adaptation to local environmental conditions (Sanford and Kelly 2011). Predicting how organisms might be able to tolerate future environmental change can be achieved through studying how

individuals of the same species are able to thrive in the extremes of current environmental conditions (DeBiasse and Kelly 2016).

Transcriptomic studies – those that examine the total mRNA transcripts produced by an individual – offer a powerful methodological approach to examine both differences in tolerance to environmental stressors among conspecifics, as well as the genetic underpinnings that give rise to the differing tolerances (Gracey 2007). Since variability in gene expression generally underlies phenotypic variation (Wray et al. 2003), transcriptomic studies can be used to understand plastic responses of organisms to environmental stress. By quantifying the expression patterns of genes or modules of co-expressed genes between populations with differing stress tolerance, the relative importance of gene expression plasticity in determining organismal tolerance can be revealed (Rivera et al. 2021).

One mechanism by which an organism may alter their gene expression to increase tolerance to environmental stressors is termed constitutive or transcriptional frontloading (Barshis et al. 2013). Transcriptional frontloading involves the increased constitutive expression of important stress-response genes in individuals with higher tolerance to an environmental stressor, allowing for quicker mobilization of the stress response in variable environments. Transcriptional frontloading can arise as a result of plastic response to environmental stress (Palumbi et al. 2014), but also has been shown to persist across generations (Maynard et al. 2018), indicating that it may reflect an adaptive response to increase stress tolerance. This phenomenon has been

studied primarily with respect to thermal tolerance (e.g., Barshis et al. 2013, Fifer et al. 2021, Gleason and Burton 2015, Mizrahi et al. 2012), but has also been observed in individual oysters with increased tolerance to low salinity (Maynard et al. 2018). Whether transcriptional frontloading is a mechanism of increased organismal tolerance to hypoxia has not been well studied. There is evidence that frontloading may be a mechanism allowing for increased hypoxia tolerance between species (Avivi et al. 1999, Shams et al. 2004), but evidence is lacking for intraspecific comparisons.

Marine bivalves provide an ideal group to study the gene expression response of organisms to low oxygen due to their comparatively high tolerance to hypoxic stress (Vaquer-Sunyer and Duarte 2008) and their sessile life history that differentially exposes individuals to fine scale spatial patterns in stressors (e.g., Altieri 2006). Their high tolerance to low oxygen means that elucidating the molecular responses that allow bivalves to tolerate extreme oxygen conditions can yield insights important for understanding the underlying genetic mechanisms of hypoxia tolerance (Gorr et al. 2010). Additionally, the variability in low oxygen availability creates conditions by which geographically proximal bivalve individuals could exhibit different plastic gene expression responses to increase their stress tolerance, as has been seen in studies of temperature (Li et al. 2018) and salinity tolerance (Eierman and Hare 2016, Maynard et al. 2018).

The general gene expression response of bivalves to hypoxia has been well-characterized (e.g., David et al. 2005, Piontkivska et al. 2011, Giannetto et al. 2015, Nie et al. 2020). For example, hypoxia alters the expression of genes for carbonic anhydrase, glycogen phosphorylase, and heat shock protein 70, associated with respiration, metabolism, and stress response respectively in *Crassostrea gigas* (David et al. 2005). Hypoxia-inducible factors (HIF), which are important transcriptional factors for vertebrate response to low oxygen (Semenza 2001), are upregulated in gill tissues of *C. virginica* when exposed to prolonged hypoxia (Piontkivska et al. 2011). In comparison, intraspecific comparisons of bivalve gene expression in response to hypoxia are rare and the studies to date provide variable results. For example, one study found no difference in gene expression under hypoxia exposure between individuals from two selectively-bred lines of *C. gigas* that differed in their phenotypic response to hypoxia (Sussarellu et al. 2010). Similarly, targeted gene expression analysis of heat shock protein 70 expression in oysters with and without prior exposure to hypoxia showed no clear pattern of expression related to historic hypoxia exposure (Ueda et al. 2009). In contrast, significant differences in gene expression under hypoxic stress were observed for genes related to antioxidant defense, oxygen sensing, stress response, and anaerobic capacity between two populations of the ocean quahog, *Artica islandica*, that differed in their tolerance to hypoxia (Philipp et al. 2012).

The eastern oyster, *Crassostrea virginica*, is a model species for use in studies of comparative transcriptomic responses to low oxygen. *C. virginica* is an ecologically

and economically important bivalve species that has suffered significant declines in abundance as a result of overfishing, habitat destruction, and disease (Rothschild et al. 1994, Jackson et al. 2001, Wilberg et al. 2011). Low oxygen events are a known threat to oyster restoration efforts being undertaken throughout the range of *C. virginica* (Johnson et al. 2009) and identification of genetic markers to low oxygen tolerance is of considerable interest to selective breeding programs of oysters (East Coast Shellfish Breeding Consortium 2018). As a bivalve species living both intertidally and subtidally, as well as in environments experiencing variable oxygen availability (Widdows et al. 1989, Keppel et al. 2016), *C. virginica* provides a useful model for studying intraspecific plasticity in gene expression response to low oxygen.

Results from prior research indicate that *C. virginica* sourced from reefs in the Chesapeake Bay that experience increased frequency of hypoxic exposure throughout the year show some degree of differing physiological response when exposed to low oxygen in the laboratory (*Chapter 2*). Using oysters collected from the same sites, the goal of the present study is to examine the gene expression response of these oysters to understand both the global response of *C. virginica* to low oxygen as well as intraspecific variability in gene expression that may arise as a result of prior hypoxia exposure. Specifically, this study aims to answer the following questions: 1) What are the major genes driving oyster response to hypoxia? 2) When exposed to low oxygen, do oysters from hypoxic and normoxic sites show differing gene expression patterns? 3) Are there differences in constitutive gene expression in oysters from hypoxic and

normoxic sites in the control treatment indicative of transcriptional frontloading (*sensu* Barshis et al. 2013)?

Methods

Site Selection

Oysters were collected from six reefs – three hypoxic reefs and three normoxic reefs – throughout the Maryland portion of the Chesapeake Bay for use in the experiment. The collection sites (Figure 3.1) were selected using long-term dissolved oxygen data from water quality monitoring stations (Maryland Department of Natural Resources Eyes on the Bay) located near known oyster reefs (Tarnowski 2017). Hypoxic sites were characterized by historic annual reductions in bottom water oxygen concentrations below 2.0 mg l^{-1} , while normoxic sites were identified based off data on bottom water oxygen concentrations that infrequently experienced concentrations less than 2.0 mg l^{-1} (Figure 3.2). Between 2013 and 2017, the five years preceding the experiment, $20.4 \pm 9.0\%$ of the monthly dissolved oxygen measurements (average of approximately 3 monthly measurements per year per site) taken at the hypoxic sites were at hypoxic levels, whereas only $1.7 \pm 2.9\%$ of the monthly measurements at the normoxic sites were at or below 2.0 mg l^{-1} (average of <1 monthly measurement per year per site). In addition to their annual dissolved oxygen profiles, the temperature (Figure 3.3) and salinity (Figure 3.4) regimes of sites were assessed to ensure that oysters were experiencing relatively similar water quality conditions.

Oyster Collection and Acclimation

Oysters used in the experiments were collected using a combination of hand-collection by divers and dredging (courtesy of the Maryland Department of Natural Resources). Approximately 100 oysters, all measuring at least 75 mm in length, from each site were collected. Only oysters greater than 75 mm in length were used in the experiments to control for the age of oysters used in experiments since different life stages may show differing responses to hypoxic exposure (e.g., Leiva et al. 2018). After collection, oysters were kept for six months suspended in the water (10.95 ± 2.96 ppt salinity) in 18 mm plastic mesh aquaculture bags at a marina in the South River, Maryland until their use in experimentation. This ensured that oysters were acclimated to the same environmental conditions prior to their use in the experiments. Twelve (12) oysters per site per treatment were used in the experiment.

Experimental Oxygen Treatments

Experimentation was carried out in July 2018. Following their acclimation in the South River, oysters were randomly selected from each of the six source sites for use in experiments. Once in the lab, fouling organisms, such as barnacles and mussels, were removed from the oysters and each oyster was scrubbed with metal brushes to remove any external debris. Oysters were then cleaned with a 10% bleach solution to aid in removal of bacteria and small fouling organisms as this was shown to aid in maintaining better water quality in the experimental tanks (*personal observation*). Shell length and total wet weight were both measured on all oysters, and each oyster was marked with a unique identifier using nail polish. The oysters were fed *ad libitum*

with commercial algal diet (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA) for at least two days in aerated artificial seawater (Instant Ocean®, Spectrum Brands, Blacksburg, VA) at room temperature (approximately 22°C) and a salinity matching the conditions from the dock at the time of the experiment (10 ppt, determined using nearby water quality monitoring station data) prior to the start of experiments.

At the end of the two-day lab acclimation, individual oysters were randomly placed into one of six 10-gallon aquaria filled with artificial seawater at room temperature ($21.8 \pm 0.2^\circ\text{C}$) and the same salinity as the holding tank (10 ppt). Each tank held a total of 24 oysters, four from each of the six sites. Three of the tanks (hereafter ‘control treatments’) were constantly bubbled with compressed air to ensure normoxic conditions in the tank. The remaining three tanks (hereafter ‘low oxygen treatments’) were sparged with compressed nitrogen gas until the dissolved oxygen was reduced to at least 2.0 mg l^{-1} . All dissolved oxygen measurements were carried out using a YSI Model 55 dissolved oxygen reader (Yellow Springs Instruments, Yellow Springs, OH). An air stone bubbling compressed air at a slow rate was also added to each of the low oxygen treatment tanks to compensate for oyster oxygen consumption and prevent oxygen concentrations from dropping into anoxic levels over the course of the experiment. The low oxygen treatment tanks were then sealed with glass lids to minimize oxygen diffusion from the surrounding air. In total, 12 oysters from each of the six sites were exposed to each of the two oxygen treatments, for a total sample size of 144 oysters.

Oysters were exposed to their treatments for a period of 96 hours (four days), with daily monitoring of oxygen concentrations and tank temperatures. Although prior research has shown differences in gene expression of Pacific oysters exposed to hypoxia in as little as one day of exposure (Samain et al. 2007), the present study focused on identifying transcriptomic responses over longer-term exposures, while balancing the need to maintain oyster survival. A 50% water change in each of the tanks was carried out every 48 hours. Oysters were not fed over the course of the experiment in order to prevent algal RNA contamination in proceeding genetic analysis (Eierman and Hare 2014).

At the conclusion of 96 hours, oysters were sacrificed and approximately 0.5 g of gill tissue was dissected from each oyster. Tissue samples were immediately placed in *RNAlater* Solution (Invitrogen, Life Technologies, Carlsbad, CA, United States) and stored at room temperature for at least 48 hours to allow for the solution to penetrate the tissue. Samples were then stored in the freezer at -80°C until subsequent extraction. Wet shell weight and dry tissue weight (assessed by drying the tissue at 55°C for one week) were measured for each oyster.

RNA Library Preparation, Sequencing, and Bioinformatics

Extractions of the total RNA pool were carried out using RNeasy Mini Kits (Qiagen, Valencia, CA, United States) following the manufacturer's protocol. RNA yield was quantified using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, United

States). Following RNA extraction, individual samples from oysters of each site by oxygen treatment group and experimental tank were pooled in equimolar amounts, resulting in 36 individual samples, 6 sites x 2 treatments x 3 replicate pools. Because of some mortality during the experimental exposure, only three oysters were used in each pool. The pools were used in order to reduce the sample size for library prep and sequencing, while allowing for gene expression representation from a greater number of individuals. Pooled samples were treated with DNase I kit (DNA-free™ DNA Removal Kit, Invitrogen, Life Technologies, Carlsbad, CA, United States) following manufacturer's protocols to remove genomic DNA from the samples.

Total, DNA-free RNA was sent to the University of Austin Genomic Sequencing and Analysis Facility for TagSeq library prep (Meyer et al. 2011, Lohman et al. 2016) and sequencing. The cDNA was sequenced on two lanes of an Illumina NovaSeq 6000 using single-end sequencing of 100 base pair read lengths. Resulting reads were then processed for downstream analyses. Trimmomatic (Bolger et al. 2014) was used to remove adapter sequences, trim low-quality reads (<30) from the 3' end of sequences using a sliding window size of five base pairs, and filter out reads shorter than 35 bp. Trimmed reads were aligned to the *C. virginica* genome (Gómez-Chiarri et al. 2015) with STAR aligner (version 2.7.3a, Dobin et al. 2013) using the single pass option and additional parameters necessary for use with RSEM downstream (`--scoreDelOpen -10000, --scoreInsOpen -10000, --alignIntronMax 1, --alignIntronMin 1, --quantTranscriptomeBan IndelSoftclipSingleend`). Gene counts were assembled using RSEM (version 1.2.28, Li and Dewey 2011).

Principle Components Analysis

A principal components analysis (PCA) and permutational multivariate analysis of variance (PERMANOVA) were used to assess global gene expression patterns, looking for clustering of expression based on treatment and source site. The principal components analysis was run on log₂-normalized read counts using the *prcomp* base function in R (RStudio Version 1.3.1073 / R Version 3.6.1). The PERMANOVA was run on log₂-normalized read counts using the *adonis* function from the R package VEGAN (Dixon 2003) based on Euclidean distances and using 10⁶ permutations.

Differential Gene Expression Analysis

Differential gene expression comparisons between treatments and sites were analyzed in two different ways. First, a single-factor negative binomial generalized linear model implemented in the R package DESEQ2 (Love et al. 2014) was used to determine global differential gene expression between the two treatments, ignoring the effect of oyster site, using a false discovery rate (FDR) of 0.05 and a minimum absolute log₂-foldchange (log₂FC) of 0.75 to identify significantly differentially expressed genes. Functional enrichment of gene ontology terms was determined using a Mann-Whitney U-Test (Wright et al. 2015) on signed log-transformed p-values for each of the significantly differentially expressed genes (n=837). Second, differentially expressed genes between sites within each of the two experimental treatments, control and low oxygen, were assessed using pairwise comparisons between each of the sites. Differentially expressed genes were identified using the same threshold FDR and

log₂FC as above. The goal of this analysis was to determine whether certain sites showed different expression profiles in the control treatment that might indicate: 1) differences in constitutive gene expression (“transcriptional frontloading”; Barshis et al. 2013) or 2) variation among sites in gene expression profiles when exposed to low oxygen indicating differential functional responses for hypoxic versus normoxic site oysters.

Weighted Gene Correlation Network Analysis

A weighted gene correlation network analysis (WGCNA) was used to compare the expression patterns of co-expressed gene networks across treatments and sites. The goal of this analysis was to determine whether any gene networks were associated with differential response to treatment between oysters from the different sites. The analysis was run on log₂-normalized gene expression data using the WGCNA package in R (Langfelder and Horvath 2008). Reads were filtered to remove genes with less than five counts in 90% of the samples in order to exclude genes with low counts for the network analysis. WGCNA was run on a total of 9560 gene features using a soft-thresholding value of 4 and a minimum module size of 30 genes. A correlation analysis was run within WGCNA to identify significant correlations between module eigengenes and oyster traits, including site type, site, and treatment. In addition, module eigengene expression values were extracted for each resulting module and analyzed using ANOVAs for the effects of treatment, site, and their interaction on eigengene expression. Functional enrichment of gene ontology (GO) terms for each of the identified modules was determined using a Mann-Whitney U-

Test (Wright et al. 2015) on unsigned eigengene-based module connectivity values (kME) for each gene.

Analysis of Transcriptional Frontloading

Transcriptional frontloading is the increased constitutive expression of stress-response genes in individuals identified as more tolerant to an environmental stressor (Barshis et al. 2013). Frontloaded genes are genes that are significantly differentially expressed under stressful conditions only for individuals deemed more sensitive to the experimental stressor. The lack of differential expression for tolerant individuals is due to the fact that they show higher levels of expression under control conditions in comparison with the sensitive individuals, but similar levels of expression under the stressor (Figure 3.5). In this study, frontloaded genes were identified as genes that responded significantly to low oxygen stress for normoxic site oysters but not hypoxic site oysters and showed higher expression levels in the control treatment for hypoxic site oysters. The goal of the first analysis was to examine the difference in the magnitude of gene response to low oxygen stress, as measured through log₂-foldchange (log₂FC), between site types for uniquely upregulated and downregulated genes. Oysters were first grouped according to the site type from which they had been collected (hypoxic and normoxic). Then, using DESEQ2, a single-factor negative binomial generalized linear model was run to identify differentially expressed genes between the low oxygen treatment and control for each site type independently. An online Venn diagram builder, jvenn (Bardou et al. 2014), was used to identify genes uniquely upregulated or downregulated in each of the two site types. Using this gene

subset, comparisons of log₂FC were made between hypoxic and normoxic sites for each gene identified as being uniquely differentially expressed for each of the site types. A chi-square goodness-of-fit test was run using the *chisq.test* base function in R to determine whether the pattern of log₂FC for each individual gene across the two site types deviated from a 50/50 distribution around a 1:1 line of equality. A lack of deviation from the line of equality would indicate a similar magnitude of response between to the site types for these genes. In contrast, deviations above the line of equality for downregulated genes and below the line for upregulated genes would indicate that those genes were showing a reduced response (i.e., less expression difference) between the two treatments. Increased response of these genes would be indicated by deviations below the line (for downregulated genes) or above the line (for upregulated genes).

The goal of the second analysis was to characterize the pattern of expression between treatments and site types to determine whether oysters from the hypoxic sites – presumably the more tolerant population – were showing increased expression of the reduced response genes in the control treatment, indicative of transcriptional frontloading. To accomplish this, comparisons were made on the log₂-normalized expression of the genes identified as uniquely upregulated or downregulated in each of the two site types. The expression levels were averaged across all genes for each replicate and ANOVAs were used to test for statistically significant differences in mean log₂-normalized gene expression between the different sites, treatments, and their interaction. Where significant differences were detected, a post-hoc Tukey HSD

test was run to determine statistically significant differences between sites within each treatment type. The test of transcriptional frontloading typically only involves genes that are uniquely differentially expressed by sensitive individuals (e.g., Collins et al. 2021, Fifer et al. 2021). In prior research, genes that are uniquely differentially expressed by tolerant individuals lack a detectable difference in expression between treatments for sensitive individuals because those genes show high variability in expression levels in the control treatment (Barshis et al. 2013). In the present study, expression levels of genes uniquely differentially expressed by tolerant (hypoxic site) oysters were also analyzed as above in order to determine whether the lack of differential expression for normoxic site oysters was due to a high variance in expression under the control treatment.

Results

Tank Conditions

Throughout the duration of the experiment, the temperature in the control tanks averaged $21.77 \pm 0.11^\circ\text{C}$ and dissolved oxygen concentrations were maintained at approximately $6.79 \pm 0.43 \text{ mg l}^{-1}$, consistent with normoxic oxygen levels. Low oxygen treatment tanks experienced slightly higher temperatures of $22.51 \pm 0.39^\circ\text{C}$ and hypoxic conditions were maintained throughout, with dissolved oxygen concentrations of $1.52 \pm 0.50 \text{ mg l}^{-1}$. The lower temperatures experienced in the normoxic tanks ($t = -7.04$, $df = 28$, $p < 0.001$) likely was the result of higher evaporative cooling from the bubbling of the tanks.

Sequencing and Mapping Results

Sequencing resulted in a total of 178 million raw reads, with an average of 4.95 million reads per sample (Table 3.1). After trimming, the mean number of reads per sample was 4.83 million. On average, 87.4% of reads mapped to the genome using STAR aligner. However, of the reads that mapped to the genome, a higher-than-expected fraction of them mapped to multiple loci (average uniquely mapped: 51.8%, average multi-mapped: 35.7%) compared to a similar study using TagSeq library prep with *C. virginica* mRNA (approximately 75% unique mapping in Johnson et al. 2020). The large number of multi-mapped reads may be a result of the significant number of duplicated genes identified in the *C. virginica* genome, particularly for genes important for functions related to hypoxia response such as metabolism and immune function (Modak et al. 2021). Regardless, mapping still resulted in approximately 2.5 million uniquely mapped reads per sample, a number sufficient for downstream differential expression analysis (Lohman et al. 2016). The reads mapped to a total of 35,203 gene features.

Principal Components Analysis

Principal components analysis revealed distinct clustering of gene expression related to treatment (Figure 3.6). However, within each treatment, sites, and site types did not show any clear clustering patterns. These patterns were confirmed by the results of the PERMANOVA, where treatment was significant ($p < 0.001$), but the interaction between site and treatment was not significant ($p = 0.63$). While not apparent from

the PCA plot, site was also significant ($p = 0.011$), likely as a result of differing expression across both treatments in oysters from Holland Point (HP; see *Differential Gene Expression* results).

Differential Gene Expression

Analysis of global differential gene expression in response to low oxygen resulted in a total of 837 differentially expressed genes, 497 upregulated and 340 downregulated in low oxygen (top fifty genes displayed in Table 3.2). The top five differentially expressed genes were persulfide dioxygenase ETHE1 ($p_{\text{adj}} = 6.78\text{e-}57$, $\log_2\text{FC} = 3.03$, LOC111122501), kelch-like ECH-associated protein 1 ($p_{\text{adj}} = 3.11\text{e-}37$, $\log_2\text{FC} = 2.05$, LOC111120128), an uncharacterized protein ($p_{\text{adj}} = 7.90\text{e-}29$, $\log_2\text{FC} = 2.50$, LOC111111326), a second uncharacterized protein ($p_{\text{adj}} = 5.03\text{e-}23$, $\log_2\text{FC} = 1.50$, LOC111109404), and egl nine homolog 1-like ($p_{\text{adj}} = 5.51\text{e-}23$, $\log_2\text{FC} = 2.43$, LOC111129345). Other notable differentially expressed genes included hypoxia-inducible factor 1- α ($p_{\text{adj}} = 5.67\text{e-}19$, $\log_2\text{FC} = 0.87$, LOC111129189), peroxiredoxin-6 ($p_{\text{adj}} = 9.28\text{e-}18$, $\log_2\text{FC} = 1.39$, LOC111127513), cytochrome p450 ($p_{\text{adj}} = 3.92\text{e-}15$, $\log_2\text{FC} = 3.11$, LOC111132807), and heat shock cognate 71 ($p_{\text{adj}} = 1.50\text{e-}11$, $\log_2\text{FC} = 0.76$, LOC111127289). A Mann-Whitney U-Test revealed 57 significantly enriched gene ontology (GO) categories (Table 3.3). Upregulated genes were significantly enriched for GO categories including signal transduction, immune system processes, tumor necrosis factor receptor, oxidoreductase activity, and tetrapyrrole binding. Downregulated genes were significantly enriched for GO

categories including nucleic acid metabolic process, fucosidase activity, and ligase activity.

Comparisons of differentially expressed genes between sites within each of the two treatments showed patterns of intraspecific variability in gene expression response to low oxygen. Within the control treatment, there were few differentially expressed genes overall (ranging between 1 and 75) and generally similar expression patterns were observed between sites (Table 3.4). However, one normoxic site, Holland Point (HP), showed overall higher numbers of differentially expressed genes when compared against the other sites, a pattern that was also apparent in the low oxygen treatment (Table 3.5). Within the low oxygen treatment, comparisons of differentially expressed genes among hypoxic sites showed few differentially expressed genes (NA vs. NM: 1, NA vs. RP: 19, NM vs. RP: 5) and therefore generally similar patterns of expression. Normoxic sites tended to have greater numbers of differentially expressed genes (CP vs. HC: 20, CP vs. HP: 43, and HC vs. HP: 185) in pairwise comparisons in the low oxygen treatment compared to the hypoxic sites. However, the larger number of differentially expressed genes between normoxic sites tended to occur in comparisons made with Holland Point.

Weighted Gene Network Correlation Analysis

The weighted gene network analysis identified eight modules of significantly co-expressed genes across the total gene expression dataset, identified according to their color assignment from WGCNA (Figure 3.7). A correlation analysis yielded one

module that was significantly negatively correlated with treatment (Blue Module: $r = -0.93$, $p = 3e-16$) and three modules that were significantly positively correlated with treatment (Midnight Blue: $r = 0.4$, $p = 0.02$, Black: $r = 0.55$, $p = 5e-04$, and Purple: $r = 0.64$, $p = 3e-05$). No modules were found to be significantly correlated with site type.

Results from the eigengene ANOVAs confirmed results from the correlation analysis and provided additional insights into how expression of genes from each of the modules related to oyster source site. Consistent with the correlation analysis, the Black, Blue, Midnight Blue, and Purple modules had statistically significant relationships between eigengene expression and treatment (Table 3.6). In addition, the eigengene ANOVA analysis identified a significant effect of treatment on expression in the Turquoise Module. The Black and Purple modules both had higher expression of the co-expressed genes in the control treatments, while the Blue, Midnight Blue, and Turquoise modules had higher expression in the low oxygen treatment (Figure 3.8).

The two modules with higher expression in the control treatment, Black and Purple modules, showed enrichment for genes relating primarily to metabolism. The Black Module tended to be dominated by genes relating to biosynthesis and metabolic processes, with significant enrichment in GO terms related to nitrogen compound metabolic process, cellular macromolecule metabolic process, and organonitrogen compound biosynthetic process (Table 3.7). The Purple Module had significant

enrichment of genes related to the transcription regulator complex and the regulation of metabolic process.

Three co-expressed gene modules showed higher expression in the low oxygen treatment – Blue, Midnight Blue, and Turquoise modules – and exhibited higher variability in the types of GO categories enriched within each module compared to the Black and Purple modules described above (Table 3.7). The Blue Module showed enrichment for genes relating to oxidoreductase activity and tetrapyrrole binding. The Midnight Blue Module had significant GO terms related to ligase activity and metabolic processes such as cellular nitrogen compound metabolic process and organic cyclic compound metabolic process. The Turquoise Module also showed enrichment for genes relating to metabolic processes, including cellular catabolic process and carbohydrate metabolic process.

While not statistically significant (Table 3.6), the Tan and Turquoise modules both showed patterns of expression that could indicate differences in expression response to low oxygen as a result of prior exposure to hypoxia (Figure 3.8g and 3.8h). In the Tan Module, oysters from hypoxic sites tended to show a lower magnitude response among module genes to the treatment in contrast to the oysters from normoxic sites. This is indicated by the smaller absolute difference in mean eigengene expression in control treatment relative to low oxygen treatment (hypoxic site oysters: 0.018 ± 0.052 , normoxic site oysters: 0.16 ± 0.061). GO enrichment analysis revealed that the Tan Module was enriched for genes related to signal transduction and microtubule-based

processes (Table 3.7). In contrast to the Tan Module, the Turquoise Module showed an opposite pattern of expression, with minimal expression differences between treatments in oysters from normoxic sites (control relative to low oxygen eigengene expression difference: 0.060 ± 0.047), but a trend towards higher expression in the low oxygen treatment for oysters from hypoxic sites (control relative to low oxygen eigengene expression difference: 0.14 ± 0.028). The Turquoise Module was enriched for genes related primarily to catabolism and metabolism (Table 3.7).

Transcriptional Frontloading

An analysis of differential gene expression was carried out on oysters grouped by site type to identify uniquely upregulated or downregulated genes for use in transcriptional frontloading analysis. For hypoxic site oysters, differential gene expression analysis between low oxygen and control treatments yielded 621 differentially expressed genes, of which 376 were upregulated and 245 were downregulated (Figure 3.9). Normoxic site oysters had 854 differentially expressed genes between treatments, 424 of which were upregulated and 430 were downregulated. Comparing across the two site types, 276 of these genes were differentially expressed across both site types, 156 upregulated and 120 downregulated.

Overall, the comparisons of gene expression magnitude between the two site types (normoxic vs. hypoxic) did not show clear evidence of transcriptional frontloading. For genes uniquely differentially expressed by normoxic site oysters, the oysters from

the hypoxic sites showed a reduced response to the low-oxygen treatment, with deviation from a 50/50 distribution about the 1:1 line in both upregulated (n=220, $X^2=216.02$, p-value < $2.2e-16$) and downregulated (n=125, $X^2=125$, p-value < $2.2e-16$) genes (Figure 3.10a). The reduced response would be predicted with transcriptional frontloading as this would indicate that there is a smaller difference in expression between the two treatments for the more tolerant hypoxic site oysters. For genes uniquely upregulated by normoxic site oysters, hypoxic site oysters appeared to show a pattern indicative of transcriptional frontloading, with higher expression levels under control conditions (Figure 3.11b). However, this pattern was not statistically supported, as the mean log₂-normalized expression levels of these genes in the control treatment for hypoxic site oysters did not differ significantly from expression in the normoxic site oysters (Table 3.8, post-hoc results on Figure 3.11a and b). Similar patterns were observed in genes uniquely differentially expressed in oysters from hypoxic sites, with reduced response of these genes in normoxic site oysters (upregulated: n=268, $X^2=268$, p-value < $2.2e-16$ and downregulated: n=310, $X^2=310$, p-value < $2.2e-16$, Figure 3.10b), again indicative of transcriptional frontloading. However, as with the analysis of uniquely expressed genes for normoxic site oyster described above, expression levels of these genes in the control treatment compared between hypoxic and normoxic sites did not differ significantly (Table 3.8, post-hoc results on Figure 3.11c and d). The expected higher level of variance in expression levels for normoxic site oysters in the control treatment was also not observed (Figure 3.11c and d).

Discussion

Hypoxia is a prevalent and increasing environmental stressor in the marine environment (Breitburg et al. 2018) and identification of the physiological mechanisms underlying differential tolerance will improve understanding about the capacity of organisms to acclimate or adapt to changing oxygen conditions. Analysis of gene expression data is one useful way to test hypotheses regarding intraspecific plasticity of tolerance because gene expression serves as a link between an organism's genotype and physiological response to environmental stress. The present study provided novel insights into the potential for gene expression plasticity in response to a low oxygen challenge for oysters from hypoxia-prone reefs vs. reefs where hypoxia rarely occurs. Results revealed global gene expression responses of *C. virginica* to low oxygen exposure that are consistent with results from previous studies of bivalves and supports current knowledge on physiological mechanisms of tolerance to hypoxia. Interestingly, differential gene expression did not indicate any site-level differences in oyster gene expression response to low oxygen as a result of prior hypoxia exposure. However, although not statistically significant, a pattern of gene expression levels consistent with transcriptional frontloading was observed. Gene network analysis identified two modules of co-expressed genes that showed differing patterns of gene expression response to low oxygen challenge between oysters with different histories of hypoxia exposure, although these patterns were not statistically significant. Discussed below are potential reasons why disparate expression patterns were not seen between the examined sites and how greater

frequency or severity of hypoxia exposure may be important in the development of plastic gene expression response to low oxygen in *C. virginica*.

Oyster genetic response to hypoxia reflects generalized bivalve hypoxia response

Regardless of source site, oysters showed a clear difference in gene expression in the low oxygen treatment compared to the control treatment, as demonstrated in the PCA analysis, differential gene expression analysis, and gene network analysis results.

Results from the gene ontology enrichment analysis and characterization of specific differentially expressed genes support findings from prior research on bivalve response to low oxygen. Functional enrichment analysis of differentially expressed genes indicated a downregulation of genes broadly related to metabolism.

Downregulation of key metabolic genes has been shown in prior studies on transcriptomic response to low oxygen in *C. gigas* (David et al. 2005) and is indicative of the lowered metabolic activity in *C. virginica* in response to low oxygen (Shumway and Koehn 1982, Willson and Burnett 2000). The function of certain observed upregulated genes in response to hypoxia were also consistent with the types of genes previously identified in studies of transcriptomic response to hypoxia in bivalves (David et al. 2005, Sussarellu et al. 2010, Nie et al. 2020). For example, similar to the response of the manila clam *Ruditapes philippinarum* to hypoxia, significant upregulation was observed for immune system processes, tumor necrosis factor receptor binding, and oxidoreductase activity (Nie et al. 2020). These findings indicate an increase in immune response, cellular apoptosis, and redox reactions, respectively, consistent with the generalized molecular stress response observed in

oysters (Zhang et al. 2016). Collectively, these gene ontology results indicate that hypoxia exposure leads to decreased metabolic activities in *C. virginica* and increased activation of genes used to mitigate against cellular damage due to environmental stress.

The differentially expressed genes identified in this study also tended to be consistent with genes known to play a role in the generalized bivalve stress response (Miao et al. 2015). Of the top five differentially expressed genes identified based off of the global gene expression response to hypoxia, only three were significantly differentially expressed when compared across all of the oyster source sites. These genes included persulfide dioxygenase ETHE1 (LOC111122501), kelch-like ECH-associated protein (LOC111120128), and an uncharacterized protein (LOC11111326). Persulfide dioxygenase plays an important role in hydrogen sulfide metabolism (Beltowski 2015). Hydrogen sulfide is produced by aerobically respiring bacteria during times of reduced oxygen availability and its presence in synergy with hypoxia has been shown to increase mortality of marine invertebrates (Vaquer-Sunyer and Duarte 2010). Given the ubiquitous upregulation of persulfide dioxygenase in the present study and the fact that it has been shown to be upregulated during reoxygenation following hypoxic stress in the Pacific oyster *Crassostrea gigas* (Sokolov et al. 2019), it likely is a key gene for oyster defense against co-occurring stressors associated with hypoxia.

In addition, the upregulation of kelch-like ECH-associated protein across all sites in response to low oxygen stress likely represents an important response of oysters for protection against oxidative damage from low oxygen exposure. Kelch-like ECH-associated protein (Keap1) is a key sensor of oxidative stress and, coupled with the Nrf2 transcription factor, plays an important role in transcribing proteins important in preventing antioxidant damage to cells (Baird and Dinkova-Kostova 2011). Hypoxia exposure commonly increases the production of reactive oxygen species in mitochondria, which can both activate important genes for hypoxia response as well as oxidize proteins and DNA, leading to cellular damage (Guzy and Schumacker 2006). Thus, the upregulation of Keap1 likely represents a response of oysters to reducing the effects of oxidative stress associated with hypoxia.

A key aspect of hypoxia response in both vertebrates and invertebrates is the activation of the hypoxia inducible transcription factor (HIF), which is a regulator of genes important to hypoxia stress response (Semenza 2001). Increased expression of HIF under low oxygen conditions has been shown in *C. virginica* (Piontkivska et al. 2011), *C. gigas* (Kawabe and Tokoyama 2012), and other bivalve species, such as mussels (Giannetto et al. 2015). In the present study, hypoxia-inducible factor 1- α (HIF 1- α) was significantly upregulated after four days of low oxygen exposure.

Previous studies of HIF 1- α expression in other bivalves have shown upregulation of HIF 1- α after one to two days of exposure to hypoxia, followed at subsequent timepoints by a decrease in expression to levels equivalent to normoxic exposure (Giannetto et al. 2015, Kawabe and Tokoyama 2012). In *C. virginica*, expression

levels of HIF 1- α in anoxia were indistinguishable from normoxic conditions after six days of exposure, although a two-week exposure to hypoxia showed significant increase in expression (Piontkivska et al. 2011). These studies indicate that the expression of HIF 1- α varies across time during low oxygen exposure and the present study indicates that four days of exposure represents a time point at which HIF 1- α expression is elevated from control conditions.

Both HIF 1- α isoforms found in the *C. virginica* genome (LOC111129189 and LOC111128831) had high module membership values in the Blue Module, which showed higher eigengene expression in the low oxygen treatment (Figure 3.8b). Overall, the genes within the Blue Module likely represent key upregulated genes for oyster hypoxia response. Many genes with high module membership in the Blue Module are regulated by the HIF pathway and some are involved in the promotion of anaerobic metabolism, such as pyruvate dehydrogenase kinase (PDK-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Greer et al. 2012). The activation of anaerobic metabolism by *C. virginica* is a well-understood response to low oxygen stress (Larade and Storey 2002), which is likely reflected in the upregulation of these genes in response to low oxygen.

Heat shock proteins (Hsp), molecular chaperones which aid in maintaining the structural integrity of proteins upon exposure to abiotic stressors, are another class of proteins that have been shown to play a key role in organismal stress response (see reviews by Feder and Hofmann 1999, Dahlhoff 2004). With respect to *C. virginica*,

Hsp70 has been implicated as a biomarker of low oxygen stress given its pronounced upregulation during low oxygen exposure (Patterson et al. 2014). Oysters from the present study support this notion, with one Hsp70 isoform (LOC111108251) showing significant upregulation in the low oxygen treatment according to differential gene expression analysis. Reiterating the importance of this Hsp70 isoform to oyster response to low oxygen, it also showed strong module membership in the Purple Module, which was significantly correlated with treatment. Interestingly, a second Hsp70 isoform (LOC111121313) was associated with the Turquoise Module, which trended toward higher eigengene expression in the low oxygen treatment for oysters from hypoxic sites and similar expression across treatments for the normoxic sites (Figure 3.8h). While this represents a trend and not a significant difference, it is possible that this particular isoform may play a role in a differing response to hypoxia between the two site types and is worth further investigation.

Overall, gene ontology analysis and patterns of individual gene expression in the present study captures much of the known physiological response of oysters to low oxygen stress and provides insights into genes potentially useful for oyster monitoring. Gene expression analysis indicated decreases in metabolic activity and increases in immune activities and generalized cellular stress response.

Transcriptomics thus provides a powerful means to characterize the generalized physiological response of oysters to low oxygen. Additionally, some of the specific genes identified as differentially expressed, such as Hsp70 and HIF, are potentially important biomarkers of hypoxic stress (Patterson et al. 2014), which could be used

as a means to assess the health of oyster reefs or identify areas suitable for oyster restoration (Sanders 1993, Bierkens 2000).

Evidence for differing intraspecific response

Gene network analyses allow for the clustering of co-regulated genes which can be used to analyze patterns of expression for sets of genes, as opposed to differential gene expression analysis that focuses on individual gene responses. Since phenotypes typically result from the combined expression of many genes (Civelek and Lusi 2014), analysis of patterns of expression of co-expressed gene networks can provide greater insight into the physiological outcome of gene expression. Additionally, collective expression patterns within gene network modules can be used to correlate gene network response with experimental variables.

In this study, gene network analysis was used to compare the plastic response of co-regulated genes to low oxygen for oysters from sites with differing prior exposure to hypoxia. Results from the gene network analysis provide some insights into potential patterns of differing gene expression between oysters from hypoxic and normoxic sites. For oysters from the normoxic sites, a decrease in expression in the low oxygen treatment of the Tan Module's (Figure 3.8g) co-expressed genes and similar patterns of expression between treatments in the hypoxic oysters indicate a potential difference in response due to prior oxygen exposure. Genes within this module include those associated with microtubule- and cytoskeleton-based processes, which play an important role in hypoxia stress response for intertidal organisms (Clark et al.

2018) and are a critical component in HIF mobilization during hypoxia exposure (Parker et al. 2014). The trend toward reduced expression of genes related to microtubule processes in the normoxic site oysters upon exposure to low oxygen is therefore surprising. Additionally, the increased expression of Turquoise Module (Figure 3.8h) genes (including the Hsp70 isoform described above) in low oxygen conditions for the hypoxic site oysters may indicate other means by which the two site types differ in their response to low oxygen stress. This module was functionally enriched for genes relating to protein and macromolecule catabolic processes, indicating that oysters from hypoxic sites may trend towards higher levels of metabolism under low oxygen conditions. For both the Tan and Turquoise modules, the lack of a significant interaction term may simply be a function of low sample size – only three replicates per site – with inter-individual noise preventing a statistically significant difference from being detected. However, the patterns described above do indicate a potential for differences in gene expression among oysters with differing histories of low oxygen exposure. Future research targeting these genes may provide insights into genes of potential importance for increased tolerance to low oxygen in *C. virginica*, which may be beneficial in selectively breeding more hypoxia-tolerant oysters.

While statistically significant differences in expression were not observed between hypoxic and normoxic sites through the gene network or differential gene expression analyses, other intraspecific differences in expression did exist between individual sites. Holland Point, one of the normoxic sites, showed a high number of

differentially expressed genes in both the control and low oxygen treatments (Tables 3.3 and 3.4), indicating an overall different expression profile both under baseline and stressful conditions. While it's possible that the difference was due to a different response of oysters from Holland Point to controlled experimental conditions – either the experimental salinity or the lack of food availability was stressful – it remains possible that this difference in gene expression could reflect population genetic differences between Holland Point and the other sites examined in this study. Two features of the Holland Point reef indicate that this could possibly be the case. First, Holland Point's upstream location has the potential to limit gene flow from other oyster reefs. Holland Point is located approximately 19 kilometers upstream from the next nearest collection site, Neal Addition (Figure 3.1). This distance is within the mean estimated oyster larval dispersal distance in the Patuxent River (20 km) according to larval dispersal models (North et al. 2008) and falls within the estimated larval dispersal area (429 km²) assessed by genetic differentiation of oysters in the Chesapeake Bay (Rose et al. 2006). However, given its upstream location, gene flow between the other study sites and Holland Point is likely limited, as models also predict upstream oyster dispersal to be limited by hydrography (North et al. 2010), therefore potentially leading to greater genetic isolation of Holland Point oysters compared to the other sites. Second, Holland Point oysters experience an overall lower salinity than the other collection sites (Figure 3.4), and may represent a marginal habitat to and from which dispersal may be limited. Parental salinity exposure affects survival of oyster larvae (Eierman and Hare 2013), potentially leading to isolation of populations existing at the lower extremes of tolerance. Thus,

if oysters from Holland Point have a lower tolerance to higher salinities, this may lead to limited dispersal to other reefs and genetic isolation. Further investigation of population genetic differentiation between oysters from the different source sites used in this study would provide greater insight into the potential for genetic isolation in the Holland Point oysters that may have led to the differences in gene expression.

Insights on hypoxia-induced transcriptional frontloading

Transcriptional frontloading has been identified as one mechanism of increased tolerance to stressful environmental conditions. A reduced gene expression response between treatments was observed in genes uniquely differentially expressed for each of the two site types, consistent with transcriptional frontloading. Additionally, for genes uniquely upregulated by normoxic site oysters, hypoxic site oysters trended towards higher constitutive gene expression levels. However, this apparent difference was not statistically significant, perhaps owing to the small sample size (three replicates per site) used in the study.

A great deal of research relating to transcriptional frontloading has centered on examination of plastic gene expression responses of thermally-tolerant and thermally-sensitive individuals to thermal challenge (e.g. Barshis et al. 2013, Fifer et al. 2021, Gleason and Burton 2015, Mizrahi et al. 2012), but there is a paucity of knowledge as to whether similar patterns of expression exist for individuals with differing tolerance to variable oxygen conditions. Current evidence for transcriptional frontloading of genes conferring increased tolerance to low oxygen is limited to interspecific

comparisons of vertebrates (Avivi et al. 1999, Shams et al. 2004), indicating that frontloading of genes for increased tolerance to hypoxia can occur as a result of longer-term evolutionary differentiation. However, evidence is lacking as to whether plasticity could also lead to gene frontloading for hypoxia tolerance.

One possible reason for the lack of observed transcriptional frontloading in the present study could be that the frequency or severity of prior hypoxia exposure in these oysters was not sufficient to elicit a sustained plastic response. Transcriptional frontloading appears to occur both as a plastic response after a one-year acclimation to a stress (Palumbi et al. 2014) as well as a sustained adaptive response to stress over generations (e.g., Maynard et al. 2018, Avivi et al. 1999, Shams et al. 2004).

However, the threshold of stress exposure that led to these changes, the durability of the effect following the stress exposure, and the underlying mechanism activating frontloaded genes are not well understood. While the hypoxic sites from this study vary in their oxygen availability, the periodic summertime exposure to low oxygen for the oysters from the hypoxic sites may not have been sufficiently stressful to elicit long-term increased transcription of stress-related genes. Furthermore, since the durability of a plastic frontloading response following a stressor exposure is not well characterized, it is unknown whether the approximately twelve months between the oyster's last exposure to hypoxia in the field and the experimental exposure could have attenuated the frontloading response. It is theorized that constitutive frontloading would be more evolutionarily beneficial in highly variable environments where the stress exposure occurs on frequent time scales that may preclude the

physiological response needed to respond (Rivera et al. 2021). For example, thermally-tolerant corals that have been shown to frontload genes experience daily fluctuations of temperature up to 6°C (Barshis et al. 2013), necessitating the need for easy mobilization of genes required for thermal stress response. In the case of the oysters from the present study, the exposure to the hypoxic stressor is far less frequent, occurring periodically during the summer and likely for short durations of 7-14 days (Lenihan and Peterson 1998, Sagasti et al. 2000, Neff et al. 2020). Upregulation of genes related to the stress response, such as Hsp70, does come at an energetic cost (Sørensen and Loeschcke 2006) and may even be harmful when expressed constitutively (Krebs and Feder 1997). Therefore, the sustained upregulation of genes necessary for the response to infrequent exposure to hypoxia may be too energetically costly and not evolutionarily favored in the oysters from this study. It is possible, however, that the frontloading response could exist with the oysters from the present study if the laboratory exposure to hypoxia had occurred temporally closer to the last field exposure.

It is plausible that oysters from environments that are exposed more frequently and predictably to low oxygen availability, such as intertidal habitats or areas with diel-cycling hypoxia, may experience transcriptional frontloading. Frontloading of genes involved in the response of organisms to oxidative stress have been shown in limpets transplanted from intertidal environments, with fluctuating oxygen availability, to subtidal habitats (Clark et al. 2018). Of course, intertidal limpets would have to deal with other stressors in the intertidal, namely thermal and osmotic stress, so whether

the upregulation of these particular genes reflects a response to low oxygen availability or one of these other co-stressors is unknown. However, the ability to rapidly mobilize a stress response in the hypoxia-prone intertidal environment may represent an important tradeoff to the energetic costliness associated with the constant expression of stress-response genes.

It also remains possible that transcriptional frontloading is not a mechanism of increased tolerance to low oxygen stress in oysters. Outside of the present study, no studies have explicitly measured whether prior exposure to hypoxia increases constitutive expression of genes important in hypoxia response within an intraspecific context (although interspecific observations have been made; see Avivi et al. 1999, Shams et al. 2004). However, it seems plausible that transcriptional frontloading could contribute to increased hypoxia tolerance for a few reasons. It is known that prior thermal stress exposure increases constitutive gene expression leading to increased cross-tolerance with hypoxia (Collins et al. 2020), thus frontloading the expression of stress-response genes does confer an advantage with respect to hypoxic response. In addition, given the acuteness of hypoxic exposure to normal organismal aerobic functioning (Larade and Storey 2002), the ability to mobilize a rapid response to low oxygen tension, as has been seen with respect to thermal challenge, would likely be energetically favorable. Additional studies are worth pursuing, particularly using organisms with a greater frequency of exposure to hypoxia, such as intertidal organisms or those experiencing diel-cycling hypoxia, to see if transcriptional frontloading does indeed play a role in increased tolerance to hypoxia.

Conclusions

Results from differential gene expression and gene network analyses indicate that *C. virginica* undergoes a distinct physiological response to hypoxia, which is consistent with prior studies of gene expression responses to low oxygen stress in other bivalve species. However, the apparent lack of statistically significant patterns of gene expression in relation to the oyster's prior hypoxia exposure indicates that short-term reductions in oxygen occurring on an annual basis may not be sufficient to elicit differing plastic gene expression responses to low oxygen. The ability to make predictions about whether *C. virginica* will be able to undergo adaptive plasticity to cope with changing oxygen availability in future climate scenarios is limited given the results of the present study. However, the lack of differing response observed in the present study does not necessarily indicate that oysters lack the capacity to respond to future oxygen conditions. Instead, it would be predicted that plasticity would be favored with the increase in frequency and severity of low oxygen conditions that are expected to occur with global climate change. Further research into how more frequent or sustained hypoxia exposure would affect the plastic responses of oysters to low oxygen may provide interesting insights into the ability of oysters to tolerate these future low oxygen conditions.

Table 3.1: Summary of raw read counts and mapping statistics.

Site	Treatment	Pool	Raw Reads	Trimmed Reads	Unique Mapped Reads	Multi-Mapped Reads
Cook Point	Low Oxygen	1	4529613	4419493	2191560	1671581
Cook Point	Low Oxygen	2	4450048	4343031	2167128	1652963
Cook Point	Low Oxygen	3	4430435	4328036	2204457	1598684
Cook Point	Control	1	4253552	4152665	2248714	1382313
Cook Point	Control	2	4826052	4713027	2507368	1681178
Cook Point	Control	3	4952414	4838282	2608624	1623096
Harris Creek	Low Oxygen	1	4790806	4664423	2272965	1817917
Harris Creek	Low Oxygen	2	5825156	5672250	2811700	2134688
Harris Creek	Low Oxygen	3	4394291	4279544	2299795	1451798
Harris Creek	Control	1	5143524	5009253	2677275	1749638
Harris Creek	Control	2	5025724	4895933	2703974	1601643
Harris Creek	Control	3	5261959	5126829	2804491	1745258
Holland Point	Low Oxygen	1	4525701	4409516	2095756	1741018
Holland Point	Low Oxygen	2	4909981	4784179	2406284	1679100
Holland Point	Low Oxygen	3	4569542	4454140	2199649	1757963
Holland Point	Control	1	4666963	4550088	2476515	1452421
Holland Point	Control	2	5566186	5430954	3017641	1729189
Holland Point	Control	3	4906530	4786436	2579377	1639451
Neal Addition	Low Oxygen	1	4989941	4860619	2312053	1912147
Neal Addition	Low Oxygen	2	5071229	4938354	2476533	1834462
Neal Addition	Low Oxygen	3	4685147	4567619	2418121	1587387
Neal Addition	Control	1	4848387	4723921	2551757	1613463
Neal Addition	Control	2	5886769	5740874	2963169	2047335
Neal Addition	Control	3	3883933	3787159	2113565	1194311
NW Middleground	Low Oxygen	1	6558898	6382798	2881625	2734667
NW Middleground	Low Oxygen	2	4215986	4094534	2048934	1521843
NW Middleground	Low Oxygen	3	4198082	4085243	2107254	1476534
NW Middleground	Control	1	4504654	4380122	2376418	1471695
NW Middleground	Control	2	5036169	4900385	2629863	1664753
NW Middleground	Control	3	5347046	5210192	2767270	1781208
Ragged Point	Low Oxygen	1	5226683	5080590	2390030	2002207
Ragged Point	Low Oxygen	2	5153946	5006372	2507419	1824112
Ragged Point	Low Oxygen	3	5835664	5678558	2762300	2154524
Ragged Point	Control	1	4563381	4434633	2323834	1530919
Ragged Point	Control	2	5699961	5544383	2933033	1903532
Ragged Point	Control	3	5766685	5609555	3067976	1752618

Table 3.2: Top fifty differentially expressed genes in low oxygen treatment relative to control.

Description	Gene ID	Log2FC	Adj. p-value
persulfide dioxygenase ETHE1, mitochondrial-like	LOC111122501	3.03	6.78E-57
kelch-like ECH-associated protein 1	LOC111120128	2.05	3.11E-37
uncharacterized protein	LOC111111326	2.50	7.90E-29
uncharacterized protein	LOC111109404	1.50	5.03E-23
egl nine homolog 1-like	LOC111129345	2.43	5.51E-23
uncharacterized protein	LOC111100512	1.77	3.67E-21
V-type proton ATPase subunit d 1-like	LOC111135828	-1.02	3.28E-20
probable thiopurine S-methyltransferase	LOC111122055	1.32	2.75E-19
sodium bicarbonate cotransporter 3-like	LOC111126568	0.75	2.75E-19
vitamin D3 hydroxylase-associated protein-like	LOC111115095	1.34	5.67E-19
hypoxia-inducible factor 1-alpha-like	LOC111129189	0.87	5.67E-19
V-type proton ATPase subunit e 2-like	LOC111137078	-0.65	5.67E-19
peroxiredoxin-6-like	LOC111127513	1.39	9.28E-18
selenoprotein N-like	LOC111129882	1.18	2.67E-17
uncharacterized protein	LOC111101449	2.88	4.80E-17
uncharacterized protein	LOC111109847	0.88	5.20E-17
major surface-labeled trophozoite antigen 417-like	LOC111137244	-1.26	1.38E-16
eukaryotic translation initiation factor 4E-binding protein 1-like	LOC111121086	-1.14	9.36E-16
alternative oxidase, mitochondrial-like	LOC111119097	2.13	1.92E-15
alpha-N-acetylgalactosaminidase-like	LOC111125097	-1.01	2.23E-15
spermine oxidase-like	LOC111134260	1.31	2.64E-15
cytochrome P450 2B4-like	LOC111132807	3.11	3.92E-15
ileal sodium/bile acid cotransporter-like	LOC111134859	1.07	5.83E-15
ATP-dependent RNA helicase DDX24	LOC111121781	-0.69	2.22E-14
glutamine synthetase-like	LOC111126492	-1.25	3.36E-14
putative serine protease K12H4.7	LOC111099446	-0.92	3.75E-14
uncharacterized protein	LOC111100513	1.63	2.59E-13
uncharacterized protein	LOC111129339	0.79	4.07E-13
uncharacterized protein	LOC111111183	1.34	5.27E-12
alpha-N-acetylglucosaminidase-like	LOC111119282	-0.96	8.13E-12
heat shock cognate 71 kDa	LOC111127289	0.76	1.50E-11
steroid 17-alpha-hydroxylase/17,20 lyase-like	LOC111135711	1.70	2.54E-11
gamma-interferon-inducible lysosomal thiol reductase-like	LOC111130501	-1.20	6.47E-11
uncharacterized protein	LOC111106775	1.01	6.78E-11
uncharacterized protein	LOC111108503	1.71	6.78E-11
WD repeat-containing protein 82-like	LOC111129838	-1.00	7.59E-11
type 2 phosphatidylinositol 4,5-bisphosphate 4-phosphatase-like	LOC111136180	-0.70	7.59E-11
uncharacterized protein	LOC111099928	0.88	8.00E-11
uncharacterized protein	LOC111110612	1.85	1.04E-10
dipeptidyl peptidase 1-like	LOC111107171	-0.91	1.16E-10
uncharacterized protein	LOC111132398	2.02	1.95E-10
uncharacterized protein	LOC111117850	1.60	2.69E-10
beta-hexosaminidase subunit beta-like	LOC111131382	-0.99	3.61E-10
kielin/chordin-like protein	LOC111116042	-0.70	5.42E-10
uncharacterized protein	LOC111099114	-0.91	1.49E-09
solute carrier family 23 member 1-like	LOC111130673	1.13	1.49E-09
putative aminopeptidase W07G4.4	LOC111101605	-0.81	1.93E-09
uncharacterized protein	LOC111100448	-1.78	1.98E-09
sphingomyelin phosphodiesterase-like	LOC111132893	-1.18	2.22E-09
solute carrier family 13 member 5-like	LOC111133119	1.08	2.34E-09

Table 3.3: Significantly enriched GO terms for the significantly differentially expressed genes identified between the control and low oxygen treatments.

GO Category	# of Seqs	GO Term	Name	Adj. p-value
<i>Downregulated</i>				
BP	455	GO:0090304	nucleic acid metabolic process	<0.001
BP	292	GO:0016070	RNA metabolic process	<0.001
BP	817	GO:0006139 GO:0006725 GO:0046483 GO:1901360	organic cyclic compound metabolic process	<0.001
BP	118	GO:0006399 GO:0034660	ncRNA metabolic process	<0.001
BP	941	GO:0034641	cellular nitrogen compound metabolic process	<0.001
MF	17	GO:0004560 GO:0015928	fucosidase activity	<0.001
MF	960	GO:0003676	nucleic acid binding	<0.001
MF	90	GO:0016874	ligase activity	<0.001
BP	155	GO:0006396	RNA processing	<0.001
BP	73	GO:0034470	ncRNA processing	<0.001
MF	66	GO:0140101	catalytic activity, acting on a tRNA	<0.001
MF	96	GO:0004553 GO:0016798	hydrolase activity, acting on glycosyl bonds	<0.001
MF	9	GO:0046961 GO:0009678 GO:0044769	ATPase activity, coupled to transmembrane movement of ions, rotational mechanism	<0.001
MF	1102	GO:0016787	hydrolase activity	<0.01
BP	22	GO:0006364 GO:0016072	rRNA metabolic process	<0.01
BP	14	GO:0006119	oxidative phosphorylation	<0.01
BP	43	GO:0006418 GO:0043039 GO:0043038	tRNA aminoacylation for protein translation	0.0105
BP	47	GO:0009451	RNA modification	0.0105
CC	5	GO:0033179	proton-transporting V-type ATPase, V0 domain	0.0192
CC	5	GO:0033180	proton-transporting V-type ATPase, V1 domain	0.0192
MF	47	GO:0004812 GO:0016875	ligase activity, forming carbon-oxygen bonds	0.0215
MF	18	GO:0015035 GO:0015036	disulfide oxidoreductase activity	0.0215
MF	154	GO:0140098	catalytic activity, acting on RNA	0.0216
BP	5	GO:0007041	lysosomal transport	0.0307

MF	26	GO:0016879	ligase activity, forming carbon-nitrogen bonds	0.0332
BP	7	GO:0007034	vacuolar transport	0.0336
MF	188	GO:0003924	GTPase activity	0.0436
MF	15	GO:0019829 GO:0042625	ATPase-coupled ion transmembrane transporter activity	0.0436
MF	5	GO:0004356 GO:0016211 GO:0016880	glutamate-ammonia ligase activity	0.0454
MF	381	GO:0003677	DNA binding	0.0460
<i>Upregulated</i>				
BP	798	GO:0007165	signal transduction	<0.001
BP	365	GO:0007186	G protein-coupled receptor signaling pathway	<0.001
MF	122	GO:0020037 GO:0046906	tetrapyrrole binding	<0.001
MF	336	GO:0004930	G protein-coupled receptor activity	<0.001
MF	498	GO:0004888 GO:0038023 GO:0060089	signaling receptor activity	<0.001
MF	121	GO:0005506	iron ion binding	<0.001
MF	44	GO:0004713	protein tyrosine kinase activity	<0.01
BP	58	GO:0002376	immune system process	<0.01
MF	84	GO:0005102	signaling receptor binding	<0.01
BP	85	GO:0015672	monovalent inorganic cation transport	<0.01
MF	5	GO:0016174 GO:0050664	oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor	0.0199
MF	37	GO:0003707	steroid hormone receptor activity	0.0215
MF	450	GO:0004672 GO:0016301 GO:0016773	kinase activity	0.0216
CC	26	GO:0005856	cytoskeleton	0.0249
MF	28	GO:0005164 GO:0032813 GO:0005126	tumor necrosis factor receptor superfamily binding	0.0284
BP	99	GO:0009966 GO:0010646 GO:0023051 GO:0048583	regulation of response to stimulus	0.0291
MF	6	GO:0003756 GO:0016864	intramolecular oxidoreductase activity, transposing S-S bonds	0.0312
MF	230	GO:0098772	molecular function regulator	0.0322
CC	113	GO:0005576	extracellular region	0.0351
MF	15	GO:0030374	nuclear receptor coactivator activity	0.0369
MF	55	GO:0003779	actin binding	0.0410
MF	142	GO:0046873	metal ion transmembrane transporter activity	0.0411
MF	39	GO:0004601 GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	0.0436

BP	6	GO:0001819 GO:0051240	positive regulation of cytokine production	0.0437
BP	90	GO:0007187	G protein-coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	0.0442
MF	42	GO:0005096 GO:0008047	enzyme activator activity	0.0460
MF	7	GO:0051015	actin filament binding	0.0460

Table 3.4: Matrix of differentially expressed genes (total, upregulated, downregulated) from pairwise comparisons of expression between sites in the control treatment. Blue cells correspond to comparisons between normoxic sites, orange cells correspond to comparisons between hypoxic sites, and light grey cells correspond to comparisons across site types. Upregulation versus downregulation of genes determined in reference to sites listed in row. Site abbreviations as specified in Figure 3.1.

	CP	HC	HP	NA	NM	RP
CP		7, +3, -4	60, +20, -40	4, +0, -4	1, +0, -1	6, +2, -4
HC			65, +33, -32	14, +8, -6	13, +9, -4	2, +1, -1
HP				17, +13, -4	14, +9, -5	75, +50, -25
NA					1, +0, -1	6, +4, -2
NM						6, +5, -1
RP						

Table 3.5: Matrix of differentially expressed genes (total, upregulated, downregulated) from pairwise comparisons of expression between sites in the low oxygen treatment. Blue cells correspond to comparisons between normoxic sites, orange cells correspond to comparisons between hypoxic sites, and light grey cells correspond to comparisons across site types. Upregulation versus downregulation of genes determined in reference to sites listed in row. Site abbreviations as specified in Figure 3.1.

	CP	HC	HP	NA	NM	RP
CP		20, +9, -11	43, +14, -29	2, +1, -1	4, +2, -2	40, +10, -30
HC			185, +71, -114	7, +4, -3	35, +15, -20	20, +4, -16
HP				63, +49, -14	51, +41, -10	42, +18, -24
NA					1, +0, -1	19, +1, -18
NM						5, +2, -3
RP						

Table 3.6: Table of p-values from ANOVAs on effect of site, treatment, and their interaction on eigengene expression for each of the eight identified modules. Asterisks denote statistically significant terms from model.

Module	Site	Treatment	Site x Treatment
Black	0.167	0.000881*	0.963
Blue	0.239	<0.001*	0.218
Green	0.578	0.112	0.731
Midnight Blue	0.160	0.00898*	0.113
Purple	<0.001*	<0.001*	0.0523
Salmon	0.00733*	0.570	0.830
Tan	0.495	0.244	0.716
Turquoise	<0.001*	0.0186*	0.886

Table 3.7: Significantly enriched GO terms for the eight modules identified by the network analysis.

Module	Category	# Seqs	GO Term	Name	Adj. p-value
Black	MF	14	GO:0016651	oxidoreductase activity, acting on NAD(P)H	0.05
Black	BP	87	GO:0006812	cation transport	<0.01
Black	BP	24	GO:0009152 GO:0006164 GO:0009260 GO:0072522 GO:0046390	purine-containing compound biosynthetic process	<0.01
Black	BP	11	GO:0009206 GO:0009145 GO:0009201 GO:0009205 GO:0009142 GO:0009144 GO:0009199 GO:0009141	purine nucleoside triphosphate biosynthetic process	<0.01
Black	BP	40	GO:0015672	monovalent inorganic cation transport	<0.01
Black	BP	9	GO:0015986 GO:0006754 GO:0015985 GO:1902600 GO:0098662 GO:0098655 GO:0098660	inorganic cation transmembrane transport	<0.01
Black	BP	19	GO:0034220 GO:0055085	transmembrane transport	0.017
Black	BP	440	GO:0034641	cellular nitrogen compound metabolic process	<0.01
Black	BP	165	GO:0034645 GO:0009059	macromolecule biosynthetic process	<0.01
Black	BP	149	GO:0043603 GO:0006518	cellular amide metabolic process	<0.01
Black	BP	115	GO:0043604 GO:0006412 GO:0043043	amide biosynthetic process	<0.01
Black	BP	318	GO:0044249 GO:1901576 GO:0009058	biosynthetic process	<0.01
Black	BP	521	GO:0044267 GO:0019538 GO:0044260	cellular macromolecule metabolic process	<0.01
Black	BP	228	GO:0044271 GO:1901566	organonitrogen compound biosynthetic process	<0.01
Black	BP	31	GO:1901137	carbohydrate derivative biosynthetic process	0.011
Black	BP	36	GO:1901293 GO:0090407 GO:0009165	organophosphate biosynthetic process	<0.01

Black	CC	135	GO:0005840	ribosome	<0.01
Black	CC	181	GO:0043232 GO:0043228	intracellular non-membrane- bounded organelle	<0.01
Blue	MF	165	GO:0016491	oxidoreductase activity	0.012
Blue	MF	51	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.025
Blue	MF	19	GO:0016779	nucleotidyltransferase activity	0.017
Blue	MF	55	GO:0020037 GO:0046906	tetrapyrrole binding	<0.01
Blue	CC	5	GO:0033177	proton-transporting two-sector ATPase complex, proton- transporting domain	<0.01
Green	MF	5	GO:0005244 GO:0022832	voltage-gated ion channel activity	0.025
Green	MF	11	GO:0005261	cation channel activity	0.05
Green	MF	131	GO:0022857 GO:0005215	transmembrane transporter activity	0.033
Green	BP	32	GO:0006022	aminoglycan metabolic process	<0.01
Green	BP	18	GO:0006030	chitin metabolic process	<0.01
Green	BP	25	GO:1901071 GO:0006040	amino sugar metabolic process	<0.01
Green	BP	21	GO:0015698	inorganic anion transport	0.044
Green	BP	165	GO:0034645 GO:0009059	macromolecule biosynthetic process	<0.01
Green	BP	149	GO:0043603 GO:0006518	cellular amide metabolic process	<0.01
Green	BP	115	GO:0043604 GO:0006412 GO:0043043	amide biosynthetic process	<0.01
Green	BP	94	GO:1901135	carbohydrate derivative metabolic process	<0.01
Green	CC	22	GO:0005576	extracellular region	0.037
Green	CC	135	GO:0005840	ribosome	<0.01
Green	CC	12	GO:0016459	myosin complex	0.033
Green	CC	181	GO:0043232 GO:0043228	intracellular non-membrane- bounded organelle	<0.01
Midnight Blue	MF	18	GO:0004812 GO:0016875 GO:0016874	ligase activity	<0.01
Midnight Blue	MF	416	GO:0005524 GO:0032559 GO:0030554	adenyl ribonucleotide binding	0.05
Midnight Blue	MF	49	GO:0140098	catalytic activity, acting on RNA	<0.01
Midnight Blue	MF	19	GO:0140101	catalytic activity, acting on a tRNA	<0.01
Midnight Blue	BP	330	GO:0006139 GO:0006725 GO:0046483 GO:1901360	organic cyclic compound metabolic process	<0.01

Midnight Blue	BP	37	GO:0006418 GO:0043039 GO:0006399 GO:0043038	tRNA metabolic process	<0.01
Midnight Blue	BP	11	GO:0006522 GO:0009078	alanine metabolic process	<0.01
Midnight Blue	BP	117	GO:0016070	RNA metabolic process	<0.01
Midnight Blue	BP	207	GO:0019752 GO:0043436 GO:0006082	carboxylic acid metabolic process	<0.01
Midnight Blue	BP	440	GO:0034641	cellular nitrogen compound metabolic process	<0.01
Midnight Blue	BP	49	GO:0034660	ncRNA metabolic process	<0.01
Midnight Blue	BP	339	GO:0044281	small molecule metabolic process	<0.01
Midnight Blue	BP	161	GO:0090304	nucleic acid metabolic process	<0.01
Midnight Blue	BP	163	GO:1901605 GO:0006520	cellular amino acid metabolic process	<0.01
Midnight Blue	CC	62	GO:0005737	cytoplasm	<0.01
Purple	MF	48	GO:0038023 GO:0060089	signaling receptor activity	<0.01
Purple	BP	252	GO:0006355 GO:0010468 GO:1903506 GO:2000112 GO:0060255 GO:2001141 GO:0010556 GO:0031326 GO:0019222 GO:0051252 GO:0009889 GO:0031323 GO:0019219 GO:0051171 GO:0080090	regulation of metabolic process	<0.01
Purple	CC	217	GO:0005634 GO:0043231 GO:0043227	membrane-bounded organelle	0.025
Purple	CC	192	GO:0005667	transcription regulator complex	<0.01
Salmon	CC	129	GO:0016021 GO:0031224	intrinsic component of membrane	0.05
Tan	MF	23	GO:0005216 GO:0015267 GO:0022803 GO:0022836	passive transmembrane transporter activity	<0.01
Tan	MF	15	GO:0005230 GO:0015276 GO:0022834	ligand-gated channel activity	<0.01
Tan	MF	416	GO:0005524 GO:0032559	adenyl nucleotide binding	<0.01

			GO:0030554		
Tan	MF	37	GO:0008017 GO:0015631	tubulin binding	<0.01
Tan	MF	46	GO:0008092	cytoskeletal protein binding	<0.01
Tan	MF	18	GO:0008378	galactosyltransferase activity	<0.01
Tan	MF	42	GO:0016758 GO:0016757	transferase activity, transferring glycosyl groups	<0.01
Tan	BP	18	GO:0006030 GO:1901071	chitin metabolic process	0.05
Tan	BP	25	GO:0006040	amino sugar metabolic process	0.033
Tan	BP	47	GO:0007018 GO:0006928 GO:0007017	microtubule-based process	<0.01
Tan	BP	12	GO:0007155 GO:0022610	cell adhesion	0.04
Tan	BP	314	GO:0007165	signal transduction	0.025
Tan	BP	14	GO:0008283	cell population proliferation	0.05
Tan	CC	54	GO:0005874 GO:0099513 GO:0099512 GO:0099081 GO:0099080	polymeric cytoskeletal fiber	<0.01
Tan	CC	24	GO:0005886	plasma membrane	<0.01
Tan	CC	97	GO:0016020	membrane	0.017
Turquoise	BP	78	GO:0005975	carbohydrate metabolic process	0.01
Turquoise	BP	28	GO:0006511 GO:0019941 GO:0043632 GO:0051603 GO:0044265	cellular macromolecule catabolic process	<0.01
Turquoise	BP	44	GO:0009057	macromolecule catabolic process	<0.01
Turquoise	BP	43	GO:0016192	vesicle-mediated transport	0.025
Turquoise	BP	7	GO:0030163	protein catabolic process	0.044
Turquoise	BP	86	GO:0044248	cellular catabolic process	<0.01
Turquoise	BP	61	GO:0046907 GO:0051641 GO:0051649	cellular localization	0.021
Turquoise	BP	124	GO:1901575 GO:0009056	catabolic process	<0.01
Turquoise	CC	5	GO:0005839	proteasome core complex	<0.01

Table 3.8: Results of ANOVAs for comparisons of mean log-normalized expression for genes uniquely differentially expressed (upregulated and downregulated) for each of the two site types.

Gene Subset	Site	Treatment	Site x Treatment
<i>Downregulated</i>			
Normoxic Sites	0.269	<0.001*	<0.001*
Hypoxic Sites	0.875	<0.001*	<0.001*
<i>Upregulated</i>			
Normoxic Sites	0.338*	<0.001*	0.003*
Hypoxic Sites	0.146	<0.001*	0.001*

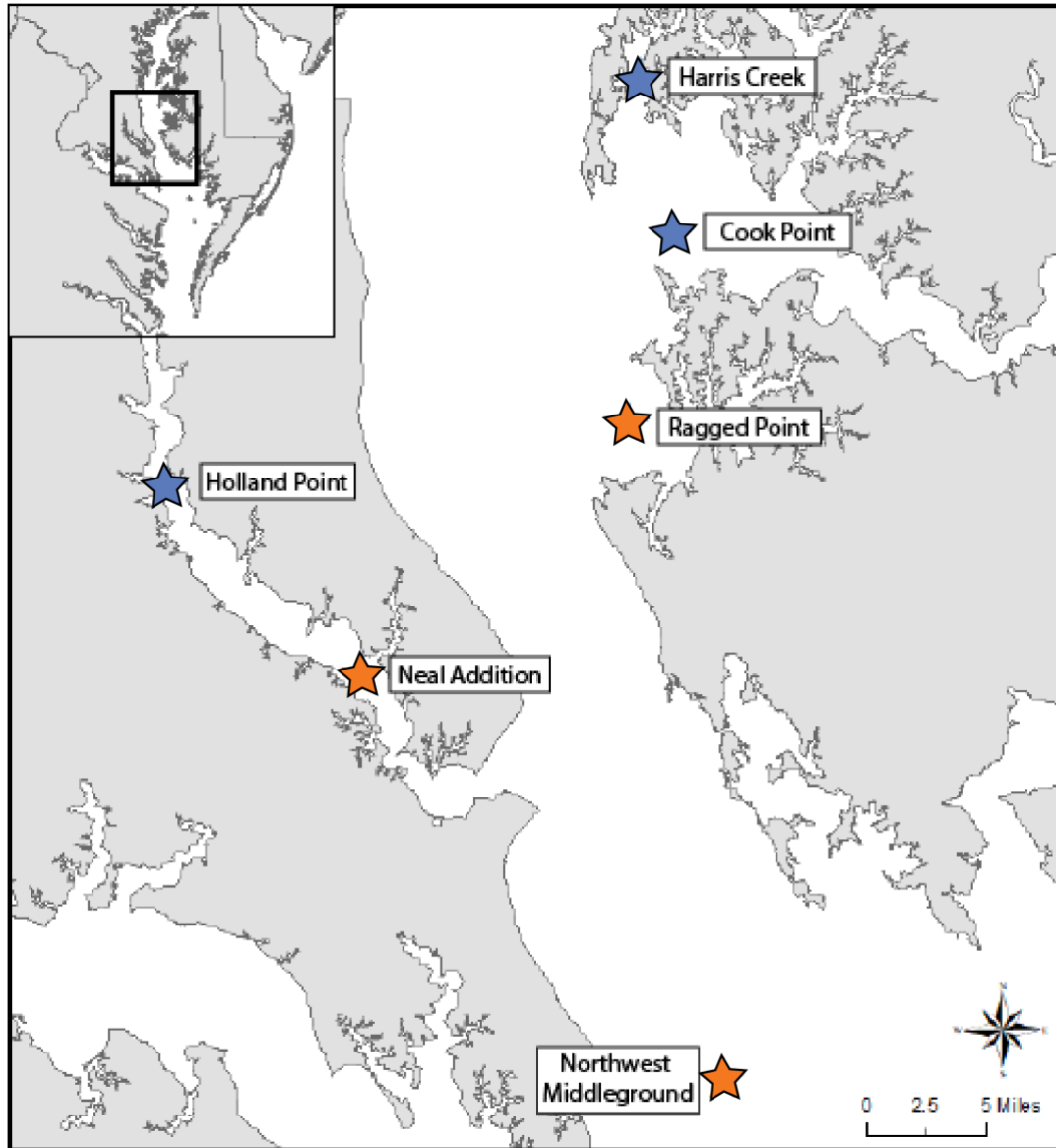


Figure 3.1: Map of oyster collection sites. Orange stars indicate sites with summertime hypoxic conditions (Neal Addition [NA], Northwest Middleground [NM], Ragged Point [RP]). Blue stars indicate sites with generally normoxic conditions (Cook Point [CP], Harris Creek [HC], Holland Point [HP]).

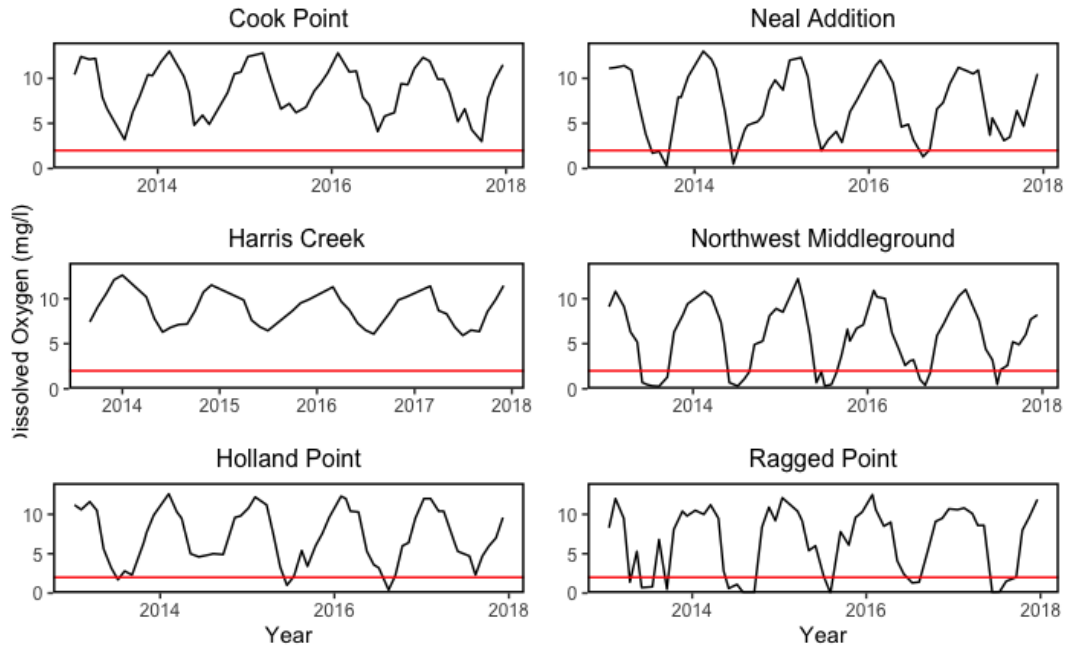


Figure 3.2: Historic dissolved oxygen concentrations (mg l^{-1}) for the six oyster collection sites from 2013-2018. Left-hand panels correspond to ‘normoxic’ sites and right-hand panels correspond to ‘hypoxic’ sites. The red lines on each panel mark 2.0 mg l^{-1} , the threshold concentration for hypoxic conditions.

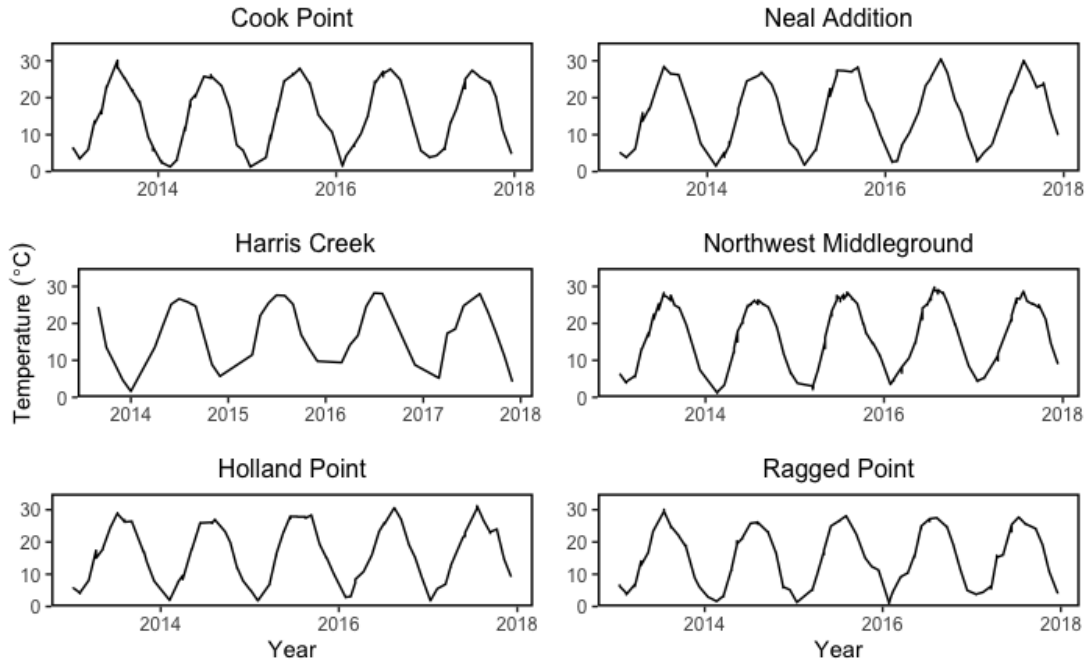


Figure 3.3: Historic temperature (°C) for the six oyster collection sites from 2013-2018. Left-hand panels correspond to ‘normoxic’ sites and right-hand panels correspond to ‘hypoxic’ sites. Temperature profiles between sites were very similar for the years compared.

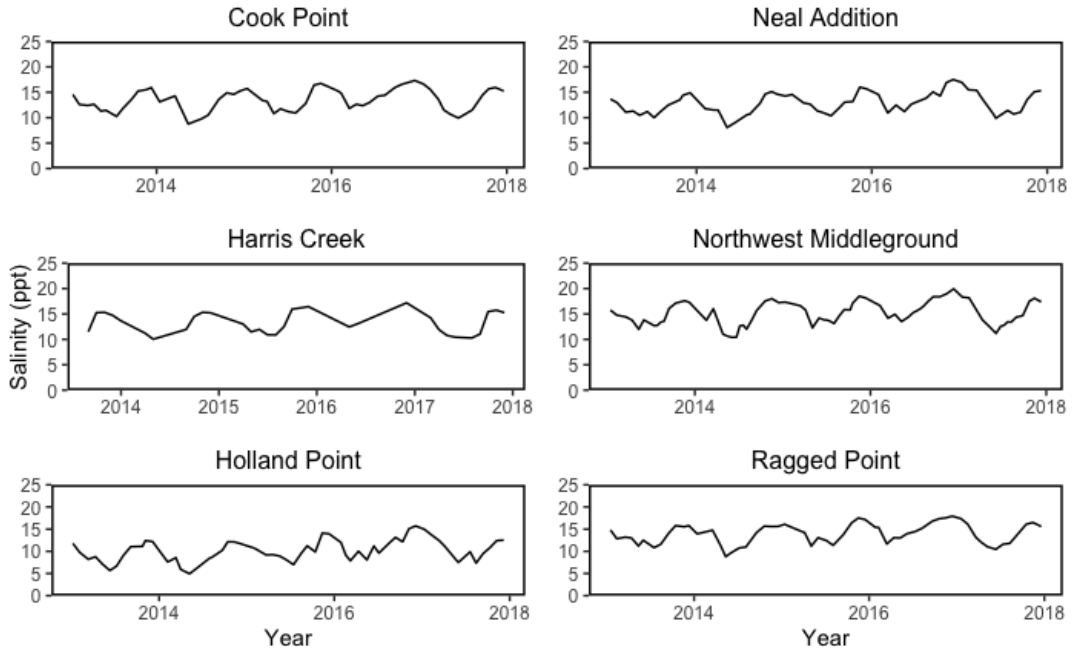


Figure 3.4: Historic salinity (ppt) for the six oyster collection sites from 2013-2018. Left-hand panels correspond to ‘normoxic’ sites and right-hand panels correspond to ‘hypoxic’ sites. Salinity profiles varied slightly between sites (e.g., slightly lower salinity at Holland Point and slightly higher salinity at Northwest Middleground) but were overall similar for the years compared.

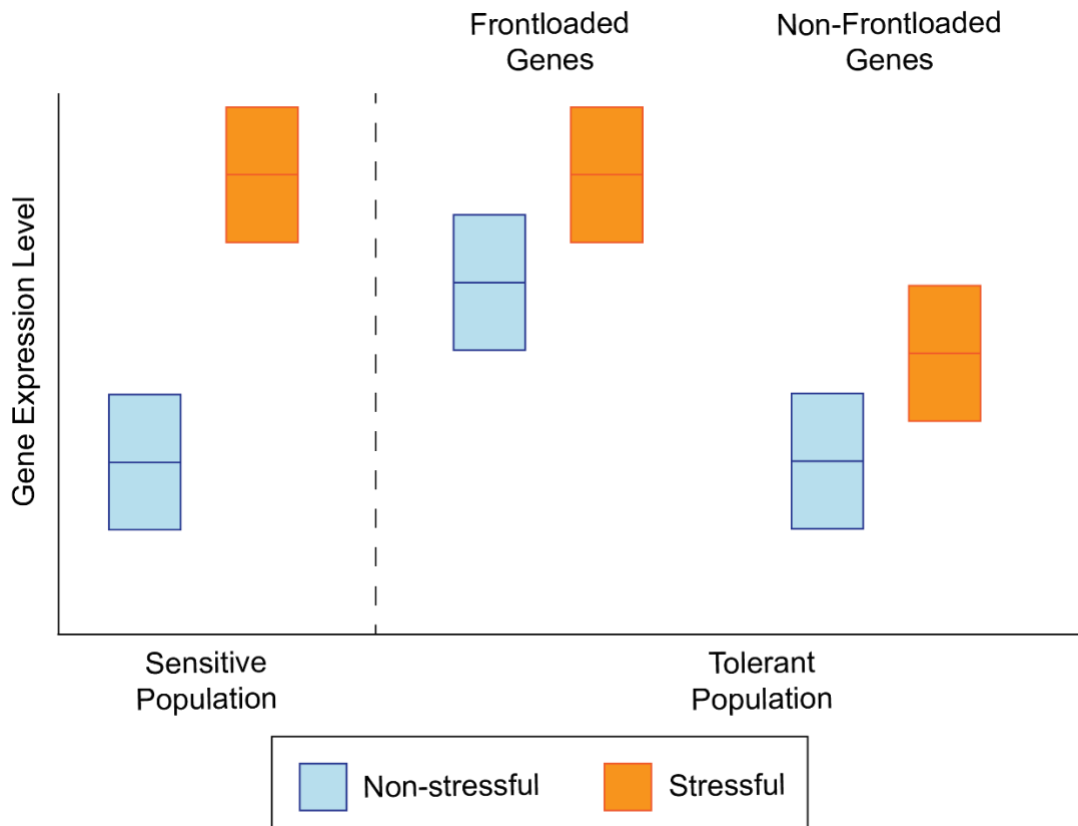


Figure 3.5: Schematic of how frontloaded genes would be identified for genes uniquely upregulated in the sensitive population. Under stressful conditions, sensitive individuals display increased expression for this set of genes in comparison to non-stressful conditions. These genes would be considered ‘frontloaded’ if the tolerant population shows higher expression levels under non-stressful conditions compared to the sensitive population and similar levels under stressful conditions. In contrast, ‘non-frontloaded’ genes would show similar expression levels in both the sensitive and tolerant populations under the non-stressful conditions, but no increase under stressful conditions for the tolerant population.

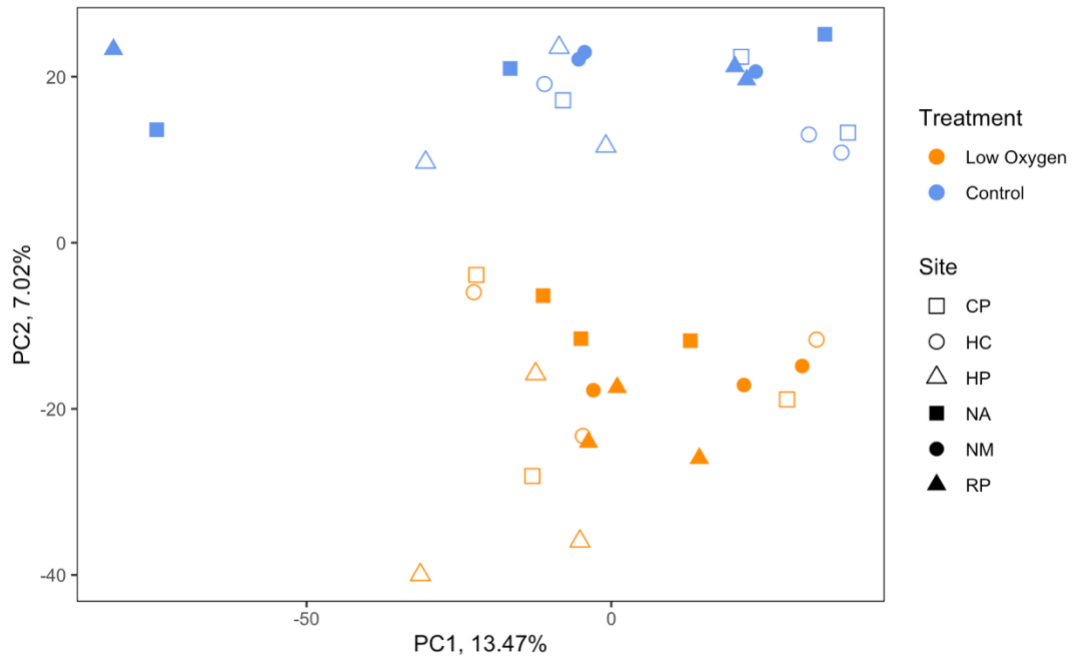


Figure 3.6: Plot of the first two components resulting from the principal components analysis of the global gene expression data. Orange points denote the low oxygen samples while blue points denote control samples. Solid points correspond to samples from hypoxic sites and unfilled points correspond to samples from normoxic sites. Site abbreviations as specified in Figure 3.1.

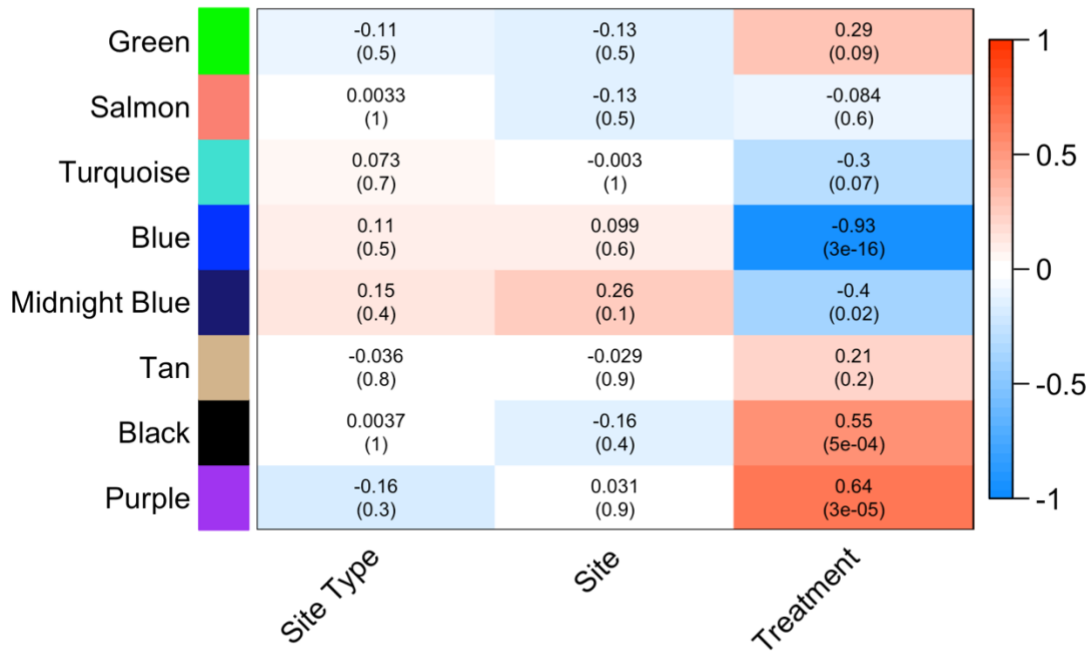


Figure 3.7: Correlation heatmap for the eight modules identified through gene network analysis. Top numbers within each cell correspond to correlation coefficients and bottom number correspond to p-values. Heatmap colors depict the correlation coefficient for each of the modules, red for positive correlations and blue for negative correlations. Four modules were significantly correlated with treatment (Blue, Midnight Blue, Black, and Purple). No significant correlations were found between site type or site and the module eigengenes.

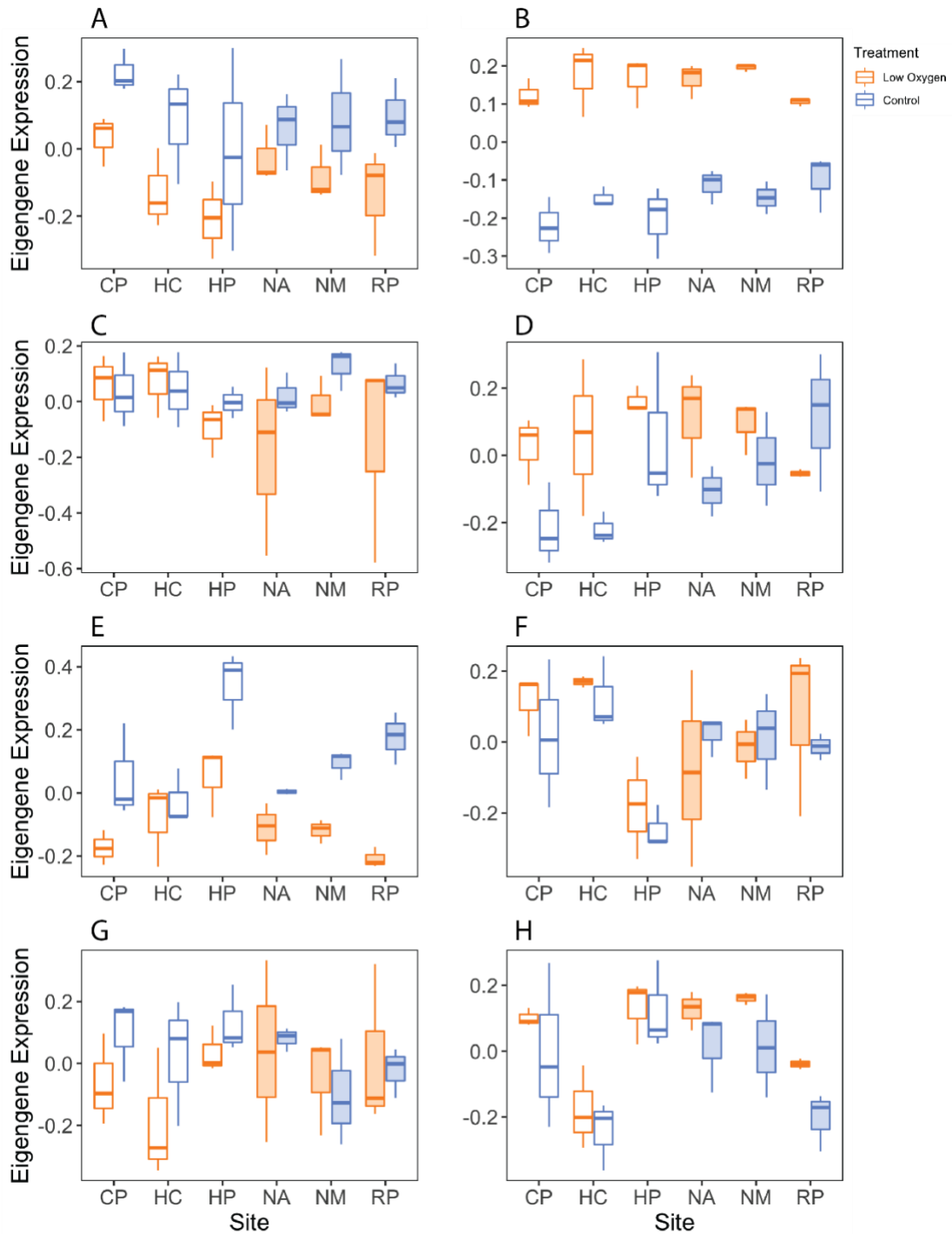


Figure 3.8: Eigengene expression by site and treatment for each of the modules identified by the gene network analysis: A) Black, B) Blue, C) Green, D) Midnight Blue, E) Purple, F) Salmon, G) Tan, and H) Turquoise. Shaded boxes correspond to oysters from hypoxic sites while open boxes correspond to oysters from normoxic sites. Orange boxes indicate low oxygen treatment and blue boxes indicate control treatment. Site abbreviations as specified in Figure 3.1.

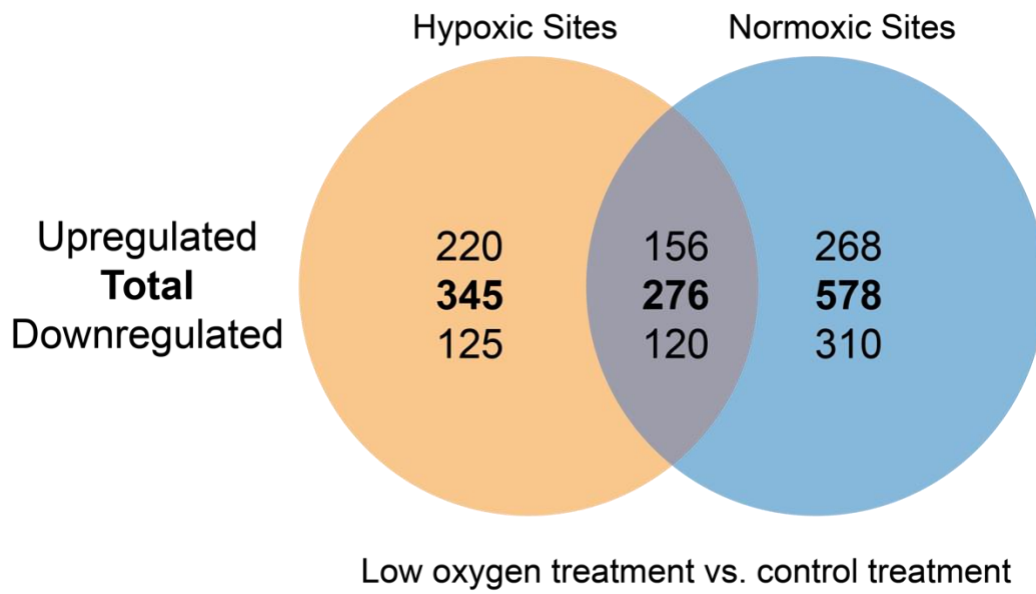


Figure 3.9: Venn diagram of uniquely differentially expressed genes for the oysters from hypoxic (orange) and normoxic (blue) sites in the low oxygen compared to control treatments.

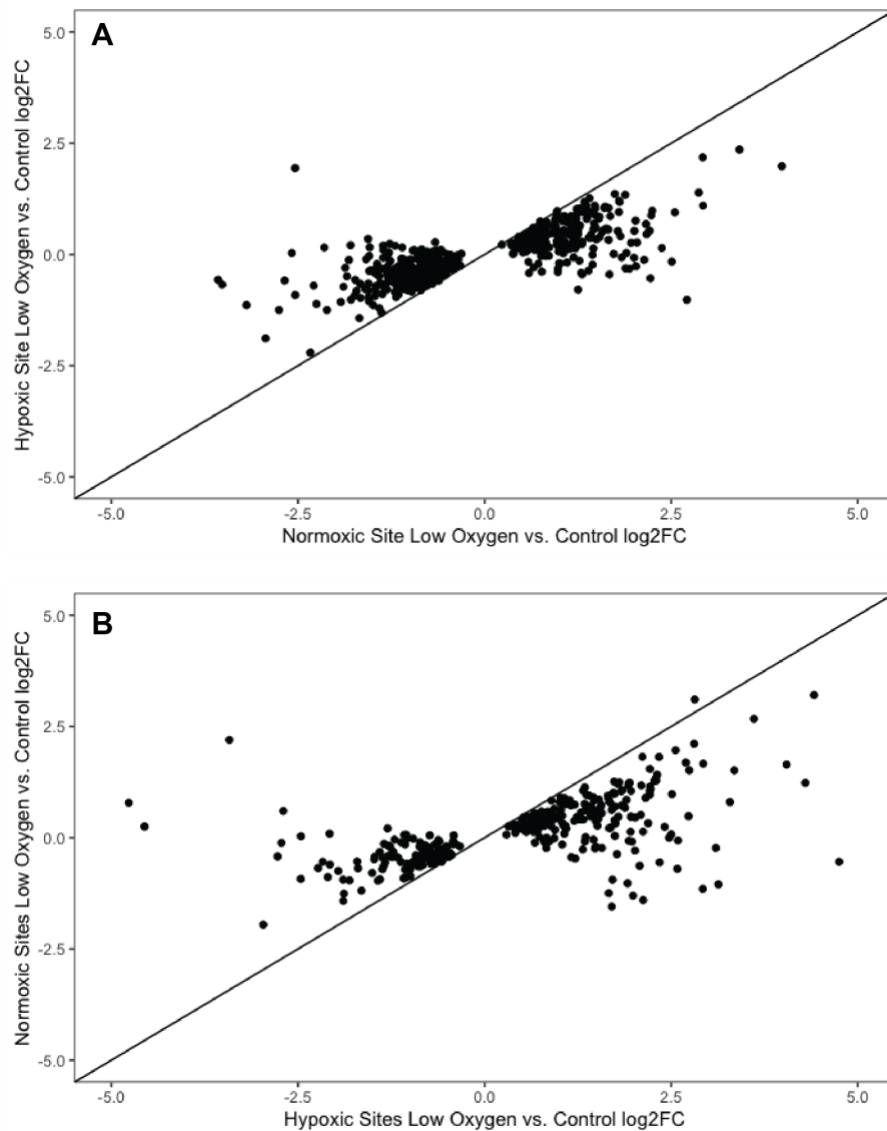


Figure 3.10: Comparison of log₂FC between low oxygen and control treatments for each site type for genes uniquely differentially expressed in A) oysters from normoxic sites and B) oysters from hypoxic sites. The lines denote a 1:1 relationship in log₂FC between the two site types for each set of genes. Both hypoxic upregulated (n=220, $X^2=216.02$, p-value < $2.2e-16$) and downregulated (n=125, $X^2=125$, p-value < $2.2e-16$) genes, as well as normoxic upregulated (n=268, $X^2=268$, p-value < $2.2e-16$) and downregulated (n=310, $X^2=310$, p-value < $2.2e-16$) genes deviate statistically from a 50/50 distribution above and below the line.

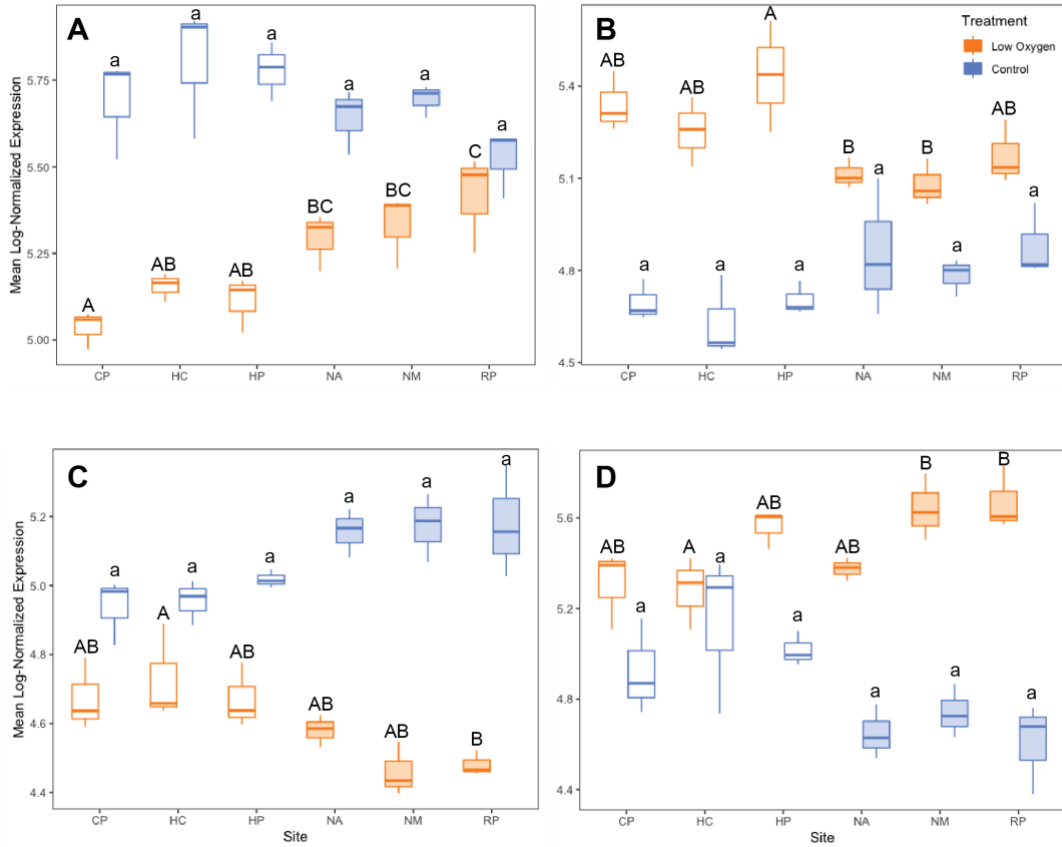


Figure 3.11: Mean log-normalized expression for differentially expressed genes identified as uniquely A) downregulated and B) upregulated in normoxic site oysters, and genes identified as uniquely C) downregulated and D) upregulated in hypoxic site oysters. Differential expression determined by comparing the low oxygen relative to the control treatment. Filled boxes correspond to oysters from hypoxic sites. Orange outlined boxes indicate low oxygen treatment and blue outlined boxes indicate control treatment. Letters above boxes denote statistically significant differences between sites within a given treatment according to a Tukey HSD post-hoc test (lowercase for control treatment, uppercase for low oxygen treatment). Site abbreviations as specified in Figure 3.1.

CHAPTER 4: PREDICTING THE EFFECTS OF HYPOXIA ON OYSTER GROWTH AND REPRODUCTION THROUGH THE DYNAMIC ENERGY BUDGET MODEL

Abstract

Hypoxia in the world's oceans poses an increasing threat to marine organisms and ecosystems, and there is a need to scale individual effects in order to make predictions about the broader ecological consequences of reduced oxygen availability. Mechanistic models, such as the Dynamic Energy Budget (DEB) model, provide a useful framework for quantifying the effects of changing environmental conditions, such as hypoxia, on individual organismal response. While the standard DEB model is forced solely by temperature and food availability, recent additions to the model for some marine species have allowed for the modulation of DEB energy fluxes in response to oxygen availability. The eastern oyster, *Crassostrea virginica*, is a sessile marine invertebrate inhabiting coastal environments experiencing variability in oxygen availability for which a DEB model has already been parameterized, but dissolved oxygen has not yet been incorporated as a forcing variable. The present study uses observed oyster growth data from sites throughout the Chesapeake Bay experiencing a range of oxygen regimes to calibrate and validate a DEB model for *C. virginica*, implementing a constraint on energy fluxes due to oxygen availability. The resulting model is then used to make predictions about hypoxia's influence on growth and reproduction over the oyster's growing season using data from two additional sites in the bay. Model outputs indicate that low oxygen exposure results in reduced growth rates for oysters, in both shell length and tissue mass, as well as reductions in

oyster fecundity and alterations to spawning frequency during the summer, which collectively has the potential to negatively affect oyster ecology. Overall, the integration of dissolved oxygen into the *C. virginica* DEB model provides an important tool to make predictions about how oysters will respond to future oceanic and coastal deoxygenation.

Introduction

Oxygen availability in coastal and open ocean waters is expected to decrease in the coming decades as a result of increased nutrient input to coastal waters and rising water temperatures, which will both reduce oxygen solubility and increase the intensity of water column stratification (Breitburg et al. 2018). Low oxygen availability or hypoxia – broadly defined as dissolved oxygen concentrations less than 2.0 mg/l (Rabalais et al. 2010) – is a significant threat to marine organisms, particularly sessile marine bivalves which lack the motility to escape deoxygenated waters. On an individual level, exposure to hypoxia in bivalves causes decreased ingestion rates (Wang and Widdows 1993, Sobral and Widdows 1997, Clark 2014), decreased respiration rates (Taylor and Brand 1975, Shumway and Koehn 1982, Hicks and McMahon 2002), decreased growth rates (Stevens and Gobler 2018), reduced reproductive potential (Long et al. 2014), and mortality (Stickle et al. 1989, Hicks and McMahon 2005, Gobler et al. 2014). These individual responses to hypoxia can collectively result in broader ecological implications, among them increased incidence of predation (Nestlerode and Diaz 1998), alterations to community diversity (Diaz and Rosenberg 1995), and effects on population structure

(Marcus et al. 2004, Eby et al. 2005). Given the growing threat of hypoxia, the ability to translate the individual effects of hypoxia into broader population and life history effects is important in order to make predictions about how reduced oxygen availability will influence organisms and ecosystems over longer timescales.

The use of mechanistic modeling offers one means to quantify and predict individual responses to hypoxia, which otherwise might be logistically prohibitive to measure empirically. One such model, the Dynamic Energy Budget (DEB) model (Kooijman 1986, Kooijman 2000), is a generalized framework for quantifying how energy is utilized in heterotrophic organisms. Because DEB theory was conceived to provide a unifying framework to describe energy utilization in all heterotrophs, it is broadly applicable for use with terrestrial or marine organisms. The DEB model quantifies organismal energy ingestion and allocation towards growth and reproduction, leading to predictions about the effects of these energetic tradeoffs on the organism's growth and reproductive potential. For the standard DEB model, food availability and temperature drive the dynamics of the system. However, recent research in marine organisms specifically has expanded the model's use to understand effects of environmental stressors on organismal population dynamics, including toxicants (Galic et al. 2017), low salinity (Lavaud et al. 2017) and low dissolved oxygen (Aguirre-Velarde et al. 2019a, Lavaud et al. 2019).

Given oxygen's importance for energy production in aerobically respiring organisms, hypoxia has the ability to affect many aspects of an organism's energy budget

(reviewed by Thomas et al. 2019). Exposure to low oxygen reduces filtration and ingestion rates in filter-feeding bivalves, reducing the influx of energy into the organism (Sobral and Widdows 1997, Hicks and McMahon 2002, Clark 2014). The assimilation of ingested energy into utilized energy can also be reduced under low oxygen exposure due to reductions in the activity of enzymes necessary for energy assimilation (Wang et al. 2011). Of the energy assimilated, hypoxia can hinder the ability of organisms to mobilize energy reserves because facultative anaerobiosis – undertaken by organisms under hypoxic stress – results in reduced energetic outputs (Aguirre-Velarde et al. 2019a). The consequences of these energetic effects are reductions in growth (Baker and Mann 1992, Gobler et al. 2017, Stevens and Gobler 2018) and reproduction (Long et al. 2014, Aguirre-Velarde et al. 2019b).

The eastern oyster, *Crassostrea virginica*, is a wide-ranging coastal bivalve providing critical ecosystem services to the waters it inhabits, such as water filtration (Coen et al. 2007), coupling benthic-pelagic dynamics (Smyth et al. 2013), and structural habitat for other marine organisms (Grabowski and Peterson 2007). Populations of *C. virginica* have suffered significant declines in abundance over the last century as a result of overharvesting, disease, and reduced water quality (Jackson et al. 2001, Kirby 2004). Throughout its range, *C. virginica* is exposed to low oxygen availability on hourly, daily, and seasonal timescales (e.g., Breitburg 1990, Dame 1979), and although oysters generally show high overall tolerances to hypoxia compared to other marine invertebrates (Stickle et al. 1989), hypoxia still poses a threat to the health of *C. virginica* populations. Hypoxia exposure has been shown to reduce oyster larval

settlement (Widdows et al. 1989), increase susceptibility of oysters to disease (Keppel et al. 2015), and lead to mass mortalities of adult oysters (Johnson et al. 2009). As the frequency of hypoxic events continues to increase in the Chesapeake Bay (Ni et al. 2019) and other areas that *C. virginica* inhabits (e.g. Laurent et al. 2018), the development of mechanistic models, such as the DEB model, to predict potential consequences of hypoxic exposure for population dynamics is critical.

Energetic constraints related to dissolved oxygen have previously been integrated into DEB models developed for Atlantic cod, *Gadus morhua* (Lavaud et al. 2019), and Peruvian scallop, *Argopecten purpuratus* (Aguirre-Velarde et al. 2019a), through the use of an oxygen correction factor. In both studies, the oxygen correction factor, driven by a species-specific critical oxygen concentration, was used to modulate energetic fluxes within the energy budget. For Atlantic cod, the oxygen correction factor acted solely on ingestion rates, reducing ingestion along with declining oxygen tension until a critical oxygen concentration, under which ingestion ceased. In contrast, for the Peruvian scallop, the energetic response to oxygen concentrations was based off of their oxygen consumption rates under declining oxygen and was treated as a two-phase response, with energy assimilation and mobilization fluxes decreasing linearly below a critical oxygen concentration. Similar to the Peruvian scallop, *C. virginica* displays a two-phase response in oxygen consumption under declining dissolved oxygen concentrations. At higher oxygen concentrations, *C. virginica* oxyregulate, maintaining relatively constant levels of oxygen uptake despite the concentration of oxygen in the environment. However, below a critical oxygen

concentration, they oxyconform, reducing their oxygen consumption as they begin to undergo anaerobic respiration (Shumway and Koehn 1982). Given the similar physiological response to oxygen in *C. virginica*, the method of modulating energy fluxes with an oxygen correction factor used in the study with *A. purpuratus* is likely transferable to the eastern oyster.

The goal of the present study is to explicitly integrate dissolved oxygen into the DEB model developed for *C. virginica* (Lavaud et al. 2017) in order to provide a tool for predicting the broader life history implications of reduced dissolved oxygen on *C. virginica*. To accomplish this, the effect of dissolved oxygen was first parameterized into the *C. virginica* DEB model using an oxygen correction factor to constrain specific energy fluxes within the energy budget. After model calibration and validation, the model was then used to predict oyster growth and reproductive output at two sites in the Chesapeake Bay that differ in summertime oxygen availability in order to understand the effects of reduced oxygen on oyster population dynamics over the course of the oyster's growing season. The development of this model and the understandings of population effects that could be gleaned from its use has the potential to yield important insights into how oyster populations in the Chesapeake Bay currently respond to hypoxic conditions and predict responses in future oxygen scenarios.

Methods

Dynamic Energy Budget Model

The model used in this study is based on the Dynamic Energy Budget (DEB) theory (Kooijman 2000). The generalized DEB theory describes the flow of energy from ingestion of food from the environment into the organism for use in growth, maintenance, development, and reproduction (Figure 4.1). The model uses four state variables to describe the flow of energy through an individual: reserves (E), structural volume (V), reproduction (E_R), and gametes (E_{GO}). A portion of ingested energy is assimilated into reserve energy. From there, a fixed fraction of energy (κ) is partitioned towards structural growth and somatic maintenance, while the remaining fraction of energy ($1 - \kappa$) is partitioned in juveniles towards maturation and in adults towards reproduction and reproductive maintenance costs. Some of the energy allocated for reproduction in adults is stored in and eventually released as gametes.

The model used in the present study builds on the standard DEB growth model parameterized for *C. virginica* (Lavaud et al. 2017). Each model run represented growth of a single individual using fixed parameters. Growth time steps occurred every fifteen minutes (in accordance with the environmental data time step) and growth was calculated using DEB model differential equations for the four state variables (reserves, structural volume, reproduction, and gametes). Specific equations used in the model are listed in Table 4.1. Parameter values were primarily sourced from Lavaud et al. 2017 and Lavaud et al. 2021, although some parameters were changed during model calibration or identified from primary literature (described

below). The parameters used in the model as well as the source from which the value was obtained can be found in Table 4.2. All statistical analyses and model simulations were carried out in R (Version 3.6.1 / RStudio Version 1.3.1073). Model simulations were run using a publicly available R script (Monaco et al. 2019), customized for use with the *C. virginica* DEB model developed here.

The standard DEB model is forced by environmental food availability and temperature, the former driving ingestion rates and the latter affecting ingestion, mobilization, and maintenance rates. Recently, salinity was incorporated as an additional forcing variable in the *C. virginica* growth model through the use of a salinity correction factor (C_S) acting on ingestion rates (\dot{p}_X , Lavaud et al. 2017). The correction factor is calculated by a sigmoidal function that reduces ingestion rates between salinities of 3 and 10 ppt and suspends ingestion under 3 ppt (threshold values identified from previous literature: Galtsoff 1964, Soniat et al. 2013, and Casas et al. 2017). The DEB model used in the present study will continue to use this correction factor to incorporate salinity effects on oyster growth and reproduction.

Gametogenesis and spawning activities vary between species and have been modeled in several different ways in DEB models developed for bivalves (e.g., Lavaud et al. 2014, Pouvreau et al. 2006, Bernard et al. 2011). *C. virginica* is known to undergo gametogenesis once they've reached a threshold size (volume at puberty [V_P]) and ambient temperatures rise above a threshold gametogenesis temperature (T_G) (Price and Mauer 1971). Spawning is triggered after an accrual of degree-days above T_G ,

ambient temperatures have reached the spawning temperature (T_{sp}) (Shumway 1996) and an individual has grown to attain a threshold gonado-somatic index (GSI) (Choi 1992), measured by:

$$GSI = \frac{GDW}{TDW} \times 100 \quad (1)$$

where GDW is equal to the gonad dry mass and TDW denotes the total dry tissue mass. In the present model, gametogenesis occurred only when oysters were above the previously parameterized volume at maturity (0.416 cm^3 , Lavaud and Kooijman 2018) and temperatures exceeded 12°C (Price and Mauer 1971). Spawning was triggered when oysters reached a threshold GSI of 30 (Barber et al. 1991), 434 degree-days above 12°C had accrued (Mann et al. 2014), and ambient temperatures were above 20°C (Shumway 1996). The gonads were completely emptied upon spawning (Kennedy and Krantz 1982).

Oxygen Correction Function

A primary goal of the present study was to incorporate dissolved oxygen as an additional forcing variable into the *C. virginica* DEB model. Similar to previous work (Aguirre-Velarde et al. 2019a, Lavaud et al. 2019), this was accomplished through the integration of an oxygen correction factor (C_{DO}) to constrain energy fluxes in the DEB model. To parameterize C_{DO} , a consideration of *C. virginica*'s response to declining oxygen tension is necessary. *C. virginica* is known to elicit a two-phase response in oxygen consumption across declining oxygen tensions, where oxygen

consumption rates are maintained above a threshold oxygen concentration and decline linearly as concentrations fall below the threshold (Shumway and Koehn 1982). Thus, similar to Aguirre-Velarde et al. (2019a), C_{DO} in the present study was modeled using a two-phase response using the following equation:

$$\text{for } C_{O_2} < C_{O_{2c}}, C_{DO} = \frac{C_{O_2}}{C_{O_{2c}}}, \text{ else } 1 \quad (2)$$

where C_{O_2} is the dissolved oxygen concentration at any given point and $C_{O_{2c}}$ denotes the threshold (or critical) oxygen concentration.

Critical oxygen concentrations (also identified as P_{crit}) vary considerably both interspecifically (Vaquer-Sunyer and Duarte 2008) as well as intraspecifically, either as a result of differential tolerance to low oxygen (e.g., Sollid et al. 2003) or as a result of the ambient environmental conditions during which it's measured (Rogers et al. 2016). With respect to *C. virginica*, oxygen uptake measured across a range of dissolved oxygen concentrations has been shown to differ depending on the ambient temperature and salinity (Shumway and Koehn 1982). Since temperature and salinity also force the DEB model, a dynamic $C_{O_{2c}}$ equation was parameterized to integrate their effect on the critical oxygen concentration. To accomplish this, critical oxygen concentrations were extrapolated from the oxygen uptake curves measured across three temperatures and three salinities from Shumway and Koehn (1982) by identifying the dissolved oxygen concentration at which oxygen uptake began to decline. Then, a linear model relating salinity and temperature to the critical oxygen

concentration was determined using the *lm* function in R (Figure 4.3). The resulting equation:

$$C_{O2c} = 0.00148 T[t] - 0.173 S[t] + 7.0756 \quad (3)$$

was used to vary the critical oxygen concentration depending on the ambient temperature (T) and salinity (S) at a given time point (t).

Oxygen correction factors have previously been used in DEB models to constrain ingestion rates for cod (Lavaud et al. 2019) or assimilation and mobilization rates for scallops (Aguirre-Velarde et al. 2019a). While prior work using the oxygen correction factor for bivalves did not include an effect of oxygen on feeding, oysters have been shown to reduce their ingestion rates during low oxygen stress (Clark 2014). Thus, in addition to effects of dissolved oxygen on assimilation (\dot{p}_A), and mobilization (\dot{p}_C) rates, the present study also applied C_{DO} to constrain ingestion rates (\dot{p}_X) of the oysters as follows (parameter definitions are listed in Table 4.2):

$$\begin{aligned} \dot{p}_X &= C_T C_{DO} C_S \{ \dot{p}_{Am} \} f V^{2/3} \\ \dot{p}_A &= C_{DO} \dot{p}_X k_A \\ \dot{p}_C &= C_T C_{DO} \left(\frac{[E]}{[E_G] + \kappa[E]} \dot{v} [E_G] V^{2/3} + \dot{p}_M \right) \end{aligned} \quad (4)$$

The result is a two-phase response to oxygen availability. In oxygen concentrations above the critical oxygen concentration ingestion, assimilation, or mobilization rates

of the oyster are not affected by oxygen availability. Below the critical oxygen concentration, the oxygen correction factor declines linearly between 1 and 0, leading to a reduction in the ingestion, assimilation, and mobilization rates.

In order to test whether these modifications to the oxygen correction factor improved the model fit, I ran four independent models on the two calibration datasets (see *Model Calibration* below) and assessed their fit to the observed oyster growth data. The first model ('No oxygen effect') was run using the standard DEB model (including the salinity correction factor), without any effect of dissolved oxygen. The second model ('Static C_{O2c} ') included an effect of oxygen on assimilation and mobilization rates, with a static critical oxygen concentration set at 2.0 mg/l, the level used to broadly define 'hypoxia' (Rabalais et al. 2010). The third model included a dynamic C_{O2c} to calculate the C_{DO} that only acted on assimilation and mobilization rates ('Dynamic C_{O2c} '). The fourth and final model ('Full model') used a dynamic C_{O2c} to calculate the C_{DO} , which was then applied to the ingestion, assimilation, and mobilization rates. The fit of these models was assessed by calculating the root-mean square error (RMSE) between the observed and simulated oyster shell lengths.

Oyster Growth and Environmental Data

Oyster growth data used in model calibration and validation was collected in 2008 and 2009 as part of a study conducted by Breitburg et al. (2015) examining the susceptibility of oysters to *Perkinsus marinus* (Dermo) infection in relation to low-oxygen exposure. The reader is referred to this study for a detailed description of the

experimental setup. Briefly, oysters were deployed suspended in polyethylene mesh cages at sites throughout tributaries of the Chesapeake Bay. Water quality parameters – temperature ($^{\circ}\text{C}$), salinity (ppt), dissolved oxygen (mg/l), and chlorophyll-*a* ($\mu\text{g/l}$) – were continuously monitored at 15-minute intervals at each site using Yellow Springs Instruments model 6600 sondes (Yellow Springs Instruments, Yellow Springs, OH). In June of each year, initial oyster shell lengths to the nearest millimeter were measured prior to the deployment of the oysters in the field. Final shell lengths were measured when oysters were retrieved in early October. In 2008, three intermediary shell length measurements – each approximately one month apart – were taken on oysters during the deployment, while only one midpoint measurement was taken in 2009. Shell lengths for all oysters were measured with a flexible ruler wrapped across the longest dimension from hinge to the tip of the valve to account for three-dimensional growth of the shell. A subset of oyster shell lengths was also measured with calipers along the same shell axis. Data were collected on both one- and two-year-old oysters, although only growth of one-year-old oysters were used for the model calibration and validation in the present study.

Two modifications were made to the oyster growth and environmental data prior to their use in modeling. First, since the DEB model output of shell length does not account for the three-dimensional curvature of the shell, it was necessary to transform the three-dimensional flexible ruler measurement to the two-dimensional caliper measurement for comparison with the model. To do so, a power function was fit to the data relating the flexible ruler and caliper measurements (Figure 4.4) and resulting

equation ($Caliper\ Length = 1.1879\ Ruler\ Length^{0.9383}$) was used to transform the three-dimensional lengths to two-dimensional lengths. Second, the continuous water quality monitoring data from the validation and calibration sites contained some gaps in data measurements, which were necessary to rectify prior to use in the model. These missing data points were interpolated using the MATLAB (version 9.10.0.1602886 R2021a) *fillgaps* function, which uses autoregressive modeling to interpolate the data. The data were interpolated using 24 data points (six hours) worth of data from either side of the gap, which visually provided the best fit of the interpolated data to the trends of the measured data. Statistics for the number of interpolated datapoints for each environmental dataset is provided in Table 4.3.

Model Calibration

Model parameters were calibrated using two sites, Chesapeake Biological Lab (CBL) and Cole’s Creek (CCR). CBL represented a ‘normoxic’ site where none of the dissolved oxygen measurements experienced by oysters during the experiment fell below 2.0 mg/l, whereas CCR represented a ‘low oxygen’ site where 2.7% of measurements were below 2.0 mg/l (Figure 4.6). For each site, the initial shell length (L_0) for the model run was set to the mean starting shell length from the oyster growth dataset and this value was used to calculate the initial total energy (TE) in an individual according to the equation (McFarland 2015):

$$TE = \frac{\{p_{AM}\}}{v} (L_0 \delta_M)^3 \quad (5)$$

where $\{\dot{p}_{AM}\}$ is equal to the maximum surface area-specific assimilation rate, \dot{v} is equal to the energy conductance, and δ_M is equal to the shape coefficient. Since simulations began in mid-June, just as oysters would have been preparing to spawn in the Chesapeake Bay (Thompson et al. 1996), the initial percentage of energy in reserves (E) was assumed at 0.7, with the remaining energy allocated towards energy in the reproductive buffer (E_R) at 0.2 and gonad energy (E_{GO}) at 0.1, following the logic of Filgueira et al. (2014). Model calibration focused on varying parameters that tended to show large variability between published studies for *C. virginica* DEB models: volume-specific somatic maintenance costs, half-saturation coefficient, and maximum surface-area ingestion rate. During calibration, initial values for these parameters were set to those from Lavaud et al. (2017) and changed individually to minimize the root mean square error between the observed and simulated shell lengths.

A sensitivity analysis was run on some model parameters using environmental data from the two calibration datasets (CBL and CCR) to assess the sensitivity of the model output to changes in parameter values. Parameters included in the sensitivity analysis included those that differed between published studies of DEB models for *C. virginica*: half-saturation constant, volume-specific somatic maintenance costs, maximum surface area assimilation rate, maximum surface area ingestion, cost for structure, energy to growth, and energy conductance. The sensitivity analysis was run

according to Filgueira et al. (2014). Sensitivity (S) was determined by changing each parameter individually by $\pm 50\%$ and calculated according to:

$$S = \frac{L_{alt} - L_{ori}}{L_{ori}} \times 100 \quad (6)$$

where L_{alt} is the shell length using the alternative parameter value and L_{ori} is the shell length using the original parameter value.

Model Validation

The model was validated against six different datasets, three generally normoxic sites (Bowler's Wharf [BWH], Mulberry Point [MUL], and Yeocomico River [YEO]) and three reduced oxygen sites (Harness Creek [HCR], Little Monie Creek [LMN], and St. Mary's College [SMC]) (Figure 4.2). The three normoxic sites all had dissolved oxygen measurements above 2.0 mg/l, whereas the three hypoxic sites had 3.1% (HCR), 0.1% (LMN), and 12.2% (SMC) of dissolved oxygen measurements below 2.0 mg/l (Figures 4.8-4.13). As with the calibration, initial shell lengths for each model simulation were set to the average observed shell length for each site and initial values for energy in reserves (E) was 0.7 total energy, energy in reproduction (E_R) was 0.2 of total energy, and energy in gonads (E_{GO}) was 0.1 of total energy. The fit of the simulated oyster shell lengths to the observed shell lengths was assessed in two ways. First, a linear model was run on the simulated and observed oyster shell lengths. The resulting slope and intercept of the equation was used to determine how close the model fit to a 1:1 relationship between observed and simulated shell lengths,

while the R^2 value was used to determine the amount of variance in the simulated shell lengths explained by the variance in the observed shell lengths. Second, deviations (F) between the simulated (L_S) and observed (L_O) shell lengths were calculated according to (Rosland et al. 2009):

$$F = \frac{100}{2T} \left(\sum_{t=1}^T \frac{|L_S(t) - L_O(t)|}{L_O(t)} \right) \quad (7)$$

where T is the total number of shell length comparisons made for each site for each given time point (t). Smaller values of F would correspond to a better fit between observed and simulated oyster shell lengths.

Predicting Oyster Growth and Reproduction

The calibrated and validated model was used to make predictions about oyster growth and reproductive output over the full growing season for oysters at two sites that differ in oxygen availability, Wicomico Beach (WIB) and Breton Bay (BBY) (Figure 4.2). Continuous monitoring data for temperature, salinity, chlorophyll- a concentration, and dissolved oxygen concentration were downloaded from Maryland Department of Natural Resources Eyes on the Bay (<http://eyesonthebay.dnr.maryland.gov> [last accessed May 25, 2021]) for each of the sites from April through October 2007, the duration of time the water quality monitoring sondes were deployed (Figure 4.16). Missing datapoints for the water quality data were interpolated using identical methods to the model calibration and

validation environmental data. Initial shell length for each model run was set to 4.5 cm, an approximate length for one-year-old oysters in the Chesapeake Bay (Coakley 2004). Oysters used in the prediction analyses were predicted to have less energy in their gonads than those used in the calibration and validation datasets since oysters typically overwinter with very little energy stored in gonads (Thompson et al. 1996) and gametogenesis typically would begin after the beginning of the model run (April or May according to Kennedy and Krantz 1982). Thus, initial values for energy allocation were slightly altered from the calibration and validation model runs, with 0.7 of total energy in reserves (E) and 0.3 of total energy in reproduction (E_R). No energy was predicted to be in the gonads (E_{GO}) and initial degree-days were set to zero since water temperatures in the Chesapeake Bay before April are typically not above 12°C, the gametogenesis temperature threshold (NOAA National Data Buoy Center [<https://www.ndbc.noaa.gov>] last accessed June 8, 2021). The model was used to predict overall shell length, total wet tissue mass, and gonad wet mass at each of the sites. Shell (cm/month) and wet tissue mass (g/month) growth rates (GR) were calculated according to the following equation:

$$GR = \frac{M_E - M_0}{7} \quad (8)$$

where M_E and M_0 denote the ending and initial size measurements, respectively, and seven corresponds to the number of months the model was run. Additionally, resulting gonad wet mass was used to assess timing of spawning and fecundity of spawning oysters (number of eggs spawned) using the equation:

$$\text{Number of eggs} = \frac{E_{sp}}{\rho_{egg}} \quad (9)$$

where E_{sp} is the energy released during spawning and ρ_{egg} is the amount of energy in a single egg. In the case of multiple spawnings, the number of eggs was totaled across the whole season.

Results

Model Calibration

In general, use of previously published parameters from the literature resulted in model outputs that closely fit empirically measured oyster growth data (Figure 4.5). During model calibration, only one primary DEB parameter – volume-specific somatic maintenance rate [\dot{p}_M] – was changed from the most recently published DEB paper for *C. virginica* sourced from the Gulf of Mexico (Lavaud et al. 2021) to improve model fit. However, the value used for the present study, 30 J/cm³d, falls within the range of values previously parameterized for *C. virginica* (4.166; Lavaud and Kooijman 2018, 24; Filgueira et al. 2014, and 38; Lavaud et al. 2021) and is therefore not an unreasonable parameter estimate for the species.

Results of the alternative model comparisons for integrating dissolved oxygen into the DEB model indicated that including the effects of dissolved oxygen on oyster growth, specifically by using a dynamic C_{O2c} , provided the greatest improvement of

model fit for the site experiencing low oxygen conditions (CCR, Figure 4.5 and Table 4.5). Overall, the best model fit for both sites was for the full model integrating a dynamic C_{O_2c} to determine C_{DO} and incorporating an effect of dissolved oxygen on ingestion rates (RMSE: CBL=0.132, CCR=0.413) while the worst model fit included no effect of dissolved oxygen on oyster growth (RMSE: CBL=0.173, CCR=0.815).

Results of the sensitivity analysis indicated variability in sensitivity among parameters tested and minimal difference in sensitivity measurements between the two site types assessed (Table 4.6). Three changed parameters resulted in greater than 10% reduction in shell length with a 50% reduction in parameter value: maximum surface area ingestion rate, energy conductance, and energy to growth. The maximum surface area ingestion rate decreased by an average of 13.58% with a 50% reduction in the parameter value. A 50% decrease in the kappa parameter value resulted in an average -17.18% change in the shell length. It should be noted that the asymmetrical effect of the parameter changes for kappa was the result of the forced setting of the +50% value to one since the parameter represented a proportion. Finally, a 50% reduction in energy conductance resulted in a -10.91% change in final shell length. One parameter, volume-specific maintenance rate, showed some intermediate levels of sensitivity, with average changes of -7.92% / 9.64% (+50% / - 50%). Three parameters, half-saturation constant, maximum surface area assimilation rate, and cost for structure, showed relatively low levels of sensitivity (<5%).

Model Validation

The model was validated against six different observed oyster growth datasets and there was strong agreement between simulated and observed shell lengths at each site (Figure 4.14). The empirical growth data indicated that oysters from the sites with low oxygen exposure grew by a smaller percent change in shell length (HCR 23.3%, LMN 23.0%, SMC 15.5%) compared to the normoxic site oysters (BWH 29.2%, MUL 33.7%, YEO 23.2%), and the model was able to predict these changes in length fairly well (Low oxygen: HCR 27.8%, LMN 21.0%, SMC 21.1%; Normoxic: BWH 28.2%, MUL 32.0%, YEO 30.1%). The linear model assessing the fit of the simulated oyster growth in shell length compared to the observed growth showed a nearly 1:1 relationship between simulated and observed data (Figure 4.15; slope=0.94, intercept=0.42, $R^2=0.71$, $p < 0.001$). Deviations between simulated and observed oyster lengths were also relatively minimal showing strong agreement between the model and observed data, with an average deviation of 2.34% across all sites (Table 4.7). One site, Yeocomico River (YEO), showed the largest deviation of 6.34%, where the model tended to overestimate the growth of oysters, particularly at the midpoint measurement (Figure 4.14C).

Predicting the Effects of Low Oxygen on Oyster Growth and Reproduction

The model predicted overall greater growth in both oyster shell length and whole tissue weight, as well as greater reproductive output for the oysters from the normoxic site, Wicomico Beach (WIB) compared to the low oxygen site, Breton Bay (BBY) (Figure 4.17). The final predicted oyster shell length for WIB was 6.88 cm, with an

average growth rate of 0.34 cm/month. The final oyster shell length for BBY was 6.41 cm, with an average growth rate of 0.27 cm/month. Oysters from WIB were approximately 7.3% longer than oysters from BBY (Figure 4.17A). Final whole wet tissue weight showed a larger difference between the two sites, with BBY predicted to have approximately 17.1% less weight in their tissues at the conclusion of the model run. The final wet tissue mass of WIB oysters was 7.06 g and the final wet tissue mass of BBY oysters was 5.86 g, with growth rates of 0.76 g/month and 0.59 g/month respectively (Figure 4.17B). Reproductive output also differed between sites (Figure 4.17C). Oysters from the hypoxic site were predicted to reproduce two times throughout the summer, in July and early September, whereas oysters from the normoxic site spawned three times, in July, August, and late September. Overall fecundity for hypoxic oysters was also predicted to be lower, with a total spawning of approximately 12.4 million eggs throughout the season compared to the normoxic site oysters with a total spawning of approximately 22.8 million eggs (a 45.9% reduction in fecundity for hypoxic site oysters).

Discussion

Mechanistic modeling, such as the Dynamic Energy Budget (DEB) model, provides a useful tool to make predictions about the effects of environmental stressors on organismal growth and reproduction. Hypoxia is a pervasive oceanic and coastal stressor that is predicted to increase in frequency in marine environments into the future (Breitburg et al. 2018). Given the importance of oxygen availability for aerobic respiration, the integration of oxygen availability into individual bioenergetics models

can provide an important method of scaling individual effects of low oxygen onto broader population dynamics. The present study provides a first step in the integration of dissolved oxygen into the DEB model for *C. virginica*. The incorporation of an oxygen correction factor on aspects of oyster energetic flux improved the fit of the model for oyster shell growth, particularly for oysters in low oxygen habitats. The model predicted empirically measured Chesapeake Bay oyster growth data well, with minimal changes to previously published parameters for *C. virginica*, indicating that the oyster DEB model is fairly well-parameterized for this species. Importantly, preliminary predictions from the model of oyster growth and reproductive output at two different sites in the Chesapeake Bay indicate that reduced oxygen availability decreases oyster's overall growth and fecundity, and alters the frequency and timing at which an oyster spawns during the summer.

Integration of Dissolved Oxygen as a Forcing Variable

A primary goal of the present study was the integration of dissolved oxygen into the DEB growth model for adult *C. virginica*. Oxygen is necessary for energy production in aerobically-respiring organisms and is therefore predicted to be an important abiotic force driving organismal bioenergetics (Thomas et al. 2019). Prior studies parameterizing the integration of dissolved oxygen into DEB models have used oxygen correction factors to constrain energetic fluxes within the model and have shown improved prediction of organismal growth in low oxygen habitats (Aguirre-Velarde et al. 2019a, Lavaud et al. 2019). By using a similar oxygen correction factor within the *C. virginica* DEB model, the model used in the present study was able to

accurately estimate differences in oyster growth between sites with differing oxygen availability (Figure 4.7). Two modifications were made to the prior integration of C_{DO} . First, a dynamic (as opposed to static) critical oxygen concentration (C_{O2c}) was used to calculate C_{DO} , as prior work indicates this value varies as a result of the other DEB model forcing variables, temperature and salinity (Shumway and Koehn 1982). The integration of a dynamic value led to the greatest improvement in model fit for the oysters from the hypoxic site, CCR (Figure 4.5 and Table 4.4). The effect of ambient temperature and salinity on the ability of marine organisms to regulate their oxygen consumption is true for many organisms beyond *C. virginica* (Rogers et al. 2016, Herreid 1980). Thus, for future integration of a C_{DO} term into DEB models, particularly for marine ectotherms, the use of a dynamic critical oxygen concentration is of great importance. Second, the model used an additional constraint on ingestion rates for *C. virginica*, compared to prior work with bivalves where C_{DO} acted only on energy assimilation and mobilization rates (Aguirre-Velarde et al. 2019a). Given the close link between oyster feeding and respiration, lowered oxygen availability reduces oyster ingestion rates (Clark 2014). For oysters from the low oxygen site, the additional constraint on ingestion rates further improved model fit (Figure 4.5 and Table 4.4).

Validation of the model against six different sites in the Chesapeake Bay indicated that the simulated oyster growth fit the observed data well, with a nearly 1:1 relationship and very small deviations between the observed and simulated shell lengths, regardless of the oxygen conditions of the site (Figure 4.15). The model

slightly overestimated growth for oysters from one normoxic site, Yeocomico River (YEO), compared to the other validation sites (Figure 4.14). This was reflected in the fact that the deviation between the simulated and observed shell lengths for YEO ($F=6.339\%$) was the largest for all of the sites (Table 4.6). There are two plausible explanations for the observed difference in fit. First, the higher deviation could have been the result of random chance in oyster sampling during measurement. The estimated oyster length for the intermediate measurement of oysters at YEO was outside of the standard deviation of observed lengths (Figure 4.14C) and thus likely drove the higher deviation for this site. Because the length measurements were reliant on a small, haphazardly collected subsample of oysters, it's possible that the smaller shell length of the collected oysters at this timepoint could have simply been due to a random sample consisting of smaller oysters. Second, it is also possible that another unmodeled environmental factor was acting on the oysters from this particular site. The focus of the study from which the oyster growth data was collected was examining susceptibility of oysters to the parasitic disease *Perkinsus marinus* in relation to hypoxia exposure. Data on infection intensity per site was not reported in the published Breitburg et al. (2015) study, but YEO did trend towards a higher mean level of infection intensity with *P. marinus* than the other normoxic sites, BWH and MUL (D. Hondorp, *personal communication*). The reduced growth rate not captured by the model may have therefore been a result of this additional stressor. Regardless of the reason for the reduced fit, the difference between the simulated and observed shell lengths is minimal at YEO and, overall, the model predicted oyster growth well

in both site types indicating its utility in predicting oyster growth in varying oxygen conditions.

Predicted Effects of Hypoxia on Oyster Growth

Environmental water quality data from two sites with differing oxygen regimes in the Chesapeake Bay were used to force the calibrated and validated model in order to make predictions about how hypoxia exposure during the growing season affects oyster growth and reproduction. One of the primary predictions from the model was that oyster shell growth rates would be reduced for oysters from the low oxygen site (Figure 4.17A). Predicted growth rates for oysters from the normoxic site, WIB (0.34 cm/month), were higher compared to the low oxygen site, BBY (0.27 cm/month). Overall, the predicted growth rates fall within the range of observed oyster growth rates in the Chesapeake Bay (0.64 ± 0.33 cm/month, Paynter and Burreson 1991; 0.11 ± 0.029 cm/month, Paynter and DiMichele 1990). Additionally, the 21% reduction in growth rate between sites is consistent with the approximately 30% reduction in growth rate that has previously been shown for *C. virginica* exposed to chronic hypoxic conditions for four weeks (Stevens and Gobler 2018).

Reduced growth was also observed in the wet tissue mass for oysters from the low oxygen site compared to the normoxic site (Figure 4.17B). It should be noted that empirical data on oyster tissue growth was not available for validation of the model for the present study. However, predicted values for wet tissue mass are close to values predicted by an allometric scaling relationship between oyster wet tissue mass

and oyster length. Final wet tissue mass for oysters from the normoxic site was calculated at 5.95 g compared to model prediction of 7.06 g for oysters from the normoxic site and calculated at 4.80 g compared to model prediction of 5.86 g for the low oxygen site (Pollack et al. 2011). A greater decrease in final tissue mass (17.1%) compared to oyster shell length (7.3%) was observed for low oxygen site oysters compared to normoxic site oysters, which may indicate a lower fitness of oysters from the low oxygen site. Tissue growth is more energetically costly than shell growth (e.g., Watson et al. 2017), thus a decrease in energy allocated to tissue growth for oysters exposed to low oxygen would likely imply the oysters' energetic demands are not being met to produce sufficient tissue growth.

Overall, the reduced growth in shell length and wet tissue mass expected for oysters from the low oxygen site should have implications for the ecology of oyster reefs in the Chesapeake Bay. Slowed growth rates and smaller size-at-age for oysters exposed to periodic hypoxia could reduce the capacity of oysters to provide ecosystem services, such as water filtration (Zu Ermgassen et al. 2013). Additionally, oysters from low oxygen sites may take longer to reach a size refuge and would be more likely to be predated upon since smaller oysters are more prone to predation (Eggleston 1990). While the model does not make predictions about juvenile oyster growth, similar patterns of reduced growth have been shown in juvenile oysters (Baker and Mann 1992), which could delay maturity for oysters.

Predicted Effects of Hypoxia on Oyster Reproduction

In addition to constraints on growth, oysters at hypoxic sites were also predicted to experience adverse effects on reproduction as a result of low dissolved oxygen availability during the summer growing season. Overall fecundity for oysters at the reduced oxygen site, BBY, was predicted to be greatly reduced, with 45.9% fewer eggs released each year compared to the normoxic site, WIB. These data align with findings from prior research on bivalve reproductive response to low oxygen as a similar 40% reduction in egg production has been reported in the clam *Macoma balthica* due to exposure to hypoxia (Long et al. 2014). In addition, the predicted number of eggs spawned at each site (WIB = 22.8 million eggs, BBY = 12.4 million eggs) is in agreement with previous estimates of fecundity for Chesapeake Bay oysters with a dry tissue weight similar to those of the present study – 21.08 ± 1.82 million eggs for WIB oysters averaging 0.77 g and 15.72 ± 1.77 million eggs for BBY oysters averaging 0.68 g (Thompson et al. 1996).

The reduced fecundity of BBY oysters compared to WIB oysters was in part the result of the fewer predicted spawnings (two versus three spawnings for BBY and WIB, respectively) over the course of the model run. The number of spawns an individual oyster undertakes under natural conditions over the course of a year in the Chesapeake Bay has not been well documented or studied empirically. However, based on temperature thresholds necessary for inducing gametogenesis, it has been predicted that oyster populations in the Chesapeake Bay can undertake up to five mass spawning events throughout the summer, and at least two of those have been

empirically observed (Mann et al. 2014). Therefore, the two to three predicted spawns from the DEB model does appear to be a reasonable estimate for oysters from the area, at least at the population level. Additionally, the model predicted that low oxygen conditions would lead to asynchrony of spawning between oysters from the two sites as reduced growth in BBY oysters prevented them from reaching a sufficient GSI to spawn. Oyster spawning in the Chesapeake Bay tends to occur in pulses throughout the summer (Mann et al. 2014). For broadcast spawners, like the eastern oyster or corals, synchronicity in spawning is critical to prevent temporal reproductive isolation (Levitan et al. 2004) as well as to allow for overall success in fertilization of gametes (Levitan 1995). Thus, asynchronous spawning of oysters from reefs prone to hypoxia could lead to reduced reproductive success among oysters from the same reef.

In the model, spawning was induced by a combination of ambient temperature, accrual of degree-days above a threshold gametogenesis temperature, and a sufficient percentage of body mass allocated towards gonads (GSI). The ambient temperature necessary for inducing spawning (Shumway 1996) as well as the number of degree-days needing to be accrued have both been studied in Chesapeake Bay oysters (Mann et al. 2014), so these parameter values are fairly well established. However, the threshold GSI used to induce spawning is less well understood in the region. The available estimates of threshold GSI values for spawning in *C. virginica* are limited to oysters sourced from the Gulf of Mexico, Lavaud et al. (2021) with a GSI of 8 and Choi (1992) with a GSI of 20. Using either of these GSI values during model

calibration induced spawning at six or more timepoints throughout the model run which exceeded the predicted number of spawns oysters undergo in the bay each summer, indicating that these GSI values may not be properly parameterized for Chesapeake Bay oysters. Characteristics of oyster reproduction do vary geographically (Loosanoff and Nomejko 1951) so it is possible that oysters from the more temperate climate of the Chesapeake Bay would be triggered to spawn at a higher threshold GSI. *Crassostrea gigas*, a congener of *C. virginica*, tends to inhabit more temperate habitats and their estimated threshold GSIs for spawning – ranging between 35-50 (Pouvreau et al. 2006, Thomas et al. 2016, van deer Veer 2006) – are much higher than those reported for Gulf of Mexico *C. virginica*. Since an accurate estimate of spawning GSI for Chesapeake Bay oysters was not available for use in the current model, a proxy of GSI was used to parameterize spawning. The GSI threshold of 30 used for this study was determined based off of a gonadal area index from a study of oyster reproduction in nearby Delaware Bay (Barber et al. 1991). In contrast to GSI – measured as the ratio of gonad dry weight to total dry tissue weight – gonadal area index is measured as a ratio of gonadal area to the entire visceral mass. While not an exact measure of GSI, the use of this value produced spawning patterns for oysters in the Chesapeake Bay more consistent with the predicted number of spawns in the bay (Mann et al. 2014).

Empirically measured data on oyster gonad mass was not available to validate the model predictions for reproductive output. Given that the reproductive output as measured by number of spawned eggs matched what would be expected based on

oyster size (discussed above), gonad masses likely reflect accurate values of reproductive outputs. However, it is plausible that hypoxia exposure could impose additional energetic stress on oyster's reproductive activities. Hypoxia has been shown to be an endocrine disruptor in fish, limiting gametogenesis and reducing the quality of gametes (Wu et al. 2003). Without observed measurements of gonad mass for oysters from sites experiencing differing oxygen availability, it is unknown whether additional constraints could be imposed on reproduction in *C. virginica*. Further parameterization of the reproductive aspect of the *C. virginica* DEB model with empirical data would provide more certainty in the model's reproductive outputs.

Overall Model Caveats and Future Directions

The overall fit of the model to measured oyster growth in the Chesapeake Bay indicates that the model provides an accurate estimate of oyster growth even though most parameters used in this DEB model were sourced from the literature and not estimated from empirical data collected on geographically proximal individuals. The energetic parameters used for the study were primarily parameterized for oysters sourced from the Gulf of Mexico (Lavaud et al. 2021) and model calibration required alterations to only one parameter, volume-specific somatic maintenance costs. In general, DEB parameters are typically relatively well-constrained for a given species (Kooijman 2000) and, for some parameters, even show similar values between closely related species (e.g., van der Veer et al. 2006). However, non-stationarity of DEB parameters between geographically distant populations has been found, owing

perhaps to local adaptation to differing environmental conditions between populations (Monaco et al. 2019). Volume-specific somatic maintenance costs have been shown to vary considerably between different parameterizations for *C. virginica*, from 4.166 J/cm³d (Lavaud and Kooijman 2018) to 38 J/cm³d (Lavaud et al. 2021), although the value used in the present study (30 J/cm³d) does fall within the range of previous values. The difference in values may indicate that this parameter is not stationary and requires additional estimation, perhaps using oysters from different geographical locations to provide an optimal estimate.

The results of the sensitivity analysis provide an assessment as to how changes in parameter values affected the final oyster shell height. Overall, variation in most parameters led to minimal changes in final oyster size. The greatest sensitivities were shown for 50% decreases in the volume-specific ingestion rate, kappa value, and energy conductance. Reductions to volume-specific ingestion rate would strongly influence oyster growth as ingestion drives energy uptake which would decrease the available energy for growth. Similarly, it is expected that the change in kappa parameter would greatly affect oyster shell length given that it controls the allocation of energy between somatic and reproductive tissue. Similar high sensitivities for kappa have been observed for DEB models developed for other species (e.g., Pouvreau et al. 2006). Finally, energy conductance plays a part in food-energy conversion, so similar to ingestion rate, it would strongly influence the ability of an oyster to grow in size. While the sensitivity of the model to changes in some parameter values may seem discouraging with respect to the utility of this model,

given the close estimation between simulated and observed oyster shell lengths, it's likely that the parameter values used in this study reflect values well-parameterized for this species.

The present model provides an important first step in modeling the bioenergetic effects of low dissolved oxygen on adult oyster growth and reproduction, but additions to the model will allow for its application more broadly. One limitation of the present model is that it only focuses on the effect of hypoxia on adult oyster growth. Hypoxia also affects bioenergetics of larval and juvenile oysters, as indicated by reduced feeding rates, reduced growth rates, increased mortality, and delayed development (Baker and Mann 1992, Baker and Mann 1994, Widdows et al. 1989). However, the current parameterization of the oxygen correction factor may not be easily transferable to earlier oyster life-stages because metabolic responses to hypoxia do appear to vary ontogenetically in other marine invertebrates (Leiva et al. 2018). The utility of DEB models is in the ability to follow an individual throughout their full life cycle, so incorporating larvae and juveniles into the model is crucial. Future work integrating additional life-stages into the model will allow for better predictions of how hypoxia exposure will affect oysters over their complete life cycle, but requires targeted experimentation at each life stage.

Additionally, the model assumes additive effects of salinity and temperature on oyster bioenergetics, which may not accurately reflect oyster response to co-occurring temperature or salinity stress. The environmental data used to force the current model

did not expose the oysters to any extremes in either temperature or salinity so interactions between stressors did not complicate the present study. However, there is potential for synergism between temperature and salinity stress and low oxygen stress on organismal energetics (e.g., Wang et al. 2011, Marcek et al. 2020) and these should be considered in future model iterations. For *C. virginica*, increases in the incidence of high temperatures and hyposalinity are both expected, which would likely coincide with periods of seasonal hypoxia (Najjar et al. 2010), but there is currently limited empirical data available on the energetic effects of co-occurring stressors with hypoxia for *C. virginica*. Future studies that examine the interactions between stressors on *C. virginica* energetics would be valuable for use with the model, allowing for more accurate predictions of long-term effects of hypoxia and co-occurring stressors on oysters.

Conclusions

Integration of dissolved oxygen into the DEB model for *C. virginica* provides an important first step in the ability to mechanistically predict the effects of hypoxic exposure on oyster growth and reproduction. The model developed in the present study indicates that exposure to hypoxia decreases oyster shell and tissue growth and leads to decreases in oyster fecundity, as well as changes to spawning frequency. Future empirical studies focusing on the potential for synergistic effects of temperature, salinity, and dissolved oxygen stress on oyster energetics, as well as influences of hypoxia on oyster reproduction would allow for more confidence in model outputs. Additionally, incorporation of early life-stages into the model will

allow for predictions to be made across the full life cycle of *C. virginica*. While this additional work will increase the versatility of the model, in its current iteration, the ability of the model to accurately predict adult oyster growth under differing oxygen regimes makes it an important tool to make predictions about how oysters will respond to the increased frequency of hypoxic exposures that are expected in the future due to climate change.

Table 4.1: Equations used in the *C. virginica* dynamic energy budget (DEB) model.

	Equation	Term	Term definition
1	$f = \frac{X}{X + X_k}$	f	Scaled functional response
		X	Food density
		X_k	Half-saturation constant
2	$C_S = \begin{cases} 1, & \text{at } S \geq S_H \\ \frac{S - S_L}{S_H - S_L}, & \text{at } S_L < S < S_H \\ 0, & \text{at } S \leq S_L \end{cases}$	C_S	Salinity correction factor
		S	Salinity
		S_L	Lower salinity threshold
		S_H	Upper salinity threshold
3	$C_T = \exp\left\{\frac{T_A - T}{T_1}\right\} \left(1 + \exp\left\{\frac{T_{AL} - T}{T_1}\right\} + \exp\left\{\frac{T_{AH} - T}{T_1}\right\}\right) \left(1 + \exp\left\{\frac{T_{AL} - T}{T_L}\right\} + \exp\left\{\frac{T_{AH} - T}{T_H}\right\}\right)^{-1}$	C_T	Temperature correction factor
		T_A	Arrhenius temperature
		T_1	Reference temperature
		T_L	Lower boundary tolerance range
		T_H	Upper boundary tolerance range
		T_{AL}	Arrhenius temperature for lower boundary
		T_{AH}	Arrhenius temperature for upper boundary
4	$\dot{p}_A = C_T C_S C_{DO} \kappa_A \{ \dot{p}_{Am} \} f V^{2/3}$	\dot{p}_A	Assimilation rate
		C_{DO}	Oxygen correction factor
		κ_A	Assimilation efficiency
		$\{ \dot{p}_{Am} \}$	Maximum surface area specific assimilation rate
		V	Structural volume
5	$\dot{p}_{C1} = C_{DO} \left([E] \left(\frac{C_T [E_G] \dot{v} V^{2/3} + C_T \dot{p}_M}{[E_G] + \kappa [E]} \right) \right)$	\dot{p}_{C1}	Mobilization rate of reserve energy
		\dot{v}	Energy conductance
		κ	Fraction of utilized energy to somatic maintenance and growth
		$[E_G]$	Volume-specific costs for structure
		\dot{p}_M	Maintenance rate
7	$\dot{p}_G = \kappa \dot{p}_{C1} - \dot{p}_{M1}$	\dot{p}_G	Structural growth rate
8	$\dot{p}_{M1} = ([\dot{p}_M] V) C_T$	$[\dot{p}_M]$	Volume specific maintenance costs
9	$\dot{p}_J = \min(V, V_p) [\dot{p}_M] \left(\frac{1 - \kappa}{\kappa} \right) C_T$	\dot{p}_J	Maturity maintenance rate

10	$\dot{p}_R = (1 - \kappa)\dot{p}_{C1} - \dot{p}_J$	V_p	Volume at puberty
11	$\dot{p}_{L1} = \max(\dot{p}_{M1} - (\kappa \dot{p}_{C1} + \dot{p}_{M2} + \dot{p}_{L2}), 0)$	\dot{p}_R	Maturation and reproduction rate
12	$\dot{p}_{C2} = E_R \left(\frac{\{\dot{p}_{Am}\}\kappa_A C_T}{[E_m]V^{1/3}} + \frac{[\dot{p}_M]C_T}{[E_G]} \right) \left(\{1 - \kappa\} \frac{E}{[E_G]V + \kappa E} \right)$ if $V \geq V_p$, otherwise 0	\dot{p}_{L1}	Lysis of structure rate
13	$\dot{p}_{M2} = \min(\dot{p}_{M1} - \kappa\dot{p}_{C1}, \dot{p}_{C2})$	\dot{p}_{C2}	Gamete mobilization rate
14	$\dot{p}_{GO} = \dot{p}_{C2} - \dot{p}_{M2}$	$[E_m]$	Maximum storage density
15	$p_{L2} = \max\left(\frac{\dot{p}_{M1} - \kappa\dot{p}_{C1} + \dot{p}_{M2}}{Y_{GO}}, 0\right)$	\dot{p}_{M2}	Emergency maintenance rate
16	$L = \frac{V^{1/3}}{\delta_V}$	\dot{p}_{GO}	Gonad allocation rate
17		p_{L2}	Gamete resorption rate
17	$DD = d(T - T_G)$	Y_{GO}	Yield of gonad tissue used for maintenance
		L	Oyster length
		δ_V	Shape coefficient
		DD	Degree-days
		T_G	Threshold temperature for gametogenesis
Differential Equations			
18	$\frac{dE}{dt} = \dot{p}_A - \dot{p}_{C1}$		Reserves
19	$\frac{dV}{dt} = \left(\frac{\dot{p}_G - \dot{p}_{L1}}{[E_G]} \right)$		Structural volume
20	$\frac{dE_R}{dt} = \dot{p}_R - \dot{p}_{C2}$		Reproduction
21	$\frac{dE_{GO}}{dt} = \dot{p}_{GO} - \dot{p}_{L2}$		Gametes

Table 4.2: State variables and parameters used in the *C. virginica* DEB model.

State Variable	Symbol	Unit		
Structural volume	V	cm^3		
Reserve energy	E	J		
Reproduction energy	E_R	J		
Gonad energy	E_{GO}	J		
Parameter	Symbol	Value	Unit	Source
Half-saturation constant	X_k	1.5	$\mu\text{g chl l}^{-1}$	Lavaud et al. 2017 Powell et al. 1995,
Shape coefficient	δ_V	0.24884	--	Lavaud et al. 2017
Maximum surface area-specific ingestion rate	$\{\dot{p}_{X_m}\}$	249.5	$\text{J cm}^{-2} \text{d}^{-1}$	Lavaud et al. 2017
Surface area-specific maximum assimilation rate	$\{\dot{p}_{A_m}\}$	187.125	$\text{J cm}^{-2} \text{d}^{-1}$	$\{\dot{p}_{X_m}\} * \kappa_A$ Gerdes 1983, Lavaud et al. 2017
Assimilation efficiency	κ_A	0.75	--	
Fraction of reproduction buffer fixed into eggs	κ_R	0.95	--	Lavaud et al. 2021
Allocation fraction	κ	0.82	--	Lavaud et al. 2017
Yield of gonad tissue used for maintenance	Y_{GO}	0.25	--	Bernard et al. 2011 Calibrated in this study
Volume-specific somatic maintenance costs	$[\dot{p}_M]$	30	$\text{J cm}^{-3} \text{d}^{-1}$	
Volume-specific cost for structural growth	$[E_G]$	5230	J cm^{-3}	Lavaud et al. 2017
Maximum reserve density	$[E_m]$	5420	J cm^{-3}	Lavaud et al. 2017
Maturity threshold at puberty	E_H^p	369.9	J	Lavaud et al. 2017
GSI threshold for spawning	GSI	30	-	Barber et al. 1991 Kennedy and Krantz 1982
Spawning efficiency	k_{sp}	1	-	
Energy conductance	\dot{v}	0.03453	cm d^{-1}	Lavaud et al. 2021
Maturity maintenance rate coefficient	\dot{k}_J	0.002	d^{-1}	Lavaud et al. 2021
<i>Salinity Function</i>				
Upper salinity threshold	S_H	10	ppt	Lavaud et al. 2017
Lower salinity threshold	S_L	3	ppt	Lavaud et al. 2017
<i>Temperature function</i>				
Arrhenius temperature	T_A	6292	K	Shumway and Koehn 1982
Reference temperature	T_I	293	K	--
Lower boundary tolerance range	T_L	283	K	Galtsoff 1964
Upper boundary tolerance range	T_H	303	K	Galtsoff 1964
Arrhenius temperature for lower boundary	T_{AL}	21820	K	Lavaud et al. 2017
Arrhenius temperature for upper boundary	T_{AH}	45380	K	Lavaud et al. 2017 Price and Mauer 1971
Temperature threshold for gametogenesis	T_G	12	$^{\circ}\text{C}$	
Temperature threshold for spawning	T_{sp}	20	$^{\circ}\text{C}$	Shumway 1996

Additional and compound parameters	Symbol	Value	Unit	Source
Structure density	d_V	0.2	gdw gww ⁻¹	Lavaud et al. 2017
Chemical potential of structure	μ_V	15600	J g ⁻¹	Bernard et al. 2011
Chemical potential of reserve	μ_E	19600	J g ⁻¹	Bernard et al. 2011
Dry weight to wet weight ratio	$dw:ww$	0.2	--	Lavaud et al. 2017
Egg energy	ρ_{egg}	$2.566 \cdot 10^{-4}$	J g ⁻¹	Lavaud et al. 2021
Dry mass of egg	W_{egg}	$4 \cdot 10^{-8}$	g egg ⁻¹	Lavaud et al. 2021

Table 4.3: Percentage of datapoints requiring interpolation for each of the environmental datasets used in model development and predictions.

Site	Use	Temperature (°C)	Chlorophyll- <i>a</i> (µg/l)	Salinity (ppt)	Dissolved Oxygen (mg/l)
CBL	Calibration	0%	0%	0%	0%
CCR	Calibration	13.2%	13.3%	13.3%	13.3%
BWH	Validation	4.3%	4.5%	4.3%	4.3%
HCR	Validation	5.0%	11.2%	1.8%	4.8%
LMN	Validation	7.5%	24.2%	7.5%	7.5%
MUL	Validation	14.1%	15.3%	0.02%	<0.01%
SMC	Validation	0%	0.72%	0%	0%
YEO	Validation	8.9%	8.9%	8.9%	8.9%
BBY	Prediction	6.1%	7.2%	6.1%	11.9%
WIB	Prediction	6.9%	13.9%	10.3%	29.9%

Table 4.4: Root-mean square error between observed and predicted oyster shell lengths for the four tested alternative models of dissolved oxygen integration. Models were run on both calibration sites Chesapeake Biological Lab (CBL) and Cole’s Creek (CCR).

	Site	
	CBL	CCR
No oxygen effect	0.173	0.815
Static C_{O_2c}	0.173	0.788
Dynamic C_{O_2c}	0.137	0.496
Full model	0.132	0.413

Table 4.5: Results of the sensitivity (S) test for the calibration datasets (CBL and CCR). Sensitivity calculated using Equation 6.

Parameter	CBL		CCR	
	+50	-50	+50	-50
X_K	-1.25%	1.36%	-1.15%	1.26%
$[\dot{p}_M]$	-7.89%	9.34%	-7.95%	9.94%
$\{\dot{p}_{Xm}\}$	9.20%	-13.99%	8.92%	-13.17%
$\{\dot{p}_{Am}\}$	2.37%	-2.72%	2.47%	-2.83%
κ	5.44%*	-17.56%	5.32%*	-16.81%
$[E_G]$	4.76%	2.76%	-4.22%	2.27%
\dot{v}	6.22%	-11.32%	5.57%	-10.50%

*Since κ represents a proportion, the +50% value was set to 1.

Table 4.6: Deviations (%) between observed and simulated shell lengths for the sites used in model validation. Values calculated using Equation 7.

Site	F
<i>Normoxic</i>	
BWH	0.783
MUL	1.456
YEO	6.339
All normoxic sites	2.508
<i>Hypoxic</i>	
BBY	0.748
HCR	2.932
LMN	1.803
All hypoxic sites	2.044
<i>All Sites</i>	2.250

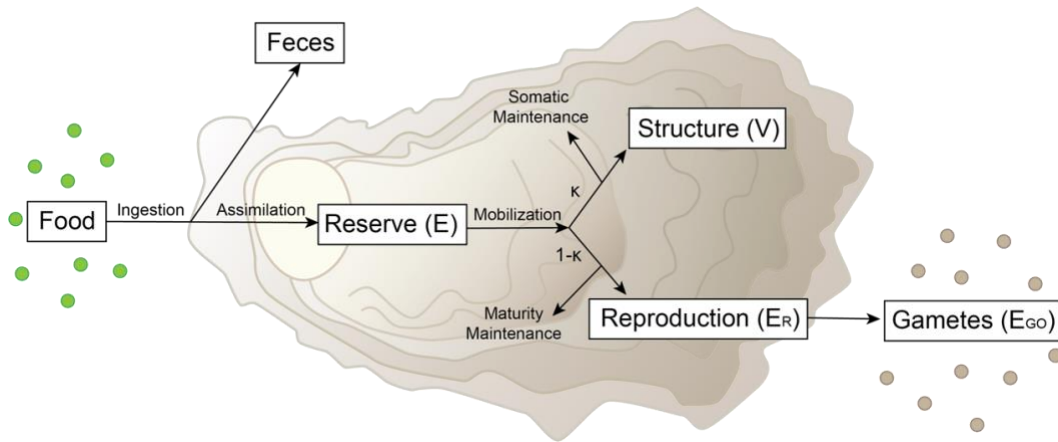


Figure 4.1: Conceptual diagram of the DEB model.

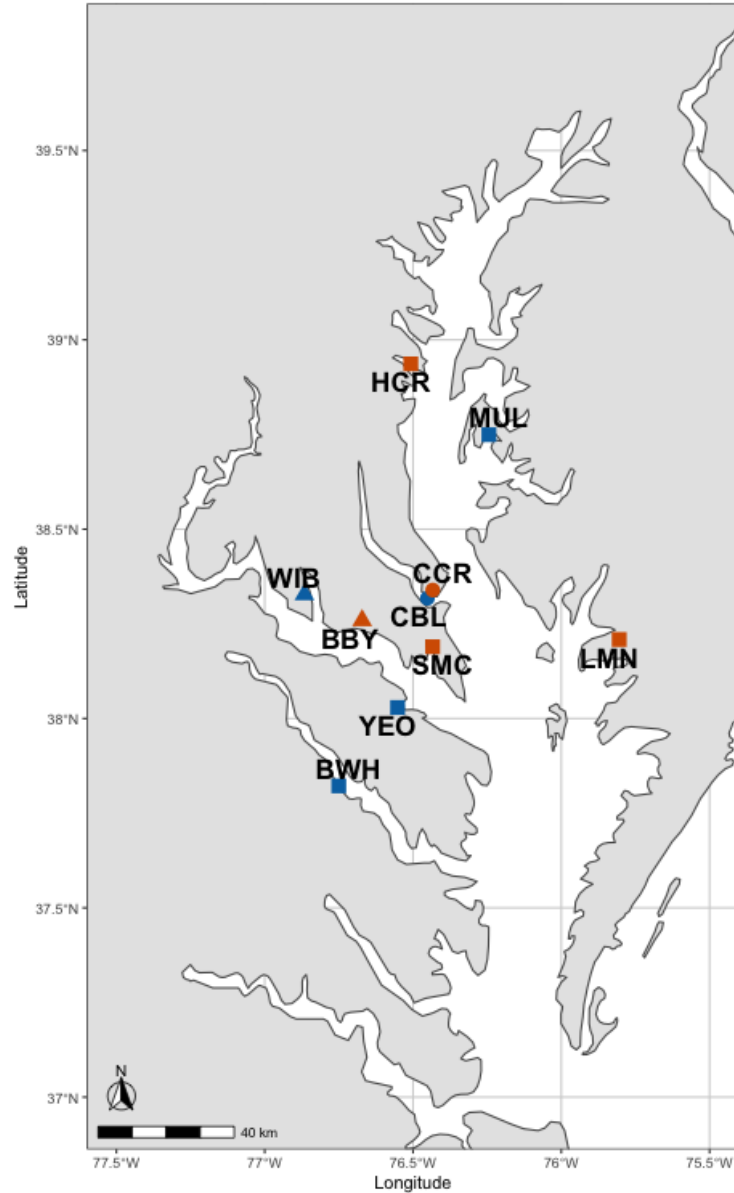


Figure 4.2: Map of sites used in the modeling analyses. Low oxygen sites are denoted by orange markers and normoxic sites are denoted by blue markers. Calibration sites (●), Chesapeake Biological Lab (CBL) and Cole’s Creek (CCR). Validation sites (■), Harness Creek (HCR), Bowler’s Wharf, Rappahannock River (BWH), Little Monie Creek (LMN), Mulberry Point (MUL), St. Mary’s College (SMC), and Yeocomico River (YEO). Prediction sites (▲), Breton Bay (BBY) and Wicomico Beach (WIB).

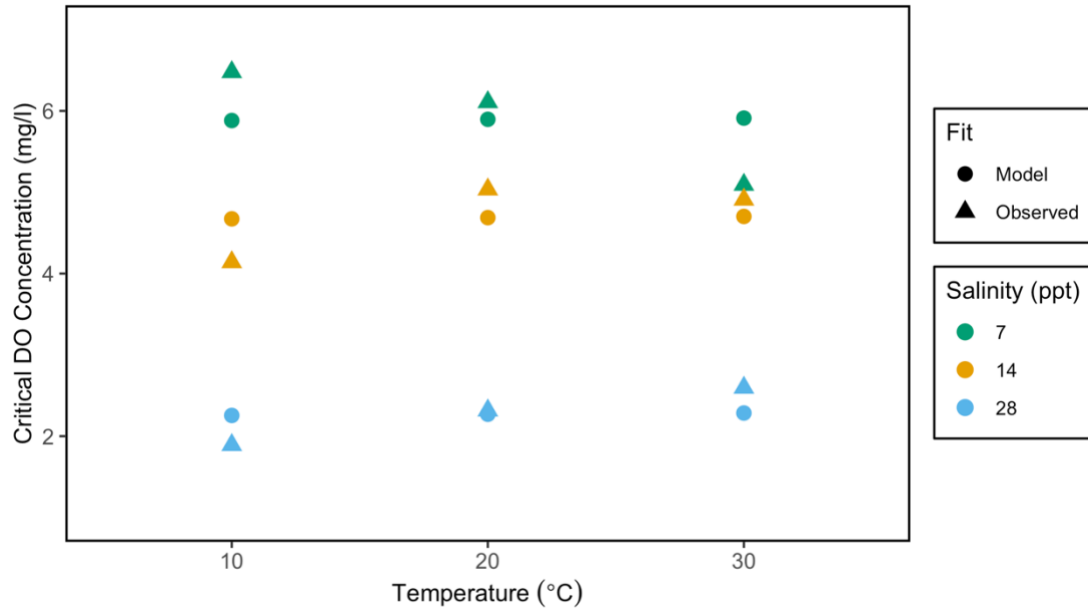


Figure 4.3: Comparison of observed critical oxygen concentrations across three temperatures and three salinities from Shumway and Koehn (1982) versus the predicted values from the linear model (Equation 3).

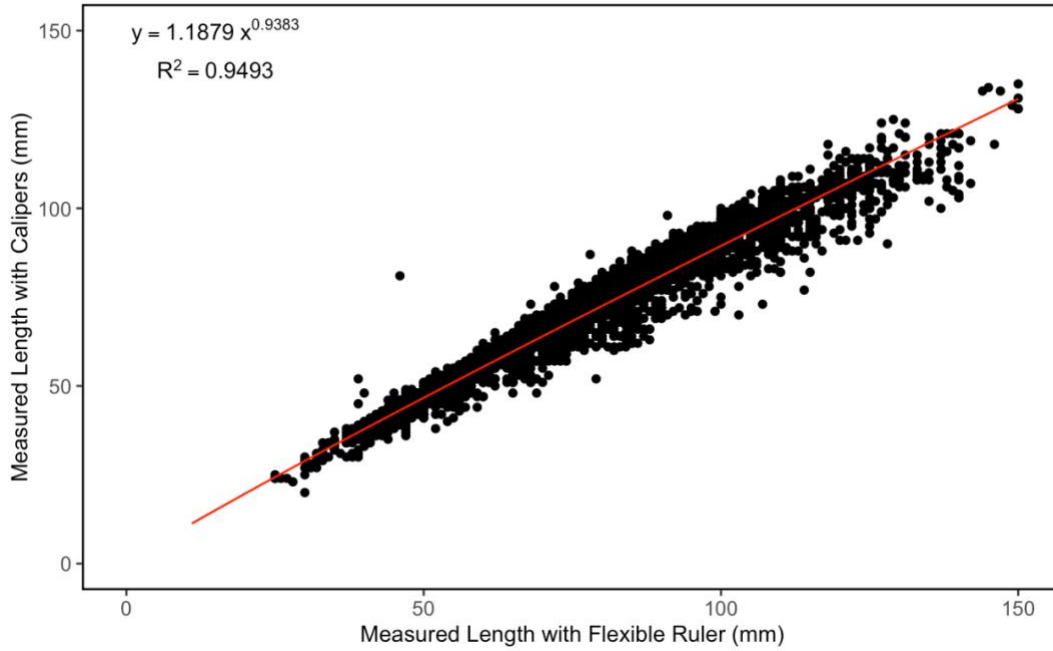


Figure 4.4: Comparison of shell lengths measured with a flexible ruler versus calipers for the calibration and validation growth data. Red line denotes best fit power function ($p < 0.001$).

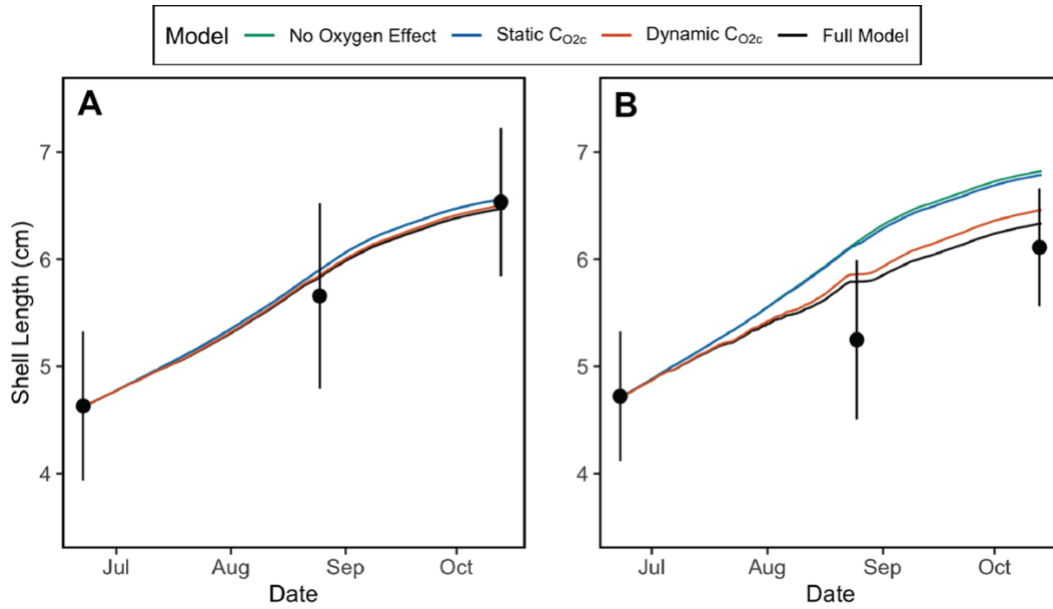


Figure 4.5: Comparisons of different integrations of dissolved oxygen effect into DEB model for the two calibration sites A) CBL and B) CCR. Black points correspond to observed shell lengths for oysters from the two sites and error bars represent standard deviation.

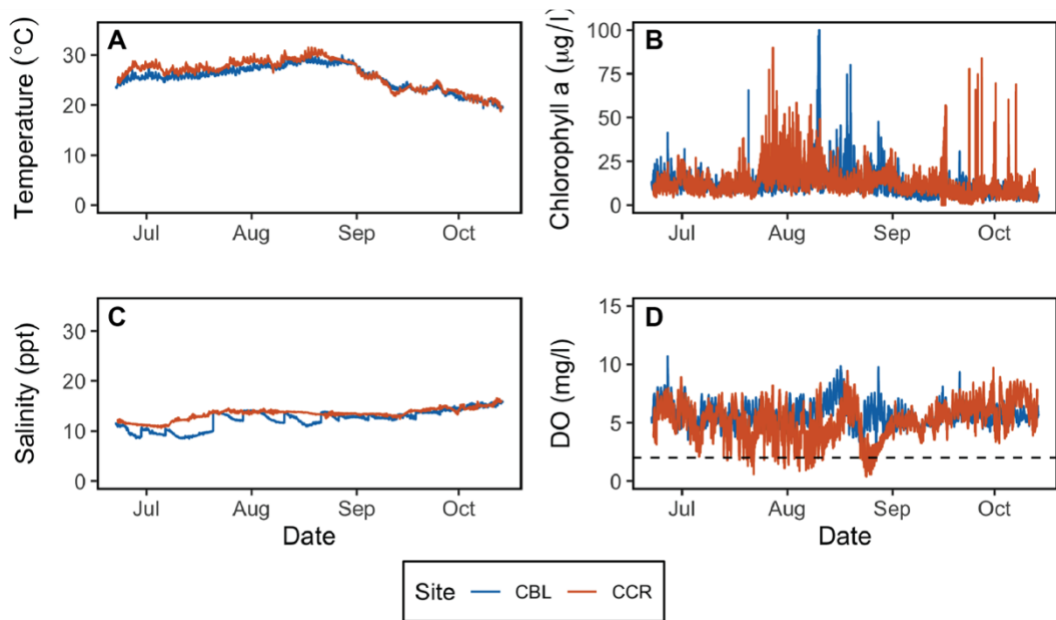


Figure 4.6: Environmental data for the calibration growth datasets at sites CBL and CCR. A) Temperature ($^{\circ}\text{C}$), B) Chlorophyll-*a* ($\mu\text{g/l}$), C) Salinity (ppt) and D) Dissolved oxygen (mg/l). Blue color denotes the ‘normoxic’ site while orange color denotes ‘low dissolved oxygen’ site.

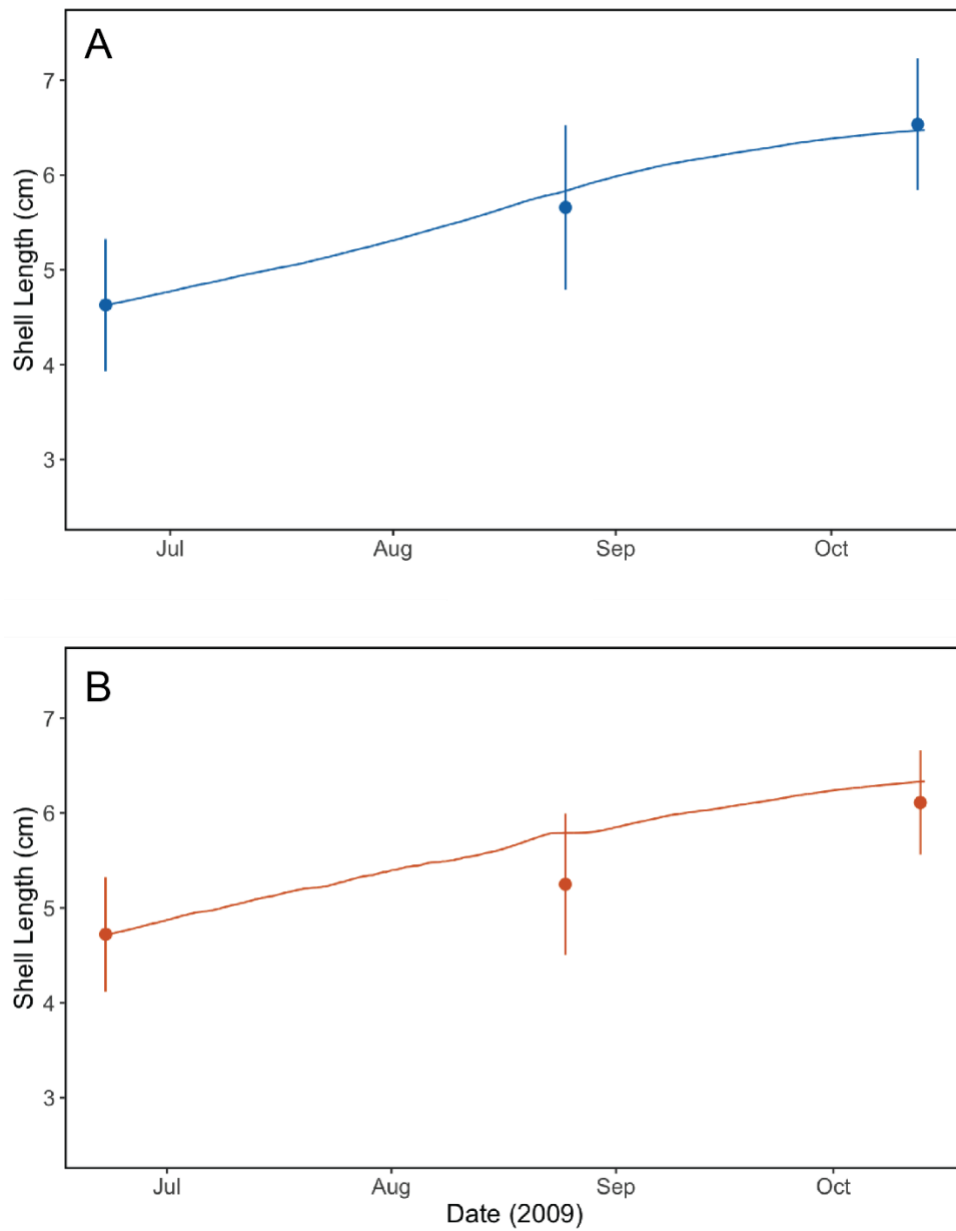


Figure 4.7: Results of the DEB model calibration showing predicted growth of *C. virginica* (line) in comparison to observed shell lengths (points) for oysters deployed at A) CBL (normoxic site) and B) CCR (low dissolved oxygen site). Bars on observed shell lengths correspond to standard deviation.

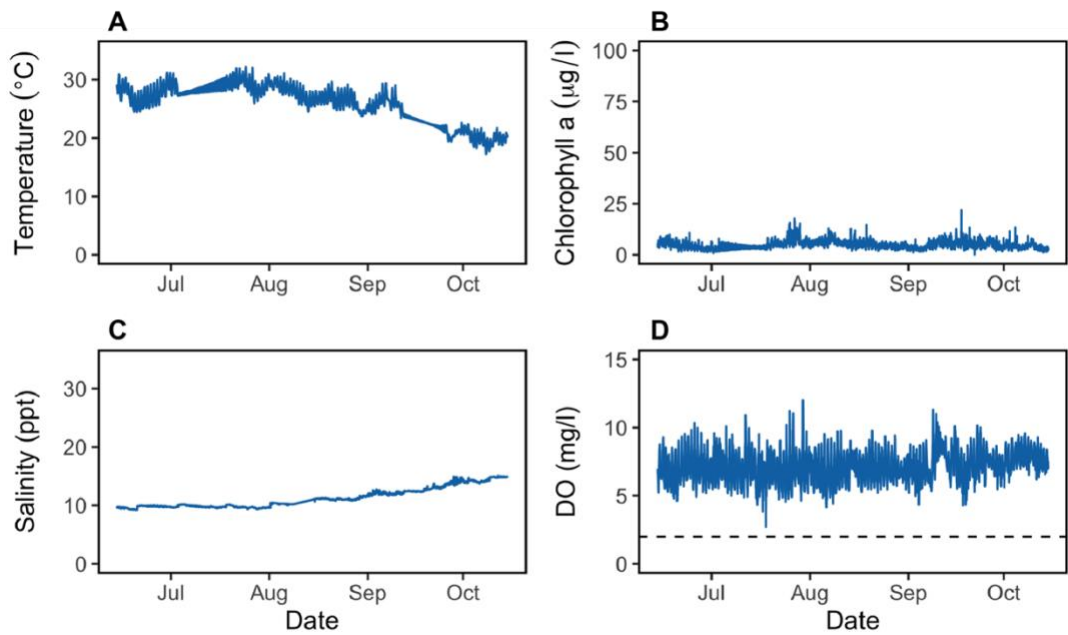


Figure 4.8: Environmental data for the validation oyster growth dataset at Mulberry Point (MUL). A) Temperature ($^{\circ}\text{C}$), B) Chlorophyll-*a* ($\mu\text{g/l}$), C) Salinity (ppt) and D) Dissolved oxygen (mg/l). The dashed line on the dissolved oxygen panel correspond to 2.0 mg/l or hypoxic conditions.

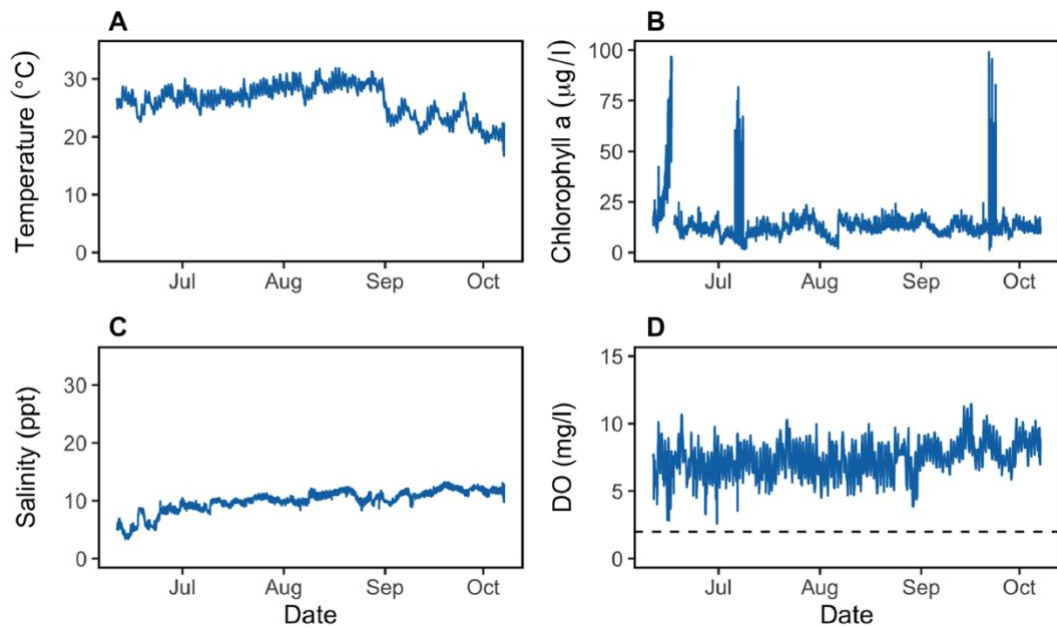


Figure 4.9: Environmental data for the validation oyster growth dataset at Bowler's Wharf (BWH). A) Temperature (°C), B) Chlorophyll-*a* (µg/l), C) Salinity (ppt) and D) Dissolved oxygen (mg/l). The dashed line on the dissolved oxygen panel correspond to 2.0 mg/l or hypoxic conditions.

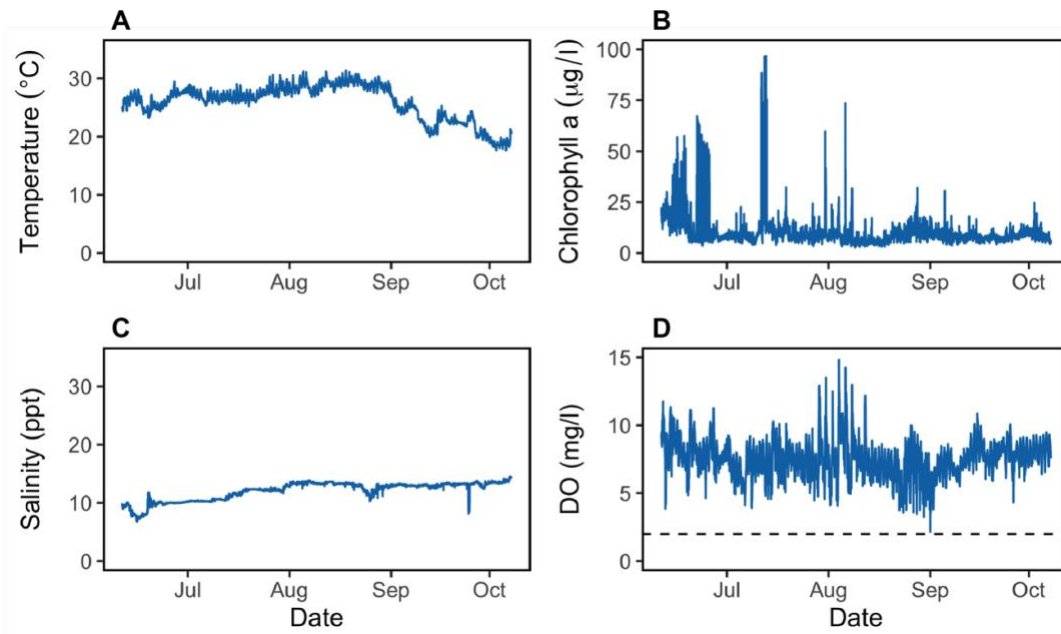


Figure 4.10: Environmental data for the validation oyster growth dataset at Yeocomico River (YEO). A) Temperature ($^{\circ}\text{C}$), B) Chlorophyll-*a* ($\mu\text{g/l}$), C) Salinity (ppt) and D) Dissolved oxygen (mg/l). The dashed line on the dissolved oxygen panel correspond to 2.0 mg/l or hypoxic conditions.

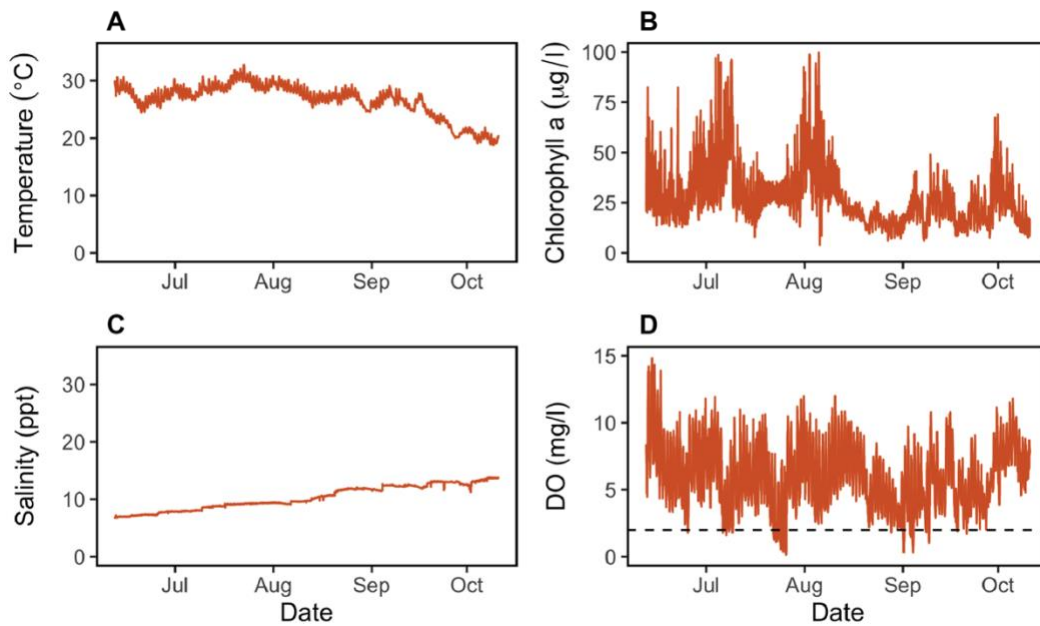


Figure 4.11: Environmental data for the validation oyster growth dataset at Harness Creek (HCR). A) Temperature ($^{\circ}\text{C}$), B) Chlorophyll-*a* ($\mu\text{g/l}$), C) Salinity (ppt) and D) Dissolved oxygen (mg/l). The dashed line on the dissolved oxygen panel correspond to 2.0 mg/l or hypoxic conditions.

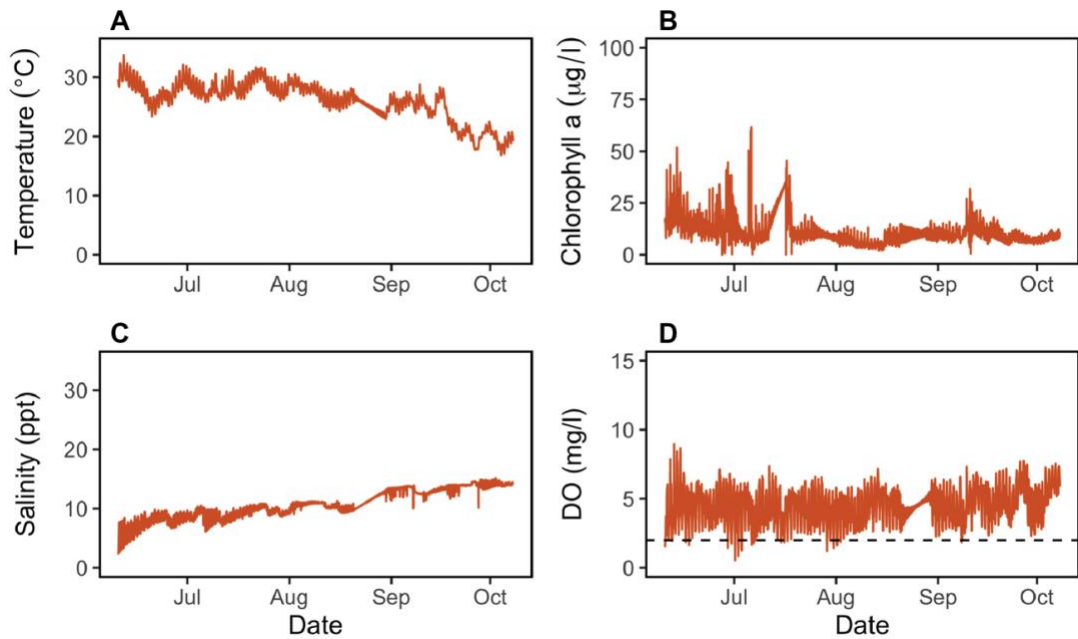


Figure 4.12: Environmental data for the validation oyster growth dataset at Little Monie Creek (LMN). A) Temperature ($^{\circ}\text{C}$), B) Chlorophyll-*a* ($\mu\text{g/l}$), C) Salinity (ppt) and D) Dissolved oxygen (mg/l). The dashed line on the dissolved oxygen panel correspond to 2.0 mg/l or hypoxic conditions.

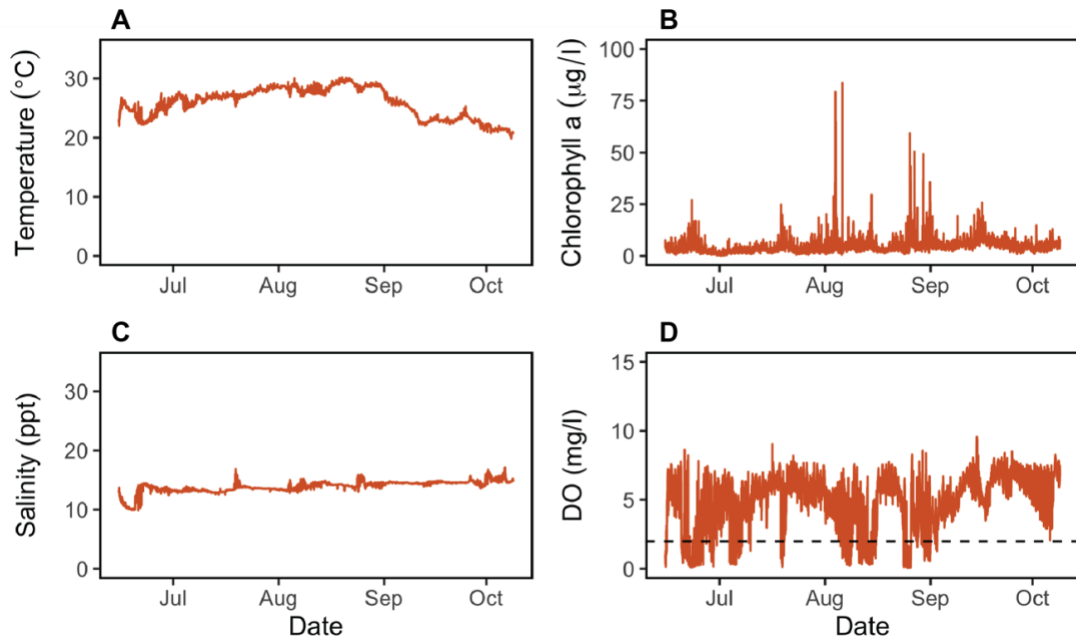


Figure 4.13: Environmental data for the validation oyster growth dataset at St. Mary's College (SMC). A) Temperature ($^{\circ}\text{C}$), B) Chlorophyll-*a* ($\mu\text{g/l}$), C) Salinity (ppt) and D) Dissolved oxygen (mg/l). The dashed line on the dissolved oxygen panel correspond to 2.0 mg/l or hypoxic conditions.

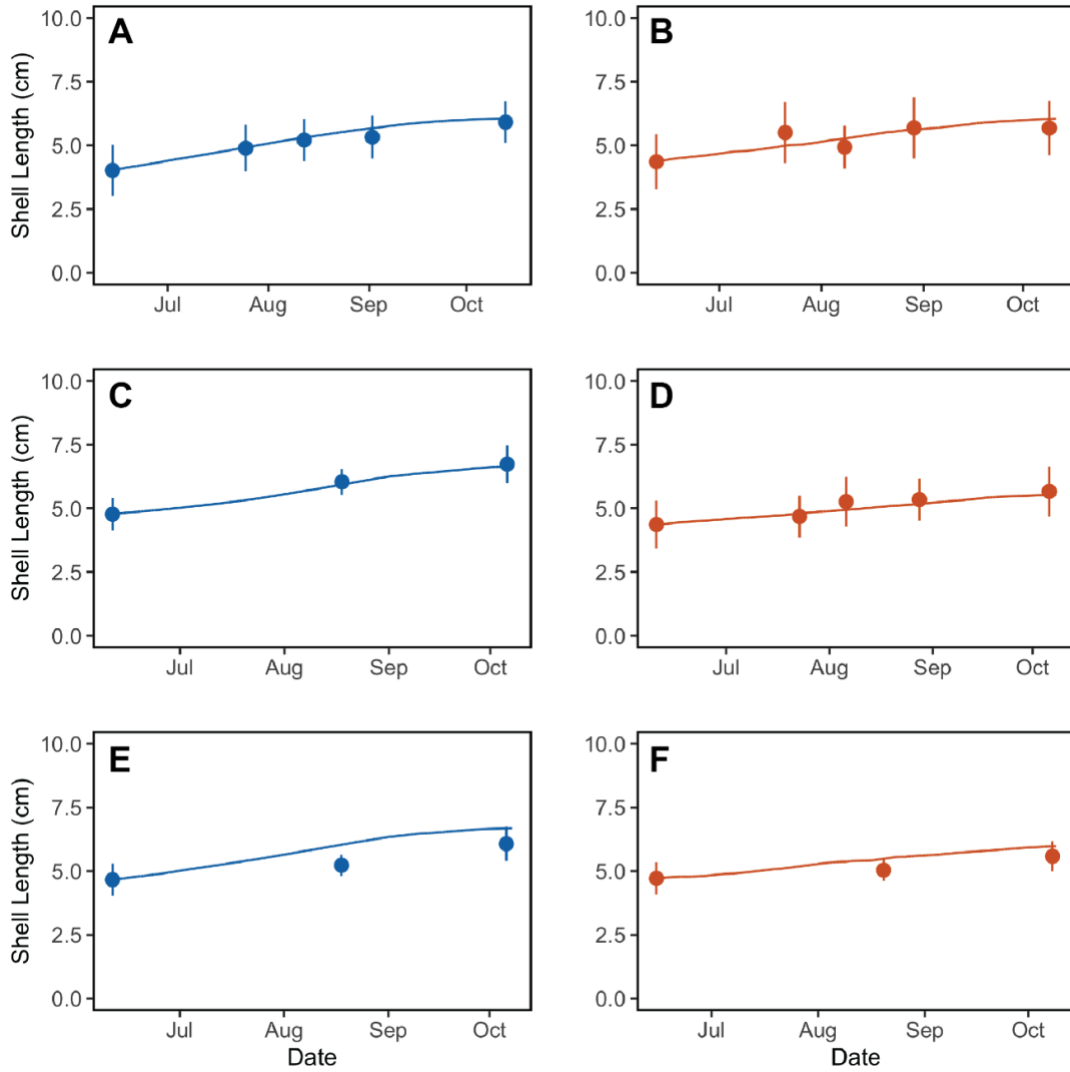


Figure 4.14: Oyster shell length (cm) growth for sites used in model validation. Normoxic sites MUL (A), BWH (C), and YEO (E) are shown in blue and hypoxic sites HCR (B), LMN (D), and SMC (F) are shown in orange. Lines denote predicted growth from model. Mean observed oyster sizes are shown as points and error bars denote standard deviation.

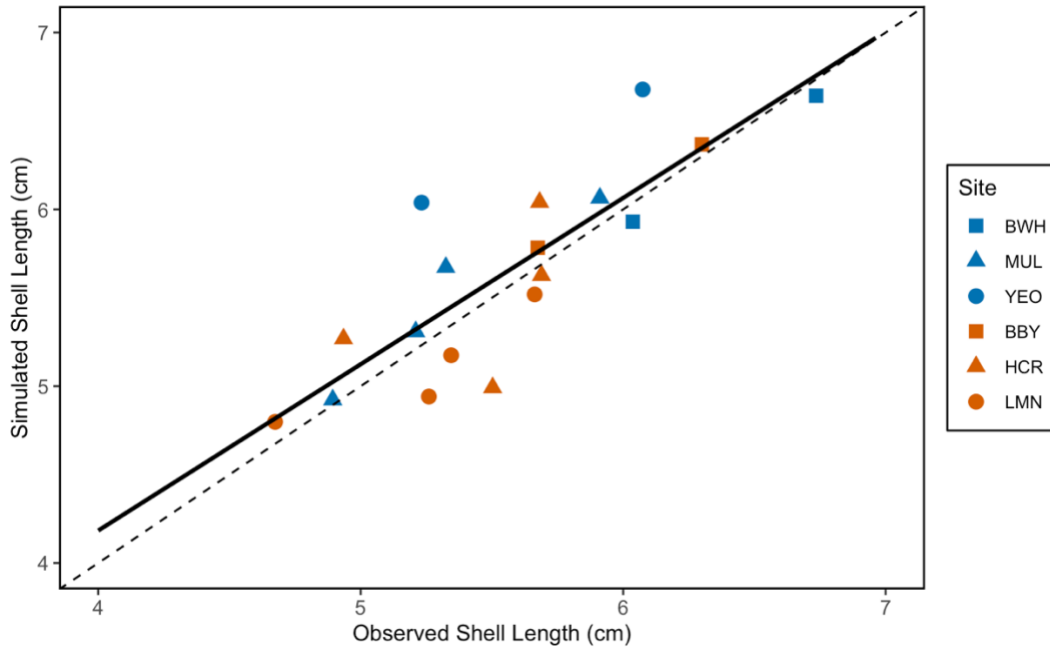


Figure 4.15: Observed versus simulated shell lengths for the six sites used in model validation. The dashed line denotes a 1:1 relationship between the modeled and observed shell lengths. The solid line corresponds to the linear regression line relating observed and simulated values (slope=0.94, intercept=0.42, $R^2=0.71$, $p < 0.001$).

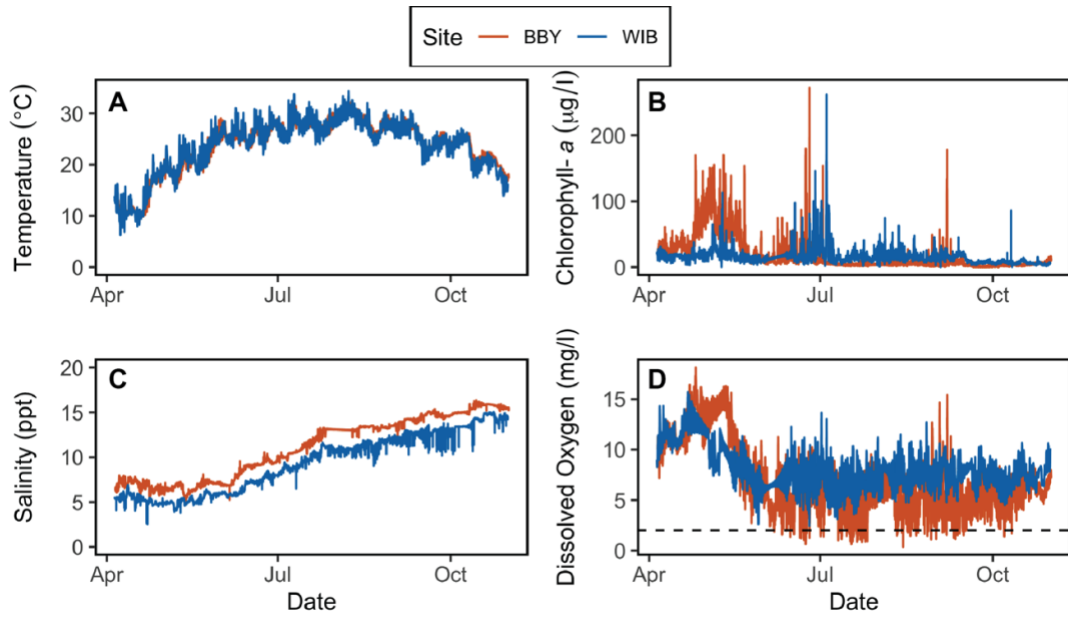


Figure 4.16: Environmental data for predicted oyster growth at Wicomico Beach (blue) and Breton Bay (orange). A) Temperature ($^{\circ}\text{C}$), B) Chlorophyll-*a* ($\mu\text{g/l}$), C) Salinity (ppt) and D) Dissolved oxygen (mg/l). The dashed line on the dissolved oxygen panel correspond to 2.0 mg/l or hypoxic conditions.

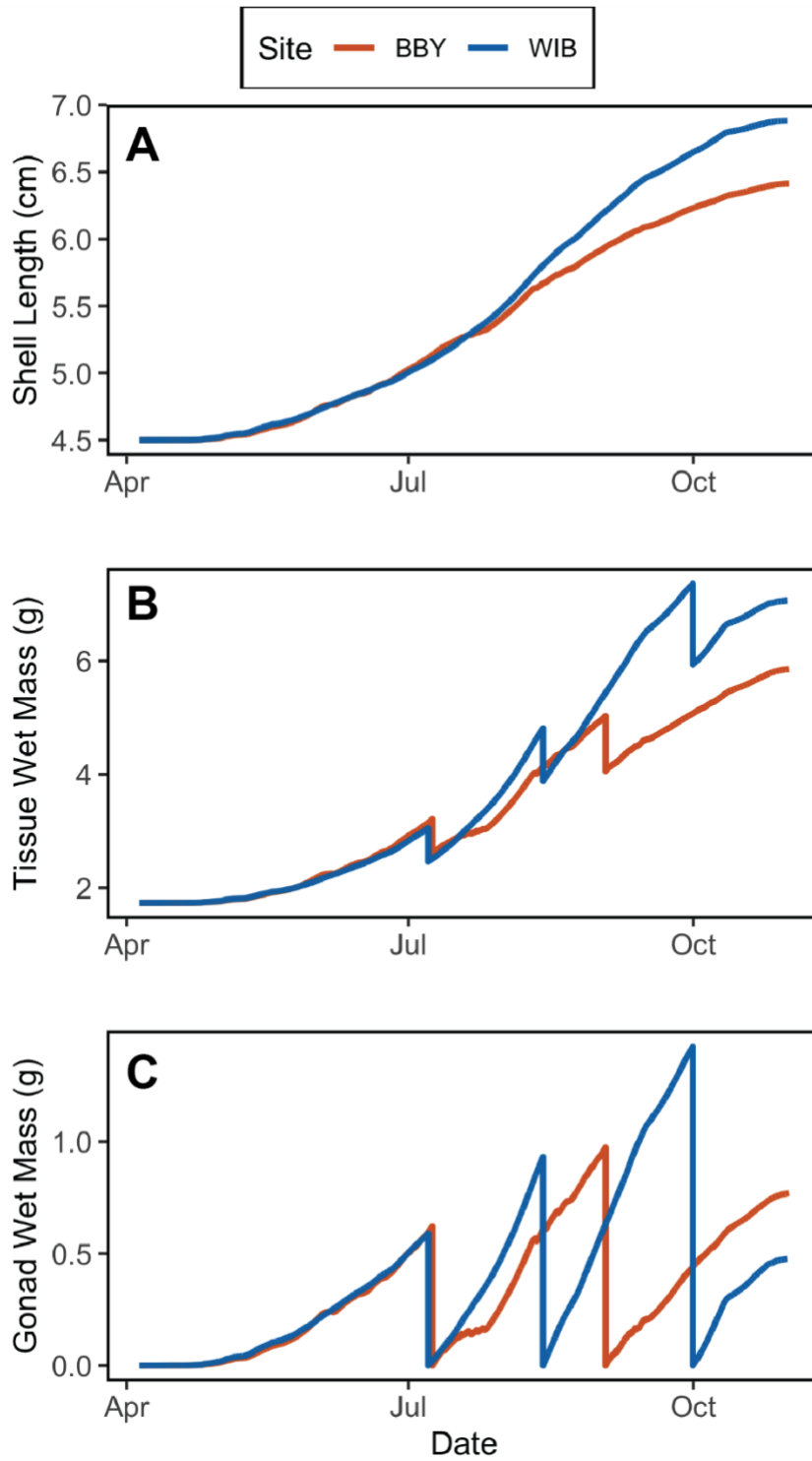


Figure 4.17: Predicted A) shell length, B) tissue wet mass, and C) gonad wet mass for Wicomico Beach (WIB) and Breton Bay (BBY)

CHAPTER 5: SYNTHESIS, CONCLUSIONS, AND FUTURE DIRECTIONS

The growing threat of hypoxia for coastal and oceanic waters (Breitburg et al. 2018) warrants the continued investigation of how organisms will mitigate the effects of this environmental change as well as making more urgent the development of novel tools to make predictions about the long-term effects of hypoxia on organismal ecology. For ecosystem engineers, such as the eastern oyster *Crassostrea virginica*, understanding the effects of hypoxia is particularly important as their ability to tolerate future conditions can influence the health and resilience of other species in the ecosystem (Thrush et al. 2009, Prather et al. 2013). This dissertation research focused on the effects of hypoxia on *C. virginica* in the Chesapeake Bay, addressing two main questions. First, to what extent do oysters in the Chesapeake Bay vary in their ability to tolerate low oxygen exposure and what mechanisms may be used to improve tolerance? Second, what are the effects of low oxygen availability on oyster population ecology?

Chapter 2 Summary

Chapter 2 focused on intraspecific variability in tolerance to low oxygen in *C. virginica* as measured through comparisons of survival and heart rate responses for oysters with varying histories of seasonal low oxygen exposure on their native reefs. There has been limited research focused on explicitly testing for intraspecific variation in bivalve tolerance to hypoxia as a result of prior hypoxia exposure (but see Ford 2005, Altieri 2006, Yamada et al. 2016), thus, this dissertation research provides

broadened knowledge and unique insights in understanding differential tolerance to low oxygen in a marine bivalve. Results of this chapter showed that hypoxia had an overall strong negative effect on oysters. Regardless of the site from which they were collected, oysters experienced significant increases in mortality and significant decreases in heart rate when challenged with low oxygen exposure in the lab. Additionally, larger oysters, as measured through wet weight, tended to die at a higher rate than smaller oysters in low oxygen. Oysters in these studies did not exhibit differing lethal tolerance to low oxygen between sites, as evidenced by the lack of significant site effect on mortality risk or LT_{50} values. However, site-level differences were observed in oyster heart rate response. Oysters sourced from two sites that experienced the greatest frequency of prior hypoxia exposure reduced their heart rate by a smaller proportion under hypoxic stress than oysters from other sites (one of the two sites showed a statistically significant difference while the other was the same trend but not statistically significant). Overall, these results indicate that oysters with prior hypoxia exposure do not have higher lethal tolerance to low oxygen but may show some degree of sublethal physiological difference in tolerance, particularly when they have previously experienced more frequent exposure to hypoxia.

Two key conclusions about oyster response to hypoxia can be drawn from the results presented in this research chapter. First, although not a novel finding, exposure to reduced oxygen availability, regardless of an oyster's prior exposure to hypoxia, has strong negative consequences for oyster survival and physiology. Second, and

perhaps more importantly, larger individuals are more susceptible to mortality under low oxygen conditions. Both of these individual effects of hypoxia on oysters have the potential to scale more broadly to influence their ecology (discussed below).

Chapter 3 Summary

In *Chapter 3*, the transcriptomic response of oysters to low oxygen was used to assess intraspecific variability in stress response between oysters with differing prior exposure to hypoxia. Results provided an understanding of the global gene expression response of oysters to low oxygen, as well as demonstrating the potential for site-level differences in gene expression. While the transcriptomic response of oysters and other bivalves to hypoxic stress has been studied elsewhere (e.g., David et al. 2005, Piontkivska et al. 2011, Giannetto et al. 2015, Nie et al. 2020), the comparison among bivalves with differing histories of low oxygen exposure conducted in this study provides novel insights into intraspecific variability in gene expression response to hypoxia. Overall, there was a strong gene expression response of oysters to low oxygen exposure, shown through principal components analysis, differential gene expression analysis, and gene correlation network analysis. Statistically significant site-specific differences in gene expression response to hypoxia were not observed through either PERMANOVA or the gene network analysis. However, expression patterns for genes from two of the gene network modules indicated that certain trends may exist related to an oyster's prior exposure to low oxygen that merits future investigation. Finally, a pattern supporting transcriptional frontloading, a mechanism of increasing tolerance to stressful conditions through the increased constitutive

expression of key stress response genes, was observed in the oysters from the low oxygen sites, although the test used to assess statistical significance of this effect was not significant.

Results from *Chapter 3* indicate that gene expression differences may exist between oysters with differing prior exposure to hypoxia and warrants further investigation. Despite the observed lack of site-level differences in response, results still provided important insights into the molecular response of oysters to low oxygen.

Characterization of specific differentially expressed genes indicates that *C. virginica* undergoes a similar molecular response to low oxygen to other bivalves studied (David et al. 2005, Sussarellu et al. 2010, Nie et al. 2020). This study was also the first to explicitly test for the presence of transcriptional frontloading in response to prior low oxygen exposure in a marine invertebrate. The lack of a statistically significant result for transcriptional frontloading may have resulted from the small sample size or an insufficient frequency of prior exposure to hypoxia to elicit a sustained upregulation of key stress response genes. It is also possible that the lack of a significant result indicates that frontloading is not a mechanism of increased tolerance to hypoxia in *C. virginica*.

Chapter 4 Summary

The primary goals of research presented in *Chapter 4* were to integrate dissolved oxygen as a forcing variable into the Dynamic Energy Budget (DEB) model parameterized for *C. virginica* and to make preliminary predictions about the effect of

low oxygen exposure on oyster growth and reproduction. DEB models are increasingly being used to make predictions about the response of *C. virginica* to environmental stressors (e.g., Steeves et al. 2018, Lavaud et al. 2021), and the growing threat of oceanic or estuarine deoxygenation is one such stressor for which DEB model development would be useful. Using empirically measured oyster growth data from sites throughout the Chesapeake Bay with differing dissolved oxygen regimes, the DEB model was successfully calibrated to include dissolved oxygen availability as a forcing variable. The calibrated model accurately predicted oyster growth across sites with varying regimes of oxygen availability. Preliminary model predictions from the calibrated and validated model indicated that low oxygen exposure resulted in reduced shell and tissue growth rates for oysters, as well as reduced oyster gonad mass and spawning frequency.

Four specific conclusions can be drawn from the results of *Chapter 4*. First, the model can replicate the observation that that hypoxia exposure reduces oyster growth rates, an effect that has been empirically measured previously (e.g., Stevens and Gobler 2018). Second, with respect to reproduction, the model predicted that hypoxia would reduce oyster fecundity and lead to asynchrony of spawning between oysters experiencing differing oxygen availability. Third, this research indicated that integrating the effects of the other forcing variables, namely temperature and salinity, into the calculation of a dynamic critical oxygen concentration is important for a more accurate assessment of the effects of dissolved oxygen on individual bioenergetics. Future DEB model development considering the effects of dissolved

oxygen on energetics should continue to apply this concept. Fourth, while this iteration of the model is useful to make predictions about how adult oysters might respond to future oceanic and coastal deoxygenation, further model parameterization is warranted to improve the versatility of the model. For example, the present model did not incorporate the effects of dissolved oxygen on larval and juvenile life stages of oysters or consider the interacting effects of co-occurring abiotic stressors with hypoxia on oyster energetics.

Overall Conclusions and Future Directions

Results presented in each of the research chapters highlight the negative implications of hypoxia on *C. virginica* health and reiterate the importance of continued research into understanding the potential effects of projected future increases in frequency and severity of hypoxia. Results from *Chapters 2* and *3* did not provide conclusive evidence that oysters are able to differentially tolerate low oxygen stress based on prior exposure to hypoxia. These results may reflect limitations of the experimental design (e.g., insufficient prior exposure to hypoxia to induce a differing response or potential interacting effects of other environmental stressors on the experimental oysters). However, if oysters are limited in their ability to acclimate or adapt to decreases in oxygen availability, results of this dissertation research indicate that oyster populations may suffer significant consequences under future ocean deoxygenation. The research from *Chapter 2* indicates that hypoxia will likely have detrimental effects on oyster survival and physiology. Increased mortality has the potential to decrease overall oyster biomass (Sturdivant et al. 2014) and limit the

ecosystem services oysters provide to their habitats (Grabowski and Peterson 2007). Similarly, the reduced metabolic activity implied by the reduction in heart rate for oysters would allow for oyster persistence during stressful conditions, but at the cost of reductions in oyster growth rates (e.g., Stevens and Gobler 2018). Additionally, the higher levels of mortality experienced by larger oysters has the potential to reduce female oyster abundance and thus overall fecundity (Rothschild et al. 1994, Powell et al. 2013). For oysters able to survive low oxygen stress, results from *Chapter 4* reveal the potential effects of hypoxic stress on oyster fecundity. The predicted reductions in the number of eggs produced by oysters throughout the growing season, as well as asynchrony in spawning as a result of hypoxia, have the potential to decrease the reproductive success of oysters from sites experiencing low oxygen stress.

Two key areas for future research emerge from the results of this dissertation. First, further investigation is necessary to determine the importance of the relative frequency of prior exposure to hypoxia in eliciting differential tolerance to low oxygen. Results from *Chapters 2* and *3* suggested that frequency of hypoxic exposure may be an important determinant in the development of differential response to low oxygen. Given that future hypoxic conditions are expected to increase in frequency and severity, gaining more knowledge on how variable frequency and duration of hypoxia exposure influence organismal response is critical. Second, in order to accurately assess how oysters will respond to the myriad of other anthropogenic stressors projected to increase in severity into the future (e.g., thermal stress, salinity stress, and ocean acidification) empirical studies on how co-occurring stressors in

combination with hypoxia will influence organismal physiology are needed (Altieri and Gedan 2015, Sampaio et al. 2021). Salinity stress potentially played a key role in influencing the results of the survival experiment from *Chapter 2*, indicating that exposure to secondary stressors may decrease tolerance to a primary stress.

Additionally, a better understanding of how co-occurring stressors interact will be important in further development of the DEB model from *Chapter 4*, providing more accurate predictions of oyster response to future environmental conditions.

An important and recurring theme throughout this dissertation research has been the investigation of how organisms are able to cope with abiotic stressors, particularly those being exacerbated by human influence. The rapid changes expected with climate change are particularly concerning for non-motile marine organisms, provoking questions about whether these organisms will be able to adapt to changing conditions over short timescales, what mechanisms will be employed to do so, and what the consequences will be for ecosystem functioning more broadly. The research presented here contributes novel findings towards understanding the effects of hypoxia on an ecologically and economically important bivalve. Continued research elucidating the responses of *C. virginica* to hypoxia will provide a deeper understanding of the oyster's ability to show mitigate against future environmental change and stress, helping to better inform oyster management, restoration, and aquaculture practices in the Chesapeake Bay and elsewhere.

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