ABSTRACT

Title of Thesis:	EFFECTS OF SHELL DAMAGE ON MORTALITY OF THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA)* IN NORMOXIC AND ANOXIC CONDITIONS
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Deoxygenation is increasingly problematic in coastal waters globally, with many costal estuaries subject to zones of hypoxia (< 2 mg/L dissolved oxygen) or anoxia (< 0.5 mg/L dissolved oxygen). The presence of hypoxic and anoxic zones can place a unique physiological burden on marine fauna and flora, potentially leading to mass mortality and resulting in dead zones. Anthropogenic stressors, such as increased nutrient input (primarily Nitrogen and Phosphorus), have led to long-term increases of hypoxia in the Chesapeake Bay over the 20th century. Although environmental management policies for the Bay have mitigated hypoxia trends, hypoxia continues to be prevalent through many parts of the Bay. While motile aquatic organisms can change locations to avoid seasonal or long-term bouts of deoxygenation, organisms with sessile adult life stages cannot move to avoid this ecological stressor. The Eastern Oyster (*Crassostrea virginica*) is a foundational species in the Chesapeake Bay's ecosystem, performing many ecosystem services such as water filtration, nutrient cycling, and fostering benthic-pelagic connectivity while also serving as an economic resource for

commercial fishing. However, long-term trends in hypoxia and anoxia, combined with other anthropogenic stressors, have contributed to a decline in Eastern Oyster in the Bay, leaving populations at a fraction of historical levels, fostering a need for research to better understand the physiological and biomechanical responses of *C. virginica* to depletion of dissolved oxygen. While the Eastern Oyster has been termed a champion of hypoxic tolerance, and studies have been published exploring the impacts of low DO on oyster mortality and sublethal responses, research is still in search of answers to whether the response of the oyster comes from shell-based behavioral resilience to isolate the animal from environmental conditions, or physiological adaptions from the tissue of the oyster. By drilling holes of three different sizes into one valve of the oyster and exposing it to anoxic external conditions, this study aims to bridge the gap in knowledge of whether anoxic tolerance is a behavioral or physiological response. Oysters with a hole drilled in the shell of any size experienced much faster mortality in anoxic environments than oysters with no hole in the shell ($\chi^2 = 8$, $p = 0.005$), while the size of the hole drilled did not impact time to death. These results shed new light on the behavioral response of the Eastern Oyster to depleted dissolved oxygen and the importance of clamping to ostracize internal tissue from environmental deviations.

EFFECTS OF SHELL DAMAGE ON MORTALITY OF THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA)* IN NORMOXIC AND ANOXIC CONDITIONS

by

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Chapter 1: Introduction and Overview of *C. virginica* in the Chesapeake Bay

Introduction

An increased prevalence of deoxygenation is a problem in coastal waters globally (Breitburg et al. 2018). These changes are frequently driven by coastal eutrophication, a direct consequence of high levels of nutrients running off costal and estuarine watersheds (Rabalais et al. 2014). Low oxygen levels, often defined as less than 5 mg/L dissolved Oxygen and termed hypoxia, increase physiological stress in estuarine and coastal organisms, and if sufficiently severe, when dissolved oxygen levels reach less than 0.5 mg/L (anoxia), can create adverse conditions in coastal and estuarine regions termed 'dead zones'. This problem is particularly relevant for organisms with sessile adult stages, such as bivalves, that cannot move to avoid such stressful or ultimately lethal environmental conditions.

Hypoxia is likely a natural feature of deeper portions of the mainstem of the Chesapeake Bay (hereafter the Bay), the largest estuary in North America. Increased nutrient loads that characterized most of the 20th Century led to an increased prevalence of hypoxia in the Chesapeake Bay (Murphy et al. 2011). Even though recent environmental policies have reduced the intensity of hypoxia, it remains a common feature of the Bay (Li et al. 2016).

The Eastern Oyster (*Crassostrea virginica,* hereafter oyster) is a foundational species in the Chesapeake Bay's ecosystem. Oyster has been a staple of the culture, economy and ecosystem of the Chesapeake Bay watershed dating back to 1608 when Captain John Smith proclaimed the "oysters lay thick as stones" upon his journey to the Chesapeake Bay (Kurlansky,

2007). Today, the oyster remains a vital element of the Chesapeake Bay ecosystem. It supports important ecosystem services including supporting services (water filtration and nutrient cycling – Newell et al. 2005), provisioning services (commercial fishing – Wilberg et al. 2013) and cultural services (supporting coastal communities – Paolisso 2012). The most significant feature of the recent history of oyster in Chesapeake Bay has been the rapid decline in commercial harvest from the late 1800s to the current day (Wilberg et al. 2011). These authors suggest abundance declined by more than 99% over this period, and the area of oyster habitat declined by more than 70%. In response to these declines, state and federal partners have implemented a massive restoration program which has sought to restore oyster reefs in multiple tributaries in Maryland and Virginia.

One of the most notable early oyster researchers was William K. Brooks. Brooks, the author of *The Oyster* - a comprehensive overview of the oyster in Chesapeake Bay, originally printed in 1891 and reprinted in 1996 - was known for his discovery of external fertilization in oyster. Brooks continued to research and advocate for oyster replenishment, noting that survey data indicate declines of oyster abundance of an average of 39% between 1879 and 1882, thereby causing oyster fisherman to harvest even more for fear of depletion and loss of livelihood, exacerbating the destruction of the oyster beds (Brooks et al., 1884). Roughly a century later, the oyster decline in the Chesapeake Bay became classified by three distinct phases, each with unique lead causes of decline: Declines during the first phase (1840-1890) were the result of overfishing and the associated destruction of oyster reef habitat. In the second phase (1900-1980), sedimentation and anoxic water in summer months have been suggested as the principal

cause for decline. During the final phase (1981-1988) exotic oyster diseases (MSX and Dermo), predation and poor management drove declines to current levels (Goulletquer et al., 1994). It is valuable to note that oyster diseases such as MSX and Dermo were prevalent in the Eastern Oyster during the 1960s as well (Crant, Undated) More recently, nutrient loading, eutrophication, hypoxia, coastal acidification and microplastic pollution contribute to current low levels of abundance (Shen et al., 2020; Waite et al. 2018; Kirby et al., 2005). The most recent assessment of patterns and trends in abundance of oyster in Maryland's portion of Chesapeake Bay (Mace et al. 2021) suggest that oyster abundance, while low by historical standards, has been relatively stable for the last 25 years, and may even be increasing.

Despite the array of publications surrounding the *C. virginica* population declines and environmental/anthropogenic stressors reviewed above, limited reports have been published relating the oyster physiology to the mitigation of environmental stressors and subsequent population declines.

Anatomy, and Physiology

The Eastern Oyster (*Crassostrea virginica:* Bivalva), occurs over a wide latitudinal range from the Gulf of St. Lawrence, Canada in the north to the Gulf of Mexico on the southern end of the range (Hoover et al., 2005). Recent next generation sequencing studies indicate multiple genetic sub populations are likely throughout this range (Thongda et al. 2018). The oyster life cycle involves motile embryonic and larval stages that last 1-2 months and a sessile, feeding adult stage that can last beyond 10 years. Dispersal only occurs during the embryonic and larval stage. Oyster embryos and larvae swim via small cilia (Brooks, 1996), and eventually settle on a

location where they undergo metamorphosis to the adult, sessile stage. Dispersal during the larval phase is likely driven more by currents and movement of larvae vertically in the water column than by swimming *per se* (Hubbard and Reidenbach, 2015). This pattern leads to a genetic structure termed "isolation by distance," in which oyster on reefs are more similar genetically to oyster on nearby reefs than those on distant reefs (Rose et al. 2006).

The gross anatomy of the adult oyster is characterized two shells (valves) that comprise the majority of the mass of the bivalve. The shells are slightly asymmetrical, with the left shell compromising a larger, more rounded shape (Galtsoff, 1964). The two valves are joined together by a hinge ligament, which serves as the pivot point for opening and closing (Mollusc, 2005). Each valve consists of four layers: 1) A periostracum formed of organic material generated by the cells of the mantle, 2) A prismatic layer of calcite crystals (Schmidt, 1931), 3) A calcite-ostracum layer comprising much of the shell and 4) The hypostracum, composed of aragonite (Galtsoff, 1964). Shell formation is a result of biomineralization originating at the epithelium of the mantle and subsequent inclusion into the hypostracum (Simkiss, 1989). Shell is constantly being created through the oyster's life, with each new layer formed slightly larger than the last, thereby contributing to the increasing size of the oyster (Brooks, 1996).

Oysters are filter feeders (Coen et al., 2007). Generally, the two valves of *C. virginica* are held closely together, thus allowing for a complete separation between the internal environment of the oyster and its surrounding, external environment. Opening and closing of the shell is accomplished via an adductor muscle located in the middle of the body of the oyster and projecting out to the inner surface of the shell (Loosanoff, 1965). When closed, the two shells form the watertight seal and when relaxed, the two valves separate, which in turn allows for entry of particulate matter (Loosanoff, 1965). The opening of the valves allows for entry of

organic and inorganic particulate matter. In general, oysters feed by creating a flow field that brings particles toward their gills (Jorgensen 1960). Subsequently, cilia select, sort and differentiate potential food particles, with rejected items being eliminated as pseudo-feces and selected items being ingested (Brooks, 1996; Loosanoff, 1949; Shumway et al., 1985; Ward et al., 1994). Oysters feeding behaviors vary with the hydrodynamic environment, food concentration as well as common environmental drivers such as temperature, salinity, and dissolved oxygen (Ehrich and Harris 2015). More recent approaches using 3-D visualization and computational fluid dynamics, indicate that feeding is a complex, energy demanding, behavioral process (Wang et al. 2020).

The physical strength of the shell plays an important role in protection of the animal against predation. *C. virginica* has been shown to have a compressive force 64% higher than that of its conspecific, the Asian oyster *C. ariakensis* (Newell et al., 2007). The valves of *C. virginica* has a 57% greater shell strength than the Asian oyster (Newell et al., 2007). Shell thickness is also plastic, with *C. virginica* in the presence of a predator displaying a thicker shell, thereby requiring more force to crush, than when in a controlled predator-free environment (Robinson et al., 2014). Additional methods of defense include a shift in habitat location, with oysters in predator rich geographic areas showing greater intertidal presence compared to those in less risky environments (Johnson et al., 2014).

The feeding behavior of oysters sets up an explicit survival trade-off, in that the valves must be open for the animal to feed, but this also exposes the animal to potentially harmful environmental conditions that characterize the intertidal habitat to which oysters are adapted. Throughout the southeastern United States, oysters populate an intertidal habitat (Dame, 1972), causing the need for mitigation tactics to survive during extended periods out of the water. In

intertidal populations, survival can come at the cost of growth. Research has shown that oyster reefs experienced the highest growth rate in subtidal locations yet experienced the lowest mortality rate in intertidal locations (Bartol et al. 1999).

It has been noted in oyster and other bivalves that a common response to external stressors is to clamp for an extended period (Lombardi et al., 2013). Shell clamping by *C. virginica* is a preferred ecological response to both predation and environmental imbalances, directly correlating with the development of thicker valves compared on non-intertidal species (Lombardi et al., 2013), likely resulting from a combination of hypoxic environmental conditions and continued predation pressures. However, clamping to mitigate external pressures can elicit a series of negative outcomes. Hypoxic conditions can result in decreased feeding, growth, and reproduction altered metabolism, and an increased susceptibility to parasitic infection (Keppel et al., 2015; Keppel et al., 2016; Jokela, 1997; Beniash et al., 2010). Additionally, the liquor of the oyster also becomes more acidic, resulting in internal erosion over the surface of the inner shell (Galtsoff, 1964). To counter these effects, *C. virginica* shifts to an anaerobic metabolic pathway (Crenshaw and Neff, 1969), during prolonged hypoxia.

Eastern oyster is understood to possess an adaptive physiology to combat dissolved oxygen, temperature, pH and salinity fluctuations (Chapman et al., 2011), resorting to an anaerobic pathway and thereby altering a metabolic pathway to combat hypoxia (Ivanina et al., 2010). It has been shown that genes controlled by a Hypoxia Inducible Factor were often downregulated in oyster exposed to anoxia, thereby indicating that the oyster exhibits a novel response when deprived of oxygen (Ivanina et al., 2010). The clamping process leads to a buildup of Carbon Dioxide, resulting in acidosis (Burnett, 1997). Therefore, by reducing

metabolism, oyster can exhibit a much lower internal acidity than its bivalve relative *C. ariakensis* (Lombardi, 2012; Hammen, 1969).

Life Without Oxygen

Recently, a proposal to introduce a nonnative, related species – the Asian oyster *C. ariakensis* led to numerous comparative physiological and behavioral studies of both the Asian and Eastern oyster to help assess the risk and potential success of the proposed introduction. These studies provided clear evidence that *C. virginica* is capable of a greater hypoxia tolerance than *C. ariakensis*, suggesting that *C. ariakensis wa*s not a suitable replacement for *C. virginica* in the Chesapeake Bay (Lombardi, 2012). The observed differences could be ascribed to both molecular tissue and shell adaptations. Ultimately, the introduction was abandoned because of numerous concerns. However, the resulting studies have shed light on the response of oysters to hypoxia, as well as larval settlement that contribute to the relative success of *C. virginica* in hypoxic waters.

There is evidence of *C. virginica* demonstrate selective settlement with a preference in normoxic waters, with settlement significantly lower in hypoxia than normoxia, and practically no settlement in anoxia (Baker & Mann, 1992). Additionally, the oysters that did settle in non-normoxic water showed significantly decreased growth when compared to samples in normoxic water (Baker & Mann, 1992). Decreased settlement in hypoxia by juvenile *C. virginica* is likely a result of the decreased survival time of newly settled oyster in hypoxia (day(s)) when compared to the adult life stage (multiple weeks) (Widdows et al., 1989; Galtsoff, 1964).

When clamped, the shell is the primary barrier used by *C. virginica* to isolate internal tissue from the surrounding environment. A better ability to clamp should therefore produce a

more successful isolation capability. Comparative clamping studies indicated that *C. virginica's* valve thickness, compressive strength, and overall clamping ability were greater than *C. ariakensis* (Lombardi et al., 2013), which is a likely result of C. virginica invading inter-tidal habitats. Additionally, environmental pressures can facilitate compensatory growth as a defense response, evidenced by juvenile oysters compensating for initial growth delays when immersed in cycled hypoxia and pH (Keppel et al., 2016). However, prolonged hypoxia can have adverse impacts on shell development, as hypoxia-based energy conservation has led to less crystalline shell formation (Leung & Cheung, 2018).

When both species were placed in hypoxic conditions, *C. ariakensis* was found to gape more often and have a wider gape than *C. virginica* (Lombardi et al. 2013). A wider gape in *C. ariakensis* would suggest that the hinge connecting the left and right valves in this species possesses more of a spring-like element. Though this could be helpful for increased oxygen consumption and feeding, it would not be beneficial in hypoxia as more energy would be required from the adductor muscle to remain clamped. Combined with lowered metabolic activity (Ivanina et al., 2010), it would be increasingly difficult for *C. ariakensis* to maintain clamping against a higher combative force when compared to *C. virginica*.

Perhaps the most unique adaptation *C. virginica* possesses are the tissue-based adaptations to hypoxic exposure. Shifting to anaerobic glycolysis allows prolonged survival in low DO conditions (Ivanina et al., 2010; Lombardi et al., 2013). The anaerobic shift allows for continued ATP turnover at rates comparable to normoxic metabolism (Ivanina et al, 2010). Interestingly, prolonged anaerobic glycolysis does not lead to the production of lactic acid, but rather a weak, non-volatile acid (Hammen, 1969; Dugal & Fortier, 1941).

The molecular response in particular poses a unique set of adaptations made to accommodate low dissolved oxygen levels for prolonged periods of time. *C. virginica* possesses upwards of 13 transcription factors all controlled by a Hypoxia Inducible Factor (HIF), allowing for molecular alterations in response to low DO (Ivanina et al., 2010; Piontkivska et al., 2011). Hypoxia is identified in the gill of the oyster, leading to a subsequent molecular shift (Piontkivska et al., 2011). The Hypoxia Inducible Factor impacts a wide array of organismal functions including embryonic development, immune responses, cell differentiation, and metabolic shifts (Greer et al., 2012).

A Need for Additional Experimentation

The changes made by *C. virginica* during prolonged hypoxia, in particular the tissue responses, present unique characteristics indicative of physiological evolutionary adaptations to low DO. Controlled experiments indicated that *C. ariakensis* has a significantly more acidic pH in its hemolymph than *C. virginica* under hypoxic conditions (Lombardi et al., 2013), indicating increased hypoxia tolerance in *C. virginica*. The metabolic response to low DO also has the capability to vary with seasonal shifts, evidenced by enzyme activity differing in winter months compared to summer months in anoxic waters (Greenway & Storey, 1999). Oyster tissue in anoxia has been shown to remain functional, with samples placed in anoxia keeping a normal ATP level and a normal tissue energy status (Ivanina et al., 2010).

With the increasing prevalence of hypoxic waters in benthic habitats, understanding the mechanism of responses by *C. virginica* with regards to hypoxia tolerance becomes increasingly important. If hypoxia tolerance is truly a behavioral component, then the size of the hole drilled

in the oyster will not impact survivorship as a hole of any size fully penetrating the shell will leave the oyster unable to successfully isolate itself from external stressors.

To test this hypothesis, holes of varied size will be drilled into the left valve of *C. virginica* of three varied sizes (Small = 1/16", Medium = ⅛" = 3.18 mm, Large = ⅜" = 9.53 mm), and those oysters will be subsequently placed into anoxic waters to determine varied levels of time to death. In conducting this experiment, the goal is to determine the necessity of clamping in anoxic water and the impacts of a stressor that renders an oyster unable to successfully clamp.

Chapter 2: Examining the Implication of Shell Damage on *C. virginica* Mortality in Hypoxic Waters

Introduction

The Chesapeake Bay offers a large source of cultural and economic value to its bordering states of Maryland and Virginia, as well as its watershed that comprises portions of Delaware, New York, Pennsylvania, West Virginia, and the District of Columbia. Estimates of the economic benefit resulting from a healthy Bay range up to $1.8 billion from swimming, boating, and fishing alone (Morgan and Owens, 2001). More recently, economic benefits resulting from the Chesapeake Bay is estimated at over $100 billion per year (Phillips & McGee, 2014). However, Bay conditions declined following European colonization, resulting in both economic and cultural losses. Anthropogenic factors have led to progressive degradation of many waters, including the Chesapeake Bay and its tributaries (Nixon, 1995; St-Laurent et al., 2020). In the Chesapeake, one primary factor is nutrient loading from the watershed, which has been the primary driver for eutrophication and hypoxia (Li et al., 2016). From 1950-2001, the volume of hypoxic water in the Chesapeake showed drastic increase and accelerated pace (Hagy et al., 2004). However, recent data indicate that Bay hypoxia has held steady and even shown trends toward DO recovery (Zhang et al., 2018).

The Eastern oyster, *Crassostrea virginica* (hereafter oyster), an ecosystem engineer of the Chesapeake Bay, providing numerous ecosystem services including commercial harvest, eutrophication mitigation, filtering of particulates from the water through feeding that leads to increased water clarity, habitat generation, and benthic-pelagic coupling (Coen et al., 2007;

Ozbay et al., 2017). Oyster can tolerate environments with low dissolved oxygen (Sokolova et al., 2019). The tolerance is accomplished by clamping both valves together using adductor muscles adhered to the inner surface of each valve. During this process, *C. virginica* utilize an anaerobic pathway to prolong survival with limited oxygen (Ivanina et al., 2010). However, this mechanism of survival, while benefiting the individual, can come at a price to the proliferation of the population. While tolerating hypoxia, both growth and reproductive energy are reallocated to survival and away from fecundity (Davis, 2021). When comparing growth of the oyster in high and low DO conditions, experimental evidence suggests that juvenile growth under hypoxia (1.5 mg/ L O_2) is only one third that of oysters placed in normoxic conditions (7.9 mg/L O_2 - Baker & Mann, 1992).

Oyster also possesses the ability to undertake shell repair. When damage is done on the shell of the oyster in normoxic waters, they will re-calcify the shell and reestablish a stable, internal environment (Cho and Jeong, 2011). However, experiments regarding recalcification have been limited to samples in environmental simulation waters with a normoxic dissolved oxygen (DO) promoting repair.

Experiments testing the response of oyster to low DO levels in the Chesapeake Bay have limited their environmental studies to the surrounding environment and not the physiology of the oyster itself, leaving a knowledge gap in physiological responses from oysters with compromised shells in low DO. This study aims to factor in the environmental factor of anoxia in conjunction with the physiological variable of shell damage to better understand *C. virginica* energy allocation and physiological responses to anoxia. This will be done via a controlled experimental

condition within which *C. virginica* will be subject to shell holes of varied sizes and either normoxic (> 5 mg/L O_2) or anoxic (< 0.5 mg/L O_2) environmental conditions.

Methods

To determine the responses of compromised *C. virginica* to anoxic waters, a controlled factorial experiment was undertaken using locally sourced oysters. One hundred oysters were purchased from Jessup Seafood Market (Jessup, MD). All oysters had been harvested commercially from the Maryland portion of Chesapeake Bay. All oysters were expected to be wild-type diploid animals. Oysters were brought directly to the Paynter Lab at the University of Maryland for environmental acclimation to prepare for experimentation. Oysters were all placed in tanks held at normoxic conditions for one day to adjust to temperature (20 C) and salinity (25-30) to reduce stress.

The experiment was conducted using a nested environmental design involving 10 2-gallon tanks. Oxygen treatments was applied at the tank level with oysters having different hole sizes nested within oxygen treatment. Tanks were maintained under static conditions. Five tanks were held under normoxic conditions created by bubbling oxygen into the bottom of the tank and leaving the tank lid off. The normoxic tanks were maintained at a dissolved oxygen (DO) of greater than 5.0 mg/L O_2. The other five tanks were prepared to represent an anoxic environment, with DO consistently below 0.5 mg/L. At the start of the experiment, N_2 was sparged into the tank via an external nitrogen source to remove dissolved O_2 from the water. Once the correct DO was obtained, and oysters were placed into each tank, the top of the anoxic

tank was capped and sealed with duct tape to minimize infiltration of oxygen. The anoxic tanks were kept at a DO less than 0.5 mg/L. Environmental conditions were measured daily to ensure proper salinity, dissolved oxygen, and water temperature of each tank using a YSI multiparameter probe (Yellow Springs Instrument, Yellow Spring, OH).

The shell hole size treatment was nested within each tank. Following the acclimation period, forty oysters were selected randomly for experimentation. Within a two-hour period, a hole was drilled with a carbide drill tip into the rounded shell of seventy oysters and they were placed in their respective environments. Each tank contained four *C. virginica* samples - one sample of each hole size treatment (no hole, 1.59 mm hole, 3.18 mm hole, 9.53 mm hole) (Figure 1). All holes that were drilled fully penetrated the rounded shell without causing damage to the body of the animal (Figure 2). Animals were replaced if there was any evidence that hole drilling had caused damage to the oyster's tissue.

Sampling Time and Quantification

Every twenty-four hours, all tanks were checked for both the appropriate dissolved oxygen level (< 0.5 mg/L DO for anoxic tanks and > 5 mg/L DO for normoxic tanks) and the response from oysters characterized following a tank disturbance. Tank disturbance response testing was done by tapping on the external glass of the front tank wall and observing if gaping *C. virginica* responded with immediate shell clamping, with clamping being denoted as a vital functioning response. An oyster that was already clamping was denoted as alive, as clamping is an active process. In the case where *C. virginica* did not respond by clamping, that sample was

denoted as dead. All dead oysters were measured lengthwise and weighed following experimentation and removed from the tank environment. At the eleven-day mark, all oysters in the anoxic treatment were dead. Sampling was carried out until three days after the last oyster in anoxic conditions had died (day 14). During experimentation, no food was provided to oysters and tanks were not cleaned or altered until all oysters in that tank were dead and removed.

Data Interpretation and Analysis

Data analysis and visualization was undertaken in Excel and R-Studio. A Kaplan-Meier survival analysis was undertaken using oxygen concentration and hole size as treatments. Kaplan-Meier explicitly accounts for censored data (i.e., animals in which death was not observed). The Kaplan-Meier approach estimates the probability of survival to time t as,

$$\widehat{S(t)} = \prod_{i:t_i<t} \left(1 - \frac{d_i}{n_i}\right)$$

Where d is the number of deaths observed at time t, and n is the number of individuals known to have survived up to time t. Survival curves can be developed for individual treatment combinations. Tests of significance can be conducted using log-rank tests, which are interpreted using a χ^2 statistic. Kaplan-Meier survival curves were fitted in R v4.03 with R-Studio v 1.1419.

A series of different comparisons were made which were 1. Survival in Anoxia vs. Normoxia, Survival with the presence or absence of a shell hole, 3. Survival based on the size of the hole in anoxia and, 4. Survival based on the combined impacts of normoxia vs. anoxia and the presence vs. absence of a shell hole. Each treatment combination was compared using log-rank tests based on the respective treatment variable.

Results

Primary Result:

Oxygen treatments were maintained throughout the experiment (Table 1). The average oxygen concentration in normoxic tanks was 6.22 mg/L O_2: the average in the anoxic treatment was 0.14 mg/ L 0_2. Oxygen treatments were significantly different (T-test: t=-71, p=0.00). All animals in the anoxic treatment died with 11 days (Table 2). The experiment was ended after 14 days, when only 3 of 20 animals in the normoxic treatment had died.

Preliminary inspection of the data indicate that the level of censoring was high in the normoxic treatment and prevented a full exploration of the effect of hole size as a gradient treatment within the normoxic treatment. According, hole size was converted to a binary variable (presence/absence) for analysis. There was a significant effect of oxygen treatment on survival, independent of the hole size treatment (χ^2= 40.3, p= 2e-10 Figure 3). The average time to 50% mortality in the anoxic treatment was 5.80 days. We note the normoxic treatment never reached the 50% mortality level. In contrast, there was no significant effect of the presence / absence of a hole in the shell, independent of oxygen treatment (χ^2= 0.2, p=0.7, Figure 4). The full analysis involving oxygen treatment and presence, or absence of a hole supported these simplified results (χ^2= 48.3, p= 2e-10, Figure 5).

When separating oysters by both the presence or absence of a hole and their environment (anoxia vs. normoxia), oysters with a shell hole of any size in anoxia died in an average 5.27 ± 0.43 days (mean ± SE), when compared to oysters with no hole in anoxia dying in an average of

8 ± 0.77 days. Oysters with a hole in anoxia died significantly faster than oysters with no holes and with holes in normoxia, as well as compared to the group with no holes in anoxia ($p=2e-10$ Figure 5). The presence of a hole of any size led to faster death by 2.74 days when compared to oysters with no hole ($p=0.005$, Figure 6).

Discussion

My results indicated that any level to which a shell is compromised in anoxia lead to significant mortality. There was no significant different in effect when the hole penetrating a shell is 1.59 mm or 9.53 mm. Yet both hole sizes lead to a far shorter time to death when the oyster is placed in anoxic environmental conditions. However, it is important to note that s shell hole does not induce mortality, but rather it is the combination of a shell hole and an anoxic environment. This perhaps indicated that clamping was a behavioral adaptation to anoxic water and the tissue itself was not functional in anoxic water for extended periods.

As it was evident that *C. virginica* shows an adverse response to shell damage in conjunction with anoxic conditions, further research must be done to understand the physiological mechanism responsible for rapid mortality. To develop a greater understanding of the impacts of hypoxia on compromised *C. virginica*, experiments could be conducted using a combination of a heart rate monitor to gather more precise data on time to mortality as well as sublethal energy allocation impacts, and shell clamping magnets to measure clamp strength to gain quantitative data on clamping energy allocation in anoxic environments. The use of shell clamping magnets would help provide insight into the behavioral response of clamping and

whether a compromised shell would lead to a need for greater energy allocation toward keeping both valves together. Current conclusions do however indicate that anoxic impacts are compounded by compromised shells by limiting the ability of *C. virginica* to maintain internal homeostasis by clamping.

The results of this research lead to the evident conclusion that without suitable DO, a compromised shell leads to rapid mortality for *C. virginica*, when compared to oysters with no shell hole in the same anoxic environment. In all treatment group oysters with a hole of any size in the shell, anoxic conditions led to relatively quick gaping, indicative of the inability to isolate environmental conditions. It has been observed that in the Bolivar Peninsula in Texas, oyster drills have left a shell hole in the Eastern Oyster of 28mm (1.1 inches) (Randolph & Maccarone, 2018), signifying that hole sizes in *C. virginica* in the field are larger than the sizes used in this experiment. If a shell hole of 9.53 mm in anoxic environments had led to much faster mortality than a shell hole of 1.59 mm, it could be concluded that there is a mechanism in place for the *C. virginica* to mitigate shell damage and maintain clamping homeostasis. However, in being that there was no significant change in time to death when comparing shell hole sizes between 1.59 mm, 3.18 mm, and 9.53 mm, it must be said that complete penetration damage of any kind, in conjunction with low DO, is sufficiently able to induce mortality and gaping.

It is possible that there is a coevolution occurring between the hinge ligament of *C. virginica* and the adductor muscle. With the hinge working with s spring-like mechanism to keep the valves apart during feeding, and the adductor muscle working to accomplish shell clamping, it would make sense that these two opposed processes coevolve to ensure clamping is done in a

metabolically efficient manner, expending as little energy as possible. Evidence of altered evolution in *C. virginica* in terms of clamping and gaping has been evidenced by *C. virginica* clamping for longer periods of time and not gaping as wide in hypoxic waters when compared to a species less tolerant to hypoxia - *C. ariakensis* (Lombardi et al. 2013). With it being understood that hypoxic tolerance involves lowered metabolic response (Ivanina et al., 2010), it would make sense to co-evolve a clamping/gaping mechanism in a manner biomechanically sustainable with lowered energy expense. Clamp strength, which is a primary product of adductor muscle strength, could be supported by a lowered internal pressure created by the more malleable outer edges of each shell forming a vacuum between both valves, containing the oyster tissue. The presence of a shell hole in this case would equalize the internal and external pressures, disrupting the coevolutionary mechanism for clamping created between the hinge ligament, adductor muscle, and pressure gradient.

Chapter 3: Implications, Interspecies Comparisons, and Future Directions on the Physiological Response of *C. virginica* to Hypoxic Waters

Implications of Research:

It took seven days for all large hole oysters in anoxic treatments to experience mortality. In environments devoid of dissolved oxygen, the ability of an individual bivalve to remain alive for seven days without the ability to properly clamp for internal homeostasis should not go ignored. It would take further experimentation to develop concrete evidence on the extent to which survival in prolonged anoxia with a compromised shell is a tissue or behavioral response, but some possibilities will be analyzed below.

One possibility is that the mantle epithelium of the oyster presses itself against the inner shell surface at the location of the shell hole, using this epithelium in place of shell to maintain internal homeostasis. The process of moving mantle epithelium across the inner surface of the shell is not novel, as this process is used to repair broken or damaged shells (Brooks, 1996). However, the mantle epithelium would not be able to permanently take the place of the shell surface as the tissue is soft and would still allow for some extent of gas exchange between the oyster's internal environment and the external environment, potentially contributing to delayed but inevitable mortality. One way to test this hypothesis is to insert microprobes between the valves of the oyster to measure fluctuations in internal DO concentrations as evidence of delayed loss of internal DO homeostasis. The use of microprobe testing with bivalve species has been

successfully done by Chaparro et al., (2009) and Gray et al., (2022), indicating it can be a reliable method for a follow-up study.

Beside the insight this experiment has given to oyster physiological evolution, there is also a curiosity in what would cause *C. virginica* to be found in the Chesapeake Bay with shell holes present. One explanation would be incomplete predation, where a predator such as *U. cinerea* and *E. depressus* would begin to predate on the oyster by compromising the shell and then leave the location due to a higher trophic predator or abrupt environmental disturbance. *C. virginica* falls prey to several predators including the mud crab (*Rhithropanopeus harrisii, Eurypanopeus depressus, Dyspanopeus sayi, Panopeus herbstii)*, oyster drill (*Stramonita haemastoma),* and the common starfish (*Asterias rubens)* (Newell et al., 2007; Brown et al., 2002). Specifically in the Chesapeake Bay, *C. virginica* predation is observed by the mud crab (*Panopeus herbstii*), blue crab (*Callinectes sapidus*), and flatworm (*Stylochus ellipticus*) (Bisker & Castagna, 1987; Eggleston, 1990; Newell et al., 2000; O'Conner et al., 2008). Common methods of predation by the mud crab include use of a crusher claw to access the edible tissue of the oyster (Milke et al., 2001), while the oyster drill uses a two-pronged predation by first secreting a chemical to dissolve the oyster shell and then a mechanical boring process to access the tissue of the oyster (Carriker, 1969). Both methods of predation have resulted in an evolutionary arms race between *C. virginica* and its predators.

Crab predation success relies greatly on the strength of the crushing claw. The Stone Crab (*Menippe spp.)* has been shown to prefer medium sized oysters when compared to small and large oysters due to its inability to 1. Mechanically handle a small shell size and 2. Crush a larger (thicker) shell (Rindone et al., 2011). Consequently, the mechanical advantage of the crushing claw becomes a significant benefit in predation success. *E. depressus,* a major predator

of *C. virginica* in the Chesapeake Bay, has a significantly higher mechanical advantage than *Rhithropanopeus harrisii* (Milke et al., 2001), signifying an evolutionary step toward more efficient predation on *C. virginica* with enhanced ability to create a hole in the oyster valve.

Predatory gastropods are known to attack the shell directly, rather than at the hinge point, through a chemo-mechanical mechanism where acid is first released to dissolve shell parts followed by a radula removing shell mechanically (Carriker, 1961). In response, *C. virginica* displays phenotypic plasticity with a thickened shell in the presence of the oyster drill (*Urosalpinx cinerea*) (Lord & Whitlatch, 2012). The Mud Crab (*E. depressus)* also has a claw adapted for crushing the shell of a bivalve and a cutter claw adapted for entering holes or small spaces (Vermeij, 1977). Both predators' tactics of *U. cinerea* and *E. depressus* involve penetration not at the hinge of the shell but rather directly on a valve surface. It could be that predatory tactics have evolved in an evolutionary predator-prey arms race in that predators have shifted shell penetration mechanisms to disturb the internal environment of *C. virginica* and compromise the ability to clamp by interfering with the ability to form a vacuum and ostracize external environments. To test the plausibility of this hypothesis, it would be beneficial to measure the internal pressure present between closed valves during clamping and determine if a pressure gradient is a factor in clamp capability.

Comparative Responses to Low Dissolved Oxygen:

Crassostrea virginica is not alone in its ability to tolerate hypoxia. Nutrient input, eutrophication, and the resulting hypoxia are globally distributed leading a wide array of marine

fauna to adapt with hypoxia-mitigation tactics. As of 2003, it is estimated that there are over one million square kilometers of permanently hypoxic sea floor (Helly & Levin, 2004). Along with widespread hypoxia on the Western Atlantic coast, human-induced hypoxia is also widespread in the Eastern Pacific, Eastern Atlantic, and portions of the Western Pacific (Rabalais et al., 2010), propagating a need for widespread global adaptation.

In aquatic turtles, hypoxia tolerance occurs in two phases. First, the need for ATP is reduced following initial exposure to hypoxia (Hochachka et al., 1996). During extended hypoxia, larger scale changes occur. During extended periods of decreased DO, hypoxia-tolerant systems of the organism activate a second 'rescue' phase where metabolic shifts occur in an attempt to keep metabolic energy demands low to accommodate ATP downregulation (Hochachka et al., 1996). The primary goal of anoxic tolerance is to alter metabolic processes where full energy demands can be met by anaerobic glycolysis (Buck, 2004).

In the case of the gastropod *Crepipatella dilatata* and the bivalve *Ostrea chilensis*, when exposed environments with reduced salinity, both species retained high salinity in the pallial cavity at the cost of oxygen availability, with oxygen levels dropping into hypoxic zones (Chaparro et al., 2009), indicating less adverse responses to hypoxia compared to reduced salinity. However, exposing juveniles to induced hypoxia can carry its own set of negative consequences. Adverse impacts include egg eviction, delayed emergence, lowered fecundity, and short-term juvenile growth (Segura et al., 2014).

The European Sea Bass (*Dicentrarchus labrax)* uses a more macroscopic physiological pathway to accommodate aquatic hypoxia. The myocardium (heart contractile muscle) of

hypoxia tolerant *D. labrax* possesses greater contractile force when compared with hypoxic-sensitive *D. labrax* (Joyce et al., 2016). Additionally, *D. labrax* uses environmental indicators, with an increase of CO2 concentration serving as a signal for increased O2 uptake (Montgomery et al., 2019).

The Striped Catfish (*Pangasianodon hypophthalmus)* utilizes both air-based and aquatic breathing (Lefevre et al., 2011). When in hypoxic environments, *P. hypothalamus* must rely on air breathing to maintain metabolism (Lefevre et al., 2011).

In British Columbia, Threespine Stickleback (*Gasterosteus aculeatus*) exposed to prolonged hypoxia can suppress metabolism by 33% compared to those not exposed to hypoxia (Regan et al., 2017). It is important to note that the Stickleback experiment did not measure time to death but rather time to a loss of equilibrium, so though there is similarity in reduction of metabolism, it is unclear how long the Stickleback are able to experience hypoxic waters.

Though there are many varied mechanisms for hypoxia tolerance in marine and aquatic species, *C. virginica* is often cited as having superior hypoxia tolerance. Perhaps this accolade is a result of superior valve thickness (Lombardi et al., 2013), indicative of prolonged ability to ostracize external stressors. Perhaps it is a result of sustained reduction in metabolism (Ivanina et al., 2010). However, it seems likely that a combination of valve biomechanics and tissue/metabolism alterations leads to a superior ability of *C. virginica* to withstand hypoxia.

Future Directions:

Though this experiment has shed light on the impacts of varied shell hole sizes on the mortality of *C. virginica* in low oxygen conditions, there remains room to continue this study and advance understanding of the physiological, biomechanical, and ecological impacts of hypoxia.

It is recommended that this experiment be completed again in the future. First, this experiment could be redone in flow through conditions, which is available in many aquaculture setups. Using flow through conditions will help shed light on the impacts of the shell hole on mortality by keeping water continuously flowing through the system. It would also be beneficial to conduct this experiment with a larger sample size. Due to financial and time constraints, only five oysters were used with each hole size in anoxic waters. Increasing the sample size could help differentiate impacts based on hole size or create more certainty on the anoxic response being a result of shell hole presence or absence alone.

This experiment used a binary classification to quality oysters as either alive or dead. Though successful in its purpose, the terms ‘alive’ and ‘dead’ do not tell the whole story. I recommend two follow up mechanisms to gain greater quantitative analysis of the sublethal impacts of hypoxia on compromised *C. virginica.* The use of heart rate monitors has been done before in numerous oyster studies and can be used to measure heart rate and thus determine alterations in heart activity resulting from an external stressor. The use of clamping magnets in determining sublethal impacts is unique in that clamp strength and gape width has been shown to vary across bivalve species (Lombardi, 2012; Lombardi et al., 2013). It may be possible that clamp strength and energy allotted to clamping is lowered in *C. virginica* when compared to *C.*

ariakensis, which could be supported by the lowered gape width in *C. virginica* (Lombardi, 2012). The use of clamping magnets could aid in verifying the plausibility of this response.

Many tests involving *C. virginica* often use the close relative of *C. ariakensis* to analyze comparative physiology. It would be beneficial to conduct the shell hole of varied size experiment on *C. ariakensis* to compare time to death and to compare gape width. Comparison of *C. virginica* to *C. ariakensis* with the following metrics allows for better understanding of the adaptations in place to withstand and tolerate environments with low DO.

One ecological factor not considered in this experiment is that of predation in hypoxia. It is possible that predation tactics may change in hypoxic conditions in order to conserve energy. Experimentation could be done using a predator in a controlled environment with compromised *C. virginica* to analyze alterations in predation tactics. Measurements would include time spent using crushing claw vs. cutting claw, or time spent on a single oyster.

Studies have been done to test the rates of regeneration in oyster shells, indicating that in response the damage *C. virginica* is capable of shell repair (Mount et al., 2004). However, there are a lack of publications identifying the efficiency or capability of shell repair following hypoxic exposure and return to normoxia. It has been identified that motile predators leave oxygen-poor waters toward oxygen-rich waters during periods of hypoxia (Breitburg, 1992). Determining the ability and efficiency of shell regeneration can lead to a better understanding of predation pressures following hypoxia.

Finally, a more anthropogenic stressor is that of dredging. Dredging is a process where a giant netted crate is dragged across the benthic surface with the intention of collecting specimens

that are partially or fully submerged (Collie et al., 2000; Lenihan & Peterson, 2004). This process has left *C. virginica* populations decimated and oyster reefs drastically lowered in elevation, which presents a problem in that oyster reefs are meant to elevate oysters above anoxic and hypoxic benthic communities (Lenihan and Peterson, 1998). However, this process, though meant to capture everything in the benthic pathway, is not one hundred percent efficient. In testing, not only did the dredge fail to capture everything in its pathway but also left widely varying samples of what was left behind (Chai et al., 1992; Webster, 1953). Though not fully able to collect everything in its path, the dredge does scrape against the benthic surface and has the potential to cause damage to specimens that it does not collect. Results from this research indicate a need to ensure dredge laws avoid hypoxic and anoxic environments to ensure that shell damage from incomplete dredging does not lead to rapid mortality.

Figures and Tables

Table 1. Dissolved oxygen levels (mg/ L O_2) in the experimental tanks.

Day 1		Day 2		Day 3		Day 4		Day 5	
Tank 1	5.87	Tank 1	5.98	Tank 1	6.1	Tank 1	5.98	Tank 1	6.26
Tank 2	0.14	Tank 2	0.05	Tank 2	0.02	Tank 2	0.01	Tank 2	0.05
Tank 3	6.25	Tank 3	6.7	Tank 3	6.75	Tank 3	7.04	Tank 3	6.98
Tank 4	0.4	Tank 4	0.06	Tank 4	0.09	Tank 4	0.01	Tank 4	0.01
Tank 5	5.56	Tank 5	5.3	Tank 5	5.65	Tank 5	6.5	Tank 5	5.94
Tank 6	0.31	Tank 6	0.09	Tank 6	0.09	Tank 6	0.02	Tank 6	0.09
Tank 7	6.1	Tank 7	5.37	Tank 7	6.12	Tank 7	6.59	Tank 7	6.45
Tank 8	0.35	Tank 8	0.18	Tank 8	0.16	Tank 8	0.05	Tank 8	0.06
Tank 9	5.75	Tank 9	6.29	Tank 9	6.55	Tank 9	6.27	Tank 9	6.13
Tank 10	0.13	Tank 10	0.32	Tank 10	0.18	Tank 10	0.16	Tank 10	0.39

Day 6		Day 7		Day 8		Day 9		Day 10		Day 11	
Tank 1	6.2	Tank 1	6.3	Tank 1	6.73	Tank 1	6.9	Tank 1	6.24	Tank 1	6.31
Tank 2	0.07	Tank 2	0.03	Tank 2	0.15	Tank 2	all dead	Tank 2	all dead	Tank 2	all dead
Tank 3	6.58	Tank 3	7.29	Tank 3	7.04	Tank 3	7.12	Tank 3	6.55	Tank 3	6.52
Tank 4	0.07	Tank 4	0.1	Tank 4	all dead	Tank 4	all dead	Tank 4	all dead	Tank 4	all dead
Tank 5	5.61	Tank 5	5.81	Tank 5	5.71	Tank 5	5.78	Tank 5	5.57	Tank 5	5.16
Tank 6	0.07	Tank 6	0.12	Tank 6	all dead	Tank 6	all dead	Tank 6	all dead	Tank 6	all dead
Tank 7	6.15	Tank 7	6.52	Tank 7	6.41	Tank 7	6.62	Tank 7	6.16	Tank 7	6.4
Tank 8	0.17	Tank 8	0.09	Tank 8	0.13	Tank 8	0.21	Tank 8	0.19	Tank 8	0.18
Tank 9	5.89	Tank 9	6.06	Tank 9	5.94	Tank 9	6.35	Tank 9	5.83	Tank 9	5.83
Tank 10	0.37	Tank 10	0.17	Tank 10	all dead	Tank 10	all dead	Tank 10	all dead	Tank 10	all dead

Table 2. Time to death of individual oyster exposed to different dissolved oxygen conditions and different shell damage treatments.

Environment	Hole Size	Tank Name	Oyster Name	Days to Death
Normoxic	Large	1N1	1N1L	
Normoxic	Large	1N2	1N2L	
Normoxic	Large	1N3	1N3L	
Normoxic	Large	1N4	1N4L	
Normoxic	Large	1N5	1N5L	10
Normoxic	Medium	1N1	1N1M	
Normoxic	Medium	1N2	1N2M	
Normoxic	Medium	1N3	1N3M	
Normoxic	Medium	1N4	1N4M	
Normoxic	Medium	1N5	1N5M	
Normoxic	Small	1N1	1N1S	
Normoxic	Small	1N2	1N2S	
Normoxic	Small	1N3	1N3S	
Normoxic	Small	1N4	1N4S	
Normoxic	Small	1N5	1N5S	11
Normoxic	None	1N1	1N1N	
Normoxic	None	1N2	1N2N	
Normoxic	None	1N3	1N3N	
Normoxic	None	1N4	1N4N	
Normoxic	None	1N5	1N5N	9
Anoxic	Large	1H1	1H1L	7
Anoxic	Large	1H2	1H2L	3
Anoxic	Large	1H3	1H3L	5
Anoxic	Large	1H4	1H4L	7
Anoxic	Large	1H5	1H5L	2
Anoxic	Medium	1H1	1H1M	6
Anoxic	Medium	1H2	1H2M	4
Anoxic	Medium	1H3	1H3M	3
Anoxic	Medium	1H4	1H4M	6
Anoxic	Medium	1H5	1H5M	6
Anoxic	Small	1H1	1H1S	4
Anoxic	Small	1H2	1H2S	6
Anoxic	Small	1H3	1H3S	6
Anoxic	Small	1H4	1H4S	7
Anoxic	Small	1H5	1H5S	7
Anoxic	None	1H1	1H1N	8

Figure 1: Photograph of tank setup in laboratory. Tank 1N1 shows uncovered top and oxygen continuously pumped in. Tank 1H1 shows lid on top of tank to isolate anoxic tank environment from normoxic external conditions. Daily YSI recordings were made to ensure anoxic tank remained anoxic and normoxic tank remained normoxic.

Figure 2: A *C. virginica* sample prepared for placement into an experimental environment. The hole was drilled with a carbide tip roughly a centimeter from the shell hinge. The body of the oyster remains undamaged with the hole only compromising the full depth of the rounded shell. This *C. virginica* sample has a hole drilled of size 3/8" (9.53 mm), which is delineated in this experiment as a large shell hole.

Figure 3: Kaplan-Meier survival curves for oyster based on environmental conditions (anoxia vs. normoxia). Data for the normoxic treatment were censored. The curves represent the average probability of survival to day t with associated estimates of standard error and shows that anoxia significantly reduces survival of all oysters.

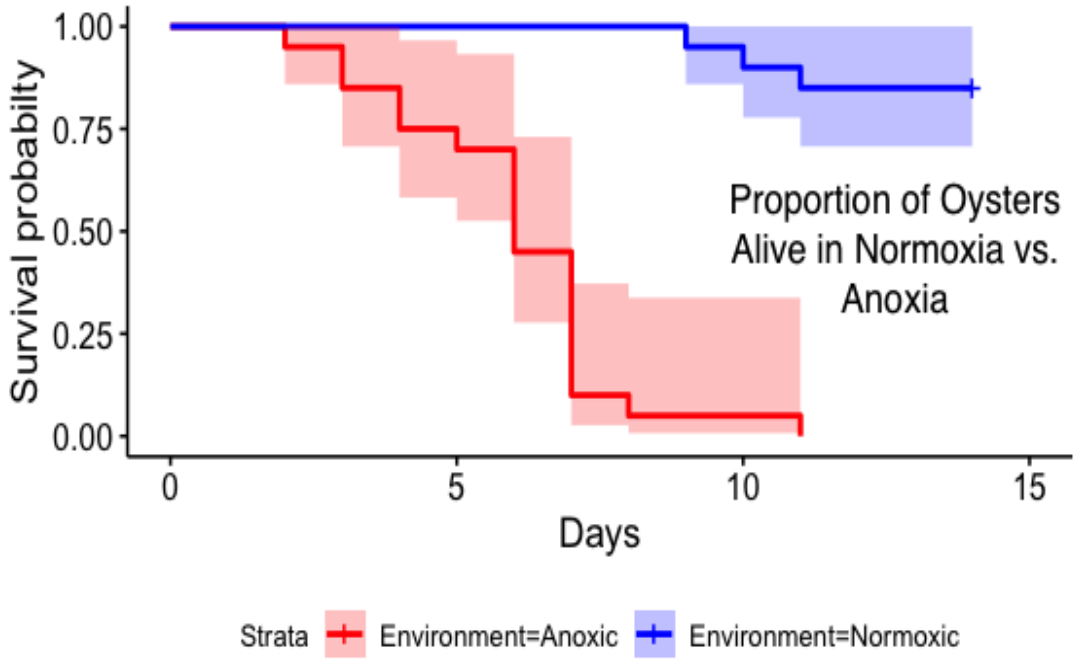


Figure 4: Graph indicating the proportion of oysters surviving after the fourteen-day experiment based only on the presence or absence of a shell hole. This graph represents the average probability of survival to day t with associated estimates of standard error and demonstrates that shell hole size alone is not a significant factor in oyster death.

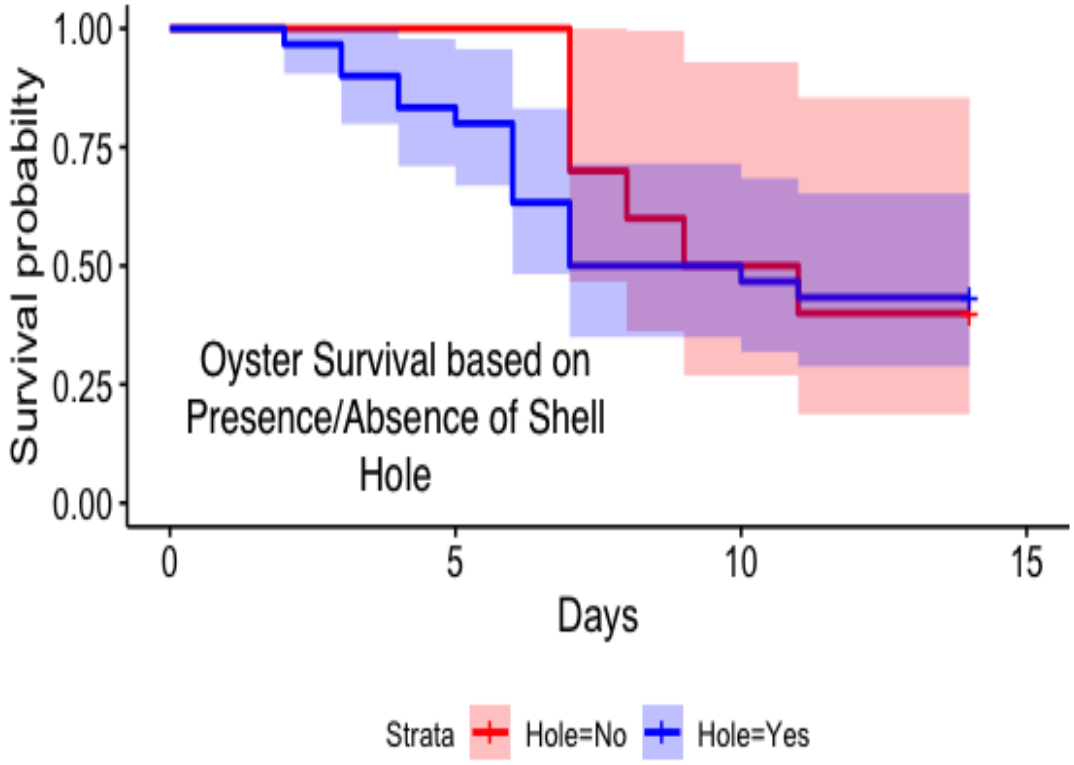


Figure 5: Graph depicting the combined impacts of both the presence or absence of a shell hole and the environment (anoxic vs. hypoxic). This graph represents the average probability of survival to day t with associated estimates of standard error. This graph demonstrates that in Normoxic conditions a shell hole does not significantly impact survivorship while having a shell hole present in anoxic conditions does lead to faster oyster death.

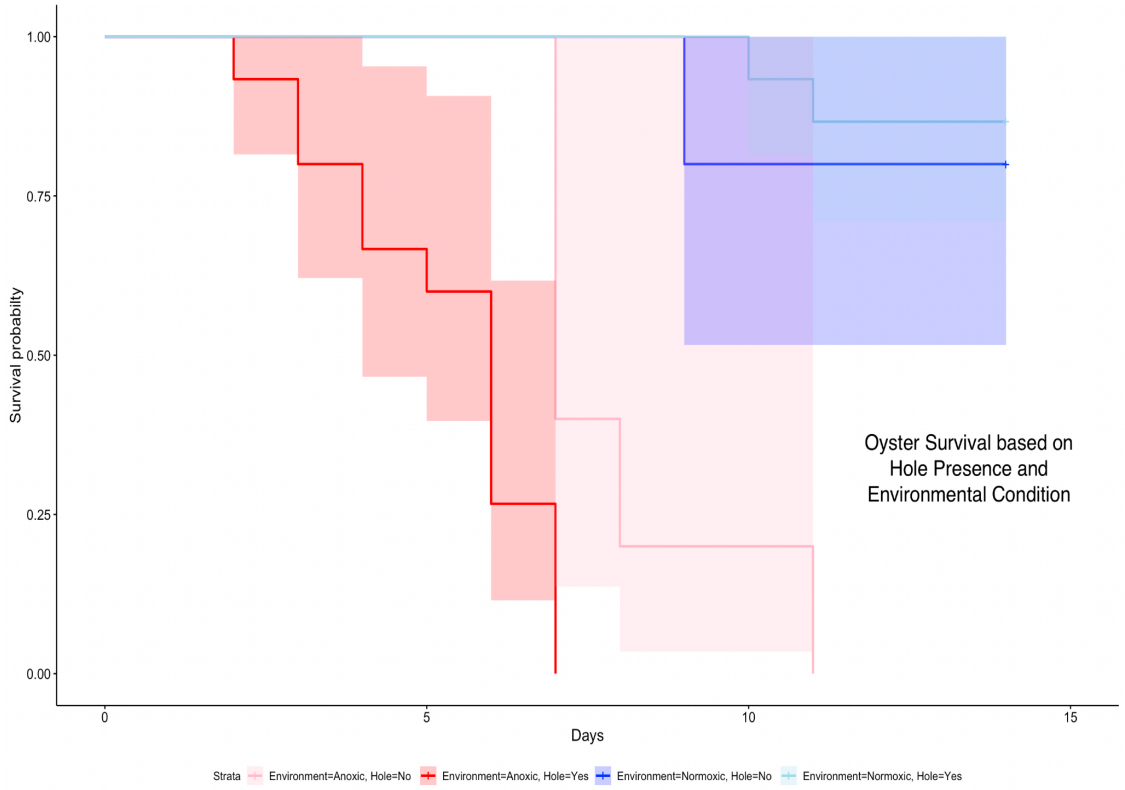
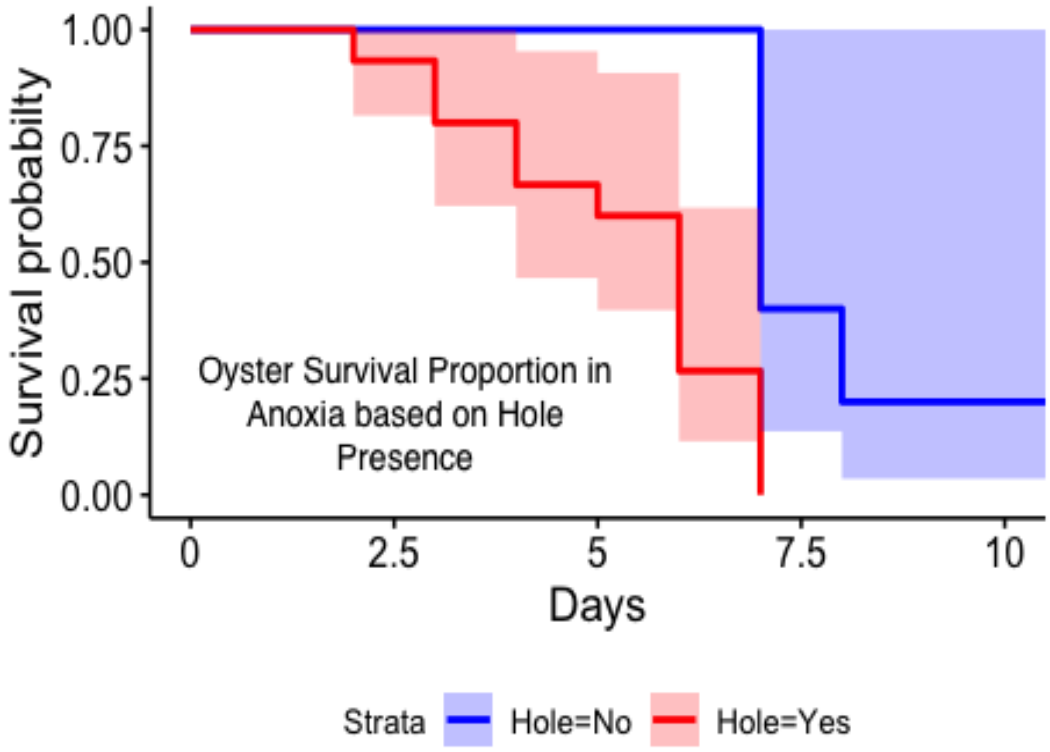


Figure 6: Graph indicating oyster survival rates based on the presence or absence of a shell hole in anoxic environmental conditions. After three days, anoxic oysters with shell holes present had dropped to 75% survival while all anoxic oysters with non-compromised shells remained alive. This graph represents the average probability of survival to day t with associated estimates of standard error, zooming in on the faster mortality of oysters with a shell hole in anoxic conditions.



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