EFFECTS OF HYPOXIA AND HIGH TEMPERATURE ON EASTERN OYSTERS: INVESTIGATING DIFFERENTIAL TOLERANCE IN POPULATIONS AND PLOIDIES

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EFFECTS OF HYPOXIA AND HIGH TEMPERATURE ON EASTERN OYSTERS: INVESTIGATING DIFFERENTIAL TOLERANCE IN POPULATIONS AND PLOIDIES

A Thesis
Submitted to the Graduate Faculty of the Louisiana State University and Agriculture and Mechanical College in partial fulfilment of the requirements for the degree of Master of Science in
The School of Renewable Natural Resources

by
Nicholas Conrad Coxe
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ABSTRACT

Increasing prevalence of hypoxia in estuarine waters can pose a serious threat to eastern oysters (*Crassostrea virginica*). While oysters are considered more tolerant to hypoxia than many other bivalves, their tolerance at temperatures of 28 °C and above, typical in northern Gulf of Mexico estuaries in the summer, is not well characterized. Moreover, it is unknown whether differences in hypoxia tolerance exist among oyster populations or between diploid and triploid oysters. To investigate population differences, wild oysters were collected from four estuaries in the northern and northwestern Gulf of Mexico and spawned. In a series of studies, the progenies were exposed to continuous normoxia (dissolved oxygen > 5.0 mg L$^{-1}$) and hypoxia (dissolved oxygen < 2.0 mg L$^{-1}$) at 32 °C. Cumulative mortality was recorded, hemolymph and tissue samples were collected to measure the sub-lethal, physiological response to hypoxia. Oyster valve movement during normoxia and hypoxia was also measured. Significant differences in hypoxia tolerance were found among populations with calculated median lethal times ($LT_{50}$) differing by population, ranging from 3.9 to 12.5 days. After 3-5 days of hypoxia exposure, the most tolerant population had elevated concentrations of plasma calcium and glutathione compared to the least tolerant population, potentially conferring better protection against hypoxia stress. To investigate ploidy differences, diploid and tetraploid oysters were spawned to produce diploid and triploid progenies. In a series of studies, the adult and seed progenies were exposed to continuous normoxia, hypoxia, and anoxia (dissolved oxygen < 0.5 mg L$^{-1}$) at 28 °C. Cumulative mortality was recorded, and hemolymph and tissue samples were collected to measure the sub-lethal physiological response to hypoxia. Differences in $LT_{50}$ were found between ploidies in adult and seed oysters, but not consistently. Adult triploid mortality was delayed by 4.6 days under hypoxia and 1.5 days under anoxia compared to diploids. In seed oysters, diploid mortality was delayed by 2.4 days under hypoxia compared to triploids, and under anoxia triploid mortality was delayed by 1.3 days compared to diploids. Despite these differences, sub-lethal responses between adult ploidies were largely comparable in all treatments, suggesting diploids and triploids use similar strategies to cope with hypoxia and anoxia stress at high temperatures. Combined, this research shows that hypoxia and high temperature (>28 °C) can cause significant oyster mortality if conditions persist longer than three weeks. Under anoxia exposure, significant mortality can occur within several days. Population-specific tolerances suggest that adaptation to local environmental conditions has occurred, and that selectively breeding populations for better environmental tolerance is possible. The relatively comparable responses between diploid and triploid oysters suggest the risks to co-occurring hypoxia and high temperature events are the same, regardless of ploidy, when considering their use in aquaculture. As environmental conditions shift due to climate change and other anthropogenic activities, testing single and multi-stressor tolerance, and potential population and ploidy tolerances will be critical to develop better restoration and production strategies.
CHAPTER 1. INTRODUCTION

In coastal ecosystems, hypoxia, functionally defined as a dissolved oxygen concentration (DO) < 2.0 mg L\(^{-1}\), is an increasing concern with potential impacts on ecosystem health and coastal aquaculture production. Continued impacts from coastal eutrophication combined with rising water temperatures from climate change influence the prevalence and severity of hypoxia (Diaz and Rosenberg 1995; Diaz 2001; Kennish 2002; Diaz and Rosenberg 2008; Rabalais et al. 2009). Understanding the impacts of hypoxia on ecosystem functioning and aquaculture production requires measuring the tolerance of key organisms and understanding potential population-specific and ploidy-specific adaptations.

Hypoxia and anoxia (severe hypoxia) are well known features in the ocean (Richards 1965; Kamykowski and Zentara 1990) and usually occur near the bottom when the water column becomes stratified. These phenomena are common in the deep ocean and fjords, but their occurrence along coastlines and in shallow estuaries is increasing (Diaz 2001). Hypoxia formation is influenced by a variety of environmental factors including nutrient load, temperature, stratification, and circulation patterns. While anthropogenic eutrophication (i.e. excess nutrient loading) is perhaps the largest contributor to increasing hypoxia around the world (Rabalais et al. 2002; Rabalais et al. 2009), increasing coastal temperature can also affect hypoxic formation (Rabalais et al. 2007) by lowering the capacity for water to dissolve and hold oxygen. Consequently, the combination of eutrophication and increasingly higher temperatures common in the summer months are extending the periods of coastal hypoxia (Rabalais et al. 1992; Diaz and Rosenberg 1995; Rabalais et al. 2009) and may increasingly impact estuarine communities. The negative consequences of hypoxia on estuarine ecosystems are well documented and can affect community structures at multiple trophic levels (Rabalais et al. 2001, Breitburg 2002, Diaz and Rosenberg 2008), but more severe hypoxia can often affect species of benthic fauna that reside at the bottom of the trophic web and lack the ability to escape hypoxic events (Baustian and Rabalais 2009; Long and Seitz 2009; Riedel et al. 2012; Riedel et al. 2014). Many benthic organisms such as mollusks can tolerate shorter periods of hypoxia by reducing energy consumption (Stickel et al. 1989), however, sustained or increasingly severe periods of hypoxia can lead to increased mortality and behavioral changes that can alter community structure (Diaz and Rosenberg 1995; Riedel et al. 2014), change ecosystem function (Norkko et al. 2015) from the bottom-up, and impact aquaculture production in commercially important species.

The eastern oyster (*Crassostrea virginica*) is a species of particular concern when considering changing hypoxia and temperature regimes because of its importance to coastal ecosystems (Coen et al. 2007; Gedan et al. 2014; Fodrie et al. 2017) and economies (Mackenzie 1996; Grabowski et al. 2012; Zu Ermgassen et al. 2012). This is especially true in coastal estuaries in the northern Gulf of Mexico (GoM), where the largest remaining natural oyster fishery in the world resides (Beck et al. 2011; Hesterberg et al. 2020). More than 65% of oysters in the U.S. are harvested from the northern GoM (NOAA 2018), and the opportunity for oyster aquaculture is growing (Maxwell et al. 2008; Walton et al. 2013; Casas et al. 2017; Hensey 2020).
Eastern oysters are remarkably tolerant to environmental variation (Casas et al. 2018a; Casas et al. 2018b; Marshall et al. 2021a; Marshall et al. 2021b) and are often more resilient to environmental stress, including hypoxia, than other benthic fauna (Stickley et al. 1989). Stickley et al. (1989) found that, among seven benthic invertebrate species, eastern oysters were the most tolerant to continuous anoxia and survived the longest, with median lethal times ($LT_{50}$) ranging from 18 days to greater than 28 days at non-stressful salinity and temperature. Similarly, Lombardi et al. (2013) showed that eastern oysters survived longer and mitigated the effects of acidosis (i.e., tissue acidification) better than the taxonomically and physiologically similar Asian oyster (Crassostrea ariakensis) when exposed to continuous hypoxia. In addition, both eastern oyster larvae (North et al. 2006) and juveniles (Matsche and Barker 2006) can tolerate hypoxia in the laboratory for significantly longer than those of the Asian oyster.

There are multiple short-term and long-term strategies that eastern oysters employ to tolerate hypoxia stress. For instance, when faced with acute onset hypoxia, eastern oysters will close their valves to effectively isolate themselves from the environment (Porter and Breitburg 2016). In the event of severe or persistent hypoxia, eastern oysters rely on mechanisms that produce energy through anaerobic pathways that produce high energy yields (Collicutt and Hochachka 1977), reduce metabolic energy demands through metabolic rate depression (Willson and Burnett 2000), and protect against harmful reactive oxygen species that accumulate in hypoxic tissue (Kurochkin et al. 2009).

Although eastern oysters are remarkably tolerant to environmental stress including low oxygen, simultaneous stress from multiple environmental parameters can reduce the tolerance to any single parameter (Cherkasov et al. 2006; Kurochkin et al. 2009; Ivanina et al. 2011; Dickinson et al. 2012; Ivanina et al. 2012). If the response to multiple stressors is greater than the sum of responses to the same individual stressors, the effect is considered synergistic (Todgham and Stillman 2013). Synergistic effects from multi-stressor events have been reported in previous laboratory and field studies with oysters (Marshall et al. 2021b; Ivanina et al. 2012) and other marine mollusks (Mohamed 2003; Ilarri et al. 2011; Tripp-Valdez et al. 2017). The co-occurrence of temperature and hypoxia stress, while individually tolerable, may cause synergistic effects in eastern oysters that severely limit their tolerance.

A considerable amount of research describes the lethal (Stickley et al. 1989; Baker and Mann 1992) and sub-lethal (Lombardi et al. 2013; Keppel et al. 2015; Khan and Ringwood 2016) effects of hypoxia stress on eastern oysters at temperatures below 30 °C, however, there is little known about effects of hypoxia at higher temperatures that approach the upper limits of an eastern oyster’s range of thermal tolerance (Marshall et al. 2021b). As estuaries in the northern GoM are predicted to be increasingly exposed to higher temperatures (Keim et al. 2008) understanding the effects that co-occurring stressors have on eastern oysters is critical. In addition, it is unclear whether differences in tolerance exist among geographically distant populations of eastern oysters in the northern GoM, or whether they exist between diploid and triploid oysters used in aquaculture. Understanding the range of tolerance and potential for adaptation to low oxygen levels, as well as potential trade-offs in tolerance resulting from differences in energy allocation between diploid and triploid oysters is critical for predicting future distribution, survival, and developing aquaculture populations to thrive under future conditions.
Populations of oysters exist in at least 28 estuaries across the northern GoM (La Peyre et al. 2021), and each estuary experiences different water quality characteristics due to differences in estuarine morphology, freshwater input, residence time, and gulf exchange (Orlando 1993). As a result, geographically distant populations can become adapted to their local environmental conditions (Burford et al. 2014) and respond differently to environmental stress (Marshall et al. 2021b). A study by Marshall et al. (2021b) shows that eastern oyster populations originating from Laguna Madre in Texas, a more southern and higher salinity estuary in the northern GoM, survived longer when exposed to extreme salinity and prolonged high temperature than populations originating from Calcasieu Lake and Vermilion Bay estuaries in Louisiana. As factors contributing to hypoxia formation including nutrient loading, residence time, stratification, and temperature (Rabalais et al. 2007) differ among northern GoM estuaries, there is potential for distinct populations to become adapted to local hypoxia regimes, or perhaps to unique patterns of co-occurring temperature and hypoxia. Understanding which populations can better tolerate specific environmental stresses will provide insight into how local adaptation develops and may improve strategies for oyster restoration and aquaculture in the northern GoM. This knowledge will lay the groundwork for future selective breeding projects.

The estuaries along the northern GoM have historically been ideal locations for eastern oysters to grow (Hayes and Menzel 1981), and there has been recent investment in off-bottom aquaculture across all GoM states. Using triploid oysters (i.e., oysters with three sets of chromosomes) is one way to improve off-bottom aquaculture production because of certain advantages triploid oysters have over traditional diploid oysters (i.e., oysters with two sets of chromosomes). Specifically, unlike diploid oysters found in the wild, triploid oysters are functionally reproductively sterile and do not develop much gonadal tissue during seasonal spawning cycles. The reduced energy investment toward reproduction (Allen and Downing 1986, 1990) leads to increased triploid growth rates compared to fertile diploid oysters (Baker and Mann 1991). This increased rate of growth allows triploids to be harvested earlier and marketed year-round (Nell 2002), qualities that often make triploids more desirable to oyster farmers. While the phenotypic differences (less gonadal tissue and faster growth) between diploid and triploid oysters are apparent, less is known about their physiological differences including environmental tolerance. Recent reports of unusual triploid oyster mass mortality in farms in the northern GoM (Wadsworth et al. 2019a) raise questions about potential trade-offs between growth and stress tolerance in diploid and triploid oysters. Investigating potential differences in diploid and triploid oyster hypoxia tolerance will help answer questions regarding the unusual triploid mortality and inform aquaculture managers concerned with site suitability.

The studies in this thesis investigated the lethal and sub-lethal responses of eastern oysters to co-occurring hypoxia and high temperature to better describe the limits of eastern oyster hypoxia tolerance, and potential differences among populations (Chapter 2) and between ploidies (Chapter 3). Measuring lethal response involves quantifying rates of mortality so that the absolute limits of tolerance can be defined and compared among different groups. This is a useful metric to determine the limits of an organism’s environmental range, but it is a binary measure that provides little insight into underlying mechanisms of stress response. By measuring sub-lethal responses to stress, including aspects of metabolism and behavior, it may be possible to identify the type (salinity, temperature, oxygen, disease) and level (mild, moderate, severe) of stress an organism is under before mortality occurs. Certain biomarkers
involved in anaerobic metabolism (pH, alanine, succinate) and cell protection (calcium, glutathione) are good indicators of stress tolerance in oysters (Booth et al. 1984; La Peyre et al. 1995; Dwyer and Burnett 1996; Zhang et al. 2006; Lombardi et al. 2013; Khan and Ringwood 2016; Noguiera et al. 2017; Haider et al. 2020). Similarly, valve movement is a good behavioral measure that can indicate temperature and hypoxia stress in oysters (Casas et al. 2018b; Porter and Breitburg 2016). Casas et al. (2018b) reported oysters exposed to less optimal temperatures (10 and 30 °C) compared to a more optimal temperature (20 °C) were closed more often, and Porter and Breitburg et al. (2016) noted that oysters responded to the onset of hypoxia by closing.

The second chapter of this thesis examines the response of four geographically distant eastern oyster populations from the northern GoM to continuous hypoxia (DO < 2.0 mg L\(^{-1}\)) and high temperature (32 °C) to measure potential differences in lethal (mortality) and sub-lethal (metabolic biomarkers and valve movement) response. This chapter examines the potential for oyster populations to adapt to increasing low oxygen events by determining whether there are population-specific differences in lethal and sub-lethal response to continuous hypoxia. This work contributes to the understanding of hypoxia tolerance and the identification of potential populations that could be used to increase tolerance in broodstocks for future restoration and aquaculture production.

The third chapter of this thesis examines the response of diploid and triploid eastern oysters from the northern GoM to continuous hypoxia (DO < 2.0 mg L\(^{-1}\)) and anoxia (DO < 0.5 mg L\(^{-1}\)) at warm water temperatures (28 °C) to measure potential differences in lethal (mortality) and sub-lethal (metabolic biomarkers) response. This work explores potential reasons for observed differences in mortality in triploid oysters in past aquaculture work in this region (Wadsworth et al. 2019a) and examines biomarkers that could indicate differences in how diploid and triploid oysters respond to low oxygen stress.

Combined, this work examines the potential for oyster populations to adapt to increasing low oxygen events through the comparison of oyster populations from different estuaries and increases our understanding of how ploidy may impact stress response or tolerance of oysters. This work provides insight that will benefit restoration of oyster reefs and the development of an off-bottom aquaculture industry by increasing our understanding of low oxygen tolerance, its population specific variation, and understanding potential mechanisms of increased tolerance through the examination of biomarkers.
CHAPTER 2. DIFFERENTIAL HYPOXIA TOLERANCE OF EASTERN OYSTERS EXPOSED TO HIGH TEMPERATURE

2.1. INTRODUCTION

Hypoxia, defined as a dissolved oxygen concentration (DO) < 2.0 mg L\(^{-1}\), is a common environmental stressor in marine environments that many organisms, including bivalves, can tolerate under specific conditions. However, continued eutrophication and warming of coastal waters caused by global climate change is increasing the spatial and temporal extent of hypoxia in estuaries and coastal ecosystems (Tilman et al. 2001; Rabalais et al. 2009). Although many intertidal bivalves have evolved strategies to cope with periodic hypoxia (Hochachka and Lutz 2001; Sokolova et al. 2019), extended hypoxic events can negatively impact organism physiological function and lead to mass mortalities (Diaz and Rosenberg 2008; Vaquer-Sunyer and Duarte 2008).

Eastern oysters (Crassostrea virginica) are among the most tolerant marine benthic organisms to low oxygen stress (Stickle et al. 1989; Matsche and Barker 2006). In bivalves, tolerance to low oxygen is achieved through several physiological mechanisms that 1) help maintain oxygen consumption at low DO (Tran et al. 2000), 2) use alternative anerobic pathways that produce higher energy yield (Collicutt and Hochachka 1977; de Zwaan 1983; Brooks et al. 1991), 3) coordinate metabolic rate suppression to limit energy consumption and production (Hochachka and Lutz 2001; Sokolova et al. 2019), and 4) reduce damage caused by harmful reactive oxygen species (Haddad and Land 2000; Michiels et al. 2002; Ivanina et al. 2016; Sokolova et al. 2019).

Although eastern oysters can tolerate short-term hypoxia (Lombardi et al. 2013; Porter and Breitburg 2016) and even extended exposure to anoxia (Stickle et al. 1989), that tolerance declines with increasing temperature (Stickle et al. 1989; Ivanina et al. 2012). Increasing temperature simultaneously increases oyster metabolic energy demands (Shumway and Koehn 1982; Hutchinson and Hawkins 1992; Shumway 1996; Cherkasov et al. 2006) and decreases the amount of dissolved oxygen available for aerobic respiration. Previous studies investigating the effects of temperature on eastern oyster hypoxia tolerance (Stickle et al. 1989; Ivanina et al. 2012) and respiration (Casas et al. 2018b) have used temperatures as high as 30 °C, however it is common for eastern oysters in southern latitudes such as the northern Gulf of Mexico (northern GoM) to experience water temperatures of 32 °C and above during the summer. Although Marshall et al. (2021b) showed that eastern oysters can tolerate extended periods (20 days) of 32 °C water temperature, it is unclear the effect that co-occurring hypoxia can have on eastern oyster physiological function and survival.

The effect of population may also impact eastern oyster tolerance to hypoxia. Geographically distant populations of oysters can become adapted to local environmental conditions (Burford et al. 2014; Marshall et al. 2021a) and respond differently to environmental stress (Marshall et al. 2021b). Marshall et al. (2021b) found that an eastern oyster population originating from Laguna Madre in Texas, a southern and high salinity estuary in the northern GoM, survived longer when exposed to extreme salinity and prolonged high temperature than two populations originating from Calcasieu Lake and Vermilion Bay, two more central and
fresher estuaries in Louisiana (Figure 2.1). Engle et al. (1999) reviewed DO data (1991-1994) from over 600 locations in estuaries along the northern GoM, finding that estuaries which experienced hypoxia regularly were primarily located east of the Mississippi River, although hypoxia was also recorded in some small Texas estuaries. Interestingly, NOAA’s 1997 Estuarine Eutrophication Survey for the Gulf of Mexico Region reported that, although hypoxia is experienced periodically in estuaries with varying frequency, the Lower Laguna Madre was the only site to experience persistent hypoxia year-round. Due to varying hypoxia regimes occurring across northern GoM estuaries, oyster populations located in different estuaries may be adapted to local conditions and possess differential tolerances to hypoxia, especially with the co-occurrence of high temperatures.

Tolerance can be measured as a lethal and/or sub-lethal response to a particular stress. Lethal responses can be measured by quantifying and comparing the rates of mortality among groups of organisms. This is useful in determining the absolute limits of tolerance, but it does not describe potential mechanisms involved in the response. Sub-lethal responses, including aspects of metabolism and behavior, can provide insight into such mechanisms and how certain populations or organisms may better tolerate stress, and in this case low DO. In this way, stress experienced from specific biotic (disease, predation) or abiotic (salinity, temperature, DO) factors can be identified before an organism dies. For instance, oysters exposed to stressful low oxygen concentrations will close their valves and transition from an aerobic to an anaerobic metabolism (i.e., anaerobiosis). By measuring the concentration of certain metabolic end-products that accumulate with increased anaerobiosis, the timing and severity of oxygen stress can be determined. Additionally, valve movement can be measured to determine the severity of stress (Porter and Breitburg 2016), and the limit to which oysters can remain closed before dying. Lombardi et al. (2013) showed how valve movement differs between Asian oysters (Crassostrea ariakensis) and more hypoxia-tolerant eastern oysters in response to anoxia. The same comparison can be made between eastern oyster populations to understand how behavior (valve movement) can contribute to increased tolerance.

While eastern oysters are well adapted to estuarine conditions and can tolerate a wide range of water temperatures and DO levels, the extent of hypoxia tolerance when also experiencing high temperature (> 30 °C) is not known; nor are there data examining population-specific hypoxia tolerance. Using oyster populations from four distinct GoM estuaries, these studies assessed oyster population-specific mortality and physiological response to continuous hypoxia at a temperature near their upper thermal critical limit (32 °C). The cumulative mortalities of the progenies of wild eastern oysters from four northern GoM estuaries differing in mean temperature and exposed to extended (12 days) hypoxia and high temperature (32 °C) were measured, and median lethal times (LT50) were determined. Concentrations of important physiological biomarkers (protein, alanine, succinate, calcium, glutathione) were also measured along with oyster valve movement to examine sub-lethal responses, and to better understand mechanisms involved in responses.
2.2. METHODS

2.2.1. Oysters
Oysters used in these studies were the progeny of wild oysters collected at four estuarine sites within the northern GoM. Sites differ in mean annual salinity (Figure 2.1, Table 2.1). The progenies were produced in August 2018 at the Auburn University Shellfish Hatchery in Dauphin Island, Alabama, as described in Marshall et al. (2021b). The F1 oysters were grown in bags on an adjustable long line system (ALS, BST Oyster Co., Cowell, South Australia) in Bayou Sullivan, Alabama (30° 21’ 52” N, 88° 12’ 57” W), before being moved in Spring 2019 to the Grand Bay Oyster Park (GBOP), Alabama (30° 22’ 15” N, 88° 19’ 0” W) for further grow-out prior to being transported to Louisiana State University Agricultural Center Animal and Food Sciences Laboratory building (AFL) in Baton Rouge, Louisiana, to measure their response to hypoxia.

![Map of sites](image)

Figure 2.1. Map of sites where the wild oyster broodstock were collected and grown. Collection sites are located in Texas (Packery Channel, PC and Aransas Bay, AB) and Louisiana (Calcasieu Lake, CL and Vermilion Bay, VB), and F1 progenies were grown at the Grand Bay Oyster Park (GBOP), Alabama.

2.2.2. Experimental design
Three studies were conducted at AFL between August and December 2019. Before the start of each study, the shell heights of 20 oysters from each F1 population used were measured using a digital caliper (ABS Coolant Proof Calipers, Mitutoyo USA, Aurora, Illinois) and mean shell heights (mm) were calculated (Table 2.1). Oysters were placed in 400-L tanks with bio-filters and filled with aerated artificial seawater (Crystal Sea Marinemix, Marine Enterprises International, Baltimore, Maryland, USA) adjusted to a salinity of 20 and temperature of 28.9 °C.
(84°F). These conditions represent common conditions experienced by northern GoM oysters during the summer. Water temperature was controlled using submersible heaters with thermostat setting in °F (Cobalt Aquatics Flat Neo-Therm 300W). Water salinity, temperature (°C) and dissolved oxygen concentration (DO, mg L⁻¹) were measured using a YSI-Pro30 handheld multimeter (YSI Incorporated, Yellow Springs, Ohio, USA). Oysters were fed daily with Shellfish Diet 1800® (Reed Mariculture Inc, Campbell, California, USA) and acclimated for about two weeks before the start each study. Normoxia was defined as DO > 2.0 mg L⁻¹, however for these studies DO was maintained > 5.0 mg L⁻¹ to prevent potential oxygen stress in control groups. Hypoxia was defined as DO < 2.0 mg L⁻¹.

2.2.3. Study 1

Oysters from Packery Channel (PC), Aransas Bay (AB), Calcasieu Lake (CL) and Vermilion Bay (VB) F1 populations were used in Study 1, with 45-50 oysters from each population placed into each of 4 tanks. After acclimation, water temperature was increased at a rate of 1.1 °C (2 °F) every other day to the target temperature of 32.2 °C (90 °F). After one week at the target temperature, air stones from two of the tanks were then removed (replicate hypoxia tanks) and left in the other two tanks (replicate normoxia tanks). Oyster feeding was ceased in all tanks once air stones were removed. The numbers of live and dead oysters from each population were counted daily for about 2 weeks and dead oysters removed. Cumulative mortalities of each population in each tank were calculated by dividing the number of oysters that died each day by the total (live + dead). The median lethal times (LT₅₀) of PC, CL, AB, and VB oysters exposed to continuous hypoxia were then determined. Temperature (°C), salinity, and DO (mg L⁻¹) were recorded daily.

Table 2.1. Geographic coordinates and annual mean ± SD salinity and temperature (°C) (2009–2018) of sites where eastern oyster broodstocks were collected and mean ± SD shell height (mm) of F1 progeny at the time of the studies. The collection sites were Packery Channel (PC) and Aransas Bay (AB) in Texas, and Calcasieu Lake (CL) and Vermilion Bay (VB) in Louisiana. Study 1 occurred in August 2019, Study 2 in October 2019, and Study 3 in November 2019.

<table>
<thead>
<tr>
<th>Population</th>
<th>Latitude, Longitude</th>
<th>Salinity (°C)</th>
<th>Temperature (°C)</th>
<th>Study 1 SH (mm)</th>
<th>Study 2 SH (mm)</th>
<th>Study 3 SH (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>27° 37' 38&quot; N, 97° 13' 59&quot; W</td>
<td>35.5 ± 5.1</td>
<td>26.1 ± 1.7</td>
<td>46.9 ± 5.1</td>
<td>59.4 ± 4.8</td>
<td>66.7 ± 7.8</td>
</tr>
<tr>
<td>CL</td>
<td>28° 7' 38&quot; N, 96° 59' 8&quot; W</td>
<td>23.0 ± 6.9</td>
<td>22.9 ± 1.1</td>
<td>45.5 ± 4.0</td>
<td>58.7 ± 8.0</td>
<td>63.1 ± 5.4</td>
</tr>
<tr>
<td>AB</td>
<td>29° 50' 58&quot; N, 93° 17' 1&quot; W</td>
<td>16.2 ± 2.8</td>
<td>21.7 ± 1.8</td>
<td>42.8 ± 4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VB</td>
<td>29° 34' 47&quot; N, 92° 2' 4&quot; W</td>
<td>7.4 ± 1.6</td>
<td>22.0 ± 1.4</td>
<td>46.3 ± 4.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.4. Study 2

Oysters from the most (PC) and least (CL) hypoxia tolerant population, as determined in Study 1, were used in Study 2. Oysters were exposed to normoxia and hypoxia, and their biomarkers were measured. After initial acclimation, oysters were then acclimated to a temperature of 32.2 °C (90 °F) as described in Study 1. After one week at the target temperature, the number of oysters in each population in each tank was adjusted to 80 and air stones from one of the tanks were removed. Feeding ceased in both tanks once air stones were removed. The numbers of live and dead oysters from each population were counted daily for 2 weeks and dead oysters removed.

Oysters were sampled on days 3-5 of normoxia or hypoxia exposure. On each of the three days, 6-8 oysters were taken out to sample. A total of 21 oysters were sampled for each population in each tank. Hemolymph was withdrawn from the adductor muscle sinus of each oyster sampled through a notch on the dorsal side of the shell, using a 3-ml syringe equipped with a 25-gauge 11/2” (3.8 mm) needle. The hemolymph was immediately placed into 1.5 ml eppendorf tubes on ice to limit hemocyte clumping and used to determine hemocyte density and granulocyte % as described below. Left over hemolymph was then centrifuged at 400 x g for 15 min at 4 °C and the supernatant was collected as plasma and frozen at -20 °C for later measurement of protein, calcium and glutathione concentrations as described below (Section 2.2.6). Oysters were then carefully opened, and sections of digestive gland were cut and stored at -20 °C to measure alanine and succinate concentrations as described below. Because oysters were destructively sampled during part of the study, interval mortalities and adjusted mortalities were first calculated to derive cumulative mortalities as described by Ragone Calvo et al. (2003).

2.2.5. Study 3

Study 3 was a repeat of Study 2, except oysters were sampled to measure biomarkers on days 1-3 of normoxia or hypoxia exposure. On each of the three days, 7 oysters were taken out to sample. A total of 21 oysters were sampled for each population in each tank. Hemolymph and tissue samples were taken following methods outlined in Study 2 and used to measure biomarkers (Section 2.2.6). In addition, shell valve movement of two oysters from each population x DO combination was measured as described below (Section 2.2.7).

2.2.6. Biomarkers/Assays

Hemocyte density (cells ml⁻¹) and granulocytes percentage (%) were determined with improved Neubauer hemocytometers (Reichert, Buffalo, NY) as described by La Peyre et al. (1995). Plasma protein concentration (mg mL⁻¹) was measured using Pierce Biotech Micro BCA Protein Assay Kit (Rockford, IL, USA) with bovine serum albumin as a standard. Plasma calcium ion concentration (mM) was measured using Sigma-Aldrich MAK022 Calcium Colorimetric Assay Kit (St. Louis, MO, USA). Plasma glutathione concentration (µM) was measured using BioAssay systems, DIGT-250 QuantiChrom™ Glutathione Assay kit (Hayward, CA, USA). All samples were adjusted to 2.5 mg mL⁻¹ with distilled water prior to measuring glutathione (La Peyre et al. 2014). Digestive gland alanine concentration (µmol gram⁻¹) was measured using Sigma-Aldrich MAK001 Alanine Assay Kit. Tissue samples were thawed, homogenized in alanine assay buffer (10 mg in 0.1 mL buffer), and centrifuged at 10,000 x g for 5 minutes. Supernatant was collected
and used to measure alanine concentration. Digestive gland succinate concentration (µmol gram⁻¹) was measured using Sigma-Aldrich, MAK184 Succinate Colorimetric Assay Kit. Samples were prepared similarly to those used in the Alanine Colorimetric Assay Kit, but with succinate assay buffer. All measurements were done in duplicate.

2.2.7. Valve movement determination
A non-invasive system to measure oyster valve movement, more thoroughly described in Casas et al. (2018a), was used to measure oyster valve movement for about 15 days during normoxia or hypoxia exposure. To compare valve movement between population and DO levels, two oysters per population were used for each DO level (2 oysters x 2 populations x 2 DO levels = 8 oysters total). For each oyster, a small magnet was glued to one valve at the maximum distance from the hinge, and a Hall element sensor (HW-300a, Asahi Kasei, Japan) coated in epoxy was glued directly across from the magnet on the other valve. The magnetic field in the form of output voltage (µV) was recorded every minute by dynamic strain recording devices (DC 204R, Tokyo Sokki Kenkyujo Co., Shinagawa-ku, Tokyo, Japan). At the end of the experiment oysters were opened, calibration wedges of known width (1-10 mm) were placed between the oyster’s valves and the output voltage was measured with each wedge to create a voltage-to-width regression equation specific to each oyster. The relationships between voltage and wedge width (i.e., valve opening) were strong (r² > 0.97). Valve opening data were converted into gape angles (θ in degrees) as described in Wilson et al. (2005). Gape angle values were expressed as a percentage of the maximum gape angle recorded before death and categorized as closed for values ≤10% (Comeau et al. 2018) or otherwise opened. These categories were defined because of the difficulty of defining a “fully closed” or “fully open” oyster due to the sensitivity of the equipment used and variations at both ends of valve amplitude. The frequency at which valves were open (%) and mean (± SD) gape angles (°) were calculated for each oyster during Day 0 to Day 2.5 of exposure. This time frame was chosen so that the movement of all oysters could be compared before mortality occurred (Figure 4). Percent (%) open was calculated by dividing the time oysters were open by the total time measured (2.5 days), and mean gape angles were calculated using only the data categorized as “open”.

2.2.8. Statistical analyses
Mortality data were subjected to probit analysis using the R package ‘ecotox’ (Wheeler et al. 2006). LT₅₀ was calculated from the day hypoxia was first measured in each tank and herein referred as Day 0. For all populations with >15% mortality in each hypoxia trial, median lethal time (LT₅₀) with 95% confidence intervals (95% CI) were determined. Confidence intervals that do not overlap are statistically different (Wheeler et al. 2006). All analyses were performed using R 3.6.3 (R Foundation for Statistical Computing, 2020).

Hemocyte density, granulocyte %, plasma protein, calcium and glutathione concentrations, digestive gland alanine and succinate concentrations, and valve movements to measure % open and mean gape angle were examined for normality and homogeneity of variance and analyzed with a two-factor (DO level x population) analysis of variance (ANOVA). When significant differences were found (P < 0.05), Tukey’s HSD test was used for pairwise multiple comparisons. Data that did not fulfill ANOVA requirements were instead analyzed with a Kruskal-Wallis non-parametric rank-sum tests, followed by pairwise t-tests with a Bonferroni
correction when significant differences were found.

2.3. RESULTS

2.3.1. Study 1

Water quality

Hypoxia (< 2.0 mg L⁻¹) was measured on the third day after air stones were removed from both replicate tanks (Figure 2.2). From that day to the end of the study water quality remained relatively consistent (Table 2.2; Figure 2.2).

Table 2.2. Mean ± SD temperature (°C), salinity, and DO (mg L⁻¹) of tanks during exposure to normoxia and hypoxia in Study 1, Study 2, and Study 3. Study 1 includes two replicates per treatment, and Study 2 and 3 have only one tank per treatment. Temperature SD is higher in Study 3 because aquarium heaters were accidentally shut off for 12 hours and temperatures briefly decreased.

<table>
<thead>
<tr>
<th></th>
<th>Study 1</th>
<th></th>
<th>Study 2</th>
<th></th>
<th>Study 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td></td>
<td></td>
<td>Normoxia</td>
<td></td>
</tr>
<tr>
<td>Rep 1</td>
<td>31.7 ± 0.5</td>
<td>Temperature (°C)</td>
<td>32.1 ± 0.5</td>
<td>3.1 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Rep 2</td>
<td>31.7 ± 0.5</td>
<td>Salinity</td>
<td>21.3 ± 0.2</td>
<td>21.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Hypoxia Rep 1</td>
<td>31.6 ± 0.4</td>
<td>DO (mg L⁻¹)</td>
<td>6.0 ± 0.1</td>
<td>5.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Hypoxia Rep 2</td>
<td>31.6 ± 0.4</td>
<td></td>
<td>6.7 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td></td>
<td></td>
<td>Hypoxia</td>
<td></td>
</tr>
<tr>
<td>Rep 1</td>
<td>20.3 ± 0.1</td>
<td></td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Rep 2</td>
<td>20.2 ± 0.1</td>
<td></td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep 1</td>
<td>20.7 ± 0.2</td>
<td></td>
<td>0.4 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep 2</td>
<td>20.7 ± 0.2</td>
<td></td>
<td>0.4 ± 0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lethal median time (LT₅₀)

The cumulative mortalities of all populations under hypoxia were > 90%, except for the PC population, which was only 43.8% in one of the two replicate tanks (Figure 2.2). Significant differences in LT₅₀ were found among populations under hypoxia with the PC population having a significantly higher LT₅₀ (i.e., lower mortality; 7.9-12.5 days) and CL population having a significantly lower LT₅₀ (i.e., higher mortality; 3.9-4.5 days) than all other populations (Table 2.3).
Figure 2.2. Cumulative mortality of the progenies (F1) of Texas oysters from Packery Channel (PC) and Aransas Bay (AB) broodstocks, and of Louisiana oysters from Calcasieu Lake (CL) and Vermilion Bay (VB) broodstocks during Study 1. Hypoxia was reached in the hypoxia tanks on Day 0, two days after all airstones delivering air to the hypoxia tanks were removed. Normoxic tanks were supplied with continuous aeration for the duration of the experiment. DO was measured in all tanks throughout the experiment but recorded only twice in normoxic tanks.
Table 2.3. Median lethal time (LT$_{50}$; days) with 95% confidence intervals (CI 95%) of the F1 progenies of Texas oyster broodstocks from Packery Channel (PC) and Aransas Bay (AB), and of Louisiana oyster broodstocks from Calcasieu Lake (CL) and Vermilion Bay (VB) from continuous hypoxia exposure.

<table>
<thead>
<tr>
<th>Population</th>
<th>Study 1</th>
<th></th>
<th>Study 2</th>
<th></th>
<th>Study 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LT$_{50}$</td>
<td>95% CI</td>
<td>LT$_{50}$</td>
<td>95% CI</td>
<td>LT$_{50}$</td>
</tr>
<tr>
<td>PC</td>
<td>7.9</td>
<td>(7.4, 8.5)</td>
<td>12.5</td>
<td>(11.5, 14.1)</td>
<td>7.9</td>
</tr>
<tr>
<td>CL</td>
<td>3.9</td>
<td>(3.5, 4.3)</td>
<td>4.5</td>
<td>(4.2, 5.0)</td>
<td>4.7</td>
</tr>
<tr>
<td>AB</td>
<td>5.4</td>
<td>(5.1, 5.9)</td>
<td>6.9</td>
<td>(6.5, 7.5)</td>
<td></td>
</tr>
<tr>
<td>VB</td>
<td>5.4</td>
<td>(5.1, 5.8)</td>
<td>6.3</td>
<td>(5.9, 6.8)</td>
<td></td>
</tr>
</tbody>
</table>

2.3.2. Study 2

Water quality

Hypoxia was measured one day after air stones were taken out. From that day to the end of the study water quality in normoxia and hypoxia tanks remained relatively consistent (Table 2.2; Figure 2.3).

Median lethal time (LT$_{50}$)

Both PC and CL populations under hypoxia experienced 100% cumulative mortality within 2 weeks (Figure 2.3). The PC population had a significantly higher LT$_{50}$ (7.9 days) than the CL population (4.7 days; Table 2.3).
Figure 2.3. Cumulative mortality of the F1 progenies of Texas oysters from Packery Channel (PC) broodstock, and of Louisiana oysters from Calcasieu Lake (CL) broodstock during Studies 2 and 3. Hypoxia was reached in the hypoxia tanks on Day 0. Normoxia tanks were supplied with continuous aeration for the duration of the experiment.

**Hemocyte density and granulocyte percentage**

There was a significant treatment x population interaction for hemocyte density (p < 0.001). For both populations, oysters under hypoxia had lower hemocyte densities than under normoxia, and CL oysters had higher hemocyte densities than PC oysters under normoxia (Table 2.4).

There was a significant treatment x population interaction for granulocyte % (p < 0.001). Under both normoxia and hypoxia, granulocyte % was greater in PC oysters than in CL oysters. Granulocyte % of PC oysters under hypoxia was also greater than under normoxia (Table 2.4).

**Plasma protein, glutathione and calcium concentrations**

Plasma protein concentration (mg mL⁻¹) differed significantly by population only (p<0.001), with CL oysters having about twice the protein concentration of PC oysters (CL: 6.3 ± 2.3, PC: 3.1 ± 2.3).

There was a significant treatment x population interaction for plasma calcium concentration (p < 0.001), with PC and CL oysters under hypoxia having 52% and 16% greater calcium concentrations than under normoxia, respectively, and with PC oysters having 28% greater calcium concentrations than CL oysters under hypoxia.

There was a significant treatment x population interaction for plasma glutathione concentration (p = 0.001). PC oysters under hypoxia had greater plasma glutathione
concentrations than PC oysters under normoxia and CL oysters under hypoxia and normoxia (Table 2.4). Under normoxia, plasma glutathione concentration of PC oysters was also greater than that of CL oysters.

**Digestive gland succinate and alanine concentrations**

Digestive gland alanine concentration did not significantly differ by DO level or population. Alanine concentration for PC and CL oysters were similar under hypoxia and normoxia (Table 2.4).

Digestive gland succinate concentration differed significantly among groups (H (3) = 17.408, p<0.001). CL oysters under hypoxia had significantly lower succinate concentration than PC oysters under hypoxia and oysters from either population under normoxia (Table 2.4).

### 2.3.3. Study 3

#### Water quality

Hypoxia was measured two days after air stones were taken out of the water (Figure 2.3). From that day (i.e., Day 0) to the end of the study water quality in normoxia and hypoxia tanks were remained relatively consistent (Table 2.2; Figure 2.3).

**Lethal median time (LT$_{50}$)**

Both PC and CL populations under hypoxia experienced ≥ 90% cumulative mortality within 2 weeks. (Figure 2.3). The PC population had a significantly higher LT$_{50}$ than the CL population (Table 2.3). All populations in normoxic tanks experienced <15 % mortality, with the highest mortality from PC (8.4%).

**Hemocyte density and granulocyte percentage**

Hemocyte density differed significantly by treatment (p = 0.027) and population (p = 0.004). Hemocyte density of oysters under hypoxia was lower than under normoxia, and greater in CL than in PC (Table 2.4).

Granulocyte % differed significantly by population (p<0.001). Granulocyte % was greater in PC oysters than CL oysters (%: CL: 26 ± 7, PC: 43 ± 13).

**Plasma protein, glutathione, and calcium concentrations**

Plasma protein concentration differed significantly by treatment (p<0.001) and population only. Plasma protein concentration in CL was approximately twice that of PC (Table 2.4).

Plasma calcium concentration differed significantly by treatment (p<0.001), with oysters under hypoxia having greater plasma calcium concentration than oysters under normoxia (Table 2.4).

There was a significant DO x population interaction for plasma glutathione concentration. In PC oysters under normoxia, glutathione concentration was lower than in CL oysters under normoxia, and lower than both populations under hypoxia (Table 2.4).

**Digestive gland alanine and succinate concentrations**
Digestive gland alanine concentration differed significantly among groups ($H(3) = 23.957, p<0.001$). CL oysters under hypoxia had significantly higher alanine concentration than PC oysters under hypoxia and oysters from each population under normoxia (Table 2.4).

Digestive gland succinate concentration differed significantly among groups ($H(3) = 12.427, p = 0.006$). CL oysters under normoxia had significantly higher succinate concentration than PC oysters under normoxia and CL oysters under hypoxia (Table 2.4).

Table 2.4. Mean ± SD hemocyte density, granulocyte percentage (%), plasma protein, calcium and glutathione concentrations, and digestive gland alanine and succinate concentrations of Packery Channel (PC) and Calcasieu Lake (CL) F1 oysters under hypoxia and normoxia in study 2 and 3. Oysters in study 2 were sampled after an average of 4 days of hypoxia (late exposure), whereas oysters in study 3 were sampled after an average of 2 days (early exposure). For each study, groups with different letters are statistically different ($p<0.05$).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Study 2 - Late Exposure</th>
<th>Study 3 - Early Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Hemocyte density ($10^6$ cells mL$^{-1}$)</td>
<td>1.9±1.2$^b$</td>
<td>1.4±0.8$^c$</td>
</tr>
<tr>
<td>Granulocyte %</td>
<td>44 ± 11$^b$</td>
<td>62 ± 13$^a$</td>
</tr>
<tr>
<td>Protein (mg ml$^{-1}$)</td>
<td>3.3 ± 2.9$^a$</td>
<td>2.9 ± 1.5$^a$</td>
</tr>
<tr>
<td>Calcium (mM)</td>
<td>5.4 ± 0.5$^c$</td>
<td>8.2 ± 1.3$^a$</td>
</tr>
<tr>
<td>Glutathione (µM)</td>
<td>36 ± 19$^b$</td>
<td>85 ± 38$^a$</td>
</tr>
<tr>
<td>Alanine (µmol g$^{-1}$)</td>
<td>17.2 ± 9.6$^b$</td>
<td>16.9 ± 10.3$^a$</td>
</tr>
<tr>
<td>Succinate (µmol g$^{-1}$)</td>
<td>8.2 ± 2.8$^a$</td>
<td>8.9 ± 3.5$^a$</td>
</tr>
<tr>
<td></td>
<td>Study 2 - Late Exposure</td>
<td>Study 3 - Early Exposure</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Hemocyte density ($10^6$ cells mL$^{-1}$)</td>
<td>4.3 ± 2.7$^a$</td>
<td>2.9 ± 2.5$^a$</td>
</tr>
<tr>
<td>Granulocyte %</td>
<td>43 ± 11$^a$</td>
<td>44 ± 16$^a$</td>
</tr>
<tr>
<td>Protein (mg ml$^{-1}$)</td>
<td>4.5 ± 1.8$^a$</td>
<td>4.1 ± 2.5$^a$</td>
</tr>
<tr>
<td>Calcium (mM)</td>
<td>5.6 ± 0.7$^a$</td>
<td>6.8 ± 1.5$^a$</td>
</tr>
<tr>
<td>Glutathione (µM)</td>
<td>19 ± 9$^b$</td>
<td>35 ± 22$^a$</td>
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<tr>
<td>Alanine (µmol g$^{-1}$)</td>
<td>11.4 ± 2.6$^a$</td>
<td>12.8 ± 2.7$^a$</td>
</tr>
<tr>
<td>Succinate (µmol g$^{-1}$)</td>
<td>7.9 ± 1.7$^a$</td>
<td>8.7 ± 3.0$^{ab}$</td>
</tr>
</tbody>
</table>
Valve movement

The percentage of time oysters were opened differed significantly by DO level \((p=0.004)\), where oysters under hypoxia were open less often than those under normoxia (Table 2.5; Figure 2.4). The mean gape ± SD angles of PC and CL oysters were similar under hypoxia \((2.85 ± 1.31°\) and \(1.23 ± 0°\), respectively) and normoxia \((1.98 ± 0.08°\) and \(2.00 ± 0.11°\), respectively) (Table 2.5).

![Diagram of valve movement](image)

Figure 2.4. Valve movement (Degrees Open) of Packery Channel (PC) and Calcasieu Lake (CL) F1 oysters under normoxia and hypoxia. Individuals numbered 1-2 were in the normoxia tank, and 2-4 were in the hypoxia tank. Hypoxia was reached in the hypoxia tank on Day 0 of Treatment Days. In the hypoxia tank, PC oysters died around day 9 (PC 3) and day 8 (PC 4), and the CL oysters died around day 3 (CL 3) and day 5-6 (CL 4). Shading indicates the time period used to calculate values for Table 2.5. Time periods were selected based on when individual oysters exposed to hypoxia, from Day 0, began to display gaping behavior preceding death.
Table 2.5. Percentage of time the valves of individual oysters were open and their mean ± SD angle (degrees) under normoxia and hypoxia in Study 3. Mean angle was calculated using only the data categorized as open. Oysters used were the progenies of broodstocks from Packery Channel (PC) and Calcasieu Lake (CL). Values were calculated using data from Day 0 to Day 2.5. The numbers 1 and 2 denote the two oysters that were in the normoxia tank, and 3 and 4 denote the two oysters in the hypoxia tank.

<table>
<thead>
<tr>
<th>Oyster</th>
<th>% Open</th>
<th>Mean angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normoxia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC 1</td>
<td>55.1</td>
<td>2.04 ± 0.54</td>
</tr>
<tr>
<td>PC 2</td>
<td>52.5</td>
<td>1.92 ± 0.53</td>
</tr>
<tr>
<td>CL 1</td>
<td>60.2</td>
<td>1.93 ± 0.48</td>
</tr>
<tr>
<td>CL 2</td>
<td>64.5</td>
<td>2.08 ± 0.55</td>
</tr>
<tr>
<td><strong>Hypoxia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC 3</td>
<td>32.2</td>
<td>1.93 ± 0.65</td>
</tr>
<tr>
<td>PC 4</td>
<td>16.4</td>
<td>3.78 ± 1.01</td>
</tr>
<tr>
<td>CL 3</td>
<td>30.2</td>
<td>1.23 ± 0.41</td>
</tr>
<tr>
<td>CL 4</td>
<td>12.6</td>
<td>1.23 ± 0.46</td>
</tr>
</tbody>
</table>
2.4. DISCUSSION

Continuous exposure to hypoxia at 32 °C resulted in high oyster mortality (< 90%) across all four populations tested over a 2-week period. The rate and final cumulative mortality differed between populations, with one population (PC) dying at a slower rate compared to the other three populations. The largest difference in LT$_{50}$ occurred between PC and CL oysters, with PC having an LT$_{50}$ 4-6 days longer than CL. Follow-up studies found similar differences in mortality between the two populations, indicating PC is more hypoxia tolerant at high temperatures. These results demonstrate potential for local adaptation to stressors that may become more frequent with climate change, as PC oysters are exposed to higher water temperatures on average, and likely more frequent low oxygen events. Differences in valve movement suggest the two populations may employ different strategies of oxygen consumption even during normoxia, which may partially explain differences in hypoxia tolerance. Moreover, biomarker analysis showed the more hypoxia tolerant PC oysters generally had greater accumulations of calcium, and glutathione than CL after longer hypoxia exposure, suggesting PC is better able to maintain metabolic function and respond to low oxygen stress. These findings identify populations potentially useful for oyster restoration or aquaculture production and for future selective breeding projects.

In all three studies, oysters exposed to normoxia experienced minimal mortality (<15%) at 32 °C. This is consistent with Marshall et al. (2021b), who reported similarly low mortality (<15%) in oysters exposed to continuously aerated waters at the same salinity and a slightly higher temperature (33.7 °C). Estuaries in the northern GoM routinely experience temperatures of 32 °C in the summer (Leonhardt et al. 2017; La Peyre et al. 2019b) where monthly oyster mortality generally does not exceed 5% unless salinity is < 5 or dermo infection is high (Wadsworth et al. 2019a; La Peyre et al. 2019a; Casas et al. 2020). While oysters can survive these high temperatures, evidence indicates they may still be experiencing sub-lethal stress. Loosanoff (1958) reported maximum pumping rates in oysters between at 30–32 °C, followed by decreased rates above 32 °C. There were also changes in shell movements and pumping intervals above 32 °C that indicated oysters were being unfavorably affected by the higher temperatures. Feng and Van Winkle (1975) observed a maximum heart rate in C. virginica at 30 °C followed by a decrease at 35 and 40 °C. Maintaining proper physiological function at 32 °C is demanding for oysters, and exposure to temperatures above the thermal optimum can limit the supply of oxygen to tissues, causing a transition from an aerobic to an anaerobic metabolism (Pörtner 2010; Pörtner et al. 2017).

When oysters were exposed to high temperature (32 °C) combined with hypoxia in these studies, cumulative mortality greater than 90% occurred in less than 14 days in all but one trial population (Figure 2.2; Figure 2.3). This suggests the oxygen concentration was too low for oysters to maintain aerobic metabolism, and that any transition to partial or full anaerobic metabolism could not compensate for an increased metabolic rate resulting from the elevated temperature. The effects on oysters were synergistic in that the mortality response was much greater than when oysters were exposed to either stressor alone (Todgham and Stillman 2013). Although the single effect of hypoxia on oyster mortality were not measured in the present studies, previous studies which measured oyster hypoxia tolerance at non-stressful temperatures (Stickle et al. 1989; Ivanina et al. 2011) can be used for comparison. Stickle et al. (1989) exposed eastern oysters to continuous hypoxia (DO < 0.5 mg L$^{-1}$) at a temperature of
20°C and salinities of 20 and 30. At salinities of 20 and 30, the reported LT50’s were 18 and 20 days, respectively. Ivanina et al. (2011) also exposed eastern oysters to continuous hypoxia (DO < 0.5 mg L\textsuperscript{-1}) at 20°C and salinity of 30 for two weeks, reporting no mortality. Similarly in the present studies, the oysters exposed to high temperature (32°C) alone experienced less than 15% after nearly two weeks. It is apparent that neither continuous hypoxia nor high temperature alone cause high mortality in eastern oysters even after multiple weeks, therefore the near-complete mortality during combined temperature and hypoxia exposure for the same amount of time may represent a synergistic effect. Synergistic effects of low DO and high temperature are reported in previous studies in a variety of mollusks (Ivanina et al. 2012; Artigaud et al. 2014; Tripp-Valdez et al. 2017; Stevens and Gobler 2018; Götz et al. 2020).

In addition to elevated temperature and decreased DO, the changing climate is increasing organisms’ exposure to other co-occurring environmental stressors that often amplify their physiological responses. Co-occurring stressors can have unpredicted interactions, and there are many unknowns about their combined effects (Pörtner and Langenbuch 2005; Stevens and Gobler 2018). Co-occurring stressor events have also been associated with mass mortalities of bivalves in the environment (Callaway et al. 2013; Malham et al. 2012; Burdon et al. 2014; Soon and Zheng 2019). These unexpected mass mortalities can result from multiple stressors acting in synergy and may not be predicted by individually tested stressors. It is therefore important to continue investigating the effects of co-occurring stressors both in the laboratory and in the environment, especially now considering the changing climate and increasing extremes in environmental conditions.

Differences in rates and cumulative mortalities between populations suggests there is potential for local adaptation to hypoxia. In these studies, PC oysters consistently died more slowly, surviving approximately 50% longer (4-6 days) in each study, and in one instance survived 3 times as long as CL oysters (i.e., Study 2). The slower rate of mortality in PC oysters may potentially be explained through exposure of the parent population to more frequent or regular hypoxia, or possibly through exposure to higher temperatures on average (Table 2.1: 26.1 °C versus < 23 °C at all three other sites). A recent study using the same populations of oysters found that PC oysters experienced lower cumulative mortality (~60% cumulative mortality) after 2 weeks exposure to a temperature of 35.6 °C (96 °F) compared to the CL oysters for example (> 80% cumulative mortality). Packery Channel is located in the southern portion of the Upper Laguna Madre estuary which experiences higher temperature and salinity than the other estuaries in this study. Higher temperature and salinity are both factors that decrease oxygen solubility. Moreover, Packery Channel’s proximity to the southeast region of Corpus Christi Bay, Texas, an area known to experience persistent hypoxia (National Estuarine Inventory 1996; Applebaum et al. 2005), supports the idea that PC oysters likely experience high temperature and low oxygen more frequently than the other populations, and may display sub-lethal stress responses that enable better tolerance when exposed to high temperature and low oxygen conditions.
Differences in behavioral responses and sub-lethal metabolic responses between PC and CL oysters may explain the observed differences in mortality. Hypoxia tolerant species have been shown to maintain oxygen consumption and aerobic metabolism at lower DO (de Zwaan et al. 1991; Eberlee et al. 1983; Marshall and McQuaid 1993; Meng et al. 2018), produce different amounts and types of anaerobic metabolites (Brooks et al. 1991; Haider et al. 2020), experience greater metabolic rate suppression (de Zwaan et al. 1991; Brooks et al. 1991), and produce more metabolites involved in cellular protection (Khan and Ringwood 2016) than less hypoxia-tolerant species.

Valve movement provides important information related to the oyster’s response to stressful conditions. Oysters exposed to hypoxia were open less often than those exposed to hypoxia, an indication that hypoxia was stressful, and that oysters were attempting to isolate themselves from exposure. Similar behaviors are reported by Casas et al. (2018a) in relation to high temperature stress, and in Porter and Breitberg (2016) in relation to hypoxia exposure. Although oysters exposed to hypoxia in the present study initially responded to the onset of hypoxia by closing, all of them opened at least once within 12 hours and continued to open and close throughout the experiment, suggesting oxygen consumption and an aerobic metabolism were ongoing to some degree. In a study by Porter and Breitberg (2016), oysters under severe hypoxia (0.6 mg DO L\(^{-1}\)) were also closed significantly longer than under normoxia, but not under mild hypoxia (1.7 mg DO L\(^{-1}\)). The oysters in Porter and Breitburg (2016) were, however, only exposed to short term fluctuations of DO through diel-cycling hypoxia, and long-term behaviors were not observed. During hypoxia exposure in the present study, PC oysters tended to gape wider than CL. In contrast to our results, Lombardi et al. (2013) reported increased opening time and wider valve gaping in the less hypoxia tolerant Asian oyster compared to the eastern oyster, perhaps to reduce excessive tissue acidosis during anoxia (0.5 mg L\(^{-1}\)). However, it is unclear whether these behaviors are always an indication of lesser tolerance. Alternatively, wider opening in combination with shorter periods of openness as seen between PC and CL oysters during normoxia may indicate greater pumping rates and oxygen uptake. It is possible PC oysters were better able to uptake oxygen during hypoxia, contributing to their longer survival. At present it is difficult to conclude much about potential differences in oxygen consumption and aerobic scope, and direct rates of oxygen consumption would need to be measured between these two populations to confirm this possibility.

Along with behavioral clues, several biomarkers can provide insight into stress response, and tolerance to specific stressors. Alanine and succinate are end-products of anaerobic glycolysis, and their accumulation has been reported in several studies measuring bivalve responses to hypoxia and anoxia (Collicutt and Hochachka 1976; de Zwaan et al. 1991; Le Moullac et al. 2007; Haider et al. 2020). The digestive gland alanine concentrations for PC were similar in both studies regardless of DO exposure, but in Study 3 were elevated in CL oysters. Succinate concentration was also unaffected by DO exposure in PC but was lower in CL exposed to hypoxia compared to normoxia. Increased alanine concentrations in Study 3 suggest CL transitioned to anaerobic metabolism earlier, or to a greater extent than PC. This explanation is supported by de Zwaan et al. (1991) who found that greater alanine accumulation occurred in the less hypoxia-tolerant Mediterranean mussel *M. galloprovincialis* compared to the arc clam *S. inaequivalvis*. Succinate was also found to accumulate to a greater extent in less hypoxia-tolerant species by de Zwaan et al. (1991) and Haider et al. (2020). For this reason, the
decreased concentration in CL was unexpected. However, in some bivalve species the accumulation of alanine and succinate are temperature dependent (Le Moullac et al. 2007). Investigations by Le Moullac et al. (2007) found that the Pacific oyster *Crassostrea gigas* exposed to 20 days of hypoxia accumulated alanine at 12 and 15 °C, but not at 20 °C. Similarly, succinate accumulated only at 12 °C, but not at 15 or 20 °C, suggesting oysters were already operating at maximum anaerobic capacity at the lower temperatures. The relatively unchanged concentrations of alanine and succinate from Study 2 was likely due to the slightly higher temperature and longer hypoxia exposure compared to Study 3 (Table 2.2). Moreover, decreased succinate concentration in CL oysters exposed to hypoxia may reflect the failure of their anaerobic pathways to function, which quickly resulted in death.

Hemolymph calcium concentration is another biomarker that may indicate hypoxia tolerance in oysters and other species of mollusk. Mollusks mitigate the effects of acidosis (decreased tissue pH) primarily by elevating the concentration of calcium ions and ammonium in the hemolymph (Booth et al. 1984; Dwyer and Burnett, 1996; Burnett 1997), and a greater accumulation of calcium in the hemolymph may indicate greater buffering capacity against acidosis. This mechanism was discussed by Lombardi et al. (2013) to explain why eastern oysters displayed improved anoxia (DO < 0.5 mg L⁻¹) tolerance and higher hemolymph pH during anoxia compared to Asian oysters. Calcium accumulated in CL oysters earlier during hypoxia exposure than PC, suggesting acidosis developed more quickly. Moreover, the greatest accumulation of calcium between normoxia and hypoxia exposure was in PC oysters during the longer hypoxia exposure. This may indicate a greater buffering capacity by PC, which could partly explain their greater hypoxia tolerance.

In general, PC had greater glutathione concentration than CL, and a greater response during exposure to hypoxia. Glutathione is the most abundant antioxidant in living cells (Kelly et al. 1998), and one primary function is to scavenge for harmful reactive oxygen species (Meister and Anderson, 1983) produced during oxygen stress (Chandel et al. 1998; Clanton 2007; Istomina et al. 2013; Gostyukhina 2021). Nogueira et al. (2017) reported that marine brown mussels (*Perna perna*) exposed to air for various amounts of time had elevated levels of glutathione in gill (6, 24, 48 h) and digestive gland (6, 12, 48 h) tissue. As well, Khan and Ringwood (2016) found that exposure to continuous hypoxia increased digestive gland glutathione levels in eastern oysters by 25-30% after 4 days compared to normoxic levels. This trend was reflected at higher magnitudes in our PC population, where oysters exposed to hypoxia had digestive gland glutathione levels of 84% (Study 3) and 136% (Study 2) higher than oysters exposed to normoxia. Although PC experienced a rather large increase in glutathione concentration during exposure to hypoxia, there were still significant mortalities after 12 days. During long-term hypoxia, the production of glutathione and other antioxidants may be overwhelmed by excessive ROS production, resulting in increased cellular damage (Khan and Ringwood 2016). Although both PC and CL populations likely experienced significant cellular damage resulting from hypoxia stress, the large glutathione accumulation by PC likely resulted in greater protection against ROS and longer survival compared to CL.

Results discussed here provide evidence of population differences in hypoxia tolerance with co-occurring temperature stress. These differences suggest that populations are affected by different natural selection pressures and have locally adapted to specific environmental regimes. This implies that populations could potentially adapt to changing temperature and
hypoxia regimes if changes do not occur too quickly. It is difficult to conclude how quick is too quick, but measuring sub-lethal biomarkers, like those used in these studies, more often in oysters growing in natural environments could indicate which adaptive mechanisms are undergoing selective pressure, and to what degree pressure is being exerted. As oyster restoration efforts continue in the northern GoM, selecting broodstock which can best withstand the effects of climate change will become increasingly important. Although local populations are often the best option when restoring a site, the dramatic anthropogenic changes in some sites may make this option unfeasible (McKay et al. 2005; Jones 2013). Alternatively, having access to populations more tolerant to specific environmental stressors would allow managers to match them with sites less suited for local populations. Aquaculture managers can benefit in the same way by matching specific environmentally tolerant populations to certain sites. They can even selectively breed for greater tolerance within a population. Temperature tolerance is particularly relevant given future predictions in the northern GoM, so PC oysters may prove to be useful if breeders decide to select for this attribute. Potentially unique genotypes in environmentally tolerant populations could also be identified for future breeding projects and significantly reduce the time required to achieve breeding goals. This strategy is becoming more common as gene sequencing technology advances, and studies to breed for salinity tolerance in eastern oysters are ongoing (La Peyre, unpub.)

There is, however, a limit to the potential of environmentally tolerant oysters, regardless of population differences. The present studies show that, although populations were different in their hypoxia tolerance, none were able to survive for long under the most extreme experimental conditions. This indicates that some sites considered for restoration and aquaculture may not be suitable if conditions are too extreme or change too rapidly. Moving forward, accurately characterizing the change in temperature and dissolved oxygen, along with other relevant factors at sites of interest will be crucial for future planning. If the changes to certain sites are more gradual or less severe, there is hope that research like this can identify oysters which can tolerate an even wider range of environmental conditions.
CHAPTER 3. COMPARISON OF HYPOXIA AND ANOXIA TOLERANCE IN DIPLOID AND TRIPLOID EASTERN OYSTERS EXPOSED TO HIGH TEMPERATURE

3.1. INTRODUCTION

The eastern oyster *Crassostrea virginica* is a commercially valuable fisheries product for economies along the United States east coast and the northern Gulf of Mexico (GoM). Landings reports from 2018 show Maryland and Virginia collectively harvested over $50 million worth of eastern oysters from the Chesapeake Bay, and over $100 million was harvested by states along the northern GoM (Texas, Louisiana, Alabama, Mississippi; NOAA 2018). The northern GoM’s warmer climate and extended growing season makes it an ideal environment for eastern oysters to grow and reproduce (Hayes and Menzel 1981), and the region currently sustains the largest natural oyster fishery in the world (Beck et al. 2011; Hesterberg et al. 2020). The ideal environment and market potential of the northern GoM has generated interest in off-bottom oyster aquaculture, and the industry is growing (Maxwell et al. 2008; Walton et al. 2013; Casas et al. 2017; Hensey 2020). Advancements in farming technology and selective breeding are key components of the industry’s growth. Off-bottom aquaculture, a method of growing oysters in cages suspended in the water column, is a relatively recent and promising change to northern GoM oyster production practices that reduces the negative impacts of traditional on-bottom culture (Walton et al. 2013; Hensey 2020). Additionally, the identification of oyster populations more resistant to disease (Casas et al. 2017) and more tolerant to environmental stress (Marshall et al. 2021b) to use as broodstock can improve growth and expand potential grow sites for aquaculture.

Triploid oysters are a key component in the growth of the aquaculture industry, both in the northern GoM and globally. Triploid oysters possess three sets of chromosomes instead of two, resulting in functional reproductive sterility. As a result, triploids develop less gonadal tissue (Allen and Downing 1986, 1990) and grow at a faster rate than traditional diploids (Stanley et al. 1984; Baker and Mann 1991). Consumers do not enjoy the taste of gonadal tissue, so triploids can be marketed year-round (Nell 2002). The faster rate of growth also allows triploids to reach market size earlier (Wadsworth et al. 2019b). These production advantages often make triploids more desirable to oyster farmers, potentially improving aquaculture landings and reducing harvest pressure put on natural oyster reefs.

Although triploid oysters have several qualities making them more desirable than diploids, certain unfavorable environmental conditions can diminish these advantages. Davis (1994) found that diploid and triploid Pacific oyster (*Crassostrea gigas*) growth rates were comparable in sites where gonadal development was not suitable. Callam et al. (2016) reported that triploid eastern oysters grew slower than diploids in low salinity. Higher rates of triploid mortality were also recently observed in Alabama (Wadsworth et al. 2019a). These reports suggest triploids may be more vulnerable to certain environmental stresses and raise questions about potential tradeoffs between oyster growth and environmental tolerance. Wadsworth et al. (2019a) measured cumulative mortality of triploid and diploid oysters at four northern GoM sites and reported higher triploid mortality at all of them. Oysters in some sites experienced different
cumulative mortalities despite the relatively similar salinity and temperature regimes. Other factors including food supply, acidification, *P. marinus* infection, and dissolved oxygen may have differed among all sites and contributed to differential oyster mortality. Low dissolved oxygen is a factor of particular concern for eastern oysters as the climate changes and low oxygen events become more frequent.

As eutrophication and climate change continue to impact coastal regions including the northern GoM, the co-occurrence of warm water and low oxygen is becoming increasingly common in areas where oysters grow (Diaz and Rosenberg 1995; Diaz 2001; Diaz and Rosenberg 2008). Dissolved oxygen concentration (DO) low enough to cause stress is known as hypoxia, a term generally reserved for DO < 2.0 mg L\(^{-1}\). As the spatial and temporal extent of hypoxia increases in estuaries and coastal ecosystems (Tilman et al. 2001; Rabalais et al. 2009), the impact to ecologically and commercially important species including the eastern oyster needs to be thoroughly evaluated.

Oysters are among the most tolerant organisms to low oxygen stress (Stickle et al. 1989; North et al. 2006; Matsche and Barker 2006). This tolerance is achieved through an ability to maintain oxygen consumption at low DO levels (Le Moullac et al. 2007), energy production through high yielding anaerobic pathways (Collicutt and Hochachka 1977; de Zwaan 1983; Brooks et al. 1991), and a strategy of metabolic avoidance, known as metabolic rate depression (Willson and Burnett 2000; Hochachka and Lutz 2001; Sokolova et al. 2011). Metabolic rate depression limits non-vital energy production and consumption and reduces the accumulation of harmful metabolic end products during anaerobiosis (Hochachka et al. 1996; Sokolova et al. 2011). Although *C. virginica* can tolerate short-term hypoxia (Ivanina et al. 2016; Porter and Breitburg 2016) and even extended exposure to anoxia (DO < 0.5 mg L\(^{-1}\); Stickle et al. 1989), that tolerance declines with increasing temperature (Stickle et al. 1989; Ivanina et al. 2012). Increasing temperature simultaneously increases oyster metabolic energy demands (Shumway and Koehn 1982; Shumway 1996; Hutchinson and Hawkins 1992; Cherkasov et al. 2006) and decreases the amount of dissolved oxygen available for aerobic respiration. Previous studies investigating the effects of temperature on eastern oyster hypoxia tolerance (Stickle et al. 1989; Ivanina et al. 2012) and respiration (Casas et al. 2018b) have used temperatures as high as 30 °C, and Chapter 2 of this thesis tested population differences during hypoxia exposure at 32 °C. Effects of hypoxia and anoxia between diploid and triploid eastern oysters have been tested in a few other studies at 20 °C, a non-stressful temperature (Harlan 2007; Lombardi et al. 2013), where differences in mortality were small. However, it is not known how diploid and triploid tolerance to hypoxia compares at higher, more physiological stressful temperatures.

There is evidence that differential hypoxia tolerance exists between diploids and triploids in other oyster species (Harlan 2007; Lombardi et al. 2013), and recent reports of unusual eastern oyster triploid oyster mass mortality in farms along the northern GoM (Wadsworth et al. 2019a) provide reason to investigate the potential causes. There are noticeable phenotypic differences between diploid and triploid oysters in terms of size and tissue proportion, and these differences may impact their tolerance to different stresses (Van der Meer 2006). Although both Harlan (2007) and Lombardi et al. (2013) reported comparable rates of mortality between eastern oyster diploids and triploids, it is possible that the addition of temperature stress may elicit differential responses to hypoxia and/or anoxia between the ploidies.
Hypoxia tolerance can be measured as a mortality response (lethal level of exposure) and as a physiological response (sub-lethal level of exposure). Mortality response is measured simply by measuring the time it takes for individuals to die and calculating the rate of mortality or survival for a group. Lethal times (LT₅₀), or the time at which 50% of the group has died, is often used to measure tolerance (Stickle et al. 1989). Quantifying mortality response is an important step to understand the absolute limits of tolerance, however it does not describe any physiological changes that occur during stress. For this reason, measuring sub-lethal physiological responses, including changes in the concentration of key metabolic biomarkers including protein, calcium, glutathione, and succinate, is a useful strategy to determine tolerance.

As oyster aquaculture increases in GoM, and elsewhere, understanding trade-offs in growing diploid versus triploid oysters requires testing their tolerance and responses to environmental stresses. Understanding differences between diploid and triploid eastern oyster physiological response to hypoxia will be an important advancement for aquaculture in the northern GoM. This knowledge could provide more accurate risk assessment and production estimates for aquaculture managers. The objective of these studies is to compare the lethal and sub-lethal physiological effects of hypoxia and anoxia on diploid and triploid eastern oysters to determine if differences in hypoxia tolerance exist. Specifically, in the first study, the cumulative mortalities of diploid and triploid adult *C. virginica* exposed to extended normoxia (DO > 5.0 mg L⁻¹), hypoxia (DO < 2.0 mg L⁻¹), or anoxia (DO < 0.5 mg L⁻¹) and high temperature (28 °C) were measured, and LT₅₀’s were determined to examine lethal responses. On day 3 of continuous exposure, hemolymph pH and concentrations of plasma calcium and glutathione, and of digestive gland succinate were measured to examine sub-lethal responses, and to help better understand mechanisms or differences in responses. In the second study, the cumulative mortalities of diploid and triploid seed *C. virginica* exposed to extended normoxia, hypoxia, or anoxia and high temperature (28 °C) were measured, and LT₅₀’s were determined to examine lethal response in reproductively immature oysters. This work will improve the understanding of potential trade-offs between growth and environmental tolerance, and how to best select oysters for optimal aquaculture production.

### 3.2. METHODS

#### 3.2.1. Oysters

In the summer of 2019, diploid females from Sister Lake, Louisiana (29° 14’ 01” N, 90° 55’ 12” W) were spawned with either diploid males from Sister Lake or tetraploid males developed on the east coast of the United States. Spawning occurred at the Michael C. Voisin Oyster Hatchery in Grand Isle, Louisiana (29° 14’ 17” N, 90° 00’ 10” W). The diploid x diploid cross produced diploid progeny, and the diploid x tetraploid cross produced triploid progeny. Progenies were maintained and grew in Grand Isle until the start of experiments. In November 2021, approximately 200 diploid and 200 triploid oysters were transported to the LSU Animal and Food Science Laboratory (AFL) for use in Study 1. About 30 diploid and 30 triploid oysters were placed in each of six 800-L aquarium systems filled with aerated artificial seawater (Crystal Sea Marinemix, Marine Enterprises International, Baltimore, Maryland, USA) adjusted to a
salinity of about 17 and temperature of about 22 °C. These conditions were similar to those of Grand Isle at the time of collection. Diploid and triploid oysters were about 2.5 years old at the time with mean shell height of 118 ± 10 mm and 124 ± 11 mm, respectively.

In May of 2021, wild diploid females originating from various locations in the northern GoM were spawned with either diploid males also from the northern GOM or with tetraploid males developed on the east coast of the United States. Spawning occurred at the Auburn University Shellfish Laboratory in Dauphin Island, Alabama (30° 14’ 50” N, 88° 04’ 42’’ W) and the diploid and triploid progenies were half-siblings. The progenies were transported to Grand Isle to grow. In January 2022, approximately 250 diploid and 250 triploid progenies from these spawnings were transported to AFL to be used in Study 2. About 40 diploid and 40 triploid oysters were placed in each of six 400-L aquarium systems filled with aerated artificial seawater (Crystal Sea Marinemix, Marine Enterprises International, Baltimore, Maryland, USA) adjusted to a salinity of about 20 and temperature of about 17 °C. These conditions were similar to those of Grand Isle at the time of collection. Diploid and triploid were about seven months old with shell heights of 42.2 ± 4.9 mm and 41.4 ± 4.2 mm, respectively.

3.2.2. Study 1 Experimental design

In Study 1, the responses of adult diploid and triploid oysters to continuous normoxia, hypoxia, and anoxia were examined. In this study, oysters were allowed to acclimate for 9 days before salinity and temperature were adjusted to 20 and 25 °C. During acclimation, oysters were fed daily with Shellfish Diet 1800® (Reed Mariculture Inc, Campbell, California, USA). Daily salinity, temperature, and DO data were recorded along with number of live and dead oysters. Dead oysters were removed upon discovery and shell height measured. After about one week at 25 °C, temperature was gradually increased to 28 °C at a rate of 1 °C per day using submersible heaters (Hygger Saltwater Tank Titanium Tube 200W). Once the target temperature was reached, air stones in four of the systems were removed and nitrogen/carbon dioxide began to be injected into two of those systems. The two systems without aeration served as the hypoxia treatment (DO < 2.0 mg L⁻¹), and the two systems with gas injection served as the anoxia treatment (DO < 0.5 mg L⁻¹). Aeration continued in the two remaining systems to serve as controls (normoxia). DO was controlled by bubbling air or gas through flow meters (Brooks Instruments 0.5 LPM). Water salinity, temperature (°C) and DO (mg L⁻¹) were measured using a YSI-Pro30 handheld multimeter (YSI Incorporated, Yellow Springs, Ohio, USA). Day 0 was recorded as the first day the tank reached the target treatment conditions. During the experiment, salinity, temperature, and DO were measured daily, along with the number of live and dead oysters. Dead oysters were removed upon discovery and shell heights measured.

After three days of treatment exposure, 30 diploid and 30 triploid oysters from each of the six systems were removed and sacrificed to measure hemocyte density, granulocyte %, hemocyte pH, plasma protein concentration, plasma calcium concentration, plasma glutathione concentration, and digestive gland succinate concentration. Hemolymph was extracted, and hemolymph pH, hemocyte density, and granulocyte percentage (%) were measured for each oyster, as indicated below (3.2.3). Separate hemolymph samples were extracted, centrifuged, and the plasma collected and stored at -80 °C to measure protein, calcium, and glutathione concentrations with assay kits, as described below (3.2.3). Digestive gland tissue from each
oyster was also dissected and stored at -80 °C to measure concentration of succinate with assay kits, as described below (3.2.3).

A remaining set of oysters were continually exposed to treatment conditions to measure rates of mortality and calculate median lethal times (LT\textsubscript{50}). Since some oysters died before the sampling period (Day 3) the number of oysters remaining in each group varied (Table 3.2)

### 3.2.3. Study 2 Experimental design

In Study 2, the responses of seed diploid and triploid oysters to continuous normoxia, hypoxia, and anoxia were examined. The acclimation and treatment exposure were the same as in Study 1, except that pure nitrogen gas was bubbled in the two systems for the anoxia treatment. Oysters were continually exposed to treatment conditions to measure rate of mortality and calculate LT\textsubscript{50}.

### 3.2.4. Assays

Hemocyte pH was measured using a micro pH meter. Hemocyte density (cells mL\textsuperscript{-1}) and granulocyte % were determined with improved Neubauer hemocytometers (Reichert, Buffalo, NY) as described by La Peyre et al. (1995). Protein concentration (mg mL\textsuperscript{-1}) was measured using Pierce Biotech Micro BCA Protein Assay Kit (Rockford, IL, USA) with bovine serum albumin as a standard. Calcium ion concentration (mM) was measured using Sigma-Aldrich MAK022 Calcium Colorimetric Assay Kit (St. Louis, MO, USA). Glutathione concentration (µM) was measured using BioAssay systems, DIGT-250 QuantiChrom\textsuperscript{TM} Glutathione Assay kit (Hayward, CA, USA). All samples were adjusted to 2.5 mg mL\textsuperscript{-1} prior to measuring glutathione. Plasma measurements were done in duplicate.

Succinate concentration (µmol gram\textsuperscript{-1}) was measured using BioVision succinate colorimetric assay kits. Tissue samples were thawed, homogenized in succinate assay buffer (10 mg in 0.1 mL buffer), and centrifuged at 10,000 x g for 5 minutes. Supernatant was collected and used to measure succinate concentration.

### 3.2.5. Statistical analyses

Mortality data were subjected to probit analysis using the R package ‘ecotox’ (Wheeler et al. 2006). LT\textsubscript{50} were calculated from the day hypoxia was first measured in each tank and herein referred as Day 0. For all groups which experienced mortality, median lethal time (LT\textsubscript{50}) with 95% confidence intervals (95% CI) were determined. Confidence intervals that do not overlap are statistically different (Wheeler et al. 2006). All analyses were performed using R 3.6.3 (R Foundation for Statistical Computing, 2020).

Data on hemolymph pH, hemocyte density, granulocyte %, plasma protein, calcium, and glutathione concentrations, and digestive gland succinate concentrations were examined for normality and homogeneity of variance. Data that fulfilled ANOVA requirements (granulocyte (%), plasma protein, plasma glutathione, digestive gland succinate) were analyzed with a two-factor (DO level, ploidy) analysis of variance (ANOVA). When significant differences were found (P < 0.05), Tukey’s HSD test was used for pairwise multiple comparisons. Data that did not fulfill the ANOVA requirements (hemolymph pH, hemocyte density, plasma calcium) were analyzed with a Kruskal-Wallis non-parametric rank-sum test, followed by pairwise t-tests with a Bonferroni correction when significant differences were found.
3.3. RESULTS

3.3.1. Study 1

Water quality
Throughout the experiment, temperature, salinity, DO, and water pH remained relatively consistent at target values (Table 3.1). Levels of ammonia, nitrite, and nitrate remained at or below 1.0, 1.0, and 25 ppm, respectively, throughout acclimation and during treatment exposures.

Mortality
By Day 3, mortality among all diploid and triploid groups was less than 4%, except for diploids in one anoxia system and triploids in one hypoxia system, which had 9% and 10% mortality, respectively (Figure 3.1). By Day 12, diploid and triploid exposed to anoxia experienced 100% cumulative mortality, whereas those exposed to hypoxia and normoxia experienced < 30% and < 20% cumulative mortality (Figure 3.1). By the end of the experiment (Day 21) diploids exposed to hypoxia experienced 86-100% mortality, and triploids experienced 75-100% mortality. By that same point, diploids exposed to normoxia experienced 14-38% mortality, and triploids experienced 10-12% mortality (Figure 3.1).
Table 3.1. Mean ± SD temperature (°C), salinity, DO (mg L⁻¹), and pH of normoxia (DO > 5.00 mg L⁻¹), hypoxia (DO < 2.00 mg L⁻¹), and anoxia (DO < 0.50 mg L⁻¹) systems during in Study 1 and Study 2. In Study 1 a mixture of nitrogen and carbon dioxide gas was bubbled in the anoxia tanks, and pure nitrogen gas was used in Study 2.

<table>
<thead>
<tr>
<th>Study 1</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Anoxia (N₂/CO₂)</th>
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<tr>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
<td>Rep 1</td>
</tr>
<tr>
<td>temperature (°C)</td>
<td>27.8 ± 0.1</td>
<td>28.0 ± 0.1</td>
<td>27.8 ± 0.2</td>
</tr>
<tr>
<td>salinity</td>
<td>20.0 ± 0.1</td>
<td>20.0 ± 0.1</td>
<td>20.2 ± 0.1</td>
</tr>
<tr>
<td>DO (mg L⁻¹)</td>
<td>5.28 ± 0.12</td>
<td>5.14 ± 0.05</td>
<td>1.34 ± 0.37</td>
</tr>
<tr>
<td>pH</td>
<td>7.87 ± 0.04</td>
<td>7.82 ± 0.8</td>
<td>7.30 ± 0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study 2</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Anoxia (N₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
<td>Rep 1</td>
</tr>
<tr>
<td>temperature (°C)</td>
<td>28.0 ± 0.2</td>
<td>27.7 ± 0.4</td>
<td>28.1 ± 0.2</td>
</tr>
<tr>
<td>salinity</td>
<td>20.2 ± 0.1</td>
<td>20.4 ± 0.1</td>
<td>20.3 ± 0.1</td>
</tr>
<tr>
<td>DO (mg L⁻¹)</td>
<td>5.47 ± 0.16</td>
<td>5.45 ± 0.23</td>
<td>1.42 ± 0.34</td>
</tr>
<tr>
<td>pH</td>
<td>7.60 ± 0.07</td>
<td>7.66 ± 0.08</td>
<td>7.25 ± 0.11</td>
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</tbody>
</table>
Figure 3.1. Cumulative mortality (%) of diploid (square) and triploid (triangle) adult oysters during exposure to normoxia (DO > 5.0 mg L\(^{-1}\)), hypoxia (DO < 2.0 mg L\(^{-1}\)), and anoxia (DO < 0.5 mg L\(^{-1}\)) at 28 °C in Study 1. Filled circles indicate DO concentration and dashed lines indicate the maximum desired DO for hypoxia and anoxia treatment systems. Hypoxia and anoxia were reached in the treatment tanks on Day 0. Normoxia systems were supplied with continuous aeration for the duration of the experiment.

**Lethal median time (LT\(_{50}\))**

Oysters exposed to anoxia died about twice as fast as those exposed to hypoxia, and three times faster than those exposed to normoxia (Figure 3.1; Table 3.2). LT\(_{50}\) differed by ploidy for one replicate within each treatment, with triploid oysters having a longer LT\(_{50}\) as compared to diploid oysters; the second replicate in each treatment indicated no significant difference by ploidy. In general, however, triploid oysters died at a slower rate than diploids (Figure 3.1).
Table 3.2. Median lethal time (LT50; days) with 95% confidence intervals (95% CI) of diploid (2N) and triploid (3N) adult oysters exposed to normoxia (DO > 5.0 mg L⁻¹), hypoxia (DO < 2.0 mg L⁻¹), and anoxia (DO < 0.5 mg L⁻¹) at 28 °C in Study 1. Oysters that remained (n) after the sampling period on day 3 were used in this calculation. Confidence intervals that do not overlap are considered statistically significant (Wheeler et al. 2006).

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Normoxia</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Hypoxia</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Anoxia</th>
<th></th>
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<tr>
<td></td>
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<td></td>
<td>Replicate 1</td>
<td>Replicate 2</td>
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</tr>
<tr>
<td></td>
<td>n</td>
<td>LT50</td>
<td>95% CI</td>
<td>n</td>
<td>LT50</td>
<td>95% CI</td>
<td>n</td>
<td>LT50</td>
<td>95% CI</td>
<td>n</td>
<td>LT50</td>
<td>95% CI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2N</td>
<td>14</td>
<td>39</td>
<td>(27.8, 78.5)</td>
<td>15</td>
<td>18.9</td>
<td>(17.5, 21.0)</td>
<td>13</td>
<td>11.9</td>
<td>(11.0, 13.2)</td>
<td>14</td>
<td>15.6</td>
<td>(14.6, 17.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3N</td>
<td>13</td>
<td>25.3</td>
<td>(21.7, 39.5)</td>
<td>12</td>
<td>37.6</td>
<td>(27.4, 71.1)</td>
<td>8</td>
<td>17.4</td>
<td>(15.4, 20.5)</td>
<td>11</td>
<td>19.4</td>
<td>(17.0, 23.3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Hemocyte pH, hemocyte density, and granulocyte %**

Hemolymph pH differed significantly by group (H (5) = 44.185, p = 2.125e-08). A follow-up test indicated that oysters under anoxia had significantly lower hemolymph pH than those under normoxia regardless of ploidy; under normoxia, diploids had significantly greater pH than triploids (Figure 3.2).

Hemocyte density significantly differed by group (H (5) = 90.652, p < 2.2e-16). A follow-up test indicated that, regardless of ploidy, oysters under anoxia had significantly lower hemocyte density than those under normoxia and hypoxia. (Table 3.3).

Granulocyte % significantly differed by single effects of treatment (F2, 165 = 5.982, p=0.0031) and ploidy (F1, 165 = 74.690, p= 4.59e-15) only. Oysters under hypoxia had lower granulocyte % than those under normoxia and anoxia. Triploids had significantly higher granulocyte % than diploids regardless of treatment (Table 3.3).
Figure 3.2. Box and whisker plots of diploid (2N) and triploid (3N) oyster hemolymph pH on day 3 of continuous normoxia (DO > 5.0 mg L\(^{-1}\)), hypoxia (Do < 2.0 mg L\(^{-1}\)), or anoxia (DO < 0.5 mg L\(^{-1}\)) exposure at 28 °C in Study 1. Vertical black lines indicate the range, the bottom and top of the bar indicate the first (lowest 25%) and third (highest 25%) quartiles, the horizontal black bars indicate the median, the open dots indicate the mean, and the closed black dots indicate outlier datapoints (falling outside 1.5 times the interquartile range) of each group plotted. Horizontal dashed lines indicate the range of water pH on day 3.

**Plasma protein, glutathione, and calcium concentration**

Plasma protein concentration significantly differed by treatment only (\(F_{2, 166} = 5.216, p=0.00635\)), where oysters under hypoxia had significantly higher protein concentration than those under normoxia and hypoxia (Table 3.3).

Plasma calcium concentration significantly differed by group (\(H (5) = 106.12, p < 2.2e-16\)). A follow-up test indicated that calcium concentration under anoxia was significantly higher than under normoxia and hypoxia regardless of ploidy (Figure 3.3; Table 3.3).

Plasma glutathione concentration significantly differed only by the single effect of treatment (\(F_{2, 166} = 5.704, p=0.00402\)). Oysters exposed to anoxia had significantly higher glutathione concentration than those exposed to normoxia and hypoxia regardless of treatment (table 3.3).
Digestive gland succinate concentration

Unexpectedly, digestive gland succinate concentrations were 10-15 times higher than previously reported for eastern oysters (Haider et al. 2020; Thesis Chapter 2), however concentrations did not significantly differ by treatment or ploidy (Table 3.3).

Figure 3.3. Box and whisker plots of diploid (2N) and triploid (3N) oyster plasma calcium concentration on day 3 of continuous normoxia (DO > 5.0 mg L\(^{-1}\)), hypoxia (DO < 2.0 mg L\(^{-1}\)), or anoxia (DO < 0.5 mg L\(^{-1}\)) exposure at 28 °C in Study 1. Vertical black lines indicate the range, the bottom and top of the bar indicate the first (lowest 25%) and third (highest 25%) quartiles, the horizontal black bars indicate the median, the open dots indicate the mean, and the closed black dots indicate outlier datapoints (falling outside 1.5 times the interquartile range) of each group plotted.
Table 3.3. Mean ± SD hemolymph pH, hemocyte density, granulocyte percentage (%), plasma protein, calcium and glutathione concentrations, and digestive gland succinate concentrations of diploid (2N) and triploid (3N) on Day 3 of continuous normoxia (DO > 5.0 mg L\(^{-1}\)), hypoxia (DO < 2.0 mg L\(^{-1}\)), or anoxia (DO < 0.5 mg L\(^{-1}\)) exposure at 28 °C in Study 1. Groups with different letters denote statistically different interactions (p < 0.05).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Anoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2N</td>
<td>3N</td>
<td>2N</td>
</tr>
<tr>
<td>Hemolymph pH</td>
<td>7.10 ± 0.30(^a)</td>
<td>6.85 ± 0.40(^b)</td>
<td>6.86 ± 0.35(^{ab})</td>
</tr>
<tr>
<td>Hemocyte density (10(^6) cells mL(^{-1}))</td>
<td>2.87 ± 2.26(^{ab})</td>
<td>1.90 ± 1.09(^b)</td>
<td>3.76 ± 2.97(^a)</td>
</tr>
<tr>
<td>Granulocyte %</td>
<td>30 ± 16</td>
<td>49 ± 16</td>
<td>20 ± 11</td>
</tr>
<tr>
<td>Protein (mg ml(^{-1}))</td>
<td>5.3 ± 2.4</td>
<td>4.9 ± 2.5</td>
<td>5.6 ± 2.6</td>
</tr>
<tr>
<td>Calcium (nM)</td>
<td>5.6 ± 0.5(^a)</td>
<td>6.3 ± 1.6(^a)</td>
<td>6.7 ± 1.0(^a)</td>
</tr>
<tr>
<td>Glutathione (µM)</td>
<td>36 ± 24</td>
<td>38 ± 26</td>
<td>36 ± 28</td>
</tr>
<tr>
<td>Succinate (µmol g(^{-1}))</td>
<td>174 ± 72</td>
<td>199 ± 52</td>
<td>173 ± 44</td>
</tr>
</tbody>
</table>
3.3.2. Study 2

Water quality
Throughout the experiment, temperature, salinity, DO, and pH remained relatively consistent at target levels (Table 3.1). Levels of ammonia, nitrite, and nitrate remained at or below 1.0, 1.0, and 25 ppm, respectively, throughout acclimation and during treatment exposures.

Mortality
Diploid and triploid seed oysters exposed to anoxia experienced 100% cumulative mortality by day 17, whereas those exposed to hypoxia and normoxia experienced < 25% and < 5% cumulative mortality by day 17 (Figure 3.4). By the end of the experiment (Day 32) diploids and triploids exposed to hypoxia experienced 100% mortality. By that same point, diploids exposed to normoxia experienced 0% mortality, and triploids experienced 2.5-5% mortality (Figure 3.4).
Figure 3.4. Cumulative mortality (%) of diploid (square) and triploid (triangle) seed oysters during exposure to normoxia (DO > 5.0 mg L$^{-1}$), hypoxia (DO < 2.0 mg L$^{-1}$), and anoxia (DO < 0.5 mg L$^{-1}$) at 28 °C in Study 2. Filled circles indicate DO concentration and dashed lines indicate the maximum desired DO for hypoxia and anoxia treatment tanks. Hypoxia and anoxia were reached in the treatment tanks on Day 0. Normoxia systems were supplied with continuous aeration for the duration of the experiment.

Lethal median time (LT$_{50}$)

Oysters exposed to anoxia died about 2-5 times faster than those exposed to hypoxia (Figure 3.4; Table 3.4). LT$_{50}$ differed by ploidy for one replicate within hypoxia and anoxia treatments, and for both replicates in the normoxia treatment. Triploids had a smaller LT$_{50}$ than diploids in one hypoxia replicate, and a shorter LT$_{50}$ in one anoxia replicate. In the normoxia treatment, triploid oysters had shorter LT$_{50}$ than diploids, however the highest triploid mortality in a replicate was only 5% (2 oysters). In general, triploid oysters died at a slower rate than diploids under anoxia, but at a faster rate under hypoxia and normoxia.
Table 3.4. Median lethal time (LT$_{50}$; days) with 95% confidence intervals (95% CI) of diploid (2N) and triploid (3N) seed oysters exposed to normoxia (DO > 5.0 mg L$^{-1}$), hypoxia (Do < 2.0 mg L$^{-1}$), and anoxia (DO < 0.5 mg L$^{-1}$) at 28 °C in Study 2. Confidence intervals that do not overlap are considered statistically significant (Wheeler et al. 2006).

| Ploidy | Normoxia | Replicate 1 | | | Replicate 2 | | |
|---|---|---|---|---|---|
| | LT$_{50}$ | 95% CI | LT$_{50}$ | 95% CI | |
| 2N | NA | NA | NA | NA | |
| 3N | 76 | (57.0, 133) | 62.2 | (50.8, 86.5) | |

| Ploidy | Hypoxia | Replicate 1 | | | Replicate 2 | | |
|---|---|---|---|---|---|
| | LT$_{50}$ | 95% CI | LT$_{50}$ | 95% CI | |
| 2N | 24.5 | (23.1, 26.2) | 25 | (23.7, 26.6) | |
| 3N | 23 | (22.0, 24.2) | 21.8 | (20.9, 22.9) | |

| Ploidy | Anoxia | Replicate 1 | | | Replicate 2 | | |
|---|---|---|---|---|---|
| | LT$_{50}$ | 95% CI | LT$_{50}$ | 95% CI | |
| 2N | 11.8 | (10.9, 13.1) | 9.5 | (9.0, 10.1) | |
| 3N | 12.1 | (11.6, 12.7) | 11.8 | (11.5, 12.2) | |
3.4. DISCUSSION

The rates of mortality in all oysters increased with decreasing DO, and adult oysters had faster rates of mortality than seed oysters at all DO levels; regardless of size however, triploid oysters died more slowly compared to diploids, with their LT$_{50}$, on average, 1.5 days longer than diploids during these experiments (8.5 days versus 7.0). Despite this small difference in rates of mortality, adult diploid and triploid sub-lethal responses were largely comparable. One distinguishing difference between ploidy responses was a significantly higher granulocyte % in triploids. Taken together, these results suggest that co-occurring hypoxia and temperature stress are not the primary cause of observed elevated triploid mortality in the environment. Potential differences in energy sources used in anaerobiosis may provide some insight into how diploid and triploid oysters exposed to different levels of DO tolerate stress.

Although oysters are remarkably resilient to hypoxia and anoxia at low temperatures, increased rates of mortality with decreasing DO was expected at this temperature (28 °C). These results are in line with Stickle et al. (1989) who found that eastern oyster LT$_{50}$ decreased when exposed to increasingly severe anoxia at 30 °C. In a similar experiment by Baker and Mann (1992), eastern oyster larval settlement decreased from normoxia (DO = 7.3 mg L$^{-1}$) to hypoxia (DO = 1.5 mg L$^{-1}$), and from hypoxia to anoxia (DO < 0.07 mg L$^{-1}$) at 25 °C. The impact of size on oyster mortality at high temperatures has also been reported in previous studies. Rybovich et al. (2016) reported increased mortality in market size (> 75 mm) oysters compared to seed (< 50 mm) and spat (< 25 mm) oysters during continuous exposure to high temperatures (30-32 °C) for 15 days. As temperature increases, oxygen supply to tissue becomes increasingly limited, with larger animals experiencing faster declines in oxygen supply and earlier transition to anaerobiosis (Pörtner 2010). In bivalves, larger individuals have a smaller gill-to-body weight ratio which makes them more susceptible to oxygen limitations than smaller individuals (Shumway 1982).

When exposed to anoxia, adult and seed triploid oysters tended to survive longer than diploids, though the differences were small. Previous studies investigating eastern oyster hypoxia tolerance reported an opposite trend where diploids died more slowly than triploids (Harlan 2007, Lombardi et al. 2013), though trends were insignificant, and experimental designs differed from those of the present studies. Harlan (2007) and Lombardi et al. (2013) exposed diploid and triploid oysters from Maryland to anoxia (DO < 0.5 mg L$^{-1}$) at 20 °C and measured rates of mortality. Both studies found that mean mortality in diploids was 17 days, and in triploids was 14-15 days. These results provide a baseline for comparing diploid and triploid anoxia tolerance without the added temperature stress. Although trends reported by Harlan (2007) and Lombardi et al. (2013) contrast with ours, temperature stress likely complicates the response to anoxia, and conclusions are largely in agreement: differences in rates of mortality between diploid and triploid eastern oysters are small during anoxia stress.

In this study, adult oysters exposed to 3 days of continuous anoxia displayed elevated concentrations of plasma calcium and glutathione compared to oysters exposed to normoxia and hypoxia, but there were no differences between ploidies. In mollusks, calcium functions as a buffer against declining tissue pH (i.e., acidosis), and increasing the concentration of calcium in the hemolymph mitigates its harmful effects (Booth et al. 1984; Dwyer and Burnett 1996; Burnett 1997). Acidosis occurs when oysters remain shut for extended periods of time, for
instance during hypoxia and anoxia when oxygen supply is limited. Porter and Breitberg (2016) reported that eastern oysters closed for a significantly longer amount of time during severe hypoxia (DO = 0.6 mg L\(^{-1}\)) compared to normoxia (DO = 7.3 mg L\(^{-1}\)) and for an intermediate amount of time during mild hypoxia (DO = 1.7 mg L\(^{-1}\)). Our results on calcium concentration suggest a similar pattern of opening where oysters were closed for longer periods of time at each decreasing DO level.

Glutathione is the most abundant antioxidant in living cells (Kelly et al. 1998), and one primary function is to scavenge for harmful reactive oxygen species (Meister and Anderson, 1983) produced during oxygen stress (Chandel et al. 1998; Clanton 2007; Istomina et al. 2013; Gostyukhina 2021). Nogueira et al. (2017) reported that marine brown mussels (Perna perna) exposed to air for various amounts of time had elevated levels of glutathione in gill (6, 24, 48 h) and digestive gland (6, 12, 48 h) tissue. As well, Khan and Ringwood (2016) found that exposure to continuous hypoxia increased digestive gland glutathione levels in eastern oysters by 25-30% after 4 days compared to normoxia. Increased concentration of glutathione only in oysters exposed to anoxia suggests anoxia, but not hypoxia, was stressful enough to cause an antioxidant response. Interestingly, significant calcium accumulation in hypoxia and anoxia suggests the mechanisms involved in buffering capacity respond more rapidly than those involved in the antioxidant response.

Digestive gland succinate concentration did not differ by ploidy during any treatment exposure and did not change among treatments. This suggests all oysters were operating under similar levels of anaerobiosis during normoxia, hypoxia, and anoxia. Succinate is a stable metabolic end-product that accumulates in mollusks during anaerobiosis as glycogen and aspartate are broken down. Elevated succinate levels have been linked to anaerobic energy production in hard clams (Lee et al. 2012), mussels (de Zwaan et al. 1991), Pacific oysters Crassostrea gigas (Haider et al. 2020), and eastern oysters (Eberlee et al. 1983; Ivanina et al. 2010) exposed to hypoxia. In some bivalve species the accumulation of succinate is temperature dependent (Le Moullac et al. 2007). Investigations by Le Moullac et al. (2007) found that the Pacific oyster exposed to 20 days of hypoxia accumulated succinate at 12 °C, but not at 15 or 20 °C, suggesting oysters were already operating at maximum anaerobic capacity at the lower temperatures. Temperature-induced succinate accumulation might explain why concentrations were relatively unchanged among DO levels in the present studies, though it is difficult to argue why concentrations in oysters in all treatments were so high. In all samples, succinate measured 10-15 times higher than previously reported for oyster species exposed to hypoxia or anoxia (Eberlee et al. 1983; Ivanina et al. 2010; Haider et al. 2020), and for eastern oysters exposed to hypoxia and high temperature (Chapter 2 of thesis). The acutely high levels reported here were first thought to be an error in methodology, but after further investigation I am confident no error occurred. Two potential factors influencing such high succinate concentrations are 1) the choice of tissue (digestive gland) and 2) oyster size. The digestive gland is an organ located roughly in the middle of an oyster’s body and can become hypoxic more quickly than superficial tissues, like the gills, under limited oxygen availability. Therefore, temperature-induced hypoxia in deeper tissues could have developed even during normoxia. In addition, oysters in this study had shell heights of 120 ± 11 mm which could have further reduced the aerobic scope compared to the smaller oysters (45.4 ± 4.5 mm) used in Chapter 2 of this thesis. Future investigations into the tolerance of adult oysters would benefit from
measuring succinate and other biomarkers at multiple temperatures in several size classes to determine potential size-dependent differences. These results could have significant implications for oyster restoration.

Differences in sub-lethal response between ploidies occurred most clearly with triploid oysters displaying higher granulocyte % than diploids regardless of treatment. Triploid oysters displayed as much as double the granulocyte percentage as diploid oysters. Granulocytes are phagocytic cells involved in the oyster immune response to the parasite *Perkinsus marinus* (La Peyre et al. 1995). It is possible higher granulocyte % provides better host defense against *P. marinus* infection, though it is not necessarily a sign of greater infection in eastern oysters (La Peyre et al. 1995). *P. marinus* infection intensity is known to accumulate as oysters age (Andrews and Ray 1988) and as oysters are exposed to severe diel-cycling hypoxia (Breitburg et al. 2015; Keppel et al. 2015). Although oysters were collected from the field in the colder season when infection usually declines (Andrews and Ray 1988), exposure to increased temperature, as well as hypoxia or anoxia, for an extended period during the experiment may have increased *P. marinus* infection intensity. The potential added stress of infection and a lower granulocyte % in diploid oysters may have reduced the capacity for immune response and contributed to the faster rates of mortality during hypoxia exposure compared to triploids. While not explored in these studies, this information could inform future studies investigating diploid-triploid disease resistance. Other sub-lethal responses were less clear. Although not significant, hemocyte density tended to be lower, and calcium concentration tended to be higher in triploids in all treatments. Similar to granulocyte %, increased hemocyte density does not necessarily indicate heavier *P. marinus* infection in eastern oysters (La Peyre et al. 1995), and it is presently unclear how disease impacted this experiment. Higher concentrations of calcium may suggest better tissue buffering capacity compared to triploids, but this claim conflicts with the data on hemolymph pH, which was higher in diploids during normoxia and hypoxia. These insignificant differences may have contributed to the different trends in mortality, but overall diploids and triploids responded similarly to low oxygen and high temperature stress.

Although few differences were found between diploid and triploid oysters based on the measurements chosen here, different amounts of energy sources may still exist between the two that could influence hypoxia tolerance. Glycogen and carbohydrates are important energy sources used in anaerobiosis (Larade and Storey 2002). Guévelou et al. (2017) found that triploid eastern oysters had twice the glycogen concentration as diploids, and Shpigel et al. (1992) found that levels of carbohydrates and protein in triploid Pacific oysters were significantly higher than in diploids when exposed to high temperature (30 °C), which most likely resulted from diploid gonadal production. In the present study, plasma protein concentration was generally comparable between ploidies and among treatments but measuring glycogen and carbohydrates could have provided additional insight. This would have been especially useful to explain the responses of oysters exposed to hypoxia, where differential mortality was most pronounced and more gradual. Energy sources may have played an important role during extended hypoxia and high temperature conditions.

Season may also have impacted diploid and triploid oyster response in relation to energy reserves and overall oyster condition. Goulletquier et al. (1996) showed that levels of glycogen, carbohydrates, and lipids fluctuate differently in Pacific oyster diploids and triploids throughout
the year, with significant declines in percent of glycogen and carbohydrates in diploids during the summer. Although our studies were performed well after the summer spawning season, diploids may not have recovered glycogen and carbohydrate levels to the same extent as triploids. This may be why triploids died consistently slower in all treatments than diploids in adult oysters, but not in seed oysters. Testing diploid and triploid environmental tolerance should therefore be done at various points throughout the year, especially during periods of high activity in the summer. I attempted this experiment three times during the summer, but there was significant mortality during the acclimation period in each attempt. Spawning prior to collection, as well as observed spawning during acclimation likely left oysters in a weakened state. Despite the difficulty of testing oysters during the summer, understanding oyster tolerance during stressful environmental conditions (i.e., higher water temperatures, increased incidences of low DO) is critical.

Overall, these studies show that anoxia at 28 °C was stressful enough to elicit strong metabolic responses and rapid mortality in diploid and triploid eastern oysters. Hypoxia did not elicit a particularly strong metabolic response in diploid or triploids; however, the more gradual mortality was still indicative of chronic stress, and partial anaerobiosis may have gradually overtaken the oyster metabolism. If plasma and tissue samples were taken throughout the experiment, potential changes to these biomarkers may have been identified.

As climate change increases the prevalence of hypoxia and elevated temperature in estuaries in the northern GoM and globally, understanding the stress on critically important species such as the eastern oyster will be important if predictions of population dynamics and management are to be reliable. The development of triploid oyster population has been advantageous to the aquaculture industry, and they are often chosen over diploids to improve yield. Reports of high triploid mortality compared to diploids (Wadsworth et al. 2019a) suggest that some physiological differences between diploid and triploid oysters exist, therefore it is imperative that diploids and triploids be comparatively tested against various environmental stressors. This study found minimal differences between diploid and triploid oysters in their sub-lethal and lethal responses to hypoxia and high temperature stress, however, given the limitations of this study, it is still unclear how these differences translate in the environment and throughout the growing season. Furthermore, the slightly improved stress responses in triploids shown in these studies suggest that co-occurring low oxygen and temperature stress is not the sole cause of elevated triploid mortality observed in the field. Other factors such as food supply, acidification, and *P. marinus* infection as proposed by Wadsworth et al. (2019a) may therefore be worth future investigations. Future studies investigating differential diploid-triploid environmental tolerance could examine the effect of temperature, food supply, acidification, and *P. marinus* infection in multi-factor combinations. Nevertheless, the prevalence of hypoxia and high temperature will increase during summer months in the northern GoM and understanding the effect these factors in conjunction with others will have on both diploid and triploid oysters in necessary for future management. These studies provide more detail to the comparison of diploid and triploid oysters and will guide managers interested in the advantages and disadvantages of both.
CHAPTER 4. SUMMARY AND CONCLUSIONS

As human land use and climate change continue to impact coastal regions, newly emergent or more frequent environmental stressors will exert increasing pressure on estuarine ecosystems. Stressful salinity, temperature, and hypoxia events, as well as the co-occurrence of multiple stress events in estuaries within the northern Gulf of Mexico (GoM) may significantly affect the physiology and survival of eastern oysters, critical ecosystem engineers. Studies by Stickle et al. (1989) and Marshall et al. (2021b) have shown how multi-stressors synergistically affect the eastern oyster stress response. Accurately describing critical thresholds of stress tolerance and potential differences in stress response within the species will therefore improve our understanding of oyster population dynamics and help inform future restoration and aquaculture practices. The work presented here explored potential differences in response to hypoxia and high temperature among different oyster populations and between oyster ploidies to further describe the specie’s full range of environmental tolerance.

To better understand the full range of environmental tolerance within a species, it is useful to investigate how different populations respond to environmental stress. Geographically distant oyster populations are often adapted to their local environment and respond differently to the same conditions (Burford et al. 2014; Casas et al. 2018a). Local adaptation is shown to influence growth and respiration rates between oyster populations (Burford et al. 2014; Casas et al. 2018a), and there is growing evidence that environmental tolerance is influenced as well. Marshall et al. (2021b) showed that a population frequently experiencing higher salinities and temperatures had greater tolerance to these co-occurring environmental extremes. Reports of frequent hypoxia in the region where this population grows (NOAA 1997; Applebaum et al. 2005) support the idea that differential hypoxia tolerance exists among populations. I therefore investigated potential differences in hypoxia and high temperature tolerance among oyster populations known to differ in their response to co-occurring temperature and salinity stress. These studies identified differential hypoxia tolerance among four oyster populations and characterized sub-lethal responses between two of them. This work suggests certain locally adapted populations are more tolerant to extreme stress events and may be better able to tolerate changing climate regimes. This will inform oyster restoration managers interested in matching environmentally tolerant populations with sites experiencing more stressful environmental conditions. Additionally, populations known to better tolerate certain stresses can be used in selective breeding projects. Selective breeding useful in aquaculture where more tolerant stocks can yield more and higher quality products, and it may prove invaluable in future restoration depending on the severity of climate changes. If oyster populations cannot naturally adapt to climate change quickly enough, selective breeding may provide the relief necessary to maintain wild populations.

Ploidy may also be an important factor in considering oyster environmental tolerance. Identifying potential differences between diploid and triploid stress response could benefit oyster aquaculture in the northern GoM and globally. Although triploid oysters have several advantages over diploids, recent reports of elevated triploid mortality (Wadsworth et al. 2019a) have raised questions into potential vulnerabilities in triploid oysters and whether trade-offs between growth and environmental tolerance exist. I therefore investigated responses to hypoxia and high temperature between diploid and triploid oysters to identify potential
differences in tolerance. These studies found small differences between diploid and triploid response to hypoxia at high temperature, but this does not explain the reports of elevated triploid mortality in the field. It is unlikely that hypoxia and high temperature alone are responsible for the field observations, however these studies will better inform aquaculture managers interested in optimizing production practices and expanding into new grow sites. Future research should investigate the effects of various combinations of stressors to identify the cause of elevated triploid mortality.

The continued benefit of eastern oysters to the natural environment and use as a human food source depend on our ability to properly characterize the conditions in which eastern oysters can and cannot tolerate. These studies show how oyster populations and ploidies can be tested for environmental tolerance and that differential tolerance among populations exists. This information can help future restoration and aquaculture practices in the northern GoM. As well, exhaustively comparing diploid and triploid performance in a variety of environmental conditions will improve our understanding on the potential for aquaculture in the northern GoM, and globally.
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VITA

Nicholas Conrad Coxe was born in Baton Rouge, Louisiana to parents Carey and Elizabeth Coxe. He has one sister, Lily. After attending Baton Rouge Magnet High School, he received his B.S. in Natural Resources Ecology and Management from Louisiana State University in Baton Rouge, Louisiana. After his graduation in May 2019, he worked as a research associate in the La Peyre lab at Louisiana State University, assisting with ongoing studies with eastern oysters. In December 2021, Nicholas enrolled in a graduate program in the School of Renewable Natural Resources at the Louisiana State University AgCenter. He anticipates earning his M.S. in Renewable Natural Resources with a concentration in Fisheries and Aquaculture in May 2022. At LSU, his research has focused on the effects of hypoxia and high temperature on eastern oyster survival to identify potential differences among oyster populations and between oyster ploidies. This research is critical to the future success of wild oyster populations and oyster aquaculture in the northern Gulf of Mexico. Nicholas plans to receive his Master’s degree in August 2022.