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The Effects Of Hypoxia And Freshwater Intrusion On The Eastern Oyster (Crassostrea Virginica)

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THE EFFECTS OF HYPOXIA AND FRESHWATER INTRUSION ON THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*)

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ABSTRACT

Recent estimates revealed a significant decrease in oyster populations worldwide. This drastic decrease has detrimental effects on coastal and estuarine ecosystems. Two environmental stressors that are thought to be contributing to the oyster population decline are hypoxia and excess freshwater intrusion. In this study, effects of hypoxia and low salinity on oysters were investigated using a combination of laboratory and field-based methods. In the laboratory, oysters were exposed to 2, 4, or 8 days of hypoxia (< 2 mg/L dissolved oxygen) followed by 6 days of recovery in normoxic conditions. At the same time, caged oysters were exposed to a naturally occurring hypoxic event in the field. After 8 days, laboratory-exposed oysters showed evidence of immunosuppression indicated by significant downregulation of the immune-related gene *thymosin-β4* (*Tβ-4*) and a significant decrease in total circulating hemocytes compared to controls. However, in field oysters exposed to a naturally occurring hypoxic event, no effect on total hemocyte counts and an upregulation of *Tβ-4* was observed. In a second field study, to investigate how oysters respond to prolonged freshwater exposure, caged oysters were placed on 23 April 2019 at six reef sites in the Mississippi Sound along with *in situ* water quality sensors. One-hundred percent mortality of caged oysters occurred at four of the six sites. Of the 6 six sites, Henderson Point Reef and Kittiwake Reef showed some caged oyster survival. At Henderson Point, where higher mortality was observed compared to Kittiwake, a significant increase in lipid peroxidation was detected. Analysis of mRNA expression of surviving caged and native oysters revealed downregulation of genes involved in immune function, low oxygen response, and osmoregulation. These results show possible evidence of energetic depression

which inhibits adequate adaptation to low salinity conditions. Energetic depression and increased oxidative damage could have contributed to higher oyster mortality. Dredge sampling of native oysters at the all Mississippi Sound field sites on 27 September 2019, following recovery to ~15 ppt salinity, still indicated 100% native oyster mortality due to the prior prolonged freshwater exposure. Continued monitoring of western Mississippi Sound oyster reefs is crucial to observe recovery of oyster populations.

DEDICATION

I would like to dedicate this work to all the friends, family members, teachers, and mentors that have believed in and supported me throughout this journey. Without you I do not think I would have been able to achieve all that I have. I am beyond thankful that I am surrounded by wonderful people, with all of you behind me the sky truly is the limit.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
DO	Dissolved oxygen
GCRL	Gulf Coast Research Laboratory
HIF	Hypoxia inducible factor
HSP	Heat shock protein
MDA	Malondialdehyde
MDMR	Mississippi Department of Marine Resources
mRNA	Messenger ribonucleic acid
MXS	multinucleated sphere X
NKA	Na^+/K^+ ATPase
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TBARS	Thiobarbituric acid reactive substances

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CHAPTER 1 INTRODUCTION

1.1 Oyster Background

1.1.1 Oysters

Oysters are a type of shellfish that typically inhabit estuarine or marine environments. Oysters belong to the class Bivalvia alongside mussels and clams. Bivalvia is nested in the phylum Mollusca, and further nested into the superfamily Ostreoidea. There are several different species of oysters, some commonly known species include the Eastern oyster (*Crassostrea virginica*) of the eastern United States and Gulf of Mexico, the Pacific oyster (*Crassostrea gigas*) of the western United States, and the Pearl oyster (*Pinctada albina*) which is known for the rare and valuable pearl that members of this species produce. Commonly identified by their bilateral shells composed of calcium carbonate, also known as valves, oysters use their valves as defense mechanisms against predation as well as against rapidly changing environmental conditions. Oysters form large colonies called reefs that act as habitat for many other organisms including crabs, snails, and mussels. Oyster reefs are generally seen in two types of environments, intertidal regions which experience tidal fluctuations exposing oyster reefs to open air from time to time, and subtidal regions. Oysters are known not only as a valuable economic resource but also for the important ecosystem services which they provide. Due to these ecosystem services, oysters earn the title of keystone species (Sanjeeva Raj, 2008)

1.1.2 Ecosystem services

Oysters are considered ecosystem engineers. These invertebrates play an important part in supporting estuarine ecosystems around the world. Ecosystem services provided by oysters not only benefit other estuarine organisms, but humans as well. While filter feeding, oysters remove contamination, suspended sediments, and toxic algae from the water. By cleaning up estuarine waters, oysters make waters safer for both humans and other marine organisms. Oyster reefs also act as buffers against incoming wave energy by providing large three-dimensional structures that are raised above the seafloor. This in turn prevents shoreline erosion and destruction of important coastal ecosystems and valuable coastal real estate (Cressman et al., 2003; Tunnell, 2017). Another ecosystem service that oyster reefs provide is fisheries habitat. Reefs provide structures for other marine organisms to reside, thus creating healthier and more productive fisheries. Finally, oysters are an important economic resource for many coastal economies. In the past, oyster harvests have supplied coastal economies with substantial economic benefits through the sale of oysters as a delicacy in restaurants around the world. Previous oyster harvests in the Chesapeake Bay were valued at around $60.1 million in 1980 (National Research Council, 2004) and have dropped to roughly $28 million in 2014 (NOAA Chesapeake Bay Office, 2019).

1.1.3 Worldwide reduction in oyster reefs

Despite the many ecosystem services that oysters provide, the worldwide oyster population has drastically declined in recent years. One study that utilized data collected from commercial oyster harvests as well as fisheries survey data from roughly 140 bays and

ecoregions around the world estimated a roughly 85% reduction in oyster reefs worldwide (Beck et al., 2011). If accurate, this estimation classifies oyster reefs as one of the most heavily impacted ecosystems in the entire world. Recently, large efforts have been made to restore oyster reefs to historic population levels, however success in these restoration efforts has lagged far behind that of other coastal ecosystems (Grabowski et al., 2012). There is an increasing need to research estuarine ecosystems so that we may improve the way we understand, maintain, and restore them.

1.1.4 Environmental stressors

There are several possible contributing factors to the massive reduction in worldwide oyster populations. Due to their proximity to land, estuarine ecosystems are constantly faced with many biotic and abiotic challenges. Contaminants from stormwater, industrial, and sewage runoff contaminate estuarine waters and bioaccumulate in filter feeding organisms (Lau et al., 2018). Sources of freshwater intrusion can cause rapid and drastic fluctuations in salinity in many estuaries. Organisms in these areas, especially benthic invertebrates, must be able to quickly acclimate to large fluctuations in salinity. With freshwater intrusion often comes a large influx of excess nutrients from untreated agricultural and sewage runoff. These nutrients trigger algal blooms that can be detrimental to estuarine systems (Ansari et al., 2011). Harmful algal blooms (HABs) inhibit sunlight from reaching bottom dwelling plants, as well as release toxins that can be harmful to humans and estuarine organisms. Eutrophication can also induce hypoxia (Ansari et al., 2011). Microbes that decompose dead algae consume oxygen, causing dangerously low dissolved oxygen concentrations (Rabalais et al., 2006). Extended hypoxic events, in turn, can cause mass mortalities of estuarine organisms (Altieri et al., 2017). On top of all of these

environmental stressors, overharvesting of oysters can prevent reefs from recovering to previous population sizes (Jackson et al., 2001). It is estimated that 75% of harvested oysters come from just five North American regions (Beck et al., 2011). Lastly, disease plays a large role in the decline of oyster populations. Increased incidences of dermo disease (*Perkinsus marinus*), amongst other diseases, have caused substantial mortality in the Eastern oyster populations (Powell, 2017). Although these stressors each have the capability to individually impact oyster reefs, it is likely that oysters are challenged by a combination of these stressors simultaneously. Studying how theses stressors impact oyster reefs individually, as well as in combination with each other, is important in determining their true impacts on oyster reefs.

1.1.5 Oyster immune system

Because oysters are sessile organisms for a majority of their life and are unable to escape non-ideal environmental conditions, they must be able to efficiently acclimate to large fluctuations in conditions. In addition to fluctuating environmental conditions, oysters inhabit very pathogen rich environments. Oysters must rely on a strong immune response to defend themselves against the many pathogens such as *Haplosporidium nelsoni*, the causative parasite for multinucleated sphere X (MSX) disease, and *Perkinsus marinus* which causes dermo disease. It is well known that molluscs, unlike humans and other vertebrates, possess only an innate immune response (Wang et al., 2018). The oyster innate immune response is divided into a cellular response and humoral response. The cellular response involves phagocytic cells called hemocytes, which are specialized cells that engulf and break down pathogens via enzyme activity and/or reactive oxygen species (ROS). The humoral response utilizes antimicrobial peptides (AMPs) (Tincu and Taylor, 2004). These peptides bind to pathogens and can then

disrupt membrane structure ultimately killing the pathogen. Several studies have investigated invertebrate immunomodulation by measuring hemocyte parameters including total hemocyte counts, phagocytic ability of hemocytes, and hemocyte ROS production. Hypoxic conditions inhibit hemocyte ROS production in oysters, causing immunosuppression (Boyd & Burnett, 1999). Total hemocyte counts in mussels are significantly reduced following exposure to hypoxia thus weakening immunosurveillance (Sui et al., 2016). Immunomodulation was also observed in oysters exposed to polycyclic aromatic hydrocarbons through the evaluation of hemocyte parameters such as phagocytic ability of hemocytes, total hemocyte counts, and hemocyte mortality (Croxton et al., 2012). With the increased incidence of disease amongst *C. virginica* populations (Powell, 2017), knowing how environmental stressors impact oyster immune function is vital for the selection of adequate restoration sites and reef management. Weakened immune function could open up the possibility of mass mortalities. In this study, total hemocyte counts were used as a measure of immune health in terms of immunosurveillance.

1.1.6 Thymosin-β4

First extracted from calf thymus, thymosins are a family of intracellular proteins with molecular masses ranging from 1-14 kDa and 40-45 amino acid residues in length. There are three defined groups of thymosins, α, β, and γ, each differentiated by their isoelectric point (Huff et al., 2001). Of these three groups, beta thymosins are the most conserved members among different species and the most abundant members in most cell types (Hannapple and Van Kampen, 1987). Functions of Tβ-4 in vertebrates include cell migration, attachment and spreading of endothelial cells, and stimulation of hormone releasing factor (Grant et al., 1995; Rebar et al., 2019). Tβ-4 plays a role in host defense, for example, human Tβ4 acts as an

antimicrobial peptide in platelets (Tang et al., 2002). In invertebrates, not much is known about how Tβ4 functions. In the Hong Kong oyster (*Crassostrea hongkongensis*), Tβ4 is involved in the production and mobilization of hemocytes (Li et al., 2016). Tβ4 isolated from the Pacific oyster (*Crassostrea gigas*) has antimicrobial activity through its ability to inhibit bacterial growth (Nam et al., 2015), and is heavily involved in the immune response of the disk abalone (*Haliotis discus*) (Kasthuri et al., 2013). Furthermore, through expressed sequence tag analysis, Tβ-4 was identified as a potential immune effector in *C. virginica* (Jenny et al., 2002). In this study, *C. virginica Tβ-4* mRNA expression was studied as a potential biomarker of immunomodulation in oysters to see how expression changes in response to the environmental stressors hypoxia and low salinity/freshwater intrusion.

1.1.7 Hypoxia inducible factor

Hypoxia inducible factor (HIF) is a critical mediator in the adaptive response to low oxygen stress. HIF is a redox sensitive transcription factor composed of an α and β subunit and is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). The α subunit is tightly regulated by the environmental concentration of oxygen and has an extremely short half-life in the presence of oxygen (Wang & Semenza, 1995). A study that investigated *HIF-1α* mRNA expression in response to exposure to different concentrations of dissolved oxygen found that *HIF-1α* was significantly up-regulated following exposure to hypoxia (Piontkivska et al., 2011). Additionally, an increase in *HIF-1α* protein levels and mRNA expression in *C. gigas* was observed during exposure to air and following 48 hours of exposure to hypoxia (Kawabe & Yokoyama, 2012). In this experiment, *HIF-1α* mRNA expression was assessed as a biomarker of hypoxic stress.

1.1.8 Heat shock protein 70

Heat shock proteins (HSPs) are highly conserved in all living organisms and are known for their larger role in the stress response. Some of the main functions of heat shock proteins include refolding of denatured proteins, cell cycle signaling, and protecting cells from damage and apoptosis (Bukau et al., 2006). HSPs are activators of the innate immune system (Tsan & Gao, 2004). Expression of the HSP-70 protein in molluscs is induced following exposure to xenobiotics (Snyder et al., 2001). Additionally, in an exposure experiment, hydrocarbon contamination caused significantly higher HSP-70 protein levels compared to control oysters (Boutet et al., 2004). Due to HSPs role in the stress response of many different organisms, it is commonly utilized as a biomarker for environmentally induced stress. In this study mRNA expression of *HSP-70* was measured to assess stress induced by hypoxia in the laboratory exposure. However, because HSP-70 is post-transcriptionally regulated, mRNA measurement is not a reliable measurement of HSP-70 stress response (Theodorakis and Morimoto, 1987). For this reason, data on *HSP-70* mRNA is only included in the appendix of this thesis (Appendix Fig. 16)

1.1.9 Na^+/K^+ ATPase

Due to large changes in salinity in estuarine environments, oysters have the capacity to acclimate to extreme changes in salinity. In molluscs, low salinity stress induces an osmotic gradient between their external environment and extracellular fluid causing swelling of the tissue. In order to counteract this swelling, water and solutes including sodium, potassium, and free amino acids are expelled from the cell. Na^+/K^+-ATPase (NKA) plays an essential role in

this recovery process by facilitating the transport of solutes out of the cell (Horisberger, 2004). The NKA complex is composed of three subunits, two essential and one non-essential. The alpha subunit contains the binding sites for Na^+, K^+, and ATP; the beta subunit is believed to be involved in the attachment of the alpha subunit to the cell membrane. Lastly, the gamma subunit is thought to modulate Na^+, K^+, and ATP binding affinities (Jorgensen et al., 2003). Analysis of *NKA-α* mRNA expression in the crab, *Pachygrapsus marmoratus,* revealed significant up-regulation of *NKA-α* following 48 hours of exposure to 10‰ salinity (Jayasundara et al., 2007). Similarly, differential transcript analysis of the Sydney rock oyster (*Saccostrea glomerata*) revealed that oysters exposed to reduced salinities showed upregulation of *NKA-α* compared to control oysters (Ertl et al., 2019). These data reveal the importance of this protein complex in osmoregulation. Due to the extreme freshwater event that occurred during our 2019 field study, mRNA expression of *NKA-α* was measured to quantify salinity stress in oysters from different field sites in Mississippi Sound.

1.1.10 Lipid peroxidation

Reactive oxygen species (ROS) are produced in living organisms as a byproduct of several endogenous processes. Even though ROS are important for some physiological functions, the over-production of ROS can cause cellular damage (Bardaweel et al., 2018). Oxidative radicals, due to their highly reactive nature, can often cause damage to cellular components such as DNA, proteins, and lipid membranes (Lobo et al., 2010). Lipid peroxidation describes the oxidative degradation of lipids. ROS react with lipids forming reactive peroxyl radicals, which in turn, causes more lipid peroxidation (Lobo et al., 2010). This destructive chain reaction can disrupt the structure of cell membranes sometimes causing irreversible cell damage and in some

cases cell death. Often, during environmental stress, an increase in oxidative damage is observed. Measurement of oxidative damage in the Hong Kong oyster (*C. hongkongensis*) revealed a positive correlation between oxidative damage and concentrations of heavy metals (Chan and Wang, 2019). It is common to use oxidative damage as a marker of environmentally induced stress (Ringwood et al., 1999). As part of the 2019 field study, lipid peroxidation was measured to assess environmentally-induced stress in caged oysters deployed in the Mississippi Sound during a larger than usual freshwater inflow event.

1.2 Oysters in the Mississippi Sound

1.2.1 Decreased oyster harvest

Compared to other regions around the world, the northern Gulf of Mexico has been classified as one of the regions where large scale oyster reef restoration is actually feasible (Beck et al., 2011). Historically, the Gulf of Mexico has been known to be extremely productive with respect to oysters and other seafood. With recent events such as hurricane Katrina (2005), the Deep-Water Horizon Oil Spill (2010), and the back to back opening of the Bonnet Carré Spillway (2018 & 2019), these estuarine systems have experienced a high frequency of environmental stress. Along the Mississippi Gulf coast, the Mississippi Department of Marine Resources (MDMR) Shellfish Bureau reports commercial oyster harvests reaching record low numbers. Harvests have gone from being as high as 400,000-500,000 sacks (1 sack ≈ 100 oysters) in 2004 to as low as 20,000 – 50,000 sacks in 2016 (https://dmr.ms.gov/shellfish/). With this drastic decline in annual oyster harvest, Governor Phil Bryant formed the Governor's Oyster Council in 2015, to work towards restoring reef populations back to historic levels. 4

1.2.2 Bonnet Carré Spillway

The Bonnet Carré Spillway (Fig. 1) is located in Louisiana, just north of New Orleans. This river control structure, built by the Army Corps of Engineers, stretches roughly a mile and a half along the east bank of the Mississippi River, and diverts flood waters from the Mississippi River

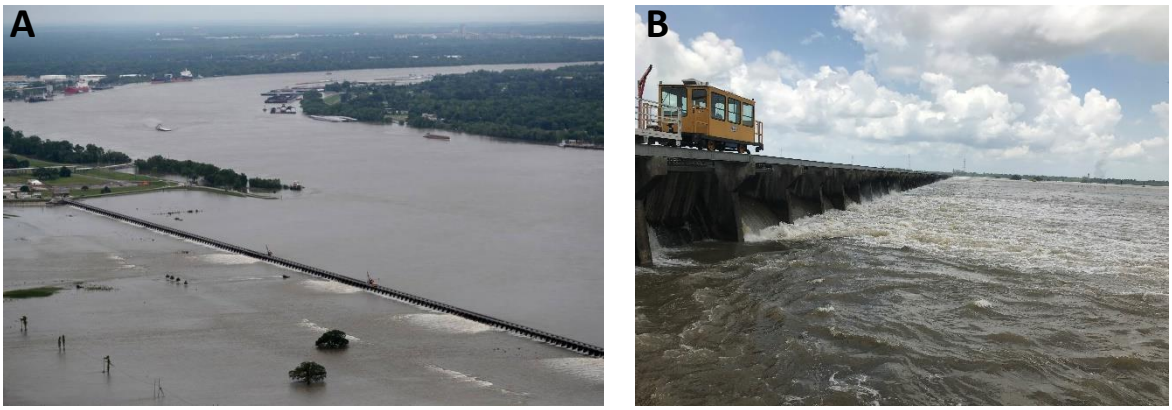
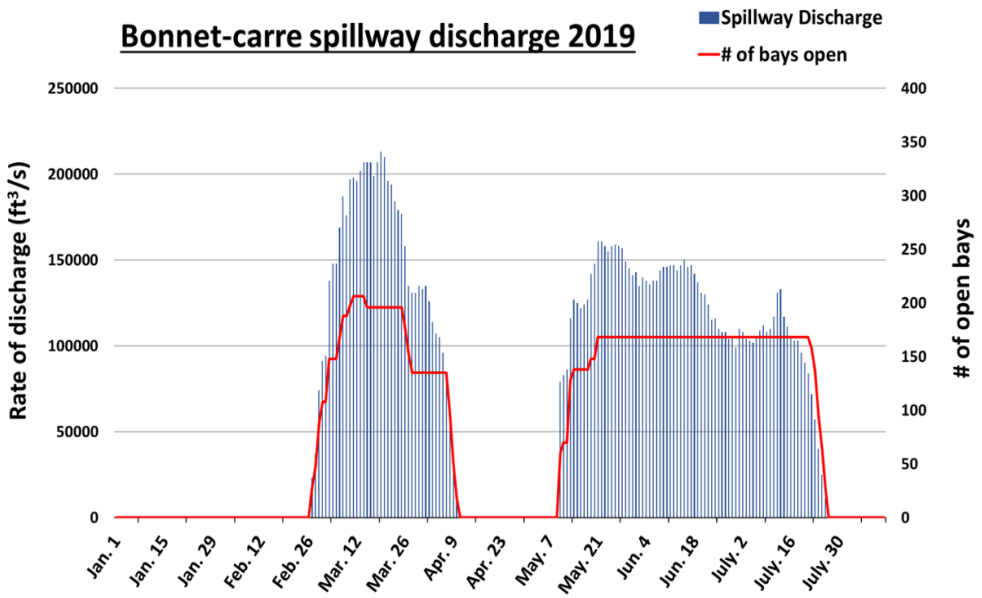


Figure 1. Bonnet Carré Spillway. **A)** Aerial view of the Bonnet Carré spillway (Photo by-Gerald Herbert, Sun Herald). **B)** Close up view of the spillway showing individual floodgates.

into Lake Pontchartrain, a brackish estuary. The spillway consists of about 350 bays/floodgates that are manually opened when floodwaters reach potentially harmful levels. When freshwater is released through the spillway, it first flows through the floodway, which runs six miles from the Mississippi River to Lake Pontchartrain, eventually making its way into the Gulf of Mexico and Mississippi Sound. This structure was originally built in response to 'The Great Mississippi Flood of 1927' when floodwaters inundated roughly 27,000 square miles with approximately 30 feet of water (Ambrose, 2001). Construction of the spillway was necessary to protect cities along the river and prevent future catastrophic flooding. Before 2019, the spillway had only opened 13 times since its construction. In 2019, for the first time in the history of the spillway, unusually large amounts of flooding of the Mississippi River warranted two spillway openings in the same year. The first opening occurred in late February and lasted until early April (44 days), and the second opening happened in early May and lasted until late July (79 days). These back to back

openings released an unprecedented amount of freshwater (Fig. 2; US Army Corps of Engineers, New Orleans District, 2019) into areas that are typically saltwater estuaries with salinities usually ranging from about 10-30 ppt (Berrigan et al., 1991). The impacts of the freshwater inflow on estuarine systems is a topic of much debate (Turner, 2006). Some studies suggest that high freshwater intrusion into the Mississippi Sound had a negative impact on oyster production (Butler & Engle, 1950). On the other hand, some studies conclude that there is no substantial data to support the conclusion that there is a negative relationship between freshwater inflow and oyster productivity, stating that freshwater influx helps mitigate predation and disease amongst oyster populations to a greater degree than we think (Loftin et al., 2011). Following the 2019 spillway opening, MDMR reported oyster mortalities as high as 100% at some reefs in the Mississippi Sound (Gulf Coast Research Lab, 2019). There is an ever increasing need to research these affected areas in order more closely understand how these massive freshwater inflows really impact our very valuable estuarine environments in our coastal waters. Figure 2 shows the

Figure 2. Discharge and the number of bays open during of both 2019 spillway openings.



length of and the discharge rates of the spillway during the 2019 spillway openings.

Because the Mississippi River Basin is so vast and drains such an expansive area, runoff water has the potential to carry contamination into the Mississippi River. If massive releases from the Bonnet Carré Spillway flush this contamination into the Mississippi Sound, this could contribute to severe eutrophication and hypoxia and further negatively impact the estuarine systems in the Mississippi Sound and Gulf of Mexico.

CHAPTER 2 LABORATORY/FIELD HYPOXIA EXPOSURE STUDY (2018)

2.1 Introduction

In recent decades, hypoxia, an indirect effect of eutrophication, has been increasingly affecting estuarine environments (Breitburg et al., 2018). Hypoxia occurs when the dissolved oxygen (DO) concentration drops below 2 mg/L (Rabalais et al., 2006, Vaquer-Sunyer & Duarte, 2008). Hypoxia negatively impacts the microbiomes of adult oysters, affecting their energy dynamics and increases their vulnerability to pathogens (Khan et al., 2018). Long-term hypoxic events can kill large numbers of marine organisms, including oysters, but even short-term hypoxic events can cause detrimental sub-lethal impacts to oysters such as lowered fecundity, growth rates, and immunosuppression, particularly at early life history stages (Baker & Mann, 1992; Johnson et al., 2009, Sui et al., 2016; Shumway, 1996).

In this study, the effects of hypoxia on the Eastern oyster *(Crassostrea virginica*) were studied by deploying caged oysters in the Mississippi Sound, during which oysters experienced a natural hypoxic event, and by exposing oysters in a laboratory setting to three different durations of hypoxia (< 2 mg/L dissolved oxygen), followed by a 6 day normoxic (>8 mg/L dissolved oxygen) recovery period. Effects were measured by analyzing mRNA expression of hypoxia inducible factor-1α (*HIF1-α*), which is known to be up-regulated under hypoxic stress (Kawabe & Yokoyama, 2012). Immune function was assessed by analyzing mRNA expression of thymosin-beta-4 (*Tβ-4*), which is involved in hemocyte production and mobilization (Li et al., 2016). We hypothesized that oysters subjected to longer durations of hypoxia would have enhanced *HIF1-α* and suppressed immune function through downregulation in mRNA expression of *Tβ-4* and reduced total hemocyte counts compared to controls.

2.2 Materials and Methods

2.2.1 Oyster collection and maintenance

Oysters (*C. virginica*) used in both laboratory and field experiments were obtained from the Auburn University Shellfish Laboratory's farm in Bayou La Batre (Dauphin Island, AL) in early July 2018. The oysters were transported to the Gulf Coast Research Laboratory (GCRL) Halstead campus (Ocean Springs, MS) and placed in flow-through holding tanks supplied with water from Davis Bayou, where salinity and temperature was similar to their site of collection. Oysters were fed *ad libitum* each day with Shellfish Diet 1800 (Reed Mariculture, Campbell, CA, USA). Oysters were kept in holding tanks for approximately one week before being transferred to exposure aquaria at GCRL's Cedar Point campus (Ocean Springs, MS) to begin both the laboratory and field experiment.

2.2.2 Experimental design

2.2.2.1 Field component

Following transport to GCRL, 20 oysters were randomly selected from holding aquaria and 10 oysters were placed in each of two oyster sensor platforms, and submerged off of the dock near GCRL (Marsh Point, MS: 30˚23'31.488"N, 88˚48'27.226"W) for 3 weeks (20 July - 10 August 2018). The sensor platforms were equipped with water quality sensors (HOBOware® loggers) that measured dissolved oxygen, conductivity, and temperature for the duration of deployment, and housed oysters on trays enclosed within crates on each platform, with perforations to allow adequate water flow (Fig. 3). Oysters were sampled from the sensor platforms following 14 days (T-1) and 21 days (T-2) of deployment. Upon sampling, five oysters were sacrificed and gill tissue was extracted for analysis of mRNA expression. At the beginning

of the experiment, five oysters were taken directly from GCRL holding tanks and used as time-zero (T-0) oysters.

Figure 3. – Images of sensor platforms containing oysters.

2.2.2.2 Laboratory component

Oysters were randomly selected from holding tanks, transferred into exposure tanks, and allowed to acclimate for 2-3 days. Treatment groups were subjected to one of three durations of hypoxia (DO < 2 mg/L): 2, 4, or 8 days (short, medium, and long, respectively; Fig. 4), beginning on 14 July 2018. Each treatment was then followed by a 6-day recovery period in normoxic conditions (DO ≥ 8 mg/L). The control group was maintained in normoxic conditions

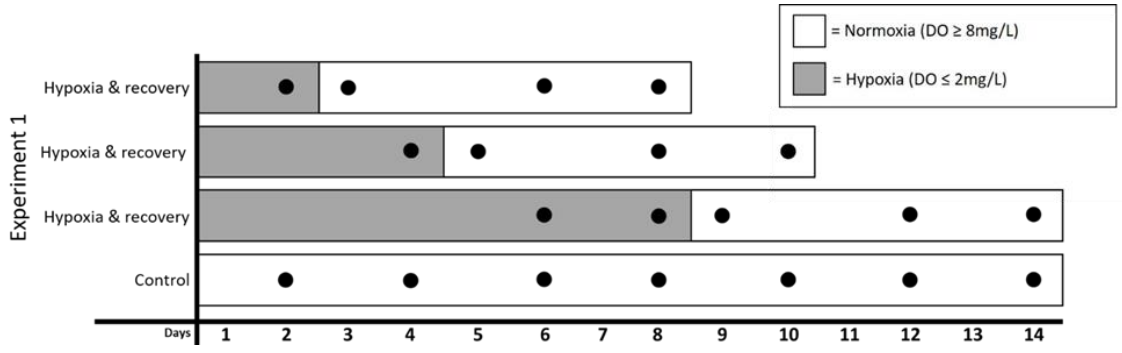


Figure 4. Experimental design of the laboratory experiment. Oysters were exposed to three durations of hypoxia, followed by 6 days of recovery in normoxic conditions. Control tanks remained normoxic for the duration of the experiment. Each tank (n = 3 per treatment) started with 20 oysters, with two oysters sampled on each sampling day (represented by black dots). A similar number of oysters was removed from all tanks on each sampling day to maintain a consistent oyster density in each tank for the duration of the experiment.

for 14 days. Each of the 4 treatments consisted of 3 replicate 65 L tanks kept at a temperature of 18°C and salinity of 15±1 ppt. Each tank started with 20 oysters, and 2 oysters were removed from all tanks on each sampling day so that oyster density remained consistent across tanks on each day of the experiment. Hypoxic conditions were created by bubbling N_2 gas into treatment water in a header tank. Fig. 4 depicts the experimental design. Upon removal from exposure tanks, gill and hemolymph samples were taken from sacrificed oysters for analysis of mRNA expression and total hemocyte counts, respectively.

2.2.3 RT-qPCR - mRNA expression

Following gill tissue collection, tissue was placed in RNAlater™ (Invitrogen) and stored at -80°C until processing. RNA isolation was achieved using TRIzol™ reagent (Invitrogen #A33251) and RNase-Free DNase set (Qiagen #74004) according to the manufacturer's protocol. RNA extract was quantified and assessed for purity using a NanoDrop 2000 spectrophotometer. Following quantification, cDNA sub-stocks were prepared via reverse transcription PCR to achieve a final concentration of 10µg/µL. Real-time Quantitative polymerase chain reaction RT-qPCR was performed using an Applied Biosystems 7200 using SYBR™ Green PCR Master Mix (Applied Biosystems #4309155) with the following parameters: 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec to generate a dissociation curve. Primer optimization was completed using the same instrumentation and procedure. Primer sequences and efficiencies are listed in Table 1. All samples were screened in duplicate and evaluated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittigen, 2001). *18s* (laboratory experiment) and *elongation factor 1-α* (*EF1-α*) were utilized as reference genes for mRNA expression analysis.

Table 1. Laboratory/field study primer names and sequences. R^2 of optimization regression and calculated primer efficiency depicted as well.

Gene	Acession	Primer sequence	r^2	Efficiency %
Tβ-4	LOC111123286	**F:** 5'- TCT GTG ATT GTG GGC TGT GTT- 3' **R:** 5'- TGG TGG GTA GAG GGT TCT TCT- 3'	0.995	101.01
Hif-1α	XM_022475425	**F:** 5'- ACC AGT GAC GCC CTG TTC TC- 3' **R:** 5'- ACA GAC TCG GTG CGA CCA A- 3'	0.999	94.67
NKA-α	XM_022468233	**F:** 5'- CGC AGT GTT TTT GGG AGT CAG T- 3' **R:** 5'- CCA TTC TCT TGG CGA TCA GTG- 3'	0.999	103.60
EF1-α	XM_022472315	**F:** 5'- GGT ATC TCG GCA AAC GGA CA- 3' **R:** 5'- TTC GTT GAA ACG GCT CTC AC- 3'	0.99	100.76
18s	EU660792	**F:** 5'- CCG TCC GTT TTG GTG ACT CT- 3' **R:** 5'- CCT TGG ATG TGG TAG CCG TT- 3'	0.991	96.06

2.2.4 Total hemocyte counts

Hemolymph was extracted from the pericardial cavity of each oyster using a sterile 16-gauge syringe and transferred to a 1.7 mL centrifuge tube. Hemolymph was diluted 1:1 with 10% formalin for preservation. Hemocyte counts were performed using a Benchtop B3 series FlowCAM® particle imaging system (Fluid Imaging Technologies, Inc., Yarmouth, ME, USA). The FlowCAM® was equipped with a 300 µm flow cell and a 20x objective lens, and was set to auto-image mode, in which photographs were taken of cells at 20 frames per second at a constant flow rate of 0.01 mL/min. Two technical replicates were counted for each sample (100 µL each) and averaged to obtain a final count for each individual oyster.

2.2.5 Data analysis

Prior to data analysis, data were tested for normality and equal variance using Shapiro-Wilk's and Brown-Forsythe's tests, respectively, in SigmaPlot version 14.0. Field data (mRNA expression and hemocyte counts) were analyzed using one-way analyses of variance (ANOVA) (n=5 per time point) followed by Tukey's post hoc tests, if appropriate. For laboratory experiment data, mRNA expression and hemocyte counts were analyzed using two-way ANOVAs with time and treatment as factors, followed by Tukey's post hoc tests, if appropriate. Control oysters were not sampled on the first day of recovery, but to evaluate acute changes in mRNA expression, treated oysters at 24 h of recovery were compared to those from the final hypoxic time point using unpaired t-tests. Hemocyte counts were also analyzed using unpaired t-tests to compare exposed oysters to controls (n = 2-3 tanks per treatment) on each day.

2.3 Results

2.3.1 Field deployment

2.3.1.1 *In situ* water quality measurements

An oyster sensor platform was deployed for three weeks near Marsh Point, MS from 20 July to 10 August 2018, which overlapped with the laboratory experiments. During deployment, the *in situ* water quality sensor data (Fig. 5) indicated fluctuating diel DO concentrations for the first 10-12 days of deployment. On day 12, DO dropped below 2 mg/L and remained near or below the hypoxic threshold for the remainder of the deployment. There was a concurrent decrease in salinity to a minimum of 4 ppt during the hypoxic event, with a subsequent return to 12-15 ppt near the end of the deployment, even while DO remained low.

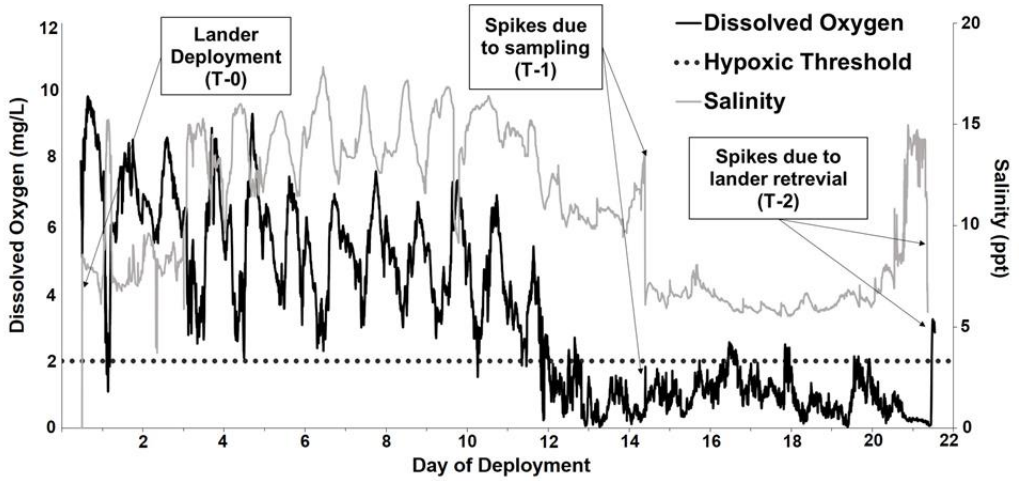


Figure 5. Dissolved oxygen (mg/L) and salinity (ppt) measured by *in situ* sensors on oyster sensor platform near Marsh Point, MS, during three-week field deployment (20 July -10 August 2018). Note the natural hypoxic event that occurred when DO fell below the hypoxic threshold (<2 mg/L) on day 12. Oysters were sampled prior to deployment (T-0), on day 14 (T-1) and on day 21 (T-2).

2.3.1.2 mRNA expression

As predicted, gill *HIF1-α* mRNA expression showed a significant response to the natural

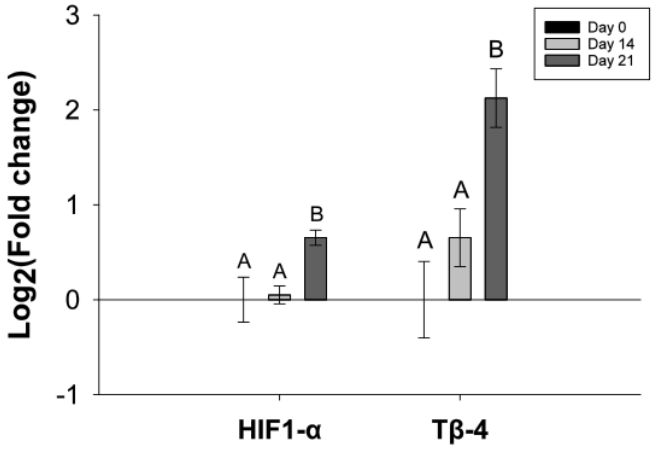


Figure 6. Relative mRNA expression of *HIF1-α*, *Tβ-4* of oysters sampled prior to deployment (T-0), on day 14 (T-1), and on day 21 (T-2) in the field deployment. A natural hypoxic event occurred on day 12 and lasted for the remainder of the deployment (Fig. 2). Bars represent means ± standard error (n = 5 oysters per time). Significant differences between time points for each gene were determined using one-way ANOVA, followed by a Tukey's post hoc test. Letters denote times that were significantly different from each other ($p < 0.05$).

hypoxic event (1-way ANOVA, $F_2 = 4.219$, $p = 0.044$; Fig. 6), with significant up-regulation on day 21 (after 9 days of hypoxia; T-2) compared to T-0 oysters (Tukey's post-hoc test, $p = 0.022$ and 0.032 for T-0 vs. T-2 and T-1 vs. T-2, respectively). *Tβ-4* mRNA expression did not vary between oysters sampled at T-0 and T-1 (day 2 of hypoxia), but showed a significant 2-fold up-regulation in oysters following 9 days of exposure to a natural hypoxic event (1-way ANOVA, $F_2 = 9.08$, $p = 0.005$; Fig. 6; Tukey's post-hoc test, $p = 0.388$ and 0.004, for T-0 vs. T-1 and T-0 vs. T-2, respectively).

2.3.1.3 Total hemocyte counts

No significant differences in total circulating hemocyte counts were found among oysters collected over the course of the field deployment (; 1-way ANOVA, $F_2 = 0.23$, $p = 0.798$, Fig. 7).

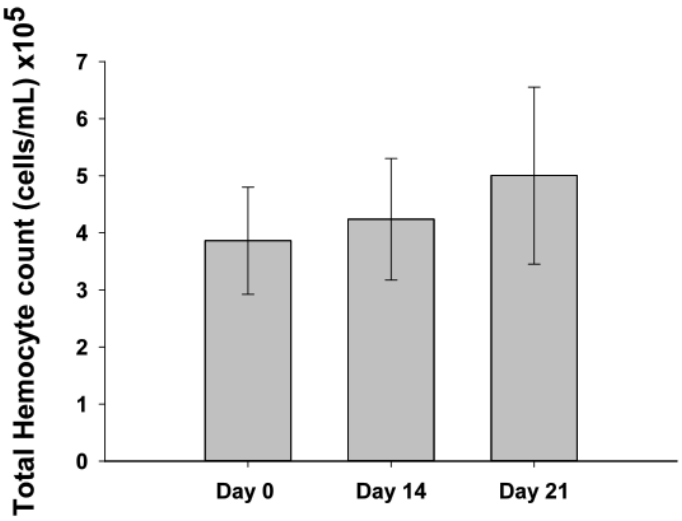


Figure 7. Total hemocyte counts from oysters sampled prior to deployment (T-0), on day 14 (T-1), and day 21 (T-2) of the field deployment. A natural hypoxic event occurred on day 12 and lasted for the remainder of the deployment (Fig. 5). Bars represent means + standard error (n = 5 oysters per time).

2.3.2 Laboratory experiment

2.3.2.1 mRNA expression

Effects on oyster gill *HIF1-α* expression were measured after different durations of hypoxia exposure, followed by 6 days of recovery in the laboratory. It is important to note that in the control treatment, *HIF1-α* and *Tβ-4* expression varied significantly over time (Fig. 8). During the 2-day exposure, there was a significant main effect of time (2-way ANOVA; $p = 0.019$) and treatment ($p < 0.001$) on *HIF1-α* expression (Fig. 8A; Table 2). In the 4-day exposure treatment, *HIF1-α* mRNA expression (Fig. 8B) showed a significant interaction between time and treatment ($p = 0.022$). During the longest hypoxic exposure (8 days), *HIF1-α* expression (Fig. 8C) had a significant time by treatment interaction ($p = 0.01$), and expression was significantly up-regulated in treated oysters compared to controls on day 14 (i.e., after 8 days of hypoxia and 6 additional days of recovery) ($p < 0.001$; Appendix Table 5c). Post hoc tests revealed a significant up-regulation of *HIF1-α* in treated oysters on day 6 of exposure compared to those on day 14, after 6 days of return to normoxia ($p = 0.013$). Acute changes were not observed in *HIF1-α* expression in oysters following 24 h in normoxic conditions after hypoxia exposures of any of the three treatment durations (unpaired t tests, $p > 0.05$).

After 2 days of hypoxic exposure, *Tβ-4* expression (Fig. 8D) showed a significant time by treatment interaction ($p < 0.022$; Table 2), with down-regulation compared to controls during each sampling point in the recovery phase (days 6 and 8; Appendix Table 5a). In the 4-day exposure, *Tβ-4* mRNA expression (Fig. 8E) exhibited main effects of both treatment ($p = 0.040$) and time ($p = 0.003$). In the longest hypoxic exposure (8 days), *Tβ-4* gill expression showed a significant time by treatment interaction ($p = 0.013$) and was significantly downregulated

compared to controls on days 6 and 8 (Appendix Table 5d). Within 24 hours of return to normoxia, mRNA expression was similar to control levels (Fig. 8F).

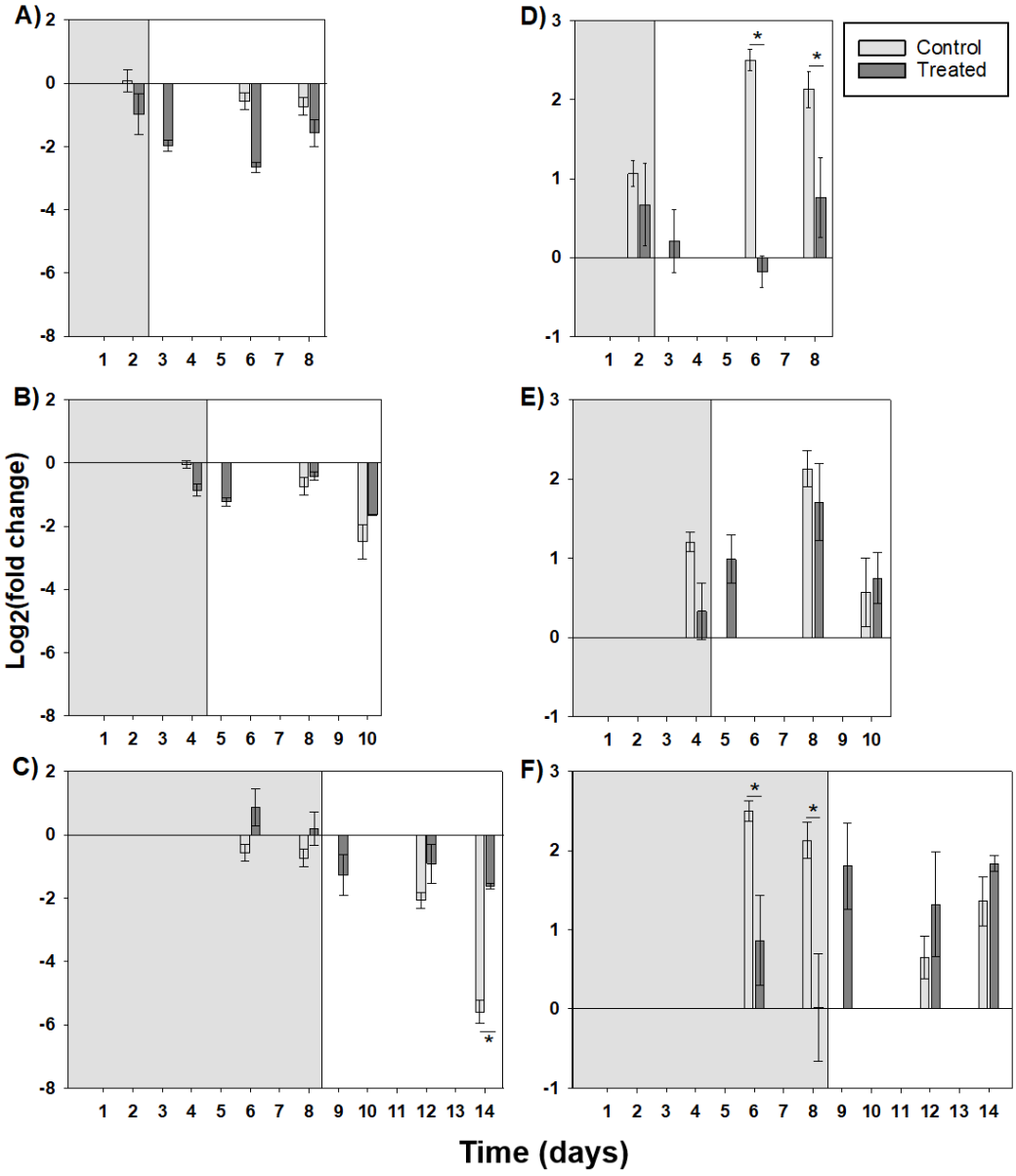


Figure 8. Oyster gill mRNA expression following different durations of hypoxia exposure (DO < 2 mg/L), followed by a 6-day recovery period (DO > 8 mg/L). HIF-1α mRNA expression during (A) 2, (B) 4, and (C) 8-day hypoxic exposures, and Tβ-4 mRNA expression during (D) 2, (E) 4, and (F) 8-day hypoxic exposures. Bars represent means ± standard errors (n = 3 tanks per treatment). Grey background represents hypoxia period, white background represents recovery period. Asterisks denote significant differences between control and treatment determined via two-way ANOVA followed by a Tukey's post hoc test ($p < 0.05$, Appendix Tables 5a-d).

Table 2. ANOVA table showing significant main effects as well as significant interaction effects of mRNA expression endpoints as well as hemocyte counts from the laboratory experiment.

Treatment	Respose/Factor	df	F	p	Figure	Respose/Factor	df	F	p	Figure
2 day hypoxic exposure	**mRNA expression**					**Physiological endpoints**				
	***HIF1-α* expression**				8a	**Hemocyte counts**				9a
	treatmet	1	43.23	<0.001*		treatmet	1	1	0.251	
	time	3	4.208	0.019*		time	2	3	0.095	
	treatment x time	3	1.959	0.154		treatment x time	2	1	0.383	
	***Tβ-4* expression**				8d					
	treatmet	1	41.87	<0.001*						
	time	3	2.811	0.067						
	treatment x time	3	5.32	0.008*						
4 day hypoxic exposure	***HIF1-α* expression**				8b	**Hemocyte counts**				9b
	treatmet	1	0.445	0.513		treatmet	1	6	0.035*	
	time	3	11.02	<0.001*		time	2	0	0.925	
	treatment x time	3	4.027	0.022*		treatment x time	2	0	0.805	
	***Tβ-4* expression**				8e					
	treatmet	1	4.856	0.04*						
	time	3	6.478	0.003*						
	treatment x time	3	1.198	0.337						
8 day hypoxic exposure	***HIF1-α* expression**				8c	**Hemocyte counts**				9c
	treatmet	1	26.19	<0.001*		treatmet	1	11	0.007*	
	time	4	17.34	<0.001*		time	3	4	0.03*	
	treatment x time	4	4.236	0.01*		treatment x time	3	1	0.311	
	***Tβ-4* expression**				8f					
	treatmet	1	2.137	0.157						
	time	4	0.957	0.45						
	treatment x time	4	4.025	0.013*						

2.3.2.2 Total hemocyte counts

In the 2-day hypoxic exposure, total circulating hemocyte counts did not differ across days or treatments (Fig. 9A; Table 2). Total hemocyte counts were significantly lower in exposed compared to control oysters after 4 days of hypoxic exposure (Fig. 9B, $p = 0.035$), but there was no significant time or interaction effect (Table 2). In contrast, during the 8-day exposure treatment (Fig. 9C), hemocyte counts showed significant main effects of both treatment ($p = 0.007$) and time ($p = 0.03$), but no significant interaction (Table 2). In the longest hypoxic exposure, there was a significant reduction in total hemocyte counts in treated oysters compared to controls on days 6 and 8 of exposure to hypoxia (unpaired t-tests, $p = 0.037$ and 0.009, respectively).

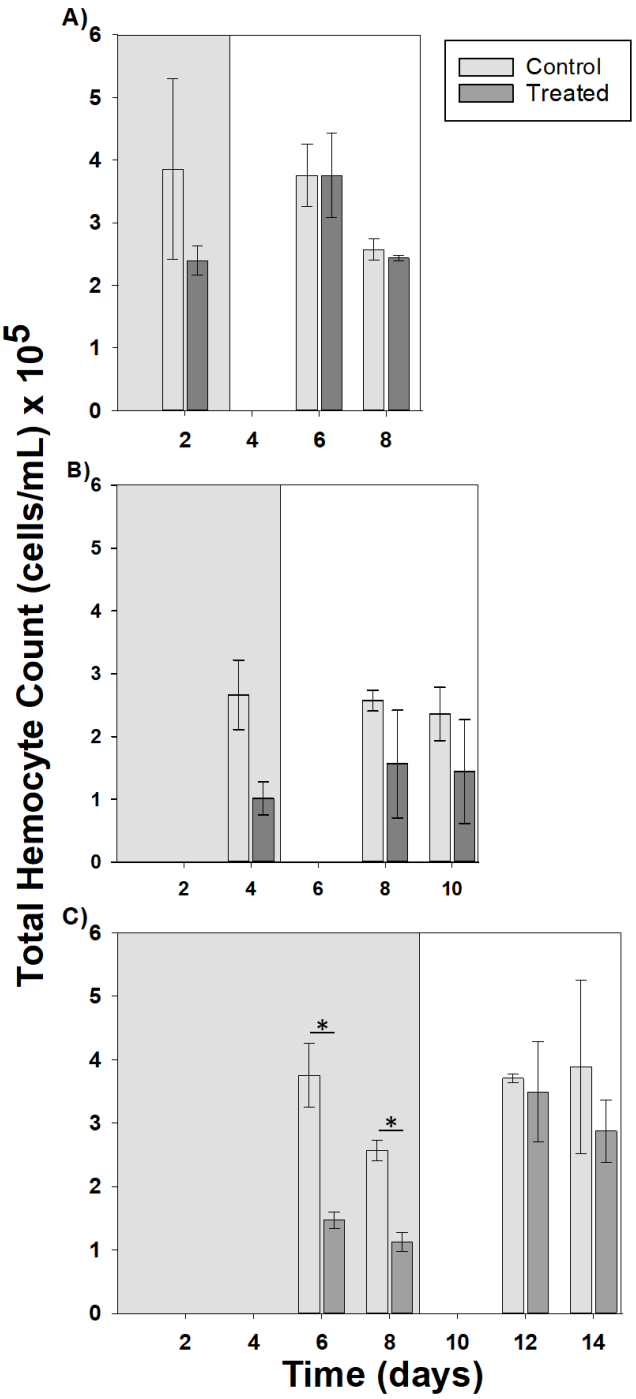


Figure 9. Total circulating hemocyte counts from hemolymph collected from oysters exposed to (A) 2, (B), 4, and (C) 8 days of hypoxia (DO $\leq$ 2 mg/L), followed by a 6-day recovery period (DO $\geq$ 8 mg/L). Bars represent means $\pm$ standard errors (n = 2-3 tanks per treatment) grey background represents hypoxia period, white background represents recovery period. Asterisk denotes significant differences between treated and control oysters on that day, as determined using t-tests ($p < 0.05$).

2.4 Discussion

Worldwide, hypoxic events are occurring in estuarine environments for longer durations and on larger spatial scales than ever before (Rabalais et al., 2006; Breitburg et al., 2018). Long term hypoxia has the potential to cause detrimental effects on aquatic organisms including suppressed immune function, decreased reproduction, and increased susceptibility to predation (Vaquer-Sunyer & Duarte, 2008; Long, et al. 2014). If oyster reefs are negatively impacted by hypoxia, amongst other environmental stressors such as disease, freshwater inflow, and eutrophication, this can have negative effects on entire ecosystems. In this study, *in situ* water quality sensors deployed in the Mississippi Sound captured the occurrence of a hypoxic event during the final nine days of the deployment (Fig. 5), which likely continued following the retrieval of the sensor platforms. The capture of this small-scale hypoxic event recognizes the relevance of persistent hypoxia as an environmental stressor in the Mississippi Sound and shows that these hypoxic events can last several days at a time.

Previously, studies have demonstrated the use of *HIF1-α* as a relevant biomarker for hypoxic exposure. Significant upregulation of *HIF1-α* mRNA expression was observed following ≥ 6 days of exposure to hypoxia (Kawabe & Yokoyama, 2012). Although similar results were observed in our field deployed oysters, which showed significant upregulation of *HIF1-α* following 9 days of exposure compared to time zero, oysters in the laboratory exposure did not yield the same results. Laboratory oysters showed no significant up-regulation of *HIF1-α* compared to controls during or following any of the hypoxic exposures. This result is potentially explained by the large amount of variance observed in our control treatment; it is likely that exposed oysters were experiencing a similar effect, which may have skewed our results.

Another aim of this study was to assess the effects of hypoxia on oyster immune function. *Tβ-4* plays an important role in immune function in that it regulates the production and mobilization of phagocytic immune cells called hemocytes (Li et al., 2016). Additionally, expressed sequence tag analysis indicated *Tβ-4* as a biomarker of immune function in the Eastern oyster (Jenny et al., 2002). In this study, we hypothesized that oysters exposed to hypoxia would show signs of immunosuppression through the downregulation of *Tβ-4* in conjunction with a reduction in the amount of total circulating hemocytes. In the laboratory exposure, oysters exposed to just two days of hypoxia showed no significant down regulation of *Tβ-4* during or following exposure to hypoxia, however on days six and eight, which were during the recovery period, significant differences in *Tβ-4* expression were observed between control and treated oysters (Fig 8d). Despite this significant difference in *Tβ-4* expression, no significant differences in circulating hemocytes were observed in the two-day exposure treatment (Fig. 9a). In the longest hypoxic exposure treatment (8 days), exposed oysters showed signs of significant downregulation of *Tβ-4* compared to control on day six and day eight of exposure to hypoxia (Fig. 8f). Interestingly, upon analysis of total hemocyte counts in oysters sampled from the longest exposure, a significant decrease in total circulating hemocytes was observed on day six and day eight of exposure to hypoxia (Fig. 9c) followed by a return to levels similar to controls during the recovery period. The reduction in circulating hemocyte counts observed in the eight-day exposure, but not the two-day exposure, could indicate that longer periods of hypoxia are necessary to induce observable physiological effects.

In contrast to the result of our laboratory study, oysters exposed to hypoxia in the field showed a significant increase in expression of *Tβ-4* compared to time zero following nine days of exposure to hypoxia. Additionally, no significant effect on total circulating hemocytes were

observed in field deployed oysters. Inconsistencies in laboratory and field deployed oysters could be explained by vastly different environmental conditions between field and laboratory settings. Oysters in the field experienced a drastic fluctuation in dissolved oxygen as well as salinity, additionally the higher microbial abundance of the field site compared to the laboratory setting may have induced an immune response in oysters deployed in the field.

CHAPTER 3 MISSISSIPPI SOUND FIELD STUDY (2019)

3.1 Introduction

Historically, Mississippi has been one of the leading producers of seafood in the United States. With the occurrence of Hurricane Katrina (2005), the Deep-Water Horizon Oil Spill (2010), and two back to back freshwater inflow events from by the Bonnet Carré Spillway (2019), the Gulf Coast and the Mississippi Sound have experienced greater than usual environmental stress. With this increased environmental stress, the Mississippi seafood industry

Figure 10. Map depicting the location of the Bonnet Carré Spillway (red arrow) in relation to our study sites (red box). Image from Google Earth.

has suffered a massive decline in productivity. Mississippi commercial oyster harvest data reveals a greater than 300,000 sack decrease (1 sack = 100 oysters) in the number of oysters harvested in 2017 compared to 2008 (https://dmr.ms.gov/shellfish/).

The Bonnet Carré Spillway, built in 1931, was designed and built for the purpose of mitigating flooding of the Mississippi River, and protecting rural and agricultural regions from

catastrophic flooding. Since its construction, the spillway has only been opened 13 times. In late February 2019, flooding of the Mississippi River reached heights great enough to warrant opening the spillway for a duration of 44 days, finally closing in early April. Approximately one month later, Mississippi River flood levels again reached dangerous heights warranting a second spillway opening in early May. The second opening lasted 79 days at an average flow rate of 120,641 ft^3/s (US Army Corps of Engineers, New Orleans District, 2019). These two openings marked the first time in the history of the spillway that it was opened twice in the same year. The spillway releases allowed the flow of an unprecedented volume of freshwater into the Gulf of Mexico and the Mississippi Sound. Oyster reef sampling by the Mississippi Department of Marine Resources (MDMR) in the Mississippi Sound on 10 June 2019, 32 days following the second spillway opening, found oyster reef mortality as high as 100% at historically productive reefs (Gulf Coast Research Lab, 2019).

This study was designed to assess the impacts of the long-term freshwater event caused by the opening of the Bonnet Carré Spillway on oysters in the Mississippi Sound. We hypothesized that extended freshwater exposure would have negative impacts on important gene expression pathways that are critical to oyster survival. This was assessed by deploying caged oysters in the field prior to the second 2019 spillway opening. Effects were measured by assessing caged and native oyster mortality at each of six field sites, and quantitating mRNA expression of stress-related biomarkers throughout the duration of the study. *HIF-1α* was measured as an indicator of oxygen deprivation stress, *Tβ-4* was used as an indicator of immunosuppression, and Na^+/K^+ *ATPase-α* subunit, involved in osmoregulation, was assessed as an indicator of low salinity stress. Throughout the study, water quality measurements were

recorded using a handheld YSI multimeter as well as through *in situ* long-term monitoring via sensor platforms equipped with dissolved oxygen, temperature, and conductivity sensors.

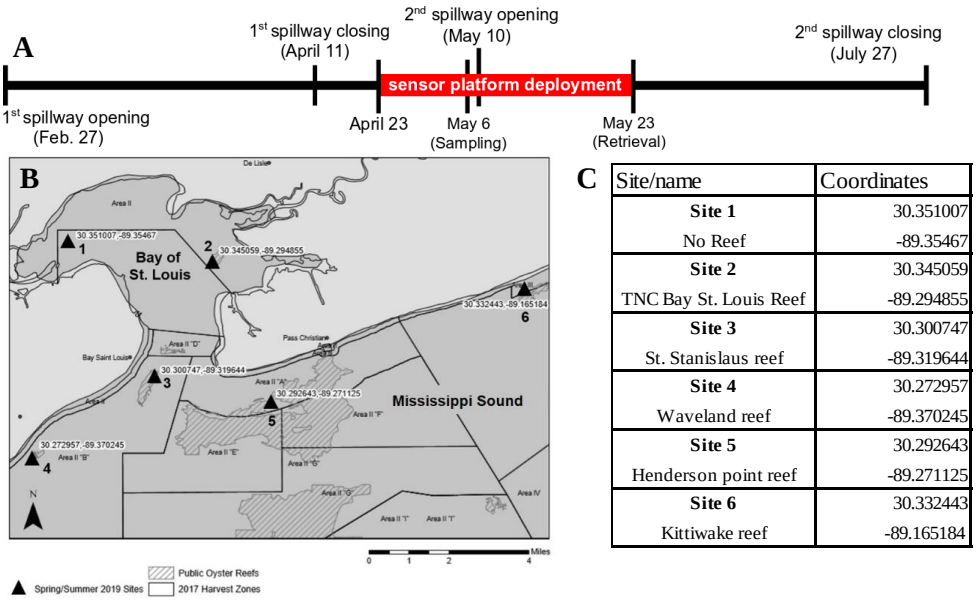
3.2 Materials and Methods

3.2.1 Oyster collection and maintenance

Oysters (*C. virginica*) were obtained from the Auburn University Shellfish Laboratory's farm in Bayou La Batré (Dauphin Island, AL) in early April 2019. Oysters were transported to the Gulf Coast Research Laboratory's (GCRL) Halstead campus (Ocean Springs, MS) and placed in a flow-through holding tank supplied with water from Davis Bayou, where salinity and temperature was similar to their site of collection. Oysters were held overnight in the tank before being placed into sensor platforms and deployed in the field on 23 April 2019.

3.2.2 Study design and field sites

Twenty oysters were placed in each of 12 sensor platforms, and placed at six different sites, in duplicate, in the Mississippi Sound and the Bay of St. Louis (Fig. 11a). All study sites, excluding our No Reef site (Site 1), were selected based on prior knowledge of oyster reefs



Site/name	Coordinates
Site 1	30.351007
No Reef	-89.35467
Site 2	30.345059
TNC Bay St. Louis Reef	-89.294855
Site 3	30.300747
St. Stanislaus reef	-89.319644
Site 4	30.272957
Waveland reef	-89.370245
Site 5	30.292643
Henderson point reef	-89.271125
Site 6	30.332443
Kittiwake reef	-89.165184

Figure 11. (A) Timeline of field deployment as it relates to the second Bonnet Carré Spillway opening. (B) Map depicting all field sites where sensor platforms were placed in the Mississippi Sound and the Bay of St. Louis. (C) Reef names and latitude and longitude coordinates of sensor platform placement.

existing at the site. The sensor platforms were equipped with water quality sensors (HOBOware® loggers; Onset Computer, Bourne, MA, USA) that measured dissolved oxygen, conductivity, and temperature for the duration of deployment, and housed oysters on trays enclosed within crates on each platform, with holes to allow adequate water flow. Following sensor platform deployment on 23 April 2019, oysters were taken directly from the holding tank at GCRL and sampled as time-zero (T-0) oysters. Oysters were sampled from the platforms on day 13 (6 May 2019: T-1) and day 31 (24 May 2018: T-2) of deployment and mortality of caged oysters was recorded. During T-2 and T-3 sampling, dredge pulls were conducted in order to retrieve native oyster samples from local reefs and assess reef mortality and population density.

At each sampling period, a subset of native and deployed oysters were sampled and placed in a bucket of site water to be transferred back to the lab. Oysters were sacrificed and gill tissue was extracted for analysis of mRNA expression.

3.2.3 Water quality measurements

A YSI™ Professional Plus handheld multimeter was used to measure water quality at each site on every sampling day. First, the probe was submerged just below the surface of the water to obtain surface water quality readings. Next, the probe was sunk to the bottom to obtain bottom water quality measurements. Measurements included temperature, conductivity, dissolved oxygen, salinity, and pH (Table 3). In addition to YSI measurements, *in situ* salinity was recorded using a HOBOware® conductivity logger (item #: U24-002-C) (Fig. 12). Data was extracted from sensors as specific conductance and converted to salinity using calibration points collected using the multimeter data from Table 3.

3.2.4 Native oyster collection by dredge

Three dredge pulls were conducted at each field site except for site 5 where six dredge pulls were conducted due to larger reef size. Each dredge pull (i.e., transect) was 305 m (1000 ft) long by 0.61 m wide. Following each dredge pull, the number of live and dead juvenile oysters (spat) were counted along with the number of live and dead adults. Oysters that were gaping, had both valves attached, and would not close valves were counted as dead. Results were reported in percent living adults or spat (Fig. 13) and living oysters per square meter (Appendix, Fig. 17). Maps and coordinates of dredge transects completed at each field site are depicted in Appendix Fig. 18-22.

3.2.5 Lipid peroxidation

Lipid peroxidation was measured in oyster gill tissue using the Thiobarbituric Acid Reactive Substances (TBARS) colorimetric assay kit (Cayman Chemicals, # 10009055). The assay was carried out in accordance with the manufacturer's protocol. A BioTek® Synergy2 plate reader was used to measure absorbance of each well at 532 nm.

3.2.6 RT-qPCR – mRNA expression

Following collection of approximately 1 mm^2 of gill tissue removed using stainless steel forceps and dissecting scissors, the gill tissue was placed in RNAlater™ (Invitrogen #AM7021) and stored at -80°C until processing. RNA isolation was achieved using TRIzol™ reagent (Invitrogen #A33251) and RNase-Free DNase kit (Qiagen #74004) according to the manufacturer's protocol. RNA was quantified and assessed for purity using a NanoDrop 2000 spectrophotometer. Following quantification, cDNA sub-stocks were prepared via reverse transcription PCR to achieve a final concentration of 10 µg/µL. qPCR was performed using an Applied Biosystems 7200 using SYBR™ Green PCR Master Mix (Applied Biosystems #4309155) with the following parameters: 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec to generate a dissociation curve. Primer optimization was completed using the same instrumentation and procedure. Primer sequences and efficiencies are listed in Table 1 All samples were screened in duplicate and fold-change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittigen, 2001). 18s was utilized as a reference gene for mRNA expression analysis.

3.2.7 Data analysis

Prior to data analysis, data were tested for normality and equal variance using Shapiro-Wilk's and Brown-Forsythe's tests, respectively, in SigmaPlot version 14.0. Lipid peroxidation results from caged oysters at Henderson Point Reef (Site 5) were analyzed using an unpaired t-test to compare mean MDA concentrations of oysters from time zero and 13-days post deployment. Kittiwake Reef (Site 6) lipid peroxidation results were analyzed using a one-way ANOVA to compare MDA concentrations from oysters collected at time zero, 13-days, and 31-days post deployment.

All gene expression data was normalized to time 0 oysters and was analyzed using a one-way ANOVA to compare sites and timepoints within deployed and native oysters separately. Significant ANOVAs were followed by Tukey's post hoc tests to identify significant differences between groups.

3.3 Results

3.3.1 Water quality measurements

Sites 1-4 all showed extremely low salinities during the study from 23 April 2019 to 25 May 2019 ranging from as low as 0.18 to as high as 0.67 parts per thousand (ppt). The two field sites

Table 3. Oceanographic data from all sites on each sampling day

		Day 0 4/23/2019		13 days post deployment 5/6/2019		31 days post deployment 5/24/2019		9/27/2019	
Site/depth	**Parameter**	**Surface**	**Bottom**	**Surface**	**Bottom**	**Surface**	**Bottom**	**Surface**	**Bottom**
Site 1	Salinity	0.67 ppt		0.4 ppt	0.39 ppt	0.18 ppt	0.18 ppt		
	temperature	21.7 °C	21.5 °C	24.4 °C	24.3 °C	27.2 °C	27.2 °C		
No reef site	Dissolved Oxygen	9.84 mg/L	9.06 mg/L	7.66 mg/L	7.2 mg/L	7.14 mg/L	7.04 mg/L		
4.4 ft	pH	7.4		7.49	7.33	7.28	7.12		
Site 2	Salinity	0.54 ppt		0.64 ppt	0.64 ppt	0.14 ppt	0.19 ppt	15.3 ppt	15.3 ppt
	temperature	22 °C	21.5 °C	24.7 °C	24.6 °C	27.4 °C	27.4 °C	28.3 °C	28.4 °C
TNC Bay St. Louis Reef	Dissolved Oxygen	10.71 mg/L	10.03 mg/L	7.5 mg/L	7.41 mg/L	7.36 mg/L	7.23 mg/L	8.55 mg/L	7.9 mg/L
4.9 ft	pH	8.5		7.61	7.6	7.07	6.94	7.7	7.8
Site 3	Salinity	0.4 ppt		0.33 ppt	0.35 ppt	0.24 ppt	0.19 ppt	16.8 ppt	16.88 ppt
	temperature	21.2 °C	21 °C	24.7 °C	24.1 °C	29.4 °C	28.6 °C	28.9 °C	28.6 °C
St. Stanislaus Reef	Dissolved Oxygen	9.71 mg/L	9.6 mg/L	7.92 mg/L	7.1 mg/L	9.29 mg/L	8.15 mg/L	9.1 mg/L	7.16 mg/L
6.2 ft	pH	8.5	7.98	7.72	7.58	8.18	7.91	7.92	7.86
Site 4	Salinity	0.32 ppt		0.25 ppt	0.33 ppt	0.18 ppt	0.19 ppt	16.39 ppt	16.34 ppt
	temperature	21.2 °C	21.1 °C	24.8 °C	24.5 °C	29.7 °C	28.9 °C	30.6 °C	28.6 °C
Waveland Reef	Dissolved Oxygen	9.54 mg/L	9.42 mg/L	7.55 mg/L	7.08 mg/L	9.21 mg/L	7.76 mg/L	10.2 mg/L	6.88 mg/L
8.4 ft	pH	7.4	7.68	7.56	7.52	8.14	7.51	8.16	7.93
Site 5	Salinity	3.14 ppt		1.24 ppt	1.3 ppt	0.18 ppt	0.18 ppt	17.2 ppt	17.3 ppt
	temperature	21.1 °C	21.6 °C	25 °C	24.9 °C	28.5 °C	28.1 °C	29.7 °C	28.7 °C
Henderson Point Reef	Dissolved Oxygen	12.5 mg/L	9.7 mg/L	8.17 mg/L	7.96 mg/L	7.38 mg/L	7.57 mg/L	12.25 mg/L	8.3 mg/L
10 ft	pH	8.8	8.8	7.89	8	7.6	7.59	8.21	8.07
Site 6	Salinity	4.21 ppt		2.31 ppt	2.45 ppt	0.48 ppt	0.48 ppt	18.9 ppt	21.06 ppt
	temperature	22.1 °C	21.7 °C	26 °C	25.8 °C	28.3 °C	28.2 °C	29.4 °C	28.5 °C
Kittiwake Reef	Dissolved Oxygen	12.75 mg/L	11.6 mg/L	8.11 mg/L	7.9 mg/L	8.29 mg/L	7.76 mg/L	11.23 mg/L	4.19 mg/L
7.4 ft	pH	8.88	8.74	8.45	8.42	7.72	7.73	8.11	7.74

furthest in position from the spillway, five and six, both showed the highest salinity measurements at the beginning of the study of 3.14 and 4.12 ppt, respectively. Despite starting out higher, sites five and six still showed a steady decrease in salinity over time reaching concentrations as low as 0.18 and 0.48 ppt, respectively by 31 days post-deployment. Measurements obtained on 27 September 2019, 63 days following the second closing of the spillway, showed complete recovery to normal salinity concentrations (15-18 ppt) at all sites (Table 3). Processing of *in situ* water quality data revealed that salinity measurements obtained via *in situ* sensors were in concordance with YSI™ multimeter measurements. Deployed sensors measuring salinity from site 2 (TNC Bay St. Louis) showed extremely low salinity conditions, ≤

2 ppt, sustained for approximately three months with a large peak in salinity occurring in early July when Hurricane Barry hit the Gulf of Mexico. Following the second closing of the Bonnet Carré Spillway on 27 July 2019, our data showed a gradual increase back to normal salinity concentration taking about 60 days (Fig. 12).

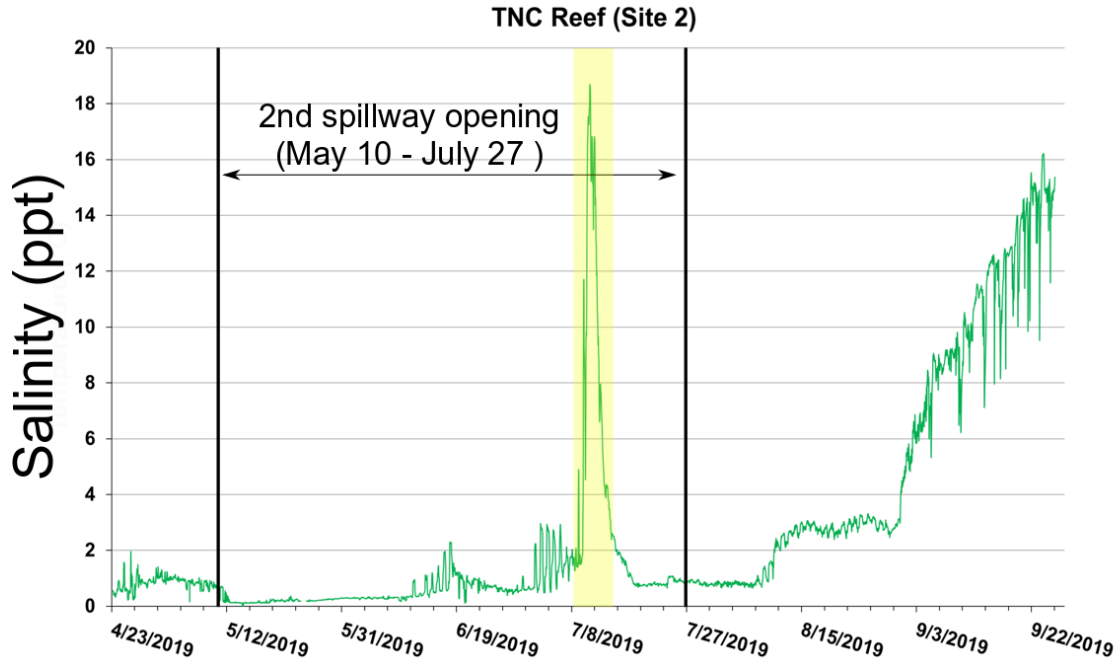


Figure 12. *In situ* salinity recorded using a HOBOware conductivity sensor at site 2. Data shows recovery to normal salinity concentrations following the second closing of the Bonnet Carré spillway. Highlighted portion denotes the occurrence of Hurricane Barry.

3.3.2 Caged oyster mortality

Caged oysters deployed at sites one through four all exhibited 100% mortality following 13 days in the field. Sites five and six had the highest salinities and the highest deployed oyster survival at 37.5% and 72.5%, respectively. Due to higher mortality at site five relative to site six, all caged oysters at site five were sampled 13 days post-deployment. Of the oysters that remained at site six 14.3% of them had died upon T-2 sampling on day 31 of deployment in the field (Table 4).

Table 4. Mortality of caged oysters recorded at each site throughout the duration of the field study.

	13 days post deployment (5/6/2019)		
	# dead	# alive	mortality
Site 1	20	0	100.0%
No reef site	20	0	
Site 2	20	0	100.0%
TNC Bay St. Louis Reef	20	0	
Site 3	20	0	100.0%
St. Stanislaus Reef	20	0	
Site 4	20	0	100.0%
Waveland Reef	20	0	
Site 5	12	8	62.5%
Henderson Point Reef	13	7	
Site 6	5	15	27.5%
Kittiwake Reef	6	14	

	31 days post deployment (6/25/2019)		
	# dead	# alive	site % mortality
Site 6	1	7	14.3%
Kittiwake Reef	1	5	

3.3.3 Native oyster collection by dredge

Dredge sampling on 26 May 2019 revealed that sites two, four, and six suffered the highest native oyster mortalities. At site two, 100% of adult oyster collected were dead while and 77.8% of collected spat was dead. Of the adult oysters collected at site four, 98% were dead while 86.4% of spat collected were dead. At site six there was 100% mortality of both adults and spat. Surprisingly, at site three, despite 100% mortality of caged oysters, dredge sampling revealed 77% of adult oysters sampled were dead and just 32.9% of collected spat were dead. Site five was found to be the most successful reef with just 9.6% dead adults and 7.5% dead spat (Fig. 13). Follow-up dredge sampling on 27 September 2019 indicated recovery of salinities back to ≥ 15 ppt at all six sites (Table 3) but still 100% native oyster mortality. Calculated population densities are shown in Appendix Fig. 17.

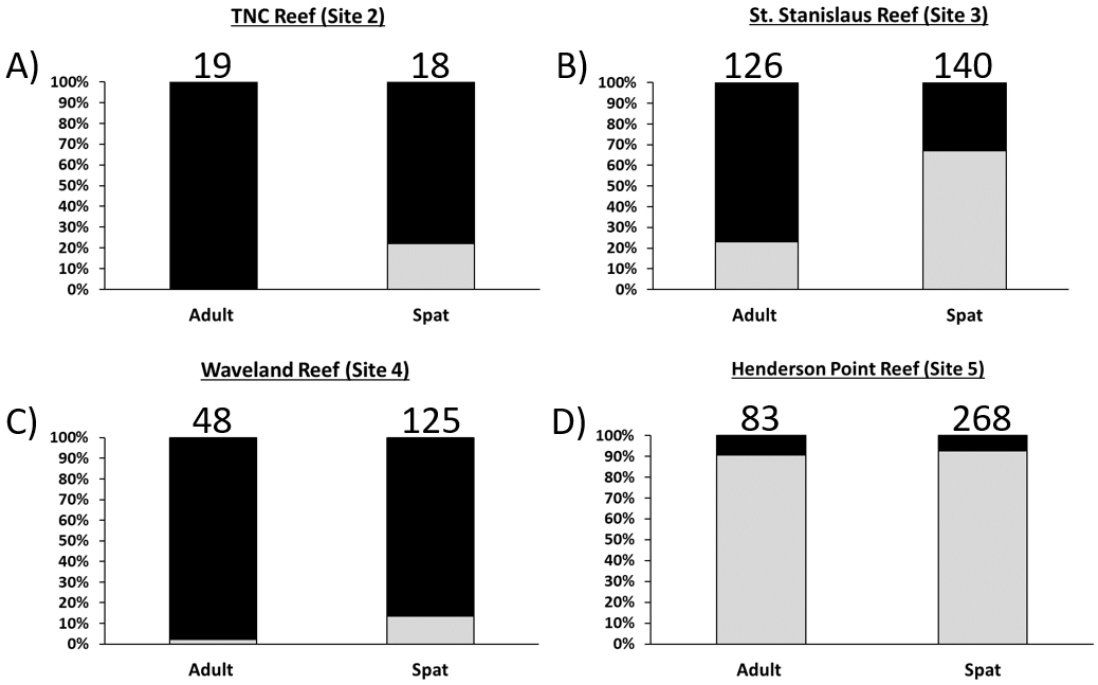


Figure 13. Native oyster mortality at each field site on 5/24/2019. Data obtained by conducting three 308 m (1000 ft) by 0.61 m (2 ft) dredge pull transects at all sites except site five where six dredge pulls were conducted due to larger reef size. Numbers above each bar represent total number of spat or adult oysters collected. Data is not shown for Kittiwake Reef (Site 6) because only one dead oyster was collected there.

3.3.4 Lipid peroxidation

Analysis of lipid peroxidation in oyster gill tissue from site five (Henderson Point Reef), the site with higher deployed oyster mortality relative to site six, revealed a significant increase in lipid peroxidation following 13 days in the field compared to time zero (T-test, $p = 0.009$). In contrast to site five, oysters from Site 6 (Kittiwake Reef), the site with the lower caged oyster mortality, did not show a statistically significant increase in lipid peroxidation following 13 or 31 days in the field (Fig. 14).

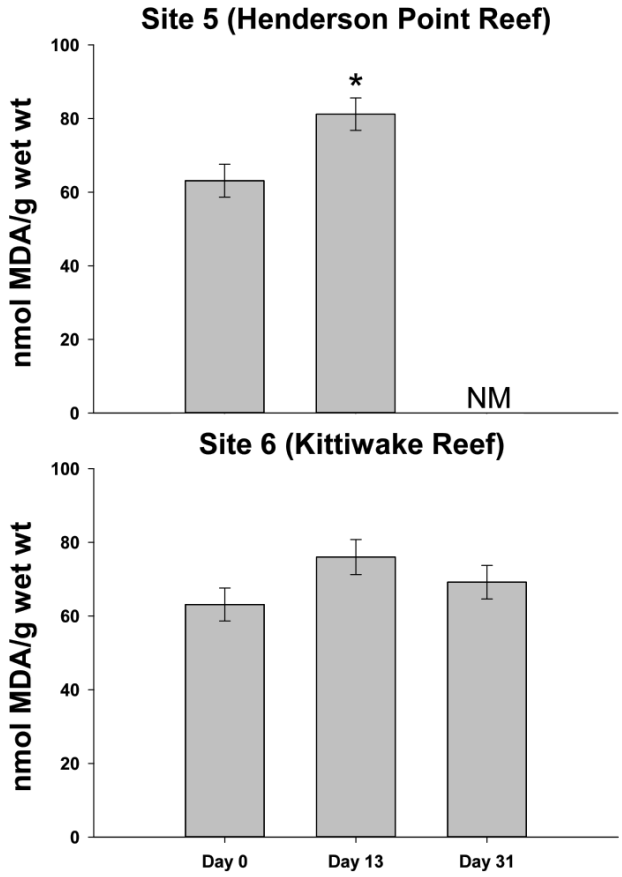


Figure 14. Lipid peroxidation measured from caged oyster gill tissue from sites five and six. Bars represent group means ± SEM. Asterisk denotes significant difference from Day 0 determined by a t-test to compare means (n = 7-15 oysters per bar, NM = not measured).

3.3.5 RT-qPCR – mRNA expression

Analysis of *HIF-1α* mRNA expression of caged oysters deployed at sites five and six for 13 and 31 days revealed significant differences between sites and time points. Oysters sampled from site five, the site with higher caged oyster mortality compared to site six, exhibited a significant down regulation of *HIF-1α* compared to time zero oysters (Tukey's post-hoc test, $F = 16.640$, $p = 0.007$; Fig 15a) and site six oysters following 13 days in the field ($p < 0.005$). Following 31 days of deployment in the field, site six oysters had a significant downregulation of *HIF-1α* compared to time zero oysters and oysters deployed for 13 days ($p < 0.001$). Native oysters collected from sites three and five showed no significant changes between time points or sites (Fig 15a).

Analysis of *Tβ-4* mRNA expression in oysters deployed in the field also exhibited significant differences between time points. After 13 days of deployment, oysters sampled from both sites five and six exhibited no significant differences in *Tβ-4* mRNA expression compared to time zero (Tukey's post hoc test, $p = 0.330$, Fig 15b). Following 31 days of deployment, there was significant down regulation of *Tβ-4* in site six caged oysters compared to time zero controls ($p = 0.025$), but expression was not significantly different than in oysters from either site after only 13 days ($p = 0.067$). Similar to *HIF-1α* mRNA expression, *Tβ-4* expression in native oysters collected from sites three and five exhibited no significant differences when compared between sites or time points (Fig 15b).

One-way ANOVA revealed no significant difference in oyster *NKA-α* expression from sites five and six following 13 days of deployment (Tukey's post hoc test, $p = 0.141$, $p = 0.189$ site five and six, respectively) (Fig. 15c). However, caged oysters at site 6 sampled after 31 days of deployment showed a statistically significant downregulation of *NKA-α* compared to time zero

oysters ($p < 0.001$) but not significantly different than day 13 oysters from site 6 ($p = 0.256$). Native oysters exhibited no significant differences between sites or time points however constitutive *NKA-α* expression was lower in native compared to deployed oysters (Fig 15c).

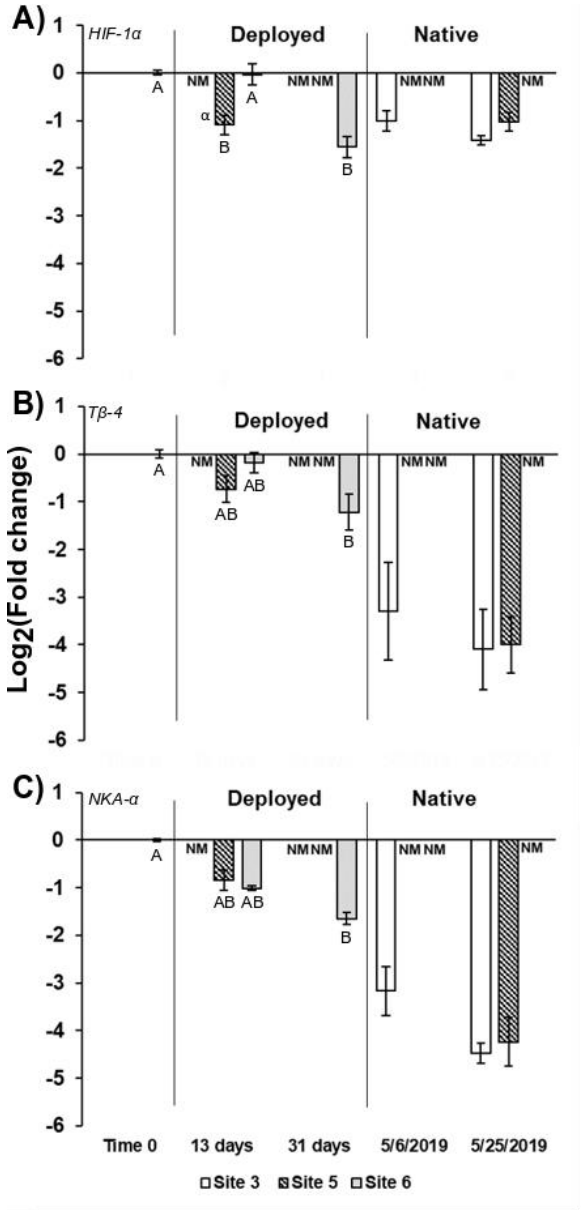


Figure 15. mRNA expression of *HIF-1α* (A), *Tβ-4* (B), and *NKA-α* (C) in caged and native oysters. Bars represent standard error of the mean. Letters represent significant differences between groups determined by one-way ANOVA followed by a Tukey's post hoc test. (n = 6-8 oysters per bar, NM = not measured)

3.4 Discussion

Oysters can tolerate a wide range of salinities ranging from roughly 5 to 40 ppt with an optimal range of 14 to 28 ppt (Galtsoff, 1964). During the present study, caged oysters were exposed for up to one month to an extreme freshwater intrusion event, which induced environmental salinities well below their known optimal range, dropping as low as 0.18 to 4.21 ppt. Recorded mortalities of caged oysters revealed that the lowest mortality (Table 4) occurred at the two sites furthest away from the Bonnet Carré Spillway, Henderson Point Reef and Kittiwake Reef, where salinities at the beginning of the study were highest (Table 4). Furthermore, Kittiwake Reef, with the highest salinity, showed a 35% lower caged oyster mortality than Henderson Point Reef following 13 days of deployment in the field. Following 31 days in the field, despite the fact that salinity continued to drop to < 1 ppt, caged oyster mortality at Kittiwake Reef was further reduced from 27.5% to 12.3%. Reduced mortality at Kittiwake Reef suggests that oysters were able tolerate extremely low salinities due to the steady decline in salinity to < 1 ppt over time at this site. On the other hand, if salinity rapidly dropped to <1 ppt oysters were not able to acclimate, shown by the 100% mortality at sites 1-4 where salinities were < 1 ppt at the on 23 April 2019 at the beginning of the study. These results are consistent with one study in which oysters were placed in exposure tanks and salinity was gradually reduced to < 1 ppt over a period of 48 hrs. Oysters in low salinity treatments showed no significant difference in mortality compared to control oysters following three weeks of exposure to freshwater (La Peyre et al., 2003).

In order to assess environmentally-induced stress in caged oysters, lipid peroxidation was measured in oyster gill tissue. Similar oxidative stress biomarkers were measured in oysters to determine the effects of environmental contaminants such as heavy metals and PAHs (Ringwood

et al., 1999). In the present study, caged oysters sampled from site five (Henderson Point Reef) exhibited significantly higher lipid peroxidation following 13 days in the field compared to time zero. Caged oysters at Kittiwake Reef (site 6), which had 35% lower percent mortality than Henderson Point Reef (site 5), showed no significant increase in lipid peroxidation. The increase in oxidative damage shows evidence that high volumes of freshwater influx that caused a rapid decrease in salinity can potentially impact oysters by inhibiting the ability of oysters to deal with increased oxidative stress. This result is similar to results observed in two studies, one involving the ark shell (*Scapharca broughtonii*) and one involving three species of clam (*Venerupis philippinarum*, *Venerupis corrugate*, and *Venerupis decussata*), where exposure to hyposaline conditions ranging from 0-7 ppt increased oxidative damage in the form of lipid peroxidation (An & Choi, 2010; Carregosa et al., 2014). In the present study, the increase in lipid peroxidation could be due to reduced energy allocation to the production of antioxidant enzymes which normally defend against oxidative damage.

To further assess the effects of the extended Bonnet Carré freshwater release on caged and native oysters, mRNA expression was utilized as an endpoint to measure oxygen deprivation stress (*HIF-1α*), immunosuppression (*Tβ-4*), and salinity stress (*NKA-α*). Oysters can respond to rapidly changing environmental conditions through the closure of their valves, while simultaneously depressing their energy usage. Valve closure results in reduced oxygen intake from the surrounding environment, in turn, activating pathways involved in the response to low oxygen. The response to low oxygen following valve closure is demonstrated in a study where oysters subjected to open air exposure induced *HIF-1α* mRNA expression (Kawabe & Yokoyama, 2012). In the present study, oysters sampled from site five showed significant down regulation of *HIF-1α* compared to time zero and site six oysters. High mortality at site five along

with a significant downregulation of *HIF-1α* could indicate metabolic depression to a point at which oysters were not able to activate important adaptive pathways. Analysis of *HIF-1α* expression in native oysters indicated a similar response to the caged oysters, suggesting that native oysters at sites three and five were experiencing a similar metabolic depression as caged oysters.

Due to the very microbe rich environment that oysters inhabit, it is vital that that oysters have strong immune function to fight invading pathogens. Determining how extended freshwater exposure impacts oyster immune function was a central aim of this study. Tβ-4 is an antimicrobial peptide that is important to the oyster immune response. The antimicrobial activity of Tβ-4 has been demonstrated by its ability to inhibit the growth of three different strains of bacteria (Nam et al., 2015). *Tβ-4* also plays an important role in the mobilization of phagocytic hemocytes, and is indicted as a biomarker for immune function (Li et al., 2016; Jenny et al., 2002). Upon analysis of *Tβ-4* mRNA expression, caged oysters collected following 31 days of deployment at site 6 exhibited significant down regulation of *Tβ-4* compared to time zero. Similarly, native oysters showed, on average, a 3-4-fold down-regulation of *Tβ-4.* Down regulation of this important immune related pathway suggests that long term exposure to freshwater can inhibit oyster immune defense mechanisms leading to possible pathogen infection and increased mortality.

In addition to mechanisms that allow oysters to adapt to hypoxic and microbe rich environments, osmoregulation is extremely important to the oyster's response to changes in salinity. One of the main mechanisms for responding to changes in salinity is by transporting solutes in and out of the cell to eliminate the solute gradient (Yancey et al., 1982). One transporter that oysters use to facilitate this is the Na^+/K^+ ATPase. Sydney rock oysters

(*Saccostrea glomerata*) exposed to low salinity stress show significant upregulation of *NKA-α* compared to control oysters (Ertl et al., 2019). Similarly, a study done on a species of crab (*Pachygrapsus marmoratus)* which found significant upregulation of this transporter subunit following exposure to low salinity. In contrast to previous results in different species, the results from the present study found a significant down-regulation in deployed and native oysters. This result further supports the hypothesis that oysters in the Mississippi Sound were experiencing energetic depression thus inhibiting oysters from activating important adaptive pathways necessary for acclimation and survival. Additionally, in both mentioned studies from literature, salinity concentrations were not < 1 ppt at any point as they were in the present study. It is also possible that different results were achieved due to the longer duration of freshwater exposure in the present study.

To determine native oyster reef population density and mortality, dredge sampling was completed on 25 May 2019. Dredge pulls revealed that Henderson Point Reef (Site 5) and St. Stanislaus Reef (Site 3) were among the more successful reefs with Henderson Point Reef showing a large proportion of living adults and spat, despite the long-term exposure to freshwater. Gene expression analysis showed evidence of energetic depression, indicating that oysters at both sites, although alive, were not adequately acclimating to the extremely low salinity conditions in the Sound. Upon return to the field sites on 27 September 2019, when salinity had recovered to > 15 ppt at all field sites (Table 4), our hypothesis of inadequate adaptation to low salinity was confirmed when dredge sampling revealed near 100% oyster reef mortality at all field sites where living oysters had been previously collected.

With large volumes of freshwater being flushed into the Mississippi Sound each year it is vital that we learn how these freshwater events impact the native oyster population and other

commercially relevant species in the Mississippi Sound. *In situ* sensors deployed in the Bay of St. Louis during the entire second spillway opening, captured a large portion of the freshwater event. Salinity remained extremely low, less than 2 ppt, for approximately 3 months, with recovery to ~ 15 ppt taking approximately two months following the second closing of the spillway (Fig. 12).

The results of this study have demonstrated some of the negative effects of the chronic freshwater event caused by the 2019 openings of the Bonnet Carré Spillway. Extended freshwater exposure caused an increase in lipid peroxidation, in unison with down-regulation of three vital adaptive pathways. The combination of these may have contributed to the mortality of the caged and native oysters. Lastly, the finding of near 100% native oyster mortality on 27 September 2019 shows that extended freshwater exposure caused a mass mortality of oyster reefs in the Mississippi Sound. Continued monitoring of Mississippi Sound oyster reefs is necessary to observe recovery of the oyster population, if any.

CHAPTER 4. CONCLUSION

Hypoxia and freshwater inflow are two very prevalent anthropogenic stressors that are occurring in the Mississippi Sound on larger scales and more frequently than in the past (Rabalais et al., 2006; Breitburg et al., 2018). Studying the occurrence of these events and their impacts on marine organisms is crucial to the management of these important ecosystems. As humans, we take on the role of ecosystem managers. It is our responsibility to protect and maintain the many important ecosystems around the world so that they will continue to provide critical ecosystem services. This thesis investigation focused on studying the adverse outcomes in oysters that result from hypoxia and freshwater intrusion.

Following eight days of hypoxic exposure in a laboratory setting, potential negative effects on oyster immune function were demonstrated by decreased mRNA expression of the immune related gene thymosin-β4 compared to control. In conjunction with the down-regulation of Tβ-4 expression, oysters also exhibited a reduction in phagocytic immune cells, hemocytes, that are vital to host defense. The immunosuppression induced by hypoxia suggests that oysters will be more susceptible to disease during and following a hypoxic event. In conjunction with this lab-based study, we wanted to see if we would observe the same effect in nature during a hypoxic event. As expected in the caged oysters, hypoxia inducible factor-1α mRNA expression was upregulated significantly in response to the hypoxic event. In contrast to the laboratory study, thymosin-β4 mRNA expression was significantly upregulated. This result was possibly caused by the naturally high microbial abundance of the field as compared to laboratory

conditions. Thus, field deployed organisms may have elicited an immune response which may explain the difference in laboratory and field responses to hypoxia. One of the more complicated issues involved in exposure research is the difficulty of mimicking field conditions in the laboratory. Oysters are constantly being exposed to several different stressors at once and determining what these stressors are and replicating these conditions in a laboratory setting is extremely difficult.

The results of my laboratory study reinforce the importance of limiting the occurrence of hypoxic events in the Mississippi Sound. Low oxygen environments can potentially cause immunosuppression in oysters and make them more susceptible to diseases such as dermo. Further studies need to be done that involve exposing oysters to these pathogens to determine how these environmental stressors affect the susceptibility to and the progression of disease amongst oyster populations.

In the Mississippi Sound field study, we aimed investigated the impacts of a chronic freshwater event on caged and native oysters in the Mississippi Sound. Dredge sampling results from sites with previously productive oyster reefs showed that the oyster population in the western Mississippi Sound and the Bay of St. Louis were completely decimated following the unprecedented freshwater release from the Bonnet Carré Spillway. Analysis of mRNA expression indicated that this mass mortality of oysters may have been due to energetic depression that inhibited induction of important adaptive pathways including those involved in host defense, osmoregulation, and low oxygen stress response. Oysters were not able to sufficiently acclimate to new conditions during the long-term freshwater event, causing mass oyster mortalities in both juvenile and adults at both historic reefs and reefs undergoing recent restoration efforts. Inadequate acclimation was also indicated in caged oysters deployed at

Henderson Point Reef through significantly increased gill lipid peroxidation suggesting oxidative damage plays a role in freshwater-mediated oyster mortalities.

In the past, the oyster reefs in the western Mississippi Sound were among the most productive reefs in the Gulf of Mexico. These reefs are extremely important to the estuarine ecosystem as well as the economy of the Mississippi Gulf Coast. Although it appears that these reefs have been severely impacted by very stressful environmental conditions, it is important that their populations recover at least to a fraction of what they once were in order to provide their ecosystem services. While restoration efforts focused on areas in the eastern Mississippi Sound may be more critical in the short term, continued monitoring of western Mississippi Sound reefs is needed to assess the extent to which these reefs are able to recover naturally. Despite the 100% mortality of adult and juvenile oysters at all field sites in this study, we also determined that the hard substrate of historic oyster reefs remains at some of these sites. This means that oyster larvae, if they encounter these reefs, will be able to settle upon the remaining dead oyster shells that are located there, given that water quality is suitable for their survival. Additionally, if restoration efforts are successful in other locations in the Sound, they could potentially help increase larval recruitment by providing larval spillover given that restored sites facilitate reef connectivity in the Mississippi Sound.

To further facilitate successful restoration efforts in the Mississippi Sound, it is necessary that we realize the importance of using scientific research to fuel our decision making when it comes to questions such as when, where, and how to best restore oyster reefs. Because hypoxia and extended freshwater inflow are proven to negatively impact oysters, we must conduct long-term water quality monitoring to identify areas that are less impacted by these stressors and focus restoration efforts there. For example, this thesis investigation has clearly demonstrated the

widespread decimation of oyster reefs caused by the extended freshwater release from the Bonnet Carré Spillway. Both Henderson Point Reef and Kittiwake Reef, the two sites furthest from the spillway, seemed to be differentially affected by the freshwater inflow event, showing higher salinity at the beginning of the study and higher caged oyster survival compared to the rest of the field locations. Ultimately, if extended freshwater events are going to be common occurrences in the Mississippi Sound, focusing restoration efforts at sites that are farthest away from the Bonnet Carré spillway will likely be most successful. If the eastern Mississippi Sound is not as heavily impacted by long freshwater releases from the Spillway, focusing restoration efforts there will likely increase the chances of restoration success. Locating areas in the Mississippi Sound that are less affected by excessive freshwater intrusion will be more effective for focused restoration efforts (e.g. cultch and oyster seed/spat deployments) and will help ensure longer term success of needed and ongoing restoration efforts.

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APPENDIX

Table 5: Results of pair-wise post-hoc tests for treatment x time interactions from 2-way ANOVAs for the Hypoxia-recovery laboratory experiment (a) *Tß-4* mRNA expression after 2 days of hypoxia exposure, (b) *HIF1- α* mRNA expression after 4 days of hypoxia exposure, (c) *HIF1- α* and (d) *Tß-4* mRNA expression after 8 days of hypoxia exposure.

A)

		Control day 2	Control day 6	Control day 8	Treated day 2	Treated day 3	Treated day 6	Treated day 8
2 day exposure (Tβ-4)	Control day 2							
	Control day 6	0.016						
	Control day 8							
	Treated day 2							
	Treated day 3							
	Treated day 6		<0.001					
	Treated day 8			0.005				

B)

		Control day 4	Control day 8	Control day 10	Treated day 4	Treated day 5	Treated day 8	Treated day 10
4 day exposure (HIF1-α)	Control day 4							
	Control day 8							
	Control day 10	<0.001	0.005					
	Treated day 4							
	Treated day 5							
	Treated day 8							
	Treated day 10							

C)

		Control day 6	Control day 8	Control day 12	Control day 14	Treated day 6	Treated day 8	Treated day 9	Treated day 12	Treated day 14
8 day exposure (HIF1-α)	Control day 6									
	Control day 8									
	Control day 12									
	Control day 14	<0.001	<0.001	<0.001						
	Treated day 6									
	Treated day 8									
	Treated day 9					0.039				
	Treated day 12									
	Treated day 14				<0.001	0.013				

D)

		Control day 6	Control day 8	Control day 12	Control day 14	Treated day 6	Treated day 8	Treated day 9	Treated day 12	Treated day 14
8 day exposure (Tβ-4)	Control day 6									
	Control day 8									
	Control day 12									
	Control day 14									
	Treated day 6	0.023								
	Treated day 8	0.005								
	Treated day 9									
	Treated day 12									
	Treated day 14									

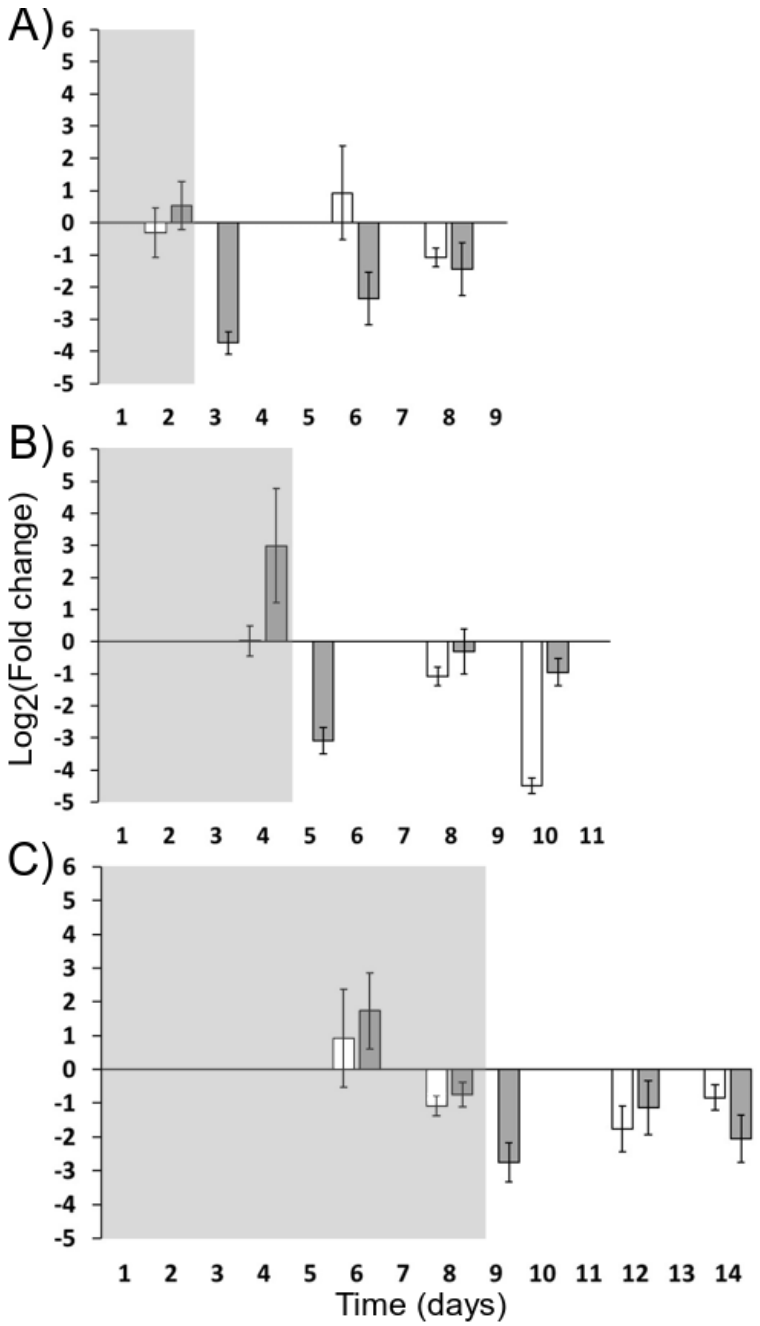


Figure 16. mRNA expression of HSP-70 during the 2-day (A), 4-day (B), and 8-day (C) exposure. Bars represent mean ± standard error.

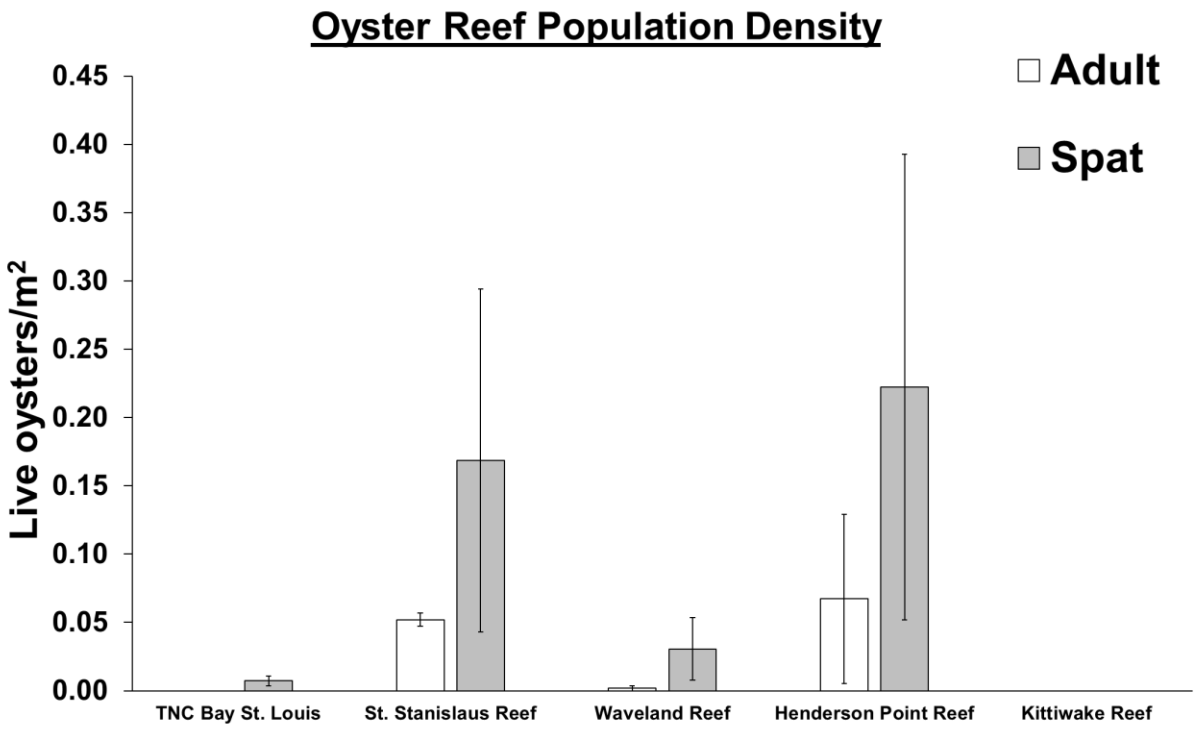
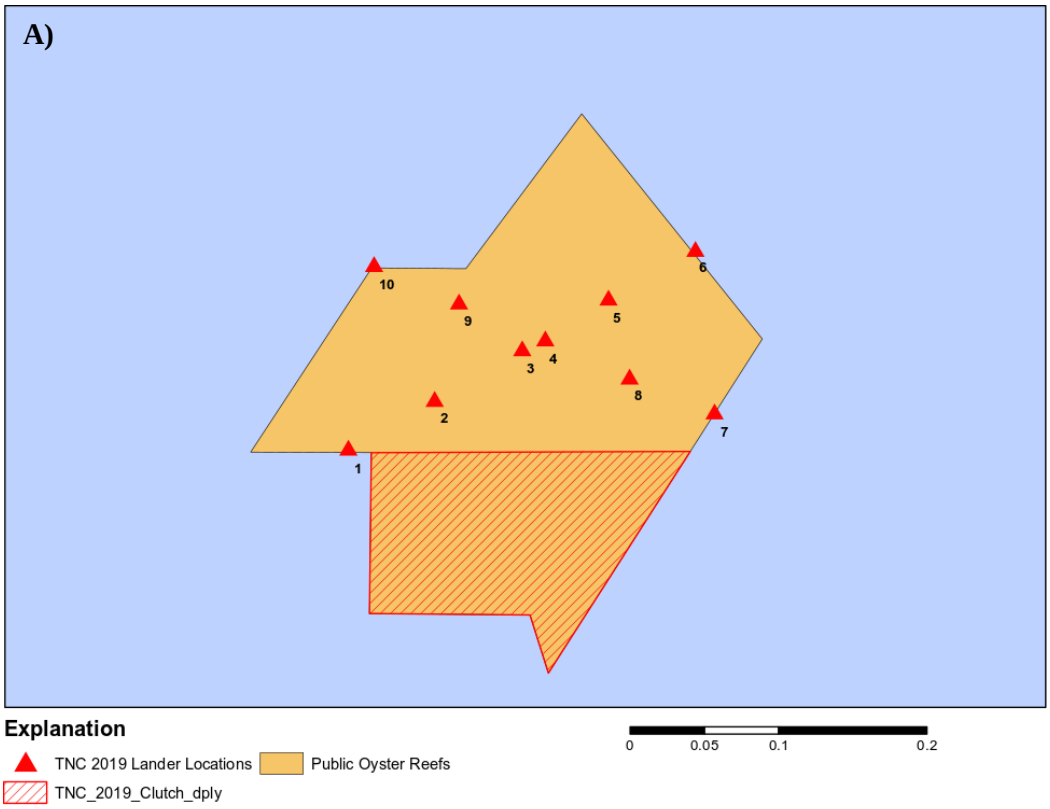


Figure 17. Dredge sampling data from all field sites in the Mississippi Sound on 25 May 2019, reported as live oysters per meter squared. Bars represent mean ± standard error.

A)



B)

Site 2 dredge transects			
Pull 1	Start	30° 20.937	89° 17.690
	End	30° 20.711	89° 17.504
Pull 2	Start	30° 20.769	89° 17.492
	End	30° 20.825	89° 17.676
pull 3	Start	30° 20.659	89° 17.569
	End	30° 20.828	89° 17.664

Figure 18. Map of TNC Bay St. Louis Reef (Site 2) (A) and starting and ending dredge coordinates (B).

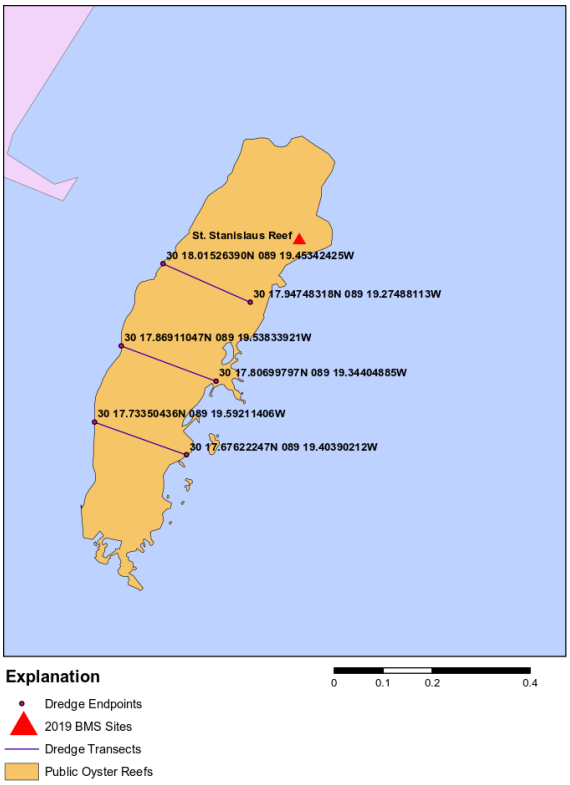


Figure 19. Map of St. Stanislaus Reef (Site 3) with starting and ending dredge coordinates.

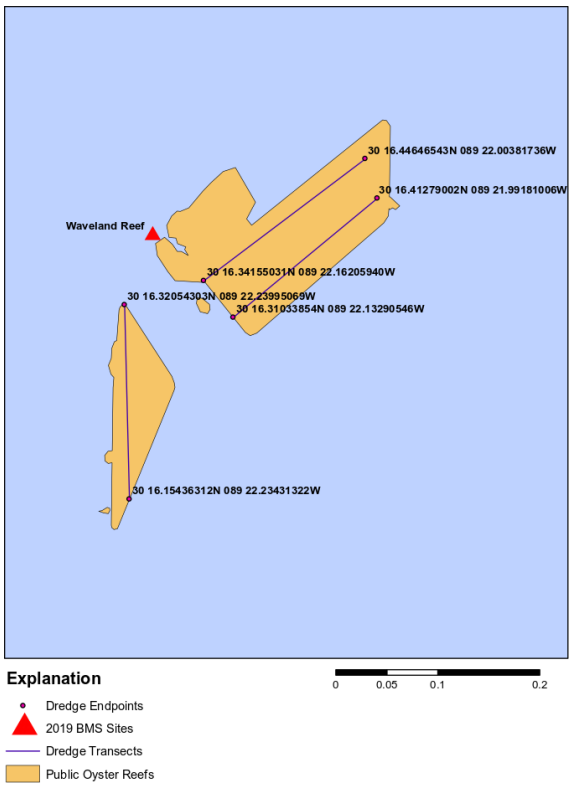


Figure 20. Map of Waveland Reef (Site 4) with starting and ending dredge coordinates.

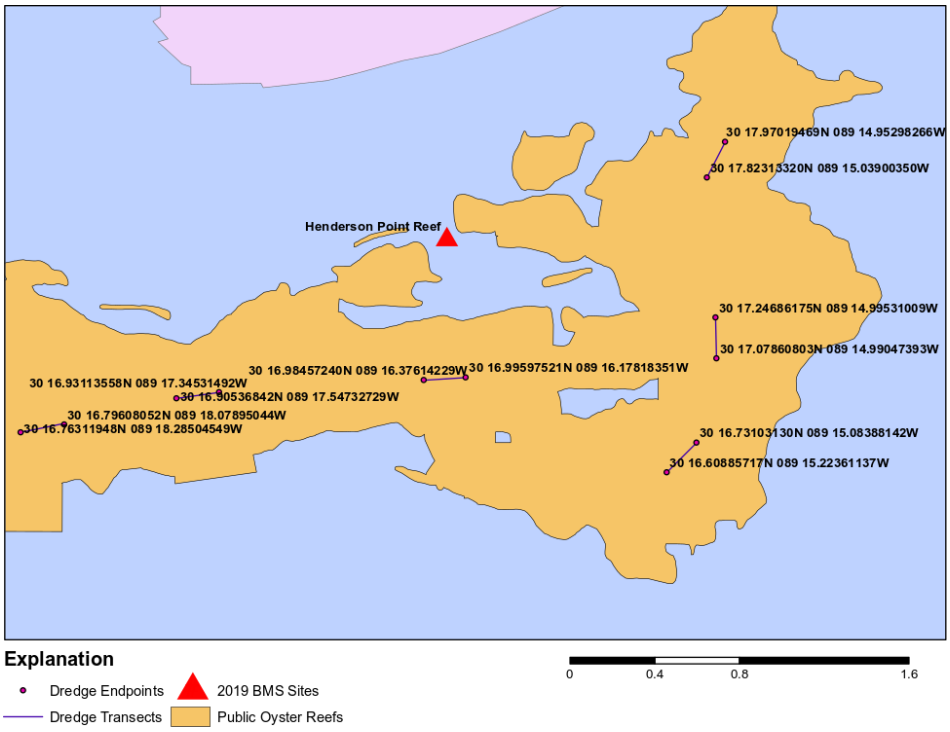


Figure 21. Map of Henderson Point Reef (Site 5) with starting and ending dredge coordinates.

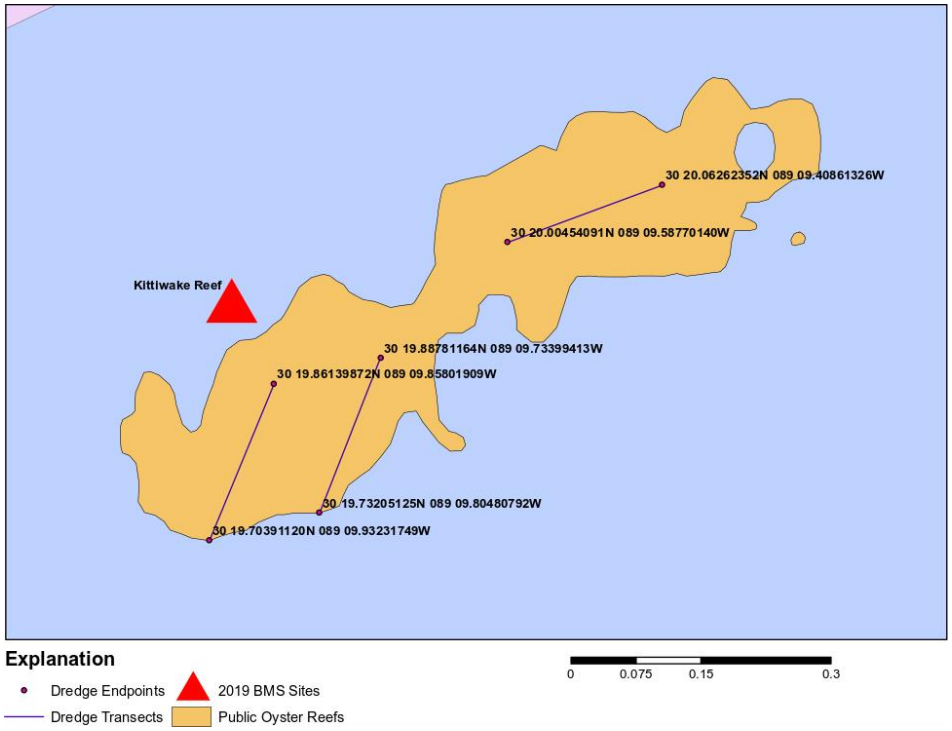


Figure 22. Map of Kittiwake Reef (Site 6) with starting and ending dredge coordinates.

VITA

EDUCATION

Bachelor of Science, The University of Mississippi -Oxford, MS **(**August 2012-May 2017)
Major: Forensic Chemistry
Minor: Spanish

RESEARCH INTERESTS

- Aquatic/environmental toxicology
- Assessing environmental health
- Studying environmental phenomena such as hypoxia and freshwater in-flow
- Studying bioaccumulation and the biochemical effects of pollutants on aquatic organisms and systems
- Investigating contaminated waste sites to assess the impact of contamination on public health and the environment
- Conducting risk assessments on contaminated waste sites
- Determining the ecosystem impacts of environmental contamination and other stressors using a combination of field and laboratory methods

WORK EXPERIECE

Graduate Research Assistant (Master's), Department of Bio-Molecular Sciences
(August 2017 – Present)
The University of Mississippi, Oxford, MS

- Design and carry out field investigations to assess the impact of hypoxia and freshwater intrusion on oyster reefs in the Mississippi Sound
- Use specialized laboratory equipment and molecular techniques such as RT-qPCR to assess the effects environmental on oysters
- Utilize *in situ* water quality sensors to study the phenomenon of freshwater intrusion in the Mississippi Sound
- Investigate oyster reef population densities and mortality
- Write in a technical manner to clearly interpret and display results

Research Intern
(May 2017 – August 2017)
United States Department of Agriculture Sedimentation Laboratory, Oxford, MS

- Used membrane inlet mass spectrometer (MI-MS) to determine the concentration of dissolved gasses in water samples
- Acid washed, cleaned, and labeled glassware to use for field and laboratory samples
- Processed sediment and soil samples for analysis of nitrogenous compounds
- Assessed the effects of wetland vegetation on the process of denitrification

Undergraduate Research Assistant
(Dec. 2015 – May 2017)
The University of Mississippi, Oxford, MS

- Studied the effects of cannabinoids tetrahydrocannabinol and cannabidiol on developing zebrafish
    - Gene expression and developmental abnormalities
- Completed RNA extraction on experimental fish tissue
- Collected, counted, and maintained zebrafish embryos and larvae during experiments
- Carried out liquid-liquid extraction of cannabinoids on water samples and prepared extracts for analysis on a gas chromatograph mass spectrometer (GC-MS) confirming experimental dosage

Publications

Carty, D.R., Thornton, C., **Gledhill, J. H.**, Willett, K. L. Developmental effects of cannabidiol and Δ9-tetrahydrocannabinol in zebrafish, Toxicological Sciences, Volume 162, Issue 1, March 2018, Pages 137–145

Manuscripts submitted for review

Barnett AF, **Gledhill J**, Willett KL, Slattery M, Griffitt RJ, Gochfeld DJ. The interactive and independent effects of hypoxia and tributyltin on physiological and molecular response of the Eastern oyster, *Crassostrea virginica*

Manuscripts in preparation

Gledhill J, Barnett AF, Gochfeld DJ, Slattery M, Bell J, Easson GL, Willett KL. Eastern oyster (*Crassostrea virginica*) mortality in the Mississippi Sound following the 2019 freshwater intrusion event