Assessing the Effects of Thermal Stress, Hypoxia, and Hydrogen Sulfide Exposure on the Survival of the Gulf Toadfish (Opsanus beta)

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ASSESSING THE EFFECTS OF THERMAL STRESS, HYPOXIA, AND HYDROGEN SULFIDE EXPOSURE ON THE SURVIVAL OF THE GULF TOADFISH (OPSANUS BETA)

By

Charles Groppe

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BETA*)

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Assessing the Effects of Thermal Stress, Hypoxia, and Hydrogen Sulfide Exposure on the Survival of the Gulf Toadfish (*Opsanus beta*)

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Northern Biscayne Bay experienced recurring mass mortality fish kill events in 2020 and 2021, largely composed of gulf toadfish (*Opsanus beta*). Low dissolved oxygen values were recorded during these periods of mortality, however, the duration and severity of hypoxia recorded in the Bay has not been confirmed as lethal to *O. beta*. Hydrogen sulfide (H\(_2\)S), a toxic gas formed naturally in aquatic sediments under hypoxic or anoxic conditions, can further inhibit respiration and decrease survival time in aerobic organisms. This study investigated how thermal and hypoxic conditions recorded in the bay affect *O. beta* survival, and whether exposure to H\(_2\)S under these same conditions altered rates of mortality. Juvenile toadfish were exposed to different combinations of thermal stress (32°C), varying levels of hypoxia (≤ 0.5 or 1 mg/L O\(_2\)), and H\(_2\)S (7.5 uM) in a non-flow-through seawater (32-35 ppt salinity) aquarium system to assess survivorship over time. Extreme hypoxia at 0.5 mg/L O\(_2\) yielded a median time till death (LT50) of 1.49 ± 0.32 hours that was significantly shorter than the survival times for hypoxia at 1.0 mg/L O\(_2\) alone (8.64 ± 1.64 hours) and hypoxia at 1 mg/L and 7.5 ppm H\(_2\)S (7.62 ± 1.29 hours). Linear regression analysis on fish survival under the 1 mg/L O\(_2\) treatment suggested larger fish by weight may survive longer under hypoxic conditions, but no significant regressions were found under the other treatments. Results from this
study confirm the lethality of hypoxia durations recorded during the 2020 fish kill, and further experimentation coupled with field collection within Northern Biscayne Bay will be useful to understand the potential effects and critical thresholds of hydrogen sulfide exposure and hypoxia in regard to *Opsanus beta* survivorship.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2 Methods</td>
<td>16</td>
</tr>
<tr>
<td>2.1 Experimental Animals</td>
<td>16</td>
</tr>
<tr>
<td>2.2 Experimental Design</td>
<td>16</td>
</tr>
<tr>
<td>2.3 Experimental Apparatus</td>
<td>18</td>
</tr>
<tr>
<td>2.4 Sulfide Infusion and Measurement</td>
<td>19</td>
</tr>
<tr>
<td>2.5 Data Analysis</td>
<td>23</td>
</tr>
<tr>
<td>3 Results</td>
<td>25</td>
</tr>
<tr>
<td>3.1 Water Quality</td>
<td>25</td>
</tr>
<tr>
<td>3.2 Mortality by Treatment</td>
<td>26</td>
</tr>
<tr>
<td>3.3 Effect of Weight on Mortality</td>
<td>30</td>
</tr>
<tr>
<td>4 Discussion</td>
<td>31</td>
</tr>
<tr>
<td>Supplemental Material</td>
<td>37</td>
</tr>
<tr>
<td>References</td>
<td>43</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Map of Northern Biscayne Bay</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Environmental parameter timelines for 2020 and 2021</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Oxygen vs. tide analysis in the Little River Canal</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>Cumulative Little River Canal flow by year</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Timeline of chlorophyll fluorescence during fish kill events</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>Hypoxia duration length vs. mean temperature at the BB14 sensor</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>Comparison of spectrophotometry calibration curves</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>Dissolved oxygen readings by experimental trial</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>Mean survival time by treatment</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>Mean survival time by experimental trial</td>
<td>28</td>
</tr>
<tr>
<td>11</td>
<td>LT50 values by experimental trial</td>
<td>29</td>
</tr>
<tr>
<td>12</td>
<td>Effect of fish weight on survival time by treatment</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Supplemental Figure 1. Temperature readings by experimental trial</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Supplemental Figure 2. Hydrogen sulfide readings by experimental trial</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Supplemental Figure 3. Removal of weight covariate from ANCOVA analysis</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Supplemental Figure 4. LT50 curves for each experimental trial</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Supplemental Figure 5. Linear regression analysis of weight by survival time</td>
<td>41</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Sample size and weight range for each experimental trial</td>
<td>18</td>
</tr>
<tr>
<td>Supplemental Table 1</td>
<td>Output of t-tests comparing treatment LT50 values</td>
<td>42</td>
</tr>
<tr>
<td>Supplemental Table 2</td>
<td>Output of t-tests comparing experimental trial LT50 values</td>
<td>42</td>
</tr>
<tr>
<td>Supplemental Table 3</td>
<td>Durations of extreme hypoxia durations at the BB14 buoy</td>
<td>42</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

Biscayne Bay is a shallow inner-shelf embayment adjacent to the Atlantic coastline of South Florida which links upland freshwater sources to oceanic saltwater flows creating a coastal lagoon (Kruczynski & Fletcher, 2012). Northern Biscayne Bay (NBB) is a segment of the bay situated north of the Rickenbacker Causeway in a heavily urbanized environment bordered by the cities of Miami along the western shoreline and Miami Beach along the eastern shoreline (Fig. 1).

![Northern Biscayne Bay](image)

Figure 1. Map of Northern Biscayne Bay using satellite imagery taken from Google Maps TerraMetrics 2022 © data.

As an estuarine system, NBB is strongly influenced by the mixing of converging freshwater and saltwater flows. Anthropogenic alterations have caused massive shifts to
historical freshwater flow regimes (Cantillo et al., 2000), changing the timing and duration of flows which led to higher average salinities and greater variability in water quality (Score & Jacoby, 2006; Langevin, 2003). Fresh water is delivered to the bay through multiple inputs including surface runoff, canal flow, and groundwater discharge (Stalker et al., 2009; Browder et al., 2005), forming spatial salinity gradients and altering nutrient loading patterns (Wang et al., 2003; Caccia & Boyer, 2005). Mainland shoreline sites in NBB with more variable salinity patterns have been found to have lower overall fish species richness, with such sites being dominated by species capable of withstanding the wide range of salinity fluctuation (Serafy et al., 1997). While the limiting nutrient in NBB is still in question (Chin, 2020), canals were found to be the largest contributor of nitrogen to the bay and provide the bulk of phosphate loading in NBB specifically (Caccia & Boyer, 2007). Shallow estuaries and lagoons are already vulnerable to eutrophication effects brought on by increased nutrient loading (Valiela et al., 1997; Whitall et al., 2007), and NBB has been shown to be the segment of Biscayne Bay at highest risk of eutrophication from nutrient pollution with the highest measurements of chlorophyll-a concentration, total phosphorus, and dissolved inorganic nitrogen (Briceño et al., 2011). NBB is also experiencing significantly higher rates of increasing chlorophyll-a and phosphate concentrations in comparison to other segments within the bay, indicating that its proximity to multiple sources of nutrient input coupled with reduced circulation and flushing are inducing greater rates of decline over time (Millette et al., 2019).

The fluctuation in nutrient availability and other water properties such as salinity and temperature drives variability in the composition of benthic vegetation (Lirman &
Cropper, 2003; Biber & Irlandi, 2006; Wingard et al., 2003) and in turn alters the health and ecological functioning of these structurally important communities (Deegan et al., 2002). NBB has experienced drastic declines recently in seagrass beds (Lirman et al., 2016) and historically in mangrove ecosystems (Peters et al., 2015), both of which are functionally vital to many species within the bay (Gilby et al., 2018; Serafy et al., 2003). Seagrass ecosystems and their associated benthic macroalgal communities serve as valuable locations of nitrogen cycling and primary production (Yarbro & Carlson, 2008; Terrados & Borrum, 2004). Shifts in these communities to greater concentrations of macrophytic algae have been linked to lower rates of photosynthesis due to higher levels of light attenuation, restricted vertical mixing of the water column, and lower levels of oxygen production and availability (Sand-Jensen, 1989; Miranda & Hodges, 2000). Additionally, blooms of macrophytic drift algae can deteriorate seagrass ecosystems and hamper their recovery and resilience, further lowering ecosystem functioning within the bay (Santos et al., 2020). Coastal zones have experienced increases in eutrophication-driven hypoxic events globally (Howarth et al. 2011, Diaz & Rosenberg, 2008), but nutrient pollution must be investigated in tandem with physical factors such as stratification and salinity to better understand the formation of hypoxia in estuarine habitats (Lowery, 1998). Temperature and freshwater discharge can exacerbate stratification effects on hypoxia along the thermocline and halocline in coastal areas (Fennel & Testa, 2019), and globally, oceans are experiencing a decline in oxygen driven by the compounding effects of rising temperatures on oxygen solubility and depth zonation (Keeling et al., 2010). If coastal hypoxia occurs for long enough durations or in
tandem with other physical stressors that exacerbate respiration, such as thermal stress, severe consequences including large scale fish die offs can occur (Breitburg, 2002).

In August 2020, a fish kill occurred in NBB resulting in an estimated mortality of at least 27,000 fish over six days (Silverstein et al., 2021). Hypoxia (\( \leq 2 \text{ mg/L dissolved oxygen} \)) was determined to be the most probable cause of mortality, and extreme hypoxic (\( \leq 1 \text{ mg/L} \)) and anoxic conditions (0 mg/L) were also detected by continuous 15-minute resolution monitoring instruments during the event. The Biscayne Bay Scientific Coordination (BBSC) group, composed of researchers and stakeholders from government, academia, and NGOs, generated a consensus statement suggesting that hypoxia as a contributing factor and that several key factors synergistically worsened these conditions. High water temperatures during the month of August may have potentially lowered the solubility of oxygen and further stratified the water column along the thermocline. Additionally, low wind movement during the fish kill may have limited surface turnover in the upper water column, a physical factor that has been shown to enhance dissolved oxygen (DO) conditions through forced re-aeration in coastal estuaries (Hull et al., 2008). Anomalously high canal flow was reported during the year of 2020, which further stratified the water column along the halocline at canal mouths and could have contributed heavily to the decline in seagrass ecosystems and their protection against extreme hypoxia in NBB. The BBSC found that an algal bloom occurred in the month of August in 2020, however, it occurred post-fish kill and was most likely a result of the die-off and subsequent nutrient release rather than a cause. Oxygen fluctuated along a diel pattern within NBB similar to patterns previously detected in nearby Florida Bay (Borum et al., 2005), indicating that remaining seagrass and algal communities were
able to offset some oxygen depletion through photosynthesis during the day. Finally, although the BBSC group suspected canal flow played a deleterious role in affecting NBB water quality, early findings suggested that hypoxic waters most likely originated within the bay itself.

In September of 2021, a fish kill event occurred once again, but at a much lesser magnitude than the previous year, with an estimated mortality of hundreds of fish across a three-day span (Irela Bagué, personal communication). Hypoxia was recorded in the bay, but to lesser extremes, as both oxygen concentrations and hypoxia episode duration lengths were not as severe as in 2020. Like in 2021, a synergistic concurrence of high temperatures, little wind action, and high canal flow occurred before and during the fish kill and may have contributed to the formation of hypoxia in the water column (Fig. 2). The Biscayne Bay Aquatic Preserve (BBAP) BB14 buoy sensor within the bay itself experienced technical difficulties during the 2021 fish kill period and was unable to provide water quality data within the basin, however, an alternative sensor from the BBAP monitoring system within the Little River canal confirmed similar physical parameters of elevated water temperature within the bay. Utilizing data collected at the LR03 sensor within the canal, it appeared yet again that hypoxic waters originated within the bay (Fig. 3). Dissolved oxygen originally showed cyclical increases with increasing salinity indicating bay waters in the canal were well oxygenated, however during the fish kill periods the synchrony between the two parameters snapped and oxygen values decreased with increasing salinity. Canal flow in 2021 was once again higher than historical averages, however, not as anomalous as in 2020 (Fig. 4). Although algae blooms were suggested to play a role in creating hypoxic conditions within the bay,
chlorophyll concentrations in both years show spikes following each fish kill event, rather than preceding them, indicating they were results and not causes of each kill (Fig. 5). Hypoxia was once again implicated to be the cause of fish mortality in the bay, however, to date, no definitive causes have been identified for either fish kill.

Figure 2. Timelines of DO, canal flow, water temperature, and wind speed for 2020 (top) and 2021 (bottom). DO and water temperature daily averages were created using data collected at the BBAP’s LR03 sensor, while daily averages for windspeed were calculated using data from NOAA’s Virginia Key Station sensor. Little River daily flows were calculated using data from SFWMD’s DBHYDRO database. Vertical solid lines outline each year’s respective fish kills, horizontal dashed lines represent the hypoxia threshold at 1 mg/L, and gray ribbons around each line represent ±1 se. Incomplete DO and temperature readings are due to limited sensor availability. The LR03 sensor did not begin taking readings until March 17, 2020, and data obtained in 2021 only had readings up to October 13.
Figure 3. DO, depth, and salinity curves before, during, and after the fish kill events in 2020 and 2021. All readings taken from recordings at the BBAP’s LR03 sensor. Oxygen originally cycles in synchrony with salinity but breaks the cycle during the fish kill to show lower oxygen values during higher salinity periods, indicating hypoxic conditions flushed into the canal from the bay.

Figure 4. Cumulative yearly canal flow in the Little River Canal measured in acre-feet. Historical data shown in the graph is an average cumulative flow value for each day of the year across all years from 1986-2019, with the gray ribbon representing ± 1 SD. Vertical black lines outline the fish kill events in 2020 (solid) and 2021 (dashed). Data taken from SFWMD’s DBHYDRO database.
While hypoxia has previously been reported as a factor in fish kills in multiple coastal ecosystems, the causes and stressors associated with each die-off can be highly variable. Along deeper coastal ecosystems, bays can experience hypoxia through upwelling and crowding of surface waters by affected fish populations, resulting in exacerbated respiration rates in oxygen-limited water and potential mass mortality events (Stauffer et al., 2012). In such cases, hypoxia not only creates a stressful environment for...
aerobic organisms, but also forces populations into altering their behavior to further decrease the potential for respiratory relief. While fish altered their behavior to surface swimming and heavily crowded upper portions of the water column in NBB, the hydrology, location, and depth of NBB essentially negate the likelihood that upwelling can be a factor. In coastal areas of Texas, the largest fish mortalities occurred in warmer months as warm temperatures and poor water circulation further stratified water columns and led to deadlier levels of hypoxia (Thronson & Quigg, 2008). While the authors noted these physical stressors are causative factors in many hypoxic fish kills, they stressed concern that further urbanization of coastal areas could influence nutrient loading patterns and further exacerbate physical conditions through eutrophication. In a tropical estuary in India, nutrient loading from freshwater inputs and lack of flushing and circulation were found to be the direct causes of hypoxia and anoxia which in turn caused mass fish mortality events (Ram et al., 2014). Nutrient inputs formed a perpetual oxygen-deficient environment within the Indian estuary, but the worst conditions of anoxia and the periods of greatest mortality occurred in between monsoon seasons when circulation was lowest. Organic matter (OM) is a byproduct of algal blooms and can form lethal levels of hypoxia in estuaries, however, OM can also be directly loaded into bays via freshwater discharge from polluted watersheds resulting in hypoxia episodes of similar severity (Paerl et al., 1998). Organic matter can also be transported from offshore locations via currents and wind, as is the case with pelagic drift algae like *Sargassum*. *Sargassum* can accumulate along nearshore habitats and induce hypoxia as well as degrade water quality, resulting in mass mortality across multiple faunal species (Rodriguez-Martinez et al., 2019). In comparison to these other ecosystems’ struggles
with hypoxia, NBB exhibits similar deficiencies in circulation and has experienced its worst hypoxia and mortality episodes in the warmer months. NBB has also experienced increases in urbanization and anthropogenic influences along its watershed and South Florida has seen recent influxes in Sargassum along its shores (Collado-Vides et al., 2020), suggesting an increase of organic matter loading to the bay. While NBB has shown higher levels of eutrophication in comparison to other segments of the bay (Millette et al., 2019), it did not experience algal blooms prior to either fish kill and does not experience continuous durations of oxygen deficiency like in the Tapi Estuary (Ram et al., 2014). Without evidence of an algal bloom driving hypoxia during these fish kills, physical stressors should be treated as potential causative factors of hypoxia acting synergistically to induce mass mortality. However, broader investigations on the parameters of the bay raises questions on the capabilities of these stressors alone being responsible for the fish kills.

Hypoxic events have been recorded at the BB14 and LR03 stations multiple times since 2019, yet only a portion of these events have occurred immediately preceding or during the fish kills. Utilizing continuous DO logging data from the 2020 fish kill, the longest duration of any hypoxic event at a 2 mg/L threshold was 18.5 hours, and the longest duration at a 1 mg/L threshold level was 10 hours. While no study has examined the lethal time threshold for toadfish at these oxygen concentrations, a previous study analyzing the effects of fluoxetine on hypoxia tolerance held the gulf toadfish (Opsanus beta) at 2.45 mg/L O₂ for 22 hours without inducing mortality, showing their capacity to handle low oxygen concentrations for long periods of time (Amador et al., 2018). There appears to be a relationship between temperature and hypoxia duration for the events
which occurred in the basin during the 2020 fish kill (Fig. 6), which might suggest that synergistic impacts of temperature and low oxygen concentrations surpassed the threshold levels of fish survival in the bay.

Recent experiments by the Grosell laboratory at the Rosenstiel School of Marine & Atmospheric Science (RSMAS) have found evidence counter to this assumption however, showing that juvenile and larval *O. beta* show innate signs of hypoxia tolerance in metabolic responses to hypoxia at a variety of temperatures (Frank et al., 2022). This finding is particularly intriguing given that *O. beta* was not only present in both fish kills, but was the predominant species killed in the 2020 event, making up an estimated 44% of
all fish mortality (Silverstein et al., 2021). Therefore, while hypoxia may have been a contributing stressor in the fish kills, it may not have been the primary cause of death, opening the investigation further for additional factors responsible for mortality.

Given the stratification of oxygen concentrations with depth, benthic substrates can have lower rates of oxygenation from a lack of mixing with surface waters. These substrates are home to a vast variety of marine bacteria, and these key bacterial and prokaryotic communities are largely responsible for the decomposition and remineralization of nutrients in coastal estuaries (Nixon, 1981). Microbial communities in the benthos vary in rates of nutrient cycling and biogeochemical capabilities, and their composition and abundance has been shown to fluctuate with elevated temperatures, organic matter loading, and oxygen concentrations (Hicks et al., 2018; Albert et al., 2021; Rousi et al., 2019). In extremely hypoxic (< 1 mg/L O2) or anoxic conditions, benthos diversity is highly reduced and dominated by specialized genera capable of tolerating low DO conditions (Levin et al., 2009), many of which utilize efficient anaerobic respiration mechanisms to survive (Coskun et al., 2019). Sulfate reducing bacteria (SRB) make up a large portion of this group, utilizing sulfate as a terminal electron acceptor for growth and produce various forms of sulfide as byproducts (Muyzer & Stams, 2008). The biodegradation of organic matter by SRBs results in production of hydrogen sulfide (H2S), a toxic gas with a complex relationship with many water quality parameters. The solubility of H2S holds inverse relationships with both temperature and salinity, and it is rapidly oxidized to sulfate in oxygenated water (Morse et al., 1987). The ionization and oxidation rates of H2S are heavily affected by pH. Both rates increase with increasing pH, thus lower percentages of total dissolved sulfide in the water column are
in the form of un-ionized H$_2$S in waters with higher alkalinity (Morse et al., 1987; Baird et al., 2017). Under healthy conditions in tropical estuaries like NBB, warm, normoxic waters with a pH of 7 - 8 will greatly limit the concentrations and toxicity of H$_2$S. However, if OM loading were to exceed the rate of consumption by bacteria, benthic waters and substrates could become hypoxic or anoxic (Levin et al., 2009). The excess OM present would then be utilized by SRBs capable of tolerating the oxygen deficient conditions, resulting in the production of H$_2$S in deoxygenated waters. Maximum H$_2$S concentrations from decomposing organic material under saline conditions are significantly higher than in freshwater environments due in part to greater availability of sulfate (Letelier-Gordo et al., 2020), and H$_2$S toxicity greatly increases in lower oxygen concentrations (Baird et al., 2017). Thus, the capacity for high H$_2$S concentrations in hypoxic waters could potentially indicate a new source of mortality during the fish kills in 2020 and 2021.

H$_2$S can induce mortality through apnea and respiratory arrest in a manner similar to hypoxia. H$_2$S inhibits cytochrome-c-oxidase and reduces its activity in the electron transport chain in many mammals including humans, thus disrupting oxidative phosphorylation in cells and limiting ATP production and cellular respiration as a whole (Guidotti, 1996). This same reduction in cytochrome-c-oxidase activity has been found in multiple fish species (Torrans & Clemens, 1982; Bagarinao & Vetter, 1989; Forgan & Forster, 2010), and further studies into H$_2$S toxicity have revealed its capability to produce detrimental reactive oxygen species which can also cause cellular damage (Truong et al., 2006). Sulfide exposure has also been shown to convert hemoglobin to sulfhemoglobin (SHb) which is incapable of carrying O$_2$ (Shen, 2015), however, while
sulfide exposure can produce SHb in fish, SHb production does not confer the same loss in O\textsubscript{2} transport in fish (Affonso \textit{et al.}, 2002; Bagarinao & Vetter, 1992). Generally, tidal marsh fishes and other fish genera accustomed to areas of higher environmental sulfide concentrations show greater tolerance to the toxic gas in comparison to more open coast and pelagic marine species (Bagarinao & Vetter, 1989). Well adapted fish can oxidize small concentrations of H\textsubscript{2}S to thiosulfate within the blood, and additional studies suggest that symbiotic relationships with sulfide-oxidizing bacteria or enhanced oxidation via mitochondrial processes increase tolerance in these species (Bagarinao & Vetter, 1989, Bagarinao, 1992). These natural capabilities are not abnormal given H\textsubscript{2}S is naturally produced in most animals as it has some uniquely beneficial uses including the inhibition of apoptosis (Sen \textit{et al.}, 2012), vasorelaxation to regulate blood pressure (Yang \textit{et al.}, 2008), and as an O\textsubscript{2} sensor in tissues of cardiovascular and respiratory systems (Olson, 2015). Advanced investigation in the medical field continues to find more cellular systems reliant on the functionality of H\textsubscript{2}S, however, these physiological processes utilize extremely small concentrations of hydrogen sulfide. As concentration grows, physiological damage increases and eventually thresholds of mortality are reached.

Sublethal concentrations of H\textsubscript{2}S caused damage to the gills and liver of pelagic Atlantic salmon smolts over both gradual long-term experiments as well as short term acute exposures, indicating the toxicity of the compound can damage multiple essential organs even in small doses (Kiemer \textit{et al.}, 1995). This physical damage can lead to indirect mortality even in the cases of organisms well adapted to sulfide environments. A recent study on H\textsubscript{2}S-tolerant fish found the molecule impaired functionality within the eyes and drastically limited vision, which could inhibit predator avoidance and ultimately
lower fitness in prey species (Allore et al., 2021). The effect of H$_2$S on respiration can also alter fish behavior as they experience lower tolerances to physical parameters such as thermal stress and hypoxia, as fish have been shown to seek cooler waters, swim along the surface where water is more oxygenated, and fully emerge from the water column to breathe atmospheric air (Skandalis et al., 2020; Bagarinao & Vetter, 1989; Rossi et al., 2019). In instances where behavioral adjustments are ineffective and tolerance thresholds are exceeded, mass mortality events may occur. H$_2$S has been ruled as the primary cause of death in a number of fish kills across a variety of marine settings including tropical fish farms and natural waters (Bagarinao & Lantin-Olaguer, 1998), shallow seas (Debol’skava et al., 2005), and coastal estuaries (Lamberth et al., 2010). Hypoxic conditions were recorded in all of these fish kills, but hypoxia could be either a facultative stressor enabling higher concentrations of H$_2$S or a result of H$_2$S reducing O$_2$ availability and redox potential within the sediments and water column (Maeda & Kawai, 1988). When coupled with hypoxia, H$_2$S acts synergistically as a catalyst for death, resulting in shorter survival times and higher rates of mortality (Bagarinao & Lantin-Olaguer, 1998; Vaquer-Sunyer & Duarte, 2010). Thus, shorter hypoxic durations that are incapable of killing fish from hypoxia alone may become much more lethal in the presence of H$_2$S, putting hypoxia-tolerant species at risk of succumbing to mortality. With this theory in mind, the present study aimed to test the factors of thermal stress, hypoxia, and H$_2$S exposure on O. beta to examine if this hypoxia-tolerant fish species shows significantly different rates of mortality in response to added H$_2$S exposure.
Chapter 2. Methods

2.1 Experimental Animals

Juvenile gulf toadfish (*Opsanus beta*) bred, fed, and maintained by the Grose laboratory at the University of Miami’s Rosenstiel School of Marine and Atmospheric Science (RSMAS) were utilized in this study. Gulf toadfish were selected for this experiment because they made up large proportions of the fishes killed in the 2020 fish kill (Miami Waterkeeper, 2020). For preliminary experiments, 20 individuals of similar size at two years of age were collected and moved to two separate tanks filled with 25 L of seawater. For all follow-up experiments ranging from the dates of April 25th to June 8th, one-year-old toadfish were selected for use due to their greater availability. Toadfish were fed every three days, with two-year-old fish receiving thawed squid and one-year-old fish receiving thawed artemia. Fish would not be fed within three days of their designated experimental trial, and all individuals were treated with malachite green immediately upon transfer to their respective aquaria to reduce risk of infection and once again every seven days. All handling and manipulation of fish were approved by the University of Miami’s Institutional Animal Care and Use Committee (IACUC) on protocol 21-193.

2.2 Experimental Design

Controls for all experimental runs were carried out during the acclimation process. The control experimental trial involved holding fish at 32 °C without any additional stress treatments for a period of 24 hours, after which the same sample of fish would be exposed to their designated treatment level. Fish were slowly acclimated to the
temperature of 32 °C by raising the temperature of their aquarium 1 °C per day, and feeding regiments were continued during the acclimation period.

For preliminary studies, oxygen was lowered gradually from 6 mg/L to 2 mg/L over 4 hours after 24 hours of 32 °C exposure. This lowering rate matched drops recorded in the natural environment by the BB14 DO sensor during the 2020 fish kill. For sulfide-infusion trials, sodium sulfide solution was gradually added upon reaching the 2 mg/L DO threshold and the 24-hour experimental window commenced. Sulfide solution was continuously pumped in over 24 hours to keep sulfide concentrations stable, and water samples were taken throughout the experiment to measure total sulfide and hydrogen sulfide concentrations. Oxygen was kept stable via controlled nitrogen and air bubbling for 24 hours and fish mortality was assessed continuously for the first eight hours. Cameras were then set up adjacent to the tank to capture images every minute for the remainder of the 24-hour window, which allowed for mortality times to be estimated overnight. After 24 hours, a water sample was taken from H₂S-infused tanks to confirm final sulfide concentrations, and any surviving toadfish from the hypoxia-only trials were remediated through gradual oxygenation and cooling of the aquarium. Surviving toadfish from hydrogen sulfide experiments were euthanized via MS-222 immersion. Preliminary trials aimed to hold a target concentration of 4.4 uM H₂S, and sulfide measurements as well as mortality rates were reevaluated after the preliminary experimental run.

Subsequent trials investigated the effects of lower oxygen concentrations and higher H₂S concentrations on *O. beta* survival utilizing the same experimental design and apparatus as preliminary studies. Experimental hypoxia and hydrogen sulfide trials were carried out at a dissolved oxygen concentration of 1 mg/L, while extreme hypoxia trials
held a target concentration of 0.5 mg/L O\textsubscript{2}. The oxygen lowering rate was extended so that O\textsubscript{2} concentration fell from 6 mg/L to 1 mg/L over the course of five hours or 6 to 0.5 mg/L over 5.5 hours, again matching observed rates of decline. Follow-up hydrogen sulfide trials were conducted at a target concentration of 8 \textmu M H\textsubscript{2}S, which was established using the same apparatus as in preliminary trials. While preliminary trials utilized sample sizes of ten fish per experimental run, sample sizes varied in follow-up experiments due to limitations in fish availability and mortality during the acclimation process (Table 1).

<table>
<thead>
<tr>
<th>Experimental Trial Date</th>
<th>Treatment</th>
<th>Sample Size</th>
<th>Fish Mortality Size Range (g)</th>
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<tr>
<td>April 25th</td>
<td>Hypoxia</td>
<td>10</td>
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<tr>
<td>May 23rd</td>
<td>Hypoxia</td>
<td>9</td>
<td>0.21 - 0.55</td>
</tr>
<tr>
<td>May 27th</td>
<td>Hypoxia</td>
<td>10</td>
<td>0.20 - 0.36</td>
</tr>
<tr>
<td>May 31st</td>
<td>Extreme Hypoxia</td>
<td>8</td>
<td>0.26 - 0.78</td>
</tr>
<tr>
<td>June 6th</td>
<td>Hydrogen Sulfide</td>
<td>7</td>
<td>0.19 - 0.79</td>
</tr>
<tr>
<td>June 8th</td>
<td>Hydrogen Sulfide</td>
<td>6</td>
<td>0.39 - 1.11</td>
</tr>
</tbody>
</table>

Table 1. Sample sizes as well as size ranges of the fish that succumbed to mortality during each respective experimental run following preliminary trials.

2.3 Experimental Apparatus

Experimental runs were conducted in 37 L glass aquaria filled with 25 L of seawater. Seawater was sourced directly from Bear Cut, a channel connecting Biscayne Bay to the Atlantic Ocean between Virginia Key and Key Biscayne (Fig. 1), then filtered and aerated through a filtration pump system at RSMAS prior to entering the aquarium. Each aquarium utilized a non-flow-through design to facilitate maintenance of sulfide concentration, oxygen saturation, and temperature. Temperature was controlled via a Finnex digital heater temperature control system, which utilized a submersible heater and thermometer sensor to actively monitor and stabilize water temperature. Water was
circulated via a submersible pump stationed alongside the heater to promote temperature uniformity throughout the tank. Oxygen concentration was controlled via an O2-stat system consisting of a Vernier LabView 3 controller, Vernier optical dissolved oxygen sensor, LabQuest digital control unit (DCU) and two solenoid valves. One solenoid valve controlled the flow of N2 gas to an airstone and the other solenoid valve controlled the flow of air to a second airstone. The LabView 3 actuated the N2 solenoid valve when O2 rose above the setpoint and actuated the air solenoid valve whenever O2 fell below the setpoint. The Vernier probe was able to measure both temperature and oxygen levels throughout the experimental run and log it in the LabQuest file system for verification after each experimental run. The surface of the water was covered as much as possible with plastic-aluminum sheeting to reduce atmospheric gas exchange and increase the efficiency of the aquarium in holding minimal oxygen levels. Air was bubbled in through an air stone via the same digital control system connected to the Vernier probe to allow for re-aeration of the water and a return to predetermined oxygen concentrations. Preliminary studies found oxygen concentrations decreased rapidly over time with the infusion of sodium sulfide solution to less than 1 mg/L O2, thus re-aeration was necessary to control for confounding factors. Hydrogen sulfide infusion was carried out via an automated pump system connected to IV bags with minimal atmospheric headspace, and pump rates were determined by sulfide concentration and IV bag volume.

2.4 Sulfide Infusion and Measurement

Sulfide concentrations were created by dissolving sodium sulfide nonahydrate (Na$_2$S•9 H$_2$O) crystals in degassed water at oxygen concentrations ≤ 1 mg/L. Sodium sulfide
crystals were rinsed in degassed water and blotted dry prior to weighing as outlined in the APHA methods for creating standard sulfide solutions (Baird et al., 2017). Sodium sulfide crystal weight for solutions were calculated using APHA calculations dependent on pH, temperature, and salinity. After weighing, crystals were dissolved in nitrogen-bubbled water to avoid oxidation of hydrogen sulfide concentrations within the solution. Sulfide solutions were then stored in separate 1 L IV bags for continuous infusion via automated pump, and an initial high concentration sulfide solution was added directly to the tank in small increments to gradually achieve the target sulfide concentration within the aquarium. Solutions were stored in IV bags to minimize headspace and oxidation throughout the experiment, and multiple IV bags were used in each experimental run due to volume constraints on solution saturation. Supersaturation in IV bags caused sodium sulfide to precipitate and settle out, resulting in a release of unequal levels of hydrogen sulfide over time. This unequal release had the potential to lower the realized concentration of sulfide within the tank, thus multiple bags of lower concentration solutions were utilized to keep realized concentrations as consistent as possible over time.

Sulfide concentrations were measured via two methods of spectrophotometry, both of which were calibrated through experimental trials without animals. Both methods required the formation of five standard sulfide concentrations ranging from 1 to 8 mg/L S\(^2\)-. To prepare sulfide standards, sodium sulfide crystals were rinsed and dried prior to weighing as described previously. First, 0.75 g Na\(_2\)S • 9 H\(_2\)O were weighed out and dissolved and mixed in 100 mL of degassed seawater to create a header solution of 100 mg/L. From this solution, 1 mL was taken and added to 99 mL of degassed seawater to create a solution of 1 mg/L S\(^2\)-. To keep the header solution from oxidizing, a nitrogen
bubbler was continuously running within the header solution while standards were being prepared. Thus, as soon as the first 1 mg/L standard was created, it was run through both spectrophotometry methods immediately. Upon finishing analysis, the next standard was created by pulling 2 mL from the header tank and adding 98 mL, and this procedure was repeated until standards of 1, 2, 4, 6, and 8 mg/L \( S^2^- \) had been analyzed to create calibration curves. During experimentation, samples were taken after the first 15 minutes to measure initial sulfide concentration, then once every half-hour for at least the first nine hours of experimentation. While samples were not taken overnight, sulfide concentrations were measured once again in the morning as soon as possible and sampling continued every half-hour until the 24-hour experimental window closed.

The first method of spectrophotometry was an adapted version of the methylene blue method from the APHA standard methods of sulfide measurement (Baird et al., 2017). The method involved treating a water sample with amine-sulfuric acid reagent, ferric chloride solution, and diammonium hydrogen phosphate solution, then using colorimetric analysis to compare the resulting solution to the calibration curves formed from the standards mentioned previously. The protocol used in this study largely follow the APHA procedure but deviated slightly by using a sample of filtered seawater as a blank rather than treating a second aquarium sample with blanking reagents. Preliminary experimental runs without animals found that utilizing a seawater blank alternative was more consistent and efficient in measuring total dissolved sulfide concentrations, thus no blanking reagents were used during actual experimental trials. The secondary method was adapted from an online study by Applied Analytics in which they showcased differences in hydrogen sulfide composition across varying pH levels (Applied Analytics, n.d.).
this method, sulfide concentration and hydrogen sulfide concentration were measured directly from tank samples using a wavelength of 229 nm and quartz cuvettes. The spectrophotometer was once again blanked with filtered seawater, however no reagents were added to tank samples prior to spectrophotometric assessment. As with the blue methylene method, calibration curves were created from the standards mentioned previously and used to calculate sulfide concentrations from tank samples. Due to higher efficiency and precision (Fig. 7), only this direct measurement method was used to measure sulfide concentrations during the June 6th and June 8th experimental trials.

![Methylene Blue Calibration Curve](image)

\[ y = 0.2736x + 0.431 \]
\[ R^2 = 0.9615 \]

![Direct Measurement Calibration Curve](image)

\[ y = 0.2061x + 0.1631 \]
\[ R^2 = 0.9996 \]

Figure 7. Comparison of calibration curves between the two spectrophotometric methods, with linear regression equations and R-squared values given for each set of standards.
2.5 Data Analysis

Temperature and oxygen concentration were continuously logged by the Vernier digital system at a rate of one sample every ten seconds. These data logs were then extracted from the logger and loaded into Microsoft Excel and RStudio for statistical analysis. Means and standard errors for temperature and DO were calculated for each trial run for comparison, and oxygen durations under specified concentration thresholds were calculated for each experimental run to assess possible differences in hypoxia severity. Sulfide concentration measurements were recorded in absorbance units and converted to total sulfide concentration in mg/L using calculations derived from standard calibration curves. The total sulfide concentrations were then converted to H\textsubscript{2}S concentrations in uM utilizing calculations from the APHA Standard Methods of the Examination of Water and Wastewater (Baird et al., 2017). H\textsubscript{2}S concentrations were plotted over time.

Fish survival was observed continuously throughout the 24-hour period, and time elapsed until mortality was recorded for each fish in hours. Survival was observed through a combination of constant in-person observation and continuous timelapse photography at a 1-minute resolution. Elapsed time was then compared to percentage of sample mortality and plotted to show rate of mortality over time. In addition to survival time, dry weight and fork length was recorded for each fish that died during experimentation. LT50 curves, which estimate the amount of time needed to induce mortality in 50% of test individuals, were calculated for each experimental run by fitting a three-parameter Weibull type 2 model with an upper limit of 1 to binomial data using the ‘drm’ function in the drc package in R (https://cran.r-project.org/package=drc, Ritz & Strebig 2016). LT50s were originally calculated using a three-parameter log-logistic regression curve
fitted to binomial data with an upper limit of 1, however, AIC model selection determined the Weibull type 2 model to have the best fit. The slopes and estimated LT50 values of each curve were then tested for significance by carrying out a t-test on each estimated LT50 parameter using the ‘compParm’ function in drc. Additionally, average mortality time was compared between treatment types as well as among experimental runs using ANCOVA with fish weight as a covariate. Linear regressions were also calculated between fish weight and survival time for each treatment group.
Chapter 3. Results

3.1 Water Quality

The preliminary hypoxia trial held an average dissolved oxygen concentration of $1.89 \pm 0.001$ mg/L O$_2$ with an average temperature of $31.7 \pm 0.003$ °C, while the preliminary hydrogen sulfide trial averaged $1.15 \pm 0.006$ mg/L O$_2$ and $31.9 \pm 0.004$ °C. Dissolved oxygen concentration ranged from $1.54 - 2.21$ mg/L O$_2$ and $0.51 - 3.32$ mg/L O$_2$ in the preliminary hypoxia and hydrogen sulfide trials, respectively. The preliminary H$_2$S trial held an average concentration of $3.47 \pm 0.15$ uM H$_2$S over the experimental trial, however, H$_2$S fell from a starting concentration of $3.85$ uM to $2.55$ uM H$_2$S over the 16-hour experimental period.

Following the preliminary trials, DO levels were dropped and maintained below target O$_2$ concentration thresholds of 1 mg/L and 0.5 mg/L across all experimental runs dating from April 25th to June 8th (Fig. 8). Initial hypoxia trials at 1 mg/L ranged in DO concentration from 0.44 to 1.44 mg/L O$_2$ over the 24-hour experimental period, while replicate trials maintained a tighter scope of oxygen with ranges of 0.76-1.06 mg/L O$_2$ and 0.69-1.16 mg/L O$_2$ on May 23rd and May 27th, respectively. H$_2$S replicate trials held lower DO concentrations on average than all 1mg/L O$_2$ hypoxia trials, and DO values fell as low as 0.48 and 0.32 mg/L O$_2$ during the June 6th and June 8th trials, respectively. Experimental runs for all treatments held average temperatures within 0.2 °C of the target 32 °C threshold (Supplemental Fig. 1). H$_2$S concentrations averaged $7.51 \pm 0.05$ uM H$_2$S and $7.64 \pm 0.05$ uM H$_2$S on June 6th and June 8th respectively (Supplemental Fig. 2). Hydrogen sulfide concentration ranged from 6.96 – 8.07 uM H$_2$S in the first sulfide infusion experiment compared to a range of 7.18 – 8.26 uM H$_2$S in the replicate sulfide
trial. Aquaria pH was measured at 8.14 and 8.16 prior to both hydrogen sulfide experiments, thus H$_2$S composed roughly 2% of all sulfide present in the aquaria.

Figure 8. Dissolved oxygen data recorded by the Vernier optical probe in each experimental trial following preliminary studies. Oxygen was recorded continuously at a ten second sampling rate, and legend captions denote the average DO ± the standard error for each experimental run.

3.2 Mortality by Treatment

Preliminary hypoxia exposure at a target concentration of 2 mg/L O$_2$ resulted in no toadfish mortality over a 24-hour period, while preliminary hydrogen sulfide infusion at the same target oxygen threshold yielded complete mortality in 13.6 hours. A three-parameter log-logistic regression calculated the LT50 of the hydrogen sulfide preliminary trial to be 9.83 ± 0.39 hours, and survival time averaged 10.3 ± 0.82 hours for the sample population.
In follow-up studies, ANCOVA analysis found extreme hypoxia resulted in a significantly lower average time until mortality than the hypoxia and hydrogen sulfide treatments when including fish weight as a covariate and a Bonferroni correction (Fig. 9, F(2, 33) = 9.89, p < 0.001). Hypoxia and H₂S treatments did not yield significantly different times until death, however, using fish weight as a covariate did alter the relationship between average survival time under the two experiments (Supplemental Fig. 3).

![Figure 9. Average time until mortality under each treatment. Error bars depict ± 1 SE using ANCOVA analysis with weight as a covariate, labels denote the average survival time ± 1 SE in hours as well as total sample size for each treatment. Survival time under the extreme hypoxia treatment was significantly lower than under the H₂S and hypoxia treatments; no other significant differences by treatment were found. ANCOVA analysis with the fish weight covariate and Bonferroni correction also found significantly different average times until mortality between individual trial runs of each treatment (Fig. 10, F(4, 31) = 8.94, p < 0.001). The hypoxia replicate from April 25th](image-url)
was excluded from ANCOVA analysis due to no mortality throughout the trial. The covariate weight was not statistically independent of the variables treatment (F(2,34) = 7.802, p=0.002) and experimental trial (F(4,32) = 5.48, p = 0.002) were not statistically significant from one another, however, nonparametric covariate analysis was not possible in R. Kruskal-Wallis rank sum tests with Bonferroni correction found significantly lower survival times under the extreme hypoxia treatment in comparison to hydrogen sulfide and hypoxia ($X^2 = 15.033$, d.f. = 2, $p = 0.001$), as well as significant differences in survival time between individual experimental runs ($X^2 = 18.147$, d.f.=4, $p = 0.001$).

![Figure 10. Average time until mortality for each replicate run. Error bars depict ± 1 SE using ANCOVA analysis with weight as a covariate, labels denote average survival time ± 1 SE in hours, letters denote significance between replicate trials, and dot colors indicate the treatment of each experimental trial.](image)

Mortality was also compared across treatments using constructed LT50 curves to incorporate surviving fish from each trial in total mortality assessment. Extreme hypoxia yielded a significantly lower estimated LT50 value of 1.49 ± 0.32 hours than the H$_2$S (7.62 ± 1.29 hours) and hypoxia (8.64 ± 1.64 hours) treatments (Supplemental Table 1).
Individual LT50 curves were calculated for each replicate run and yielded significantly different LT50 values between and within treatments (Fig. 11, Supplemental Table 2). The April 25th hypoxia trial was removed from LT50 analysis as zero mortality within the 24-hour study window cannot be used to compute an estimated lethal time until death. Slopes of LT50 curves were compared both by treatment and by replicate run, and no significant differences were found. Individual LT50 curves were graphed to portray significant differences in LT50 by replicate trial (Supplemental Fig. 4).

Figure 11. Comparison of estimated LT50 values by individual experimental trial. Models were fitted to proportion of mortality data over time, and error bars depict 1 SE around the estimated LT50 value. Letters denote significance from pairwise t-tests on estimated LT50 parameters, labels list LT50 ± 1 SE.
3.3 Effect of Weight on Mortality

Fish weights and lengths were not collected following preliminary trials but were recorded for every fish that died in follow-up experiments. Fish weight data violated Bartlett tests for homogeneity of variance among trials (p < 0.001) and among days (p = 0.001). Kruskal Wallis tests found fish weight was significantly lower in hypoxia trials ($X^2 = 10.75$, d.f. = 2, $p = 0.005$) and significantly different among replicate trial days ($X^2 = 13.803$, d.f. = 4, $p = 0.008$). For all trials at 1 mg/L O$_2$ regardless of treatment, fish survival time increased with increasing fish weight (Supplemental Fig. 5, F(1,27) = 5.194, $p = 0.03$). Likewise, survival time increased with fish weight within the hypoxia treatment at 1 mg/L O$_2$ (F(1,14) = 5.286, $p = 0.04$). However, no such relationships emerged in the other two treatments (Fig. 12).

![Fig. 12. Effect of fish weight (g) on survival time in each respective treatment group. Data only reflect fish that died within each experiment; surviving fish were not weighed. Lines reflect linear regressions for each treatment type, ribbons illustrate 95% confidence bands. Equations and r-squared values are shown in the top left for each line, while P-values are shown in the bottom right corner.](image)
Chapter 4. Discussion

Comparison of mortality between the three treatments found that extreme hypoxia at 0.5 mg/L $O_2$ yielded significantly faster rates of mortality in juvenile toadfish than hypoxia at 1 mg/L $O_2$ as well as hypoxia with hydrogen sulfide infusion with an average concentration of around 7.5 uM $H_2S$. While previous studies have shown that *Opsanus beta* exhibits similar cardiovascular and respiratory responses to acute hypoxia as other teleost species (McDonald *et al.*, 2010), toadfish are generally viewed as a hypoxia-tolerant species (Frank *et al.*, 2022; Ultsch *et al.*, 1981; Amador *et al.*, 2018). Total survival of juveniles during the preliminary hypoxia trial at 2 mg/L $O_2$ and initial follow-up experiment at 1 mg/L $O_2$ supports the notion that gulf toadfish have high tolerances to hypoxia under the generally accepted dissolved oxygen threshold of 2 mg/L. However, the rapid decline in survival time following minimal decreases in oxygen concentration past 1 mg/L supports studies contending such universal levels of hypoxia thresholds are not applicable to all species (Vaquer-Sunyer & Duarte, 2008). The fourfold difference in survival time under extreme hypoxia conditions compared to hypoxia illustrates a stark threshold in survivorship over a range of just 0.4 mg/L $O_2$. Comparatively, *Leistostomus xanthurus* and *Brevoortia tyrannus*, which occupy similar occasionally hypoxic estuarine habitats as *Opsanus beta* during juvenile stages, showed similar exponential drops in LT50 across a narrower range of dissolved oxygen values at overall higher levels of oxygen (Shimps *et al.*, 2005). Thus, while *Opsanus beta* may show greater hypoxia tolerance at oxygen levels that are harmful for co-inhabitant estuarine fishes, lethality still increases at an exponential rate once a threshold is reached. During the 2020 fish kill in North Biscayne Bay, durations of oxygen concentrations $\leq$ 0.5 mg/L were recorded on
consecutive days from August 10 – 12, with multiple durations surpassing the estimated LT50 time derived from this experiment (Supplemental Table 3). Environmental levels of hypoxia should not be examined at all-encompassing baseline concentrations, but rather at species-specific critical thresholds derived from research on the physiological processes, survival and behavioral capabilities of focal organisms (Farrell & Richards, 2009).

The lack of significant differences between pooled hypoxia and hydrogen sulfide treatments was surprising, especially given the differences in oxygen concentration between the two treatments. Not only did the sulfide infusion treatment introduce concentrations of toxic H₂S, but both trials had significantly lower levels of dissolved oxygen than either hypoxia trial. The preliminary hydrogen sulfide trial experienced a drastic drop in oxygen over the experimental period due to the rapid oxidation rates of sulfide which have been shown to reduce oxygen concentrations along sulfidic redox boundary layers (Morse et al., 1987; Maeda et al., 1988). Follow-up trials used the adjusted Vernier system with a minimal oxygen threshold to bubble in oxygen when DO concentrations dropped too low, and this remediation of oxygen directly to the tank may have presented a confounding variable on fish survival. Behaviorally, fish exhibited similar responses of surface swimming and loss of equilibrium across both treatments prior to death, and some fish were also observed to detect areas of higher oxygen concentration through their movement to the oxygen bubbling air stone over time. It is difficult to discern whether apneic responses and mortality were due to the toxicity of H₂S or the effect of the chemical on oxygen concentrations, so future studies utilizing a flow-through system for oxygen maintenance might help to address these difficulties.
Additionally, while this pilot study was unable to utilize power analyses to estimate adequate sample size and had a limitation of fish availability, further replication of experimental trials with greater sample sizes may result in clearer statistical distinctions among treatments.

Another potential reason for lack of differences between hydrogen sulfide and hypoxia trials could be the concentration of H$_2$S was too minimal to detect significant toxicity effects. Previous studies on species along the San Diego coast show tidal marsh species such as killifish could survive longer than 20 hours at concentrations up to 50 uM H$_2$S; however, these studies were not done in tandem with hypoxia (Bagarinao & Vetter, 1989). Environmental data reports from fish kills in the canal tributaries of a bay in Delaware showed H2S concentrations reached millimolar levels at depths of 4.5m within the canals (Luther et al., 2004), indicating canal systems can foster extremely high levels of H2S production. In the *Thalassia* die-offs in Florida Bay in 1990, porewater total sulfide levels were millimolar which indicates H$_2$S porewater concentrations could have been as high as 45 uM, assuming a pH of 8.14 and temperature of 32 °C (Carlson et al., 1994). A 2017 Florida Power and Light (FPL) assessment report found porewater sulfide concentrations ranging from 0 - 400 mg/L S$^2$ across sample sites in southern Biscayne Bay, however, no specific site locations or other available water quality data was provided in this report (FPL, 2017). There are currently no available data on hydrogen sulfide concentrations in northern Biscayne Bay during these recurring fish kill events, however, pH levels during the August 2020 event ranged from 7.5 to 7.7 and thus almost 10% of available sulfide was available as unionized H$_2$S within the porewater compared to the 2.5% proportion of sulfide used in laboratory experiments (pH 8.1-8.15). Bottom
water surveys collected by the Florida Department of Environmental Protection did not record quantifiable concentrations of sulfide, however, these collections were taken several days after the fish kill as part of a pilot field survey (Aliza Karim, personal communication). Field surveys projected for the summer and fall months of 2022 plan to take more accurate porewater and bottom water samples throughout the bay on a regular basis, providing potentially useful spatiotemporal datasets on realized sulfide concentrations in Northern Biscayne Bay. Additionally, further experiments investigating the release of porewater sulfide concentrations into the bottom layer of the water column may offer more insight on the potential sulfide concentration maxima in demersal fish habitats. Utilizing these field data, future studies could use natural sulfide concentrations in tandem with pH levels during fish kill events to produce a more accurate environment for comparison of hypoxia and hydrogen sulfide on toadfish survival.

Survival time did not vary with fish weight under extreme hypoxia conditions, but fish weight held a significantly positive linear relationship with survival time across all mortalities during 1 mg/L O₂ trials, including those with hydrogen sulfide infusion. Additionally, accounting for weight in covariate analysis shifted the comparison of survival times between hypoxia and hydrogen sulfide trials, indicating weight may have played a role in survivorship under both treatments. Size as a factor of fish hypoxia tolerance has long been debated, and a metanalysis study found that while body size had no impact on oxygen uptake ability under hypoxia, survival time under severe hypoxia does increase exponentially with body mass due to scaling of anaerobic metabolism and glycolysis (Nilsson & Ostlund-Nilsson, 2008). In another demersal Batrichoidid species, Porichthys notatus, males exhibited greater tolerances to hypoxia stress than their female
counterparts due to higher glycolytic potential with size (LeMoine et al., 2014), suggesting the idea of larger size advantages could apply to the related *Opsanus beta* as well. However, studies on juvenile estuarine fish found species-specific differences in relationships between size and hypoxia survival times (Shimps et al., 2005), and larval and juvenile gulf toadfish have been found to exhibit similar capacities for hypoxia tolerance despite differences in size by utilizing differing metabolic strategies (Frank et al., 2022). Thus, assessing a wider range of body sizes is necessary to fully understand how fish weight correlates with hypoxia tolerance in *Opsanus beta*. It is important to note that no juvenile fish floated to the surface after succumbing to mortality, even those that died overnight and had multiple hours within the tank to float to the surface. If larger toadfish are more resilient to hypoxia but were still observed floating along the surface following the recent Biscayne Bay fish kills, it can be assumed that a large quantity of juvenile toadfish also died during these fish kills and remained unaccounted for in carcass surveys.

While this study was unable to significantly differentiate the effects of hydrogen sulfide and hypoxia on mortality in toadfish, it did verify that *Opsanus beta* can succumb to mortality in durations of low oxygen concentrations recorded in northern Biscayne Bay. Seasonal recurrence is a common trait detected in many of the world’s hypoxic zones (Rabotyagov et al., 2014), with many aquatic ecosystems forming hypoxic bottom waters during summer months when stratification within the water column naturally intensifies (Klump et al., 2018; Bishop et al., 2006; Fennel & Testa 2019). Recurring hypoxia can impact demersal fish species at sublethal levels through the reduction of habitat availability and the increase in density-dependent interactions including an
increase in predation risk on smaller, younger organisms (Orio et al., 2019; Eby et al., 2005). Population studies on *Opsanus beta* in the 1990s found abundance to correlate positively with nocturnal dissolved oxygen and seagrass cover (Serafy et al., 1997), indicating gulf toadfish exhibit similar traits of preferential avoidance of lower oxygenated environments. Juvenile estuarine species have been found to behaviorally prioritize predator avoidance over hypoxia avoidance; however (Froeschke & Stunz, 2012), and it is unclear how juvenile toadfish behave during these intense hypoxia episodes. Multiple fish species show greater hypoxia tolerances and altered behavioral reactions and physiological processes with increased preconditioning and acclimatization (Nilsson & Renshaw, 2004; Rees et al., 2001), thus local *Opsanus beta* populations may start to exhibit phenotypical differences with recurring fish kill events in northern Biscayne Bay. Ultimately, ecological assessments in NBB should investigate the spatial and temporal distributions of *Opsanus beta* and other demersal species to determine whether recurring hypoxia and previous fish kills have significantly altered local populations.
Supplemental Figure 1. Temperature data recorded by the Vernier optical probe in each experimental trial following preliminary studies. Temperature was recorded continuously at a ten second sampling rate, and legend captions denote the average temperature in Celsius ± the standard error for each experimental run.
Supplemental Figure 2. Measured hydrogen sulfide concentrations over time from each replicate experimental run. Target concentration was 8 uM H2S; average concentration ± standard error for each trial are listed under their respective graphs.
Supplemental Figure 3. Average time until mortality under each treatment using ANCOVA analysis without weight as a covariate. Error bars depict ± 1 SE; labels denote the average survival time ± 1 SE in hours as well as total sample size for each treatment.
Supplemental Figure 4. Proportion of mortality over time calculated by fitting three-parameter Weibull type 2 models with an upper limit of 1 to binomial data using the drc package in R. An additional line is included to show proportion of mortality for the April 25th Hypoxia trial in which no fish died over the span of 24 hours. Labels denote the estimated LT50 for each curve ± standard error.
Supplemental Figure 5. Linear regression of time until death by fish weight for all fish that died during hypoxia and hydrogen sulfide treatments under a target oxygen threshold of 1 mg/L. Gray shading depicts the 95% confidence bands around the regression; $R^2$ and p-value are shown in the upper left and lower right corners respectively.
## TABLES

### Comparison of LT50 Parameter Estimates by Treatment

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Supplemental Table 1. Output table of t-test comparisons on estimated LT50 parameters for each treatment generated by the compParm function in R.

### Comparison of LT50 Parameter Estimates by Day

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<td>M23-J8</td>
<td>12.79582</td>
<td>2.62663</td>
<td>4.8716</td>
<td>1.11E-06</td>
<td>***</td>
</tr>
<tr>
<td>M27-M31</td>
<td>5.68897</td>
<td>0.76904</td>
<td>7.3975</td>
<td>1.39E-13</td>
<td>***</td>
</tr>
<tr>
<td>M27-J6</td>
<td>-3.15998</td>
<td>2.45935</td>
<td>-1.2849</td>
<td>0.1988324</td>
<td></td>
</tr>
<tr>
<td>M27-J8</td>
<td>-1.14319</td>
<td>1.18128</td>
<td>-0.9678</td>
<td>0.3331653</td>
<td></td>
</tr>
<tr>
<td>M31-J6</td>
<td>-8.84895</td>
<td>2.38752</td>
<td>-3.7063</td>
<td>0.0002103</td>
<td>***</td>
</tr>
<tr>
<td>M31-J8</td>
<td>-6.83216</td>
<td>1.02337</td>
<td>-6.6761</td>
<td>2.45E-11</td>
<td>***</td>
</tr>
<tr>
<td>J6-J8</td>
<td>2.01679</td>
<td>2.55034</td>
<td>0.7908</td>
<td>0.4290666</td>
<td></td>
</tr>
</tbody>
</table>

Supplemental Table 2: Output table of t-test comparisons on estimated LT50 parameters for each experimental trial generated by the compParm function in R.

### Extreme Hypoxia Durations (≤ 0.5 mg/L) Recorded at BB14

<table>
<thead>
<tr>
<th>Date</th>
<th>Start Time</th>
<th>Duration (hr)</th>
<th>Average DO (mg/L)</th>
<th>Average Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/10/20</td>
<td>6:15:00</td>
<td>3</td>
<td>0.16538462</td>
<td>31.55315</td>
</tr>
<tr>
<td>8/10/20</td>
<td>19:30:00</td>
<td>1.25</td>
<td>0.27333333</td>
<td>32.74983</td>
</tr>
<tr>
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<td>2:15:00</td>
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<td>0.06051282</td>
<td>32.28777</td>
</tr>
<tr>
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<td>33.37333</td>
</tr>
<tr>
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<td>4:45:00</td>
<td>0.75</td>
<td>0.3</td>
<td>32.12125</td>
</tr>
<tr>
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<td>6:00:00</td>
<td>2.25</td>
<td>0.066</td>
<td>32.0286</td>
</tr>
<tr>
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<td>31.6586</td>
</tr>
</tbody>
</table>

Supplemental Table 3. Extreme hypoxia durations under a threshold level of 0.5 mg/L O₂ recorded at the BB14 sensor. Average dissolved oxygen concentration and temperature were calculated for each duration.
REFERENCES


Briceño, Henry O., Joseph N. Boyer and Peter W. Harlem. 2011. Ecological Impacts on Biscayne Bay and Biscayne National Park from Proposed South Miami-Dade County Development, and Derivation of Numeric Nutrient Criteria for South Florida Estuaries and Coastal Waters. NPS TA# J5297-08-0085, Florida International University, Southeast Environmental Research Center Contribution # T- 530, 145 p


