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The Physiological Stress Response caused by Hypoxia and Reperfusion Injury in Striped Bass (Morone saxatilis) and Nile Tilapia (Oreochromis niloticus)

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THE PHYSIOLOGICAL STRESS RESPONSE CAUSED BY HYPOXIA AND REPERFUSION INJURY IN STRIPED BASS *(Morone saxatilis)* AND NILE TILAPIA *(Oreochromis niloticus)*

by

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(Under the Direction of Johanne M. Lewis)

ABSTRACT

Approximately five million people in the United States are affected by cardiovascular related diseases yearly contributing to 300,000 annual deaths, making cardiovascular disease (CVD) the leading cause of mortality worldwide. It has been suggested that apoptosis (programmed cell death) contributes to the pathogenesis of CVD. When blood flow is reduced or cut off from the heart, usually by a thrombus, it results in oxygen deprivation (hypoxia) to the cardiomyocytes (heart cells). In response to this hypoxic stress, cardiomyocytes will undergo apoptosis. Since many species of fish can survive levels of hypoxia that would be fatal to mammals, fish are an ideal model system to study changes at the cellular and molecular level that prevent or repair hypoxia-induced damage in cardiomyocytes. For this study, the hypoxia-tolerant Nile Tilapia (*Oreochromis niloticus)* and the hypoxia-sensitive Striped Bass (*Morone saxatilis)* were subjected to gradual hypoxia exposure and their cardiac tissue was tested for key markers of apoptosis. We hypothesized that tilapia will have lower levels of cellular markers of apoptosis due to higher anti-apoptotic and repair gene expression in their cardiomyocytes when compared to Striped Bass, which we hypothesized will have higher levels of cellular markers of apoptosis and pro-apoptotic gene expression levels. By elucidating the cellular and molecular mechanisms present in tilapia cardiomyocytes during hypoxia exposure and reoxygenation, this study could potentially aid in the

future development of therapeutic strategies for cardiovascular disease in humans. Evidence of apoptosis at the cellular level was determined by observing DNA fragmentation (late stage apoptosis) and measuring caspase-3/7 activity (early stage apoptosis) in heart tissue. DNA fragmentation was not observed in any samples across timepoints, even in our sensitive species. Additionally, there was no significant difference across timepoints and species in caspase 3/7 levels. To back up the results obtained at the cellular level, RT-qPCR was used to quantify changes in the cardiomyocyte transcriptome of pro-apoptotic (*bax, caspase3,* and *fasl*), anti-apoptotic (*bcl2, flip,* and, *p53*) and repair (*hsp70*) genes. However, primers for *hsp70* were the only ones that were successfully developed to measure gene expression. Therefore no data was collected on pro or anti-apoptotic gene expression in the hearts of Striped Bass and Nile Tilapia. Additionally, RT-qPCR efficiency for *hsp70* primers were too high to collect accurate data. In conclusion, there was no cellular evidence of apoptosis in tissue samples across timepoints and between species, and no data was collected to measure pro and anti-apoptotic gene expression. The lack of cell death in Striped Bass heart samples at the cellular level could be explained by a limited capacity for hypoxia tolerance or that the fish was undergoing another mechanism in place of apoptosis such as necrosis.

INDEX WORDS: Cardiovascular Disease, Apoptosis, Hypoxia, Reperfusion Injury, Hypoxia Sensitivity, Hypoxia Tolerance, Striped Bass, Nile Tilapia

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CHAPTER 1

INTRODUCTION

Objective

One out of three Americans will die from cardiovascular disease (CVD), averaging one death every 40 seconds, making CVD the leading cause of death in the United States (Go et al. 2013; Singh and Kang 2011). In 2009, the United States spent an estimated $312.6 billion on costs associated with the morbidity and mortality of CVD compared to the $228 billion spent on all cancers (Go et al. 2013). Research has been directed at understanding the pathogenesis of CVD and associated physiological pathways to combat CVD. It has been suggested that apoptosis (programmed cell death) is the main contributor to cardiovascular disease development (Chiong et al. 2011; Gustafsson and Gottlieb 2006; Neuss et al. 2001). This occurs when blood flow is reduced or cut off from the heart by a thrombus (blood clot), resulting in oxygen deprivation (hypoxia) to the cardiomyocytes. When cardiomyocytes encounter hypoxia, the cells can no longer produce sufficient ATP to keep up with the heart's workload demands and will initiate apoptosis (Fago and Jensen 2015; Olivetti et al. 1997). Sufficient cardiomyocyte loss can be fatal, due to human cardiomyocytes' limited ability to regenerate once damaged (Chiong et al. 2011). Current treatments and therapies for CVD patients are targeted at restoring oxygen back to the cardiomyocytes either by removing the thrombus or bypassing the artery (Yellon and Hausenloy 2007). However, reoxygenation of the ischemic (reduced blood supply) tissues can result in reperfusion (reoxygenation) injury due to reactive oxygen species formation (Fago and Jensen 2015; Yellon and Hausenloy 2007) and an increased ATP supply for apoptosis initiation (Elmore 2007).

Apoptosis

Apoptosis is one of three forms of cell death and is distinguished from necrotic and autophagic cell death by ATP dependence and unique morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation (Elmore 2007; Portt et al. 2011). Generally apoptosis is triggered by various stimuli such as heat stress, hypoxic stress, oxidative stress, heavy metals, or viruses (Elmore 2007; Favaloro et al. 2012; Gulbins et al. 2000; Krumschnabel and Podrabsky 2009; Portt et al. 2011). Therefore, apoptotic cell death is intended to promote overall tissue and organism health by removing damaged cells and leaving neighboring healthy cells (Takle and Andersen 2007). For various reasons the apoptotic pathway can become dysregulated and can lead to the pathogenesis of many types of cancer, neurodegenerative disorders, and cardiovascular diseases (Elmore 2007; Favaloro et al. 2012). In the case of CVD, DNA damage sustained during hypoxia and ATP influx during reoxygenation causes the dysregulation of normal apoptotic function that ultimately leads to organ failure (Sendoel and Hengartner 2013).

Apoptotic Pathways

This highly regulated cellular process is triggered when stimuli reach the cell, activate the ATP dependent caspase cascade, and initiate morphological changes within the cell (Dos Santos et al. 2008; Gulbins et al. 2000). Caspases can be classified as either initiators (caspase 8 and 9) or effectors (caspase 3 and 7) and during homeostasis they reside in the cytosol of the cell in an inactive form (Favaloro et al. 2012). When a stimulus reaches the cell, the initiator caspases are cleaved (an ATP dependent process) into an active form that acts on effector caspases (Elmore 2007). The two signaling pathways for apoptosis, the receptor/ligand (extrinsic) mediated pathway

and the mitochondrial (intrinsic) mediated pathway, converge on effector caspase 3 activation (fig. 1.1).

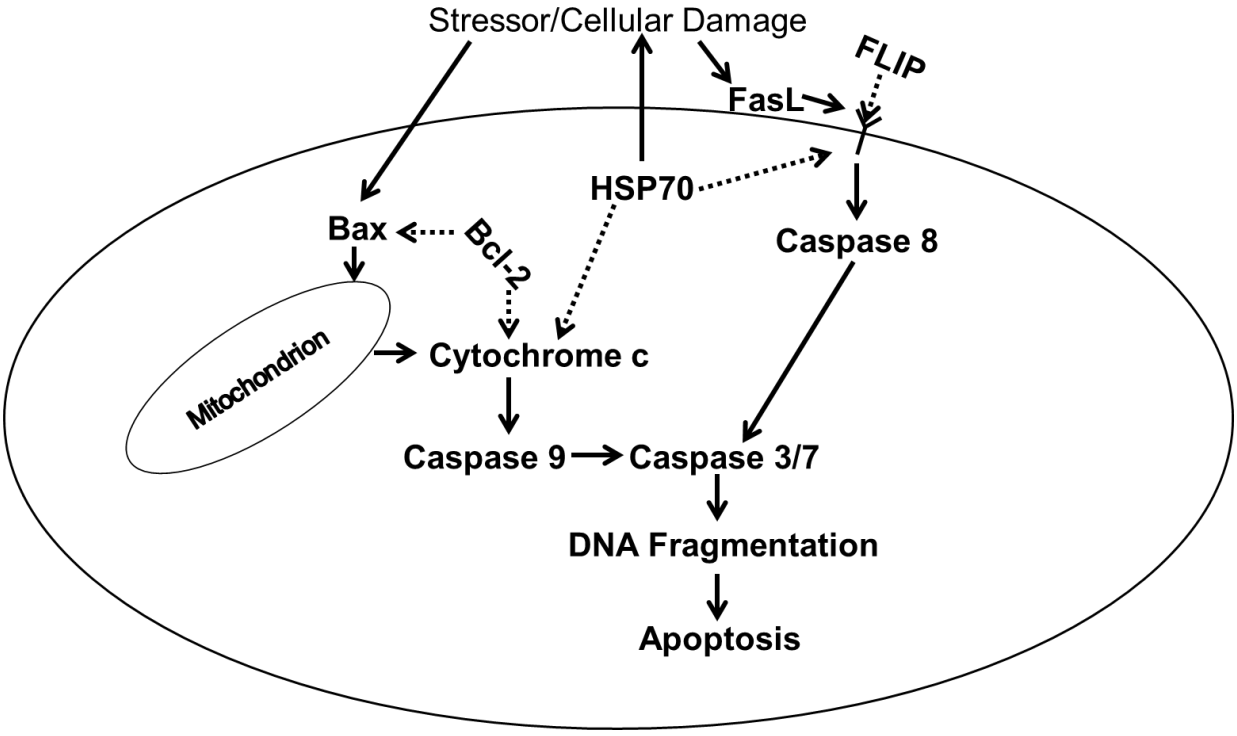

Figure 1.1 Representative image of the two apoptotic pathways that show apoptotic initiation (solid arrows) and suppression (dotted arrows) and the point of convergence at caspase 3/7.

The extrinsic (receptor/ligand) pathway is controlled by the Tumor Necrosis Factor Receptor (TNFR) superfamily and consists of receptors and ligands that control the initiation or suppression of apoptosis (fig. 1.1; Dos Santos et al. 2008). A common death receptor system in the TNFR super family is the fatty acid synthase (Fas) system. The Fas Ligand (FasL) is pro-apoptotic and binds to the Fas receptor causing caspase 8 (initiator) activation, leading to caspase 3 (effector) activation and cell death (table 1.1; Prott et al. 2011). The anti-apoptotic protein FLIP (FLICE-like inhibitory protein) inhibits apoptosis by competing with inactive caspase 8 and thus preventing its activation (table 1.1; Feng et al. 2008; Prott et al. 2011). The intrinsic (mitochondrial) pathway is initiated

via the release of cytochrome from mitochondria during stress, the release of cytochrome c during stress is mediated by the Bcl-2 family of proteins (fig. 1; Gustafsson and Gottlieb 2007; Prott et al. 2011). The pro-apoptotic member bax behaves as a second messenger causing cytochrome c release from mitochondria and cell death, while bcl-2 will prevent bax activation to suppress apoptosis (table 1.1; Prott et al. 2011).

Cellular Repair

Since initiating apoptosis in cells can be lethal, there are cellular mechanisms present outside of the apoptotic signaling pathways to prevent premature apoptosis (Prott et al. 2011). Heat shock proteins (Hsps) are one of the more commonly known cellular protective mechanisms and circumvent apoptosis through limiting cellular damage or repairing sustained cellular damage by restoring enzymatic activity to denatured proteins (table1.1; Benjamin and McMillan 1998; Arya et al. 2007). Additionally, Hsp70 inhibits the activation of the caspase cascade by preventing cytochrome c release in the intrinsic pathway or by preventing ligand receptor binding in the extrinsic pathway (table 1.1, Delogu et al. 2002; Arya et al. 2007).

Table 1.1 Summary of the function and mechanism of either apoptotic initiation or suppression by key players in the pathways.

Player	Function	Mechanism
bax	Pro-Apoptotic	Initiates apoptosis by triggering cytochrome c release from the mitochondrion.
bcl2	Anti-Apoptotic	Prevents apoptosis by suppressing cytochrome c release of bax expression.
caspase3	Pro-Apoptotic	Initiates apoptosis by triggering the caspase cascade the ultimately leads to cell death.
fasl	Pro-Apoptotic	Initiates apoptosis by binding to the fas receptor and activing caspases.
flip	Anti-Apoptotic	Prevents apoptosis by inhibiting fasl binding.
hsp70	Repair	Actively repairs cellular damage caused by physiological stressors.
p53	Pro/Anti-Apoptotic	Will either suppress apoptosis to allow the cell to be repair or initiate apoptosis if cell is too damaged.

CVD and Apoptosis

Understanding cellular coping mechanisms, i.e. regulators of apoptotic pathways or cellular protectors, during hypoxia exposure is key to developing therapeutic strategies for CVD (Choing et al. 2011; Gustafsson and Gottlieb 2007; Singh and Kang 2011). Traditionally, CVD was treated by removing clots or restoring oxygen to tissues, which we now know causes more damage via reperfusion injury (Elmore 2007; Yellon and Hausenloy 2007). Therefore, research has been dedicated to developing therapies that disrupt apoptosis by investigating the role of anti-apoptotic (Chiong et al. 2011; Gustafsson and Gottlieb 2007; Micheau 2003) and repair genes in both the extrinsic and intrinsic pathways of apoptosis (fig. 1.1; Benjamin and McMillan 1998; Gupta and Knowlton 2005; Martin et al. 1997) during hypoxia and reperfusion.

In the extrinsic pathway, is has been shown that *fasl* is not expressed in non-diseased cardiomyocytes and found to be only expressed during hypoxic or reperfusion stress (Feng et al. 2008). Additionally, elevated *fasl* expression has been measured in blood and heart samples of CVD patients (Abbate et al. 2002; Setsuta et al. 2004). High levels of the antagonist protein, FLIP, are shown to be present in healthy cardiomyocytes but at a decreased rate of expression in diseased cardiomyocytes (Micheau 2003). In the intrinsic pathway, Houchhauser et al. (2003), surmised infarction size and mitochondrial damage was reduced in *bax*-deficient mice compared to wild type in hypoxic and reperfusion injury. Reduced infarct size, cardiac myocyte apoptosis, and cardiac dysfunction after hypoxic and reperfusion injury has been observed in overexpression of *bcl2* in cardiomyocytes (Chiong et al. 2011). In repair pathways, hsp70 has been suggested by Delogu et al. (2002) to offer myocardial protection during ischemic injury. Hsp70 is expressed at low levels during normal conditions but is turned on in response to hypoxic injury as proteins are

denatured, and elevated during reperfusion injury since ATP is required to repair denatured proteins (Sharp et al. 2013).

To understand how apoptotic gene regulation can aid in the treatment of CVD in humans, these pathways need to be studied in an ideal model organism. Apoptosis has been mostly studied in invertebrate models, like *C.elegans* (Arvanitis et al. 2013) or *D.melangaster* (Denton et al. 2013) to establish the pathways controlling apoptosis. However, they lack the carryover necessary to understand the role apoptosis plays in vertebrate organ function and disease pathogenesis (Dos Santos et al 2008). In mammalian models, most research is directed at the role specific genes play in diseases development but not the role they play in apoptotic signaling and pathways. Therefore, to help bridge the knowledge gap between invertebrate models and mammalian models, researchers are using non-mammalian vertebrates such as fish to extend the information available on apoptosis and diseases like CVD (Krumschnabel and Podrabsky 2009).

Fish as Model Organisms

Fish are practical model organisms to study hypoxia-induced physiological changes because they inhabit environments that can vary in dissolved oxygen availability, ranging from persistent hypoxia to infrequent hypoxic episodes (Diaz and Breitburg 2009). Therefore, some fish possess a wide variety of physiological adaptions for hypoxic survival. Without oxygen transfer and circulation, aerobic respiration and ATP production would cease leading to eventual death. Since the cardiovascular system is vital for oxygen transfer and circulation, research has been directed at physiological adaptations within the heart (Gamperl and Driedzic 2009). Predatory or athletic fish, such as the carp and tuna, require stronger hearts and as a byproduct tend to be more hypoxia

tolerant in comparison to sedentary or cold adapted fish (Gamperl and Farrell 2004). For example, the bluefin tuna (*Thunnus orientalis)* is a large predatory fish in the Pacific Ocean that is known for exceptional cardiac performance due to a higher cardiac output (up to two time greater) than other teleosts living in comparable environments (Shiels et al. 2003). Overall, compared to non-predatory teleost, bluefin tuna have larger ventricle size in relation to body mass for increased blood and oxygen flow. A more developed circulatory system for higher cardiac output and increased mitochondrial oxygen consumption for ATP production (Shiels et al. 2003). These are some examples of adaptive cardiac physiology that enable fish to survive in their environments. In addition to organ level enhanced physiology, there are cellular and molecular coping mechanisms as well. Such as increased antioxidant production, reduced reactive oxygen species formation, metabolic rate suppression, and enhanced ATP storage and utilization (Gamperl and Farrell 204; Lague et al. 2012; Richards 2011; Wu et al. 202).

Study Species

The purpose of this comparative study is to investigate cellular and molecular apoptotic responses as a survival strategy during hypoxia exposure and reoxygenation, in both the hypoxia-sensitive Striped Bass (*Morone saxatilis)* and the hypoxia-tolerant Blue Nile Tilapia (*Oreochromis niloticus)*. Traditionally, Striped Bass are a migratory species that migrate towards colder waters in the Atlantic during the summer, but now exist in fragmented populations as a result of decreased oxygen availability from eutrophication (Price et al. 1985). The resulting lower oxygen shift in the Striped Bass' natural habit has caused decreased food consumption and growth rate and increased mortality in select populations (Brandt et al. 2009). Therefore, utilizing the sensitivity of Striped Bass would be beneficial to study hypoxia-induced physiological changes that occur after

exposure. Nile Tilapia belong to a group of cichlids that inhabit estuaries along the east African coast. These estuaries frequently fluctuate in their oxygen availability daily, causing the organisms that inhabit them possess adaptations for hypoxia survivability (Gupta and Acosta, 2004). Since Nile Tilapia are able to successfully survive hypoxic environments and potentially can prevent organ failure as a means of survival, they would be ideal to study the coping mechanism the heart undertakes after hypoxia exposure and reperfusion injury.

Project Goals

To uncover the coping mechanisms utilized in the hearts of Nile Tilapia that are preventing cardiomyocyte loss, the hypoxia tolerance threshold (HTT) in Striped Bass and Nile Tilapia was determined. Then each species was subjected to gradual hypoxia and subsequent reoxygenation to investigate the cellular and molecular mechanism of apoptotic cell death. Caspase 3/7activity (an early marker of apoptosis) and DNA fragmentation (a late stage marker of apoptosis) were used to detect the presence of apoptosis in tissue samples for both Striped Bass and Nile Tilapia. The final objective was to quantify changes in the transcriptome of Striped Bass and tilapia cardiomyocytes during hypoxia exposure for pro-apoptotic genes (*bax*, *caspase3,* and *fasl)*, anti-apoptotic genes (*bcl2*, *flip*, and, *p53),* and a repair gene (*hsp70).* We hypothesized that Nile Tilapia are preventing cardiomyocyte loss by suppressing apoptosis through increased expression of anti-apoptotic and repair genes during hypoxia exposure. Therefore, Nile Tilapia will have lower levels of cellular apoptotic markers due to higher anti-apoptotic and repair gene expression in their cardiomyocytes when compared to Striped Bass, which we hypothesized would have higher levels of cellular apoptotic markers and pro-apoptotic gene expression levels. By investigating the mechanisms

preventing cardiomyocyte loss in Nile Tilapia during hypoxia, this study has the potential to contribute to developing therapeutic and preventive treatments for CVD in the future.

CHAPTER 2

BEHAVIORAL RESPONSES OF STRIPED BASS (*Morone saxatilis*) AND NILE TILAPIA (*Oreochromis niloticus*) TO GRADUAL HYPOXIA EXPOSURE

INTRODUCTION

Hypoxia is a common occurrence in aquatic environments that can vary in range or duration (Diaz and Breitburg 2009). Bodies of water that are shallow, poorly circulated, or located in tropical climates are prone to constant fluctuations in dissolved oxygen availability (Richards 2011). Additionally, increased anthropogenic activities like fertilizer use and sewage dumping, generate increased rates of hypoxia in all aquatic systems, including ones that were previously stable (Sunyer and Duarte 2008).

Consequently, fish have evolved a whole suite of behavioral, physiological, and molecular adaptations in response to hypoxia exposure, with much research directed at the hypoxia tolerance capabilities of various fish species (Bickler and Buck 2007; Mandic et al. 2009; Nilsson and Renshaw 2004; Richards 2011). Behavioral responses in fish, such as air-breathing or aquatic surface respiration (ASR), can be triggered rapidly to provide short term relief of hypoxia exposure by allowing the fish to access areas with greater oxygen availability (Chapman and McKenzie 2009). These behavioral responses can not only affect the overall behavior (i.e. agitation, aggression, or the tendency to escape) but are often linked with physiological or biochemical responses as well (Chapman and McKenzie 2009). Some physiological adaptations include gill hyperventilation for increased oxygen intake (Perry et al. 2009), bradycardia for reduced ATP demands or metabolic waste (Lague et al. 212), or cardiac tissue and mitochondrial remodeling for

surface area for better oxygen diffusion (Gamperl and Farrell 2004). Some biochemical or metabolic adaptations include increased oxygen binding affinity of red blood cells (Wells 2009) or increased antioxidant production (Bickler and Buck 2007). All of these responses are different strategies for the organism to either maximize oxygen intake, conserve ATP expenditure, or reduce metabolic waste production for optimal survival during hypoxic conditions (Richards 2011; Wu 2002). Therefore, depending on the evolutionary history of the species, some are better suited than others to cope with dissolved oxygen (DO) fluctuations in their environment (Diaz and Breitburg 2009).

The objective of this study was to determine the hypoxia tolerance threshold (HTT) of the hypoxia-sensitive Striped Bass (*Morone saxatilis)* compared to the hypoxia-tolerant Blue Nile Tilapia (*Oreochromis niloticus)*. To determine HTT, both species were subjected to gradual hypoxia exposure and monitored for characteristic behavioral changes such as discomfort, agitation, listing, and loss of equilibrium. By doing this, a gradual hypoxia exposure protocol was developed for each species that would sufficiently challenge each species, yet allow for survival during rapid reoxygenation and sustained normoxia.

Striped Bass are an anadromous species of fish with a native range along the Atlantic coast spanning from the St. Lawrence River in Canada to the St. John River in Florida. There is evidence that the northern (Chesapeake Bay and higher) population of Striped Bass will migrate towards colder waters after summer spawning and before returning to home streams, whereas the southern population (particularly around the Savannah River) do not migrate to colder waters during the summer months (Dudley et al. 1977). Traditionally, Striped Bass will migrate to cooler

temperatures and oxygen rich habitats for optimal growth and recovery after spawning (Cech et al. 1984), and Striped Bass that tend to stay in oxygen-deprived, warmer waters either die or have reduced growth, metabolic function, and fitness (Brandt et al. 2009). These studies lend support to the idea that Striped Bass, in general, tend to be sensitive to environmental hypoxia.

Nile Tilapia belong to a group of cichlids that inhabit estuaries along the east African coast. These estuaries frequently fluctuate in their oxygen availability (Gupta and Acosta, 2004). Considering their evolutionary history, Nile Tilapia are known for being incredibly resistant to changes in dissolved oxygen levels, with ongoing research dedicated to uncovering their physiological coping mechanisms. For example, during hypoxia exposure Nile Tilapia will reduced cardiac output by up to 50% via bradycardia to limit the use of stored glycogen and metabolic waste accumulation during anaerobic respiration. (Lague et al. 2012; Speers-Roesch et al. 2010). Given the evolutionary history of Striped Bass and Nile Tilapia, we expected that Striped Bass would show signs of physiological stress sooner at higher levels of DO than Nile Tilapia during hypoxia exposure.

METHODOLOGY

Animals

All experiments were conducted following the guidelines set by IACUC and protocols were approved by the Georgia Southern University Animal Care Committee (IACUC # I14003). Striped Bass (*Morone saxatilis)* and Blue Nile Tilapia (*Oreochromis niloticus*) were given at least one month to recover from handling stress and adjust to their new environment before experimentation began. All fish were kept on a 14 hour light:10 hour dark photoperiod and were fed commercial

fish pellets ad libitum every other day, except 24 hours prior to experimentation. Since fish were housed in a static environment, 25% of aquarium water was changed daily. In addition, water quality (pH [6.8-7.5], ammonia [< 5 ppm], nitrate [< 40 ppm], nitrite [< 1.0 ppm]) was monitored daily and when levels exceeded these limits, 50% of the aquarium water was changed.

Juvenile, mixed-sex Striped Bass (n = 6, mean mass: 102.6 ± 43.2 g, mean caudal length: 17.9 ± 2.2 cm) were obtained from the Georgia Department of Natural Resources Richmond Hill Hatchery (Richmond Hill, Georgia, USA). Striped Bass were kept at Georgia Southern University in aerated 378.5 L tanks with 22 °C static brackish water (3 ppt salinity). Brackish water was mixed in lab using dechlorinated city of Statesboro tapwater with Instant Ocean® Sea Salt (United Pet Group, Blacksburg, VA). Striped Bass were fed Aquamax® Growers 500 fish pellets (Purina Mills, St. Louis, MO).

Juvenile, mixed-sex Nile Tilapia (n = 6, mean mass: 69.5 ± 23.0 g, mean caudal length: 13.8 ± 1.8 cm) were obtained from a local supplier in Bulloch County, Georgia, USA. Nile Tilapia were kept at Georgia Southern University in aerated 378.5 L tanks with 22 °C static freshwater (dechlorinated city of Statesboro tapwater). Nile Tilapia were fed Aquamax® Pond Fish 4000 fish pellets (Purina Mills, St. Louis, MO).

Hypoxia Tolerance Threshold (HTT)

To determine the HTT for Striped Bass and Nile Tilapia (n = 6, n = 5 respectively) an individual fish was placed into a 100 L tank with 22 °C static fresh water or brackish water at least 12 hours prior to the onset of the experiment. Dissolved oxygen (DO) was decreased approximately 10%

(Striped Bass) or 20% (Nile Tilapia) every 40 minutes by bubbling the water with nitrogen, until mortality or O_2 saturation percent reached zero. DO levels were continuously monitored with an YSI Pro2030 oxygen probe (YSI Inc., Yellow Springs, OH). A waterproof barrier was placed at the top of the water column, to prevent oxygen diffusion from the surrounding air and also to restrict fish engaging in ASR during hypoxia exposure. Every five minutes DO levels were recorded, as well as any changes in behavior. A series of hypoxia induced behaviors observed were: a) baseline swimming and ventilation, b) exaggerated ventilation (point of discomfort), c) increased movement in tank (point of agitation), d) slowed ventilation observed through opercular movement and listing (point of impending mortality), and e) loss of equilibrium (point of mortality).

Using the above behavioral observations, a gradual hypoxia step-down exposure was designed that both species could tolerate (no mortality but still stressed) for four hours plus reoxygenation and recovery (fig. 2.1). Therefore, each species were subjected to prolonged hypoxia exposure at DO ranges where they exhibited the most severe behavioral responses without mortality (table 2.1). Striped Bass and Nile Tilapia (n = 6, n = 5 respectively) were subjected to the following hypoxia step-down (fig. 2.1): DO was steadily decreased by approximately 10% (Striped Bass) or 20% (Nile Tilapia) every 40 minutes to each species' tolerance level, held there for four hours, followed by an hour reoxygenation to normoxia, and an hour recovery period in normoxia. Behavioral changes were continually observed to monitor levels of physiological stress (slowed ventilation and listing but no mortality) of both species during hypoxia exposure (fig. 2.1; table 2.1).

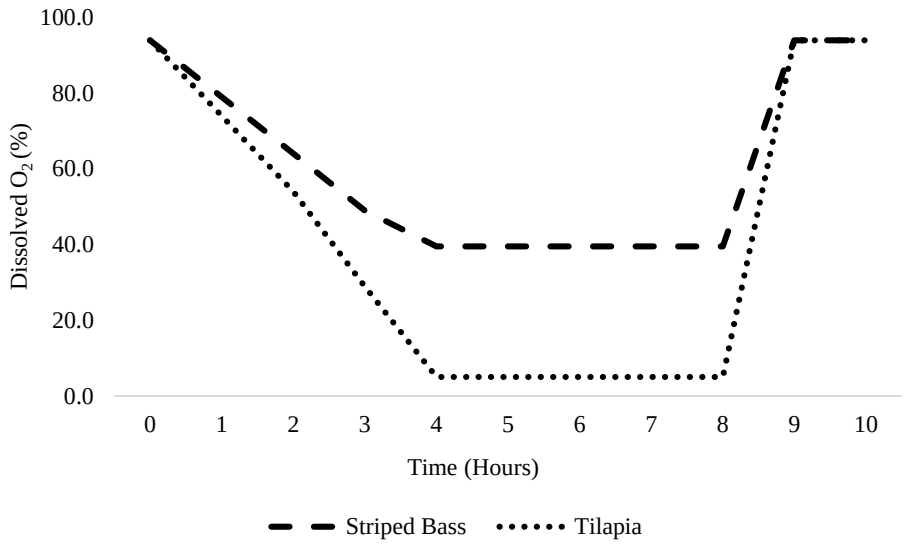

Figure 2.1: Experimental hypoxia exposure for Striped Bass (n = 6) and Nile Tilapia (n = 5). During hours 0-4 oxygen levels were decreased to either 40.0% for Striped Bass or 5% for Nile Tilapia. At hours 4-8 fish were held at their respective levels (hypoxia), hour 8-9 oxygen levels were increased to normoxia (reperfusion), hour 9-10 sustained normoxia (recovery).

Table 2.1 Observed behavioral responses in Striped Bass and Nile Tilapia during hypoxia exposure. Data is presented as means ± s.e. Values in parenthesis indicate sample size. A dashed line indicates that behavior was not observed during the trial.

	Striped Bass	**Tilapia**
Behavior	**Average Dissolved Oxygen (% SAT)**	
Normal Swimming	91.3 ± 1.9 (6)	96.5 ± 0.6 (5)
Exaggerated Ventilation	73.9 ± 2.2 (6)	6.4 ± 1.2 (5)
Increased Movement	62 ± 3.4 (6)	-
Decreased Ventilation	45.5 ± 0.9 (6)	-
Listing	34.4 (1)	-
Loss of Equilibrium	30.1 (1)	-

RESULTS

Striped Bass (total n = 6) started to show signs of exaggerated ventilation (point of discomfort) at 73.9 ± 2.2 % DO, increased movement (point of agitation) at 62 ± 3.4 %, and slowed ventilation coupled with listing (point of impending mortality) at 45.5 ± 0.9 % (table 2.1, fig. 2.2). At 34.4%, Striped Bass (n = 1) settled to the bottom of the aquarium and began leaning side to side, indicating the fish was starting to lose equilibrium (table 2.1). HTT was determined to be at 30.1% when Striped Bass (n = 1) lost equilibrium (table 2.1). Impressively, Nile Tilapia (total n = 5) only began to show signs of discomfort at 6.4 ± 1.2 % DO (table 2.1, fig. 2.2). Being a hypoxia tolerant species, Nile Tilapia did not lose equilibrium during hypoxia exposure and therefore HTT was not determined.

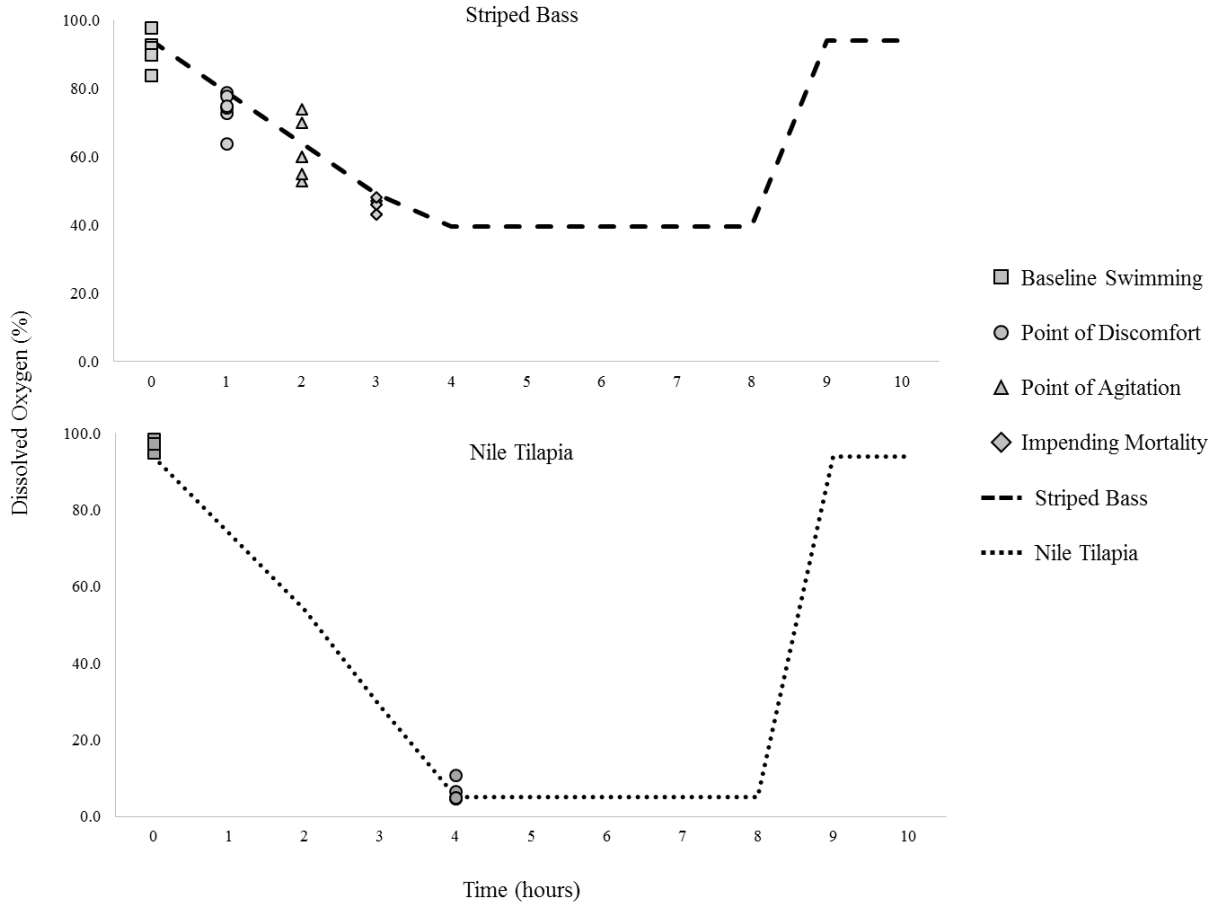

Figure 2.2. Initial onset of behavioral responses to hypoxia for individual Striped Bass *(Morone saxatilis)* (n = 6) and Nile Tilapia *(Oreochromis niloticus)* (n = 5).

DISCUSSION

As a point of departure for this project, it was critical to ascertain the ideal DO range to hold Striped Bass (hypoxia-sensitive species). HTT (point of mortality) for Striped Bass (n = 1) was determined to be 30.1% DO (table 2.1). The individual Striped Bass displayed characteristic behavioral responses (table 2.1) leading to mortality. It was suggested that similar behavioral responses (i.e. agitation and listlessness) are present in animals as a response to reduced oxygen availability (Mandic et al. 2009). Mandiac et al. 2009 tested this in various species of intertidal sculpin that were prevented from aerial surface respiration (ASR) during hypoxia exposure.

Intertidal zones fluctuate daily in oxygen availability, so it is expected that their behavioral responses would mimic the Nile Tilapia in this study. When these intertidal sculpin were prevented from (ASR), a behavioral adaptation for hypoxia survival (Chapman and McKenzie 2009), they showed signs of agitation and listlessness. These same signs were observed in the Striped Bass as oxygen levels decreased. For the remainder of the study, the observed behavioral responses in Striped Bass were used as stress indicators to determine an ideal DO range for hypoxia exposure (fig. 2.1). This ideal range for Striped Bass was empirically decided to be 10% above HTT (i.e. around 30% DO). Therefore, at 40% DO Striped Bass would be held at a range where they exhibited behaviors of impending mortality (46.2%), but did not show signs of losing equilibrium (34.4%)(table 2.1; fig. 2.2). Finally, Striped Bass (n = 5) can tolerate being decreased to and held at 40% for at least four hours without mortality (table 2.1; fig. 2.2). It was not anticipated that Striped Bass could survive such a low DO without mass mortality; since numerous environmental impact and Striped Bass health assessment studies have suggested negative impacts on the Striped Bass population in response to an increasing hypoxic habitat (Coutant 1985; Setzler-Hamilton et al. 1988). Additionally, this conflicts with previous finding by Cech et al. (1984), which showed that approximately half of Striped Bass in their study (18 out of 36) died when dissolved oxygen levels neared 32% DO or 3.5 mg/L O_2. Furthermore, they found that Striped Bass were unresponsive and showed little behavioral changes during hypoxia exposure (Cech et al. 1984). To understand the contradicting behavioral responses and hypoxia sensitivity that was found in our study, we must consider population bias. The Striped Bass used in Cech et al. 1984's study, was a northern population (New Jersey area) of Striped Bass that were introduced to California in 1879 (Fry 1973). Northern populations of Striped Bass are sensitive to DO changes which is partly the motivation for post-spawning migration during the summer. Our study used Striped Bass that

were obtained from a southern population near the Savannah River in Georgia. It is important to note that southern populations of Striped Bass do not migrate to cooler, more-oxygenated waters during the summer (May-August), even when the ocean temperatures near the Savannah River ranges between 22 to 29 °C. This sedentary response is uncommon in other populations of Striped Bass, as northern populations (Chesapeake Bay area) will migrate further north during the summer towards cooler water temperatures (8 to 16 °C) for post-spawning recovery (Dudley et al., 1977). Typically, water temperatures near the northern population spawning sites range from 16 to 25.5 °C (Dudley et al. 1977), which is on average 5 °C cooler than the waters that the non-migratory southern populations inhabit. This could explain why we did not observe rapid mortality, as southern Striped Bass do not migrate to cooler waters during the summer (Dudley et al. 1977), they may possess a limited ability for hypoxia tolerance. The reason why southern Striped Bass do not migrate during the summer months and the mechanisms that allow for limited hypoxia capabilities, could be a new direction to explore in future research projects. Additionally this could offer new insight into the physiology of hypoxia tolerant and sensitive species of fish since a natural comparison exists in one species.

Nile Tilapia (n = 5) began to show the first signs of discomfort at 6.4 ± 1.2 % DO (table 2.1, fig. 2.2). This finding is supported by previous studies suggesting Tilapia do not show signs of mortality for at least 24 hours at 5% DO (Speers-Roesch et al. 2010). Therefore, Nile Tilapia would be held at 5% DO during hypoxia exposure (fig. 2.2) to ensure they were mildly challenged. Further studies would need to be done to see if Nile Tilapia would show the other behavioral responses (i.e. agitation or impending mortality) if they were held at 5% DO for longer than four hours. Given this information, Nile Tilapia are great models to study the physiological coping

mechanisms that allow survival during hypoxia exposure. In this study, Nile Tilapia were not pushed past 5% DO because they would then be exposed to anoxia (a total lack of oxygen) and could potentially trigger different cellular and molecular responses (Fago and Jensen 2015).

CONCLUSION

In conclusion, Striped Bass began showing signs of stress at higher DO levels, suggesting that Striped Bass are more sensitive to hypoxia exposure than Nile Tilapia. However, we found that southern populations of Striped Bass do not appear as sensitive as previously thought, evident by the lack of mortality during hypoxia exposure and subsequent reoxygenation. Even though the Striped Bass in this study are not as sensitive as the literature suggested and would be considered mildly tolerant; they are sensitive enough in comparison to Nile Tilapia and would make good model organisms for comparing physiological changes during hypoxia and reperfusion.

CHAPTER 3

INVESTIGATION OF THE APOPTOTIC PATHWAYS IN STRIPED BASS *(Morone saxatilis)* AND NILE TILAPIA *(Oreochromis niloticus)* VENTRICLES DURING HYPOXIA AND REPERFUSION

INTRODUCTION

Cardiac tissue is particularly susceptible to reduced cellular ATP levels, because of the energy requirements necessary to sustain workload demands (Gamperl and Driedzic 2009). Hypoxic stress in the heart triggers a caspase cascade within cardiomyocytes that cause damage and if this damage cannot be repaired, it will eventually lead to cell death (Olivetti et al. 1997; Fago and Jensen 2015). Additional damage to cardiomyocytes can occur once oxygen supply is restored causing reperfusion injury. This sudden influx of oxygen levels results in an increased reactive oxygen species formation that causes cellular membrane damage or provides ATP for the initiation of apoptosis (Elmore 2007; Yellon and Hausenloy 2007). In humans, sufficient cardiomyocyte loss is fatal not only because cardiomyocytes possess a limited ability to regenerate once damaged (Chiong et al. 2011) but also because the cardiovascular system is vital for oxygen transfer and circulation throughout an organism (Gamperl and Driedzic 2009).

Nile Tilapia (*Oreochromis niloticus)* are known for being incredibly resistant to changes in dissolved oxygen levels, with ongoing research dedicated to uncovering their physiological coping abilities. For example, during hypoxia exposure Nile Tilapia will reduce cardiac output up to 50% via bradycardia to limit the use of stored glycogen and metabolic waste accumulation during anaerobic respiration. (Lague et al. 2012; Speers-Roesch et al. 2010). Measured responses (such

as DNA fragmentation, caspase 3/7 activity, and gene expression of target genes (table 3.1)) in the mildly, hypoxia-sensitive Striped Bass *(Morone saxatilis)* were compared to the hypoxia-tolerant Nile Tilapia (*Oreochromis niloticus),* to uncover if Nile Tilapia suppressed apoptosis as a survival mechanism during hypoxia exposure and subsequent reoxygenation. Our hypothesis is that Nile Tilapia, are able to survive hypoxia exposure because they are preventing apoptosis and/or actively repairing damage. Therefore, we expected to see less evidence of cellular apoptosis in Nile Tilapia cardiomyocytes when compared to Striped Bass cardiomyocytes after hypoxia and reperfusion injury. Additionally, we expected to find higher anti-apoptotic (*bcl2*, *flip,* and, *p53)* and repair (*hsp70)* gene expression and lower pro-apoptotic (*bax, caspase3,* and *fasl)* gene expression in the cardiomyocytes of Nile Tilapia compared to Striped Bass.

Table 3.1 List of target genes to be analyzed during RT-qPCR and justification for their selection.

Genes	**Function**	**Justification**
actb	Reference	Commonly used in RT-qPCR.
bax	Pro-Apoptotic	Initiates apoptosis by triggering cytochrome c release from the mitochondrion.
bcl2	Anti-Apoptotic	Prevents apoptosis by suppressing cytochrome c release of bax expression.
caspase3	Pro-Apoptotic	Initiates apoptosis by triggering the caspase cascade the ultimately leads to cell death.
ef1a	Reference	Shown to have the most stability in heart tissue exposed to stress.
fasl	Pro-Apoptotic	Initiates apoptosis by binding to the fas receptor and activing caspases.
flip	Anti-Apoptotic	Prevents apoptosis by inhibiting fasl binding.
hsp70	Repair	Actively repairs cellular damage caused by physiological stressors.
p53	Pro/Anti-Apoptotic	Will either suppress apoptosis to allow the cellular repair or initiate apoptosis if cell is too damaged.

METHODOLOGY

Animals

All experiments were conducted following the guidelines set by IACUC and protocols were approved by the Georgia Southern University Animal Care Committee (IACUC # I14003). Striped Bass (*Morone saxatilis)* and Blue Nile Tilapia (*Oreochromis niloticus*) were given at least one month to recover from handling stress and adjust to their new environment before experimentation began. All fish were kept on a 14 hour light:10 hour dark photoperiod and were fed commercial fish pellets ad libitum every other day, except 24 hours prior to experimentation. Since fish were housed in a static environment, 25% of aquarium water was changed daily. In addition, water quality (pH [6.8-7.5], ammonia [< 5 ppm], nitrate [< 40 ppm], nitrite [< 1.0 ppm]) was monitored daily and when levels exceeded these limits, 50% of the aquarium water was changed.

Juvenile, mixed-sex Striped Bass (n = 24, mean mass: 137.6 ± 36.0 g, mean caudal length: 19.9 ± 2.0 cm) were obtained from the Georgia Department of Natural Resources Richmond Hill Hatchery (Richmond Hill, GA). Striped Bass were kept at Georgia Southern University in aerated 378.5 L tanks with 22 °C static brackish water (3 ppt salinity). Brackish water was mixed in lab using dechlorinated city of Statesboro tapwater with Instant Ocean® Sea Salt (United Pet Group, Blacksburg, VA). Striped Bass were fed Aquamax® Growers 500 fish pellets (Purina Mills, St. Louis, MO).

Juvenile, mixed-sex Nile Tilapia (n = 24, mean mass: 86.5 ± 26.3 g, mean caudal length: 14.4 ± 1.4cm) were obtained from a local supplier in Bulloch County, Ga. Nile Tilapia were kept at Georgia Southern University in aerated 378.5 L tanks with 22 °C static freshwater (dechlorinated

city of Statesboro tapwater). Nile Tilapia were fed Aquamax® Pond Fish 4000 fish pellets (Purina Mills, St. Louis, MO).

Sampling

At least 12 hours prior to hypoxia exposure, either four Striped Bass or tilapia were transferred from the holding tanks to 100 L experimental tanks (with 22 ºC static, aerated freshwater or brackish water). Dissolved oxygen (DO) was decreased by switching the air supply from O_2 to N_2 following the exposure protocol shown in fig. 3.1. DO levels were monitored every five minutes with an YSI Pro2030 oxygen probe (YSI Inc., Yellow Springs, OH). A waterproof barrier was placed at the top of the water column, to prevent environmental oxygen diffusion and restricted fish from accessing the oxygenated surface water for aerial surface respiration during hypoxia exposure. Fish (n = 24 per species; with 6 biological replications across four timepoints) were sampled at four key time points: 1) prior to hypoxia exposure (control), 2) after four hours at each species' tolerance level (hypoxia), 3) immediately upon return to normoxia (reperfusion), and 4) after four hours at normoxia (recovery) (fig. 3.1). At each time point, fish were netted and anesthetized in a 2 mg/L clove oil (9 parts ethanol: 1 part clove oil) solution and terminated via cervical displacement. Ventricles were extracted, frozen in liquid nitrogen, and stored at -80 °C. Ventricle samples were divided among the following analyses: DNA fragmentation assay, Caspase 3/7 assay, and gene expression analysis with RT-qPCR.

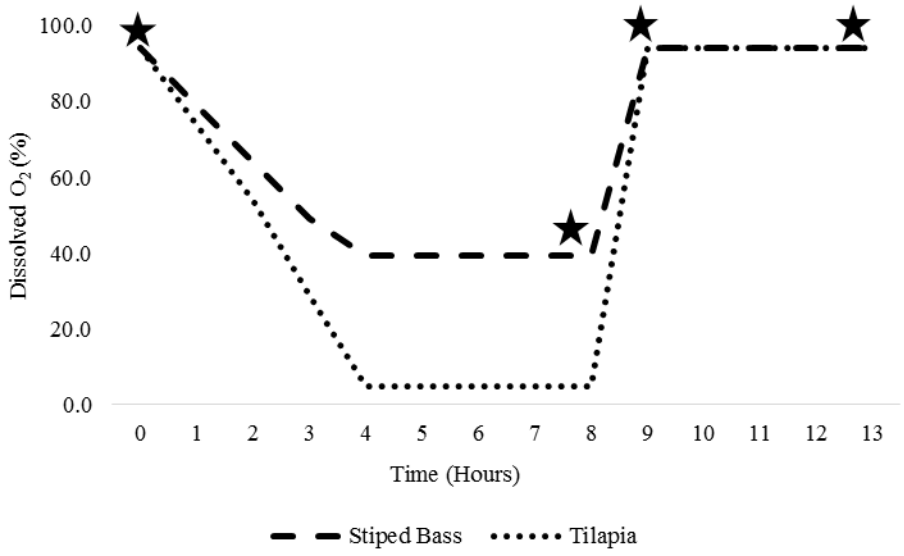

Figure 3.1. Schematic of hypoxia exposure and recovery protocol for Striped Bass (*Morone saxatilis)* and Nile Tilapia (*Oreochromis niloticus*). Stars indicate sampling timepoints: control/0h, hypoxia/8h, reperfusion/9h, and recovery/13h (n = 6/species/timepoint).

DNA Fragmentation Assay

Ventricles were homogenized in 0.5 ml of DNAzol® (Life technologies, Grand Island, NY) reagent with a motorized hand pestle. Genomic DNA was extracted following manufacture's protocol with the following modifications: during the DNA precipitation step, the homogenate/ethanol solution was instead incubated at 4 ºC for 30 minutes and then centrifuged at 10,000 x g for 30 minutes at 4 ºC. Genomic DNA (2 µg/12 µl) and a 1 kb DNA ladder (control) were examined on a 2% agarose gel stained with ethidium bromide (2h at 100v). Fragmentation was assessed qualitatively by the presence or absence of a laddering effect in the sample.

Caspase 3/7 Assay

Ventricles were homogenized in 25 mM HEPES (pH 7.5), 0.1% Triton X-100, 5 mM $MgCl_2$, 2 mM DTT, 74 µM antipain, 0.15 µM aprotinin, 1.3 mM EDTA, 1.0 mM EGTA, 15 µM pepstatin and 20 µM leupeptin (1 mg tissue:10 µl buffer) with a motorized hand pestle. Homogenate was centrifuged at 50,000g and the supernatant was collected. The supernatant was measured for caspase 3/7 activity using the Apo-ONE® Homogenous Caspase-3/7 Assay (Promega, Madison, WI) following manufacture's protocol. Homogenate incubated with caspase 3/7 reagent at room temperature (22 °C) and then measured for fluorescence every 30 minutes. Data was selected from the linear portion of the trace before enzyme activity plateaued and stabilized. Fluorescence of each sample was measured on a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 521nm at room temperature (22 °C). Wagner et al. 2003 provided the protocol to use tissue samples with Apo-ONE® Homogenous Caspase-3/7 Assay. Activity of Caspase 3/7 was expressed as relative fluorescence units (RFU) per mg of tissue.

RNA Extraction

Ventricles were homogenized in 0.5 ml of RNAzol®RT reagent (Molecular Research Center Inc., Cincinnati, OH) with a motorized hand pestle. Messenger RNA (mRNA) was extracted following the manufacturer's protocol with the subsequent modification: total RNA supernatant was mixed with 75% ethanol (0.4 ml ethanol:1 ml of RNAzol®RT) and stored in -20 °C for 48 hours before proceeding to RNA washes and solubilization. Extracted mRNA was stored at -20 °C until reversed transcribed into cDNA.

cDNA synthesis

To degrade residual genomic DNA, mRNA was treated with DNase 1 following the manufacturer's protocol (Invitrogen™, Life Technologies, Grand Island, NY). mRNA was reversed transcribed into cDNA using RevertAid Reverse Transcriptase (Invitrogen™, Life Technologies, Grand Island, NY) according to the manufacturer's protocol. cDNA was stored at -20 °C until used for target gene primer testing or RT-qPCR (Reverse Transcription-quantitative Polymerase Chain Reaction) analysis.

Primers for RT-qPCR

Primers to measure gene expression in target genes (table 3.2) were either manually designed, or obtained from literature. To test primers, PCR was performed using Platinum®Taq (Life Technologies, Grand Island, NY), pooled cDNA (mixture of cDNA from all time points) and 10µM of forward and reverse primers of either target or reference genes. Duplicate reactions were completed on a BIO-RAD T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) for 3 minutes at 94 °C, followed by 38 cycles of 30 seconds at 94 °C, 30 seconds at 58 °C, and 30 seconds at 72 °C, and a final extension of 15 minutes at 72°C. Negative controls were included during each run and contained all components of the reaction except the cDNA template, which was replaced with DEPC-treated nuclease-free water (Life Technologies, Grand Island, NY). PCR results were visualized on a 1% agarose gel stained with ethidium bromide. PCR products of 150-300 bp (fig. 3.4) were exercised from the gel and stored at 4 °C until ready for sequence verification.

Table 3.2. List of primer sequences and sources of primers that were tested in this study to isolate target genes.

Primer Name	Sequence	Source	Accession #	Designed For
18s-FWD	5-GGCAACCAACGGTAAAACAA-3	Huang et al. 2015	DQ397879	Nile Tilapia
18s-RVS	5-AGCTAGCTGCGTTCTTCATTG-3			
actb-FWD	5-GTGCAAAGCCGGATTCGC-3	Heino and Lewis 2013	N/A	Longhorn Sculpin
actb-RVS	5-CAATACCGTGCTCAATGGG-3			
bax-FWD	5-TACTTGACTTGGGAGCTGCA-3	Self-Designed	NM131562	Zebrafish
bax-RVS	5-TTACCATACTTAGCACGTGG-3			
bax-FWD	5-TACGGACATGGCTTGTGAAG-3	Self-Designed	DQ860150	Zebrafish
bax-RVS	5-AGCTGAGAGAACAAATACGT-3			
bax-FWD	5-ATGACCAGATACTTGACTTG-3	Self-Designed	NM131562	Zebrafish
bax-RVS	5-TACTTAGCACGTGGGATTCC-3			
bax-FWD	5-TTCCCCATACACGTTTTAGC-3	Self-Designed	DQ860150	Zebrafish
bax-RVS	5-CACGAATCTGTTATTCGAAC-3			
bax-FWD	5-GCCGAATCCTTATCCTAATGG-3	Mai et al. 2010	N/A	Nile Tilapia
bax-RVS	5-AGGGACTTCCACCAGATG-3			
bax-FWD	5-GGCTATTTCAACCAGGGTTCC-3	Zhange et al. 2012	AF231015	Goldfish
bax-RVS	5-TGCGAATCACCAATGCTGT-3			
bax-FWD	5-GATACGGGCAGTGGCAATGA-3	Ding et al. 2013	N/A	Zebrafish
bax-RVS	5-ACTCCGGGTCACTTCAGCAT-3			
bax-FWD	5-AGTGTTTGCAGCAGATCGGAGATG-3	Ji et al. 2011	N/A	Zebrafish
bax-RVS	5-TGACAAGGCGACAGGCAAAGTAGA-3			
bax-FWD	5-AAGCTGAGCGAGTGTCTCCGGCG-3	Suo et al. 2015	N/A	Yellow Croacker
bax-RVS	5-CAGATGCCGGTTCAGGTACTCAGTC-3			
bcl2-FWD	5-CGAGTGTGTGGAGAAGGAGATG-3	Ding et al. 2013	N/A	Zebrafish
bcl2-RVS	5-TGGTTGTCTAGGTAGACGGTCAT-3			
bcl2-FWD	5-ACCTGCACACCTGGATCCA-3	Chang et al. 2010	N/A	Tilapia Mossambic
bcl2-RVS	5-AGAGACAGCCAGGAGAAATCAAA-3			

Primer Name	Sequence	Source	Accession #	Designed For
bcl2-FWD	5-TCTTCGAGTTTGGTGGGACCATGT-3	Ji et al. 2011	N/A	Zebrafish
bcl2-RVS	5-TACATCTCCACGAAGGCATCCCAA-3			
bcl2-FWD	5-CTCGTCGCTACCGTCGTGACTTGG-3	Suo et al. 2015	N/A	Yellow Croacker
bcl2-RVS	5-CAGATGCCGGTTCAGGTACTCAGTC-3			
bcl2-FWD	5-AAGCGAGGATATGTGTGGAA-3	Self-Designed	NM001030253	Zebrafish
bcl2-RVS	5-TCGAGAGTAAGAAGTATACC-3			
bcl2-FWD	5-TGTGGAGAAATACCTCAAGC-3	Self-Designed	NM001030253	Zebrafish
bcl2-RVS	5-CTGAAAACTGACACTACGAG-3			
bcl2-FWD	5-ATGTCATCCCAGGTGGACAA-3	Self-Designed	XM003452417.2	Nile Tilapia
bcl2-RVS	5-TCCATCAAGACAGTTTTCGGCT-3			
caspase3-FWD	5-AAAGGATCCCAGTGGAGGCAGATT-3	Ji et al. 2011	NM131877	Zebrafish
caspase3-RVS	5-TGGTCATGATCTGCAAGAGCTCCA-3			
caspase3-FWD	5-CTGCTGGGGATGGCCACTGTG-3	Suo et al. 2015	N/A	Yellow Croacker
caspase3-RVS	5-TCGCCTCGAGGACATCGCTCTC-3			
ef1a-FWD	5-CAGGTCATCATCCTGAACCA-3	Aursnes et al. 2011	N/A	Atlantic Cod
ef1a-RVS	5-ATCCAGGACTGGGGCATAG-3			
ef1a-FWD	5-CTTCTCAGGCTGACTGTGC-3	McCurley and Callard 2008	N/A	Salmon
ef1a-RVS	5-CCGCTAGCATTACCCTCC-3			
ef1a-FWD	5-TGACTGCGCTGTGCTGATC-3	Speers-Roesch et al. 2010	KJ123689.1	Tilapia Hybrid
ef1a-RVS	5-CTTGGAGATACCAGCCTCGA-3			
fasl-FWD	5-GGATTGGGCCTGGGGATGTTTCA-3	Suo et al. 2015	N/A	Yellow Croacker
fasl-RVS	5-TTGTGGCTCAGGGGCAGGTTGTTG-3			
fasl-FWD	5-GTCCCAACCAGTGTTCATGG-3	Self-Designed	DQ812115.1	Zebrafish
fasl-RVS	5-CGGTTGAGCCAAGAGAAGTC-3			
fasl-FWD	5-GTGTTCATGGTGGATTCTGC-3	Self-Designed	DQ812115.2	Zebrafish
fasl-RVS	5-ACCGACAAGCAGTTACAGAG-3			
fasl-FWD	5-CCAAGGGCGATTCTAAGGTC-3	Mai et al. 2010	N/A	Nile Tilapia
fasl-RVS	5-ATCTCCCTGAGTGGCTGTGC-3			

Primer Name	Sequence	Source	Accession #	Designed For
fasl-FWD	5-ACTTCCTCTAAAATCTTGGCAC-3	Domenico et al. 2013	N/A	European Seabass
fasl-RVS	5-GCATCACAGTCTTCTCCTCTC-3			
flip-FWD	5-TGTCAGCAAAAGATGAAGCT-3	Self-Designed	BC116570.0	Zebrafish
flip-RVS	5-GAATCGTATCGATGTATGAC-3			
flip-FWD	5-AAGATGAAGCTCGCTGCTGG-3	Self-Designed	BC116570.1	Zebrafish
flip-RVS	5-AGTGAACACCGTCGATTATG-3			
flip-FWD	5-CGTCGTAGAGGAGTGCTTGAG-3	Self-Designed	XM005453062	Nile Tilapia
flip-RVS	5-AGAAGTGAGCCCACGCATAGG-3			
flip-FWD	5-TTGGGCACAGATTCGACTCCA-3	Self-Designed	XM005453062	Nile Tilapia
flip-RVS	5-ACAACGTACCTAAGTTGTCCC-3			
hsp70-FWD	5-GACATGAAGCACTGGC-3	Mladineo et al. 2009	NM001279671.1	Sculpin
hsp70-RVS	5-AGGACCATGGAGGAG-3			
hsp70-FWD	5-CATCCTTTCTGGGGACAAGTCAG-3	Geist et al. 2007	AY423555	European Seabass
hsp70-RVS	5-ACACCTCCAGCGGTCTCAATAC-3			
hsp70-FWD	5-GGAGTTCAAGCGGAAGTTCA-3	Aursnes et al. 2011	N/A	Atlantic Cod
hsp70-RVS	5-AGCCTCCTCAAAGCCCTCT-3			
hsp70-FWD	5-CAACAACCTGCTGGGCAAA-3	Keegan et al. 2002	N/A	Atlantic Cod
hsp70-RVS	5-GCGTCGATGTCGAAGGTCA-3			
p53-FWD	5-CTCAGAGGAGGCGCCATG-3	Suo et al. 2015	N/A	Yellow Croacker
p53-RVS	5-GGGCGGATTAGGGCTTCC-3			
p53-FWD	5-ATAAGAGTGGAGGGCAATCAGCGA-3	Ji et al. 2011	NM131327	Zebrafish
p53-RVS	5-AGTGATGATTGTGAGGATGGGCCT-3			
p53-FWD	5-GGACTGGCAATGAGAAGG-3	Mai et al. 2010	N/A	Nile Tilapia
p53-RVS	5-CTGGTTGTTGACGTAATGCT-3			

Table 3.3. Results from PCR analysis of primer sets from table 3.2.

Gene	# of sets tested	Results
actb	1	100-150 base pair band was visible for **both** Striped Bass and Nile Tilapia
bax	9	100-150 base pair band was visible for Striped Bass **only**
bcl2	7	100-150 base pair band was visible for Striped Bass **only**
caspase3	3	100-150 base pair band was **not** visible for both Striped Bass and Nile Tilapia
ef1a	2	100-150 base pair band was visible for **both** Striped Bass and Nile Tilapia
fasl	5	100-150 base pair band was visible for Nile Tilapia **only**
flip	4	100-150 base pair band was **not** visible for both Striped Bass and Nile Tilapia
hsp70	4	100-150 base pair band was visible for **both** Striped Bass and Nile Tilapia
p53	2	100-150 base pair band was **not** visible for both Striped Bass and Nile Tilapia

Primer Verification with Blue/White Screening

Extra measures like primer verification and sequencing are critical because correctly designed primers are imperative to obtain accurate gene expression values from RT-qPCR (Quellhorst and Rulli 2008).Following the manufacturer's protocol, PCR products were extracted and purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The extracted PCR products were cloned into DH5α competent cells (Life Technologies, Grand Island, NY) using the QIAGEN PCR cloning kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Transformed cells were plated at 50 and 100 µl densities on LB agar plates (100 µg/ml ampicillin, 50 µg/ml kanamycin, 20 mg/ml X-gal, and 10mM IPTG) for blue/white screening. All antibiotics and chemicals for plates were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Plates were incubated for 16 hours at 37 °C and then placed in 4° for at least 24 hours to enhance blue color in cells. An individual white colony was picked and placed in 5 ml of LB broth (100 µg/ml ampicillin, 50 µg/ml kanamycin) and shook at 250 rpm for 16 hours at 37 °C. Plasmid DNA was extracted and purified from the bacterial cells grown from white colonies using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), following the manufacturer's protocol. Extracted plasmid DNA was digested with EcoRI (Life Technologies, Grand Island, NY; digested plasmid DNA were visualized on a 1% agarose gel stained with ethidium bromide to verify that the plasmid DNA had two fragments: one of approximately 3500 bp (cloning vector) and 150 bp (PCR product). Purified recombinant plasmids were sequenced (Clemson University Genomics Institute) using T7 and SP6 forward and reverse primers and inserts (PCR products) were verified using BLASTx at the National Center for Biotechnology Information (NCBI).

Quality testing of primers for QRT-PCR analysis

Primers that were verified via sequencing and subsequent nucleotide blast search were checked for ideal primer and template concentrations by setting up a standard curve with template cDNA dilutions. A standard curve was established using a 1:5 serial dilution with template cDNA concentrations ranging from 1:1 to 1:3125. Two controls were used: 1) a non-template control that contained all the components for reverse transcription but replaced template with DEPC-treated nuclease-free water and 2) a no reverse transcription control that had all the components for reverse transcription but replaced transcriptase with DEPC-treated nuclease-free water. To test efficiency of primers, RT-qPCR was performed using SYBR® Green Mastermix (Applied Biosystesm, Grand Island, NY), serial dilutions of template cDNA with wither actb, ef1a, or hsp70 FWD and RVS primers at concentrations of 1.0, 2.0, and 2.5 µM. Reactions were completed in duplicate using a Mastercycler RealPlex2 (Eppendorf North America, Hauppauge, NY) for 15 minutes at 95 °C, followed by 38 cycles of 30 seconds at 95 °C, 30 seconds at 58 °C, and 30 seconds at 72 °C, and a final extension of 15 minutes at 72°C. Quantification cycle (C_q) values obtained from the mastercycler were used to generate a standard curve and to calculate R^2 values, amplification efficiency, and efficiency percent. Primers with a high efficiency (<120%) were checked for primer dimers via a melt curve analysis.

Statistical Analysis

All data was checked for normality ($P > 0.05$). Nonparametric data was log transformed and reanalyzed. For caspase 3/7 analysis, comparisons across time and species were made using a Kruskal-Wallis test. All statistical tests were performed using JMP Pro Version 10.0

RESULTS

DNA Fragmentation

DNA extracted from Striped Bass and Nile Tilapia ventricles at control/0h, hypoxia/8h, reperfusion/9h, and recovery/13h were analyzed by gel electrophoresis for evidence of DNA fragmentation, a marker of late stage apoptosis. If DNA fragmentation is present in the samples, then fragmented pieces of approximately 150 bp will be present on the gel rather than a continuous smeared band of DNA or an individual band of DNA at the top of the gel. In the control/0h samples, there was a continuous smeared band of DNA that was repeated across all timepoints (representative results are pictured in fig. 3.2). For all samples, the DNA showed a continuous smeared band, rather than distinct multiple fragments. Therefore, no differences in DNA integrity were observed across timepoints within a species or between Striped Bass and Nile Tilapia.

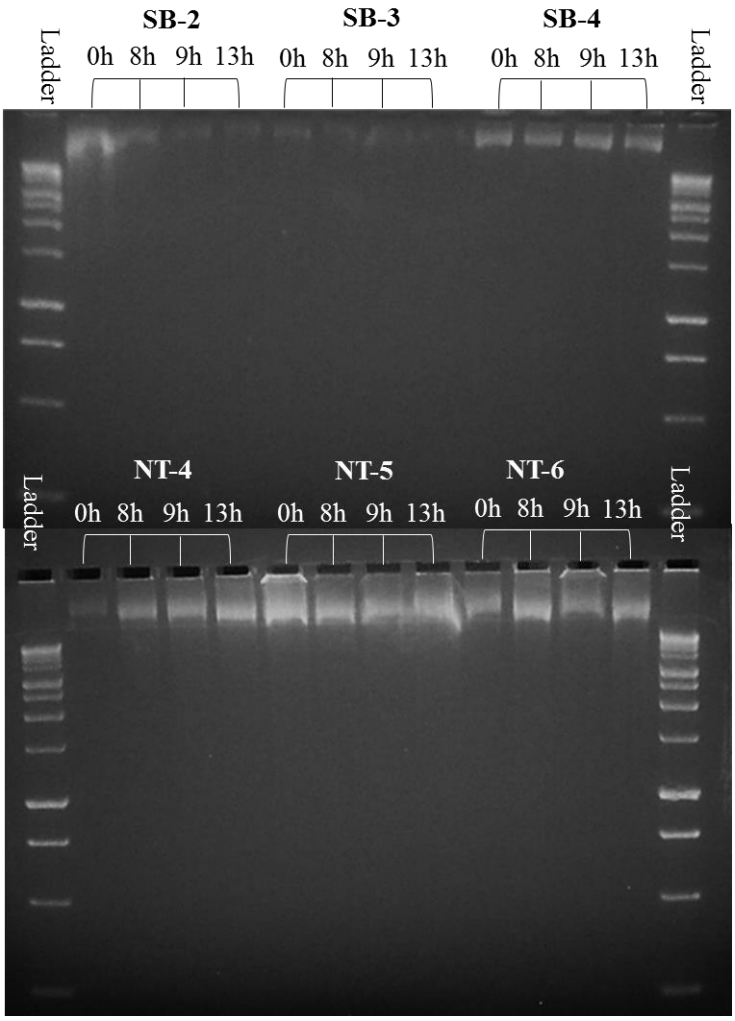

Figure 3.2. Representative gels from the DNA fragmentation assay. Striped Bass (SB) and Nile Tilapia (NT) ventricles (n = 6 per species per timepoint) were dissected at control/0h, hypoxia/8h, reperfusion/9h, and recovery/13h.

Caspase 3/7 Activity

Homogenized Striped Bass and Nile Tilapia ventricles at control/0h, hypoxia/8h, reperfusion/9h, and recovery/13h were measured for caspase 3/7 activity, a marker of early stage apoptosis. Activity of Caspase 3/7 was expressed as relative fluorescence units (RFU) relative to background fluorescence (107.6 ± 6.2 RFU/mg for both species) per mg of tissue. Caspase 3/7 activity at the

control/0h timepoint in Striped Bass was 1028.35 ± 80.2 RFU/mg (fig. 3.3). Each subsequent timepoint in Striped Bass ventricles decreased in caspase activity from the control: hypoxia/8h at 980.5 ± 99.7 RFU/mg; reperfusion/9h at 808.12 ± 73.7 RFU/mg; and recovery/13h at 769.9 ±169.3 RFU/mg (fig. 3.3). However, there was no significant difference between control/0h samples from the hypoxia/8h, reperfusion/9h, and recovery/13h samples (Kruskal-Wallis, $p = 0.86$). Caspase 3/7 activity at the control/0h timepoint in Nile Tilapia was 306.6 ± 38.6 RFU/mg (fig. 3.3). Caspase activity in both the hypoxia/8h timepoint (291.2 ± 65.2 RFU/mg) and reoxy/9h timepoint (280.6 ± 36.0 RFU/mg) decreased from the control/0h timepoint (fig. 3.3). The recovery/13h timepoint (350.6 ± 44.1 RFU/mg) in Nile Tilapia had higher levels of caspase 3/7 activity than the control/0h, hypoxia/8h, and reoxy/9h timepoints (fig. 3.3). Again there was no significant difference between control/0h samples from the hypoxia/8h, reperfusion/9h, and recovery/13h samples (Kruskal-Wallis, $p = 0.86$). However, caspase 3/7 activity were on average 2.5 to 3-fold higher in Striped Bass samples than in Nile Tilapia samples (fig. 3.3; Kruskal-Wallis, $p = 0.0001$).

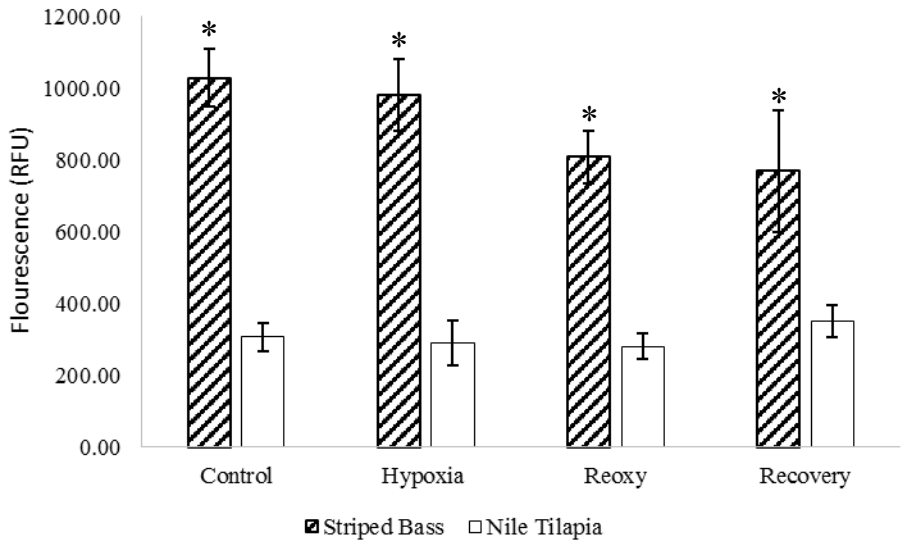

Figure 3.3. Caspase 3/7 activity (RFU/mg) for Striped Bass and Nile Tilapia. Ventricles (n = 6 per species per timepoint, except for control Striped Bass (n = 5)) were dissected at control/0h, hypoxia/8h, reperfusion/9h, and recovery/13h. Data is presented as mean ± s.e. Asterisk denotes significant difference between species ($p<0.0001$, Kruskal-Wallis).

Primers for RT-qPCR

Of the 78 total primer sets tested (table 3.2) on both Striped Bass and Nile Tilapia cDNA, only nine primer sets were successful in amplifying a product of the correct amplicon size (table 3.3; fig. 3.4). These were the PCR products associated with primer sets: *actb* (Striped Bass and Nile Tilapia), *bax* (Striped Bass), *bcl2* (Striped Bass), *ef1a* (Striped Bass and Nile Tilapia), *fasl* (Nile Tilapia), and *hsp70* (Striped Bass and Nile Tilapia). Of those nine primer sets, only five primers sets (*actb* (Striped Bass and Nile Tilapia), *ef1a* (Nile Tilapia), and *hsp70* (Striped Bass and Nile Tilapia)) were confirmed, using DNA sequencing, to have correctly isolated target genes from cDNA of either Striped Bass or Nile Tilapia samples (table 3.4).

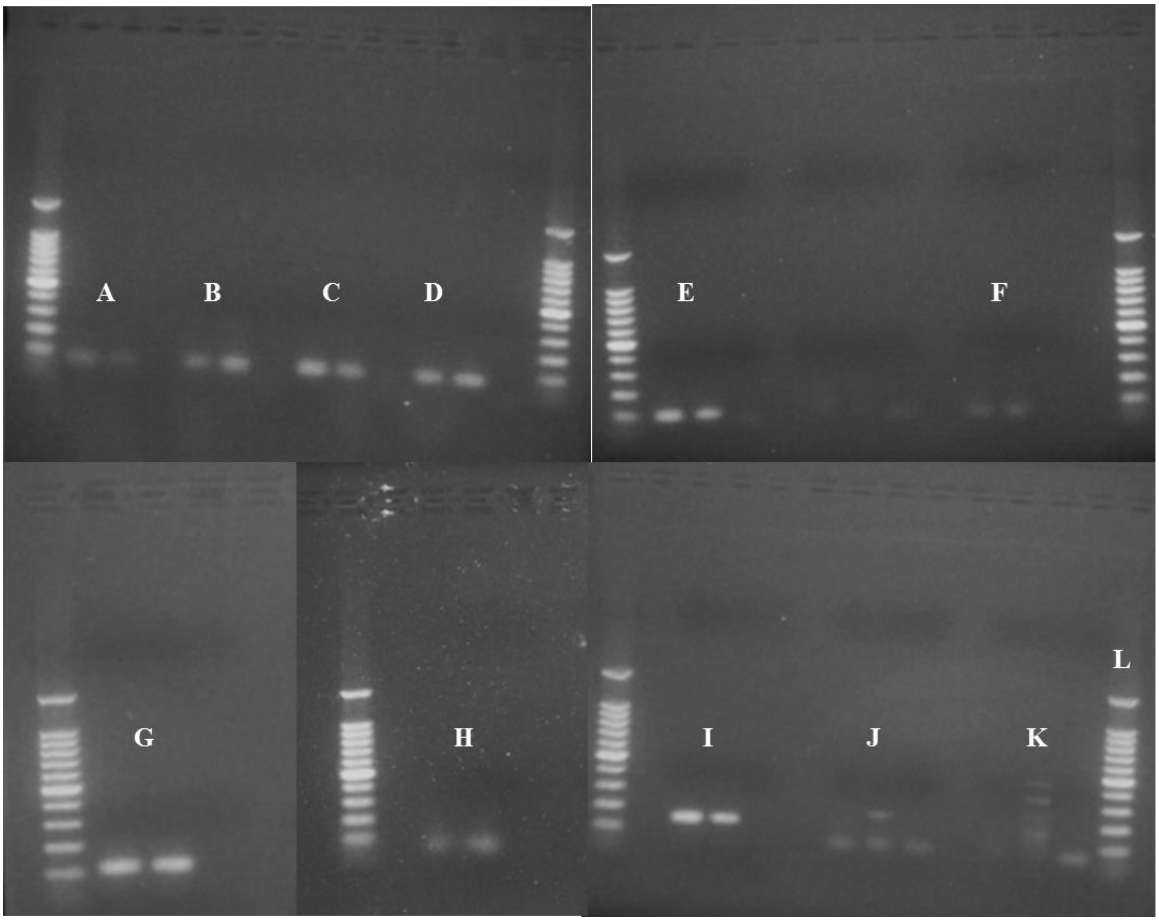

Figure 3.4. Gel images from successful primer sets. Gel images from successful primer sets: A) *actb* (Striped Bass), B) *actb (*Nile Tilapia), C) *ef1a* (Striped Bass), D) *ef1a (*Nile Tilapia), E) *hsp70* (Striped Bass), F) *hsp70 (*Nile Tilapia), G) *bax* (Striped Bass), H) *bcl2* (Striped Bass), I) *fasl (*Nile Tilapia), J) example of primer dimers, and K) example of non-specific annealing. L) 100bp molecular weight DNA ladder

Table 3.4. Results from sequenced plasmid DNA and NCBI nucleotide blast search.

Name	Sequence 5'→3'	Source	Species	Accession #	Amplicon
actbF	GTGCAAAGCCGGATTCGC	Heino and Lewis 2013	Striped Bass	AJ537421.1	179
actbR	CAATACCGTGCTCAATGGG				
actbF	GTGCAAAGCCGGATTCGC	Heino and Lewis 2013	Nile Tilapia	KJ126772.1	179
actbR	CAATACCGTGCTCAATGGG				
ef1aF	TGACTGCGCTGTGCTGATC	Speers-Roesch et al. 2010	Nile Tilapia	KJ123689.1	61
ef1aR	CTTGGAGATACCAGCCTCGA				
hsp70F	GACATGAAGCACTGGC	Mladineo et al. 2009	Nile Tilapia	NM_001279671.1	116
hsp70R	AGGACCATGGAGGAG				
hsp70F	CATCCTTTCTGGGGACAAGTCAG	Geist et al. 2007	Striped Bass	AY423555	93
hsp70R	ACACCTCCAGCGGTCTCAATAC				

Quality testing of primers for RT-qPCR analysis

Each verified primer set (*actb* (Striped Bass and Nile Tilapia), *ef1a* (Striped Bass and Nile Tilapia), and *hsp70* (Striped Bass and Nile Tilapia)) was quality tested to determine ideal assay conditions for RT-qPCR analysis. The following criteria were checked for ideal assay conditions: 1) high r^2 value (>0.99), 2) an efficiency range 85-115%, 3) all genes amplifying around the same Ct value, and 4) no amplification in NTC and No RT controls. Each verified primer set was tested at a starting primer concertation of 2.5 µM and then subsequently decreased until ideal primer efficiency was reached. Primer sequences and respective calculated efficiencies are reported in table 3.5 and representative images from melt curve analysis for primers with high efficiencies (< 120%) are presented in fig. 3.5. Of the five verified primers, only *ef1a* and *hsp70* Nile Tilapia primers (table 3.5) landed within the ideal efficiency (85-115%) for RT-qPCR (Quellhorst and Rulli 2008).

Table 3.5. Results from primer efficiency and optimization.

Primer	Species	Conc.	R^2	Amplification	Efficiency Percent
ef1a	Nile Tilapia	2.5 µM	0.974	2.03	102.91%
hsp70	Nile Tilapia	1.0 µM	0.994	2.13	112.70%

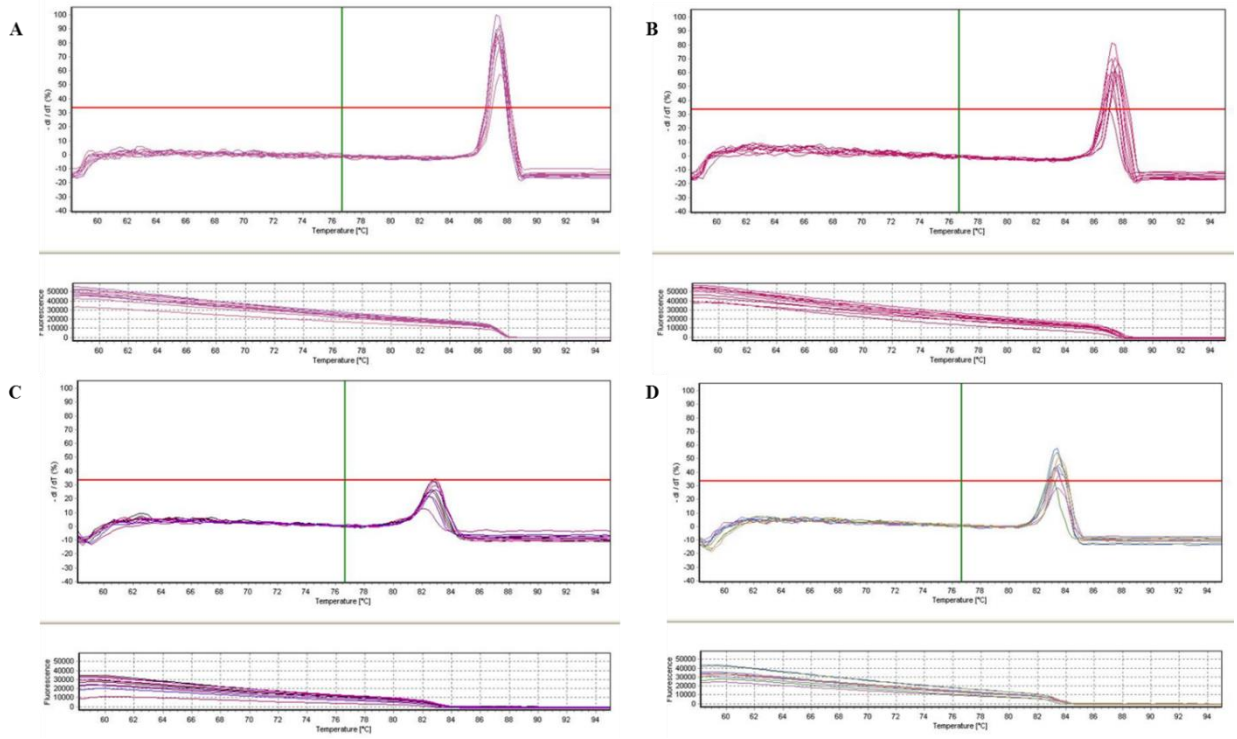

Figure 3.5. Melt curve analysis of a) *actb* (Nile Tilapia), b) *actb* (Striped Bass), c) *ef1a* (Striped Bass), and d) *hsp70* (Striped Bass). All forward and reverse primers were tested at a concentration of 1.0 µM, the lowest concentration possible for RT-qPCR analysis.

DISCUSSION

Cellular Investigation of Apoptotic Pathways

Apoptosis is crucial to removing cells that have been damaged by physiological stress but did not reach the damage threshold for other forms of cell death like necrosis or autophagy to occur (Krumschnabel and Podrabsky 2008). Once cell death has been initiated in the cell, the DNA begins to break down into approximately 50-300 bp fragments. This irreversible process is considered a characteristic marker of apoptosis and occurs in the final stages of apoptosis after the cell has already committed to death (Elmore 2007). Both Striped Bass and Nile Tilapia

cardiomyocytes were checked for DNA fragmentation caused by hypoxia exposure and reperfusion injury. However, in both Striped Bass (hypoxia-sensitive species) and Nile Tilapia (hypoxia-tolerant species) there was no evidence of DNA fragmentation, even at the 8h (hypoxia) and 9h (reperfusion) timepoints (fig. 3.2). This finding goes against our stated hypothesis, since some cellular damage was expected to occur in Striped Bass during hypoxia exposure and subsequent reoxygenation, because preliminary behavior results indicated that Striped Bass were not tolerant of low oxygen levels. Since DNA fragmentation is a downstream response in the apoptotic pathways (Elmore 2007), this does not mean that damage or cell death was not incurred by the cardiomyocytes. Therefore, levels of caspase 3/7 were measured to determine if cellular indicators were triggered at earlier points in the apoptotic pathways.

Caspase 3/7 activity was measured to test if any cellular damage manifested earlier at critical timepoints, hypoxia/8h and reperfusion/9h. Caspases are cysteine proteases that exist in an inactive state within the cell (Takle and Andersen 2007). Once mitochondrial injury or ligand/receptor binding occurs in response to physiological stress, initiator caspases are cleaved into an active form causing a cascading effect throughout cell which converges with caspases 3 and 7 (Dos Santos et al. 2008). If there is enough ATP present in the cell, these caspase will initiate apoptosis (Elmore 2007). Results from the caspase 3/7 assay show that there was no significance difference in activity across timepoints in both species (fig. 3.3; Kruskal-Wallis, $p = 0.86$). These results were unexpected because cellular evidence of apoptosis was hypothesized to be observed in Striped Bass cardiomyocytes since they are more sensitive to hypoxia than Nile Tilapia. Interestingly, caspase 3/7 activity was on average 2.5 to 3-fold higher in Striped Bass samples than in Nile Tilapia samples (fig. 3.3; Kruskal-Wallis, $p = 0.0001$). It was unexpected to find that the more

sensitive species of the two had overall higher levels of caspase 3/7 activity. After reviewing the current literature, it was found that caspase 3/7 levels in unstressed Climbing Perch (*Anabas testudineus*) are around 800 RFU/mg (Ching et al. 2013) and in unstressed Pacific Oysters (*Crassostrea gigas*) are around 1500 RFU/mg (Rolland et al. 2014). These studies suggest that caspase 3/7 activity is species-specific, and that overall higher levels of caspase 3/7 in a species is not necessarily indicative of presence of cell death. Caspase 3/7 data coupled with data from the DNA fragmentation assay suggest that apoptotic cell death did not occur in either Striped Bass or Nile Tilapia cardiomyocytes in response to hypoxia or reoxygenation. Since cellular indicators of apoptosis were not observed in either Striped Bass or Nile Tilapia cardiomyocytes, is it possible that changes were manifested at the molecular level but did not arise at the cellular level. Or cellular damage was being actively repaired during hypoxia exposure, reoxygenation and recovery, which could prevent apoptotic cell death from occurring. Or lastly, other non-apoptotic cellular and molecular mechanisms were activated in cardiomyocytes. Analyzing the gene expression of target genes in the apoptotic pathways could distinguish which of the possibilities is likely and decipher the mechanisms triggered in cardiomyocytes during hypoxia.

Molecular Investigation of Apoptotic Pathways

To rule out if cellular damage is being actively repaired by molecular chaperons or if changes that occurred at the molecular level were not being manifested at the protein level, gene expression in target genes (table 3.1) was evaluated by RT-qPCR. Before gene expression was measured, primers of target genes were designed or obtained from published papers. Of the 78 primers that were tested, only *actb, ef1a, hsp70* primer sets for Striped Bass and Nile Tilapia isolated a PCR product and were successfully verified (table 3.4). All the other primer sets resulted in primer

dimer formation, non-specific annealing, or did not produce a PCR product (data not shown). Those six primer sets were verified by sequencing (table 3.4) and then checked for ideal primer and template concentrations by establishing a standard curve using either Striped Bass or Nile Tilapia template cDNA. Four primer sets (*actb* (Striped Bass and Nile Tilapia), *efla* (Striped Bass), *hsp70* (Nile Tilapia)) had an efficiency of higher than 120% (fig. 3.4). Primer efficiency that is below 80% or above 120% is indicative of bad primer design (i.e. secondary structures, primer dimers, or incorrect annealing temperature), template contamination, or impurity of samples which (Quellhorst and Rulli 2008). Those four primer sets were checked for primer dimers using a melt curve analysis (fig. 3.5). Results from the melt curve analysis suggest that primer dimers are not the cause for the high efficiencies in (*actb* (Striped Bass and Nile Tilapia), *efla* (Striped Bass), *hsp70* (Nile Tilapia). RNA and cDNA templates were checked for contamination and impurities but samples yield a 260/280 ratio of 2.0 and 1.8 respectively (data not shown). Further studies need to be conducted to see if the efficiencies of those four primer sets can be lowered by trying different annealing temperatures or using a different reaction mastermix (i.e. using additives like DMSO). If not, (*actb* (Striped Bass and Nile Tilapia), *efla* (Striped Bass), *hsp70* (Nile Tilapia) are not ideal primers to be used for future RT-qPCR analysis with Striped Bass and Nile Tilapia ventricle tissue. Preliminary data can be collected on *hsp70* gene expression in Nile Tilapia during hypoxia and subsequent reoxygenation using the primer sets in table 3.5, but data will be limited since there will not be any Striped Bass gene expression values to compare too.

Future projects could measure gene expression using alternative methods to determine if Nile Tilapia suppress apoptosis in cardiomyocytes during hypoxia exposure and reoxygenation. Microarrays evaluate whole transcriptomal changes in response to a stimulus. This technique

allows for a large amount of sequences (up to 40,000) to be quantitatively measured simultaneously. However, the drawback to this technique is that microarrays do not allows for gene expression to be measured in targeted genes and the data provides an overall picture of transcriptomal changes (Schulze and Downward 2001). Another alternative is a relatively new technique called RNA-seq, RNA-seq can measure the complete transcriptomics of a whole organism like microarrays, but can provide precise quantitative data on targets genes in specific tissues in responses to certain stimulus, however the major drawback to this technique is the cost for processing samples in this way (Wang et al. 2009).

CONCLUSION

In conclusion, there was no cellular (DNA fragmentation and caspase 3/7 activity) evidence of apoptosis detected in both Striped Bass and Nile Tilapia cardiomyocytes after hypoxia and reperfusion injury. There are several possibilities why cellular apoptosis was not detected in Striped Bass (hypoxia-sensitive) samples: a) southern populations of Striped Bass may possess a limited ability for hypoxia tolerance, b) Striped Bass cardiomyocytes do not undergo apoptosis during but rather go through necrosis, or c) Striped Bass were not held in hypoxia long enough for any damage to occur at the cellular level. Further studies need to be done to rule out these possibilities. We expected Nile Tilapia samples to not show signs of apoptosis because our hypothesis was that Nile Tilapia are preventing apoptosis as a means of hypoxia survival. However, since apoptosis was not detected in Striped Bass, we cannot confirm that apoptosis is being suppressed in Nile Tilapia cardiomyocytes especially without quantitative molecular work to back the cellular results.

CHAPTER 4
SUMMARY AND FUTURE DIRECTIONS

The purpose of this study was to uncover the potential coping mechanisms that Nile Tilapia (*Oreochromis niloticus)* utilize to protect their cardiomyocytes from damage and subsequent death during hypoxia exposure, with the hope that the information gained would elucidate potential therapeutic strategies for CVD. To reiterate, it was hypothesized that Nile Tilapia are preventing cardiomyocyte loss by suppressing apoptosis through increased expression of anti-apoptotic and repair genes during hypoxia exposure, and therefore would have less evidence of cellular and molecular apoptosis. To be able to uncover the Nile Tilapia coping mechanisms during hypoxia exposure, their responses need to be compared to a hypoxia sensitive species. Striped Bass (*Morone saxatilis)* are a species of fish known to be hypoxia-sensitive and will migrate towards cooler water during the summer months (Cech et al. 1984). Striped Bass and Nile Tilapia were subjected to gradual hypoxia exposure and monitored for key behavioral changes (i.e. discomfort, agitation, and loss of consciousness). It was determined that Nile Tilapia showed limited physiological stress during hypoxia exposure which suggest that they had extreme hypoxia tolerance capabilities. Striped Bass showed increased physiological stress during hypoxia sooner and at a higher dissolved oxygen level than Nile Tilapia which suggest that they were sensitive to hypoxia changes in their environment. Therefore, at the conclusion of the behavioral studies it was determined that Striped Bass (hypoxia-sensitive) and Nile Tilapia (hypoxia-tolerant) are ideal model organisms to study hypoxia-induced physiological changes in cardiac tissue.

However, during this study it was discovered that Striped Bass were not are hypoxia-sensitive are previous thought from previous literature (Coutant 1985; Setzler-Hamilton et al. 1988). The Striped Bass obtained for this study was from a southern population of Striped Bass near the Savannah River, Ga. There is evidence that suggests that the southern population of Striped Bass do not migrate during the summer months, which suggests that southern Striped Bass have a limited capacity for hypoxia tolerance (Dudley et al. 1977). A novel future project could explore the extent of hypoxia tolerance in Striped Bass by comparing the HTT and subsequent behavioral responses across populations (from the southern Atlantic, mid-Atlantic, and northern Atlantic).

After Striped Bass and Nile Tilapia were subjected to hypoxia and reperfusion injury, their cardiomyocytes were checked for DNA fragmentation (a late stage marker of apoptosis) and caspase 3/7 activity (an early stage marker of apoptosis). There was no difference observed in DNA fragmentation or no significant difference in caspase 3/7 activity between Striped Bass and Nile Tilapia cardiomyocytes across timepoints. This was surprising, given that Striped Bass seemed to be physiological stressed during hypoxia exposure, so we expected to see evidence of DNA fragmentation and significantly higher caspase 3/7 activity. This suggests that no apoptosis is occurring in both Striped Bass and Nile Tilapia cardiomyocytes, however this cannot be definitively confirmed without measuring anti-apoptotic, pro-apoptotic, and repair gene expression. Since changes could have started to occur at the molecular level but did not have time to manifest at the protein level (Lewis et al. 2012). Unfortunately, problems occurred in developing and optimizing primers to measure gene expression levels in apoptotic and repair pathways. Quantitative gene expression data would have been useful to determine what happened at the cellular and molecular level after hypoxia and reperfusion injury, but without that data the project

hypothesis could not be confirmed or denied. Future research can be done to collect *hsp70* gene expression data from Nile Tilapia cardiomyocytes. However, it will only be preliminary in nature and will not paint a complete picture since there is no Striped Bass gene expression data to compare too, therefore primers for Striped Bass need to be developed as well.

At the conclusion of this study, it cannot be definitively said that Nile Tilapia are preventing apoptosis during hypoxia exposure in cardiomyocytes as a means of survival, because cellular and molecular evidence of apoptosis was not detected. It is possible that cellular evidence of apoptosis could not be detected in all samples regardless of timepoint because either a) cellular damage is actively being repaired by molecular chaperons (Delogu et al. 2002), b) the fish were not exposed long enough for cellular damage to occur (Speers-Roesch et al. 2010), c) changes started occurring at the molecular level but did not have time to manifest at the protein level (Lewis et al. 2012), or d) a combination of all three. Another hypothesis proposed by various researchers and introduced by Richards (2009), suggest that hypoxia-sensitive species trigger a hypoxia-induced response that leads directly to necrotic cell death while hypoxia-tolerant species can prevent apoptotic events by stabilizing cellular ATP levels or through other non-apoptotic mechanisms (Boutilier and S-Pierre 2000). Additionally, other mechanisms could be responsible if Nile Tilapia are not undergoing apoptosis as a means of survival, such as the hypoxia-inducible factor pathway that repairs tissue damaged by hypoxia and reperfusion injury (Zimna and Kurpisz 2015) or increased antioxidant production to fight reactive oxygen species formation during reperfusion injury (Bickler and Buck 2007). Finally, since DNA fragmentation and caspase 3/7 activity only dictate the presence or absence of apoptotic markers, they are not sensitive enough to rule out the above listed possibilities. Therefore, future studies can be conducted to ascertain if Striped Bass

cardiomyocytes are undergoing necrosis rather than apoptosis, if Nile Tilapia are stabilizing cellular ATP levels, if hypoxia-induced damage began to manifest only at the protein level because both species were not subjected to hypoxia long enough, or if there are other protective mechanisms or pathways utilized for hypoxia survival.

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