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## Cellular Coping Mechanisms to Hypoxia in the Longhorn Sculpin (*Myoxocephalus octodecemspinosus*) Brain

Kelly Heino

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CELLULAR COPING MECHANISMS TO HYPOXIA IN THE LONGHORN SCULPIN  
(*MYOXOCEPHALUS OCTODECEMSPINOSUS*) BRAIN

by

KELLY F. HEINO

(Under the Direction of Johanne M. Lewis and Karin Scarpinato)

ABSTRACT

The loss of neurological function due to hypoxia remains to be a challenge in many species due to the lack of knowledge and understanding of cellular responses. To investigate cellular responses and survival strategies of the brain during hypoxia and post-hypoxia recovery in a moderate hypoxia-tolerant species, with the hopes of identifying possible therapeutic remedies, thirty Longhorn sculpin, *Myoxocephalus octodecimspinosus*, were challenged with acute hypoxia (40% O<sub>2</sub> saturation for 1 h) and twelve additional sculpin were used as controls (~90% O<sub>2</sub> saturation). Fish were sampled at each of the following time points: control/normoxic conditions (T=0 h); hypoxic conditions (T=3 h); early (T=7 h total; 4 hours post-hypoxia), and late (T=11 h total; 8 hours post-hypoxia) normoxic recovery. The importance of hypoxia-induced apoptotic pathways has been suggested in other species; therefore, they were the focus of the present study of the Longhorn sculpin to explore their cellular coping mechanisms to hypoxia. The expression levels of hypoxia-response elements HIF-2 $\alpha$  and Hsp70 were examined at the transcriptome level at each time point using RT-QPCR, and HIF-1 $\alpha$ , active caspase 3, and survivin, were explored at the protein level using immunohistochemistry and fluorescence microscopy. HIF-2 $\alpha$  mRNA levels decreased significantly after hypoxic exposure, compared to normoxic levels, while Hsp70 levels remained unchanged. Using immunohistochemistry, we also ascertained that HIF-1 $\alpha$ , active caspase 3, and survivin proteins are present in Longhorn sculpin brain tissue,

however, acute hypoxic exposure did not alter protein levels significantly in this study between treatment groups or between regions of the brain. The Longhorn sculpin's moderate hypoxia tolerance may be due to a lack of apoptotic cell death, however, more testing of apoptotic factors on both the transcriptome and protein level is required to fully elucidate the hypoxia survival strategies of the Longhorn sculpin. Investigating the survival strategies of hypoxia-tolerant species could provide for an effective treatment against hypoxia-induced neurological damage in other, more sensitive species by assisting in the identification of target genes and pathways for therapeutic intervention.

INDEX WORDS: Hypoxia, Apoptosis, HIF, Hsp70, Survivin, Caspase-3

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B.A., Mercer University, 2004

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial  
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

STATESBORO, GEORGIA

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Electronic Version Approved:

May 2014

DEDICATION

I would like to dedicate this work to my loving family for all of their support and encouragement.

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## TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS .....	7
LIST OF FIGURES .....	9
LIST OF TABLES .....	10
INTRODUCTION .....	11
METHODS AND MATERIALS .....	23
RESULTS .....	32
DISCUSSION .....	38
CONCLUSIONS .....	47
FUTURE DIRECTIONS .....	48
REFERENCES .....	51
APPENDICES .....	59

## LIST OF FIGURES

	Page
Figure 1: [Schematic illustrating suggested apoptotic pathways that focus on genes of interest of the present study.] .....	13
Figure 2: [Diagram of a typical teleost fish brain with functional similarities to a human brain.] ... .....	16
Figure 3: [Graph illustrating our experimental oxygen exposure over time.] .....	24
Figure 4: [Identification of HIF-1 $\alpha$ protein expression in Longhorn sculpin brain tissue.] .....	35
Figure 5: [Identification of active caspase 3 protein expression in Longhorn sculpin brain tissue.] .....	35
Figure 6: [Identification of survivin protein expression in Longhorn sculpin brain tissue.] .....	36
Figure 7: [HIF-1 $\alpha$ Mean Protein Intensity Values.] .....	36
Figure 8: [Survivin Mean Protein Intensity Values.] .....	37
Figure 9: [Active Caspase 3 Mean Protein Intensity Values.] .....	37
Figure 10: [Schematic illustrating apoptotic pathways that focus on our genes of interest in the present study, and examples of suggested genes for future directions of study.] .....	50

## LIST OF TABLES

	Page
Table 1: [RT-QPCR Primer Sequences.] .....	26
Table 2: [Nucleotide BLAST Results of PCR Products.] .....	27
Table 3: [Gene expression of $\beta$ -actin.] .....	33
Table 4: [Relative Gene expression of HIF-2 $\alpha$ .] .....	33
Table 5: [Relative Gene expression of Hsp70.] .....	34

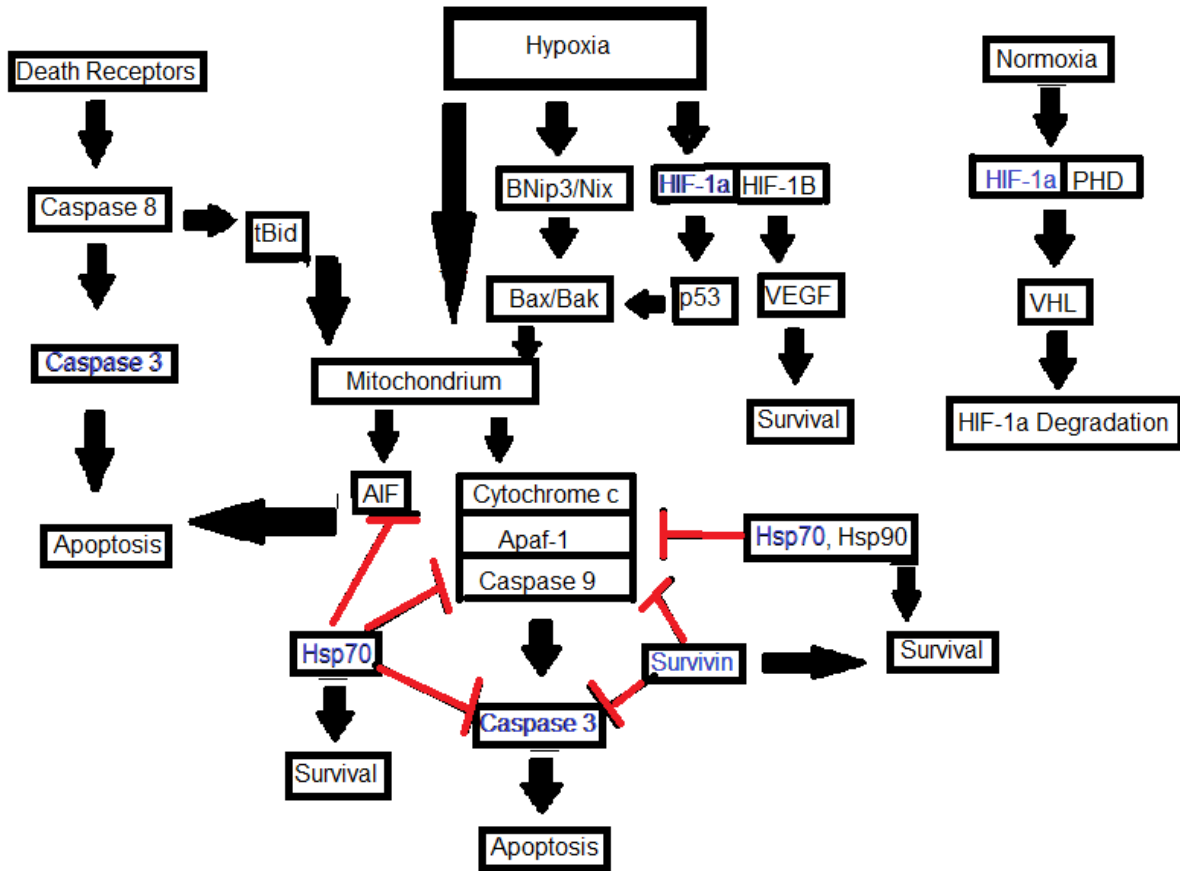
## INTRODUCTION

Diverse internal conditions such as stroke, embryonic development, tumor development, and neurodegenerative disease, as well as external conditions, such as high altitude, traumatic brain injury, blood loss, near-choking, near-drowning, and near-strangulation, amongst several others, expose neural cells to significantly lowered oxygen levels or “hypoxia” (Gopalani et al., 2012). Neural hypoxia can lead to brain damage, neurological deficits, coma, and death; however, the treatment and prevention of hypoxic cell death remain a challenge due to the quick onset and currently irreversible effects of neural damage. Thus, emphasis should be focused on investigating the physiological and cellular processes involved in the destruction of neural tissue following hypoxic exposure in the hopes of therapeutic advancement.

Apoptosis, a complex mechanism of cell death, has been well documented as a major contributor to the brain damage of many hypoxia-sensitive species after oxygen deprivation. For example, extrinsic and intrinsic apoptotic pathways were suggested to contribute to cell death following traumatic brain injury (TBI) (using a parasagittal fluid-percussion brain insult) in Sprague-Dawley rats, as indicated by the immunohistochemical staining that displayed the upregulation of active caspase 8 and 9 proteins by 6 hours post-insult, followed by an upregulation of active caspase 3 protein by 3 days post-insult (Keane et al., 2001). Apoptotic cell death has also been confirmed in the hypoxia-sensitive sturgeon, *Acipenser shrenckii*, following 30 minutes of hypoxia (15% oxygen) and 6 or 30 hours of reoxygenation, as suggested by significantly elevated active caspase 3 protein expression examined by western blotting (Lu et al., 2005). Not only has apoptosis been strongly associated with hypoxic cell death in literature, but research has also implied that apoptotic cell death, as opposed to other modes of cell death, such as necrosis and necroptosis, may be more amenable for medicinal options following

hypoxia due to its longer duration and larger window of opportunity for preventional treatment (Turkyilmaz et al., 2010).

Apoptosis is a crucial biological process of programmed cell suicide that plays an essential role in regulating development, homeostasis, and immune defense by clearing redundant or abnormal cells in organisms (Wei et al., 2008). This genetically controlled program is associated with specific morphological changes, including cell rounding and shrinkage, chromatin condensation, nuclear and DNA fragmentation, plasma membrane blebbing, and the eventual formation of apoptotic bodies, which are engulfed by phagocytes (Malhotra et al., 2001; Tzifi et al., 2012). Unfavorable conditions, such as hypoxia, can also trigger apoptotic cell death, and thus lead to brain damage and detrimental health risks in hypoxia-sensitive species, such as humans. Hypoxia-induced apoptosis can occur through several pathways, such as the hypoxia-induced inhibition of the electron transport chain at the inner membrane of the mitochondria, which causes membrane hyperpermeability, leads to the release of cytochrome c, and causes eventual apoptotic cell death via caspase 3 (Greijer et al., 2004). Apoptosis can also be triggered when death receptors on the cell surface are activated by extracellular ligands, for example, the Fas-ligand (FasL) commonly expressed on activated T-cells, can bind to the Fas receptor on a compromised cell's surface upon encounter, and recruit the adapter protein Fas-associated death domain (FADD). FADD activates caspase 8 upon oligomerization, which can in turn lead to cell death by the direct activation of caspase 3 or by the indirect activation of downstream effector caspases by cytochrome c release from mitochondria (Maher, et al., 2002). There are several other possible pathways involved in the complicated process of apoptosis, and a few of the common pathways associated with our particular genes of interest are further discussed in Figure 1.



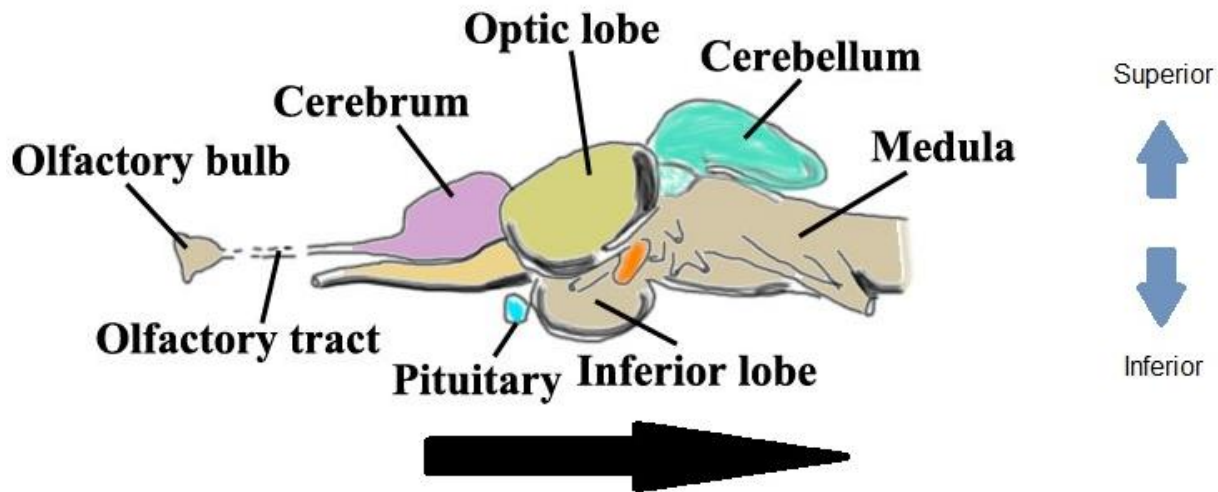
**Figure 1: Schematic illustrating suggested apoptotic pathways that focus on genes of interest of the present study (Labeled in blue).** Extrinsic pathway: Death receptors on the cell surface are triggered by extracellular ligands, and in turn activate caspase 8 intracellularly, which in turn activates caspase 3, and leads to apoptosis. Intrinsic pathway: The hypoxia stimulus is received, then multiple pathway options lead to either mitochondrial damage towards apoptotic cell death, including an HIF-1 pathway, or to cell survival, which can also include routes via HIF-1. Mitochondrial damage can then lead to the release of cytochrome c, which next can join Apoptosis protease activating factor-1 (Apaf-1) and activate caspase 9, which then can activate caspase 3, and lead to apoptotic cell death. Note that both survivin and Hsp70 can inhibit apoptosis in several locations along the cascade, and lead to cell survival. In Normoxic conditions: HIF-1 $\alpha$  is hydroxylated by prolyl hydroxylase enzymes (PHD), ubiquitinated by the von Hippel Lindau tumor suppressor gene (VHL), and degraded. (Bleackley et al., 2001; Greijer et al., 2004; Harris et al., 2002; Ischia et al., 2013; Klettner et al., 2004; Lee, J-J et al., 2011; Maher et al., 2002; Peng et al., 2005; Rerole et al., 2010; Tilly et al., 2001).

Apoptosis has not only been studied for its role in cell death, but the lack of hypoxia-induced apoptosis has also been explored as part of survival strategies to hypoxic environments in more hypoxia-tolerant species (Malik et al., 2012) by several mechanisms. For example, in the anoxia-tolerant turtle brain (*Trachemys scripta*), cell survival has been attributed to both the enhancement of pro-survival factors, such as elevated heat shock proteins 72, 60, and 27, as well as the suppression of pro-apoptotic pathways, such as the decreased expression of Bax protein (factor that encourages cytochrome c release from mitochondria in the apoptotic pathway) examined by western blot analyses following early (1 hour) and long-term (4, 24 hours) anoxia (Kesaraju et al., 2009). The lack of apoptotic cell death in several other hypoxia-tolerant species such as the mole rat (*Spalax ehrenbergi*), has been suggested to be contributed to a large conglomerate of factors and pathways that may reflect a fine balance between inducers and suppressors of apoptotic pathways in brain and muscle tissue subjected to acute (3% or 6% O<sub>2</sub> for 6 hours) and chronic (10% O<sub>2</sub> for 44 hours) hypoxia, as measured at the transcriptome level using QPCR and microarray analysis (Malik et al., 2012). There is a growing interest in understanding the factors that govern the interplay between cell death and proliferation under various conditions (Mashanov et al. 2010), such as hypoxia, with emphasis placed heavily on ways to potentially exploit these mechanisms for human health benefits. Therefore, exploring genes of apoptotic interest in hypoxia-tolerant species that possess a natural solution to such problems as hypoxia-induced cell death, could be a reasonable approach to identifying ways to prevent cell death in others during hypoxic exposures. The objective of the present study was to explore this fertile area of research and assist in the identification of target genes and pathways for therapeutic intervention by investigating the expression of apoptotic factors following hypoxia and post-hypoxic recovery in the brain tissue of a moderately hypoxia-tolerant species.

Oxygen, an essential element for all aerobic organisms, varies dramatically in aquatic environments. Water contains only 1/30<sup>th</sup> of the oxygen concentration compared with the same volume of air at the same partial pressure, and the rate of oxygen diffusion in water is only 1/10,000<sup>th</sup> of that in air (Rytkonen et al., 2007). Thus, changes in oxygen consumption or availability can dramatically decrease the oxygen tension in aquatic environments, and fish that live in these environments have developed various physiological mechanisms for surviving aquatic hypoxia (Rimoldi et al., 2012). For comparative studies of oxygen-dependent systems, fish are therefore the primary choice among vertebrates.

The Longhorn sculpin, *Myoxocephalus octodecemspinosus*, is a teleost fish commonly found both inshore and offshore in northwest Atlantic waters. To our knowledge, hypoxia-induced apoptotic factors have not previously been investigated in the Longhorn sculpin; however, Longhorn sculpins have previously been determined to have a moderate hypoxia tolerance as they can withstand exposure to oxygen levels as low as 40% for one hour without mortality and still fully recover after reoxygenation (Wilbur et al., 2012). The teleost fish brain, as shown in Figure 2, also demonstrates functional similarities to the human brain, and therefore, was a good model system of study for this project. We hypothesized that the survival mechanisms against hypoxia of the Longhorn sculpin may involve a lack of apoptotic cell death, and to investigate this hypothesis, we aimed to examine the expression levels of common apoptotic factors at both the transcriptome and protein level in response to acute hypoxia and hypoxia/reoxygenation in the Longhorn sculpin brain.





**Figure 2:** Diagram of a typical teleost fish brain with functional similarities to a human brain. Regions: **Cerebellum:** controls motor conditioning, memory, and learning; **Cerebrum:** is primarily responsible for the fish's sense of smell; **Medula:** controls the operations of the inner organs such as heart rate, blood pressure, digestion and waste disposal. The black arrow indicates the direction of horizontal sectioning. The blue arrows denote superior versus inferior brain regions. Original Image from: <http://www.fishtanksandponds.co.uk/fish-physiology/nervous-system.html>.

A delicate balance between pro-apoptotic and anti-apoptotic mechanisms determines whether a cell death signal can execute apoptosis (Wei et al., 2008); therefore, both pro- and anti-apoptotic genes were examined in the present study to investigate the Longhorn sculpin's natural survival strategies against hypoxia-induced apoptosis. Our selected genes to study were a hypoxia-induced transcription factor, HIF-1 $\alpha$ , which has been suggested to induce the transcription of both pro- and anti-apoptotic genes; a protective and anti-apoptotic heat shock protein, Hsp70; an inhibitor of apoptosis, survivin; and a pro-apoptotic effector, caspase 3. These genes of interest have been suggested in involvement of several different apoptotic

pathways, as previously illustrated in Figure 1, and will be further discussed in the sections that follow.

Hypoxia-inducible factors (HIF) are a family of heterodimeric transcriptional factors consisting of two subunits: a hypoxia-regulated  $\alpha$  subunit, (HIF-1 $\alpha$ , -2 $\alpha$ , -3 $\alpha$ ), and a constitutively expressed  $\beta$  subunit (Shen et al., 2010). Both HIF- $\alpha$  and HIF- $\beta$  belong to the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors, and are crucially involved in maintaining oxygen homeostasis (Chen et al., 2012). The main role of HIF is to regulate responses to fluctuating oxygen levels, and HIF-1 $\alpha$  specifically has been suggested as an important transcriptional factor for coordinating adaptive responses to hypoxia in both mammals and fish (Giusi et al., 2012); thus HIF-1 $\alpha$  was an important factor to explore in the present hypoxia study of the Longhorn sculpin. For example, HIF-1 $\alpha$  has been reported as a key regulator of apoptosis induced by hypoxia/reoxygenation (H/R) (cultured cells subjected to 5 hours of 5% CO<sub>2</sub> and 95% N<sub>2</sub> hypoxia followed by 2, 6, or 12 hours of reoxygenation) in primary neonatal rat cardiomyocytes, as suggested by an increase in both mRNA and protein expression of HIF-1 $\alpha$  following H/R, with a subsequent significant decrease in apoptosis shown by Annexin V-FITC apoptosis assay after HIF-1 $\alpha$  inhibition by siRNA; thus leading them to conclude that HIF-1 $\alpha$  may be a possible therapeutic target to limit injury after infarction (Wang et al., 2012). HIF-1 $\alpha$  has also been suggested to be involved in controlling gene responses to oxygen in hypoxia-tolerant crucian carp, as indicated by the upregulation of HIF-1 $\alpha$  protein on immunoblot and immunoprecipitation analyses following hypoxia (6, 24, or 48 hours of 6-8% O<sub>2</sub>) at every temperature tested (Rissanen et al., 2006).

In normoxic conditions, HIF-1 $\alpha$  typically is rapidly degraded by prolyl hydroxylases (PHD), however, in hypoxic conditions, HIF-1 $\alpha$  stabilization and accumulation occurs. HIF-1 $\alpha$

then translocates to the nucleus and dimerizes with HIF-1 $\beta$ , forming the active transcription factor, HIF-1 (Rimoldi et al., 2012). HIF-1 may then lead to cell death by activating different target, proapoptotic genes such as: BNIP3, Nix, and the caspases (Chen et al., 2009), or lead to cell survival through recruitment of other neuroprotective factors such as HSPs (Giusi et al., 2012) and survivin (Wu et al., 2010); thus, HIF-1 $\alpha$  has been considered as a regulator of both pro-death and pro-survival pathways (Chen et al., 2008). For example, in the cortical neurons of rats, mild hypoxia (cultured cells exposed to 3% O<sub>2</sub> for 3, 6, 18, 24, or 48 hours) with Lentivirus-mediated HIF-1 $\alpha$  knockdown was reported to markedly increase neuronal death involving free radical production, mitochondrial depolarization, cytochrome c release, and caspase-3 activation in comparison to cell death minus HIF-1 $\alpha$  inhibition, thereby suggesting a neuroprotective role of HIF-1 $\alpha$  in hypoxia-mediated neuronal death (Lopez-Hernandez et al., 2012). In contrast, HIF-1 $\alpha$  mRNA and protein inhibition due to HIF-1 $\alpha$  siRNA in rat brains following ischemic damage by middle cerebral artery occlusion/reperfusion has been shown to decrease mortality by suppressing HIF-1 $\alpha$ , VEGF, p53, and caspase 3, thus suggesting a pro-apoptotic role for HIF-1 $\alpha$  (Chen, C. et al., 2009). This complex activity of HIF in any species is suggested to overall be dependent on the cell type, duration of hypoxic exposure, and severity of experimental conditions (Piret et al., 2002). This dual role and specificity of apoptotic regulation by HIF-1 $\alpha$ , as well as its suggested association with our other genes of interest, Hsp 70, caspase 3, and survivin is another reason for HIF-1 $\alpha$  selection into our study to assist in identifying sculpin survival strategies against apoptosis, and was expected to be upregulated following hypoxic exposure, given its known role in hypoxia response.

An anti-apoptotic, or cellular protective factor, Heat shock protein 70 (Hsp70), was also measured in the present study following hypoxia and post-hypoxic recovery. Heat shock

proteins are highly conserved molecular chaperones that respond to stressful conditions, such as heat shock, hypoxia, osmotic stress, and metabolic abnormalities (Giusi et al., 2012). These “conformational repair agents” (Giusi et al., 2012) play critical roles in the folding and unfolding of proteins, protein assembly, and cell cycle control and signaling (Li et al., 2004). Their protective role in response to hypoxia and ischemia has also been well documented; for example, in a study of mice subjected to focal ischemia for 120 minutes by middle cerebral artery occlusion, significantly greater DNA fragmentation was indicated by TUNEL staining in Hsp70 knockout mice as compared to wildtypes, additionally, western blotting and immunohistochemistry indicated enhanced cytochrome c release into the cytoplasm, as well as a significantly enhanced active caspase 3 protein expression in Hsp70 knockout mice as compared to wildtypes (Lee et al., 2004). HSPs in the hypoxia-tolerant lungfish have also been proposed as determining elements for the physiological success of hypoxic aestivations by executing a precise balance between cell death and neurogenesis, as indicated by high Hsp70 mRNA levels (measured by qPCR and *in situ* hybridization analyses) overlapping apoptotic events (measured by TUNEL analyses) following exposure to varying aestivation methods and durations (Giusi et al., 2012). This surprising abundance of apoptotic positive cells following lungfish aestivation, despite overall fish survival, was proposed to promote the recruitment of HSPs and protective factors to prevent neuronal damage against subsequent insults in a similar manner to that reported after preconditioning in hypoxia-sensitive species (Giusi et al., 2012). Therefore, Hsp70 expression was also of some interest in our hypoxia study of a moderately tolerant species, and was expected to be upregulated after hypoxic exposure and post-hypoxic recovery.

Another anti-apoptotic gene was also desired for the present study to further investigate the possibility that sculpin brains may be preventing cell death by inhibiting the completion of

the apoptotic process in response to hypoxia. Survivin, the smallest member of the “Inhibitor of apoptosis protein” (IAP) family is associated with cell death, cell proliferation, neurogenesis, angiogenesis, and hematopoiesis (Mashanov et al. 2010). Studies have shown that IAPs can inhibit the activity of caspases, as shown in Figure 2, by interaction of their conserved BIR domain with the active sites of caspases, and promoting the degradation of active caspases, or by sequestering the caspases away from their substrates (Wei et al. 2008). Survivin has been reported to inhibit both the Bax and Fas-induced apoptosis pathways and to bind specifically with caspases 3 and 7, inhibiting activation of these two caspases (Li et al., 2012). In addition to being associated with caspase 3 during apoptosis, the expression of survivin has also been correlated in numerous studies with HIF-1 $\alpha$  under both normoxic and hypoxic conditions. For example, Chen, Y-Q et al. 2009 indicated a correlation between HIF-1 $\alpha$  and survivin protein expression by immunohistochemical staining in non-small cell lung cancer (NSCLC), additionally reported a significant increase in both mRNA and protein expression of both genes in lung adenocarcinoma cell lines under hypoxic conditions (1% O<sub>2</sub>/5% CO<sub>2</sub>/94% N<sub>2</sub> for 24 hours), and finally concluded, after a decrease of survivin expression following HIF-1 $\alpha$  inhibition by RNAi, that the binding of HIF-1 $\alpha$  to the survivin promoter increases the transcription of the survivin gene. Similar findings have also been discovered in colon adenocarcinoma cells (Wu et al., 2010), and this HIF-1 $\alpha$  and survivin correlation enhanced our interest to include survivin in the present study.

Survivin has been demonstrated in a wide range of species, from being abundantly expressed and involved in the regulation of neural cell proliferative responses in brain tissues of adult rats subjected to traumatic brain injury (Johnson et al. 2004), to being suggested as involved in angiogenesis during hypoxia-tolerant zebrafish development (Ma et al. 2007). These

proliferative and pro-mitotic roles of survivin have been well established in addition to its known function of inhibiting apoptosis (Li et al., 2012), and its significant response to hypoxia (Chen et al., 2009); however, the anti-apoptotic/pro-survival role of survivin after hypoxic exposure in the brain tissue of tolerant species has not been well documented to our knowledge. Thus, the present study explored the protein's possible role in sculpin neural cell survival, and expected to see an upregulation of survivin following hypoxia if this IAP member is involved in sculpin neural cell survival. A possible additional increase in expression post hypoxia recovery was also expected due to the protein's proliferative functions and involvement in the compensatory/reparative process (Johnson et al., 2004). Although permanent cell damage following hypoxic exposure was not expected given the Longhorn sculpin's tolerance, some repair and cell proliferation may take place to combat any possible injury acquired from the stress.

A pro-apoptotic gene was also required for the present study to fully investigate apoptosis in the Longhorn sculpin brain, and caspase 3, a known effector of apoptosis was chosen for this role. The caspases are a family of cysteine proteases that are grouped by structure and function, and act in a tissue-specific manner (Kim et al., 2010). Caspases fall into two categories: inflammatory caspases and apoptotic caspases (Elvitigala et al., 2012), the latter of which is subdivided into initiator caspases, which act upstream to initiate and regulate apoptosis, and downstream to activate effector caspases; and effector caspases, which function downstream in the apoptosis cascade (Martin et al., 2005). In general, caspase-mediated apoptosis occurs by either extrinsic (involving death receptors) or intrinsic (mitochondria-mediated) pathways, and these pathways usually converge on a common effector caspase, such as caspase 3 (Kim et al., 2010). Caspase 3 has been suggested numerous times to execute the final morphologic and

biochemical alterations of apoptosis (Kim et al., 2010; Martin et al. 2005). For example, caspase 3 has been proposed as a main effector of hypoxia-induced apoptosis in cultured neurons and astrocytes from embryonic rat forebrains, based on immunohistochemical and western blot analyses indicating both the increase of active caspase 3 protein and apoptotic cells following 6 hours of hypoxia (95% N<sub>2</sub>/5% O<sub>2</sub>), and the contrasting reduced number of apoptotic cells following hypoxia with caspase 3 inhibition by the peptide inhibitor, DEVD-CHO (Bossenmeyer-Pourie et al., 1999). One study has also suggested that during progression of apoptosis, the immunostaining pattern of caspase 3 translocates from the cytosol to the nucleus, paralleling the appearance of apoptotic bodies (Rajendran et al., 2008).

Caspase 3 has been so frequently documented as a key player in late apoptosis, that performing immunochemistry and western blotting against caspase 3 protein provides a means to confirm the presence of apoptosis (Lu et al., 2005; Rajendran et al., 2008). Hence, the inclusion of this important apoptotic marker in the present study was to signify if an upregulation of apoptosis is present in response to hypoxia and hypoxia/reoxygenation in the sculpin brain. Given the hypoxia tolerance of the Longhorn sculpin (Wilbur et al., 2012), a rapid increase in active caspase 3 expression after hypoxic treatment was not expected if the sculpin's neural cell survival is in fact due to a lack of hypoxia-induced apoptosis.

Due to the severity of health risks associated with brain damage due to hypoxia, and the lack of preventional treatment and therapeutic remedy for such problems, the present study set out to aid in filling this gap in knowledge. Given that apoptosis is one of the main processes that destroys tissue after hypoxic exposure in mammals and hypoxia-sensitive species (Meller et al., 2013), exploring these genes linked to apoptosis in hypoxia-tolerant species, such as the Longhorn sculpin, could be a step towards identifying ways to prevent cell death in the brains of

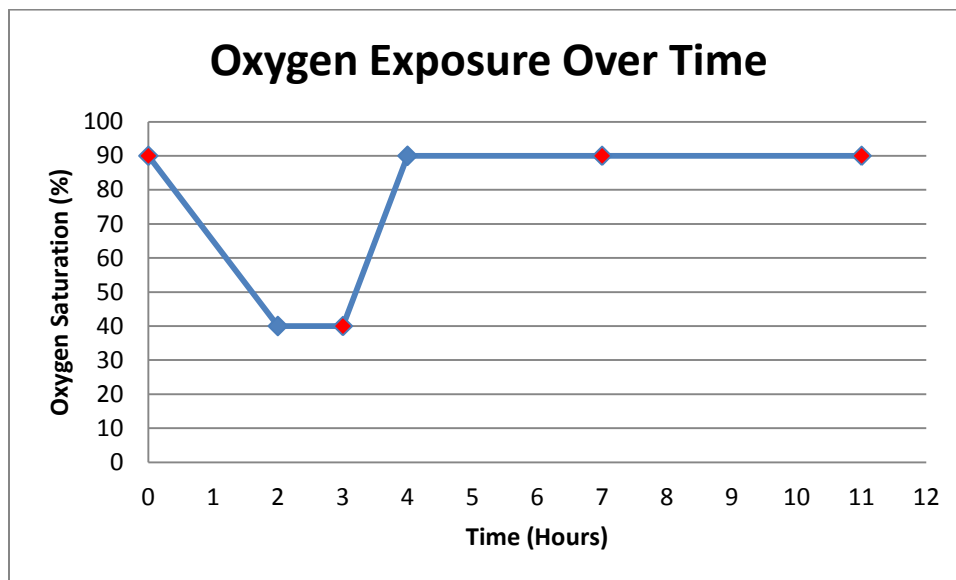
hypoxia sensitive species. We hypothesized that the Longhorn sculpin, since they show some hypoxia tolerance, has developed some cellular or physiological mechanisms for survival. If so, these mechanisms should be explored and potentially exploited for the treatment of unintended hypoxic brain damage in sensitive species such as humans.

## METHODS AND MATERIALS

To investigate cellular responses to hypoxia and post-hypoxia recovery, thirty Longhorn sculpin, *Myoxocephalus octodecemspinosus*, which were previously determined to have a moderate hypoxia tolerance (Wilbur et al., 2012) were challenged with acute water hypoxia (40% O<sub>2</sub> saturation for 1 h) and twelve additional sculpin were used as controls (~90% O<sub>2</sub> saturation). The fish were collected during the summer seasons in both 2011 (24 sculpin total, 6 in each of 4 sampling groups: control, hypoxic, early recovery, and late recovery) and 2012 (18 sculpin total, 6 in each of only 3 sampling groups: control, hypoxic, and early recovery), with only gene expression analysis completed on the 2011 samples, and immunohistochemical analysis completed on the 2012 samples. Both seasons, the Longhorn sculpin used for experimentation were mature fish (mean weight = 201.8g ± 8.2) collected from Frenchman's Bay, ME and housed at the Mount Desert Island Biological Lab (MDIBL) where the use of experimental animals was approved under MDIBL IACUC protocol #12-07. The sculpin were housed in 1m<sup>2</sup> flow-through seawater tanks with exposure to ambient seawater conditions (10-12°C) and natural photoperiods. The fish were fed a diet of chopped squid and herring ad libitum twice a week, and were transferred to individual 3L boxes with flow-through seawater access for acclimation over-night prior to experimentation. During experimental exposure, their dissolved oxygen concentration was gradually reduced over a two hour time period (Figure 3) by



a combination of the addition of nitrogen gas and the removal of the natural seawater flow-through system, to a 40% oxygen saturation. All sculpin, with the exception of the control fish, were held at the 40% dissolved oxygen saturation for one hour with continuous monitoring with a YSI Pro2030 handheld dissolved oxygen probe, and reoxygenation for the recovery samples was achieved by the immediate replacement of the seawater flow-through access.



**Figure 3: Graph illustrating our experimental oxygen exposure (% Saturation) over time (Hours).**

Note: Red diamonds indicate time points when fish were sampled: Control/Normoxic conditions (T=0 h), Hypoxic conditions (T=3 h), Early Normoxic Recovery (T=7 h), and Late Normoxic recovery (T=11 h).

In both study years, six fish were sampled at each of the following time points (Figure 3): control/normoxic conditions (T=0 h), hypoxic conditions (T=3 h), and early normoxic recovery conditions (T=7 h total; 4 h post-hypoxia); however, in 2011, there was an additional sampling at a late normoxic recovery time point (T=11 h total; 8 h post-hypoxia). In all cases, fish were

anesthetized at sampling points with a 1:9 part clove oil/ethanol solution (80mg/L of water), and their spinal cords were immediately severed. Brain tissues were harvested, weighed, and preserved in either liquid nitrogen for gene expression studies, or fixed in 2mL of 4% paraformaldehyde for 48 hours at 4° C, and then transferred to a 10 mL PBS/0.01% sodium azide solution at 4°C for approximately 2 months until they were embedded in paraffin for immunohistochemical analysis.

### **Real Time-QPCR**

The sculpin brains harvested in 2011 were preserved in liquid nitrogen and stored at -80°C until analysis. Individual brains were homogenized and total RNA was isolated using RNeasy RLT (Life Technologies, cat# RN 190), following the manufacturer's instructions. Briefly, the DNA/protein was precipitated by adding DEPC water to the RNeasy RLT tissue sample to stand at room temperature for 15 minutes, and then centrifuged at 12,000g for 15 minutes. The RNA was precipitated by adding ethanol to the supernatant at room temperature for 5 minutes, and then centrifuged at 12,000g for 8 minutes. Next, to wash the RNA, ethanol was added to the pellet twice and centrifuged at 4,000g for 3 minutes each time. Finally, RNA solubilization was achieved by dissolving the pellet in DEPC water for 5 minutes before vortexing and storing the samples at -80°C until further analysis.

The RNA was later quantified on a Nanodrop ND-1000 spectrophotometer, and cDNA was synthesized using a protocol for real time PCR. Briefly, the RNA samples were diluted with DEPC water for a total volume of 5µl and 2µg of RNA. 5µl of a master mix including 10x buffer, DEPC water, and DNase (amplification grade, Invitrogen, cat #18068-015) was added to each sample and they were incubated at room temperature for 12 minutes. The reaction was

terminated by the addition of 1µl of 25mM EDTA and incubating at 65°C for 10 minutes in a thermal cycler. Next, the samples were chilled at 4°C for 60 minutes before the addition of a random hexamer (Invitrogen, cat# 48190-011) and heating at 65°C for 5 minutes. The samples were then briefly held at 4°C until adding 7.5µl of a master mix consisting of 5x 1<sup>st</sup> strand buffer, 10 nM dNTP mix (Invitrogen, cat#18427-013), RNase out (Invitrogen, cat#1-0777-019), and DEPC water. 0.5µl of RevertAid H-MuLV Reverse Transcriptase was then added at room temperature for 10 minutes before incubating at 42°C for 60 minutes. The reaction was finally terminated by heating the samples at 70°C for 10 minutes, and then they were stored at -20°C.

**Table 1: RT-QPCR Primer Sequences.** \* sequences obtained from Giusi et al., 2012; ° sequences obtained from Mladineo et al., 2009; + sequences manually designed from alignments of known sequences in the NCBI nucleotide database: *L. crocea* Accession #GU584189.1, *D. labrax* Accession #AJ537421.1, *D. rerio* Accession#AF057040.1.

Gene of Interest	Primer Sequence	Efficiency %	Amplicon Size
HIF-1α Fwd*	5'-CGC ACC GTC AAT ATC AAG TCT G-3'	100.9	199
HIF-1α Rvs*	5'-GCT GAG GAA GGT CTT GCT GTC-3'		
Hsp70 Fwd°	5'-GAC ATG AAG CAC TGG C-3'	93.4	117
Hsp70 Rvs°	5'-AGG ACC ATG GAG GAG-3'		
B-Actin Fwd+	5'-GTG CAA AGC CGG ATT CGC-3'	108.7	180
B-Actin Rvs+	5'-CAA TAC CGT GCT CAA TGG G-3'		

Primer sets (Table 1) for each gene of interest were tested using a protocol for PCR of real time primers. Briefly, a master mix consisting of 20.3ul DEPC water, 2.5ul 10X Buffer, 0.5ul dNTP mix, 0.5ul 10uM Forward Primer, 0.5ul 10uM Reverse Primer, 0.2ul Taq, and 0.5ul

of a template random cDNA pool was made for each primer set. 25ul of each master mix were placed in PCR tubes and run on a Bio-RAD T100 thermal cycler. The PCR running conditions were 94°C for 3 minutes; 38 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; followed by 72°C for 15 minutes and indefinitely holding at 4°C.

Agarose gel electrophoresis was next performed to size separate the products using a 1% agarose/TAE solution and ethidium bromide staining to view the PCR products. The products of expected size were then excised from the gel and gel extracted using a QIAquick Gel Extraction kit and following the instructions of the manufacturer. The PCR products were then cloned using *Escherichia coli* and blue/white screening. The cloned plasmid DNA product was then purified using a QIAprep miniprep kit and following the instructions of the manufacturer. The products (See Appendix for sequences) were sent to the CUGI sequencing facility at Clemson University for sequencing, and the results were BLASTed using the NCBI Nucleotide megablast program to compare the products against known sequences for each gene of interest (Table 2).

**Table 2: Nucleotide BLAST Results of PCR Products.**

<b>Gene of Interest</b>	<b>BLAST Result</b>	<b>Accession # of Result</b>
HIF-1 $\alpha$	Micropogonias undulatus hypoxia-inducible factor 2 alpha mRNA, complete cds	DQ363932.1
Hsp70	Oligocottus maculosus heat shock protein 70 (Hsp70) mRNA, partial cds	DQ013309.1
B-Actin	<i>Gasterosteus aculeatus</i> Beta-actin mRNA, partial cds.	DQ018719.1

Real time-Quantitative Polymerase Chain Reaction (RT-QPCR) with SYBR green was conducted next for each sculpin brain using an Eppendorf realplex2 mastercycler to examine the expression levels of HIF at each time point sampled.  $\beta$ -actin, a commonly used housekeeping gene (Invitrogen 2008), was chosen as a reference gene for normalization for this study due to its constitutive and expected consistent expression, as it functions as a structural constituent of the cytoskeleton (Tan et al., 2012; Sandvik et al., 2012). Briefly, standard curves were generated for all three genes using a 1:5 serial dilution of a cDNA pool of the samples as the template. 2 ul of the template were pipetted with 10.5 ul of a master mix into each well. The master mix consisted of 0.5 ul of 2.5uM forward primer, 0.5 ul of 2.5 uM reverse primer, 6.25 ul of SYBR Green mix, and 3.25 ul of DEPC water. All five dilutions, as well as, two negative controls, a negative control reverse transcriptase (NRT) and a no-template control (NTC), were run in duplicates with the gene of interest (GOI) and normalizer gene on the same plate. The running conditions for the reactions consisted of heating to 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealment at 58°C for 1 minute, and extension at 72°C for 1 minute. Once the cycles were completed, the samples were heated again to 95°C for 1 minute, lowered to 55°C for 30 seconds, and lastly heated to 95°C for 30 seconds. A melting curve step was added to the protocol for 20 minutes during the ramp time between the 55°C step and the last 95°C step. The melting curves were used to assess the number of products accomplished in each reaction, and the linear statistics (slope and  $R^2$  values) of the standard curves were used to assess the efficiency of each reaction. The standard curves were generated by plotting the log of each concentration in the dilution series (x-axis) against the threshold cycle values (Ct values) for that concentration (y-axis). Once the standard curves were completed, analysis of mRNA expression with QRT-PCR was conducted for sculpin brain samples from each time point in 2011.

The threshold cycle values (Ct values) for each gene of interest were compared with the Ct values of the reference gene,  $\beta$ -actin, to calculate the relative expression ratios used for statistical analysis. The specific equation ( $\text{Relative Values} = E_{\text{target}}^{\Delta\text{Ct}_{\text{target}}(\text{control} - \text{sample})} / E_{\text{ref}}^{\Delta\text{Ct}_{\text{ref}}(\text{control} - \text{sample})}$ ) used for the calculation of the relative expression values comes from the Pfaffl Method of mathematical models for relative quantification in real-time RT-PCR (Pfaffl 2001). The ratio of the target gene is expressed in samples versus a control in comparison to the reference gene  $\beta$ -actin.

### **Immunohistochemistry**

Eighteen of the harvested sculpin brains from 2012 were immediately fixed in 4% paraformaldehyde for 48 hours at 4°C. After fixation, the samples were transferred to a 1x PBS/0.01% sodium azide solution and stored at 4°C for approximately two months. The brains were then dehydrated and embedded in paraffin for preservation. Briefly, the dehydration protocol consisted of a 70% ethanol bath for 30 minutes, two 95% ethanol baths for 30 minutes each, two 100% ethanol baths for 30 minutes each, two xylene baths for 30 minutes each, and lastly two 65°C paraffin baths for 30 minutes each. The brains were then embedded in paraffin blocks, and stored at room temperature. The samples were next cut into 25  $\mu\text{m}$  horizontal sections (Figure 2) on a Leica microtome (Model-RM 2235), fixed on slides, deparaffinized, rehydrated, and stained for immunohistochemistry and florescent microscopy to view the protein expression of HIF-1 $\alpha$ , active caspase 3, and survivin at each time point sampled in 2012.

Briefly, the steps for deparaffinization and rehydration included: two xylene baths for 5 minutes each, two 100% ethanol baths for 2 minutes each, a 95% ethanol bath for 2 minutes, a 70% ethanol bath for 2 minutes, a 50% ethanol bath for 2 minutes, a 35% ethanol bath for 2

minutes, and two deionized water baths for 1 minutes each. The slides were then marked with a pap pen, and placed into a sealed plastic container that was lined with wet paper (0.1% sodium azide/deionized water solution) to provide a chamber that would prevent the slides from drying out. For staining, the slides were washed twice for 1 minute each in 100 ul of a PBS/0.1% sodium azide buffer. After washing, tissues were blocked for 30 minutes at room temperature in 100 ul of a Fetal Bovine serum/PBS/0.1% sodium azide solution. Next, 100 ul of primary antibody (HIF-1 $\alpha$ : 1/50 Novus Biologicals mouse monoclonal anti-HIF-1 $\alpha$  antibody Catalog#NB100-105, caspase 3: 1/100 abcam rabbit polyclonal anti-active caspase 3 antibody #Ab13847, survivin: 1/500 abcam rabbit polyclonal anti-survivin antibody #Ab24479) was applied for 45 minutes at room temperature. The slides were then washed three more times for 1 minutes each, and 100 ul of secondary antibody (HIF-1 $\alpha$ : 1/5000 Alexa Fluor 594 donkey anti-mouse IgG, caspase 3: 1/7500 Alexa Fluor 647 goat anti-rabbit IgG, survivin: 1/4000 Alexa Fluor 647 goat anti-rabbit IgG) was applied for 30 minutes at room temperature. The slides remained in the dark for the duration of the staining procedure to avoid tissue bleaching from the fluorescent markers. Finally, the tissues were washed for five more cycles of 1 minute each before applying Invitrogen Prolong Gold antifade reagent and cover slips, and sealing the edges of the slides with clear fingernail polish. Sections immunostained without exposure to primary antibodies were used as negative controls. After staining, the tissues were viewed and analyzed on a Zeiss 710 LSM Confocal microscope.

Quantification was performed retroactively in this study, and the immunohistochemical data deviated from traditional quantification methods due to a lack of clearly defined cells. The quantitative data presented was instead based solely on simple intensity measurements from a randomly selected region, chosen from a visually ubiquitous expression in each sample that

made this type of quantification possible using optimal saturation conditions for standardization. The tissues were assessed for both treatment affect and comparison of protein expression differences between superior, middle, and inferior brain regions sampled (Figure 2).

### **Statistical Analysis**

All statistical analyses were conducted using a JMP Pro10-X64 program with values considered significant at  $p \leq 0.05$ . The relative RT-QPCR values were tested for assumptions of parametric testing, and both HIF- $\alpha$  and Hsp70 data sets failed to fit a normal distribution according to Shapiro-Wilk Goodness-of-Fit Tests with p-values  $< 0.0001$ . Logarithmic, square root, and arcsine transformations were performed in an attempt to have the data fit a normal distribution, however, none were successful, and the data was finally analyzed using non-parametric testing, such as Kruskal-Wallis and Wilcoxon Mann-Whitney U-Tests. The experiment-wise error rates for the Wilcoxon Mann-Whitnet U-Tests were adjusted using the Bonferroni method to account for non-independent sampling (Sokal et al., 2012).

The protein data, according to Shapiro-Wilk Goodness-of-Fit tests with significant p-values  $< 0.0001$ , and equal to 0.0089, also failed to fit a normal distribution. Square root transformation of values for all three proteins did, however, fit a normal distribution on Goodness-of-Fit tests with p-values of 0.1026, 0.8055, and 0.2221. The square root transformed values for HIF-1 $\alpha$  by both treatment group and brain region with p-values of 0.5487 and 0.7558 on Levene tests, and caspase 3 by both treatment group and brain region with p-values of 0.8579 and 0.3379 (see Appendix I for all critical values), also met the assumption of equal variances; therefore, Model I two-way ANOVA testing was performed for statistical analysis of each HIF-1 $\alpha$  and caspase 3 protein expression. A Levene test with a p-value of 0.9486, indicated that the



mean intensity values for survivin protein expression by brain region also met the assumption of equal variances; therefore, a Model I one-way ANOVA test was conducted for statistical analysis. A Levene test on survivin intensity values by treatment group with a significant p-value of 0.0052, failed to meet the assumption of equal variances for parametric testing; therefore, a Welch's ANOVA test was performed on this data.

## RESULTS

### RT-QPCR

RT-QPCR analysis was planned in the present study to examine the mRNA expression levels of our apoptotic genes of interest following hypoxic exposure and post-hypoxic recovery in the Longhorn sculpin brain. HIF- $\alpha$  and Hsp70 were successfully analyzed in our examination; however, both caspase 3 and survivin mRNA proved difficult to detect in the Longhorn sculpin brain in our experimental conditions. Although a considerable amount of effort was put into the testing of primers and cloning for caspase 3 and survivin, we were not able to successfully find primers for these two genes. Our trouble began during the cloning process, and whether this is due to undetectable levels expressed in the Longhorn sculpin brain, problematic primer sequences, or experimenter error has yet to be determined, and would need to be better addressed in future work in order to draw conclusions about caspase 3 and survivin at the transcriptome level.

It is also important to note that the HIF product sequenced for mRNA expression in this study actually displayed a higher similarity to known sequences for HIF-2 $\alpha$  in the NCBI database during verification. Despite our plan to explore HIF-1 $\alpha$  at both the transcriptome and

protein level, this surprise during product verification led us to only be able to draw conclusions about HIF-2 $\alpha$  and Hsp70 at the transcriptome level.

A Kruskal-Wallis analysis of the raw Ct values for the reference gene  $\beta$ -actin (Table 3) did not show a statistically significant difference in expression level between treatment groups in this study (p-value of 0.277, see Appendix for all critical values), providing validation for its use as a reference gene for the quantitative PCR analysis of Hsp70 and HIF-2 $\alpha$ . A significant down-regulation of HIF-2 $\alpha$  mRNA expression (Table 4) after hypoxic exposure was observed compared to normoxic values (p-value of 0.0367); however, there was no significant change in the mRNA expression levels of Hsp70 (p-value of 0.3511) (Table 5) in response to hypoxia or during post-hypoxic recovery in our study.

**Table 3: Gene expression of  $\beta$ -actin** from RT-QPCR analysis on Longhorn sculpin (*M. octodecemspinosus*) brain tissue sampled at normoxia, immediately post hypoxia and post-hypoxia recovery in normoxic conditions (early and late recovery). Data is presented as means  $\pm$ SE. Significant differences ( $p \leq 0.05$ ) from normoxic levels indicated with \*.

Treatment Group	$\beta$ -actin Mean Ct-values	Standard Error ( $\pm$ SE)
Control	15.216	0.201
Hypoxia	17.113	0.130
Early Hypoxic Recovery	20.286	0.837
Late Hypoxic Recovery	16.589	0.295

**Table 4: Relative Gene expression of HIF-2 $\alpha$**  from RT-QPCR analysis on Longhorn sculpin (*M. octodecemspinosus*) brain tissue sampled at normoxia, immediately post hypoxia and post-hypoxia recovery in normoxic conditions (early and late recovery). Data is presented as means  $\pm$ SE. Significant differences ( $p \leq 0.05$ ) from normoxic levels indicated with \*.

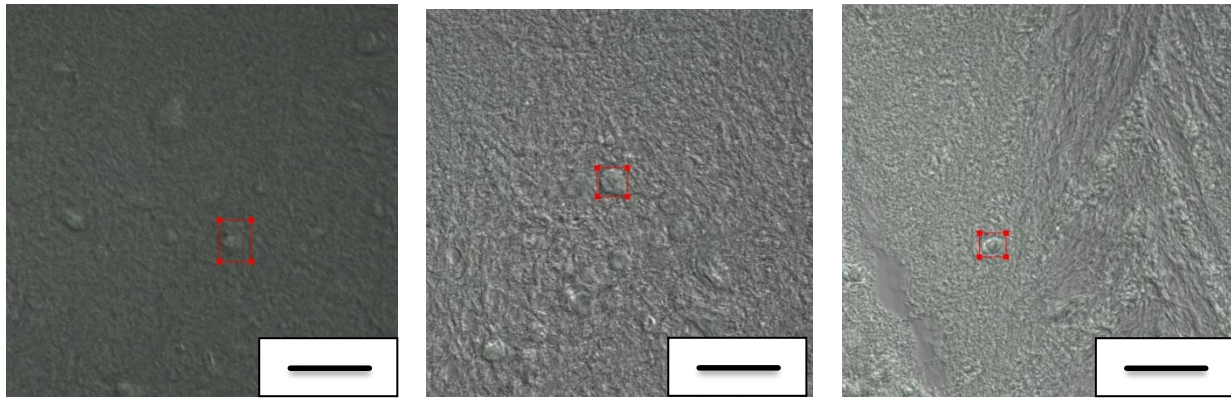
Treatment Group	Mean Relative HIF-2 $\alpha$ Gene Expression	Standard Error ( $\pm$ SE)
Control	0.00086	0.00078
Hypoxia	0.00002*	0.00001
Early Hypoxic Recovery	0.00003	0.00001
Late Hypoxic Recovery	0.01720	0.01688

**Table 5: Relative Gene expression of Hsp70** from RT-QPCR analysis on Longhorn sculpin (*M. octodecemspinosus*) brain tissue sampled at normoxia, immediately post hypoxia and post-hypoxia recovery in normoxic conditions (early and late recovery). Data is presented as means  $\pm$ SE. Significant differences ( $p \leq 0.05$ ) from normoxic levels indicated with \*.

<b>Treatment Group</b>	<b>Mean Relative Hsp70 Gene Expression</b>	<b>Standard Error (<math>\pm</math>SE)</b>
Control	0.00094	0.00090
Hypoxia	0.00003	0.00002
Early Hypoxic Recovery	0.00006	0.00005
Late Hypoxic Recovery	0.00514	0.00311

### **Immunohistochemistry (IHC)**

The Longhorn sculpin brains collected in 2012 were stained for fluorescent microscopy, and the tissues were viewed (Figures 4, 5, and 6) and quantitated for analysis by a Zeiss 710 LSM Confocal microscope. The intensity values for HIF-1 $\alpha$  (Figure 7), survivin (Figure 8), and active caspase 3 (Figure 9) protein expression were then analyzed for statistical significance and no significant differences were found between treatment groups (p-values: HIF-1 $\alpha$  = 0.1184, survivin = 0.2950, caspase 3 = 0.2812) or brain regions analyzed (p-values: HIF-1 $\alpha$  = 0.1468, survivin = 0.6948, caspase 3 = 0.6860).

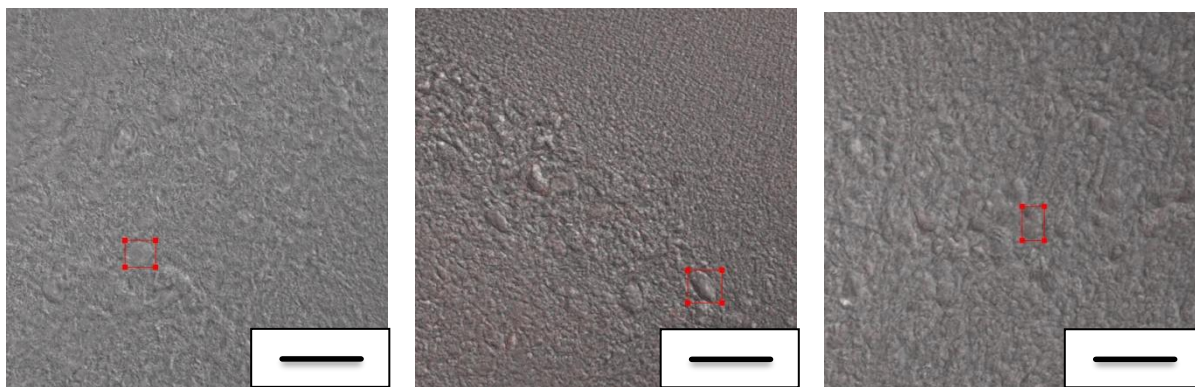


Left: Control Tissue

Middle: Hypoxia Tissue

Right: Recovery Tissue

**Figure 4: Identification of HIF-1 $\alpha$  protein expression (Green fluorescence) in Longhorn sculpin brain tissue.** Boxes indicate the random region used per section for simple intensity measurement quantification by analysis on a Zeiss 710 LSM Confocal Microscope. Magnification: 40X. All scale bars are 1mm.

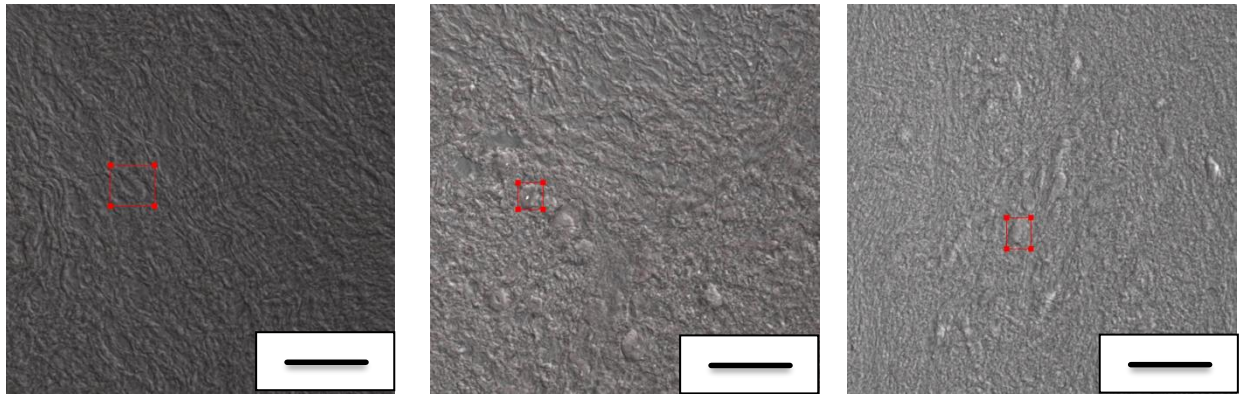


Left: Control Tissue

Middle: Hypoxia Tissue

Right: Recovery Tissue

**Figure 5: Identification of active caspase 3 protein expression (Red fluorescence) in Longhorn sculpin brain tissue.** Boxes indicate the random region used per section for simple intensity measurement quantification by analysis on a Zeiss 710 LSM Confocal Microscope. Magnification: 40X. All scale bars are 1mm.

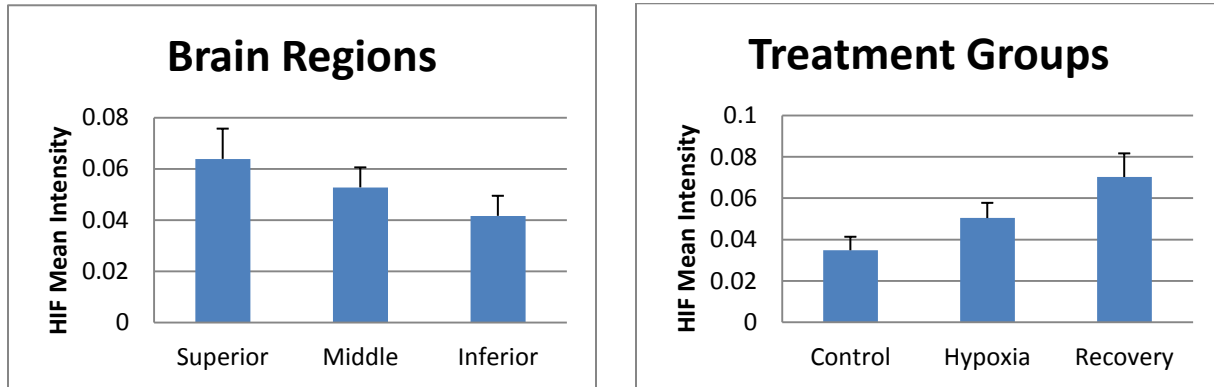


Left: Control Tissue

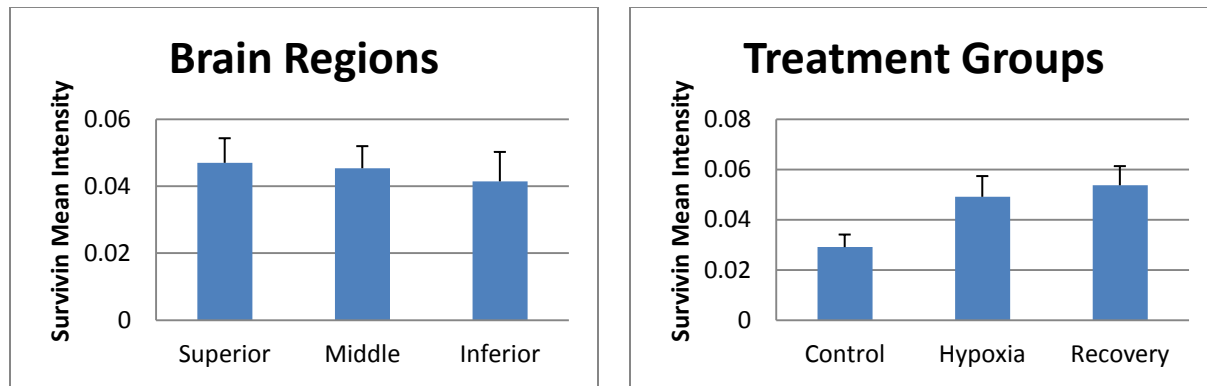
Middle: Hypoxia Tissue

Right: Recovery Tissue

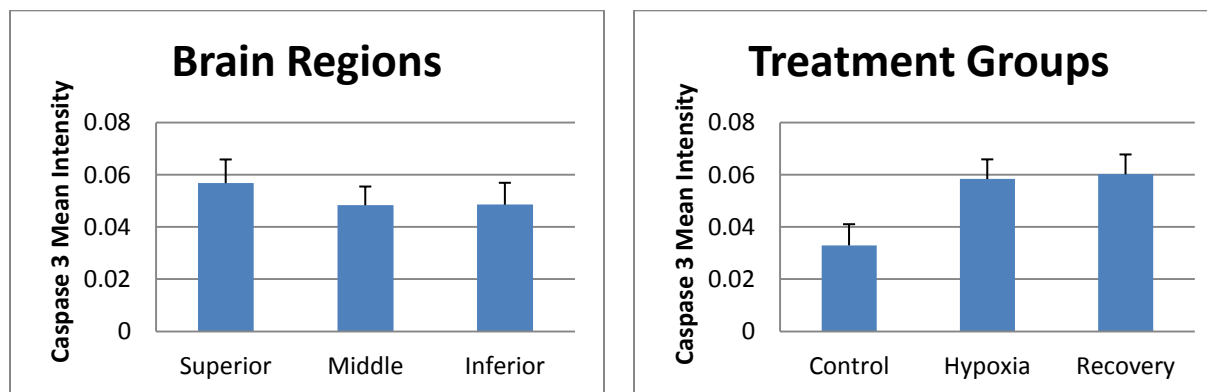
**Figure 6:** Identification of survivin protein expression (Red fluorescence) in Longhorn sculpin brain tissue. Boxes indicate the random region used per section for simple intensity measurement quantification by analysis on a Zeiss 710 LSM Confocal microscope. Magnification: 40X. All scale bars are 1mm.



**Figure 7:** HIF-1 $\alpha$  Mean Protein Intensity Values from IHC analyses of Longhorn sculpin (*M. octodecemspinosus*) brain tissue sampled at normoxia, immediately post hypoxia and post-hypoxia recovery in normoxic conditions (early recovery). Data is presented as means  $\pm$ SE. Significant differences ( $p \leq 0.05$ ) from normoxic levels indicated with \*.



**Figure 8: Survivin Mean Protein Intensity Values** from IHC analyses of Longhorn sculpin (*M. octodecemspinosus*) brain tissue sampled at normoxia, immediately post hypoxia and post-hypoxia recovery in normoxic conditions (early and late recovery). Data is presented as means  $\pm$ SE. Significant differences ( $p \leq 0.05$ ) from normoxic levels indicated with \*.



**Figure 9: Active Caspase 3 Mean Protein Intensity Values** from IHC analyses of Longhorn sculpin (*M. octodecemspinosus*) brain tissue sampled at normoxia, immediately post hypoxia and post-hypoxia recovery in normoxic conditions (early and late recovery). Data is presented as means  $\pm$ SE. Significant differences ( $p \leq 0.05$ ) from normoxic levels indicated with \*.

## DISCUSSION

The present study investigated Hypoxia inducible factors (HIF), caspase 3, survivin, and Heat shock protein 70 (Hsp70) in Longhorn sculpin brains under normoxic, hypoxic, and post-hypoxic recovery conditions, using RT-QPCR and immunohistochemistry. Given that apoptosis is one of the main processes that destroys tissue after hypoxic exposure in mammals and hypoxia-sensitive species (Meller et al., 2013), exploring these genes linked to apoptosis in hypoxia-tolerant species, such as the Longhorn sculpin, which hold a natural solution to such problems could be a reasonable approach to identifying ways to prevent cell death in the brains of hypoxia sensitive species by assisting in the identification of target genes and pathways for therapeutic intervention. The Longhorn sculpin's hypoxia tolerance may be in part due to a lack of significant apoptotic cell death; however, our results are not conclusive, and more research is needed for full resolution. Our semi-quantitated protein analyses were not strong enough in the present study to draw inferences from due to the inability to differentiate individual cells, and definite measurements of all apoptotic factors explored would need to be obtained at both the transcriptome and protein levels in the future to better interpret the Longhorn sculpin's survival strategies against hypoxia.

Although definitive conclusions about the Longhorn sculpin's neural cell survival during hypoxic exposure could not be stated from our results, our RT-QPCR data did reveal a significant and interesting finding about HIF-2 $\alpha$  expression in our study as discussed, in addition to our other findings, in the sections that follow.

## HIF

Hypoxia inducible factors are crucially involved in regulating responses to fluctuating oxygen levels in order to maintain oxygen homeostasis (Chen et al., 2012), and HIF-1 $\alpha$  specifically has been suggested as an important transcriptional factor for coordinating adaptive responses to hypoxia in both mammals and fish (Giusi et al., 2012); thus, initial expectations in our hypoxia study were to see an increase in HIF-1 $\alpha$  expression immediately post hypoxic exposure. Both HIF-1 $\alpha$  and HIF-2 $\alpha$  are regulated through oxygen-dependent proteolysis, and both transcription factors are suggested to mediate responses to environmental oxygen levels, as concluded in a study of rats in which marked upregulation of HIF-2 $\alpha$ , at both the transcriptome and protein level, was found following hypoxia (8% O<sub>2</sub> for 6 hours), in comparison to normoxic samples in the brain, heart, lung, kidney, liver, pancreas, and intestine, comparable to known HIF-1 $\alpha$  responses (Wiesener et al., 2002). Hence our expectation in our hypoxia study would also be to see an immediate upregulation of HIF-2 $\alpha$  after hypoxic exposure. Contrary to initial expectations, there was a significant decrease in HIF-2 $\alpha$  at the transcriptome level (Table 4) in the brain of the sculpin in response to hypoxia. A similar response has been found in HIF- $\alpha$  isoforms in human neuroblastoma and glioblastoma cells in which a consistent downregulation of HIF-1 $\alpha$  mRNA expression was discovered, coupled with an upregulation of HIF-2 $\alpha$  mRNA expression and strong protein inductions measured for both isoforms following hypoxic exposure (1% or 21% O<sub>2</sub> for 24, 48, or 72 hours). Further investigations indicated by luciferase reporter activities revealed that transcriptional activities of the HIF-1 $\alpha$  promoter/enhancer fragments were also decreased under hypoxic conditions, in contrast to HIF-2 $\alpha$  promoter/enhancer fragments which increased under hypoxia compared to normoxia. These findings were further investigated to the level of histone acetylation in HIF-1 $\alpha$  and HIF-2 $\alpha$  promoter/enhancer regions using ChIP



analyses, and the results correlated with the other findings of downregulation and upregulation respectively; therefore, they concluded that the decrease in HIF-1 $\alpha$  mRNA expression following hypoxia was most likely due to the decreased acetylation of histones that rendered the promoter of HIF-1 $\alpha$  less accessible to transcription cofactors, and resulted in suppressed HIF-1 $\alpha$  mRNA expression (Lin et al. in 2011).

This significant decrease of HIF-1 $\alpha$  mRNA expression after hypoxic exposure reported in Lin et al., 2011, and our significant decrease in HIF-2 $\alpha$  mRNA expression in the present study could likely be due to a tightly controlled balance of HIF- $\alpha$  expression and may constitute a mechanism to fine-tune hypoxic response dependent on the intensity and/or duration of the hypoxic exposure (Kopp et al., 2011; Li et al., 2006; Li et al., 2011; Lin et al., 2011; Soitamo et al., 2001). HIF-1 $\alpha$  and HIF-2 $\alpha$  are both regulated through oxygen-dependent proteolysis, and both mediate responses to environmental oxygen levels (Wiesener et al., 2002). These two isoforms have also both been shown to be very similar in sequence, as discussed in a study on Rainbow trout where it was reported that HIF-1 $\alpha$  and HIF-2 $\alpha$  have a strong homology with 70-90% similarity between them (Soitamo et al., 2001); however, these two HIF- $\alpha$  isoforms have also been reported to differ in their transactivation domains. This implies that they have similar functions, but could have unique target genes, as investigated in a study of renal carcinoma cells, human umbilical vein endothelial cells, and human microvascular endothelial lung cells that used DNA microarray analysis to evaluate a large selection of hypoxic genes induced in cells expressing HIF-2 $\alpha$  but not HIF-1 $\alpha$  (gene knockout) and vice versa; concluding that HIF-1 $\alpha$  and HIF-2 $\alpha$  have both common and unique target genes. In that study, both isoforms targeted hypoxia- inducible genes involved in respiration, immune response, and regulatory processes such as Glucose transporter 1, ADRP, NDRG-1, DMXL-I, IL-6, Carbonic anhydrase XII,

filaggrin, ADM, and VEGF; however, HIF-1 $\alpha$  uniquely targeted most glycolytic genes such as Hexokinase 2, glucosephosphate isomerase, phosphofructokinase, aldolase A, aldolase C, triosephosphate isomerase, glyceraldehydes-3-phosphate dehydrogenase, PGK-1, PGM-1, enolase 1, and LDHA (Hu et al., 2003). This idea of a difference in target gene could possibly explain the controlled balance of HIF- $\alpha$  expression, and why one isoform may be upregulated in one instance as opposed to the other isoform, and vice versa. Specificity regarding hypoxic response gene upregulation could potentially save on energy expenditure from upregulating redundant or unnecessary response elements at the same time. Thus our decrease in HIF-2 $\alpha$  mRNA expression could have been to conserve energy for respiration during the upregulation of other hypoxia response isoforms.

Another theory for explaining decreases in HIF- $\alpha$  mRNA expression was discussed in a study of human alveolar epithelial cells, in which HIF-1 $\alpha$  protein levels increased following acute hypoxia (4 hours at 0.5% O<sub>2</sub>), accompanied by a reduction in HIF-1 $\alpha$  mRNA, and an increase in natural antisense HIF-1 $\alpha$  (aHIF) mRNA expression. These HIF-1 $\alpha$  protein levels were suggested to negatively regulate HIF-1 $\alpha$  mRNA expression after hypoxic exposure through the increase in aHIF and destabilization of HIF-1 $\alpha$  mRNA. These conclusions were supported by the HIF-1 $\alpha$  mRNA decrease being ablated after the addition of cycloheximide, an inhibitor of protein synthesis; and through the transient transfection of cells with the dominant-negative HIF-2 $\alpha$  mutant, which also prevented the down-regulation of HIF-1 $\alpha$  mRNA (Uchida et al., 2004). These ideas of an auto-negative feedback by HIF itself, could also possibly explain our results in the Longhorn sculpin brain; however, we cannot make any substantial inferences without more research. Further analyses including both HIF-1 and -2 $\alpha$  mRNA and protein explorations is needed to fully elucidate the complicated mechanisms of hypoxia inducible factors, as well as

research to further investigate the adaptive benefits of this feedback system. Compared to mammals, the information available regarding fish HIF is very limited (Chen et al., 2012), and should be considered for future work.

## **Hsp70**

Hsp 70 is a member of the heat shock protein family that are highly conserved molecular chaperones that respond to stressful conditions, such as heat shock, hypoxia, osmotic stress, and metabolic abnormalities (Giusi et al., 2012) by facilitating the synthesis and folding of proteins. In addition to a known role in repair mechanisms (Li et al., 2004), Hsp70 has also been suggested numerous times as an important anti-apoptotic factor in both fish and mammals (Kim et al., 2006). For example, Hsp70 protein expression measured by immunoblotting was reported to be elevated in sea bream fibroblast cells following heat shock, coupled with stable mitochondrial membrane potential (as measured using a fluorescent probe, Mitotracker Red CMXRos), and a lack of significant caspase 3 protein upregulation (measured by a Sigma caspase 3 activity kit), whereas, cells treated with quercetin, an Hsp70 inhibitor, revealed a decrease in mitochondrial membrane potential, followed by an increase in caspase 3 protein activity; thus indicating an anti-apoptotic role of Hsp70 (Deane et al., 2012). An increase in Hsp70 mRNA expression has also been reported significantly at both the transcriptone and protein level (measured using high density oligonucleotide array and western blot analyses) in rat gastric epithelial cells following 2 hours of hypoxia (95% N<sub>2</sub>/5% CO<sub>2</sub>) and 2 hours of reoxygenation, and was suggested to play a role in maintaining cell survival (Katada et al., 2004). Thus, our initial expectation in the present study was to see an upregulation of Hsp70 in response to hypoxia; however, our results revealed that Hsp70 mRNA levels (Table 5) did not

vary significantly in response to hypoxia or reoxygenation post hypoxia in the Longhorn sculpin brain.

Though contrasting to the studies mentioned above, our results are consistent with a study of human myocardial tissue exposed to ischemic stress following surgery (cardiopulmonary bypass (CPBP) was established with a crystalloid cold cardioplegic solution, and cross-clamping (CC) time was  $75 \pm 26$  minutes), which reported no significant change in Hsp70 or Hsp90 mRNA expression (Storti et al., 2003). The most likely possibility for this lack of significant change in Hsp70 mRNA expression following hypoxia is that other protective factors, instead of Hsp70, such as other heat shock proteins or other anti-apoptotic genes could be responsible for aiding in cell survival in these cases. This idea is in line with the observations noted in a study of the slightly hypoxia-sensitive ruffe (*Gymnocephalus cernua*) and the hypoxia-tolerant flounder (*Platichthys flesus*) exposed to mild (52% oxygen), moderate (36% oxygen), and severe (15% oxygen) hypoxia for 48 hours, which also resulted with a lack of significant change in Hsp70 mRNA levels in brain tissue at all exposures, but did report a significant increase in Hsp27 and Ngb (Neuroglobin) mRNA levels following severe hypoxia in the ruffe, and a significant increase in GbX (Gastrulation Brain Homeobox) mRNA levels following mild hypoxic exposure in the flounder (Tiedke et al., 2014). Overall, these results suggest that Hsp70 may not play a significant role in neural cell survival against hypoxia in all species, as is indicated with our results in the Longhorn sculpin brain.

Another possibility for our lack of significant Hsp70 response following hypoxia and post-hypoxia recovery could be due to the regional vulnerability of the Central Nervous System (CNS). For example, hypoxia-tolerant lungfish, as previously mentioned, showed significantly increased Hsp70 mRNA levels in brain tissue following exposure to 6 days or 6 months of

hypoxic mud aestivation (30% water content of mud). These increased Hsp70 mRNA levels were, however, only found to be significant in the dorsal pallium (similar to the mammalian isocortex which functions for cognitive and emotional mental processes), optic tectum (neural processor for sensory information; controls eye, approach, and avoidance movements), and corpus cerebella (part of the cerebellum which controls motor conditioning, memory, and learning) following hypoxic aestivation, as compared to other brain regions which did not reveal a significant Hsp70 response (Giusi et al., 2012; Ikenaga 2013; Mueller et al., 2011). These results suggest that some regions of the CNS may be more vulnerable to hypoxia than others; thus, our lack of significant Hsp70 response to hypoxia in the Longhorn sculpin brain could be dependent on the differential expression pattern. Though our reported Hsp70 mRNA expression levels are based on RT-QPCR analyses using whole brain homogenization, it is possible that our results could be attributed to a watering-down effect of expression levels, and the regions that were not significantly affected by hypoxia could overall be masking the significant response from more susceptible regions. Further investigations which clearly differentiate between sampling regions, would be required to eliminate this as a possibility.

## **Survivin**

Another anti-apoptotic gene was also desired for the present study to better investigate the possibility that sculpin brains may be preventing cell death by inhibiting apoptotic pathways in response to hypoxia. A member of the Inhibitor of Apoptosis Protein (IAP) family, survivin, was selected to fulfill this role in our hypoxia study of the moderately hypoxia-tolerant, Longhorn sculpin. Our analyses confirmed the presence of survivin protein in the sculpin brain; however, there was no significant change in survivin protein expression after hypoxic or

hypoxic/reoxygenation treatments (Figure 8). An obvious possibility for our survivin findings, could be again that this protein does not play an important protective role in the Longhorn sculpin's hypoxia tolerance. Cell death, repair, and survival mechanisms are complicated processes with many associated genes involved, and other genes, as opposed to survivin and Hsp70, such as Bcl-2 or Bcl-XL, may be the important players in sculpin neural cell survival.

### **Caspase 3**

Caspase 3, a known effector of apoptosis, has been suggested numerous times to execute the final morphologic and biochemical alterations of apoptosis (Kim et al., 2010); hence, the inclusion of this important apoptotic marker in the present study was to signify if an upregulation of apoptosis is present in response to hypoxia and hypoxia/reoxygenation in the Longhorn sculpin brain. Unfortunately, we were unsuccessful in our attempt to measure caspase 3 expression at the transcriptome level in the Longhorn sculpin, and as a result, we will be limited to discussion of caspase 3 only at the protein level. We were able to confirm the presence of active caspase 3 protein in the sculpin brain, however, our quantitative protein analysis was based solely on simple intensity measurements, and though discussed below, we must exercise caution in drawing absolute conclusions in reference to caspase 3 in the Longhorn sculpin without further investigation.

Our results indicated that there was no significant change in the protein expression of active caspase 3 between treatment groups, or between brain regions analyzed (Figure 9). The most likely possibility as to the reasoning behind the lack of significant protein expression change in our study, specifically in active caspase 3, a known effector in late apoptosis (Martin et al., 2005), could be due simply to the lack of apoptosis occurring; hence the Longhorn sculpin's

moderate tolerance to hypoxia. This idea is consistent with several studies of hypoxia-tolerant species, for example, a study of the extremely hypoxia-tolerant mole rat subjected to acute hypoxia (3%), natural mole tunnel environmental hypoxia (6%), and mild long term hypoxia (10%) for up to 44 hours suggested the suppression of apoptosis for enhanced cell survival, as demonstrated by a highly significant overrepresentation of groups of genes involved in anti-apoptotic pathways out of the outrageously large number of genes mapped, microarrayed, and measured using RT-QPCR (Malik et al., 2012). These ideas are further supported by a previously mentioned study of anoxia-tolerant turtle brains that revealed a lack of significant change in active caspase 3 protein levels measured by western blot analyses following early (1 hour) and long-term (4, 24 hours) anoxia. This same study did, however, report an increase in procaspase 3 protein expression, which suggested that apoptosis may be initiated, but not executed (Kesaraju et al., 2009) in species which exhibit a tolerance to low oxygen environments.

A study of the hypoxia-sensitive sturgeon exposed to hypoxia (15% O<sub>2</sub> for 30 minutes) and reoxygenation (6 or 30 hours of recovery) also found no significant change in caspase 3 protein expression measured by western blot analyses in the olfactory lobe, cerebellum, or pons/medulla; however, this same study did find a significant change in caspase 3 protein expression in the retina, optic tectum, pituitary, and spinal cord, which led them to conclude that apoptotic factor expression is not only dependent on the severity of hypoxic exposure, but that cell vulnerability to hypoxia also differs between brain regions (Lu et al., 2005). Therefore, our findings of unaltered active caspase 3 protein expression, though both expected and beneficial to sculpin survival, could possibly be attributed to the particular brain regions sampled, and further research with enhanced specificity and measurement of brain regions, as opposed to our

generalized superior, middle, and inferior regions sampled, would be required to eliminate this as a possibility.

## CONCLUSIONS

There is a growing interest in understanding the factors that govern the interplay between cell death and proliferation under various conditions, such as hypoxia (Mashanov et al. 2010), with emphasis placed heavily on ways to potentially exploit these mechanisms for human health benefits. Neural hypoxia can lead to brain damage, neurological deficits, coma, and death, yet the treatment and prevention of hypoxic cell death remain a challenge due to the lack of knowledge and understanding of cellular responses. Apoptosis has been suggested as one of the main processes that destroys tissue after hypoxic exposure in hypoxia-sensitive species (Meller et al., 2013); thus, exploring these genes of apoptotic interest in hypoxia-tolerant species that possess a natural solution to such problems as permanent tissue damage and mortality, could be a reasonable approach to identifying ways to prevent cell death in others following hypoxic exposures. After the exploration of apoptotic factors such as hypoxia inducible factors, active caspase 3, survivin, and Hsp70 exposed to hypoxia and post hypoxia recovery in this study, we cannot definitively conclude whether or not the Longhorn sculpin's moderate hypoxia tolerance is due to a lack of apoptotic cell death. More testing of apoptotic factors on both the transcriptome and protein level is required to determine if apoptosis is being avoided in these brains. Other physiological and biochemical adaptations, such as decreased metabolic rate, increased ventilation rate, increased hematocrit and haemoglobin O<sub>2</sub> affinity, and increased anaerobic respiration, have also been reported in fish as part of their survival strategies to hypoxia (Chen et al., 2012), and should also be investigated in future studies of the Longhorn

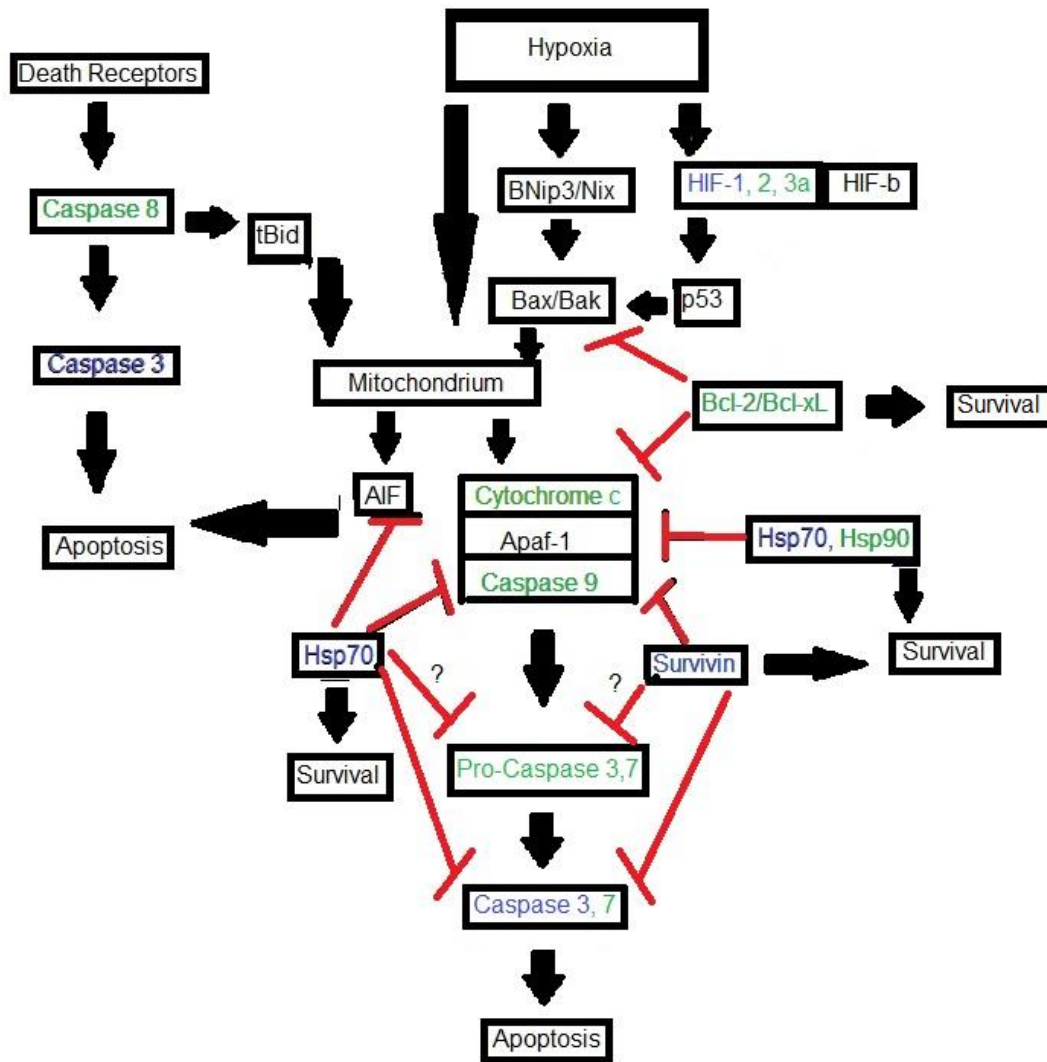


sculpin. As we gradually achieve a better understanding of the mechanisms that regulate cell death and cell survival processes, future medical advances, may become possible.

### FUTURE DIRECTIONS

Retrospectively speaking, several changes could have improved and added extra depth to this study, and should be considered for future work. In a general sense, broadening the amount of factors explored (Examples are illustrated in Figure 10) at both the transcriptome and protein level, such as HIF-1, -2, and -3 $\alpha$ ; more pro-apoptotic factors such as cytochrome c, several procaspases (inactive precursors of caspases), and active caspases, such as 3, 7, 8, and 9; and more anti-apoptotic factors such as Bcl family members and more heat shock proteins would greatly enhance the knowledge gained from this type of study. Increasing the specificity and identification of brain regions chosen to analyze, along with including more tissue types to study, as well as varying the durations of hypoxic exposure would intensify the results and inferences made possible. Overall, these future directions and continued research of hypoxia tolerant species and their survival strategies against hypoxia-induced apoptosis could drastically advance therapeutic strategies against apoptotic cell death in more sensitive species. For example, traumatic brain injury has been reported to be a leading cause of death and functional disability in western countries due to modes of both necrosis and apoptosis (Soustiel et al., 2005), and could possibly be prevented by enhancing anti-apoptotic factors following injury. The acute neuronal degeneration in the ischemic core upon stroke has also been reported in a review to be followed by a second wave of cell demise by apoptosis in connected sites that often exceeds the initial damage of stroke and contributes pivotally to significant losses in neurological functions (Rami et al., 2008). This same review also discussed the benefits of “patronizing the neuronal

endogenous anti-apoptotic machinery”, such as inhibitors of apoptosis (IAPs) (survivin is a member) against cell death following stroke (Rami et al., 2008). A resounding conclusion and remark consistent in these and many other studies is that more knowledge is needed in this field to significantly make a difference.



**Figure 10: Schematic illustrating apoptotic pathways that focus on our genes of interest in the present study (Labeled in blue), and examples of suggested genes for future directions of study (Labeled in green).** Extrinsic pathway: Death receptors on the cell surface are triggered by extracellular ligands, and in turn activate caspase 8 intracellularly, which in turn activates caspase 3, and leads to apoptosis. Intrinsic pathway: The hypoxia stimulus is received, then multiple pathway options lead to either mitochondrial damage towards apoptotic cell death, including an HIF pathway, or to cell survival, which can also include routes via HIF. Mitochondrial damage can then lead to the release of cytochrome c, which next can join Apoptosis protease activating factor-1 (Apaf-1) and activate caspase 9, which then can activate caspase 3, and lead to apoptotic cell death. Note that Bcl-2, Bcl-xL, survivin, Hsp70, Hsp90, and etc. can inhibit apoptosis in several locations along the cascade, and lead to cell survival. (Bleackley et al., 2001; Greijer et al., 2004; Harris et al., 2002; Ischia et al., 2013; Klettner et al., 2004; Lee, J-J et al., 2011; Maher et al., 2002; Peng et al., 2005; Rerole et al., 2010; Tilly et al., 2001).

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## APPENDICES

**Table 1: Kruskal-Wallis Analysis of  $\beta$ -actin**

H-value	3.8571
DF (Degree's of Freedom)	3
p-value	0.2773

**Table 2: Kruskal-Wallis Test for HIF-1 $\alpha$  Relative Values**

H-value	8.0211
DF	3
p-value	0.0456

**Table 3: Wilcoxon Mann-Whitney U-Tests Comparing HIF-1 $\alpha$  Treatment Groups**

In the figure below, Group 1 = control group, Group 2 = hypoxia group, Group 3 = early normoxic recovery group, and Group 4 = late normoxic recovery group.

Groups	Sample Size (N <sub>1</sub> )	Sample Size (N <sub>2</sub> )	U-Value	p-Value
1 vs 2	5	5	21	0.0367
1 vs 3	5	4	18	0.1113
1 vs 4	5	4	18	0.5403
2 vs 3	5	4	18	0.7133
2 vs 4	5	4	18	0.0662
3 vs 4	4	4	15	0.1124

**Table 4: Kruskal-Wallis Test for Hsp70 Relative Values**

H-value	3.2750
DF	3
p-value	0.3511

**Table 5: Levene Tests of HIF-1 $\alpha$  Values to test for Equal Variances**

	Treatment Group	Brain Region
F-Ratio	0.6076	0.2816
DF Num	2	2
DF Den	49	49
Prob > F	0.5487	0.7558

**Table 6: A Model I, two-way ANOVA of HIF-1 $\alpha$  Square Root Transformed Mean Intensity Values****Analysis of Variance**

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	0.06388301	0.007985	1.4230
Error	43	0.24130739	0.005612	<b>Prob &gt; F</b>
C. Total	51	0.30519040		0.2146

**Effect Tests**

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatment	2	2	0.02517531	2.2431	0.1184
Region	2	2	0.02252185	2.0067	0.1468
Region*Treatment	4	4	0.01543654	0.6877	0.6044

**Table 7: Critical Values on Levene Tests on Caspase 3 Values to test for Equal Variances**

	Treatment Group	Brain Region
F-Ratio	0.1537	1.1094
DF Num	2	2
DF Den	49	49
Prob > F	0.8579	0.3379

**Table 8: A Model I, two-way ANOVA of Caspase 3 Square Root Transformed Mean Intensity Values****Analysis of Variance**

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	0.05131403	0.006414	1.1235
Error	43	0.24550033	0.005709	<b>Prob &gt; F</b>
C. Total	51	0.29681436		0.3672

**Effect Tests**

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatment	2	2	0.01492073	1.3067	0.2812
Region	2	2	0.00434190	0.3802	0.6860
Region*Treatment	4	4	0.03122087	1.3671	0.2612

**Table 9: Levene Tests on Survivin Intensity Values**

	Brain Region	Treatment Group
F-Ratio	0.0528	5.8564
DF Num	2	2
DF Den	49	49
Prob > F	0.9486	0.0052

**Table 10: A Model I, one-way ANOVA for Survivin Values by Brain Region****Oneway Analysis of Survivin Root Trans By Region****Oneway Anova****Analysis of Variance**

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Region	2	0.00383717	0.001919	0.3669	0.6948
Error	49	0.25624967	0.005230		
C. Total	51	0.26008684			

**Table 11: Welch's ANOVA Test for Survivin Values By Treatment Group****Welch's Test**

Welch Anova testing Means Equal, allowing Std Devs Not Equal

F Ratio	DFNum	DFDen	Prob > F
1.2732	2	29.312	0.2950

**Table 12: Partial Longhorn sculpin Gene Sequences**

Gene	Partial mRNA sequence
B-actin	GTGCAAAGCCGGATTTCGCCGGAGACGACGCCCTCGTGCTGTCTTTCCCTC CATCGTCGGTCGCCCCAGACATCAGGGAGTGATGGTGGGTATGGGCCAGA AGGACAGCTACGTTGGTGATGAAGCCAGAGCAAGAGAGGTATCCTGACT CTGAAGTACCCATTGAGCACGGTATTGA
HIF- $\alpha$	TCGCACCGTCAATATCAAGTCTGCCAGCTGGAAGGTGCTGCACTGCACCG GACACCTGCAGATGTACAACAGCTGCCCGCCGCACGTGATGTGCGGCTTC ACGGAGCCCCCGCTCACCTGCGCCATCCTGATGTGCGAACCCATCGCACAC CCGTCCAACATCGACACGCCGCTGGACAGCAAGACCTTCCTCAGCAAT
Hsp70	GACATGAAGCACTGGCCCTTCAAGCTGGTGGGAGACGGAGGGAAGCCCAA GATTCAGGTGGAGCACAAGGGGAGGACAAAACCTTCTCCCCGAGGAGA TCTCCTCCATGGTCCTA