Spring 5-22-2020

# Physiological and behavioral correlates of HIF-1 alpha protein levels in the Gulf killifish, Fundulus grandis

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# Physiological and behavioral correlates of HIF-1 alpha protein levels in the Gulf killifish, Fundulus grandis

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biological Sciences

by

Jasmine C. Harris

B.S. University of New Orleans, 2010

May, 2020

Acknowledgements

I sincerely thank my advisor, Dr. Bernard Rees, for welcoming me into his lab and showing me the importance of appreciating detail and the big picture. I would also like to thank Drs. Anthony and Clancy for their availability and constructive suggestions that improved the quality of my work. I am grateful to Dr. Schluchter for her support and providing reagents without hesitation.

I would also like to acknowledge Rees lab members, Emily, Mohammad and Jessica, who contributed to this work. Kes Lynn graciously provided plates and teamed up with Courtney to salvage freezer samples. I thank Annie, GinaMarie, Fadeke, and all fellow graduate students for their encouragement throughout the process. Last, but not least, I would like to thank my family and friends for their continued support. I've limited my words for the sake of brevity, but I appreciate you all more than these words express.

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Abstract

Although the hypoxia-inducible transcription factor (HIF) orchestrates molecular responses to low oxygen, the amount of HIF-1α protein expressed during hypoxia varies among species, individuals, and tissues. This study measured HIF-1α protein levels along with several physiological and behavioral variables in the estuarine fish *Fundulus grandis* under normoxia (> 7 mg $l^{-1}$ dissolved oxygen) and hypoxia (1 mg $l^{-1}$ dissolved oxygen). Fish under hypoxic conditions had higher tissue levels of HIF-1α, hematocrit, blood glucose, blood lactate, frequency of aquatic surface respiration (ASR), and lower activity than normoxic controls. Under hypoxia, HIF-1α abundance in gill was positively correlated with body mass, HIF-1α abundance in liver was positively correlated with blood lactate, and HIF-1α abundance in brain was negatively correlated with time the fish was quiescent. Therefore, this work reveals that heterogeneity in HIF-1α protein in tissues of fish is related to select physiological and behavioral variables under hypoxia.

Keywords: oxygen, fish, metabolism, stress, physiology, behavior

# Introduction

## 1.1 Aquatic hypoxia

Aquatic hypoxia, the depletion of dissolved oxygen concentration in water, is a naturally occurring phenomenon, but in recent decades, human activity has resulted in increased eutrophication and elevated temperatures, exacerbating its geographic scope and severity (Diaz and Rosenberg, 2008; Jenny *et al*., 2016; Breitburg *et al*., 2018). Anthropogenic stressors have doubled the number of hypoxic zones each decade since the 1960s, resulting in over 400 systems comprising a total area of approximately 250,000 $km^2$ being affected by low oxygen, and the impact is steadily increasing (Diaz and Rosenberg, 2008; Conley *et al*., 2011). One well-documented area where the reaches of hypoxia have heavily disturbed ecosystems is the Gulf of Mexico, one of the largest hypoxic zones worldwide, second only to the Baltic Sea (Rabalais *et al*., 2014; Dzwonkowski *et al*., 2018; Carstensen and Conley, 2019).

Decreased oxygen levels frequently have dramatic effects on aquatic organisms, including avoidance of the hypoxic area, altered reproduction, and, in some instances, mortality, all of which may contribute to altered or decreased biodiversity in impacted habitats (Vaquer-Sunyer and Duarte, 2008; Small *et al*., 2014). On the other hand, certain hypoxia-tolerant species employ behavioral, physiological, and molecular responses that allow them to withstand low oxygen. Air breathing is one behavioral adaptation exhibited in nearly 400 fish species (Graham, 1997). Fish may also respond physiologically, for example, by increasing oxygen uptake and transport via changes in respiration, circulation,

and the amount or oxygen affinity of hemoglobin (Nikinmaa and Salama, 1998; Gilmour and Perry, 2007; Wells, 2009). At the molecular level, many fish show changes in gene expression that may improve their ability to tolerate aquatic hypoxia (Gracey *et al*., 2001; Ton *et al*., 2002; Richards *et al*., 2009). These changes have been found in genes associated with glycolysis (e.g. lactate dehydrogenase A, enolase), locomotion (e.g. α-tropomyosin, skeletal α-actin) as well as other biological functions.

1.2 Hypoxia-inducible factor (HIF)

The hypoxia-inducible transcription factors (HIFs) are evolutionarily conserved, central regulators of the molecular responses to low oxygen in animals (Semenza, 2012). HIF-1 was identified in mammalian cell culture as a protein required for the hypoxic induction of erythropoietin (Semenza *et al*., 1991; Maxwell *et al*., 1993). It was later found to be a heterodimer composed of an oxygen-dependent HIF-1α subunit (Fig. 1.1) and a constitutively expressed HIF-1β subunit (also known as ARNT; aryl hydrocarbon receptor nuclear translocator) (Wang *et al*., 1995).

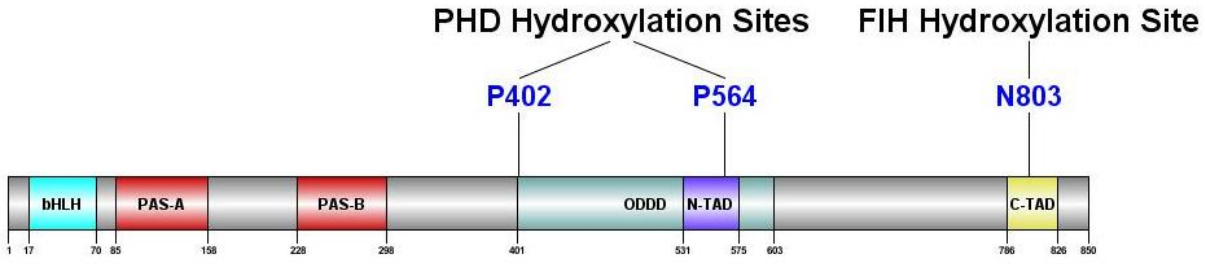


Figure 1.1: Schematic representation of HIF-1α domains. Basic helix-loop-helix (bHLH), Per-ARNT-Sim (PAS), oxygen-dependent degradation domain (ODDD), N transactivation domain (N-TAD), C transactivation domain (C-TAD), Proline residue (P), Asparagine residue (N), prolyl hydroxylase (PHD), factor inhibiting HIF (FIH). Figure modified from Kaelin, 2005.

Changes in the abundance of HIF-1α protein in mammalian cells are post-translationally controlled. During normoxia, there are two levels of oxygen-dependent regulation of the HIF-1α pathway (Fig. 1.2). In the von Hippel-Lindau protein (pVHL) dependent pathway, prolyl hydroxylase (PHD) hydroxylates HIF-1α at conserved proline residues in the oxygen-dependent degradation (ODD) domain (Maxwell *et al*., 1999; Kaelin, 2005). pVHL is a ubiquitin ligase that recognizes hydroxylated HIF-1α (Kaelin *et al*., 2005) and targets it for proteasomal degradation. In the pVHL-independent pathway, factor inhibiting HIF (FIH) hydroxylates HIF-1α at a conserved asparagine residue near the C-terminus. This hydroxylation prevents the interaction between HIF-1α and the accessory proteins CBP/p300, thereby suppressing transcriptional activity of any HIF-1α that escapes proteasomal degradation (Mahon *et al*., 2001; Cai *et al*., 2018). As oxygen levels decrease, the activities of PHD and FIH become substrate-limited, resulting in the stabilization and activation of HIF-1α protein. HIF-1α travels to the nucleus, binds to constitutively expressed HIF-1β to form an active heterodimer, and along with CBP/p300, binds to hypoxia response elements (HREs) in target genes, regulating their expression (Wenger and Gassmann, 1997). Target genes include those related to improvement of oxygen delivery systems, for example erythropoietin (Semenza and Wang, 1992) and vascular endothelial growth factor (Forsythe *et al*., 1996), increased glycolysis, for example lactate dehydrogenase (Firth *et al*., 1995; Rees *et al*., 2001), and several other processes thought to improve hypoxia tolerance of cells and tissues.

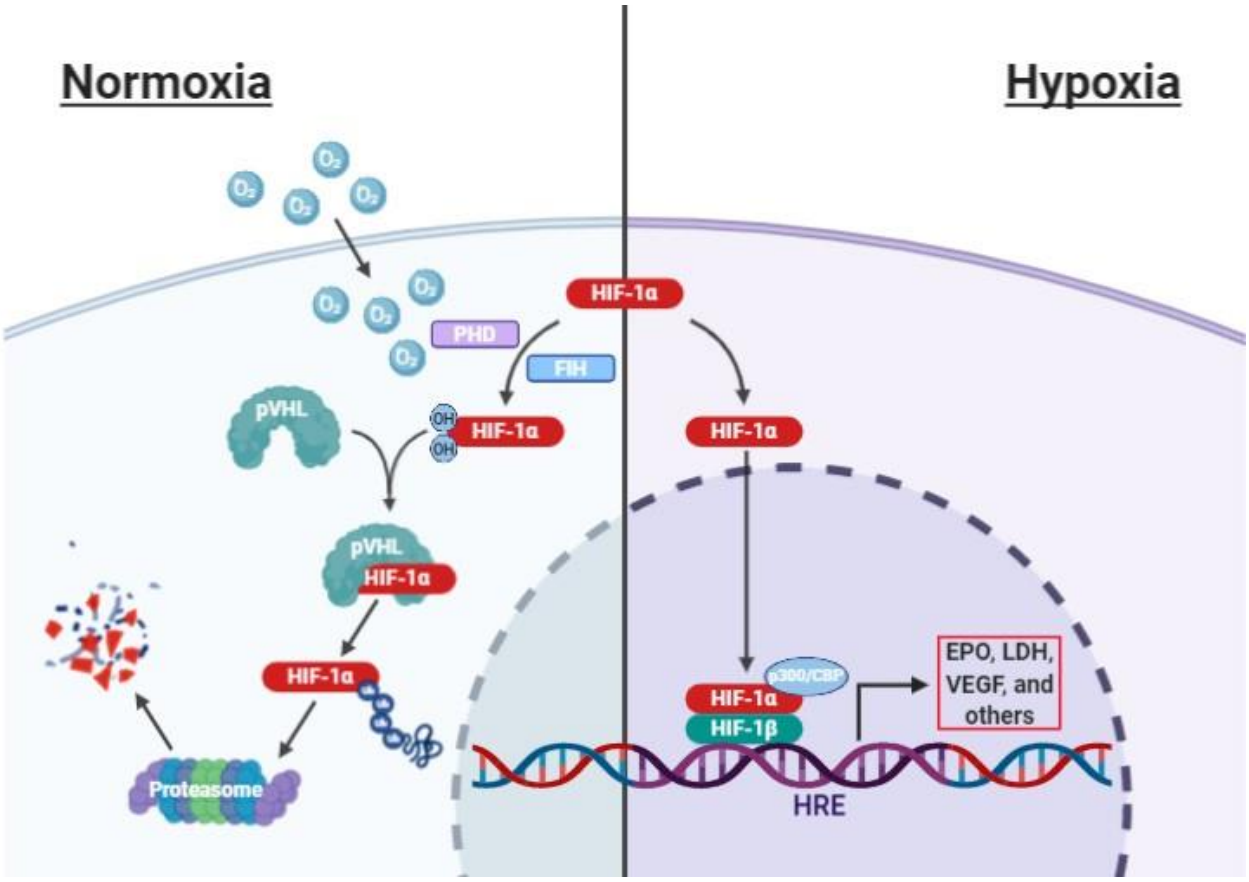


Figure 1.2: Simplified representation of HIF-1α regulation during normoxia and hypoxia. Under normoxic conditions, prolyl hydroxylase (PHD) hydroxylates specific proline residues within the HIF-1α oxygen-dependent degradation domain. This targets HIF-1α for ubiquitination by the von Hippel-Lindau protein (pVHL) and degradation by the 26S proteasome. Factor inhibiting HIF (FIH) may also prevent transcriptional activation by hydroxylating an asparagine residue in the HIF-1α C-TAD, thus preventing recruitment of p300/CBP coactivators.

Soitamo *et al*. (2001) were the first to report the presence of HIF-1α in fish. They sequenced HIF-1α from rainbow trout (*Oncorhynchus mykiss*) and showed that HIF-1α protein increased in abundance during short-term (4 h) hypoxic exposure of salmonid cells in culture (liver, gonad, and embryonic cells). Since then, changes in HIF-1α protein abundance have been observed during hypoxic exposure of zebrafish embryos (Kopp *et al*., 2011; Köblitz *et al*., 2015), as well as in tissues and cells from adult fishes (Rissanen *et al.* 2006a; Gonzalez-Rosario, 2016; Guan *et al*., 2017; Borowiec *et al*., 2018). Based upon these studies, it

appears that the protein levels of HIF-1α in fishes depend on the species, the tissue, and severity and duration of low oxygen exposure. Interestingly, within a given experiment, there is also variation in the tissue levels of HIF-1α protein among individuals, both during normoxia (Rissanen *et al.*, 2006b; Sollid *et al*., 2006) and hypoxia (Gonzalez-Rosario, 2016).

### 1.3 Individual variation in hypoxia tolerance

There is an increasing appreciation that individuals vary in their physiological capacities (Roche *et al*., 2016); among fishes, this includes their responses to and tolerance of hypoxia (Joyce *et al*., 2016; Metcalfe *et al*., 2016; Rees and Matute, 2018). Presumably, some of this variation is based upon heritable, genetic differences among individuals (Nikinmaa and Waser, 2007), although the links to specific genes have only been recently addressed (Healy *et al*., 2018). Though HIF-1α protein abundance may vary by individual, its relationship to other physiological associated with oxygen transport and carbohydrate metabolism, and behavioral variables is unknown.

### 1.4 Body mass

Body mass is a property of all organisms that affects many aspects of their physiology (Schmidt-Nielsen, 1984), including the hypoxia tolerance of fishes (Nilsson and Östlund-Nilsson, 2008). Rissanen *et al.* (2006b) found that smaller crucian carp (*Carassius carassius*) tended to have more HIF-1α protein in the liver, heart, gills, and kidney and that the increase in HIF-1α protein during hypoxia occurred faster than in larger fish. Thus, the present study tested the hypothesis that variation in body mass would explain, in part, differences

measured in HIF-1α protein levels in the brain, ovaries, skeletal muscle, liver, and gills of *F. grandis*.

1.5 Oxygen transport

One well-described physiological response of fishes to hypoxia is an increase in their capacity for oxygen uptake and transport (Gallaugher and Farrell, 1998; Nikinmaa and Salama, 1998). Oxygen delivery by blood to metabolically active tissues can be influenced by changes in hematocrit, hemoglobin concentration or type, intra-erythrocytic pH, and allosteric effectors of oxygen binding by hemoglobin (Jensen *et al*., 1998; Verde *et al*., 2002). In the context of tissue levels of HIF-1α, whether there is an increase or decrease relative to blood oxygen transport capacity is unknown. On one hand, because low oxygen is the signal for HIF-1α protein stabilization (Eltzschig and Carmeliet, 2011; Semenza, 2012), individuals with relatively lower oxygen transport capacity might have higher levels of HIF-1α protein in their tissues. On the other hand, because one target of HIF is the glycohormone erythropoietin (EPO), then those individuals with higher levels of HIF-1α protein would presumably respond by erythropoiesis, thereby increasing the number of red blood cells (RBCs) and hematocrit in whole blood samples. To distinguish which of these scenarios apply, if either, we measured hematocrit, RBC number, blood hemoglobin (Hb), and mean corpuscular hemoglobin concentration (MCHC) in the same fish that were measured for tissue levels of HIF-1α protein. These measurements also allowed us to determine mean corpuscular volume (MCV) to assess the contribution of RBC swelling to any observed changes in hematocrit, further

testing the hypothesis that individuals with differing oxygen transport capacities express different levels of HIF-1α protein in their tissues.

### 1.6 Carbohydrate metabolism

There are also changes in carbohydrate metabolism by fishes during exposure to hypoxia (Greaney *et al*., 1980; Kraemer and Schulte, 2004; Martínez *et al.,* 2006). As oxygen levels decrease, there is an increased reliance upon anaerobic glycolysis, especially in extra-hepatic tissues (Knox *et al*., 1980, Mandic *et al*., 2013, Wang and Richards, 2011). In order to support this increase, liver glycogen stores are mobilized and, combined with gluconeogenesis, frequently result in an increase in blood glucose levels (van Raaij *et al*., 1996; Petersen and Gamperl, 2010). Because glucose transporters are among the targets of HIF-1α (Ebert *et al*., 1995; Semenza, 2003; Bruckner *et al*., 1999), we predict that there will be a positive relationship between tissue levels of HIF-1α and blood glucose concentrations. Glycolytic enzymes are also targets of HIF-1α and low oxygen could stimulate expression of these enzymes, increasing glycolytic flux and lactate accumulation. Thus, we predict that there will be a positive relationship between tissue levels of HIF-1α and blood lactate concentrations. In addition, the major glucocorticoid in fishes is cortisol, which increases in the plasma during hypoxic exposure and, at least in some species, contributes to the increase in glycogenolysis and gluconeogenesis (Milligan, 2003; van Raaij *et al*., 1996; Wendelaar Bonga, 1997). If the effects of low oxygen on carbohydrate metabolite concentrations in the blood are mediated by

cortisol, then its concentration in the plasma might also be positively related to tissue levels of HIF-1α.

1.7 Behavioral responses

Finally, not all fish behave similarly in the presence of a stressor, including low oxygen. van Raaij *et al*. (1996) found that some rainbow trout try to escape when exposed to hypoxia, whereas others did not increase activity over normoxic controls. This behavioral variation was linked to whether the fish survived the exposure or not: fish that responded with an increase in activity either died during the hypoxic exposure or shortly thereafter, whereas all fish that did not increase in activity survived the entire experiment. The behavioral difference was correlated with differences in hormone signaling (high activity fish had higher norepinephrine and lower cortisol than low activity fish) and plasma lactate concentrations (higher in high activity fish). Another behavioral response to hypoxia is aquatic surface respiration (ASR), in which fish ventilate their gills with the surface layer of water, which is more likely to be higher in oxygen due to diffusion from the atmosphere (Chapman and McKenzie, 2009). Recently, Rees and Matute (2018) showed that *F. grandis* differ from one another in their propensity to conduct ASR under controlled low oxygen conditions. In light of these studies, we hypothesize that individuals in the current experiment may differ in their overall activity or their tendency to conduct ASR in response to hypoxia. If either affects tissue oxygenation, e.g., increasing activity causing physiological hypoxia exacerbating the environmental hypoxia, those individuals with greater activity might also display higher levels of HIF-1α in their tissues.

## 1.8 Fundulus as a model system

The Fundulus genus is composed of small, teleost fishes, specifically topminnows and killifish that dwell in coastal marshes along the eastern coast of North America, the Gulf of Mexico and inland waterways. *F. heteroclitus* is an important model in field research because this species can endure fluctuations in temperature, salinity, oxygen concentrations, pollution, and they exhibit hallmark responses to hypoxia (Burnett *et al*., 2007). Their sister species, *F. grandis* Baird and Girard 1853, resides in the Gulf of Mexico and is also an abundant, robust model that can tolerate hypoxia and is easily maintained in a laboratory setting (Everett and Crawford, 2010; Everett *et al*., 2012; Virani and Rees, 2000). Also, HIF-1α protein abundance has been measured in the tissues of both *F. heteroclitus* (Borowiec *et al*., 2018) and *F. grandis* (Gonzales-Rosario, 2016). Thus, *F. grandis* and the closely related *F. heteroclitus* are valuable models for assessing molecular responses of fish to aquatic hypoxia, which may help determine their resilience in the current context of changing aquatic habitats.

## 1.9 Research goals and objectives

The goals of the current study were to measure HIF-1α protein abundance in various tissues, specifically the brain, ovaries, skeletal muscle, liver, and gills, of the Gulf killifish, *F. grandis*, under normoxia and hypoxia, and explore the links between individual variation of HIF-1α protein abundance in the context of the broader physiological and behavioral responses of fish to low oxygen. Specifically, this thesis assesses:

1. The time- and tissue-dependence of HIF-1α protein abundance during short-term exposure to hypoxia (24 h),
2. Whether individual variation in HIF-1α protein correlates with variables related to blood oxygen transport,
3. Whether individual variation in HIF-1α protein correlates with variables related to carbohydrate metabolism,
4. And, whether individual variation in HIF-1α protein correlates with behavioral responses during the initial exposure of fish to hypoxia.

# Materials and Methods

*NOTE: Two series of experiments were conducted for this thesis: Series I was a pilot study performed in the spring of 2018; Series II was an expanded study performed in the spring of 2019. Except where indicated, Materials and Methods in the two series were the same*.

## 2.1 Fish collection and maintenance

All *F. grandis* were adult, female fish purchased from Joe's Landing (Barataria, LA, USA). Fish were transported to the University of New Orleans and treated within one week for bacterial infection and ectoparasites with API Furan-2 and API General Cure (Chalfont, PA, USA). Fish for Series I (n=12) were obtained in August 2017 and housed in 36-l (51 cm x 25 cm x 30 cm) aquaria that were part of an 8-tank recirculating system that shared a common sump (total system volume = ca. 350 l). Fish were held in the lab approximately 6 months prior to experiments. Fish for Series II (n=27) were obtained in February 2019 and housed in two independent 38-l (62 cm x 22 cm x 32 cm) aquaria for approximately 1 month prior to experiments.

Water used during maintenance and experiments was New Orleans city water treated with API Marine Stress Coat (Chalfont, PA, USA) to remove chlorine, chloramines, and ammonia, and adjusted to 1/3 strength seawater (1/3 SW; 10-12 ppt) by the addition of Instant Ocean Synthetic Sea Salt (Blacksburg, VA, USA). Water was aerated (>80% air saturation, as this is easily maintained and acceptable for normal feeding and metabolic processing in most aquatic organisms), filtered through biological and chemical media, and maintained at approximately 25°C. Temperature, dissolved oxygen (DO, in % saturation and mg $O_2$ $l^{-1}$), and salinity were monitored using a YSI Pro2030 oxygen-temperature-

salinity probe (Yellow Spring, OH, USA). Nitrates, nitrites, pH, and ammonia were determined weekly (API Master Test Kit; Chalfont, PA, USA) and partial water changes were performed as needed to maintain acceptable values. Fish were fed dried flake food once per day and the photoperiod was 12L:12D. All procedures with live fish adhered to established guidelines and were approved by the University of New Orleans Institutional Animal Care and Use Committee (University of New Orleans IACUC Protocol 18-006; see Appendix).

2.2 Experimental exposures

In Series I, fish were randomly assigned to one of three treatments of 4 fish each: normoxia; 6 h hypoxia; or 24 h hypoxia. Fish for each treatment were transferred to independent 38-l aquaria (62 cm x 22 cm x 32 cm), where they were held without food and allowed to adjust to their new surroundings overnight. The aquaria were wrapped in opaque plastic to minimize disturbance (Fig. 2.1) and the water was continuously aerated to maintain > 80% air saturation. The next day, 2 fish were sampled from one tank, which then remained aerated for 48 h (DO = 99.7 ± 0.5% air-saturation). At this time, 2 more fish were sampled, and data for these 4 fish were combined as the normoxic sample. Concurrently, the DO of the hypoxic tanks was lowered to ~12% air-saturation (approximately 1 mg $l^{-1}$ at experimental salinity and temperature) by the addition of nitrogen gas via a computer-controlled solenoid valve (SNS Industrial Group, Ontario, Canada). The water surface was partially covered by plastic “bubble wrap.” Data-acquisition software (AutoResp; Loligo Systems, Viborg, Denmark) continuously monitored DO in both tanks via optical oxygen sensors and controlled the initial

drop and subsequent maintenance of DO in the hypoxic treatments (11.9 ± 0.7%). Fish for the hypoxia treatments were sampled at 6 and 24 h after achieving the target DO level, and all exposures ended between 12:00 and 19:30.

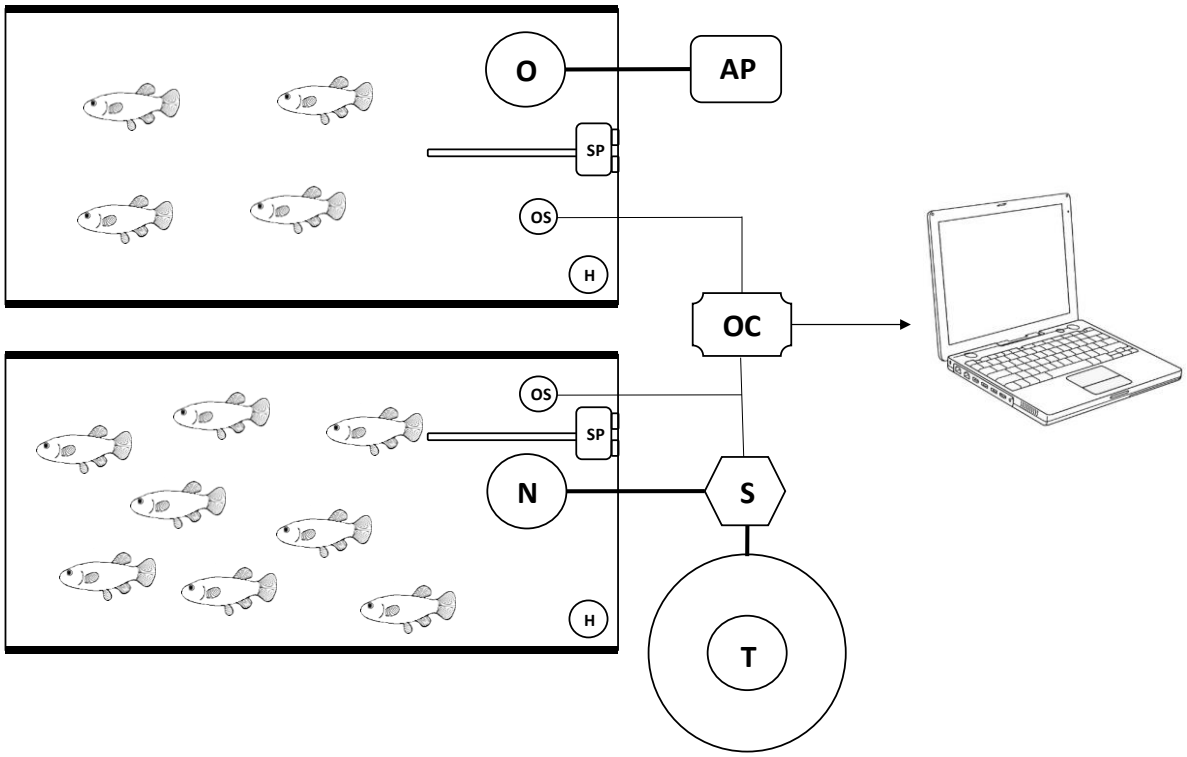


Figure 2.1: Apparatus used to expose *Fundulus grandis* to normoxia and hypoxia during Series I. Each aquarium (62 cm x 22 cm x 32 cm) contained an oxygen sensor (OS), a heater (H), and a submersible water pump (SP) to ensure homogenous water circulation. In the normoxic tank, air was continuously introduced from an air pump (AP) through a gas diffusing stone (O). In the hypoxic tank, the oxygen controller (OC) opened or closed a solenoid valve (S) to deliver nitrogen gas from a tank of compressed nitrogen (T). Nitrogen was introduced into the hypoxic tank via one large air stone (N), to lower and maintain DO at the target level. The OC monitored dissolved oxygen in both tanks, but only controlled DO in the hypoxic tank.

In experimental Series II, exposures were conducted on 4 fish at a time in a 76-l exposure tank (77 cm x 32 cm x 32 cm). This tank was subdivided by polystyrene grate (ca. 8 mm opening) into 4 fish compartments (19 cm x 15 cm x 22 cm each) and a back section that ran the entire width of the tank (77 cm x 16 cm x 32 cm). Opaque dividers separated the 4 fish compartments from one another, and each had a removable polystyrene grate below the air-water interface to prevent surface access by the fish. The rear compartment contained filters, heaters, and aerators, and communicated with each fish compartment by submersible water pumps to promote water circulation (Fig. 2.2).

Fish were assigned to one of four treatments: 6 h normoxia (n=6); 24 h normoxia (n=4); 6 h hypoxia (n=9); 24 h hypoxia (n=8). Fish were placed individually into the fish compartments of the exposure tank and fasted 22-28 h. For normoxic exposures, water was continuously aerated with room air to maintain > 80% air saturation. Normoxic fish were sampled 6 and 24 h after the initial adjustment period. In separate exposures, hypoxia was achieved by bubbling nitrogen gas into the rear compartment via a solenoid valve (SNS Industrial Group, Ontario, Canada) controlled by a CanaKit Raspberry Pi 3 Model B+ (North Vancouver, BC, Canada). The controller was programmed to take input from a galvanic oxygen electrode (Atlas Scientific, Long Island City, NY, USA) every 15 s and introduce nitrogen until the target DO (~12% air-saturation or 1 mg $l^{-1}$ DO) was reached. The target DO was achieved within 60 min and then maintained for the duration of the hypoxic exposure (a further 6 or 24 h). Plastic bubble wrap was placed on the surface of the water to minimize diffusion

of oxygen from ambient air during all exposures. DO was checked intermittently with a YSI handheld oxygen-temperature-salinity sensor. These measures showed that DO, salinity, and temperature were uniform throughout the system and that DO was within 4% of the value determined by the galvanic electrode. Exposures were timed such that all treatments ended between 13:30 and 16:30.

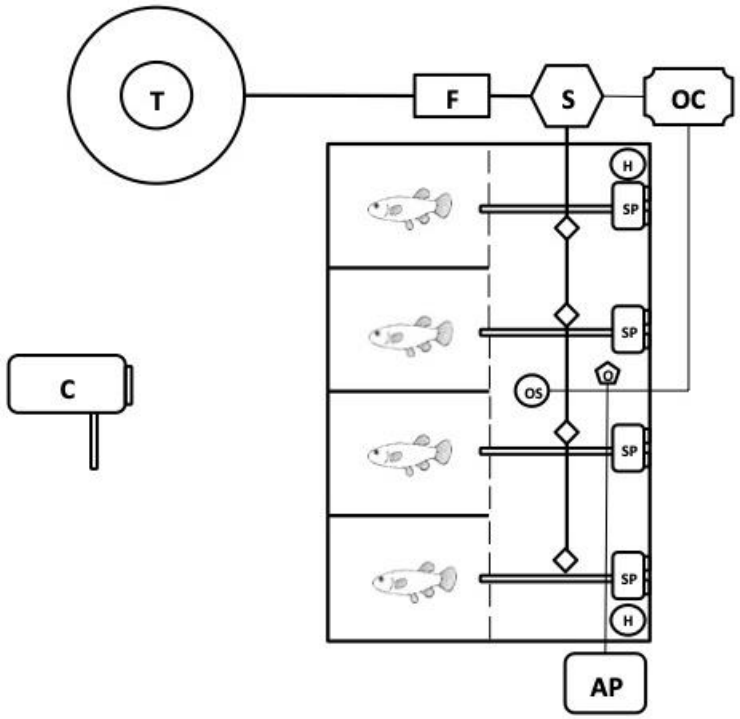


Figure 2.2: Apparatus used to expose *Fundulus grandis* to normoxia and hypoxia in Series II. A thick outline displays the perimeter of the entire tank (77 cm x 32 cm x 32 cm). The dashed line shows the partition between the front and back sections of the tank. The front section was divided into four compartments (19 cm x 15 cm x 22 cm each), each holding one fish. The back section (77 cm x 16 cm x 32 cm) accommodated an oxygen sensor (OS), two heaters (H), one air stone bubbling air (O) and four submersible water pumps (SP) to ensure homogenous water circulation. The oxygen controller (OC) measured DO every 15 s and opened or closed a solenoid valve (S) to deliver nitrogen gas from a tank (T) via a flowmeter (F). During hypoxic exposures, four air stones (small diamonds) introduced nitrogen, to gradually lower and maintain DO levels. Another air stone received air from an air pump (AP). The behaviors of all fish were simultaneously recorded with a camcorder (C).

2.3 Behavioral analysis

In Series II only, fish were recorded with an HD camcorder (Sony Handycam, Tokyo, Japan) during the first 6 h of exposures and their behaviors during this time were analyzed using EthoVision XT 14.0 tracking software (Puglis *et al*., 2018). Video files were analyzed for the following variables: mean velocity (in cm $s^{-1}$); duration of time attempting aquatic surface respiration (ASR); and duration of time immobile. The duration of attempted ASR was determined as the percentage of total time analyzed that a fish spent within 3.5 cm of the top grate (approximately 1.5 x body depth), and the duration immobile was the percentage of time an individual's velocity fell within the lowest 5% of all observed velocities for all fish.

2.4 Euthanasia and tissue sampling

After experimental exposures, fish were netted and immersed in a 1/3 SW ice-water slurry (< 2°C) until loss of equilibrium (within 3 min) (Larter and Rees, 2017). Fish were briefly blotted and bled by severing the caudal peduncle. Multiple blood samples (30-60 µl) were collected in heparinized capillary tubes and analyzed immediately for hematocrit or saved for determination of other blood variables (see below). Euthanasia was confirmed by severing the spinal cord immediately behind the head, after which, fish were weighed and dissected for brain, ovaries, skeletal muscle, liver, and gills. Tissues were frozen in liquid nitrogen, immediately placed on dry ice and transferred within 2 h to -80°C, where they were kept until analysis (within 9 months).

2.5 Blood analyses

Blood was measured for hematocrit, plasma cortisol, blood glucose, and blood lactate, essentially as described by Larter and Rees (2017). Hematocrit was determined by centrifugation in a microhematocrit centrifuge (BD Clay Adams AutoCrit Ultra 3, Franklin Lakes, NJ, USA) for 3 min. The same microcapillary tube was broken at the erythrocyte-plasma interface and the plasma was collected into a pre-weighed microcentrifuge tube and frozen at -80°C for plasma cortisol analysis (Cayman Cortisol EIA Kit, Ann Arbor, MI, USA).

A second blood sample was collected in a pre-weighed microcentrifuge tube containing 300 µl of ice-cold 6% perchloric acid (PCA), immediately vortexed, and placed on ice. Sample volume was determined as the mass of the whole blood sample divided by 1.1 g $ml^{-1}$ (the density of whole blood; Virani and Rees, 2000). The acidified whole blood sample was centrifuged at 15,000 x g at 4°C for 15 min to remove potassium perchlorate salts. Three hundred µl of supernatant was transferred to a clean microcentrifuge tube, combined with 2 µl of 0.05% methyl orange, and titrated with 2 M KOH in 0.4 M imidazole until neutral. The neutralized samples were stored at -20°C until analyzed for blood glucose and blood lactate.

Immediately prior to analysis, thawed samples were centrifuged as above. Glucose was determined in the supernatant using a glucose oxidase-peroxidase coupled colorimetric assay according the manufacturer's directions (Glucose Colorimetric Assay Kit, Ann Arbor, MI, USA). Lactate was determined in the supernatant using an enzyme-linked assay (Gutmann and Wahlefeld, 1974;

Virani and Rees, 2000) modified for a 96-well plate. Briefly, each 250-µl assay contained glycine (0.192 M), hydrazine (0.160 M), $NAD^+$ (5 mM), LDH (12 units/ml), and lactate standard or sample. Each standard and sample was assayed in quadruplicate, with an equal volume of water replacing LDH in one well to account for non-specific absorbance. Plates were agitated for 5 s, incubated at 37°C for 1 h, and read at 340 nm.

In Series II only, an undergraduate assistant collected a third blood sample for additional analyses (Emily Martin). This sample was immediately diluted into 1.0 ml of saline in a separate pre-weighed microcentrifuge tube. The composition of the saline was based upon the plasma concentrations of major inorganic ions and pH reported for *F. heteroclitus* (Genz and Grosell, 2011; Wood *et al*., 2010), with sodium citrate added to prevent clotting. The saline consisted of 145 mM NaCl, 5 mM KCl, 3 mM sodium citrate (dihydrate), 12 mM $NaHCO_3$, and 3 mM $NaH_2PO_4$, pH 7.6. Erythrocytes were counted in freshly diluted blood using a Neubauer hemocytometer. The same diluted blood sample was frozen at −20°C, thawed, and vortexed vigorously to lyse red blood cells. Lysates were clarified by centrifugation at 16,000 x g for 60 s at room temperature. Hemoglobin concentration was determined with a 96-well plate hemoglobin assay (Cayman Hemoglobin Colorimetric Assay Kit, Ann Arbor, MI, USA). Erythrocyte count (cells per ml), blood hemoglobin (g $dl^{-1}$ blood), and mean corpuscular hemoglobin concentration (MCHC; g $l^{-1}$ cells) were determined after accounting for dilution of whole blood by saline.

2.6 Tissue lysate preparation

Frozen tissues were rapidly weighed and approximately 100 mg was ground under liquid nitrogen in pre-cooled mortars and pestles. Frozen tissue powder was added to 1 ml lysis buffer [137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1mM sodium orthovanadate, 50 µg/ml MG-132 (Fisher scientific, Lenexa, KS, USA) and 1% (v/v protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)] and homogenized with 2 x 10 strokes using Teflon-glass homogenizers (Thomas Scientific, Houston, TX, USA) on ice. Lysates were centrifuged at 4°C for 10 min at 10,000 x g, and the supernatants were frozen at -80°C. Protein concentration was determined by the bicinchoninic acid protein assay (Smith *et al*., 1985) using bovine serum albumin as the standard (Pierce, ThermoFisher, Rockford, IL, USA).

2.7 Immunoprecipitation and detection of HIF-1α

HIF-1α was immunoprecipitated from tissue lysates containing 1-2 mg total protein (1 mg in brain; 2 mg in ovaries, liver and gills; 1.25 mg in skeletal muscle) using chicken polyclonal antibodies developed against HIF-1α from *F. heteroclitus* (Townley *et al*., 2017) as described in Gonzales-Rosario (2016). The final immunoprecipitates were resuspended in Laemmli sample buffer (Laemmli, 1970) containing 50 mM dithiothreitol, heated at 95°C for 3 minutes, clarified by centrifugation, and stored at -20°C.

Upon thawing, immunoprecipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 50 – 60 min

(Laemmli, 1970). Gels included molecular weight markers (Cell Signaling Biotinylated Protein Ladder, Danvers, MA, USA; Novex Sharp Pre-stained Protein Standard, Waltham, MA, USA) and positive HIF-1α controls (in vitro transcribed and translated *F. heteroclitus* HIF-1α; Townley *et al*., 2017). After electrophoresis, proteins were transferred to polyvinylidine difluoride (PVDF) membranes by tank (wet) electrotransfer at 100 V for 1 h at 10°C in 25 mM Tris, 192 mM glycine, 20% methanol, 0.05% SDS (Towbin *et al*., 1979). Electrotransfer was verified by staining the gels with modified colloidal Coomassie blue composed of 2% phosphoric acid, 10% ammonium sulfate, 0.1% Serva Blue G and 20% methanol (Neuhoff *et al*., 1988).

PVDF blots were blocked in TBS-T (20mM Tris, pH 7.6, 150 mM NaCl, 0.05% (v/v) Tween-20) containing 5% (w/v) non-fat dry milk at room temperature for 1 h, followed by incubation in the same buffer containing 1:500 dilution of chicken anti-HIF-1α. After overnight incubation in primary antibody at 4°C, blots were washed with TBS-T 3 times and then incubated for 1 h in TBS-T containing 5% non-fat dry fat dry milk and anti-biotin horseradish peroxidase (Cell Signaling, Danvers, MA, USA) and HRP-conjugated donkey anti-chicken antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted to 1:2000 and 1:5000, respectively. Blots were washed with TBS-T 5 times, developed in enhanced chemiluminescent detection reagents (Harlow and Lane, 1988) at room temperature for 60 s, and imaged with a ChemiDoc MP (Bio-Rad, Hercules, CA, USA). The resulting images were analyzed with ImageLab (Bio-Rad), after automatic background subtraction using a rolling disk size of 15 mm. In order to account for minor

variation in immunoprecipitation efficiency among samples, the intensity of HIF-1α band was expressed relative to the intensity of the IgY band for the same sample (see Results).

2.8 Analysis of tissue LDH activities

In Series I only, tissue activities of lactate dehydrogenase (LDH), a potential target of HIF signaling (Rees *et al*., 2009), were determined by an undergraduate assistant (Mohammad Hamed). A small portion of frozen tissue was homogenized as in Martinez *et al*. (2006) and analyzed for LDH activity as described therein. LDH activities were determined at 1 mM pyruvate to avoid substrate inhibition and data were expressed as specific activity (units mg protein$^{-1}$). LDH activity was not measured in the brain because the entirety of this tissue was required for immunoprecipitation and detection of HIF-1α.

2.9 Statistical analyses

Normality of the response variables was tested by Shapiro-Wilk tests and equality of group variance was tested using Bartlett's tests. Variables that were not normally distributed were transformed using the Box-Cox technique (Box and Cox, 1964). In Series I, analysis of variance (ANOVA) was used to test for effects of treatment (normoxia, 6 h hypoxia, and 24 h hypoxia) and Tukey's test was used for multiple comparisons. In Series II, the effects of treatment (normoxia and hypoxia), exposure time, and the interaction between treatment and time were assessed by two-way ANOVA. Because HIF-1α abundance in skeletal muscle was not normally distributed after Box-Cox transformation in either experimental series, non-parametric Kruskal-Wallis tests were used to assess

the effects of treatment (Series I) or the effects of oxygen treatment, exposure time, and the interaction between these two factors (Series II). Plasma cortisol data from Series II was also analyzed with Kruskal-Wallis tests due to its non-normal distribution. Relationships between levels of HIF-1α in various tissues, as well as between levels of HIF-1α and the other response variables, were assessed by Spearman's rank order correlation. Statistical analyses were performed in R version 3.6.1 and graphs were created in GraphPad Prism version 7.01.

# Results

## 3.1 Series I

### 3.1.1 Blood variables

Blood variables known to be influenced by low oxygen exposure of fishes were measured to ensure that the experimental conditions used here promoted physiological hypoxia in *F. grandis* (Fig. 3.1). By 6 h exposure, hematocrit exhibited a statistically significant increase in response to hypoxia that was maintained up to 24 h (Fig. 3.1 A; $F_{2,9} = 6.169$, $p_{Trx} = 0.021$). Hypoxia also led to significantly elevated blood glucose concentration by 6 h that was sustained throughout the duration of the experiment (Fig. 3.1 B; $F_{2,9} = 17.38$, $p_{Trx} < 0.001$). There was variation in the degree of hyperglycemia among fish, with one fish exposed to hypoxia for 24 h having twice that of any other fish (see individual symbols in Fig. 3.1 B). Although there was a trend toward higher levels of blood lactate, the effect of hypoxia was not significant (Fig. 3.1 C; $F_{2,9} = 2.867$, $p_{Trx} = 0.11$). There was considerable variation among individuals in blood lactate concentrations, as there was for blood glucose concentrations, especially at 24 h, which likely contributed to the lack of a treatment effect. Plasma cortisol levels were highly variable, especially in normoxia, and were not significantly affected by low oxygen (Table A1). The mean plasma cortisol value (± 95% C.I.) across all fish was 63 ± 50 ng $ml^{-1}$.

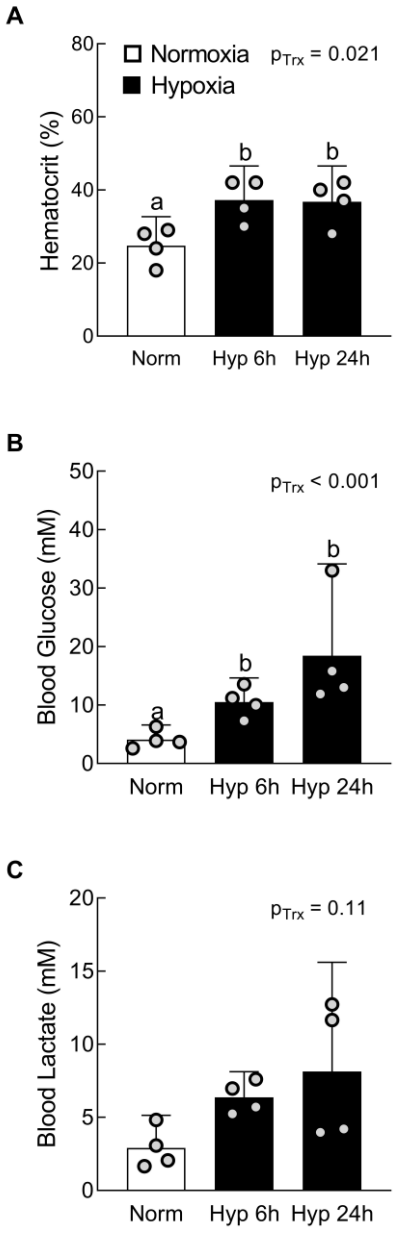


Figure 3.1: Hematocrit (A), blood glucose (B), and blood lactate (C) measured in *F. grandis* exposed to normoxia (> 7 mg $l^{-1}$ DO; open bars), or hypoxia for 6 h or 24 h (1 mg $l^{-1}$ DO; filled bars). Untransformed values are shown as means and the error bars represent the 95% confidence interval. The effects of treatment ($p_{Trx}$) were determined by ANOVA, using Box-Cox transformed values as necessary to achieve a normal distribution. Lowercase letters indicate significant differences between groups ($p < 0.05$).

### 3.1.2 Tissue HIF-1α levels

Levels of HIF-1α protein were determined in five tissues of fish that were maintained under normoxia or exposed to hypoxia for 6 or 24 h. The time between loss of equilibrium by a fish and freezing the last tissue (skeletal muscle) averaged 15 ± 4 min for Series I. HIF-1α protein levels were near the limit of detection in normoxia and increased during hypoxia, but to a degree that varied among time points, individuals, and tissues within given fish (Fig. 3.2). This variation is shown for skeletal muscle (Fig. 3.2 A), where HIF-1α in extracts from normoxic fish was very low and increased to a variable extent during hypoxia (compare lanes 7-14 with lanes 3-6). The relative abundance of HIF-1α for each sample was determined as the ratio of the intensity of the HIF-1α band (ca. 100 kD) to the intensity of the IgY band (ca. 68 kD). This approach showed that HIF-1α was significantly affected by experimental treatment in skeletal muscle (Fig. 3.2 B; $\chi^2 = 6.57$, df = 2, $p_{Trx} = 0.037$), ovary (Fig. 3.2 D; $F_{2,9} = 11.32$, $p_{Trx} = 0.004$), and brain (Fig. 3.2 E; $F_{2,9} = 28.31$, $p_{Trx} < 0.001$). In skeletal muscle, pairwise comparisons among treatments failed to distinguish either the 6 h or 24 h hypoxic values from normoxia, likely due to small sample sizes and variability within groups. In ovary and brain, on the other hand, HIF-1α abundance was significantly elevated by 6 h of hypoxia and remained significantly higher than normoxia at 24 h exposure. The effect of treatment on liver HIF-1α abundance was not significant (Fig. 3.2 C; $F_{2,9} = 2.136$, $p_{Trx} = 0.17$), despite a trend towards increased levels under hypoxia. Levels of HIF-1α in gill were low, variable, and unaffected by treatment (Fig. 3.2 F; $F_{2,9} = 0.7532$, $p_{Trx} = 0.50$).

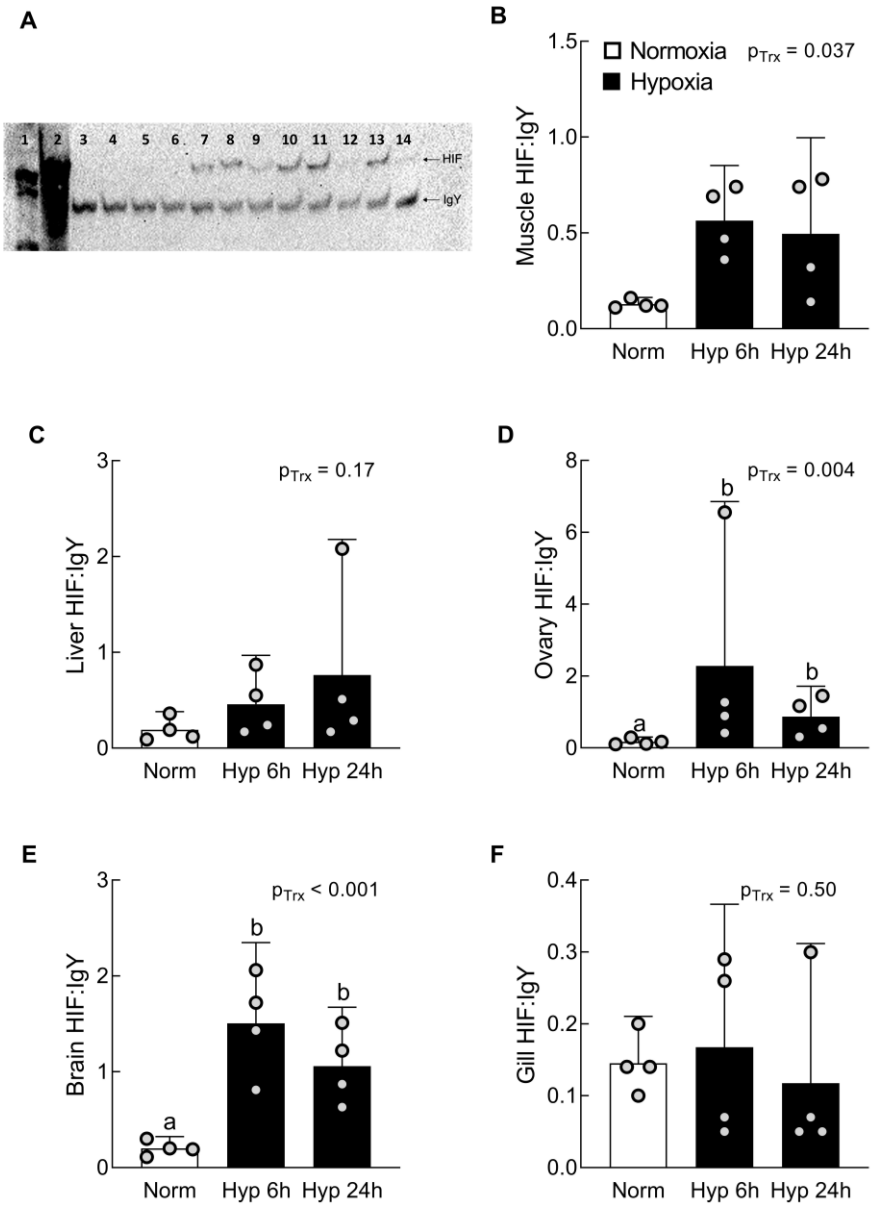


Figure 3.2: HIF-1α protein levels in tissues of *F. grandis* exposed to normoxia (> 7 mg $l^{-1}$ DO; open bars) or hypoxia for 6 h or 24 h (1 mg $l^{-1}$ DO; filled bars). Panel A shows a western blot of HIF-1α immunoprecipitated from skeletal muscle lysates of fish exposed to normoxia (lanes 3-6), 6 h hypoxia (lanes 7-10) or 24 h hypoxia (lanes 11-14). A positive HIF-1α control is shown in lane 2 and arrows to the right indicate migration of HIF-1α and chicken IgY. Relative HIF-1α protein abundance (the ratio of the HIF-1α band intensity to the IgY band intensity) was determined for skeletal muscle (B), liver (C), ovary (D), brain (E), and gill (F). Untransformed values are shown as means and the error bars represent the 95% confidence interval. Statistical analyses (ANOVA) used Box-Cox transformed values as necessary to achieve a normal distribution. Skeletal muscle HIF-1α could not be transformed to a normal distribution and was evaluated by Kruskal-Wallis test followed by Dunn's test. Lowercase letters indicate significant differences between groups ($p < 0.05$).

### 3.1.3 LDH activity

The specific activity of LDH (units mg protein$^{-1}$) was measured in tissues of *F. grandis* because it is a potential target of transcriptional control by HIF-1α. However, there were no effects of hypoxia over this period on LDH activity in skeletal muscle (Fig. 3.3 A; $\chi^2 = 1.077$, df = 2, $p_{Trx} = 0.58$), liver (Fig. 3.3 B; $F_{2,9} = 2.386$, $p_{Trx} = 0.15$), ovary (Fig. 3.3 C; $F_{2,6} = 1.892$, $p_{Trx} = 0.23$), and gill (Fig. 3.3 D; $F_{2,7} = 0.477$, $p_{Trx} = 0.64$). LDH activity was not measured in brain due to limited amounts of this tissue.

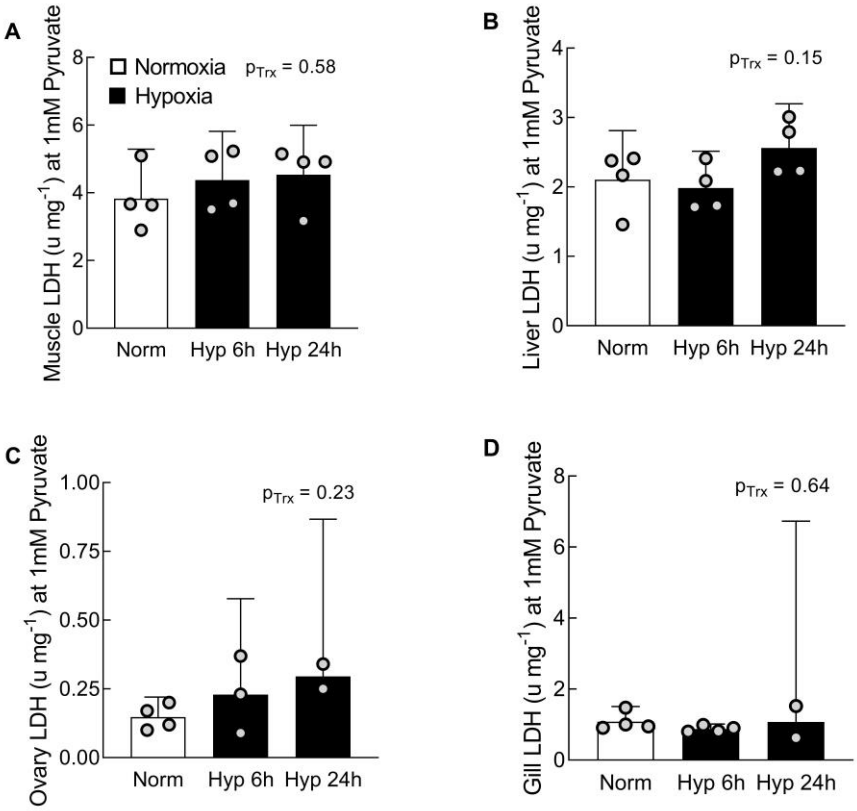


Figure 3.3: LDH activity in *F. grandis* exposed to normoxia (> 7 mg l$^{-1}$ DO; open bars) or hypoxia for 6 h or 24 h (1 mg l$^{-1}$ DO; filled bars). Units of LDH activity were measured per millligram of protein in skeletal muscle (A), liver (B), ovary (C), and gill (D). Untransformed values are shown as means and the error bars represent the 95% confidence interval. Statistical analyses (ANOVA) used Box-Cox transformed values as necessary to achieve a normal distribution. Skeletal muscle LDH could not be transformed to a normal distribution and was evaluated by Kruskal-Wallis test followed by Dunn's test.

## 3.2 Series II

### 3.2.1 Blood variables

In Series II, other indicators of oxygen transport capacity in *F. grandis* augmented measures of hematocrit during hypoxic exposure (Fig. 3.4). As seen in Series I, hypoxia resulted in a statistically significant increase in hematocrit (Fig. 3.4 A; $F_{3,23}$ = 3.03, $p_{Trx}$ = 0.006), although both the absolute hematocrit values and the change during hypoxia were smaller than in Series I. During these short-term hypoxic exposures, there was no significant increase in erythrocyte count (Fig. 3.4 B; $F_{3,23}$ = 1.187, $p_{Trx}$ = 0.31), but rather a significant increase in mean corpuscular volume (MCV; Fig. 3.4 C; $F_{3,23}$ = 3.299, $p_{Trx}$ = 0.015). Consistent with no significant change in erythrocyte count, there was no significant change in blood hemoglobin (Hb) concentration (Fig. 3.4 D; $F_{3,23}$ = 0.556, $p_{Trx}$ = 0.40). Because hypoxia led to an increase in MCV with no increase in blood Hb, low oxygen caused a significant decrease in mean corpuscular hemoglobin concentration (MCHC) when expressed per liter of cells (Fig. 3.4 E; $F_{3,23}$ = 3.457, $p_{Trx}$ = 0.004). The inverse relationship between MCHC and MCV was highly significant (Fig. 3.4 F; rho = -0.73, $p < 0.001$), illustrating that changes in MCHC were due to changes in cell volume, rather than changes in hemoglobin content per cell, which was not affected by hypoxia (Table A2). There was no effect of time of exposure, nor any significant interaction between exposure type and time, on any of these variables, indicating the effects of hypoxia occurred within the first 6 h of exposure.

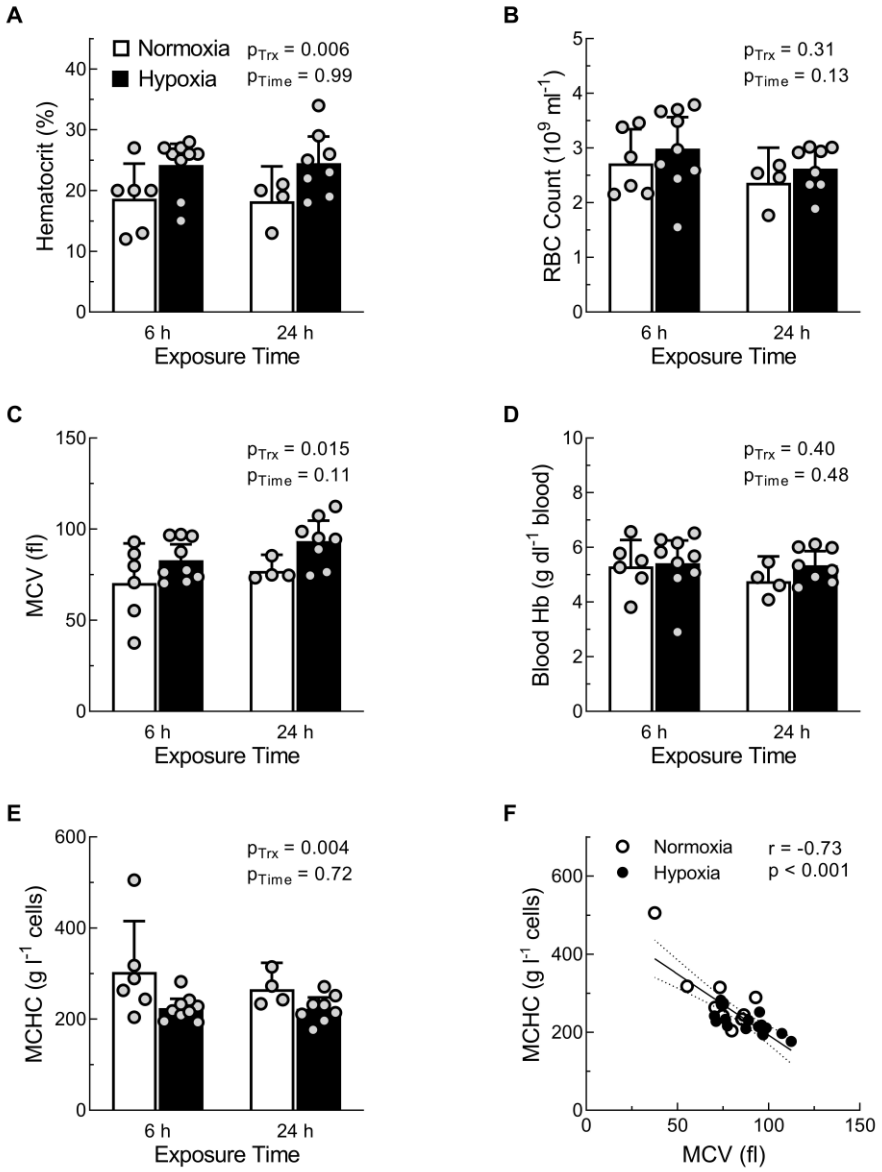


Figure 3.4: Blood oxygen transport variables in *Fundulus grandis* during short-term hypoxia. Hematocrit (A), red blood cell (RBC) count (B), mean corpuscular volume (MCV) (C), blood hemoglobin (Hb) (D), and mean corpuscular hemoglobin concentration (MCHC) (E) were measured in *F. grandis* exposed to normoxia (> 7 mg $l^{-1}$ DO; open bars) or hypoxia (1 mg $l^{-1}$ DO; filled bars) for 6 or 24 h. Untransformed values are presented as means and the error bars are the 95% confidence interval (sample sizes in Table A2). The effects of oxygen treatment, time, and the interaction between treatment and time were determined by 2-way ANOVA (MCHC was Box-Cox transformed). Treatment by time interactions were not significant for any variable (see Table A2 for $p_{Intrxn}$). Panel F shows the relationship between MCV and MCHC for all normoxic (○, n = 10) and hypoxic (●, n = 17) fish. The line of best-fit ± 95% C.I. is shown along with Spearman's rho and corresponding p value.

Blood variables related to carbohydrate metabolism were also measured. As seen in Series I, hypoxia led to moderate hyperglycemia (Fig. 3.5 A; $F_{3,23}$ = 9.553, $p_{Trx}$ < 0.001). In contrast to Series I, though, the increase in blood lactate in Series II was significant (Fig. 3.5 B; $F_{3,23}$ = 34.157, $p_{Trx}$ = 0.002), presumably reflecting an increase in anaerobic glycolysis. As observed for variables related to oxygen transport capacity, there was neither an effect of duration of exposure nor an interaction between exposure type and time for either variable. Blood glucose and blood lactate were positively correlated across all fish (Fig. 3.5 C; rho = 0.62, $p$ < 0.001). There was substantial overlap between normoxic and hypoxic fish with low blood concentrations of glucose and lactate. The variability in responses to hypoxia among individuals could explain, in part, the differing effects of hypoxia in Series I and Series II. Plasma cortisol, the predominant glucocorticoid in fishes, was highly variable and not significantly affected by either hypoxic treatment or time of exposure (Table A2). The mean plasma cortisol value (± 95% C.I.) across all fish was 123 ± 55 ng $ml^{-1}$.

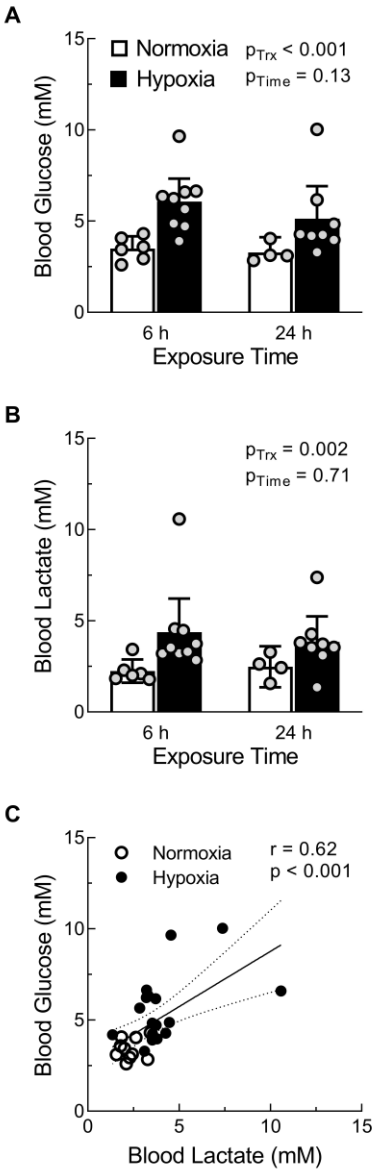


Figure 3.5: Blood variables related to carbohydrate metabolism in *Fundulus grandis* during short-term hypoxia. Blood glucose (A) and blood lactate (B) were measured in *F. grandis* exposed to normoxia (> 7 mg $l^{-1}$ DO; open bars) or hypoxia (1 mg $l^{-1}$ DO; filled bars) for 6 or 24 h. Untransformed values are presented as means and error bars represent the 95% confidence interval (sample sizes in Table A2). The effects of oxygen treatment, time, and the interaction between treatment and time were determined by 2-way ANOVA after Box-Cox transformation. Treatment by time interactions were not significant for either variable (see Table A2 for $p_{Intrxn}$). Panel C shows the relationship between blood glucose and blood lactate for all normoxic (○, n = 10) and hypoxic (●, n = 17) fish. The line of best-fit ± 95% C.I. is shown along with Spearman's rho and corresponding p value.

### 3.2.2 Tissue HIF-1α levels

The time between loss of equilibrium by a fish and freezing the last tissue (skeletal muscle) averaged 10 ± 1 min for Series II. As seen in Series I, HIF-1α protein increased in tissues of *F. grandis* exposed to hypoxia (Fig. 3.6). An example image of a western blot of HIF-1α protein immunoprecipitated from brains of fish exposed for 6 h (Fig. 3.6 A) shows that most hypoxic samples (lanes 3-4, 6-7, and 9) had more HIF-1α than corresponding normoxic controls (lanes 2, 5, 8, and 10-11), although there was appreciable variability in HIF-1α protein abundance in both hypoxic and normoxic fish. After normalizing HIF-1α band intensity for slight differences in immunoprecipitation efficiency, the effects of hypoxia were significant in brain (Fig. 3.6 B; $F_{3,18} = 14.85$, $p_{Trx} < 0.001$) and ovary (Fig. 3.6 C; $F_{3,19} = 2.295$, $p_{Trx} = 0.02$). In brain, mean HIF-1α protein levels were about 3-times higher in hypoxia than normoxia, and in ovaries, the increase in mean HIF-1α protein abundance was about 2-fold. In these two tissues, there was no effect of exposure time nor was there an interaction between exposure type and time. In skeletal muscle, there was a significant interaction between treatment and exposure time (Fig. 3.6 D; $p_{Intrxn} = 0.006$), with significantly elevated HIF-1α protein levels in hypoxic fish at 6 h compared to corresponding controls. For liver (Fig. 3.6 E) and gill (Fig. 3.6 F), there were no significant effects of treatment, exposure time, or their interaction. Of note, levels of HIF-1α protein were highest in brain, followed by ovary and liver (see Fig. 3.6 y-axis scales and Table A2). High levels of HIF-1α protein were detected in brain despite using the lowest amount of protein from lysates of this tissue in

immunoprecipitation (see Methods). HIF-1α protein abundance was extremely low in skeletal muscle and gill.

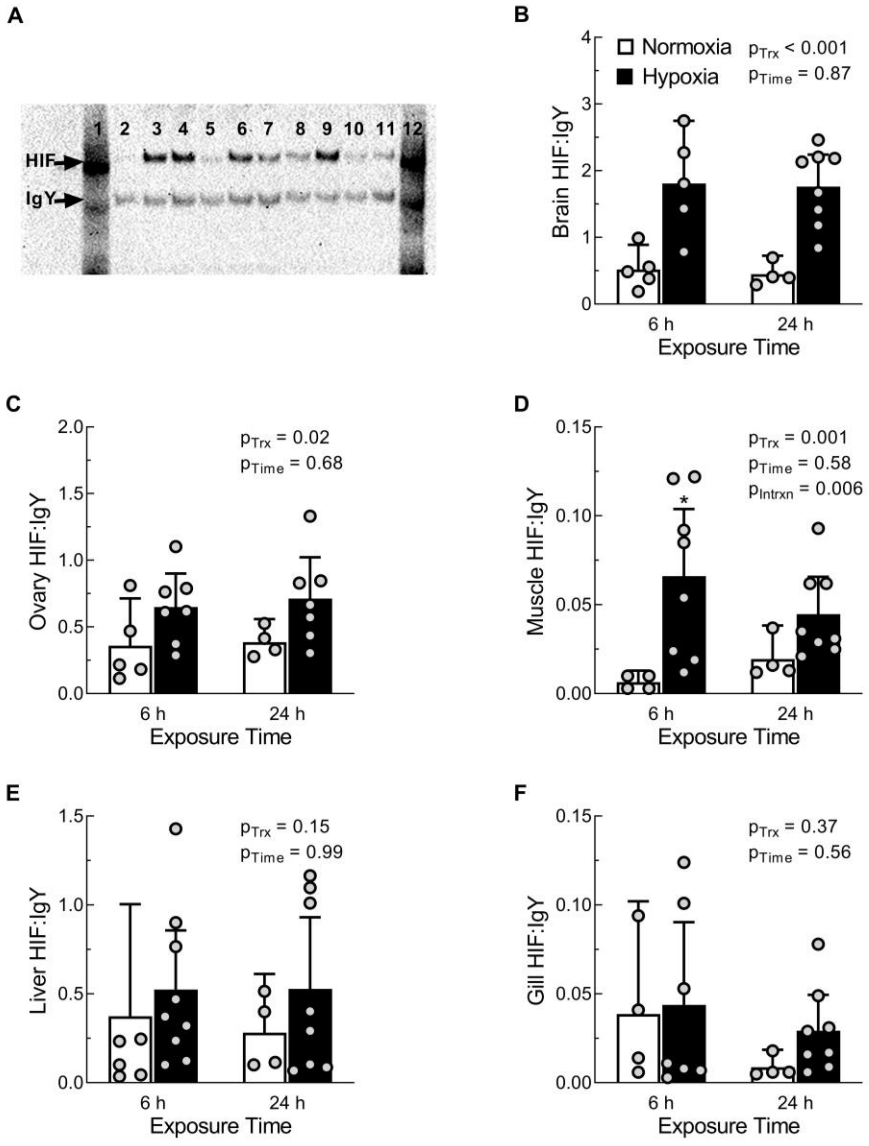


Figure 3.6: HIF-1α protein levels in tissues of *Fundulus grandis* during short-term hypoxia. Panel A shows a western blot of HIF-1α immunoprecipitated from brain lysates of fish exposed for 6 h to normoxia (> 7 mg $l^{-1}$ DO; lanes 2, 5, 8, 10, and 11) or hypoxia (1 mg $l^{-1}$ DO; lanes 3-4, 6-7, and 9). A positive HIF-1α control is shown in lane 1 and arrows show the mobility of HIF-1α and chicken IgY (to left). HIF-1α abundance in each sample was expressed as the ratio of the HIF-1α band intensity to the IgY band intensity. HIF-1α abundance was determined for brain (B), ovary (C), skeletal muscle (D), liver (E) and gill (F) in normoxia (> 7 mg $l^{-1}$ DO; open bars) or hypoxia (1 mg $l^{-1}$ DO; filled bars) at 6 and 24 h. Untransformed values are presented as means and the error bars represent the 95% confidence interval (sample sizes in Table A2). The effects of oxygen treatment ($p_{Trx}$), time ($p_{Time}$), and the interaction between treatment and time ($p_{Intrxn}$) were determined by 2-way ANOVA (data for all tissues except ovary were Box-Cox transformed). The treatment by time interaction was significant only for skeletal muscle (see Table A2 for $p_{Intrxn}$ of other tissues), for which the effect of hypoxia on HIF-1α abundance was significant at 6 h exposure (asterisk).

### 3.2.3 Behavioral responses

In Series II, the following behaviors of *F. grandis* were measured during normoxic or hypoxic exposures: the percentage of time a fish spent attempting aquatic surface respiration (ASR; Fig. 3.7 A), mean velocity (Fig. 3.7 B), and the percentage of time a fish was relatively immobile (Fig. 3.7 C). Because the onset of ASR is coupled with the initial decrease in oxygen concentration, this response is shown for the first hour of exposure to normoxia or hypoxia. Mean velocity and relative quiescence were analyzed during the first six hours of exposure. Due to unintentional loss of video data, there are no results for normoxic fish that were sampled at 6 h. Also, hypoxic fish that were ultimately sampled at 6 and 24 h experienced identical conditions during the first 6 h; hence the responses of both groups of hypoxic fish were pooled for visualization and statistical analysis.

As expected, the percentage of time spent attempting ASR was significantly higher for hypoxic fish during the initial onset of hypoxia (Fig. 3.7 A; $F_{1,15} = 8.559$, $p_{Trx} = 0.01$). This was effect, though, was driven by one particular fish whose attempted ASR duration was more than three times that of any other individual. In addition, exposure to hypoxia led to a reduction in mean velocity over 6 h (Fig. 3.7 B; $F_{1,15} = 10.15$, $p_{Trx} = 0.006$). While there was a corresponding increase in the percentage of time fish were immobile (Fig. 3.7 C), this was not a significant change due to the large variability and small sample sizes for behavioral measurements (Table A2).

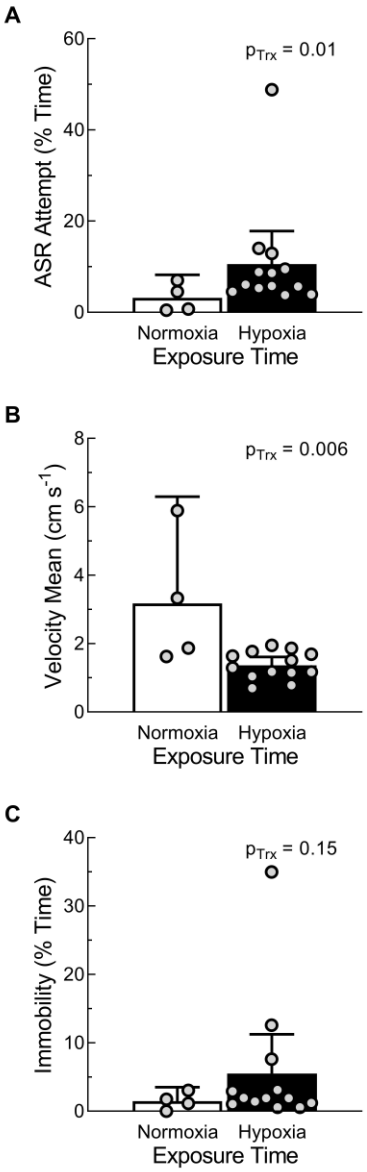


Figure 3.7: Behavioral responses of *Fundulus grandis* during initial exposure to hypoxia. Percentage of time attempting aquatic surface respiration (ASR) (A) was determined for *F. grandis* during the first hour of exposure while mean velocity (B) and percentage of time immobile (C) were determined for *F. grandis* during the first six hours of exposure to normoxia (> 7 mg $l^{-1}$ DO; open bars) or hypoxia (1 mg $l^{-1}$ DO; filled bars). Untransformed values are presented as means and the error bars represent the 95% confidence interval (sample sizes in Table A2). The effects of treatment ($p_{Trx}$) were determined by 1-way ANOVA.

### 3.2.4 Correlation analyses of HIF-1α levels

In both Series I and II, there was considerable variation in HIF-1α protein levels, blood variables, and behavior (Series II only) among fish during normoxia and hypoxia. Particularly in hypoxia, this variation suggested that the response to low oxygen differs among individual fish. Using Series II data, in which sample sizes were twice as large, the correlations between HIF-1α levels and other variables were assessed by Spearman's rank order correlation. Because the majority of variables were not affected by treatment time, values from the 6 and 24 h samples were pooled.

The first question addressed was whether the levels of HIF-1α protein covaried among tissues, either during normoxia or hypoxia (Table A3). Only a single correlation in normoxia, between ovary and liver HIF-1α (rho = 0.66), was significant at the 0.05 level (Table A3 A). The levels of HIF-1α protein were not correlated among any tissues of hypoxic fish (Table A3 B).

The second question addressed was whether individual variation in HIF-1α in any tissue correlated with other variables measured in this study (Table A4). Because variation in the response to hypoxia was of most interest, these analyses were performed only on data from fish exposed to hypoxia, pooling the data collected at 6 and 24 h. The only significant correlations were the positive relationships between HIF-1α protein levels in gill and body mass (Fig. 3.8 A; rho = 0.69, p = 0.004), between HIF-1α protein levels in liver and blood lactate concentration (Fig. 3.8 B; rho = 0.64, p = 0.006), and the negative correlation between HIF-1α protein levels in brain and the percent time a fish was immobile

(Fig. 3.8 C; rho = -0.75, *p* = 0.02). The last of these was influenced by a single fish that was very inactive (> 35% of the total time it had a velocity of < 5% of the overall mean). Removal of this individual, however, did not alter the negative relationship between brain HIF-1α protein and immobility (rho = -0.74, p = 0.04; data not shown). Variation in other blood or behavioral variables was uncorrelated with variation in tissue HIF-1α protein levels.

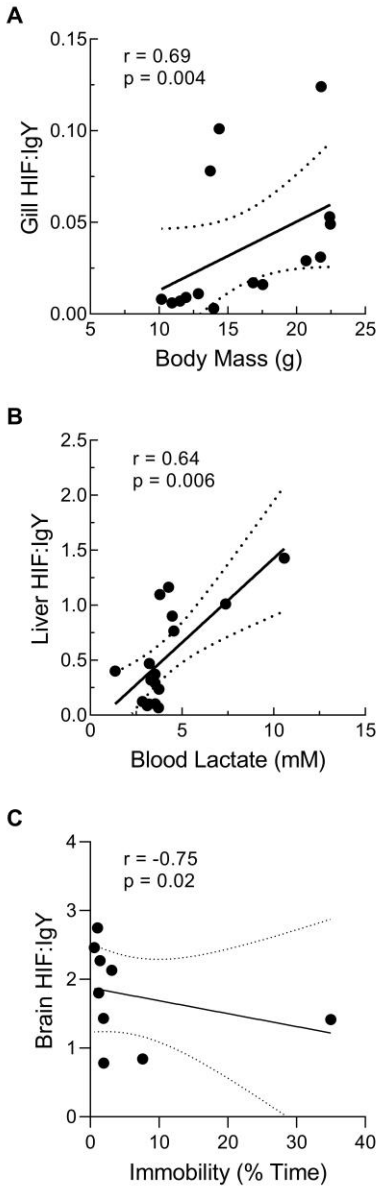


Figure 3.8: Relationships between gill HIF-1α abundance and body mass (A), liver HIF-1α abundance and blood lactate concentration (B), and brain HIF-1α abundance and time spent immobile by *Fundulus grandis* during short-term hypoxia. Values are shown for hypoxia-exposed fish from series II, pooling 6 and 24 h exposures. The line of best-fit ± 95% C.I. is shown with Spearman's rho and the corresponding p value.

# Discussion

## 4.1 Series I and Series II experimental differences

The results reported in this thesis came from two experiments designed to measure the effects of short-term (up to 24 h) exposure to hypoxia (1 mg $l^{-1}$) in the Gulf killifish, *Fundulus grandis*. Of primary interest was the time- and tissue-dependence of changes in HIF-1α, as well as the observation that tissue levels of HIF-1α vary among fish exposed to the same level of low oxygen (Gonzalez-Rosario, 2016). The two experimental series employed similar hypoxia exposures and they were conducted at the same time of year (March) with fish matched for size and sex. However, there were subtle differences in the maintenance of fish prior to experimental exposures, the control of DO during the exposure, the design of the exposure tank, and some of the experimental variables measured. For these or other unknown reasons, values of some variables differed between years (e.g., hematocrit, skeletal muscle HIF-1α levels), which prevented combining the two data sets. Because the sample sizes were larger and the control over experimental conditions was better in Series II, results from this series forms the basis of this discussion.

## 4.2 Blood variables

As previously observed in many fish species, hematocrit increased significantly during exposure to low oxygen (Gallaugher and Farrell, 1998; Lai *et al*., 2006; Baldisserotto *et al*., 2008; Larter and Rees, 2017; Li *et al*., 2018; Cadiz *et al*., 2019). The absolute value of hematocrit and the extent of the increase due to hypoxia were both higher in Series I than in Series II. In both series, however,

hematocrit was significantly higher by 6 h of hypoxia and remained elevated for the duration of the 24 h experiment.

An increase in hematocrit can result from an increase in red blood cell number, an increase in red blood cell volume, or both (Gallaugher and Farrell, 1998). Series II measurements showed that short-term exposure of *F. grandis* to 1 mg $l^{-1}$ DO (6 or 24 h) did not result in significant changes in red blood cell number; rather the increase in hematocrit under these conditions was explained by an increase in erythrocytic volume. Red blood cell swelling during hypoxia has been attributed to an increase in circulating catecholamines, which bind to β-adrenergic receptors and activate sodium-proton exchange across the erythrocyte cell membrane (Berenbrink and Bridges, 1994; Nikinmaa, 1997). Protons are moved from the cytoplasm to the plasma, leading to an increase in erythrocytic pH and an increase in oxygen binding by hemoglobin through the Bohr effect. Movement of $Na^+$ into cells, along with $Cl^-$, draws water into cells by osmosis, leading to an increase in cell volume (Bianchini and Wright, 2013). A consequence of cell swelling was a significant dilution of the intracellular concentration of hemoglobin (MCHC), as well as a dilution of intraerythrocytic modulators of hemoglobin affinity (primarily ATP in fishes; Gillen & Riggs, 1971; Powers and Edmundson, 1972; Powers, 1974; Jensen, 2004). A decrease in intraerythrocytic ATP concentrations would also tend to increase oxygen affinity of hemoglobin (Gillen & Riggs, 1972; Powers & Edmundson, 1972; Wood & Johansen, 1973). Data from Series II also showed that the hemoglobin concentration of blood remained unchanged during hypoxia, supporting the idea

that the increase in hematocrit was not due to an increase in red blood cell count. This experiment, lasting only 24 h, cannot rule out the possibility of increased red blood cell number and blood hemoglobin content during longer or more severe hypoxic exposures, as reported for other fishes (Baptista *et al*., 2016; Zhang *et al*., 2017).

Another response of fish to hypoxia is mobilization of glucose reserves and an increase in anaerobic carbohydrate metabolism (Nilsson and Östlund-Nilsson, 2008; Omlin and Weber, 2010). In the current study, short-term exposure of *F. grandis* to 1 mg $l^{-1}$ DO led to increases in both blood glucose and blood lactate. The extent of hyperglycemia during hypoxia varies among fishes and even for a given species under similar experimental conditions (MacCormack and Driedzic, 2007; Neiffer and Stamper, 2009; Larter and Rees, 2017). Indeed, blood glucose concentrations, under both normoxia and hypoxia, tended to be higher in Series I than in Series II, even though fish were held under similar conditions. Variability in blood glucose concentrations among fish can be attributed to differences in food intake, glucose storage in the form of glycogen, and the utilization of blood glucose by tissues.

In both series, hypoxia caused an increase in blood lactate, although this effect was not statistically significant in Series I, likely due to small sample sizes. While the increase in Series II was significant, it was smaller in magnitude than previously documented for this species held under similar conditions (Virani and Rees, 2000; Larter and Rees, 2017). This difference may reflect a lower reliance upon anaerobic metabolism by fish in the current study. Blood glucose and blood

lactate concentrations were positive correlated (Series II), indicating that those fish that responded to hypoxia by mobilizing glucose stores were the same ones that demonstrated increased glycolytic production of lactate. The overlap observed between fish exposed to normoxia and those exposed to hypoxia with low blood concentrations of blood glucose and blood lactate suggests that fish respond to hypoxia to differing degrees, including some that do not increase blood glucose and lactate values above normoxic controls.

In some fishes, the stress hormone cortisol has been implicated in the mobilization of glucose stores (Randall and Perry, 1992; Barton, 2002), although a link between hyperglycemia and cortisol concentration is not always seen (Larter and Rees, 2017; Reemeyer *et al*., 2019). In the current study, changes in blood glucose levels cannot be ascribed to changes in plasma cortisol, which was highly variable and not significantly affected by hypoxia.

4.3 Tissue HIF-1α levels

The present study found increased levels of HIF-1α protein in issues of *F. grandis* exposed to short-term hypoxia, although the timing and tissue responsiveness varied between experimental series. Specifically, HIF-1α protein was significantly elevated in brain, ovary, and skeletal muscle by 6 h of hypoxic exposure, compared to normoxic controls. In brain and ovary, the levels remained elevated at 24 h exposure, although there was a trend in Series I, but not in Series II, toward lower levels by 24 h. On the other hand, data from Series II, but not Series I, suggested a decrease in muscle HIF-1α protein abundance by 24 h hypoxia. For these three tissues, therefore, the effect of hypoxia was to

increase tissue proteins levels of HIF-1α, typically within the first 6 h, and whether those levels were maintained for 24 h or decreased depended upon the tissue and the experimental series. In liver, there was a trend toward higher levels in both experimental series, although neither was statistically significant primarily due to variability in the response among fish. The results for muscle mirror those recently described in the literature. Borowiec *et al.* (2018) showed that HIF-1a protein abundance elevated in the skeletal muscle of *F. heteroclitus* after 12 h hypoxic exposure. In *F. grandis*, Gonzales-Rosario (2016) found that HIF-1α protein levels increase in skeletal muscle and ovary during hypoxia.

In this study, there were no effects of hypoxic exposure on levels of HIF-1α protein in gill. In contrast, HIF-1α protein abundance increases in gills of crucian carp in response to hypoxia with the greatest accumulation occurring at 6 h when compared to 24 and 48 h of exposure (Rissanen *et al*., 2006b). Another study using crucian carp also determined that HIF-1α protein had accumulated in gills with the highest amounts at the same exposure time (6 h) and found that levels returned to those of fish exposed to normoxia by 48 h (Sollid *et al*., 2006). In rainbow trout, hypoxia was induced in the gill by exposure to copper, and HIF-1α protein increased in abundance in gills after 4 h (van Heerden *et al*., 2004)

There were also differences in HIF-1α abundance among tissues of hypoxic fish, with the highest levels measured in brain, followed by liver and ovary, and very low levels in skeletal muscle and gill. As mentioned above, there was considerable variation among individuals in tissue HIF-1α protein levels (also see below). In crucian carp, the amount of HIF-1α was positively correlated

among tissues during both normoxia and hypoxia (Rissanen *et al*., 2006b). In the current study, a positive correlation was only seen in normoxic fish between HIF-1α levels in liver and ovary. This result was marginally significant ($p = 0.05$), and considering the large number of tests performed, should be viewed with caution. Hence, the current study does not provide strong support for coordinated variation in tissue HIF-1α levels in either normoxia or hypoxia.

4.4 LDH activity

The enzyme LDH is a potential target of regulation by HIF-1α in mammals (Semenza *et al*., 1996) and fishes (Rees *et al*., 2001; Rees *et al*., 2009) In several species of fish, including *F. grandis*, LDH activity increases during hypoxia, at least in some tissues, e.g., liver (Kraemer and Schulte, 2004; Martinez *et al*, 2006). In the present study (Series I), however, hypoxia did not result in any change in LDH activity in the tissues examined. In tissues where HIF-1α protein was increased during hypoxia, it is possible that LDH mRNA expression increased, but because translation lags behind transcription and because translation is also subject to regulation, LDH protein, and hence activity, did not increase as expected. On the other hand, fish in this experiment could have depressed metabolism during hypoxia to an extent that increased LDH expression was unnecessary.

4.5 Behavioral responses

The behaviors of fish range from very stereotypical behaviors (e.g., aquatic surface respiration), to a general increase in activity (escape), to an overall suppression of activity (quiescence). It is common for fish to initially

attempt escaping low oxygen environments. In sole, escape and burst activity were used only when depressing metabolism below the standard metabolic rate and employing anaerobic metabolism were inadequate under hypoxia (Dalla Via *et al.*, 1998). As previously mentioned, rainbow trout that increased activity in response to hypoxia died shortly after the experiment (van Raaij *et al*., 1996). If escape attempts are unsuccessful, fish may reduce movement to lower oxygen demands (Wu, 2002). To assess these behaviors in *F. grandis*, the present study documented the percentage of time attempting ASR, mean velocity, and percentage of time fish were immobile. During the first hour of exposure, fish exposed to hypoxia spent a larger proportion of time attempting ASR than normoxic fish, which agrees with previous studies of this and other fish species (Love and Rees, 2001; Rees and Matute, 2018; Chapman and McKenzie, 2009). Mean velocity over the first 6 h of hypoxia was significantly lower than normoxic controls. This decrease in activity was reflected by a trend, albeit not statistically significant, toward increased proportion of time fish were immobile. All behavioral measures showed considerable variation among individuals, suggesting that individuals differed in their initial reactions to hypoxia (e.g., ASR, escape, or quiescence).

4.6 Correlation analyses of HIF-1α levels

By measuring multiple physiological and behavioral variables on the same fish for which HIF-1α levels were determined, the present study was able to address the question, “Is variation in tissue levels of HIF-1α protein correlated with measures of oxygen transport, carbohydrate metabolism, or initial behavior

in hypoxia?" This analysis demonstrated significant positive correlations between HIF-1α protein abundance in gill and body mass and between liver HIF-1α protein abundance and blood lactate concentration, and a negative correlation between brain HIF-1α protein abundance and the percentage of time a fish was relatively immobile (velocity was < 5% of the overall mean velocity). The present results on the relationship with body mass contrasts those of Sollid *et al*. (2006) and Rissanen *et al*. (2006), who observed negative relationships between body mass and HIF-1α protein abundance in several tissues of in crucian carp, including gill, during both normoxia and hypoxia. In the present study, the levels of HIF-1α were very low in gill. It is possible, therefore, that small increases in HIF-1α abundance in a small number of fish under hypoxia could have driven the observed positive relationship. Nevertheless, the present data do not support a negative relationship between HIF-1α abundance and body mass.

Although the positive correlation between liver HIF-1α protein and blood lactate does not mean that one variable causes the other, previous work from this lab has shown that the promoter of the liver form of lactate dehydrogenase (LDH) in the closely-related *F. heteroclitus* contains a functional hypoxia response element (Rees *et al*., 2009). While it is possible that HIF-1α mediated an increase in liver LDH, potentially contributing to the increase in blood lactate during hypoxia, there was no increase in LDH activity in Series I, nor was there any relationship among hypoxic fish between LDH activity and liver HIF-1α abundance (unpublished data). Consequently, it appears that low oxygen

independently increases both the level of liver HIF-1α protein and blood lactate such that these variables change in concert in fish exposed to low oxygen.

The final correlation observed here was a negative correlation between brain HIF-1α protein abundance and the percentage of time fish were quiescent. Although the value of rho was rather large (-0.75), the p value was only 0.02, meaning that this result could occur by chance when conducting the large number of correlations performed in this study (45). Also, the correlation appeared to be influenced by a single high value, although removal of this individual did not materially alter the result. While speculative, this relationship suggests that fish that have a lower level of HIF-1α in their brain are less active (i.e., more time immobile). If supported by further study, this relationship would demonstrate a link between a molecular indicator of hypoxia in the brain and a behavioral adjustment to hypoxia.

Counter to expectations, the data obtained here do not support the hypothesis that individuals with different oxygen transport capacity (high or lower) express different levels of HIF-1α protein in their tissues. Similarly, our data do not support a relationship between the hyperglycemic response or anaerobic metabolism and tissue HIF-1α protein levels (other than the relationship between liver HIF-1α protein and blood lactate), nor a relationship between circulating cortisol levels and HIF-1α protein abundance in tissues.

One reason that more correlations failed to rise to the level of statistical significance is sample size. This study was designed to have sample sizes similar to previous studies, which were generally sufficient to show statistical

differences among group means. For correlation analysis, however, sample sizes are limited to the number of fish for which both variables were measured. Occasional missing data (e.g. plasma cortisol, brain HIF-1α levels, and behavioral variables) resulted in smaller than anticipated sample sizes, and a decrease in statistical power to test correlations. These limitations could partly explain why so few correlations were significant in the present study and highlight the challenges in studying inter-individual physiological variation (see also Nikinmaa and Waser, 2007).

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# Appendices

## IACUC Approval

### Institutional Animal Care and Use Committee

UNIVERSITY OF NEW ORLEANS

**DATE:** **September 6, 2018**
**TO:** **Bernard B. Rees**
**FROM:** Simon Lailvaux
**RE:** *IACUC Protocol* #18-006
*Entitled*: Population and individual level variation of hypoxia tolerance in the Gulf killifish, Fundulus grandis

Your application for the use of animals in research (referenced above) has been approved beginning 9/6/2018 and expiring 9/5/2021. The initial approval period is one year. Near the end of this period, you will be asked to complete and submit an annual review in order to continue animal activities.

The University of New Orleans has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is **D16-00191**.

❖UNIVERSITY OF NEW ORLEANS Institutional Animal Care and Use Committee❖

Table A1. Series I means ± 95% C.I. of body mass, blood variables and tissue HIF-1α levels in *Fundulus grandis* exposed to normoxia (> 7 mg $l^{-1}$) or hypoxia (1 mg $l^{-1}$) for 6 or 24 h. The last column shows results of ANOVA assessing the effects of hypoxia treatment. Sample size was 4 fish per treatment unless otherwise indicated in parentheses.

| | Normoxia | Hypoxia | | p values |
|---|---|---|---|---|
| Variable | | 6 h | 24 h | Trx |
| Body Mass (g) | 19.5 ± 8.7 | 17.5 ± 1.9 | 16.7 ± 11.2 | 0.73 |
| Blood Variables | | | | |
| Hct (%) | 25 ± 8 | 37 ± 9 | 37 ± 10 | 0.021 |
| Blood Glucose (mM) | 4.1 ± 2.5 | 10.5 ± 4.2 | 18.4 ± 15.7 | $8.1x10^{-4}$ |
| Blood Lactate (mM) | 2.9 ± 2.2 | 6.4 ± 1.8 | 8.1 ± 7.5 | 0.11 |
| Plasma Cortisol (ng $ml^{-1}$) | 101 ± 210 | 21 ± 17 | 67 ± 59 | 0.34 |
| HIF-1α Relative Abundance | | | | |
| Brain | 0.20 ± 0.12 | 1.50 ± 0.84 | 1.06 ± 0.62 | $1.3x10^{-4}$ |
| Ovary | 0.17 ± 0.14 | 2.28 ± 4.57 | 0.87 ± 0.85 | 0.0035 |
| Skeletal Muscle | 0.13 ± 0.035 | 0.56 ± 0.29 | 0.50 ± 0.50 | 0.037[a] |
| Liver | 0.19 ± 0.19 | 0.46 ± 0.51 | 0.76 ± 1.42 | 0.17 |
| Gill | 0.15 ± 0.066 | 0.17 ± 0.20 | 0.12 ± 0.19 | 0.50 |
| LDH Activity (u/mg) | | | | |
| Skeletal Muscle | 3.83 ± 1.46 | 4.38 ± 1.44 | 4.54 ± 1.46 | 0.58[a] |
| Liver | 2.10 ± 0.71 | 1.98 ± 0.53 | 2.56 ± 0.64 | 0.15 |
| Ovary | 0.15 ± 0.073 (4) | 0.23 ± 0.35 (3) | 0.30 ± 0.57 (2) | 0.23 |
| Gill | 1.08 ± 0.42 (4) | 0.88 ± 0.14 (4) | 1.08 ± 5.65 (2) | 0.64 |

[a]Kruskal-Wallis test was used to assess the effects of treatment on skeletal muscle HIF-1α relative abundance and LDH activity

Table A2. Series II means ± 95% C.I. of body mass and variables related to blood oxygen transport, carbohydrate metabolism, tissue HIF-1α abundance, and behavior of *Fundulus grandis* exposed to normoxia (> 7 mg $l^{-1}$) or hypoxia (1 mg $l^{-1}$) for 6 or 24 h. The last three columns show results of ANOVA assessing the effects of hypoxia treatment, exposure time, and the interaction between treatment and time. Sample sizes are shown in parentheses.

| | Normoxia | | Hypoxia | | *p* values | | |
|---|---|---|---|---|---|---|---|
| Variable | 6 h | 24 h | 6 h | 24 h | Trx | Time | Intrxn |
| Body Mass (g) | 16.7 ± 4.8 (6) | 13.8 ± 2.4 (4) | 16.5 ± 4.1 (9) | 17.0 ± 3.7 (8) | 0.51 | 0.67 | 0.36 |
| Hct (%) | 19 ± 6 (6) | 18 ± 6 (4) | 24 ± 4 (9) | 25 ± 4 (8) | 0.0062 | 0.99 | 0.86 |
| RBC Count (cells $nl^{-1}$) | 2.7 ± 0.6 (6) | 2.4 ± 0.6 (4) | 3.0 ± 0.6 (9) | 2.6 ± 0.4 (8) | 0.31 | 0.13 | 0.98 |
| MCV (fl) | 71 ± 22 (6) | 77 ± 9 (4) | 83 ± 9 (9) | 94 ± 11 (8) | 0.015 | 0.11 | 0.73 |
| Blood Hb (g $dl^{-1}$) | 5.3 ± 1.0 (6) | 4.8 ± 0.9 (4) | 5.4 ± 0.8 (9) | 5.4 ± 0.5 (8) | 0.40 | 0.48 | 0.52 |
| Cellular Hb (umole per $10^9$ cells) | 0.31 ± 0.06 (6) | 0.32 ± 0.05 (4) | 0.29 ± 0.02 (9) | 0.32 ± 0.02 (8) | 0.46 | 0.10 | 0.39 |
| MCHC (g $l^{-1}$) | 304 ± 111 (6) | 266 ± 58 (4) | 224 ± 21 (9) | 223 ± 25 (8) | 0.0040 | 0.72 | 0.87 |
| Blood Glucose (mM) | 3.5 ± 0.7 (6) | 3.3 ± 0.8 (4) | 6.1 ± 1.3 (9) | 5.1 ± 1.8 (8) | $3.8 \times 10^{-5}$ | 0.13 | 0.55 |
| Blood Lactate (mM) | 2.2 ± 0.6 (6) | 2.5 ± 1.1 (4) | 4.4 ± 1.8 (9) | 3.8 ± 1.4 (8) | 0.0023 | 0.71 | 0.44 |
| Plasma Cortisol (ng $ml^{-1}$) | 97 ± 132 (6) | 173 ± 298 (4) | 74 ± 113 (5) | 149 ± 103 (8) | 0.76[a] | 0.24[a] | 0.64[a] |
| HIF-1α Relative Abundance | | | | | | | |
| Brain | 0.52 ± 0.37 (5) | 0.45 ± 0.28 (4) | 1.81 ± 0.94 (5) | 1.76 ± 0.48 (8) | $2.9 \times 10^{-6}$ | 0.87 | 0.86 |
| Ovary | 0.36 ± 0.36 (5) | 0.39 ± 0.17 (4) | 0.65 ± 0.25 (7) | 0.71 ± 0.31 (7) | 0.018 | 0.68 | 0.88 |
| Skeletal Muscle | $0.0065 \pm 6.4 \times 10^{-5}$ (4) | 0.020 ± 0.019 (4) | 0.066 ± 0.038 (8) | 0.045 ± 0.021 (8) | 0.001[a] | 0.58[a] | 0.006[a] |
| Liver | 0.37 ± 0.63 (6) | 0.28 ± 0.33 (4) | 0.52 ± 0.33 (9) | 0.53 ± 0.40 (8) | 0.15 | 0.99 | 0.58 |
| Gill HIF-1α | 0.039 ± 0.063 (4) | 0.0088 ± 0.0098 (4) | 0.044 ± 0.046 (7) | 0.029 ± 0.020 (8) | 0.37 | 0.56 | 0.17 |
| ASR (% Time) | n.d. | 3.2 ± 5.0 (4) | 10.6 ± 7.2 (13) | | 0.01[b] | n.a. | n.a. |
| Velocity (cm $s^{-1}$) | n.d. | 3.2 ± 3.1 (4) | 1.4 ± 0.2 (13) | | 0.006[b] | n.a. | n.a. |
| Immobility (% Time) | n.d. | 1.5 ± 2.0 (4) | 5.5 ± 5.7 (13) | | 0.15[b] | n.a. | n.a. |

[a]Plasma cortisol and skeletal muscle HIF-1α: non-parametric tests used Kruskal-Wallis

[b]Behavioral variables: both hypoxic time points were pooled and compared to normoxic values

Table A3. Spearman's correlation coefficient matrix comparing HIF-1α abundance among tissues in *Fundulus grandis* exposed to A. normoxia (>7 mg $l^{-1}$) or B. hypoxia (1 mg $l^{-1}$). Values from fish exposed for 6 or 24 h were pooled within treatments. For each comparison, Spearman's rho is shown above the diagonal and *p* value is shown below the diagonal. Sample sizes are shown in parentheses. Correlations with $p \leq 0.05$ (unadjusted) are shown in bold font.

A.

| **Tissue** | **Brain HIF-1α** | **Ovary HIF-1α** | **Muscle HIF-1α** | **Liver HIF-1α** | **Gill HIF-1α** |
|---|---|---|---|---|---|
| Brain HIF-1α | --- | 0.43 (8) | 0.10 (8) | 0.58 (9) | 0.51 (8) |
| Ovary HIF-1α | 0.29 | --- | -0.04 (8) | **0.66 (9)** | -0.12 (8) |
| Muscle HIF-1α | 0.82 | 0.93 | --- | -0.07 (8) | -0.33 (8) |
| Liver HIF-1α | 0.10 | **0.05** | 0.88 | --- | 0.44 (8) |
| Gill HIF-1α | 0.19 | 0.77 | 0.42 | 0.27 | --- |

B.

| **Tissue** | **Brain HIF-1α** | **Ovary HIF-1α** | **Muscle HIF-1α** | **Liver HIF-1α** | **Gill HIF-1α** |
|---|---|---|---|---|---|
| Brain HIF-1α | --- | 0.43 (11) | 0.37 (12) | 0.14 (13) | 0.10 (11) |
| Ovary HIF-1α | 0.19 | --- | -0.23(14) | -0.38 (14) | 0.48 (12) |
| Muscle HIF-1α | 0.23 | 0.43 | --- | 0.31 (16) | -0.37 (14) |
| Liver HIF-1α | 0.64 | 0.18 | 0.24 | --- | -0.13 (15) |
| Gill HIF-1α | 0.77 | 0.11 | 0.19 | 0.64 | --- |

Table A4. Spearman's correlation coefficients (rho) and p values comparing body mass, blood variables, and behavioral variables to tissue HIF-1α abundance in *Fundulus grandis* exposed to hypoxia (1 mg $l^{-1}$) for 6 or 24 h. Correlations with p ≤ 0.05 (unadjusted) are shown in bold font.

| | **Brain HIF-1α** | | | **Ovary HIF-1α** | | | **Muscle HIF-1α** | | | **Liver HIF-1α** | | | **Gill HIF-1α** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **rho** | **p** | **n** | **rho** | **p** | **n** | **rho** | **p** | **n** | **rho** | **p** | **n** | **Rho** | **p** | **n** |
| Body Mass | 0.34 | 0.26 | 13 | 0.27 | 0.35 | 14 | -0.15 | 0.57 | 16 | 0.25 | 0.32 | 17 | **0.69** | **0.004** | 15 |
| Hematocrit | 0.38 | 0.20 | 13 | 0.23 | 0.43 | 14 | -0.06 | 0.82 | 16 | 0.05 | 0.86 | 17 | 0.41 | 0.13 | 15 |
| Blood Hemoglobin | 0.05 | 0.87 | 13 | 0.06 | 0.84 | 14 | -0.36 | 0.18 | 16 | -0.09 | 0.73 | 17 | 0.54 | 0.04 | 15 |
| Blood Glucose | 0.40 | 0.18 | 13 | -0.02 | 0.96 | 14 | 0.43 | 0.09 | 16 | 0.23 | 0.38 | 17 | -0.11 | 0.70 | 15 |
| Blood Lactate | -0.21 | 0.48 | 13 | -0.08 | 0.78 | 14 | 0.27 | 0.32 | 16 | **0.64** | **0.006** | 17 | 0.02 | 0.93 | 15 |
| Plasma Cortisol | 0.27 | 0.37 | 13 | 0.11 | 0.75 | 11 | 0.30 | 0.34 | 12 | 0.52 | 0.07 | 13 | 0.27 | 0.42 | 11 |
| Velocity Mean | -0.15 | 0.70 | 9 | -0.38 | 0.25 | 11 | 0.35 | 0.27 | 12 | 0.29 | 0.33 | 13 | -0.21 | 0.54 | 11 |
| ASR Attempt | 0.13 | 0.73 | 9 | -0.24 | 0.48 | 11 | 0.11 | 0.73 | 12 | -0.03 | 0.93 | 13 | -0.29 | 0.39 | 11 |
| Immobility | **-0.75** | **0.02** | 9 | -0.31 | 0.35 | 11 | 0.15 | 0.65 | 12 | 0.19 | 0.53 | 13 | -0.28 | 0.40 | 11 |

# Vita

Jasmine C. Harris was born in New Orleans, LA. She earned her bachelor's degree in Biological Sciences from the University of New Orleans in 2010. She was admitted into the Graduate School in the Department of Biological Sciences at the University of New Orleans in the fall of 2017, joining the research laboratory of Dr. Bernard Rees.