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# Protein Expression of Hypoxia-Inducible Factor 1-Alpha (HIF-1α) in Spot (Leiostomus xanthurus) Under Acute Exposure to Hypoxic Treatments : A Laboratory-Field Comparison Study

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# PROTEIN EXPRESSION OF HYPOXIA-INDUCIBLE FACTOR 1-ALPHA (HIF-1α) IN SPOT (*LEIOSTOMUS XANTHURUS*) UNDER ACUTE EXPOSURE TO HYPOXIC TREATMENTS: A LABORATORY-FIELD COMPARISON STUDY

by

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Dedication

This thesis is dedicated to my son, Arthur James Smith, who was born during the completion of this work on April 20th, 2011. I also express my deepest gratitude for my wife, Alycia Smith, and my parents, Paul and Gail Smith, for all of their love and support throughout my graduate career. Thanks to my primary advisor Dr. Kelly Smith for all of her support and guidance, as well as committee members Dr. Jim Gelsleichter and Dr. Courtney Hackney.

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Abstract

Hypoxia in coastal estuaries is a topic of increasing concern, as the magnitude and frequency of hypoxic events have increased over the past several decades. These hypoxic events are highly detrimental to the coastal biota, particulary fish. The hypoxia-inducible factor 1α (HIF-1α) protein was used as a candidate biomarker for deciphering exposure of fish to hypoxic events. In chapter one, Spot (*Leiostomus xanthurus*) were exposed to three treatment groups of DO (means 7.32 ± 0.18, 5.15 ± .37, and 2.57 ± 0.01 mg $L^{-1}$ DO) and sampled (n = 5) at time zero, 36 and 72-h for each treatment. The results of the laboratory trials suggested that duration had a significant effect ($F = 28.9$, $p < .001$) on concentration of HIF-1α protein, however, the DO treatment group did not have a significant effect ($F = .739$, $p = .546$) on the concentration of HIF-1α protein. *L. xanthurus* were also analyzed for HIF-1α from field sites of varying DO concentrations (7.0, 5.2, 4.8, and 3.3 mg $L^{-1}$ DO), with no significant differences ($F = 1.621$, $p = .208$) between sample sites, and with a negative relationship between DO and HIF-1α protein ($p = .197$) from these sites.

*Leiostomus xanthurus* were exposed to either constant or diel-cycling hypoxia, and HIF-1α expression was compared to normoxic control over three days. The results indicated that HIF-1α protein significantly ($p = 0.02$) increased in muscle tissue after three days exposure to both constant and a simulated diel-cycling hypoxic event in a laboratory setting when compared to normoxic control animals. It was also found that body mass (measured in wet weight, grams) was a significant covariate for the concentration of HIF-1α produced under normoxia ($p = 0.04$) and constant hypoxia ($p = 0.03$), but did not affect the diel-cycling ($p = 0.83$) groups, suggesting that body mass is a

confounding factor when measuring HIF-1α. The correlation of HIF-1α with body mass is likely due to the different tolerances to hypoxia between small and large young-of-year *L. xanthurus*, an effect that was possibly overshadowed by the acclimation response under diel-cycling hypoxia.

# Introduction

## Hypoxia

Hypoxia in estuarine ecosystems is a problem of growing concern worldwide. Hypoxia is generally considered a condition in which a water column contains less than 2.0 mg $L^{-1}$ dissolved oxygen (DO), the point where the majority of aquatic organisms can no longer survive (Chesney et al. 2000; Goodman & Campbell 2007). It has recently been suggested, however, to define hypoxia as any level of DO low enough to negatively impact the behavior and (or) physiology of an organism (Pollock et al. 2007). Recent evidence suggests that both the frequency of occurrence and surface area of hypoxic zones have increased in recent decades due to eutrophication of coastal waters (Rabalais et al. 2007). Eutrophication-induced hypoxic events are responsible for approximately half of the known 'dead zones' across the world, a phrase describing coastal waters with oxygen too low to sustain life (Diaz & Rosenburg 2008). Hypoxia-induced dead zones are the product of decaying biomass of animals such as sediment-dwellers, which cannot leave the hypoxic zone, most of which die below 1.5 ppm, and span areas of tens of thousands of kilometers for months at a time (Dybas 2005). In 2005, there were 146 known dead zones, 43 of which were in the United States in key areas crucial to fisheries such as the Chesapeake Bay and Gulf of Mexico (Dybas 2005). Estuarine dead zones are expected to increase in area and magnitude in future years (Diaz & Rosenburg 2008).

In a healthy estuarine ecosystem, the average summer DO concentrations typically range from 5.0-15.0 mg $L^{-1}$. Unfortunately, many estuaries are highly eutrophic from anthropogenic sources, such as agricultural runoff, industrial effluent, and maritime activity (Howarth 2008; Lotze et al. 2006; Rabalais et al. 2007). This leads to the primary

cause of estuarine hypoxic events: algal bloom die-offs (Anderson 2002). Nitrogen-limited alga and cyanobacteria thrive in eutrophic waters during summer and fall months, leading to massive algal blooms (Anderson 2002); after the die-off of such a bloom, the decomposition of the large amount of organic matter by heterotrophic bacteria typically depletes the water column of oxygen leading to hypoxia and sometimes anoxia (Anderson et al. 2002; Dybas 2005).

Hypoxic conditions are highly detrimental to coastal biota, particularly fish. In a recent study reviewing coastal fish kills in the Gulf of Mexico from over fifty years of data (1951-2006), hypoxia was found to be the greatest cause of death in comparison to other causes such as pollutants, toxins, and disease (Thronson and Quigg 2008). This may have been an underestimate, since fish which lack swim bladders are much less detectable than the fish which end up on the surface and shores (Breitburg 1992). Diver observations following an 18-h hypoxic event in the Chesapeake Bay revealed the benthos littered with dead fish (Breitburg 2002). Hypoxic fish kills such as these most likely occur when DO decreases at a rate which fish are unable to escape due to physical inability or behavioral confusion (Breitburg 2002).

Just as ecologically important, are the sub-lethal effects hypoxia has on fish. In response to hypoxia, some female fish produce fewer eggs with smaller yolks, decreasing their fecundity (Zhang et. al 2009). In addition, females produce less vitellogenin, the precursor to yolk, under hypoxic conditions, resulting in unviable eggs (Thomas et al. 2006). Reduced growth rates, particularly of juvenile size classes, occur in many species when exposed to low DO (Chabot 1999; Wu 2002). Behavioral responses include a change in predator-prey interactions involving cost-benefit decisions, which alter major

pathways of energy flow, as predators reduce foraging to conserve energy (Breitburg 2002). In addition, male individuals of oyster reef-inhabiting species of fish, including gobies, blennies, and clingfish abandon their nests under hypoxic conditions resulting in the predation of eggs (Breitburg 1992) as energetic costs are increased as the amount of fanning to supply oxygen to the eggs increases (Jones and Reynolds 1999). These responses to hypoxia have the potential to be detrimental to coastal fish populations by decreasing population size and genetic diversity, or altering community structure. Such a decrease in genetic diversity leads to a lowered resilience to future environmental stresses (Lande 1988) and may lead to future population crashes.

Another indirect, yet detrimental, effect of hypoxia on fish is habitat loss. Structures such as seagrass beds, which provide shelter from predators, nesting sites, and feeding grounds, are vulnerable to decreased growth rates and mortality under hypoxic conditions. A study by Borum et al. (2004) showed that low DO allows the invasion of sulfide into seagrass tissue, resulting in massive die-offs in the tropical seagrass *Thalassia testudinum* in the Florida Bay. High sulfide concentrations have been shown to disrupt photosynthetic activity in seagrass, and in combination with hypoxia reduce growth rates of elongation and production of new leaves (Holmer and Bondgaard 2001). Oyster reefs are also negatively impacted under hypoxia; oysters create biogenic reef habitat important for estuarine biodiversity due to the increased complexity in structure compared to the otherwise flat bottom (Breitburg 1992; Lenihan 1998). Larval settlement, juvenile growth, and juvenile survival of oysters are all significantly reduced under hypoxia when compared under normoxic conditions (Widdows et al. 1989; Baker and Mann 1992). In addition, the response of high numbers of fish seeking refuge in the

more oxygenated oyster reef systems can alter the trophic dynamics of the oyster reef systems (Lenihan et al. 2001).

## Biomarkers

With such detrimental effects on fish, DO is monitored directly by water management bodies using portable or permanent data loggers. As a different approach, biomarkers are used to monitor organisms' response to environmental stress. Hypoxia-inducible factor 1-alpha (HIF-1α) has been named the master regulator involved in the homeostasis of cells under hypoxic conditions, and has been found to be up-regulated in fish exposed to hypoxia (Nikinmaa and Rees 2005; Thomas and Rahman 2009). HIF-1α controls over 100 different target genes whose products are involved in angiogenesis (generation of new blood vessels), erythropoiesis (formation of red blood cells), glycolysis (energy metabolism), and glucose transport into the cells (Weinberg 2006). Under normoxic conditions, HIF-1α is synthesized at a high rate, but is almost immediately degraded through a pathway involving an oxygen-dependent step leading to destruction. A molecule called prolyl hydroxylase oxidizes the HIF-1α molecule which is later tagged with ubiquitin, a tag for a cell destined for apoptosis (Weinberg 2006). Under hypoxic conditions however, prolyl hydroxylase fails to oxidize HIF-1α due to the lack of oxygen and HIF-1α escapes ubiquitation, resulting in a drastic increase of the protein (Weinberg 2006). This pathway of hypoxia-induced expression makes HIF-1α a strong hypoxia biomarker candidate.

## Local Study System

The St Johns River (SJR) is one of the largest blackwater systems in the United States, flowing northward approximately 500 km from its headwaters near Vero Beach to just east of Jacksonville, Florida where it empties into the Atlantic Ocean (Morris 1995). The study system included only the Lower St. Johns River (LSJR), Florida, which comprises the estuarine portion of the SJR from the confluence of the SJR and the Ocklawaha River to the mouth at Mayport, Florida. The LSJR estuary is characterized by approximately 684 $km^2$ of surface area, an average depth of 3.4 m, and an average tidal range of 0.7 m (Dame et al. 2000). Based on chlorophyll *a* and trophic state index (TSI) values, the majority (11 out of 15 segments) of the LSJR has been verified as impaired by nutrients (Magley and Joyner 2008). Because of the high nutrient availability, chlorophyll *a* concentrations in the LSJR region often exceed 100µg/L (Hendrickson et al. 2003). The eutrophication-induced algal growth in the LSJR has been shown to be the primary cause of low oxygen stress; Hendrickson et al. (2003) found that chlorophyll *a* concentration had a significant negative correlation with DO in the LSJR. Over two-thirds of the variation in DO deficit in the LSJR was accounted for by changes in the algal community (Hendrickson 2003). The slow nature of the LSJR results in increased residence time for nutrients such as nitrogen and phosphorus, which presents the photosynthetic community the opportunity to fully exploit nutrients for growth (Hendrickson et al. 2003). Large population crashes in the algal communities of the LSJR primarily occur following periods of phosphorus limitation (Hendrickson 2003).

## Species of Interest

Spot croaker (*Leiostomus xanthurus*) was chosen as the primary model organism because wild populations of *L. xanthurus* are quite commonly found in hypoxic waters (Bell and Eggleston 2005; Montagne and Froeschke 2009; Tremain and Adams 1995), and even exploit hypoxic areas for vulnerable benthic organisms (Pihl et al. 1991). In laboratory studies, *L. xanthurus* had a 12h LC50 of 1.10 and 1h LC50 of 0.49 mg/L DO, suggesting high tolerance to hypoxia (Shimps et al. 2005; Burton et al. 1980). These behaviors and physiological adaptations towards hypoxia suggest *L. xanthurus* as an excellent bio-monitoring species of hypoxic exposure in estuaries.

Field sampling by FWRI from 2006 to 2007 found 886 *L. xanthurus* in the LSJR within two of the three zones that experienced persistent hypoxic events (Brodie & DiGirolamo 2008). This indicates that these species are being persistently exposed to hypoxic conditions.

## Goals and Objectives

The following work evaluated the use of HIF-1α in detecting exposure to low oxygen in *L. xanthurus*. Specifically, the objectives were to (1) determine the rate of expression of the HIF-1α protein based on both concentration of DO and duration of exposure by maintaining fish in a controlled laboratory experiment, (2) compare wild fish of known environmental conditions to laboratory individuals to determine if HIF-1α is an accurate predictor of DO exposure, and (3) determine how diel-cycling hypoxia affects the expression of the HIF-1α protein modeling.

Chapter 1: Hypoxia inducible factor (HIF-1α) protein expression in spot (*Leiostomus xanthurus*) exposed to hypoxia: a laboratory study with field comparison.


Abstract

Hypoxia in coastal estuaries is a topic of increasing concern as the magnitude and frequency of hypoxic events have increased over the past several decades. These hypoxic events are highly detrimental to the coastal biota, particulary fish. The hypoxia-inducible factor 1α (HIF-1α) protein was used as a candidate biomarker for determining exposure of fish to hypoxic events. Spot (*Leiostomus xanthurus*) were exposed to three treatment groups of DO (means 7.32 ± 0.18, 5.15 ± 0.37, and 2.57 ± 0.01 mg $L^{-1}$ DO) and sampled (n = 5) at time zero, 36 and 72-h for each treatment. The results of the laboratory trials suggested that duration had a significant effect ($F = 28.9$, $p < 0.001$) concentration of HIF-1α protein, however, the DO treatment group did not have a significant effect ($F = 0.739$, $p = 0.546$) on the concentration of HIF-1α protein. *L. xanthurus* were also analyzed for HIF-1α from field sites of varying DO concentrations (7.0, 5.2, 4.8, and 3.3 mg $L^{-1}$ DO), with no significant differences ($F = 1.621$, $p = 0.208$) between sample sites, and with a negative relationship between DO and HIF-1α protein ($p = 0.197$) from these sites.

## Introduction

Hypoxic conditions are highly detrimental to the biota of coastal waters, particularly fish. In a recent study reviewing coastal fish kills in the Gulf of Mexico from over 50 years of data (1951-2006), hypoxia was found to be the greatest cause of death compared to other causes such as pollutants, toxins, and disease (Thronson and Quigg 2008). Hypoxic fish kills occur when DO decreases at a rate at which fish are unable to escape due to physical inability or behavioral confusion (Breitburg 2002).

Just as ecologically important, are the sub-lethal effects on fish. In response to hypoxia, female fish produce fewer mature ovaries and fewer eggs with smaller yolks (Zhang et. al 2009) and less vitellogenin, the precursor of yolk (Thomas et al. 2006). Reduced growth rates, particularly of juvenile size classes, occur in many species when exposed to low DO (Chabot 1999; Wu 2002; Del Toro-Silva et al. 2008). Behavioral responses include a change in predator-prey interactions as predators reduce foraging to conserve energy (Breitburg 2002), and parental care, as some fish abandon their nests under hypoxic conditions resulting in the predation of eggs (Breitburg 1992).

The study system included only the Lower St. Johns River (LSJR), Florida, which comprises the estuarine portion of the SJR from the confluence of the SJR and the Ocklawaha River to the mouth at Mayport, Florida (Hendrickson et al. 2003). The LSJR has large urban land use, as high as to 95 percent in many areas (Chadwick et al. 2006). Based on chlorophyll *a* and trophic state index (TSI) values, the majority (11 out of 15 segments) of the LSJR has been verified as impaired by nutrients (Magley and Joyner 2008). Because of high nutrient availability, chlorophyll *a* (a measure of algal biomass) concentrations in the LSJR region often exceed 100μg/L (Hendrickson et al. 2003). The

eutrophication-induced algal growth in the LSJR is the primary cause of low oxygen stress (Hendrickson et al. 2003).

Molecular biomarkers are a recent approach to monitoring environmental exposure. Hypoxia-inducible factor 1-alpha (HIF-1α) has been named the master regulator involved in the homeostasis of cells under hypoxic conditions, and has been found to be up-regulated in fish exposed to hypoxia (Nikinmaa and Rees 2005; Thomas and Rahman 2009). HIF-1α controls over 100 different target genes whose products are involved in angiogenesis (generation of new blood vessels), erythropoiesis (formation of red blood cells), glycolysis (energy metabolism), and glucose transport into the cells (Weinberg 2006). Under normoxic conditions, HIF-1α is synthesized at a high rate but is almost immediately degraded through a pathway involving an oxygen-dependent step leading to destruction; a molecule called prolyl hydroxylase oxidizes the HIF-1α molecule which is later tagged with ubiquitin, a tag for a cell destined for apoptosis (Weinberg 2006). This up-regulation under hypoxia, and subsequent down-regulation under normoxia makes it a strong candidate for a hypoxia biomarker. Field site analysis by Thomas and Rahman (2009) found that HIF-2α protein was significantly elevated in their hypoxic site (2.1 mg $L^{-1}$ DO) than a formerly hypoxic site (4.5-5.8 mg $L^{-1}$ DO). They did not report, however, on HIF-1α protein expression differences between field sites.

The primary goal of this experiment was to identify the biomarker hypoxia-inducible factor 1-alpha (HIF-1α) as a direct indicator of hypoxia in spot (*Leiostomus xanthurus*), a common estuarine fish. This was achieved by exposing fish to varying levels of DO for a determined amount of time in a controlled laboratory setting and

analyzing the protein expression of HIF-1α through immunoblotting methods, and thus determining relationships between DO, exposure duration and HIF-1α. The first objective was to determine whether treatments and controls were significantly different in HIF-1α protein concentration, dependent upon both the intensity and duration of the hypoxia, analyzed via a Two-Way Analysis of Covariance (ANCOVA). The specific pairs of hypotheses tested directly by the Two-Way ANCOVA included the following: (1) whether DO has a significant effect on HIF-1α expression, (2) whether duration has a significant effect on HIF-1α expression, and (3) whether the interaction of DO and duration has a significant effect on HIF-1α expression. The second objective was to determine whether field samples of fish caught in various concentrations of DO differed in HIF-1α protein concentration. This was achieved by sampling *L. xanthurus* from four sites ranging from 7.0 to 2.2 mg $L^{-1}$ DO and comparing the means using a one-way ANCOVA. It was predicted that lower DO sites would produce significantly higher HIF-1α concentration than higher DO sites, and that a negative relationship would exist between DO and HIF-1α protein concentration.

## Methods

### Species Selection

Spot croaker (*Leiostomus xanthurus*) was the primary model organism based on field availability and background information on reaction to hypoxic exposure: In wild populations, *L xanthurus* is commonly found in hypoxic waters (Bell and Eggleston 2004; Montagne and Froeschke 2009; Tremain and Adams 1995), and will exploit hypoxic areas for vulnerable benthic organisms (Pihl 1991). In laboratory studies, *L. xanthurus* had a twelve hour LC50 of 1.10 and one hour LC50 of 0.49 mg/L DO, suggesting high tolerance to hypoxia (Shimps *et al.* 2005; Burton *et al.* 1980). These behaviors and physiological adaptations towards hypoxia make *L. xanthurus* an excellent bio-monitoring species for hypoxic exposure in estuaries.

### Fish Care

Fish were collected from local sites within the Lower St. Johns River using a 21.3m x 1.8m center bag seine with assistance from the Florida Fish and Wildlife Fisheries-Independent Monitoring (FIM) program. The fish were kept in an aerated cooler until they reached the lab. During field collections, water quality measurements such as DO, temperature, salinity and conductivity were recorded at each sampling site using YSI® (Yellow Springs Instruments, OH) model 85 multi-meter to determine proper acclimation needed to laboratory conditions. The YSI meter was calibrated daily before each sampling trip. Prior to treatments, fish were acclimated for a minimum of one week after capture to ensure that no animals used in the experiment were damaged from collection. Methods and research procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at UNF.

Water quality in all aquaria was maintained using 150L Fluval® submersible filters and artificial seagrass constructed from activated carbon, keeping ammonia at a minimum (0.09 ±0.06 ppm). Water quality was maintained at ambient summer field conditions for Southeastern estuaries (temperature 26.9 ± 0.4 °C; salinity 17.9 ± 0.3 ppt, pH 8.21 ± 0.20, ammonium 0.08 ± .02) by recording approximately every three hours using a YSI® Professional Plus meter/Quattro Cable (Yellow Springs Instruments, OH) throughout the experiments (Table 1). Fish were fed frozen *Artemia* daily throughout the entire course of the study. To reduce stress on fish during the experiment, a framework consisting of PVC was constructed to cover each tank with shade cloth.

Hypoxic Exposure Trials

Fish (n = 218, mean standard length (mm) = 51.62 ± 6.09, mean wet weight (g) = 2.72 ± 1.05) were randomly assigned to one of three treatment groups of DO: (1) normoxia (goal of 7.5 mg $L^{-1}$ DO), (2) moderate hypoxia (goal of 5.0 mg $L^{-1}$ DO), and (3) hypoxia (goal of 2.5 mg $L^{-1}$ DO), and sampled (n = 5) at time zero, 36 and 72-h for each treatment (Figure 1). A subsequent 24-h recovery period was also sampled following each trial. A total of three trials were conducted to achieve truly independent replicates, where all five individuals per sample were averaged into a single value per trial (replicate). To achieve the desired DO concentrations for each treatment, air flow was controlled using air flow meters (Aquatic Ecosystems, Inc., Apopka, FL) attached to the primary aerators of each individual aquarium, and when necessary, nitrogen gas bubbling replaced ambient air, quickly reducing the DO.

At time zero, 36-h, 72-h, and a 24-h recovery period for all treatment groups, fish (n = 5 to 7 per treatment group) were euthanized, weighed (wet weight), measured

(standard length), and white muscle tissue obtained. Tissues were immediately frozen with liquid nitrogen and stored at -80°C until protein analysis could be completed. For each treatment group, three replicates were conducted, each a month apart (April, May, and June) due to the acclimation times and water chemistry stabilization necessary for laboratory housing.

Field Sample Analysis

Field samples were obtained from sites of varying DO levels to determine whether a relationship existed between DO and HIF-1α protein concentration in wild fish. Sample sites were limited to mesohaline areas of similar water quality (Table 2). Only young-of-year fish of similar standard length (mean 51.45 ± 3.30 mm) and body mass (mean 3.12 ± .67) were sampled from each site to avoid possible confounding effects from varying size classes.

HIF-1α Analysis

Relative intensities of HIF-1α were measured via immunoblotting with a Bio-Dot® Microfiltration Apparatus (Bio-Rad, Inc., Berkeley, Ca). Total protein concentrations were first calculated using the Bradford method (Bradford 1976). Samples were prepared in 4 μg total protein aliquots in Tris-Buffered Saline (TBS- 8g NaCl; 3g Trisbase; 0.2g KCl; pH 7.4) and bound to a nitrocellulose membrane via gravity filtration. The membrane was then rinsed using TBS-T, and incubated at room temperature for 60 min in a 10% NFDM blocking solution. The membrane was next incubated in primary antibody (1/500 anti-HIF-1α goat polyclonal IgG; Santa Cruz Biotechnology) in TBS-T for 60 min at room temperature and the membrane washed five times with TBS-T at room temperature, and incubated in secondary antibody (1/30,000

rabbit anti-goat IgG; Sigma-Aldrich, St. Louis, MO) in TBS-T for 1 h at room temperature. The membrane was again rinsed five times with TBS-T and exposed to a nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) as a colorimetric substrate for the indication of protein expression.

Protein Specificity

Specificity of the aforementioned antibodies was determined using western blot using protocol described in the previous section, which showed a strong band of HIF-1α (Figure 2). A standard curve using partial HIF-1α protein (human, 576-785aa, Novus Biological, Inc.) was produced using a serial dilution of known concentrations (Figure 3). HIF-1α expression was calculated for each individual by applying the linear equation of the standard curve to the mean intensity values for each sample; intensities were measured using KODAK® molecular imaging software.

Data Analysis

Laboratory treatments were compared using a Two-Way Analysis of Covariance (ANCOVA) to determine if the mean HIF-1α relative intensities of any treatment variables differed significantly based upon hypoxic extent and/or duration with body mass (g) as a covariate. Field samples were compared using a one-way Analysis of Covariance (ANCOVA) using body mass (g) as a covariate due to the variation from treatment sites.

## Results

### Hypoxia Exposure

DO values across each treatment group per trial were consistent with goal values: 7.32 (± 0.18) mg $L^{-1}$ DO for control, 5.15 (± .37) mg $L^{-1}$ DO, and 2.57 (± 0.01) mg $L^{-1}$ DO for constant hypoxia (Table 1). Mortality was higher in trial 1 (4 normoxic, 4 hypoxic, and 6 diel hypoxic deaths) compared to trials two and three, both of which experienced no mortality. The high mortality in trial one was due to a shorter acclimation time, and thus only samples from trials two and three were included in the analysis.

### Experimental Treatments

HIF-1α protein concentrations of all treatment groups ranged from 0.035 to 0.203 µg per 4µg sample. After passing all assumptions of normality, the two-way ANCOVA determined that duration had a statistically significant ($F = 28.9$, $p < .001$) effect on the concentration of HIF-1α protein, however, the DO treatment group did not have a significant ($F = .739$, $p = .546$) effect on the concentration of HIF-1α protein (Table 3).

All treatment groups including control showed a large increase in HIF-1α concentration after 36-h of exposure, and a subsequent sharp decrease after 72-h of exposure, and a slight decrease after a 24 hour recovery period (Figure 4).

### Field Analysis

*L. xanthurus* were collected from four field sites including a 7.00 mg $L^{-1}$ DO (site 1), a 5.20 mg $L^{-1}$ DO (site 2), a 4.80 mg $L^{-1}$ DO (site 3), and a 3.30 mg $L^{-1}$ DO (site 4) site, collected with assistance by the FWC FIM program from July to August (Table 2).

HIF-1α protein concentrations of all sites ranged from 0.065 to 0.079 µg per 4µg sample (Figure 5). After passing assumptions of normality, the one-way ANCOVA found

no significant differences ($F = 1.621$, $p = 0.208$) in HIF-1α protein concentration between sites (Table 4). Linear regression analysis found a negative trend that was not significant ($R = .059$, $p = .197$) (Figure 6).

## Discussion

### Experimental Treatments

Under acute exposure for 72 h, HIF-1α did not predictably increase based on hypoxia or duration, rejecting the hypothesis that both hypoxia and duration would affect HIF-1α in a linear manner. The increase in HIF-1α after 36 h was likely due to an initial shock after the initiation of the experiment, since control individuals expressed elevated HIF-1α to the same degree. This pattern was certainly unexpected as all individuals were acclimated to the experimental tanks for 48-h prior to the initiation of the experiment. It is possible that after 84-h (48-h acclimation plus 36-h treatment), there was a change in water chemistry leading to the spike in HIF-1α. No changes in pH, ammonia, salinity, or temperature were observed throughout the experiments (table 1). Nitrite and nitrate were not measured during the study. These nutrients would not have spiked without an initial spike in ammonia, which was measured approximately every 2 to 3 h during the course of the experiments.

In a closely related fish, Atlantic croaker (*Micropogonias undulatus*), HIF-1α protein was significantly elevated compared to control animals after two weeks in hypoxia, yet was not significantly elevated compared to control animals after four weeks (Thomas and Rahman 2009). The results from this study mimic that using *M. undulatus*, but on a much shorter time scale. Thomas and Rahman (2009) did not analyze HIF-1α protein at the time scale of acute exposure, so it is not certain whether there was an initial increase in their animals after 24 to 36 h of hypoxic exposure. This nonlinear pattern of expression makes it difficult to determine the extent of hypoxic exposure using HIF-1α protein as a biomarker.

After 72-h of exposure, the hypoxic treatment groups were significantly elevated above the control group, but were not significantly elevated when compared to time zero animals. Thus, after three days of exposure to hypoxia, a significant difference in HIF-1α protein occurred between treatment groups. The first 36 h of exposure created a large flux in HIF-1α protein, which is highly problematic in predicting the exposure extent or duration, as the general stress of the initiation of the experiment led to the largest increases in HIF-1α. Therefore, it is not possible to determine the cause or duration of a hypoxic event leading to an elevated level of HIF-1α protein in wild fish.

Basal Level of HIF-1α

It was apparent that a basal level of HIF-1α protein exists in the muscle of *L. xanthurus*. A similar observation was made in Crucian carp (*Carassius carassius*) by Rissanen et al. (2006), which suggested that this basal level in fish may be due to a relatively high responsibility of HIF-1α in the maintenance of energetic homeostasis in tissues. In mammals, recent studies have shown that the deletion of HIF-1α in mice have detrimental effects on the animal even during normoxia (Huang et al. 2004, Mason et al. 2004). Cardiac deletion of HIF-1α in mice resulted in a reduction in vascularization, ATP, phosphocreatine, lactate levels and overall contractile function in normoxic hearts (Huang et al. 2004). The loss of HIF-1α in skeletal muscle in mice led to a reduction in glycolytic activity and an increase in fatty acid oxidation, resulting in increased muscle damage (Mason et al. 2004). With such functions even during normoxia, it is clear that a basal level would exist in some fish. Estuarine fish are constantly exposed to fluctuating salinities, temperatures, and DO concentrations, as estuaries are impacted by daily cycles

of ultraviolet radiation, tidal cycles, changes in rain/freshwater inflows and changes in photosynthetic/respiration rates of aquatic organisms (Hackney et al. 1976).

Thomas and Rahman (2009) also found a basal expression of HIF-1α in control animals of *M. undulatus*. This basal requirement of HIF-1α protein may be responsible for the unpredictable nature of HIF-1α in fish. The basal level (i.e. mean of control animals before and after experiment) of HIF-1α protein from our experiment appeared to be 0.075μg, with concentrations above considered elevated.

Field Analysis

The one-way ANCOVA suggested no significant differences ($F = 1.621$, $p = 0.208$) between sample sites (Table 4), rejecting the hypothesis that lower DO sites would have individuals with significantly higher concentrations of HIF-1α protein. However, the 7.0 mg $L^{-1}$ DO treatment site had the lowest HIF-1α protein concentration (Figure 5). A negative correlation existed between HIF-1α protein concentration and DO of the sampling site, however was not statistically significant ($p = 0.197$) (Figure 6).

HIF-1α concentrations from field sites did not deviate significantly from the basal level around .075μg determined by the laboratory experiment. This suggests that these animals were either: (1) not affected by the low DO concentrations from which they were found, or (2) already acclimated to the point where HIF-1α protein was not needed.

Other biological and environmental factors may have contributed to the lack of variation in HIF-1α protein concentration between sites. The mean body mass of the hypoxic site (site 4) was 2-fold higher (mean 9.67 ± 1.82 g) than all other field sites (sites 1-3) (mean 3.12 ± 0.67 g). In a recent review by Nilsson and Nilsson (2008), it was concluded that for species of fish which utilize anaerobic metabolism under hypoxic

conditions, as does *L. xanthurus* (Cooper et al. 2002), larger fish have an advantage over smaller, because the smaller individuals run out of glycogen and accumulate lethal levels of anaerobic end-products such as lactate and H+ faster than larger individuals. Since these larger fish from site 4 were more tolerant to hypoxia than the smaller fish from sites 1 to 3 to which they were compared, the HIF-1α protein concentration of site 4 would have likely been higher if these animals were of the same weight class. Unfortunately, only these larger individuals were found within this hypoxic zone, further indicating that larger individuals are more tolerant than small. Avoidance behavior may have also influenced the results of this study. Hanke and Smith (In press) showed that Silver perch (*Bairdiella chrysoura*), a close relative of *L. xanthurus,* is capable of avoiding hypoxia given an appropriate escape route. The animals used in this study may have been avoiding the hypoxia for the majority of time, resulting in the lower-than-expected HIF-1α protein concentrations. Field studies with *L. xanthurus* have shown hypoxia avoidance to be the case, with evidence of exploiting hypoxic sites for foraging for short periods of time (Eby et al. 2005; Pihl et al. 1991).

Environmentally, temperature did not vary between field sites (range 30.6 – 31.3 °C). Salinity concentrations did vary between site (range 8.6 – 15.1 ppt), but likely had no contribution to such an effect, since juvenile *L. xanthurus* are well-adapted to withstand extreme changes in salinity. In laboratory studies juvenile *L. xanthurus* showed either no difference in oxygen consumption, a measure of a metabolic response, to extreme changes in salinity (Moser and Gerry 1989), or a decrease in oxygen consumption with decreasing salinity (Moser and Hettler 1989). Other factors such as

nitrite might be more important as nitrite converts hemoglobin to methemoglobin (which does not carry oxygen), and thus causes hypoxic effects on fish (Lewis and Morris 1986).

Field site analysis data by Thomas et al. (2007) concluded both HIF-1α and HIF-2α mRNA expression significantly increased in ovaries of *M. undulatus* from hypoxic sites (2.2-3.5 mg $L^{-1}$ DO), while expression was not significantly increased in formerly hypoxic (4.7 mg $L^{-1}$ DO) and transition sites (6.7 mg $L^{-1}$ DO) compared to normoxic sites. Field analysis from our data set included a similar range of DO concentrations with little variation in HIF-1α protein expression, suggesting HIF-1α mRNA as a better candidate for hypoxia exposure. In laboratory experiments, HIF-1α mRNA began to increase after 12-h, and was significantly elevated after three and seven days, respectively (Rahman and Thomas 2007), further suggesting HIF-1α mRNA as a more effective marker than HIF-1α protein for acute exposure.

Conclusions and Management Applications

The results of our study suggest that HIF-1α is not a quantitatively predictable biomarker of hypoxic exposure in *L. xanthurus*. It is however, reliable as a measure for general hypoxic stress, and the elevation of protein concentration (i.e. above .075μg) can be used as a measure for such stress. Wild-caught animals exhibited similar HIF-1α protein concentrations as laboratory control animals, suggesting little hypoxic stress in these wild populations.

Tables and Figures

**Table 1** Water quality data across all experimental trials conducted from 5-10-2011 to 6-25-2011 (initiation dates). DO treatment groups were grouped into individual trials as only DO varied across these groups.

| Water Parameter (Initiation Date) | Trial 1 (5-10-2011) | Trial 2 (6-4-2011) | Trial 3 (6-25-2011) |
|---|---|---|---|
| Salinity (ppt) | 17.6 ± .28 | 17.9 ± .08 | 18.0 ± .08 |
| Temperature (°C) | 26.7 ± .39 | 26.8 ± .34 | 27.1 ± .32 |
| pH | 8.24 ± .16 | 8.23 ± .18 | 8.18 ± .22 |
| NH4+ | .06 ± .03 | .08 ± .04 | .10 ± .07 |

**Table 2** Water quality data across all field sites which *Leiostomus xanthurus* was obtained from the Lower St Johns River, Florida. Field data was collected from July to August 2011 by Florida Fish and Wildlife's Fisheries-Independent Monitoring program at the time of fish collection.

| Water Parameter (Collection Date) | Site 1 (07-0-2011) | Site 2 (07-08-2011) | Site 3 (07- 09-2011) | Site 4 (08-08-2011) |
|---|---|---|---|---|
| DO (mg $L^{-1}$) | 7.00 | 5.20 | 4.80 | 3.30 |
| Temperature (°C) | 30.6 | 31.0 | 30.9 | 31.3 |
| Salinity (ppt) | 8.6 | 15.1 | 13.3 | 11.5 |

**Table 3** Two-way ANCOVA between experimental treatments (DO and Duration) using mean body mass as covariate on HIF-1α expression in *Leiostomus xanthurus*. DO treatment groups consisted of means 7.30, 5.12, and 2.57 mg $L^{-1}$ DO. Duration treatment groups consisted of samples taken from each DO treatment at 0, 36, and 72 hours exposure.

| Source | Type III Sum of Squares | Df | Mean Square | F | Sig. (p) | Partial $Eta^2$ |
|---|---|---|---|---|---|---|
| Corrected Model | 0.028[a] | 16 | 0.002 | 6.181 | 0.001 | 0.876 |
| Intercept | 0.005 | 1 | 0.005 | 16.964 | 0.001 | 0.548 |
| Mean body mass (g) | 1.628E-5 | 1 | 1.628E-5 | 0.057 | 0.816 | 0.004 |
| Duration | 0.025 | 3 | 0.008 | 28.973 | 0.000 | 0.861 |
| DO Treatment | 0.001 | 3 | 0.000 | 0.739 | 0.546 | 0.137 |
| DO*Duration | 0.001 | 9 | 0.000 | 0.503 | 0.849 | 0.244 |
| Error | 0.004 | 14 | 0.000 | | | |
| Total | 0.298 | 31 | | | | |
| Corrected Total | 0.033 | 30 | | | | |

**Table 4** One-way ANCOVA between *L. xanthurus* HIF-1α protein taken from field sites (7.0, 5.2, 4.8, and 2.2 mg $L^{-1}$ DO) within the Lower St Johns River, Florida. Samples taken were constrained to young-of-year individuals found in mesohaline areas from July to August 2011.

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. (p) | Partial $Eta^2$ |
|---|---|---|---|---|---|---|
| Corrected Model | 0.001 | 4 | 0.000 | 1.568 | 0.211 | 0.188 |
| Intercept | 0.003 | 1 | 0.003 | 14.558 | 0.001 | 0.350 |
| Body Mass (g) | 0.000 | 1 | 0.000 | 1.493 | 0.232 | 0.052 |
| Field Site | 0.001 | 3 | 0.000 | 1.621 | 0.208 | 0.153 |
| Error | 0.005 | 27 | 0.000 | | | |
| Total | 0.179 | 32 | | | | |
| Corrected Total | 0.007 | 31 | | | | |

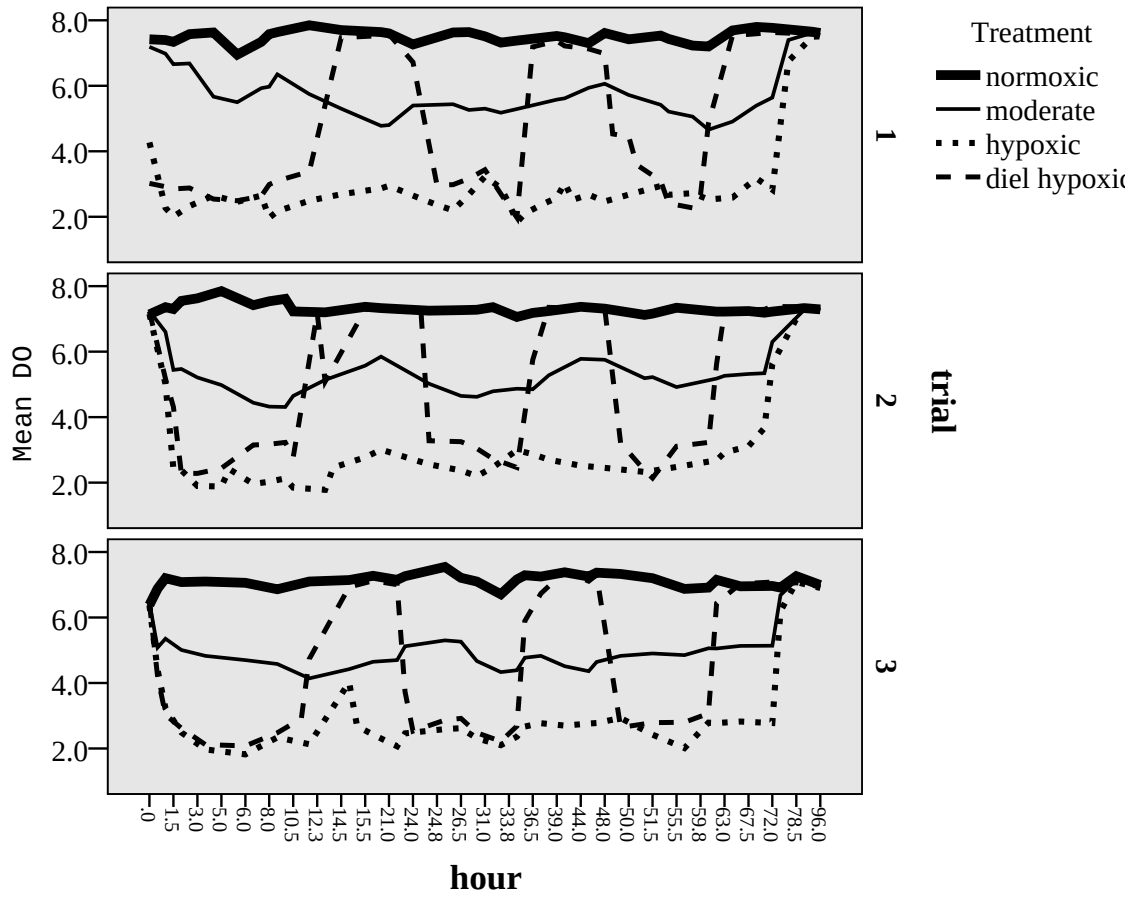


**Fig 1** Dissolved oxygen (DO) concentrations for all treatment groups over the 96 h experimental periods. Each columned graph represents one independent trial: initiation dates of 5/10/2011, 6/4/2011, and 6/25/2011.

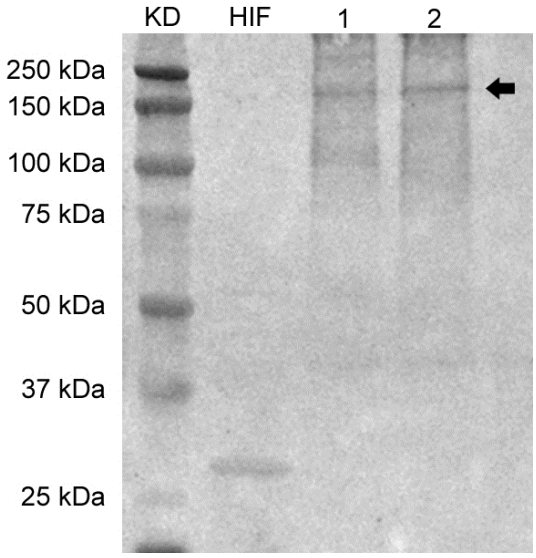


**Fig 2** Western blot using 20µg total protein from gill (1) and liver (2) tissue of *L. xanthurus*, showing a single main band of HIF-1α in area of interest (likely forming heterodimer with β subunit in samples leading to slightly higher molecular weight than the typical 120kDa). Positive control of partial protein (human, 576-785aa, Novus Biological, Inc.), labeled "HIF," showing specificity of binding. Polyclonal primary anti-HIF-1α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using 1/500 dilution was used for protein binding.

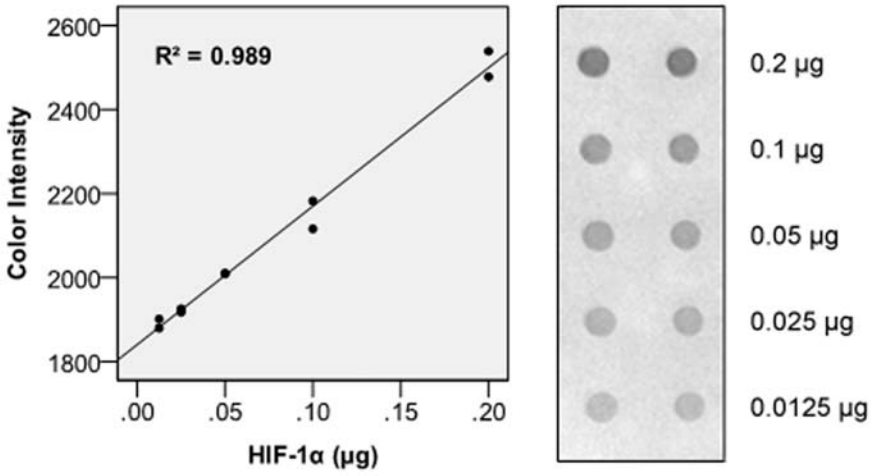


**Fig 3** HIF-1α protein standard curve using partial recombinant protein (human, 576-785aa, Novus Biological, Inc., Littleton, CO) on Bio-Dot® microfiltration apparatus (Biorad, Inc., Berkeley, CA) Polyclonal primary anti-HIF-1α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using 1/500 dilution was used for protein binding.

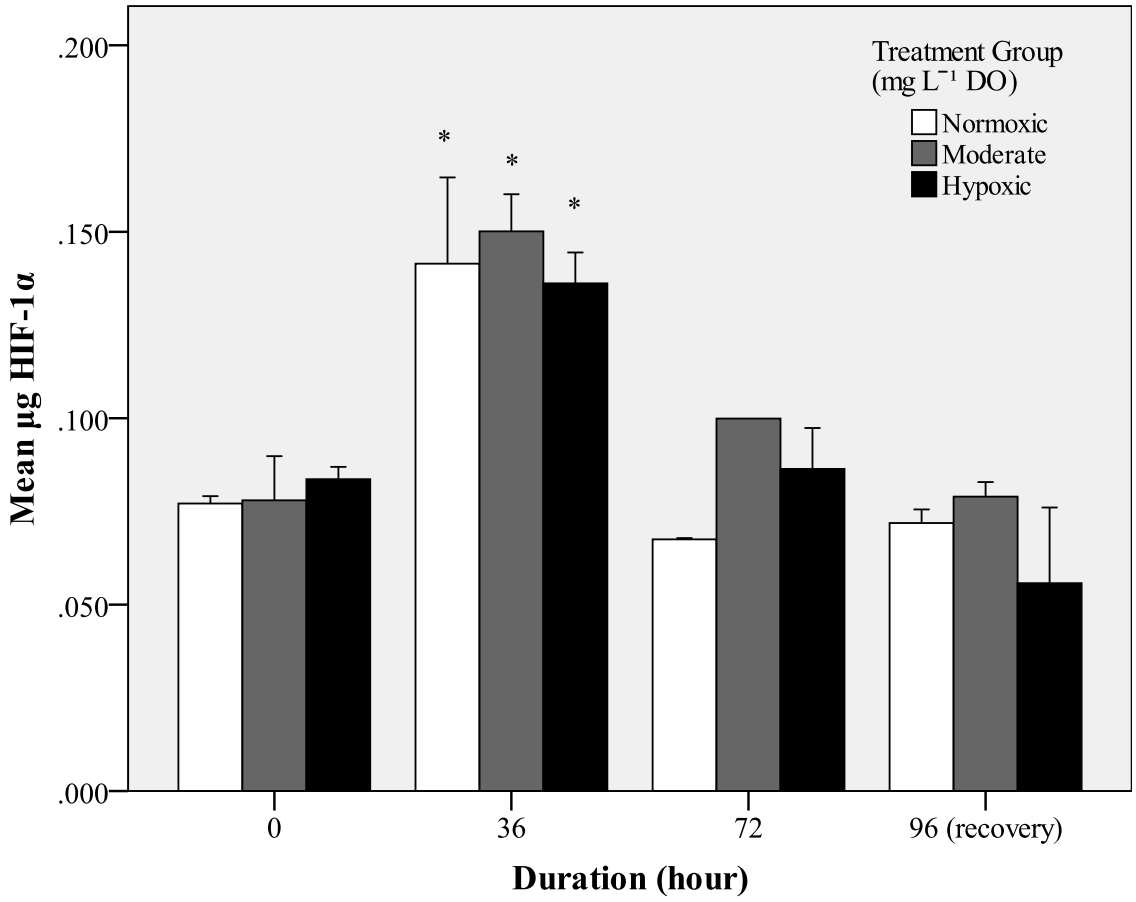


**Fig 4** Mean HIF-1α protein concentrations (µg per 4µg sample) for each treatment group-duration combination. Means represent two independent experimental trials conducted 30-d apart from one another, each with individual n = 5 per treatment group-duration combination, which were averaged into one independent value for total mean calculation. Error bars represent 1 standard error.

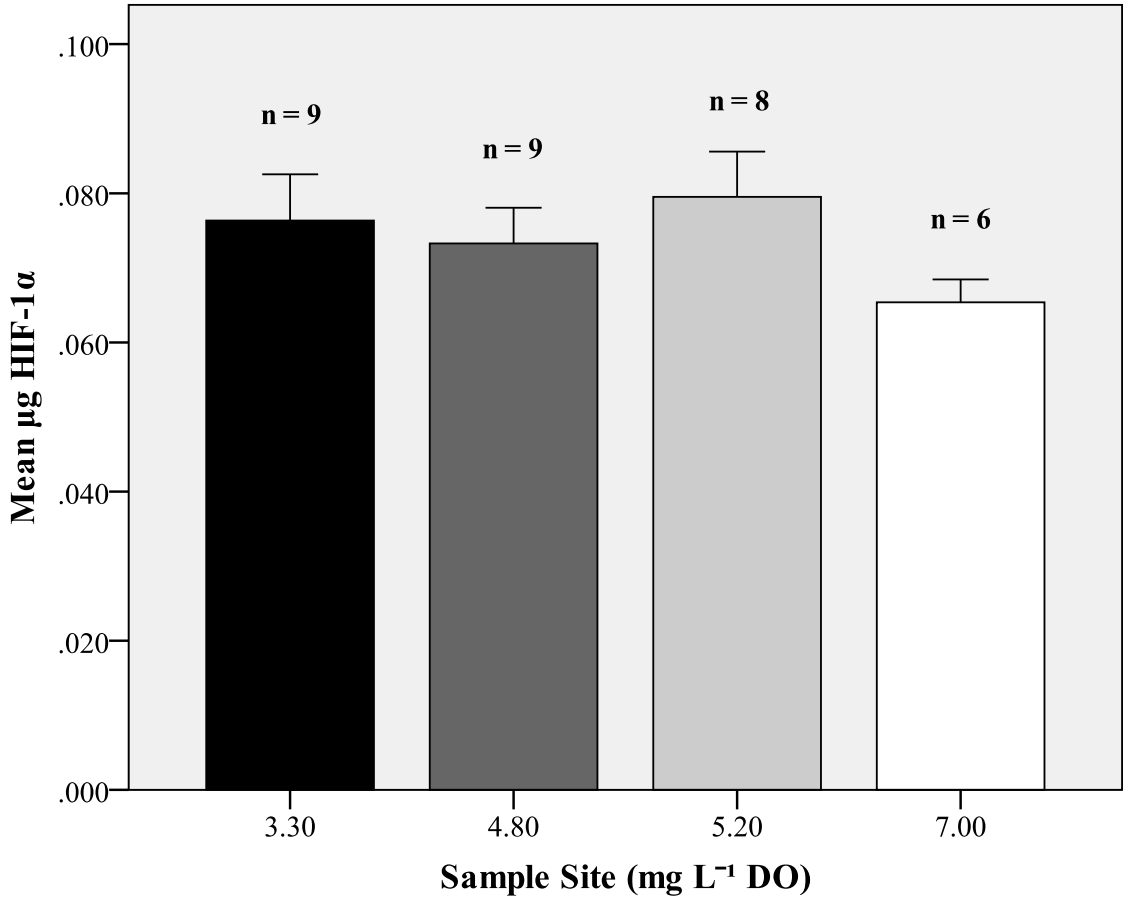


**Fig 5** Mean HIF-1α protein concentrations (µg per 4µg sample) from individuals from different field sites of varying DO concentrations caught in the Lower St Johns River estuary, Florida. Error bars represent 1 standard error.

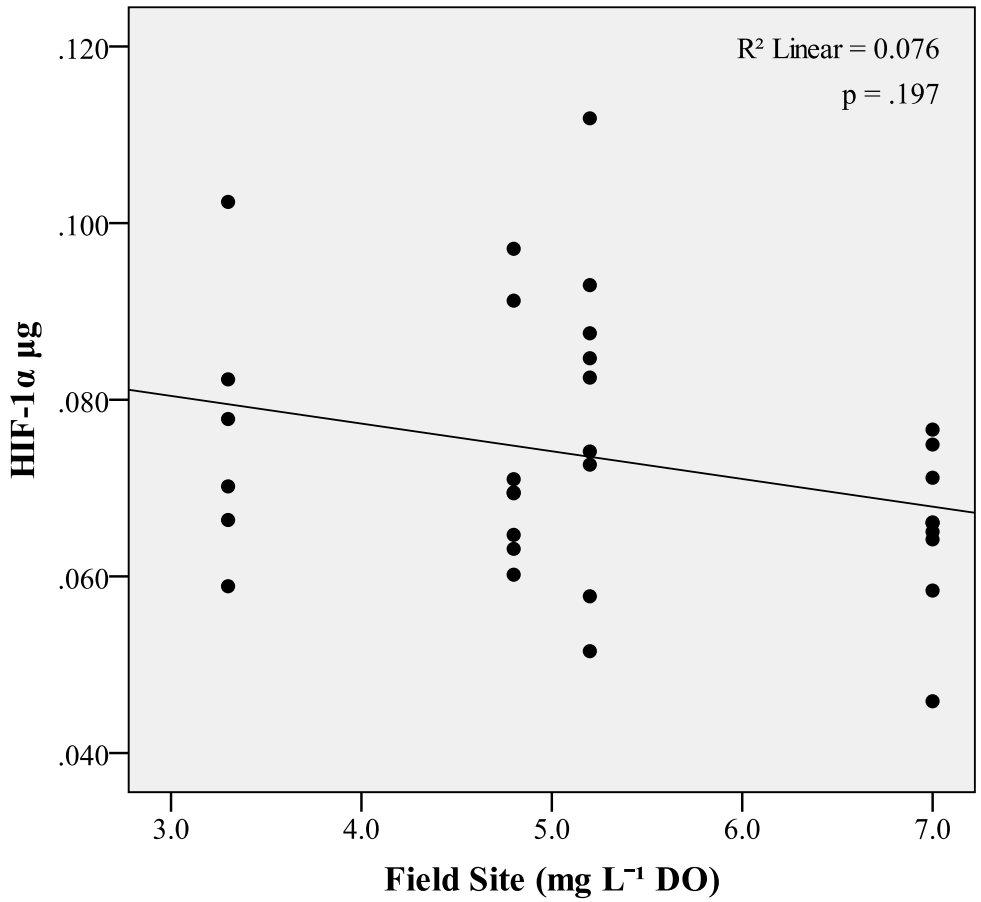


**Fig 6** Pearson's correlation showing a negative trend of HIF-1α protein concentration with increasing DO from wild populations of spot (*L. xanthurus*) from areas of varying DO levels along the mesohaline reaches of the LSJR estuary. Each DO level represents an independent sampling site.

# Chapter II: Protein expression of hypoxia-inducible factor 1-alpha (HIF-1α) under constant and diel-cycling hypoxic exposure


## Abstract

Fish kills often occur overnight from low dissolved oxygen (DO) events, however many mortality events are of unknown cause, since little monitoring occurs overnight. The hypoxia-inducible factor 1α (HIF-1α) protein is an excellent candidate as a biomarker for deciphering idiopathic fish kills. In this study, spot (*Leiostomus xanthurus*) were exposed to either constant or diel-cycling hypoxia, and HIF-1α expression was compared to normoxic control over three days. The results indicated that HIF-1α protein significantly ($p = 0.02$) increased in muscle tissue after three days exposure to both constant and a simulated diel-cycling hypoxic event in a laboratory setting when compared to normoxic control animals. Body mass (measured in wet weight, grams) significantly affected the concentration of HIF-1α produced under normoxia ($p = 0.04$) and constant hypoxia ($p = 0.03$), but did not affect the diel-cycling ($p = 0.83$) groups, suggesting that body mass is a confounding factor when measuring HIF-1α. The correlation with body mass is likely due to the developmental role of HIF-1α, a role that was possibly overshadowed by the acclimation response under diel-cycling hypoxia.

## Introduction

Hypoxia in coastal environments is a topic of increasing concern, with the potential to worsen (Diaz and Rosenberg 2008). Hypoxia is commonly described as having equal or less than 2 mg/L dissolved oxygen (DO), and can be examined at three scales: seasonally (months to weeks), episodic (weeks to days), and diel cycling (hours to minutes). Episodic and seasonal hypoxic events are commonly caused by eutrophication-induced algal blooms and their subsequent population crashes (Anderson 2002), while diel-cycling hypoxia is often driven by the photosynthesis and respiration cycles of aquatic bacteria and algae over a 24-hour period. DO concentrations are typically at their lowest at sunrise, as high amounts of respiration occur overnight without new oxygen production via photosynthesis (Beck and Bruland 2000; D'Avanzo 1994).

Hypoxia has been directly linked to fish kills: a recent study by Thronson and Quigg (2008) found that low levels of DO concentrations accounted for the majority (57%) of fish kill events in Texas bays between 1951 and 2006. Cyclic hypoxia following periods of sunny, then cloudy days with low photosynthesis/respiration ratio has also been linked with fish kills (D'Avanzo and Kremer 1994). Given the strong avoidance behavior towards low DO (Eby et al. 2005), hypoxia-related fish kills likely occur when fish are unable to escape the affected area, due to the large extent or rapid development of the event.

With such detrimental impacts, frequent monitoring of DO is of obvious importance since diel-cycling hypoxia occurs overnight, it often goes undetected by daily monitoring practices commonly used, and only becomes noticeable with permanent (and expensive) data loggers in place. For this reason, there has been growing interest over the

last decade to find biomarkers for hypoxia to detect these unnoticed exposures on wildlife (Zhang et al. 2009).

The hypoxia-inducible factor 1-alpha (HIF-1α) protein has been named the master regulator involved in the homeostasis of cells under hypoxic conditions, and has been found to be up-regulated in fish exposed to hypoxia (Nikinmaa and Rees 2005; Thomas and Rahman 2009). HIF-1α is a transcription factor which targets genes involved in three main groups of low oxygen homeostasis: vascular development, production of blood cells, or altering energy metabolism (Zagorska and Dulak 2004). Under normoxic conditions, HIF-1α is synthesized at a high rate, but is almost immediately degraded through a pathway involving an oxygen-dependent step leading to the ubiquitin-proteosome pathway after forming a complex with the von Hippel-Lindau (pVHL) tumor suppressor protein (Huang et al. 1998; Maxwell et al. 1995). Under hypoxic conditions however, HIF-1α protein escapes forming the complex with pVHL, and thus escapes destruction, leading to an increase in its abundance and an increase in HIF-1α-regulated gene expression (Maxwell et al. 1995).

HIF-1α protein has been shown to increase under constant hypoxic exposure in laboratory populations of Atlantic croaker (*Micropogonias undulatus*), supporting its use as a biomarker of hypoxia in estuarine fish (Thomas and Rahman 2009). This experiment was conducted to determine how the expression of the HIF-1α protein in spot (*Leiostomus xanthurus*) was affected by diel-cycling hypoxia in comparison to a simulated episodic hypoxic event for three days. It was hypothesized that (1) both constant and diel-cycling hypoxia would produce higher HIF-1α than normoxic control, and (2) that diel-cycling hypoxia would produce approximately one-half the HIF-1α

protein than constant hypoxia of the same degree. This the first experiment to investigate the expression of HIF-1α under diel-cycling hypoxia. The results presented here represent the ability of HIF-1α to differentiate between constant and diel-cycling hypoxia in fish.

## Methods

### Species Selection

Spot croaker (*Leiostomus xanthurus*) was the primary model organism based on field availability and background information on reaction to hypoxic exposure: In wild populations, *L xanthurus* is commonly found in hypoxic waters (Bell and Eggleston 2004; Montagne and Froeschke 2009; Tremain and Adams 1995), and even exploit hypoxic areas for vulnerable benthic organisms (Pihl 1991). In laboratory studies, *L. xanthurus* had a twelve hour LC50 of 1.10 and one hour LC50 of 0.49 mg/L DO, respectively, suggesting high tolerance to hypoxia (Shimps et al. 2005; Burton et al. 1980). These behaviors and physiological adaptations towards hypoxia make *L. xanthurus* an excellent bio-monitoring species of hypoxic exposure in estuaries.

### Fish Care

Fish were collected from local sites within the Lower St. Johns River using a 21.3m x 1.8m center bag seine. The fish were kept in an aerated cooler until they reached the laboratory. During field collections, water quality measurements such as DO, temperature, salinity and conductivity were recorded at each sampling site using YSI® (Yellow Springs Instruments, OH) model 85 multi-meter to ensure proper acclimation to laboratory conditions. The YSI meter was calibrated daily before each sampling trip. Prior to treatments, fish were acclimated for a minimum of one week after capture to ensure that no animals used in the experiment were damaged from collection. Methods and research procedures were approved by the University of North Florida's Institutional Animal Care and Use Committee (IACUC).

All aquaria were maintained using 150 L Fluval® submersible filters and artificial seagrass constructed from activated carbon, keeping ammonium at a minimum ($NH_4^+$ - 0.09 ±0.06). Water quality was maintained at ambient summer field conditions for southeastern estuaries (temperature 26.9 ± 0.4 °C; salinity 17.9 ± 0.3 ppt, pH 8.21 ± 0.20), and recorded along with ammonium approximately every three hours using a YSI® Professional Plus meter throughout the experiments. Fish were fed frozen *Artemia* daily throughout the entire course of the study. To reduce the stress on fish during the experiment, a framework consisting of PVC was constructed to cover each tank with shade cloth.

Hypoxia Exposure

Fish (total n = 39) were randomly assigned to one of three treatment groups of DO exposure designed to compare diel-cycling hypoxia to episodic: (1) a fully aerated 'normoxic' treatment (goal = 7.5 mg $L^{-1}$ DO), (2) a continuously hypoxic 'episodic' treatment (goal = 2.5 mg $L^{-1}$ DO), and (3) a 12-hour fluctuating 'diel cycling' hypoxic treatment (goal range = 7.5 to 2.5 mg $L^{-1}$ DO). To achieve the desired DO concentrations for each treatment, air flow was controlled using air flow meters (Aquatic Ecosystems, FL) attached to the primary aerators of each individual aquarium. In this way, diel cycles were able to be produced due to the respiration of the fish when air flow was reduced. When necessary, nitrogen gas bubbling replaced ambient air, quickly reducing the DO. Following each experiment, fish (n = 5 to 7 per treatment group) were euthanized, weighed (wet weight), measured (standard length), and white muscle tissue obtained. Tissues were immediately frozen with liquid nitrogen and stored at -80°C until protein analysis could be completed. For each treatment group, three replicates were conducted,

each a month apart (April, May, and June) due to the acclimation times and water chemistry stabilization necessary for laboratory housing.

HIF-1α Analysis

Relative intensities of HIF-1α were measured via immunoblotting with a Bio-Dot® Microfiltration Apparatus (Bio-Rad Inc., Berkeley, CA) as described in Chapter I Methods: Total protein concentrations were first calculated using Bradford method (Bradford 1976). Samples were then prepared in 4 μg total protein aliquots in Tris-Buffered Saline (TBS-8g NaCl; 3g Trisbase; 0.2g KCl; pH 7.4) and bound to a nitrocellulose membrane via gravity filtration. The membrane was then rinsed using TBS-T, and incubated at room temperature for 60 minutes in a 10% nnonfat dry milk (NFDM) blocking solution. The membrane was then incubated in primary antibody (1/500 anti-HIF-1α goat polyclonal IgG; Santa Cruz Biotechnology) in TBS-T containing 1% NFDM for 60 minutes at room temperature. Then, the membrane was washed five times with TBS-T at room temperature, and incubated in secondary antibody (1/30,000 rabbit anti-goat IgG; Sigma-Aldrich) in TBS-T for 1 hour at room temperature. The membrane was once again rinsed five times with TBS-T and exposed to a nitro-blue tetrazolium chloride/5-bromo-4-chloro-3’-indolyphosphate p-toluidine salt (NBT/BCIP) as a colorimetric substrate for the detection of antigen-antibody complexes.

HIF-1α Protein Determination

A standard curve using partial HIF-1α protein (human, 576-785aa, Novus Biological, Inc.) was produced using a serial dilution of known concentrations (Figure 3). HIF-1α expression was then calculated for each individual by applying the linear

equation of the standard curve to the mean intensity values for each sample; intensities were measured using KODAK® molecular imaging software.

## Statistical Analyses

A one-way between-groups analysis of covariance (ANCOVA) was conducted to compare HIF-1α protein between the three treatment groups (normoxic, hypoxic, and diel-cycling hypoxic) using SPSS statistical software. Preliminary checks were conducted to ensure that there was no violation of the following assumptions: normality, linearity, homogeneity of variances, homogeneity of regression slopes, and reliable measurement of the covariate. A stepwise multiple regression was first performed to determine which covariates were significant for use in the ANCOVA. The weight (g) of each individual was the only covariate used as the covariate of standard length (mm) was highly significantly correlated ($R^2 = 0.927$, $p < 0.001$) to weight (g). Pearson's correlations were used to determine specific relationships within each treatment group between HIF-1α and abiotic factors.

## Results

### Hypoxia Exposure

DO values across each treatment group per trial were consistent with goal values: 7.32 (± 0.18) mg $L^{-1}$ DO for control, 2.57 (± 0.01) mg $L^{-1}$ DO for constant hypoxic, and 4.54 (± 0.15) mg $L^{-1}$ DO (range 7.3 to 2.5 mg $L^{-1}$ DO) for diel-cycling hypoxic group. Mortality was higher in trial 1 (4 normoxic, 4 hypoxic, and 6 diel hypoxic deaths) compared to trials 2 and 3, both of which experienced no mortality.

### HIF-1α Protein Expression

#### Effect of Treatment Groups

After adjusting for body mass, there was a significant difference between the three treatment groups, $F(1,35) = 4.39$, $p = 0.02$, partial $eta^2 = 0.20$ (Table 5, Figure 7). The covariate of body mass contributed significantly, $p = 0.002$, partial $eta^2 = 0.24$, to the variance in the expression of HIF-1α between treatment groups. To investigate this relationship further, the body mass of each individual was regressed against that same individual's HIF-1α protein expression (Figure 8). Pearson's correlation indicated a highly significant positive correlation between body mass and HIF-1α protein ($p = 0.004$) for all individuals ($n = 39$). When investigated at the level of individual treatment groups, the weight-HIF-1α relationship was similar and significant when all normoxic ($R^2 = 0.343$, $p = 0.036$) and hypoxic individuals ($R^2 = 0.337$, $p = 0.030$) were pooled, but was not significant for diel-cycling hypoxic individuals ($R^2 = 0.005$, $p = 0.833$) (Figure 8).

Effect of Trial on Body Mass

To investigate the factors of body mass and trial period, a two-way analysis of variance was conducted to explore the differences, if any, between fish mass among the three trials and treatment groups. There was a highly significant effect of trials ($p < 0.001$) on the weights of fish used; however, neither of the variables: treatment group ($p = 0.584$), or the interaction of treatment groups and trials ($p = 0.468$), was statistically significant. Post hoc comparisons using the Tukey HSD test indicated that trials one and two were significantly different from one another ($p < 0.001$ ), as well as trials one and three ($p < 0.001$); however trials two and three were not significantly different ($p = 0.400$).

## Discussion

### Diel-Cycling versus Constant Hypoxia

HIF-1α of both constant and diel-cycling individuals was elevated compared with controls. Individuals exposed to diel-cycling hypoxia had equally high or higher concentrations of HIF-1α protein, rejecting the hypothesis that diel-cycling hypoxia would produce approximately half of the protein produced under constant hypoxia. This was unexpected since the half-life of HIF-1α in mammalian tissues under normoxia is under five minutes (Anastasiadis et al. 2002).

It may be possible that diel-cycling hypoxia had an acclimation-like effect on HIF-1α protein due to the 12 h fluctuations of low and high DO, allowing the animals to recover for 12 h while building a store of HIF-1α protein over time. Mortality experiments by Shimps et al. (2005) indicated that a 24 h acclimation period to low DO (1.2 mg/L) significantly increases survival of *L. xanthurus* under severe hypoxic conditions (0.6 mg/L) at 25°C in comparison to normoxia-acclimated individuals. Similarly, zebrafish (*Danio rerio*) acclimated to nonlethal hypoxia (10% air saturation) for a period of 48 hours significantly increased survival under lethal hypoxia (5% air saturation) (Rees et al. 2001). HIF-1α protein was likely involved in the hypoxic acclimation of those studies, allowing the fish to survive longer after a build-up of the protein. Mortality in the experiment (occurring only in trial one) however, was slightly higher under diel-cycling hypoxia, possibly due to the stress of a fluctuating environment on such small YOY individuals.

## Role of Body Size

The weight-adjusted model suggested that the different DO treatment groups had significantly different expression of HIF-1α. However, the covariate of weight appeared to contribute more significantly (24%) to the variance of HIF-1α expression, than did the treatment groups (20%), as indicated by partial eta squared values of 0.24 and 0.20, respectively. Similar to our study, hypoxia tolerance studies by Shimps et al. (2005) found that body weight (range 1.98 – 8.01g), significantly affected the survival of *L. xanthurus* under hypoxic conditions, with larger individuals being more tolerant than smaller. In a recent review, Nilsson and Nilsson (2008) concluded that for species of fish which utilize anaerobic metabolism (i.e. glycolysis) under hypoxic conditions, larger fish have an advantage over smaller, because the smaller individuals run out of glycogen and accumulate lethal levels of anaerobic end-products such as lactate and H+ faster than larger individuals. *L. xanthurus* does rely on anaerobic metabolism as part of its hypoxic tolerance. Fish exposed to hypoxia (0.8 mg/L DO) for 12 h showed an increase in lactate dehydrogenase (LDH) enzyme activity in gill and muscle, an indicator of anaerobic metabolism in fish, while there was no change in the activity of citrate synthase enzyme activity, an indicator of aerobic metabolism (Cooper et al. 2002).

## Role of Age

Because the trials were conducted approximately one month apart, it is possible that the correlations between body mass and HIF-1α were also affected by the age of the fish due to the role of the HIF complex in developing juvenile animals. In mammalian tissues, HIF-1 targets the following growth-related genes: Erythropoietin (Jiang et al.

1996), Vascular endothelial growth factor and VEGF receptor 1 (Gerber et al. 1997), Insulin-like growth factor 2, and Insulin-like growth factor binding proteins 1, 2 and 3 (Feldser et al. 1999). Responsible for targeting genes involved in angiogenesis and erythropoeisis, as well as growth factors, HIF-1α protein is likely highly involved with a developing juvenile, explaining the presence of HIF-1α in control normoxic animals.

Due to the acclimation (two weeks), experimental (four days), and preparatory (two weeks) periods of our experiment, these replicates were conducted approximately one month apart. It may not be until the year's cohort became of certain age (trial three) that HIF-1α expression decreased in the normoxic individuals. Unfortunately the fish were not aged in this study. Future studies involving age-related mechanisms in relation to HIF-1α protein would help clarify the optimum size and/or age class of fish for biomarker monitoring of HIF-1α protein.

## Conclusions

### HIF-1α Protein

HIF-1α was significantly higher in hypoxic and diel-cycling hypoxic treatments compared with control animals, but only after the covariate of body mass was included in the ANCOVA model. It is recommended then, that size-related data be precisely accounted for when analyzing HIF-1α expression in teleost fishes. It was also found that diel-cycling hypoxia produced individuals with HIF-1α expression equally as high as individuals exposed to constant hypoxia of the same degree (approx. 2.5 mg $L^{-1}$ DO), and future studies on the half-life of HIF-1α protein in fish need to be conducted.

## Management Applications

The results of this study have implications for management, as diel-cycling hypoxia can be difficult to observe. It is therefore possible to obtain individuals from "normoxic" field sites with high HIF-1α expression caused by overnight low DO. At sublethal DO concentrations, diel-cycling hypoxia reduced growth rates by up to 35% in summer flounder at both 25 and 30° C (Stierhoff et al. 2006), reduced the reproductive organ size and steroid concentration of both male and female gulf killifish (*Fundulus grandis*) (Cheek et al. 2009), and shifted distributions of juvenile weakfish (*Cynoscion regalis*) with a threshold around 2.0 mg/L DO (Tyler and Targett 2007). The utilization of HIF-1α may help managers determine whether such strong ecological effects of cyclic hypoxia are occurring in wild populations while going undetected by diurnal monitoring practices.

## Acknowledgments

Support for this project was funded by the University of North Florida Coastal Biology Program at Jacksonville, Florida. We also include a personal thanks to Alycia Smith, Ryan Ford, Mike McAllister, and all members at the Florida Fish and Wildlife Conservation Commission's Fisheries-Independent monitoring lab in Jacksonville, Florida for all the assistance and support.

Tables and Figures

**Table 5** One-way analysis of variance (ANOVA) of HIF-1α protein expression in spot (*Leiostomus xanthurus*) between DO treatment groups: normoxic control (mean, 7.32 ± 0.18 mg $L^{-1}$), hypoxic (mean, 2.57 ± 0.01 mg $L^{-1}$) and diel-cycling hypoxic (range, 7.3 – 2.5 mg $L^{-1}$), after 72 hours of exposure.

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. (p) | Partial Eta Squared |
|---|---|---|---|---|---|---|
| Corrected Model | 0.002 | 3 | 0.001 | 6.626 | 0.001 | 0.362 |
| Intercept | 0.026 | 1 | 0.026 | 264.887 | 0.000 | 0.883 |
| Body Mass (g) | 0.001 | 1 | 0.001 | 10.928 | 0.002** | 0.238 |
| Treatment Group | 0.001 | 2 | 0.000 | 4.388 | 0.020* | 0.200 |
| Error | 0.003 | 35 | 9.941E¯5 | | | |
| Total | 0.099 | 39 | | | | |
| Corrected Total | 0.005 | 38 | | | | |

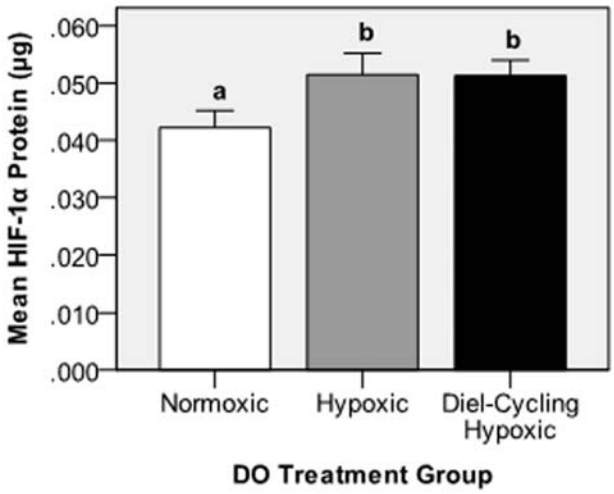


**Fig 7** Mean HIF-1α concentrations (µg) per sample for each treatment group: normoxic (mean, 7.32 mg/L), hypoxic (mean, 2.57 mg/L), and diel-cycling hypoxic (range, 7.3 – 2.5 mg/L). One-way between groups ANCOVA determined significant differences in mean between DO treatment groups ($p = .02$). Error bars represent ± 1 standard error. Sample sizes (n) for each treatment group were three after all individuals (n = 4 or 5) were averaged for each trial. Combined sample sizes were 14, 14, and 13 for normoxic, hypoxic, and diel hypoxic groups, respectively.

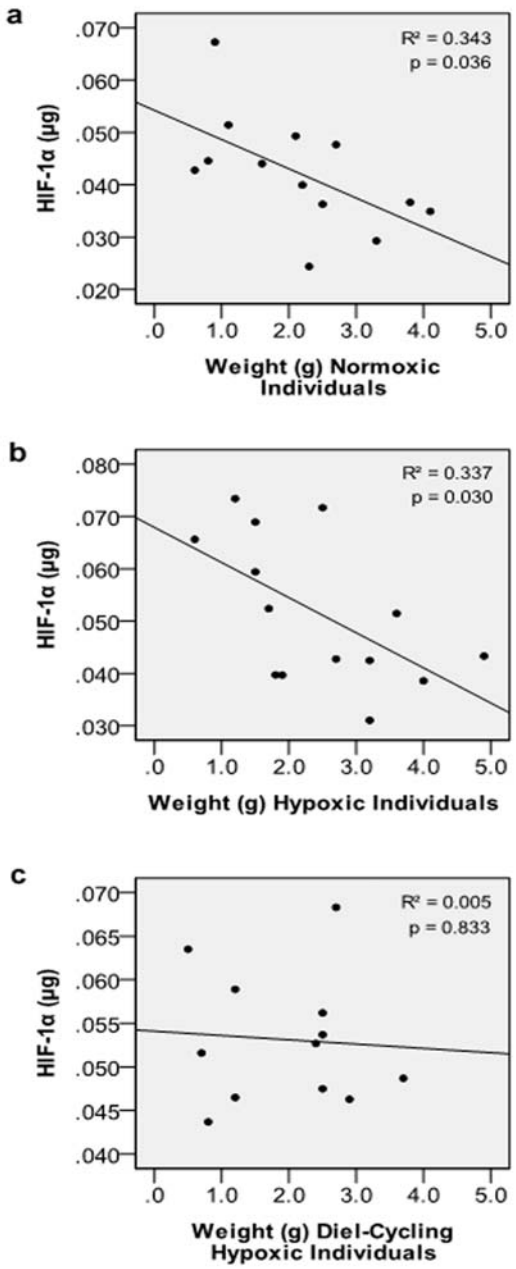


**Fig 8** Scatterplots showing correlations between HIF-1α protein expression and weight for each treatment group independently (A-normoxic, B-hypoxic, and C-Diel-cycling hypoxic). P values represent Pearson's correlation significance. Samples sizes for treatment groups were as follows: normoxic (n = 13), hypoxic (n = 14) and diel-cycling (n = 12) groups.

## Primary Conclusions

It is crucial to the management of coastal environments to continue to progress in the development of novel water quality monitoring techniques such as biomarker analysis using HIF-1α. With an ever-increasing human population, particularly surrounding coastal areas, it is predicted that estuaries will become eutrophied further in the years to come, resulting in higher occurance, frequency, intensity and duration of hypoxic events (Rabalais et al. 2009). The general hypothesis is that each year which nutrient loads increase, a gradual increase in net system heterotrophy will occur, causing long-term decreases in DO (Verity et al. 2006). In fact, a long-term study conducted on the Skidaway estuary in Georgia showed a decrease in DO by 15 to 30% from 1986 to 2004, with summer DO concentrations below 3 mg $L^{-1}$, despite strong vertical mixing, and was strongly correlated with increased nutrients, chlorophyll a, and bacterial abundance (Verity et al. 2006).

## Conclusions

Coastal estuarine habitat is crucial to the survival of the aquatic wildlife which fuels the fisheries economy as many recreationally important species such as red drum, spotted sea trout, southern flounder, common snook and striped mullet spawn into coastal estuarine environments (Reyier et. al 2008; Holt 2008; Powell 2003) and also utilize estuaries as feeding grounds (Stevens et. al 2006). In Florida alone, roughly $25 billion annually is collected by the Florida Fish and Wildlife Conservation Commission (FWC) through recreational activities such as saltwater fishing, thus showing a clear public concern for the health of coastal fishery habitat (Small 2009). The results from these experiments support the use of HIF-1α as general indicator of low oxygen exposure in *L.*

*xanthurus*, however, HIF-1α did not predictably respond to hypoxia and duration as expected. It is therefore unclear at this time to determine whether the presence of high HIF-1α protein is the result of a sudden acute stressor, or a longer term chronic stress, and thus the use of HIF-1α should only be used as a general measure of hypoxic stress. In addition, the weight of fish used in the experiments had a highly significant effect of the expression of HIF-1α protein, and much care should be taken to control for body size when analyzing the protein.

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Vita

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