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THE INFLUENCE OF BODY SIZE AND HEMOGLOBIN MULTIPLICITY ON CRITICAL OXYGEN THRESHOLD IN RED DRUM (*SCIAENOPS OCELLATUS*)

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In partial fulfillment of the requirements for graduation with the Dean's Scholars Honors Degree in Biological Sciences

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The influence of body size and hemoglobin multiplicity on critical oxygen threshold in red drum (*Sciaenops ocellatus*)

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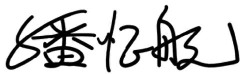

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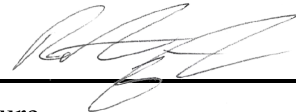

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Abstract

Hypoxia is common in marine environments and fishes use a suite of cardiorespiratory adjustments to defend aerobic metabolism, including reducing standard metabolic rate (SMR), the minimum metabolic rate needed to sustain life at a specified temperature, or increasing hemoglobin (Hb)-O_2 affinity. Nonetheless, hypoxia can constrain oxygen transport whereby fish cannot accommodate standard metabolic rate; a point known as critical oxygen tension (P_{crit}). Currently, it is unclear how life history traits may impact P_{crit}, but available data on red drum (*Sciaenops ocellatus*) suggest that its SMR decreases with size, and its transcriptome contains multiple Hb-α and Hb-β subunits. Therefore we sought to explore the influence of body size and acclimation to hypoxia. Critical oxygen tension (P_{crit}) was measured for fish over a 2500-fold range in mass (0.26 - 686 g) and surprisingly showed an increase (P_{crit} = 3.15 log$M$ + 16.19; R^2 = 0.44) despite decreasing SMR. Two groups of *S. ocellatus* (90.96 ± 5.00 g ranging from 69.7 g to 141.9 g) were also subjected to either normoxia (> 95% P_{O_2}) or hypoxia ($30\% \pm 5\%$ P_{O_2}) treatment for two weeks. Only fish subjected to hypoxia treatment showed a statistically significant decrease in P_{crit} after the treatment. Acclimation had no impact on gill surface area, diffusion distance or relative ventricular mass, but mRNA expression levels of the major Hb-α subunit switched from Hbα-3.1 in the normoxia group to Hbα-3.2 in the hypoxia treatment group and expression levels of Hbα-2, Hbα-3.2 and Hbβ-3.1 showed a statistically significant increase in the hypoxia treatment group. Decrease in P50 and thus an increase in Hb-O2 binding affinity was observed for fish subjected to hypoxia treatment. Taken together these data indicate that hypoxia tolerance is affected by both developmental stage and hypoxia acclimation.

Background

Water is intrinsically poor in dissolved O_2 and this has been recognized as the most important environmental factor limiting the range of possible oxidative metabolism from rest to maximal exercise in fishes (Fry 1971). As partial pressure of O_2 (PO_2) in the water drops, the scope of possible oxidative metabolism gradually decreases to zero until only standard metabolic rate (SMR) can be maintained (Farrell & Richards 2009). The water PO_2 at which this transition occurs is defined as the critical oxygen tension (P_{crit}) (Farrell & Richards 2009). If water PO_2 falls below P_{crit}, fish would have to maintain metabolic rate through anaerobic metabolism (Nilsson & Östlund-Nilsson 2008). This is generally not sustainable as fish have a finite storage of glucose and a limited tolerance for lactate, the end-product of anaerobic metabolism (Nilsson & Östlund-Nilsson 2008). Aside from direct mortality caused by suffocation, long-term exposure to low oxygen conditions, or hypoxic conditions, can also have sublethal effects such as reduced feeding and growth in fish (Flint et al. 2015). Due to the adverse effects of hypoxia, much effort is put into understanding how fish are able to tolerate hypoxic conditions.

The red drum *Sciaenops ocellatus* is an estuarine-dependent fish commonly found in most of the Gulf of Mexico and along the southeastern coast of the United States (Hollenbeck et al. 2015). As *S. ocellatus* has high economic value in the recreational saltwater fishing industry, its population generates major concerns from many fishing stakeholders (Camp et al. 2013). Strict management actions have been taken in the United States to mitigate significant population declines observed since the late 1980s, including the designation of *S. ocellatus* as a federally protected game species in 2007. Juvenile *S. ocellatus* typically inhabit estuarine habitats along the Gulf of Mexico (Rooker & Holt 1997), where hypoxia is a common stressor at night due to it being a highly productive habitat. Once sexually mature, adult *S. ocellatus* move to offshore waters along the Gulf of Mexico continental shelves, where the second largest human-caused hypoxic zone is located (Rabalais et al. 2007). As *S. ocellatus* of different life stages are commonly exposed to hypoxic conditions, they make a good model for studying the mechanisms fish use to adapt to hypoxic conditions.

For fish species such as the case of *S. ocellatus* that occupy different environments

during different life stages, their survival rate may depend on the hypoxia tolerance of the life stage that inhabits the environment with the lowest dissolved O_2 levels (Nelson & Lipkey 2015). Survival rate should increase with a declining P_{crit} (Nilsson & Östlund-Nilsson 2008) as fishes with a lower P_{crit} can occupy lower dissolved O_2 environments without becoming reliant on anaerobic metabolism. This makes scaling of P_{crit} in fishes of particular interest as it can identify potentially susceptible life stages and subsequent developmental bottlenecks that may be caused by hypoxia.

Although studies have examined the effect of body size on various indices of hypoxia tolerance in a wide range of fish species, the results have been variable (Nilsson & Östlund-Nilsson 2008). Smale & Rabeni (1995) conducted hypoxia tolerance studies using lethal [O_2] on common fish species that inhabit small streams in Missouri and concluded that hypoxia tolerance did not vary with fish size. The authors defined lethal [O_2] as the oxygen tension where fish stopped ventilating during a 4-6 h exposure of progressively decreasing oxygen. As such, these results are more indicative of the combined aerobic and anaerobic capacity of the tested species. Existing studies using P_{crit} as a measurement for hypoxia tolerance were mostly conducted on freshwater fish species over a limited size range, and the results also varied. Larger oscar cichlid *Astronotus ocellatus* were found to be more hypoxia tolerant (Sloman et al. 2006), yet Nile tilapia *Oreochromis niloticus* showed no difference in hypoxia tolerance between the smaller and larger individuals (Verheyen et al. 1994). For saltwater fish species, P_{crit} for damselfishes from the Lizard Island reef was strikingly constant over a 4000-fold range in mass (Nilsson & Östlund-Nilsson, 2008; Nilsson et al., 2007; Nilsson & Östlund-Nilsson, 2004), but this data set is a compilation of 15 different damselfish species with none exceeding 41 g. These results may not represent the scaling of hypoxia tolerance in a single species, especially non-reef species with much larger home ranges and size ranges.

Other than size as an influencing factor for hypoxia tolerance, existing studies mostly on fresh water species have shown that fish are capable of increasing hypoxia tolerance when given time to adapt to hypoxic environments (Lomholt & Johansen 1979; Rees et al. 2001; Stecyk & Farrell 2002; Thillart & Smit 1984; Lewis et al. 2007). After more than 4 weeks of hypoxia acclimation, carp *Cyprinus carpio* was shown to both decrease

oxygen consumption rate (Lomholt & Johansen 1979) and cardiac output (Stecyk & Farrell 2002) by up to 50%, and juvenile carp exposed to hypoxic conditions also experienced hormonal disruptions leading to decreased reproductive performance (Zhou et al. 2003). In the hypoxia-tolerant goldfish *Carassius auratus*, acclimation to hypoxic conditions resulted in changes in liver enzyme activities, suggesting depressed protein synthesis and enhanced gluconeogenesis (Thillart & Smit 1984). Similarly in the Oscar cichlid *Astronotus ocellatus*, hypoxia acclimated fish decreased liver, heart and gill protein synthesis by 50-60% (Lewis et al. 2007). Few acclimation studies carried out on saltwater fish showed that for Atlantic stingray *Dasyatis sabina*, hypoxia acclimated fish began to show uncoordinated swimming and onset of muscle spasms at lower O_2 concentrations compared to non-acclimated fish (Dabruzzi & Bennett 2014). All of the above point to metabolic depression or increased anaerobic capacity as a mechanism for adapting to hypoxic conditions. In contrast to metabolic depression and increased anaerobic capacity, some fish species increase their aerobic capacity to adapt to hypoxic conditions. Hypoxia acclimation experiments conducted on both *C. auratus* and southern catfish *Silurus meridionalis* showed a significant decrease in P_{crit} after acclimation (Fu et al. 2011; Yang et al. 2013).

It is also interesting to consider how fish increase their aerobic scope after acclimation. Various studies have confirmed that phenotypic plasticity both at the physiological and molecular level is commonly found in fish. Changes in gill morphology had been observed under hypoxia exposure (Dabruzzi & Bennett 2014; Fu et al. 2011; Dhillon et al. 2013; Tzaneva et al. 2014; Mitrovic et al. 2009) or acute freshwater transfers of saltwater fish (Watson et al. 2014), either to regulate oxygen uptake or ionic balance. Likewise, hematocrit levels were also observed to increase in *D. sabina*, rock perch *Scorpaena porcus*, and sea carp *Diplodus annularis* when exposed to hypoxic conditions (Silkin & Silkina 2005; Dabruzzi & Bennett 2014). On the molecular level, hypoxia exposure had been found to induce the expression of carbonic anhydrase IX in normal functioning tissues including the eye, brain, and muscle of zebrafish *Danio rerio* possibly to protect tissues against detrimental effects of acidosis resulting from increased reliance on anaerobic metabolism (Esbaugh et al. 2009). Thus it is highly likely that phenotypic plasticity contributes to the increased hypoxia tolerance of acclimated fish.

One hypothesized theory for increased hypoxia tolerance in acclimated fish is the increase of blood hemoglobin (Hb)-oxygen binding affinity (Dhillon et al. 2013; Rees et al. 2001). Extensive studies have shown that increase in Hb-O_2 affinity can be achieved by lowering the concentration of allosteric modulators such as ATP and GTP, whose binding to Hb causes a decrease in Hb–O_2 affinity (Frey et al. 1998; Mandic et al. 2009; Val 2000; Weber & Jensen 1988; Weber 1996; Weber 2000), and fish do adopt this pathway when exposed to hypoxic conditions (Mandic et al. 2009; Frey et al. 1998). Alternatively, Hb isoform switching involving the preferential synthesis of high-affinity Hb, or a reduction in the amount of low-affinity Hb could also achieve an increase in Hb-O_2 affinity (Rutjes et al. 2007). Thermo-acclimation has been shown to alter Hb composition, with *C. auratus* increasing the number of Hb isoforms in higher temperatures and rainbow trout *Salmo gaidnerii* showing temperature-related variation in the abundancies of specific Hb isoforms (Houston & Cyr 1974). The only definitive evidence of Hb isoform switching in response to hypoxia that we know of was obtained from Lake Victoria cichlid *Haplochromis (Labrochromis) ishmaeli*. In a split brood hypoxia exposure experiment, hypoxia treatment fish lacked four Hb isoforms that were present in normoxia treatment siblings. Analogously, five new Hb were seen in hypoxia treatment specimens that were lacking in normoixa treatment *H. ishmaeli* (Rutjes et al. 2007).

Thus the purpose of this study is to look at how the size of fish and acclimation to hypoxic conditions might influence P_{crit} in *S. ocellatus*. We hypothesize that larger fish would have a lower P_{crit} due to their lower SMR. A transcriptome analysis of *S. ocellatus* also revealed that it has at least six different forms of Hb-α subunits and five different forms of Hb-β subunits (unpublished data), so we hypothesize that *S. ocellatus* could decrease P_{crit} through acclimation, and hemoglobin isoform switching would account for the reduction in P_{crit}.

Materials and Methods

Experimental fish.

All experimental procedures were performed under the auspices of the University of Texas at Austin Institutional Animal Care and Use Committee and unless specified all

chemicals were obtained from Fisher Scientific. *S. ocellatus* used in the current study were raised on-site at the Fisheries and Mariculture Laboratory (FAML) and the University of Texas Marine Science Institute (Port Aransas, TX, USA), according to previous established protocols (Holt, Godbout, et al. 1981; Holt, Johnson, et al. 1981). Fish were held in recirculating in-door 150 L tanks supplied with filtered, running seawater originating from the Corpus Christi ship channel maintained at 22 ℃. The recirculation system was equipped with a common biological filter tank to control ammonia levels. Fish were fed daily with commercial fish pellets (Aquafeed, Cargill, USA) and tanks were siphoned periodically to remove debris.

SMR and P_{crit} measurement.

Fish were starved for at least 48 h prior to measurements. Standard metabolic rate (SMR) of fish was measured in advance of P_{crit} measurement. Oxygen uptake (MO_2) of the fish was measured using computerized intermittent-flow respirometry (Loligo Systems, Denmark) for SMR calculations (Lefevre et al. 2011; Steffensen et al. 1984) at 24 ℃ for 8 - 24 h. Each MO2 measurement cycle consisted of a flushing period followed by a closed period. MO2 was calculated from the decline in PO2 during the closed period according to Eqn 1:

$$\mathrm{MO2} = \frac{-\delta \mathrm{PO2} \times \alpha \mathrm{O2H2O} \times (\mathrm{Vchamber} - \mathrm{Mf})}{\mathrm{Mf}} \qquad (1)$$

where $\dot{M}O_2$ is oxygen uptake (µmol kg^{-1} min^{-1}), δPO_2 is slope of the decline in oxygen tension of the water (mmHg min^{-1}) during a closed respirometer cycle, αO_2H_2O is the solubility of oxygen in the water at the relevant temperature (µmol L^{-1} $mmHg^{-1}$), $V_{chamber}$ is the volume of the respirometer (L) and M_f is the mass of the fish (kg). Values for MO_2 were omitted when the R^2 of the linear regression for the decline in PO_2 was less than 0.95. Background tests were conducted for 0.5 h-2 h both before the fish was placed into the respirometer chamber and after the fish was removed from the respirometer chamber. Background MO_2 was then subtracted from the calculated MO_2, to control for bacterial oxygen uptake. SMR was determined as the average value of the lowest 10% MO_2 values obtained from the measurement period. Directly following SMR measurement, the fish was subjected to a closely monitored closed period in the respirometer chamber until PO_2 drops

below 8%. MO_2 was calculated from the decline in PO_2 in 0.5 min - 2 min intervals depending on the size of the fish during the closed period. P_{cirt} was then determined as the PO_2 where MO_2 was reduced below the SMR (Affonso & Rantin 2005; Thuy et al. 2010).

Scaling of SMR and P_{crit} with size

SMR and P_{crit} measurements were made for 55 *S. ocellatus* ranging from 0.26 g to 686 g. Regression analysis was used to explore relationships between SMR, P_{cirt} and body size using R (https://cran.r-project.org/).

Hypoxia acclimation experiment.

Fourteen fish (90.96±5.00 g ranging from 69.7 g to 141.9 g) were used in the experiment. Exposures were performed in two 150 L tanks, with one maintained at hypoxic condition (PO_2 values of 30% ± 5%) through injection of ultra-high purity N_2 gas (Matheson Tri-Gas, USA) and the other at normoxic condition ($PO_2 > 95\%$) through aeration. Both tanks were monitored on a daily basis to ensure a constant salinity and pH. 14 fish were tagged with VI alpha tags (Northwest Marine Technology, USA) 1 week prior to the experiment for identification purposes and randomly assigned to either hypoxia or normoxia group. P_{crit} of all experimental fish were measured before subjecting them to their respective treatments for 2 weeks. Fish were fed ad libitum during the acclimation period. At the end of the 2 week acclimation, P_{crit} of fish from both groups were measured for a second time. They were then placed back in their respective treatments for 1 week to recover from the P_{crit} measurement, after which individuals were removed and tissues and fluids sampled as described below.

Sampling and analysis techniques.

Sampling was conducted according to previously established protocols in the lab (Watson et al. 2014). Fish were euthanized by full immersion in a MS-222 bath (250 mg/L; 500 mg $NaHCO_3$ per L) followed by spinal transection. Body mass of the fish was weighed. Blood was sampled by caudal puncture using a 23-gauge needle pre-rinsed in heparinized saline (50 units/mL). Two 0.5 ml blood samples were transferred into 1.5 ml microcentrifuge tubes, centrifuged at 12,000 rpm for 1 min to remove the plasma, and preserved in -80 ℃ for

further analysis. Another small portion was transferred into a 40 mm heparinized plastic microhematocrit tube and sealed at one end with Critoseal®. The blood sample was then centrifuged using a CritSpin centrifuge at 15,800 rpm for 2 min, after which hematocrit was read using a microhematocrit capillary tube reader. An incision was then made above the anal vent of the fish and the body cavity opened to expose the heart. The heart was excised out, and the ventricle of the heart was weighed. The first gill arch on the left side of the fish was also sampled for gill surface area and diffusion distance analysis. Gill samples were placed immediately in zinc formalin fixative (Z-Fix; Anatech Ltd, USA), stored overnight, and subsequently transferred to 70% ethanol for long-term storage.

Gill surface area and gas diffusion distance.

Measurements of gill surface area were carried out according to methods outlined by Hughes (Hughes 1984). Briefly, the total number of filaments on the gill arch was counted and the length of every tenth filament was measured under a dissecting microscope. The linear spacing between lamellae along the filament was measured along at least 8 lamellae at 100X magnification under a light microscope (Nikon Eclipse TE2000-U) at the base, midsection and tip of 5 filaments evenly distributed along the gill arch using digital image capture software (QED Capture). An image of a stage micrometer was also digitally captured to calibrate the measurements.

Approximately 10 filaments from the midsection of the gill arch were dehydrated first in an ethanol series (95%, 3*60 min; 100%, 3*45 min), then in butanol for 1 h, and finally left in butanol overnight (12-16 h). Filaments were then treated twice with Histochoice clearing reagent (Amresco, USA) for 90 min, followed by two 60 min washes in Paraplast Plus embedding media (Leica Biosystems Richmond Inc., USA) at 58 ℃. The samples were embedded in casting cubes at room temperature and stored at 4 ℃ until sectioned. Samples were sectioned at 6 μm using a microtome and mounted onto Superfrost Plus slides (VWR, USA). At least 5 sections were mounted onto each slide. Slides were then deparaffinized with two 5 min washes in Histochoice clearing agent and rehydrated in an ethanol series (100%, 2*5min; 95%, 1*5 min; 70%, 1*5 min). Slides were stained with hematoxylin solution, gill no. 2 (Sigma-Aldrich, USA) for 120 s, soaked in 0.3% HCl-EtOH solution for 60 s, blued in

Scott's tap water substitute (RICCA Chemical, USA) for 45 s and counterstained with eosin y solution, alcoholic (Sigma-Aldrich) for 60 s. Slides were rinsed with tap water in between the different solutions and soaked additionally for 30 s in 95% ethanol before counterstaining. Coverslips were then applied with Permount® as a mounting medium after the slides were dehydrated.

Stained slides were viewed under a light microscope (Nikon Eclipse TE2000-U) at 400X magnification. Digital images of 20 lamellae were captured for each fish to quantify lamellar area. All lamellae were selected from the midsection of the filament and no more than 4 lamellae were chosen from a single filament. Images were assigned random numbers before surface areas were scored to minimize bias. Total surface area was calculated as $A = LfB$ where L is the total filament length (mm), f is the number of lamellae per millimeter on both sides of the filament, and B is the average bilateral surface area of a single lamella (mm^2). Surface area was standardized to the mass of the fish for comparisons between different fish.

Fifteen digital images of lamellae were then taken for each fish under 1000X magnification and 10 measurements of lamellar blood-to-water diffusion distance were taken to estimate the lamellar blood-to-water diffusion distance of each fish. Similar to surface area measurements, images were also assigned random numbers before diffusion distances were scored.

RNA analysis.

RNA analysis was conducted using pre-established protocols in the lab (Watson et al. 2014). Total RNA for each experimental fish was extracted from red blood cells obtained during the sampling using TRI Reagent (Molecular Research Center Inc., USA) according to manufacturer guidelines, with homogenization performed using a 18 gauge and then a 23 gauge needle. Total RNA was quantified using an ND-1000 (Thermo Scientific, USA) spectrophotometer at a wavelength of 260 nm and sample purity was assessed using 260:280 ratios. cDNA synthesis was performed using RevertAid reverse transcriptase (Thermo Scientific) according to the manufacturer specification, using 1 μg of DNase I (Thermo Scientific) treated total RNA as template. Relevant Hb-α and Hb-β gene sequences were identified from a commercially developed (LC Sciences, USA) RNA-Seq transcriptome

library for gill, intestine and red blood cells. Primer sets were then designed using the Primer3: WWW primer tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and specificity of each set was verified using standard PCR and gel electrophoresis procedures. All primers are listed in Table 1. Real-time PCR was performed on an Mx3000P real-time PCR system (Stratagene, USA) using the Maxima SYBR green master mix kit (Thermo Scientific; 12.5 µL reactions). Thermocycler program and reaction composition were optimized for the highest PCR efficiency calculated using a cDNA standard curve (94 ℃ 15s, 60 ℃ 30s, 72 ℃ 30s, 40 cycles; 0.3 µM primer pair), and primer specificity was accessed using the disassociation curve of each reaction. PCR efficiencies ranged from 77 to 96% with an $R^2 \geq 0.99$. Relative mRNA expression was calculated using the delta-delta ct method using elongation factor 1α (ef1α) as an internal control (Tang et al. 2007) and the normoxia treatment as the relative control (Pfaffl 2001). Successful DNase treatment was verified using a no reverse transcriptase control for each treatment.

Statistical Analysis.

Data are presented as means ± SE. P_{crit} from hypoxia acclimation experiment was analyzed using one-sided paired t-test for changes before and after acclimation in both groups. Parameters measured after the acclimation experiment was analyzed using unpaired t-test to identify differences between the normoxia and hypoxia group. Statistical significance was assumed at $P < 0.05$.

Results

Scaling of SMR and P_{crit} with size

SMR in fish typically decreases with body mass (M) according to the allometric equation:

$$SMR = k \cdot M^{b-1} \quad (1)$$

where k is a species-specific scaling constant and b is the scaling exponent (Lucas et al. 2014). Fitting the SMR obtained from *S. ocellatus* into the model yielded a scaling exponent of 0.79 (Fig. 1). Since the model is in the form of a power function, it can be linearized with a $\log_e$ transformation:

$$\ln(SMR) = \ln(k) + (b - 1) \cdot \ln(M) \qquad (2)$$

to give a R^2 value of 0.86 (Fig.1).

Even though SMR scaled negatively with mass, P_{crit} increased over the 2500-fold range in mass. Log_{10} transformed body mass and P_{crit} (%) follows a linear relationship where P_{crit} = 3.15 log$M$ + 16.19 (Fig. 2).

Hypoxia acclimation

Two sets of seven fish were exposed to either normoxia or hypoxia conditions for 2 weeks. Fish subjected to hypoxia treatment exhibited a statistically significant decrease ($P < 0.05$) in P_{crit}. However, no changes in P_{crit} were observed for fish in normoxia group (Fig. 3).

Physiological changes after acclimation

A sutie of cardiorespiratory parameters capable of impacting oxygen delivery were assessed between the two groups. The total gill surface area standardized over the fish's mass (Nilsson & Östlund-Nilsson 2008) and the lamellar blood-to-water diffusion distance were measured and no difference were observed between the two groups (Table 2). Hematocrit and relative ventricular mass were also examined for changes in the efficiency of oxygen delivery in the fish between the two treatment groups. Hematocrit within the normoxia group was highly variable and the changes were not statistically significant ($P = 0.706$) between normoxia and hypoxia treatment groups (Table 2).

Hemoglobin isoform changes after acclimation

. Hemoglobin α and β subunits' gene expression were assessed for both normoxia and hypoxia treatment groups (Fig. 4). All subunit nomenclature is based on placement of the sequence within a maximum likelihood phylogenetic analysis (data not shown). Expression levels for one Hbα and one Hbβ subunit were too low for an accurate measurement in both normoxia and hypoxia treatment groups and thus were omitted from the analysis. The hypoxia treatment group showed a significant increase in Hbα-2 ($p < 0.01$) and Hbα-3.2 ($p < 0.05$) compared to the normoxia treatment group. A more interesting set of data was also obtained when the expression levels of Hbα-3.1 in the normoxia group was used as the

relative control (Fig. 4b) for the calculation of mRNA expression. In the normoxia group, Hbα-3.1 was the most dominant α subunit, while in the hypoxia treatment group, Hbα-3.2 replaced Hbα-3.1 as the most dominant α subunit. β subunit wise, Hbβ-3.1 was the most dominant β subunit in both the normoxia and hypoxia treatment group, with expression levels more than two orders of magnitude higher than the other β subunits (Fig. 4d). Expression levels of Hbβ-3.1 were also significantly higher ($p < 0.01$) in the hypoxia treatment group compared to the normoxia treatment group. This switch in mRNA expression levels was also reflected in functional blood oxygen binding properties, as reduction in non-stripped hemolysate P_{50} were observed for fish in the hypoxia treatment group (Fig. 5).

Discussion

Impact of Size on Hypoxia Tolerance

Fitting the SMR obtained from *S. ocellatus* into the model yielded a scaling exponent of 0.79 (Fig. 1), falling at the lower end of the 0.79 to 0.88 range for teleost fishes (White et al. 2006; Clarke & Johnston 1999). This indicates that the scaling of SMR in *S. ocellatus* follows the same pattern as other fishes. However, the increase of P_{crit} over the 2500-fold range in mass (Fig. 2), contradicts with the results of Nilsson & Östlund-Nilsson (2008). This relationship was strongly significant ($P < 0.001$) with 44% of the variation in P_{crit} directly attributable to size. It is interesting to consider how smaller fish are able to maintain a lower P_{crit} despite having a higher SMR. Gill surface area is closely tied to oxygen uptake, but is also an unlikely explanation as it scales linearly with metabolic rate (Nilsson and Östlund-Nilsson 2008). Similarly, it has been demonstrated that branchial diffusion distance is unaffected by body size (Kisia & Hughes, 1992). P_{crit} is also related to hemoglobin oxygen binding affinity, so it may be possible that smaller fish have higher affinity hemoglobins that are more efficient at transporting O_2 in hypoxic environments. Finally, it is possible that the magnitude of hypoxia induced hyperventilation (Ern & Esbaugh 2016), or other such cardiorespiratory responses in red drum, vary based on size. Regardless, deciphering the underlying mechanisms for scaling of Pcrit in red drum is clearly an area of future study.

Ecologically, it is advantageous for smaller fish to have a lower P_{crit}. Smaller fish are less able to meet energetic demands through anaerobic metabolism due to their higher SMR and

more limited glycogen storage capacity (Nilsson & Östlund-Nilsson 2008). Thus having a lower P_{crit} may increase their survival rate in hypoxic environments. Additionally, smaller fish that have a lower P_{crit} could potentially use hypoxic environments as a refuge from predators, increasing their survival rates. In a study comparing hypoxia tolerance in yellow perch *Perca flavescens* and its prey fathead minnow *Pimephales promelas*, smaller *P. flavescens* were more tolerant to hypoxic conditions than larger ones, and comparing between species, *P. promelas* were more tolerant to hypoxic conditions than similar-sized *P. flavescens* (Robb & Abrahams 2003). Similarly in a behavioral preference study in largemouth bass *Micropterus salmoides*, smaller fish were less confined to higher oxygenated waters (Burleson et al. 2001). Thus the increase in *S. ocellatus'* P_{crit} as size increase may reflect the life history of the fish. Smaller *S. ocellatus* typically recruit to seagrass habitats during the early life settlement stage. These areas are most likely to experience diel fluctuations in oxygen saturation related to plant respiration. The lower P_{crit} would not only allow smaller fish to more successfully tolerate transient hypoxia, but also could result in these habitats acting as a refuge to avoid predators. As fish grow larger and subsequently move up the food web, there is no longer a necessity for maintaining a low P_{crit}.

Impact of Acclimation on Hypoxia Tolerance

The acclimation experiment demonstrated that *S. ocellatus* increases its hypoxia tolerance when acclimated to hypoxic conditions. P_{crit} of *S. ocellatus* in the hypoxia treatment group showed a 5.1% decrease, from 22.8% ±2.1% to 17.7% ±1.3%, after acclimation while P_{crit} of fish in the normoxia treatment group remained the same. Thus *S. ocellatus* is capable of increasing its oxygen transport capacity when acclimated to hypoxic conditions. Similar findings of increasing aerobic capacity to increase hypoxia tolerance were also documented in *C. auratus* and *S. meridionalis* (Fu et al. 2011; Yang et al. 2013), with *C. auratus* capable of decreasing P_{crit} by up to 49% after acclimating to hypoxic conditions (Fu et al. 2011).

Previous studies have shown that an increase in aerobic capacity is often linked to changes in gill morphology (Dabruzzi & Bennett 2014; Fu et al. 2011; Dhillon et al. 2013; Tzaneva et al. 2014; Mitrovic et al. 2009), such as an increase in gill surface area or a decrease in lamella thickness to facilitate the diffusion of oxygen into the bloodstream. The

increase in aerobic capacity in *S. ocellatus* however was found to be not linked to gill morphology changes. Gill surface areas were not different between the normoxia and hypoxia treatment groups, even when the non-linear relationship between gill surface area and the mass of the fish (Nilsson & Östlund-Nilsson 2008) was taken into consideration. Similarly, lamella thickness was shown to be almost identical between the two treatment groups.

In addition to gill remodeling, some fish have been shown to maximize blood oxygen-carrying capacity in response to hypoxia by increasing hematocrit level (Silkin & Silkina 2005; Dabruzzi & Bennett 2014). However, this was also not seen in *S. ocellatus* as no changes in hematocrit were observed between the normoxia and hypoxia treatment groups. An increase in heart mass leading to an increase in the maximal oxygen uptake (Young et al. 2002) was also ruled out as the cause for the increased aerobic capacity in this study. Thus for this experiment, it is more likely that the increase in aerobic capacity was caused by changes at the molecular level.

Hemoglobin isoforms had previously been shown to be present in both reptiles and birds, and different isoforms were shown to exhibit different O_2 binding properties. In the turtle *Trachemys scripta*, two isoforms HbA and HbD had been characterized with HbD demonstrating a consistently higher O_2 affinity compared to HbA (Damsgaard et al. 2013). Similar results were also found in 11 different bird species (Grispo et al. 2012). The fact that different hemoglobin isoforms have different O_2 binding affinities makes hemoglobin isoform switching a possible mechanism for the increased aerobic capacity after acclimation. Hemoglobin multiplicity is also a commonly observed phenomenon in fish species (Fago et al. 2001; Shimada et al. 1980; Borza et al. 2009; Olianas et al. 2011); its function however is not as well known (Rutjes et al. 2007). In a study examining hemoglobin multiplicity in over 80 different Amazonian fishes, Fyhn et al. (Fyhn et al. 1979) found an average of four different hemoglobins per species. It was hypothesized that species with multiple hemoglobins acting in concert would have better blood gas transport capabilities during environmental fluctuations than would a species with only one type of hemoglobin, and this could likely be the case in *S. ocellatus*, accounting for the increase in aerobic capacity when acclimated to hypoxic conditions. When acclimated, the dominant form of Hb-α subunit transcribed was found to switch from Hbα-3.1 to Hbα-3.2. This would likely result in a

switch in the most dominant hemoglobin isoform as well. Hb-O_2 binding curves of non-stripped hemolysate supports this claim, as a decrease in P_{50} and thus an increase in Hb-O_2 binding affinity, was observed for fish subjected to hypoxia treatment (Fig. 5). Taken together the Hb mRNA expression levels and whole blood P_{50} data, it can be concluded that hypoxia acclimated fish are switching to higher O_2 affinity hemoglobin isoforms to increase their hypoxia tolerance. Similar result of utilizing hemoglobin isoform switching to increase hypoxia tolerance was only confirmed in one other fish species to our knowledge. In hypoxia acclimated *H. ishmaeli*, five new hemoglobins were seen that were lacking in normoixa acclimated fish, explaining the higher oxygen affinity of the hemoglobins from hypoxia acclimated fish (Rutjes et al. 2007).

Since oxygen binding curves were measured were for non-stripped hemolysate, the possibility that hypoxia acclimated fish are also manipulating allosteric factors to increase Hb-O_2 binding affinity cannot be ruled out. In vertebrates, organic phosphates are important allosteric factors and provide a rapid means of adapting hemoglobin function to ambient oxygen tension and tissue oxygen demand (Val 2000), with fish using polyanionic nucleoside triphosphates that bind in a positively charged cavity between the β-chains of the hemoglobin (Rutjes et al. 2007) to decrease Hb-O_2 binding affinity. While all fish have high ATP levels in the red blood cells, some species also have a significant amount of GTP, which binds in the phosphate pocket with an additional hydrogen bond compared with ATP and decreases Hb-O_2 affinity more potently (Gronenborn & Clore 1984; Weber 1996). Thus in hypoxia tolerant fish such as anguilliforms and cichlids, they have high GTP to ATP ratios in red blood cells and are able to decrease GTP concentrations when exposed to hypoxic conditions (Weber 2000; Rutjes et al. 2007). It is likely that this type of change in GTP concentration could also be happening in *S. ocellatus* to increase their Hb-O_2 binding affinity when acclimated to hypoxic conditions.

Future work for this study could focus on two parts – examining the effects of allosteric modulators on Hb-O_2 binding curves and examining the Hb-O_2 bidning curves of individual Hb isoforms. The first part could be easily conducted by extracting red blood cells from normoxia and hypoxia acclimated fish, and stripping the red blood cells of allosteric modulators. Measurement of ATP and GTP concentrations could be conducted with a typical

spectrophotometric assay. The second part requires generating recombinant isoforms to test in isolation, as isolating out the similar Hb isoforms from *S. ocellatus* could be difficult due to their similarity in structure. Obtaining the individual Hb-O_2 binding curves for the different Hb isoforms would provide more concrete evidence for Hb isoform switching. This could be further combined with assessing the impacts of ATP/GTP on the different hemoglobin isoforms.

In conclusion, the current study has demonstrated a relatively strong allometric relationship for hypoxia tolerance in the estuarine *S. ocellatus* and shown that they are capable of switching hemoglobin isoforms to increase hypoxia tolerance. The idea that hypoxia tolerance is affected by developmental stage is in stark contrast to previous work in marine systems, as well as the prevailing view that hypoxia tolerance is unaffected by body size. While the underlying physiological mechanisms that explain the change in hypoxia tolerance with body size are unclear and clearly of interest for further study, the known habitat usage of *S. ocellatus* across their life history would suggest that greater hypoxia tolerance in earlier life stages would be beneficial. Similarly, this experiment provided the first piece of evidence suggesting that fish past the juvenile stage are still capable of switching hemoglobin isoforms to tolerate hypoxic conditions. Future studies investigating the Hb-O_2 binding curves of each possible hemoglobin isoform would provide more concrete evidence to the story.

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Tables and Figures

Table 1. List of real-time PCR primers used for Hb subunit gene expression analysis. All sequences listed 5′ to 3′ with the reverse primer sequences listed as the reverse compliment of the gene sequence.

Gene	Orientation	Sequence
EF1α	F	GTT GCT GGA TGT CCT GCA CG
	R	GTC CGT GAC ATG AGG CAG ACT G
Hbα-2	F	TTT CAG GTG CTG TGA GAG AGA G
	R	GCC AGA GTT TTG ACT CAG GTC T
Hbα-6.1	F	AAA TAC CGA TAA ACT GCA AAC AGG
	R	AGA GTA TCC GAG CTT TTG GTA TTG
Hbα-short	F	ATG CTC TCA AAG AAG GAG AAA GAG
	R	GAT GGG AAA AGT ATG TTT TTG TGC
Hbα-3.1	F	GTA GGT GCT TCT TCC CCA CA
	R	CTT AAG CCA CCG ACA AGG TC
Hbα-3.2	F	TAA TCT TGT CGG TGC TAT GAA GG
	R	CCA GGG AAG TAC ATG CTG ATT AC
Hbα-27680	F	TAA ACA GCA GGA GAA GAT GAT GG
	R	CGT TTT TGC ATT CAT GTG TTT AT
Hbβ-1	F	AAA GTT GGG TAA AGC CTT CAC TG
	R	GTC TTC TGT TGC AGC TTT CTA GTG
Hbβ-4	F	GCT GTT TGG GAA AAG GTT GTA A
	R	TAT CCC CAA AAC TTC CGA AAT A
Hbβ-35878	F	AAC TCT TCA TCT CCA GCC TAT CAC
	R	TGC CAA AGA TCT TGG TGA TGA T
Hbβ-3.1	F	GCT TGC TAT CAG AGA ACT CGT TTG
	R	TGT TGA TGG TGT GAA AGT CTT CTT
Hbβ-3.2	F	TTA ATA AAA GCC TCC AAA GGA CTG
	R	CAT GTT GAC GAG GTT TAG GTT TAA G

Table 2. Gill surface area standardized to mass based on $A_{gill} = a \cdot M^b$ (mean mm^2/g ± SE), diffusion distance (mean μm ± SE), hematocrit (mean μm ± SE) and ventricle weight (mean μm ± SE) of *S. ocellatus* subjected to 3 weeks of nomorxia and hypoxia treatment. No difference was observed for all measured parameters.

Parameter Measured	**Normoxia Group**	**Hypoxia Group**	**P-value**
Gill Surface Area (mm^2/g)	348.9 ± 18.9	369.9 ± 24.1	0.499
Diffusion Distance (μm)	1.22 ± 0.04	1.18 ± 0.03	0.515
Hematocrit (%)	37.6 ± 2.4	36.6 ± 0.8	0.706
Relative Ventricular Mass (%)	$7.3 * 10^{-4} \pm 3 * 10^{-5}$	$7.3 * 10^{-4} \pm 3 * 10^{-5}$	0.905

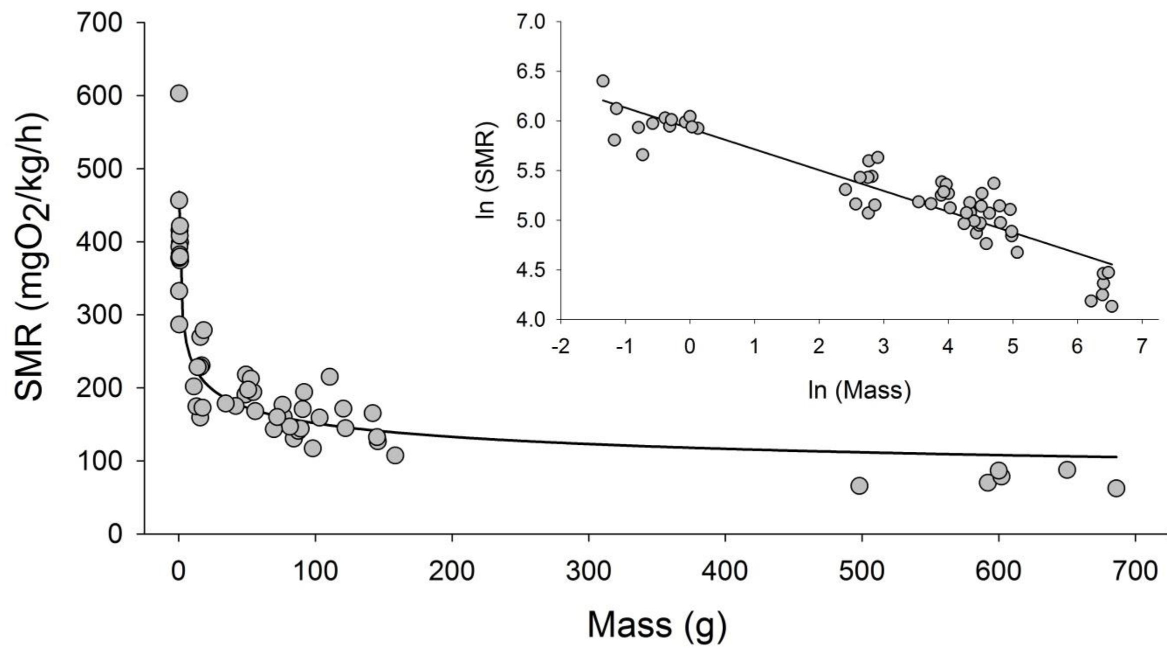

Figure 1. The relationship between body mass [M (g)] and standard metabolic rate [SMR (mgO_2 kg^{-1} h^{-1})] for *S. ocellatus*. Results are $SMR = 373.91M^{-0.21}$; linear regression on logarithmically transformed data points: $R^2 = 0.86$.

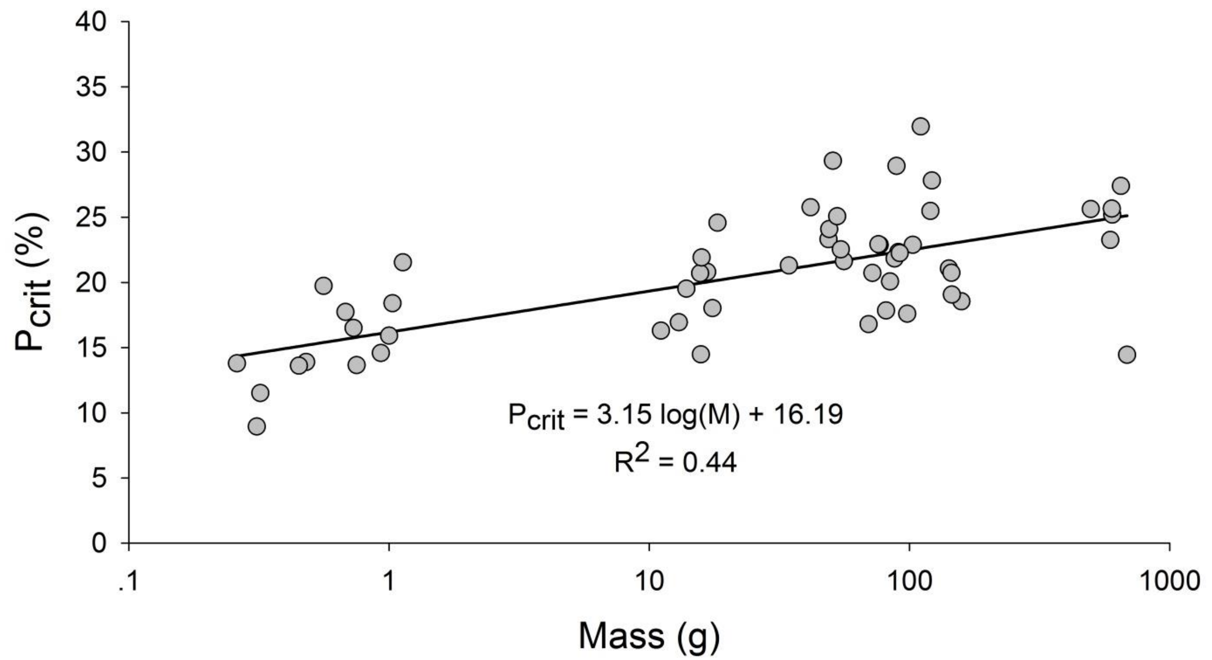

Figure 2. Relationship between $\log_{10}$ transformed body mass [M (g)] and critical oxygen tension [P_{crit} (%)] for *S. ocellatus*. P_{crit} increased over the 2500-fold range in mass: $P_{crit} = 3.15 \log M + 16.19$, $R^2 = 0.44$, $p < 0.001$.

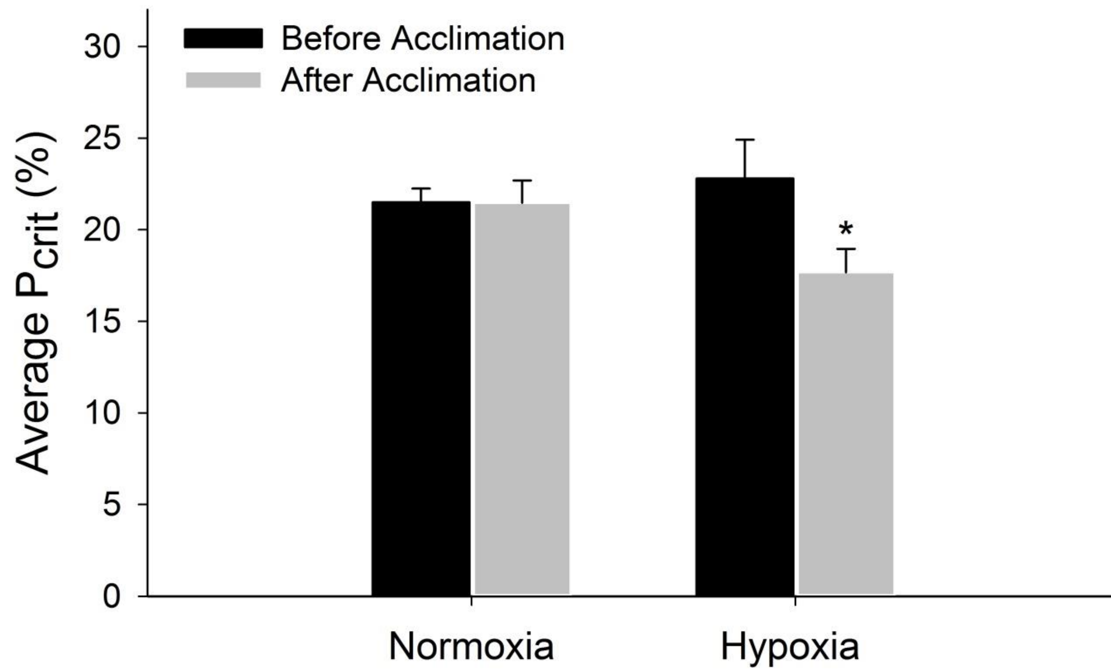

Figure 3. Average P_{crit} of fish in normoxia and hypoxia treatment groups before and after 2 weeks of acclimation in their respective treatments. Statistically significant decrease ($p < 0.05$) in P_{crit} was observed for the hypoxia treatment group, from 22.79% ± 2.12% to 17.67% ± 1.28% (mean ± SE).

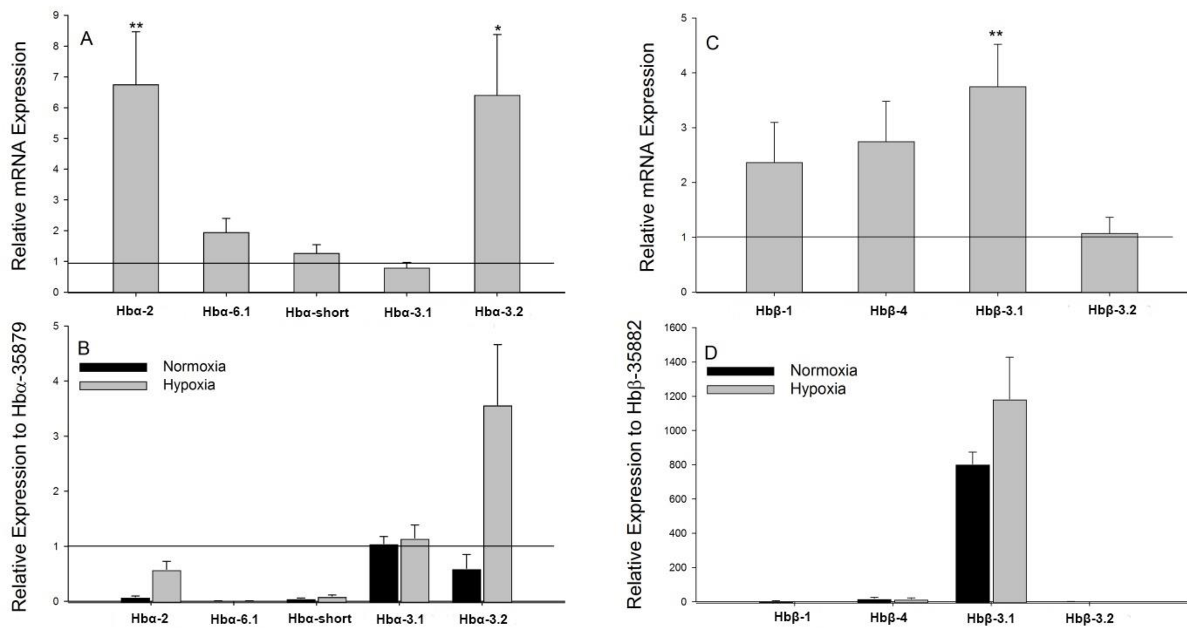

Figure 4. The effect of hypoxia acclimation on gene expression of Hbα and Hbβ subunits in the red blood cells of *S.ocellatus* as detected by real-time RT-PCR. A significant difference between hypoxia and normoxia treatments is denoted by an asterisk (unpaired Student's t-test, $P < 0.05$). All values are mean ± S.E. N = 6–7

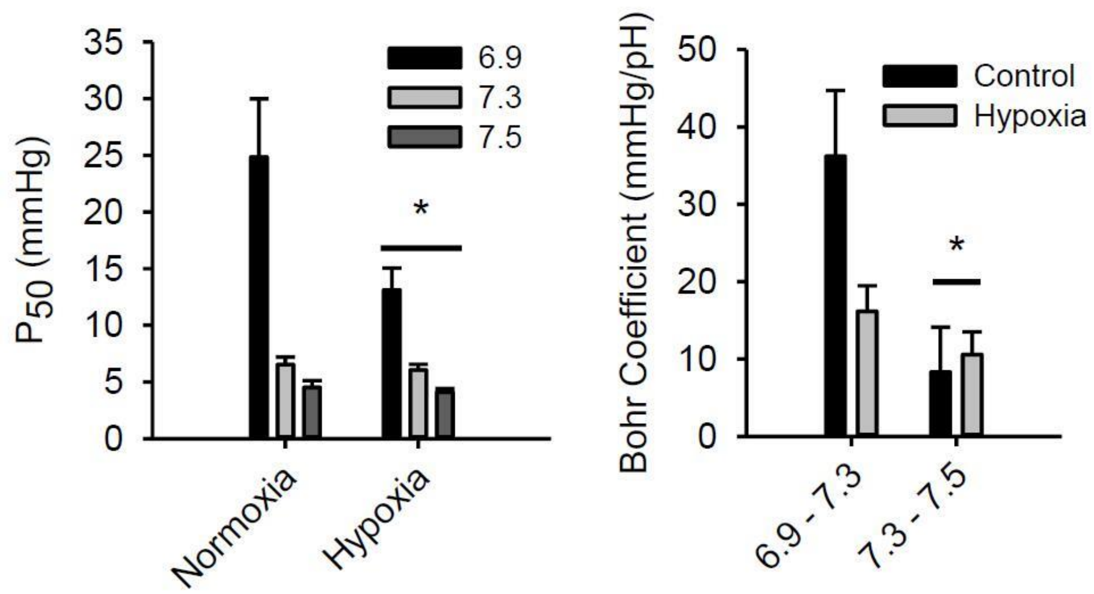

Figure 5. Significant decrease in P_{50} was observed for fish in the hypoxia treatment group. Bohr effect is magnified between pH 6.9 and 7.3 as compared to 7.3 to 7.4.