Fish Physiol Biochem (2014) 40:129–140
DOI 10.1007/s10695-013-9830-6

Acclimation to a low oxygen environment alters the hematology of largemouth bass (*Micropterus salmoides*)

Greg L. Gaulke · Clark E. Dennis III · David H. Wahl · Cory D. Suski

Received: 11 December 2012 / Accepted: 5 July 2013 / Published online: 13 July 2013
© Springer Science+Business Media Dordrecht 2013

Abstract One of the most severe impacts of urbanization on aquatic systems is the increasing presence of low oxygen environments caused by anthropogenic sources of pollution. As urbanization increases nationally and globally, it is becoming exceedingly important to understand how hypoxia affects aquatic fauna, especially fish species. In an effort to better understand the impacts of prolonged hypoxia on fishes, largemouth bass were held at 3.0 and 9.0 mg L^{-1} for 50 days, which has previously shown to be temporally sufficient to impart plastic phenotypic changes. Following the holding period, fish from each group were subjected to a low dissolved oxygen (DO) challenge of 2.0 mg L^{-1} for 6 h, and their physiological and hematological parameters were compared with control fish held for 6 h with no change in DO. There were no differences in the physiological stress responses between the two holding groups; however, the low oxygen holding group had increased hemoglobin and hematocrit levels following the 6-h low oxygen challenge compared with the high oxygen group. These results suggest largemouth bass exposed to chronic low oxygen conditions, either naturally or anthropogenically, may possess a beneficial advantage of increased oxygen uptake capacity during periods of low oxygen.

Keywords Stress · Physiology · Hematology · Hypoxia · Acclimation

G. L. Gaulke · C. E. Dennis III · C. D. Suski (✉)
Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana–Champaign, Urbana, IL, USA
e-mail: suski@illinois.edu

G. L. Gaulke · D. H. Wahl
Illinois Natural History Survey, University of Illinois at Urbana–Champaign, Urbana, IL, USA

Introduction

Animals acclimated to a particular environment are predicted to have an advantage over animals not acclimated to that environment, defined as beneficial acclimation (Leroi et al. 1994). While the majority of studies investigating beneficial acclimation have examined thermal acclimation (Deere and Chown 2006; Geister and Fischer 2007; Wilson et al. 2007; Lurman et al. 2009), few studies have investigated the effect of hypoxia, especially in terms of the physiological and hematological response. Previous research has shown that extended exposure to low oxygen can result in improved swimming performance, reduced metabolic rates, and altered gill morphology of fish (Timmerman and Chapman 2004; Fu et al. 2011; Zhao et al. 2012), all of which can provide performance advantages. However, most studies do not attempt to provide a link between the potential mechanisms behind hypoxia tolerance and improved performance, particularly in terms of the structure/function of

hematological characteristics (Nikinmaa and Rees 2005).

Aquatic organisms possess a suite of potential mechanisms to overcome environmental challenges related to low oxygen environments. More specifically, behavioral shifts, such as avoidance of hypoxic conditions (Kramer and Mehegan 1981), are a common mechanism utilized to overcome environmental hypoxia. Blue crabs (*Callinectes sapidus*), for example, avoid hypoxic conditions during periods of low dissolved oxygen (DO) and choose to inhabit more oxygenated water, thereby avoiding hypoxia-related challenges (Bell et al. 2003). Similarly, the release of stress hormones (e.g., cortisol) typically starts a physiological chain reaction of subsequent responses (i.e., secondary and tertiary stress responses), which enable an animal to maintain homeostasis and cope with a hypoxic disturbance (Barton 2002). Finally, genetic mechanisms, such as the up-regulation of hypoxia-inducible factor 1α (HIF-1α), can trigger several physiological responses to improve oxygen transport and carbohydrate metabolism that can allow an individual to cope with hypoxic conditions (Soitamo et al. 2001; Nikinmaa and Rees 2005; Richards 2009).

Although hypoxia can occur naturally in the environment (e.g., stratification, low light, reduced mixing; Wetzel 1983; Officer et al. 1984; Chapman et al. 1998), anthropogenically driven hypoxia is becoming increasingly abundant in natural ecosystems. The sources of pollutants driving environmental hypoxia can include combined sewer systems (Zhu et al. 2008; Baker 2009), agricultural runoff (Bernhardt et al. 2008), and urbanization (Paul and Meyer 2008; Zhu et al. 2008). These factors typically increase nutrients (e.g., nitrogen and phosphorous), containing oxygen-depleting substances, or both, which lowers DO in the aquatic environment. These low oxygen events can persist for weeks (Burton and Pitt 2002; Alp 2006) and can have negative impacts on fish populations, such as mass mortalities, extirpation, and population declines (Pollock et al. 2007). Despite the quantity of research conducted on hypoxia response and tolerance, few have attempted to determine the mechanism behind the observed responses. Because of these links between urbanization and the increase in adverse effects on aquatic systems, there is a critical need to understand (1) how these stressors (e.g., chronic hypoxia) affect the species therein, (2) if prolonged exposure to sublethal conditions can actually impart coping mechanisms that are of benefit to the animal's performance, and (3) the mechanism behind the observed effects of hypoxia and improved hypoxia tolerance.

The objective of this study was to quantify the impacts of extended exposure to low DO (3.0 mg L^{-1}) on the subsequent oxygen tolerance of largemouth bass (*Micropterus salmoides*), an ideal species to address hypoxia tolerance mechanisms as it is a robust species that can be found in hypoxic waters (Hasler et al. 2009) and is a commercially viable and important species. Largemouth bass were held at low oxygen for an extended period of time and then given a low oxygen challenge. Blood physiology parameters were quantified and compared with a control group not held in a low oxygen environment. Results have important implications for understanding how fish respond to chronic hypoxia, and how these changes may provide a potential advantage(s) that help elucidate how similar species are able to withstand such environments.

Methods

Fish collection and husbandry

Largemouth bass (range 150–180 mm) were acquired from the Logan Hollow Fish Farm (Murphysboro, IL, USA) in October 2011 and transported to the University of Illinois Aquatic Research Facility (Champaign, IL, USA). Upon arrival at the facility, fish were transferred into two 1,136-L plastic holding tanks where they were held for 48 h to recover from hauling and handling stressors. Largemouth bass were fed floating pelleted food (Aquatic Eco-Systems Inc., Apopka, FL, USA) ad libitum once per day, and solid wastes were removed from the tank by a siphon every other day. A 10 L min^{-1} pump was placed inside the tank to ensure adequate water mixing.

Acclimation treatments

Following the 48-h recovery period, each of the two tanks was assigned to one of two acclimation treatments: high DO (H) or low DO (L). The H tank was aerated continuously with air stones attached to a compressed air blower (Sweetwater Linear II, Aquatic

Eco-Systems, Apopka, FL, USA), and a 10 L min^{-1} pump was placed in the tank to ensure adequate water mixing. Throughout the duration of the study, nitrogenous waste was removed from the tanks using a combination of powdered ammonia remover (Pro-Line®, AquaticEcosystems, Apopka, FL, USA) and a container of activated carbon; ammonia levels did not exceed 1 ppm (Model # 33D, Aquarium Pharmaceuticals Inc., Chalfont, PA). Additionally, half of the water in each tank was removed weekly and refilled with fresh water from outdoor experimental holding ponds on site to avoid increase in ammonia or detrimental changes in pH. DO was measured daily with a handheld DO meter (YSI 550A, Yellow Springs Instruments, Yellow Springs, OH, USA) and remained at 9.0 mg L^{-1} ± 0.25 [$\bar{x}$ ± standard error (SE); 97 % DO saturation]. The L treatment held fish at 3.3 mg L^{-1} ± 0.07 (36 % DO saturation) by bubbling nitrogen gas (N_2) into the tank to displace oxygen, and a 10 L min^{-1} pump was again used to ensure homogeneous oxygen concentrations throughout the tank. DO concentrations were held constant by a 1-channel oxygen analyzer (OXY-REG) (Loligo Systems, Tjele, Denmark), which regulated oxygen levels using a DO probe connected to a solenoid switch, which, in turn, was attached to a tank of nitrogen gas. The oxygen analyzer was programmed such that when DO increased above a threshold of 3.5 mg L^{-1}, nitrogen gas would diffuse into the tank. Once DO fell to a target concentration (~2.8 mg L^{-1}), the OXY-REG would shut off the solenoid, ending nitrogen gas diffusion. DO for the L treatment was also quantified daily with the same handheld meter. Each tank was held at these respective DO concentrations for 50 days, as previous work with low oxygen acclimation has shown 6 weeks to be a sufficient duration of time to induce plastic physiological changes and cause acclimation to low oxygen conditions (Timmerman and Chapman 2004; Petersen and Gamperl 2010). The temperatures in the L tank (15.0 ± 0.4 °C) were not statistically different than the temperatures in the H tank (14.6 ± 0.28 °C) during the 50 days acclimation period ($t_{48} = 0.82$, $P = 0.42$).

Oxygen shock experiment

Following the 50 days holding period, largemouth bass from both acclimation groups were subjected to a low oxygen shock experiment to quantify the physiological response to prolonged holding at low oxygen. For this, 16 largemouth bass (8 from each oxygen group) were randomly assigned to a treatment (i.e., oxygen shock or control) and placed in individual, aerated, sensory-deprived chambers continuously supplied with freshwater from a central basin in a flow-through, closed system maintained at approximately 8.0 mg L^{-1} (Suski et al. 2006). This experimental design (i.e., holding the L group at a "neutral" condition following the extended acclimation period) is similar to previous studies investigating the effect of acclimation on fish (Timmerman and Chapman 2004; Zhao et al. 2012) and likely did not negate any potential physiological changes induced during the acclimation period. Additionally, largemouth bass in the control H group were subjected to identical protocols and handling, isolating any impacts of the low holding group. Following a 24-h acclimation period to the chamber, 8 of the largemouth bass were subjected to a DO shock of 2.0 ± 0.01 mg L^{-1} for 6 h by pumping N_2 gas into the central basin to displace oxygen (Suski et al. 2006). This oxygen level was chosen as previous studies have shown this concentration (i.e., 2.0 mg L^{-1}) to induce physiological responses in largemouth bass (Hasler et al. 2009; VanLandeghem et al. 2010). DO concentration in each individual chamber typically fell from 8.0 to 2.0 mg L^{-1} in <3 min. Following this 6 h exposure to 2.0 mg L^{-1} DO, largemouth bass were killed with an overdose of tricaine methanesulfonate [MS-222 (250 mg L^{-1} buffered with 250 mg L^{-1} sodium bicarbonate)] added directly to each chamber. Following cessation of ventilation, fish were measured to the nearest mm (total length, TL), weighed to the nearest g, and then sampled for blood and tissues. Blood was extracted from the caudal vessel using a 1-mL syringe and a 21-guage needle rinsed with lithium heparin. A small volume of whole blood was placed in a capillary tube and spun for 2 min using a hematocrit spinner to quantify percent hematocrit (i.e., packed red cell volume). A second small aliquot of whole blood was placed in a 1.5-mL centrifuge tube for subsequent quantification of hemoglobin. The remaining whole blood was centrifuged for 2 min at 2,000×g to separate erythrocytes from plasma, stored in a 1.5-mL centrifuge tube, and both blood plasma and red cells were flash frozen in liquid nitrogen until further processing. A section of epaxial muscle above

the lateral line was then excised with a razor blade, placed in a 1.5-mL centrifuge tube, and stored in liquid nitrogen until further processing. Control fish remained in the individual, aerated chambers for 6 h (following the 24 h acclimation to the chambers) with no change in DO (8.13 ± 0.01 mg L^{-1} O_2), and were sampled in a manner identical to fish exposed to the low oxygen shock.

Quantification of physiological parameters

Cortisol concentrations were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Kit # 900-071, Farmingdale, NY, USA; Sink et al. 2008), while plasma glucose and plasma lactate concentrations were quantified enzymatically using methods described in Lowry and Passonneau (1972). Plasma sodium (Na^+) and potassium (K^+) concentrations were quantified using a flame photometer (Model 2655-00; Cole-Parmer Instrument Company, Chicago, IL, USA), and plasma chloride (Cl^-) was quantified enzymatically with a commercially available kit (DICL-250, BioAssay Systems, Hayward, CA). Whole blood hemoglobin concentration (Hb) was quantified using a commercially available kit (DIHB-250, BioAssay Systems, Hayward, CA, USA). Mean cell hemoglobin concentration (MCHC) was calculated as [(% Hb ÷ Hct) × 100] (Houston 1990). Relative weight (W_r) was calculated by: [$W \div W_s \times 100$], where W is the weight of the fish and W_s is a length-specific weight standard (Anderson and Neumann 1996). Muscle water content [(wet weight − dry weight) ÷ 100] was quantified by obtaining wet weights and subsequent dry weights of the muscle samples excised during sampling after being placed in a drying oven for 72 h.

Quantification of HIF-1α mRNA

RNA isolation and cDNA synthesis

All procedures below adhere to current guidelines for the publication of quantitative real-time PCR studies (Bustin et al. 2009). Red blood cell total RNA was isolated using an Ambion RiboPure Blood Kit (AM1928, Life Technologies, Grand Island, NY, USA). Changes were made to the protocol to maximize extracted RNA purity and quantity: (1) red blood cells were thawed on ice as RNAlater® (i.e., RNA stabilizing reagent) was not used prior to storage in liquid nitrogen, and 2) extracted RNA was treated with Ambion DNase (AM1928, Life Technologies, Grand Island, NY, USA) to eliminate genomic DNA. Following RNA isolation, yield and purity of the RNA were determined using a Nanodrop ND-1000 UV–Vis spectrophotometer (Peqlab, Erlangen, Germany). Extracted RNA was subsequently stored at −80 °C until cDNA synthesis.

To synthesize cDNA, MultiScribe Reverse Transcriptase (Life Technologies, Grand Island, NY, USA), RNase Inhibitor, and random primers were used following the manufacturer's instructions of the High-Capacity cDNA Reverse Transcription kit (ABI # 4374966, Life Technologies, Grand Island, NY, USA) using 1 μg of total RNA for a reaction volume of 20 μL. The enzymes were then activated using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) set at 25 °C for 10 min, followed by an incubation period at 37 °C for 2 h, and then stopping the reaction at 85 °C for 5 min. All cDNA was then stored at −20 °C until qPCR analysis.

Creation of species-specific qPCR primers

Species-specific quantitative real-time polymerase chain reaction (qPCR) primers were obtained using degenerate primers manufactured from conserved regions of the gene from multiple species to obtain partial sequences for the target (HIF-1α) and reference (18S) genes. HIF-1α primers were determined using sequences from silver carp (*Hypophthalmichthys molitrix*) (accession no. HM146312), zebra fish (*Danio rerio*) (accession no. AY326951), and European perch (*Perca fluviatilis*) (accession no. EF100706). Primers for 18S were determined using sequences from mummichog (*Fundulus heteroclitus*) (accession no. M91180), Japanese killifish (*Oryzias latipes*) (accession no. AB105163), and the European perch (*P. fluviatilis*) (accession no. FJ710876).

All PCRs were performed using the Eppendorf Mastercycler with Taq DNA polymerase (M0285S, New England BioLabs, Ipswich, MA, USA; a thermally stable DNA polymerase), the extracted red blood cell cDNA, and each gene primer pair (i.e., HIF-1α, 18S). Each PCR reaction (25 μL) underwent the following conditions: one cycle at 95 °C for 30 s followed by 40 cycles of 95 °C for 25 s, 1 min at

58 °C, and 68 °C for 45 s. At the conclusion of the 40 cycles, the reaction ended at 68 °C for 5 min. Products of the PCR procedure (i.e., the DNA fragments created between the forward and reverse primers) were then separated using gel electrophoresis (1.5 % agarose gel containing ethidium bromide). The target DNA fragments were then excised from the gel using a MinElute Gel Extraction kit (#28606, QIAGEN Inc., Valencia, CA, USA) and purified using a QIAquick PCR Purification kit (#28106, QIAGEN Inc., Valencia, CA, USA). The purified fragments were sequenced using a capillary sequencer (ABI 3730XL, Life Technologies, Grand Island, NY, USA) at the Core DNA Sequencing Facility at the University of Illinois at Urbana–Champaign (Urbana, IL, USA). The resulting sequence information for largemouth bass HIF-1α and 18S was submitted to the National Center for Biotechnology Information, National Institute of Health's GenBank (accession no. JX901057 and JQ896299, respectively). The sequences were subsequently used to create largemouth bass-specific qPCR primers[1] and were designed using Primer Express software (v2.0.0.0, Life Technologies, Grand Island, NY, USA).

qPCR analysis

All qPCR reactions were performed using 2 μL of stock cDNA diluted 1:10 using RNase-free water, 2 μL of each qPCR primer at a 1 μM concentration, 4 μL of RNase-free water, and 10 μL of 2× Power SYBR Green Master Mix (Life Technologies, Grand Island, NY, USA), for a total reaction volume of 20 μL. The gene expression analysis was then conducted using an ABI 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA) under the following conditions: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at (1) 95 °C for 15 s, and (2) 60 °C for 1 min. At the conclusion of the cycles, the PCR results underwent a melt curve analysis (1 cycle at 95 °C for 15 s, 1 cycle at 60 °C for 15 s, and then 1 cycle at 95 °C for 15 s) to confirm the presence of a single amplicon. To confirm that the amplicon was the correct length and the only product generated in the reaction, gel electrophoresis was conducted on a 3 % agarose gel containing ethidium bromide.

Relative standard curves for HIF-1α (i.e., the target gene) and 18S (i.e., the reference gene) were created using multiple, highly induced samples to compare the threshold cycle to cDNA amount for each primer pair. The subsequent results were normalized using the 18S reference gene due to the mRNA levels of 18S remaining constant between controls and treatments for red blood cells [analysis of variance (ANOVA) $P > 0.05$]. To detect potential genomic DNA contamination, several RNA samples were chosen, and a qPCR was conducted with each primer pair. Results from this showed genomic contamination for 18S primers ranging from 1:500 to 1:1,000 indicating a negligible source of error, while genomic contamination for the HIF-1α primers was found to be 1:32. Despite this, the Ct levels for samples that contained no reverse transcriptase (i.e., 35–36 Ct) were outside the detection limit of the standard curve (i.e., 30 Ct), and this level of contamination can be considered nonexistent (Lewis et al. 2010; Caraguel et al. 2011).

Statistical analyses

A two-way ANOVA was used to quantify differences between the two acclimation treatments for all quantified parameters. The main effects in the ANOVA were acclimation treatment (i.e., high or low DO group), experimental treatment (i.e., control or oxygen shock), and their interaction. Initially, lengths and weights were added as covariates to determine if size of the fish influenced the results. If the interaction was significant, or if the interaction was not significant but at least one of the main effects was significant, a Tukey's post hoc test was used to separate the means. All tests were performed using JMP version 9.0 (SAS Institute Inc., Cary, NC, USA), and significance was assessed at $\alpha = 0.05$.

Results

Exposure of largemouth bass to water at 2.0 mg L^{-1} DO for 6 h did not have a significant effect on concentrations of plasma cortisol (acclimation: $F_{1,23} = 0.20$, $P = 0.66$; treatment: $F_{1,23} = 1.83$,

[1] Sequences 5′ → 3′:
18S: F-GCAAAGCTGAAACTTAAAGGAATTG; R-CCC GTGATTGAGTCAAATTAAGC
HIF-1α: F-AACACAGAGCGCAGCTTCTTC; R-CGGCC CCTGCTTGTGA.

$P = 0.19$; interaction: $F_{1,23} = 2.35$, $P = 0.14$; Fig. 1a) or plasma glucose (acclimation: $F_{1,23} = 1.14$, $P = 0.30$; treatment: $F_{1,23} = 0.25$, $P = 0.62$; interaction: $F_{1,23} = 3.17$, $P = 0.09$; Fig. 1b). Plasma lactate increased threefold as a result of the exposure to the low oxygen for both groups compared with controls ($F_{1,23} = 22.1$, $P = 0.0001$; Fig. 1c); however, there was no significant effect of either acclimation or the interaction of acclimation and treatment (acclimation: $F_{1,23} = 0.63$, $P = 0.44$; interaction: $F_{1,23} = 0.085$, $P = 0.77$; Fig. 1c).

Prior to exposure to low oxygen, there were no significant differences in hematocrit values between largemouth bass in the H and L treatments (Fig. 2a). After 6-h exposure to water at 2.0 mg L^{-1}, however, hematocrit values for the L treatment fish were 22 % higher than the H treatment fish (Tukey–Kramer HSD, $P = 0.03$; Fig. 2a). Similarly, hemoglobin values for the L group treatment fish were 14 % higher than H group treatment fish following 6 h exposure to water with 2.0 mg L^{-1} DO (Tukey–Kramer HSD, $P = 0.046$; Fig. 2b). MCHC, however, did not differ significantly across acclimation, treatment, or their interaction (acclimation: $F_{1,23} = 1.53$, $P = 0.23$; treatment: $F_{1,23} = 0.17$, $P = 0.68$; interaction: $F_{1,23} = 0.44$, $P = 0.51$; Fig. 2c). In red blood cell samples, the relative expression of HIF-1α was similar across all acclimations and treatments (acclimation: $F_{1,23} = 0.21$, $P = 0.65$; treatment: $F_{1,23} = 0.51$, $P = 0.48$; interaction: $F_{1,23} = 0.17$, $P = 0.68$; Fig. 3).

Plasma Na^+ and K^+ were not significantly affected by acclimation, the treatment, or their interaction (Na^+: acclimation: $F_{1,23} = 0.63$, $P = 0.44$; treatment: $F_{1,23} = 0.25$, $P = 0.62$; interaction: $F_{1,23} = 0.0078$, $P = 0.93$; K^+: acclimation: $F_{1,23} = 0.06$, $P = 0.81$; treatment: $F_{1,23} = 0.3$, $P = 0.60$; interaction: $F_{1,23} = 2.3$, $P = 0.15$; Fig. 4a, b). Plasma Cl^- increased significantly as an effect of the treatment after exposure to water at 2.0 mg L^{-1} DO ($F_{1,23} = 4.9$, $P = 0.038$; Fig. 4c). Muscle water content was not significantly affected by the acclimation, treatment, or their interaction (acclimation: $F_{1,23} = 3.97$, $P = 0.060$; treatment: $F_{1,23} = 0.11$, $P = 0.74$; interaction: $F_{1,23} = 0.095$, $P = 0.76$).

At the conclusion of the 50 days acclimation period, largemouth bass in the L group ($L_{Tlow} = 172.8$ mm ± 2.8; mean ± SE) were 8 % longer than the H group fish ($L_{Thigh} = 159.2$ mm ± 2.5). Fish from the L group ($T_{Wlow} = 77.4$ g ± 3.7) were also approximately one-third heavier than in the H group ($T_{Whigh} = 62.4$ g ± 3.3). Relative weights were similar across acclimations and treatments ($F_{3,25} = 0.55$, $P = 0.65$).

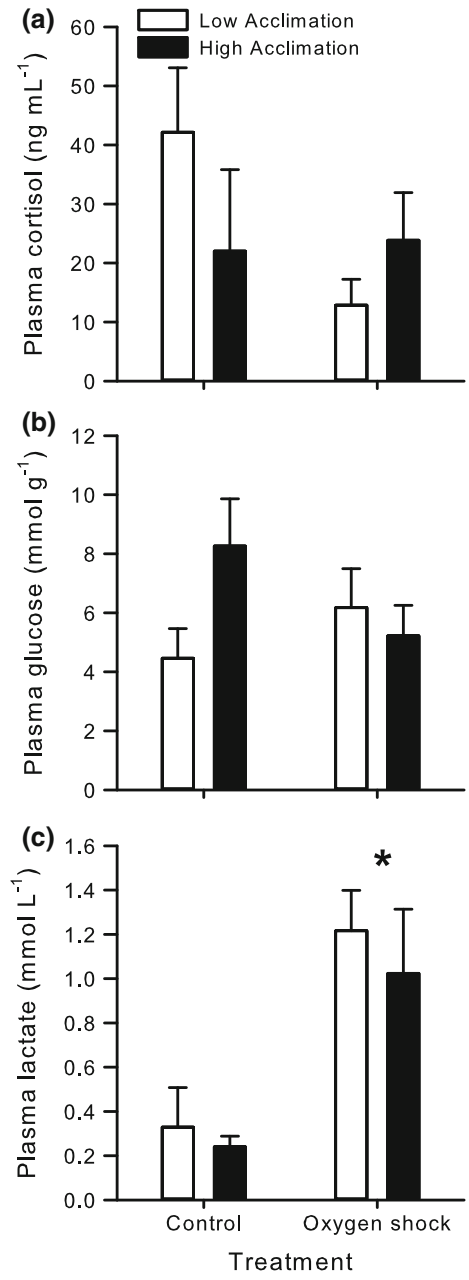

Fig. 1 Concentrations of plasma cortisol (**a**), plasma glucose (**b**), and plasma lactate (**c**) for largemouth bass held for 50 days at high DO (8.0 mg L^{-1}—H) or 50 days at low DO (3.0 mg L^{-1}—L) and then subjected to a low oxygen shock (2 mg L^{-1} for 6 h). Control fish for each group were held for 6 h at 8.0 mg L^{-1}. *Error bars* show ±1 SE, and an *asterisk* (*) indicates a significant treatment effect

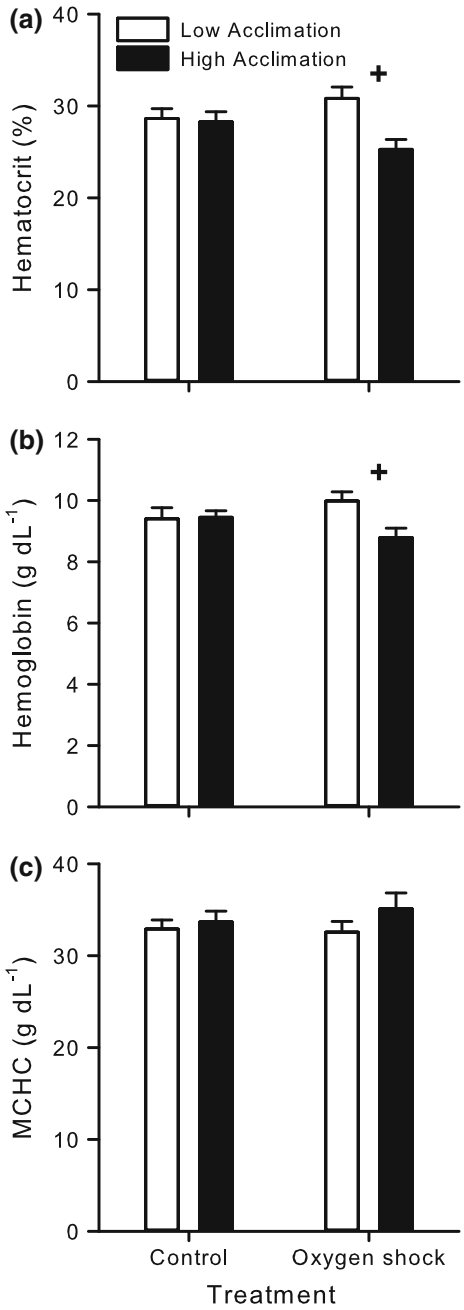

Fig. 2 Concentrations of hematocrit (**a**), hemoglobin (**b**), and MCHC (**c**) for largemouth bass held for 50 days at high DO (8.0 mg L^{-1}—H) or 50 days at low DO (3.0 mg L^{-1}—L) and then subjected to a low oxygen shock (2.0 mg L^{-1} for 6 h). Control fish for each group were held for 6 h at 8.0 mg L^{-1}. *Error bars* show ±1 SE, and *plus sign* (+) indicates a statistically significant difference between low and high acclimation means after a low oxygen shock

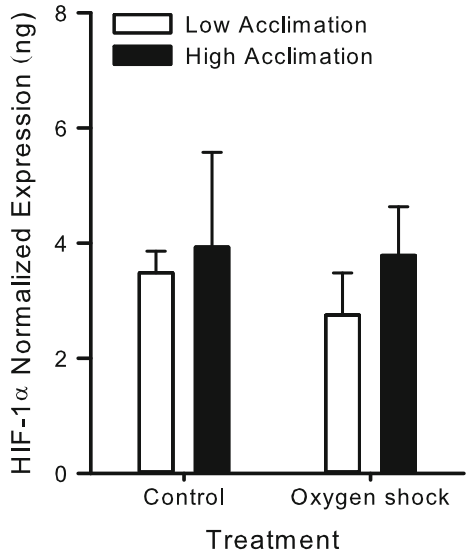

Fig. 3 Gene expression of HIF-1α from real-time quantitative PCR analysis of largemouth bass held for 50 days at high DO (8.0 mg L^{-1}—H) or 50 days at low DO (3.0 mg L^{-1}—L) and then subjected to a low oxygen shock (2 mg L^{-1} for 6 h). Control fish for each group were held for 6 h at 8.0 mg L^{-1}

Discussion

Extended holding of largemouth bass at low DO induced an improved ability to transport oxygen in blood relative to fish held at higher oxygen concentrations. Concentrations of both Hct and Hb were significantly higher in largemouth bass held at low oxygen for 50 days relative to fish held at higher oxygen. Hct is the percentage of packed red blood cells relative to the whole volume of blood, but does not account for the size or number of erythrocytes. Hb is a quantification of the O_2 binding protein found in red cells, whereas MCHC is a measure of the Hb in a given volume of packed erythrocytes (Houston 1990). Increases in Hct and/or Hb are typically caused by an increase in the production of erythrocytes, swelling of the erythrocytes, or a combination of both. These changes are typically a result of catecholamine releases that induce the release of erythrocytes from the spleen (Jensen et al. 1993), or acidosis in the blood, which alters the affinity of Hb to bind oxygen, and can stimulate an increase in erythrocytes (Wells 2009). Increases in Hb and Hct concentrations between the L and H groups during an oxygen challenge may have

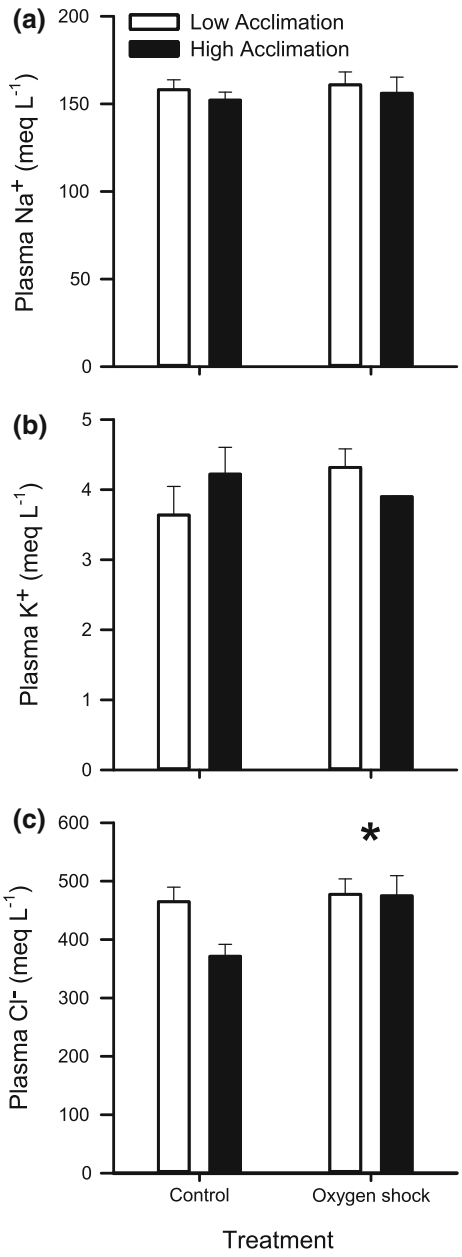

Fig. 4 Concentrations of plasma Na^+ (**a**), plasma K^+ (**b**), and plasma Cl^- (**c**) for largemouth bass held for 50 days at high DO (8.0 mg L^{-1}—H) or 50 days at low DO (3.0 mg L^{-1}—L) and then subjected to a low oxygen shock (2 mg L^{-1} for 6 h). Control fish for each group were held for 6 h at 8.0 mg L^{-1}. *Error bars* show ±1 SE, and an *asterisk* (*) indicates a significant treatment effect

been driven by the release of erythropoietin, the hormone responsible for synthesizing erythrocytes and releasing erythrocyte stores from the spleen. This is evidenced by the increase of erythrocytes numbers (i.e., increase in HCT and Hb) without increasing the amount of Hb per cell volume (i.e., no change in MCHC). This is only offered as a potential mechanism as erythropoietin was not quantified. Rainbow trout (*Oncorhynchus mykiss*) subjected to sustained hypoxia (maximum 216 h) had persistent increases in erythropoietin, as well as increased Hb levels (Lai et al. 2006), thereby providing an improved ability for oxygen uptake. Additionally, long-term exposure to hypoxia increases both Hb and Hct concentrations for numerous fish species, both air and water breathers (Scott and Rogers 1981; Tun and Houston 1986; Petersen and Petersen 1990; Timmerman and Chapman 2004). These changes typically confer an increase in oxygen-binding affinity or increased substrata for oxygen binding on the erythrocyte, improving performance of fish in low oxygen conditions. Despite Hct and Hb concentrations not differing between control treatments for the H and L group, largemouth bass acclimated to a low oxygen environment were able to increase those hematological variables relative to the high oxygen group following a low oxygen challenge. It is likely that this increase in Hb and Hct provided an increase in performance during hypoxia, but additional work measuring blood gas concentration and/or Hb/O_2 affinity would be necessary to confirm this. However, based on previous research, the prolonged exposure of largemouth bass to a low oxygen environment may have conferred a beneficial advantage by improving the fish's ability to transport oxygen via erythrocytes (e.g., more red blood cells means increased surface area to bind oxygen) when exposed to an oxygen challenge.

The oxygen-dependent transcriptional factor HIF-1α is produced during normoxia in many tissues and is quickly degraded (Soitamo et al. 2001; Nikinmaa and Rees 2005; Richards 2009). During hypoxia, the HIF proteins stabilize and accumulate within tissues, targeting genes involved with hypoxia-adapting mechanisms, such as erythropoiesis, carbohydrate metabolism, vascularization, etc. (Bracken et al. 2003; Nikinmaa and Rees 2005). We did not observe a significant increase in HIF-1α expression in red blood cells between acclimation groups or treatments, indicating the genetic function of red blood cells was likely not altered as a result of acclimation or the treatment. Previous work on HIF-1α has shown expression levels to increase in the liver tissue of sea bass (*Dicentrarchus labrax*) when exposed to

moderate hypoxia (4.3 mg L^{-1}) for 15 days compared to sea bass held under normoxia (Terova et al. 2008). Under acute hypoxia, HIF-1α is up-regulated in brain and liver tissue of European perch (*P. fluviatilis*) and not muscle tissue; however, under chronic hypoxia, HIF-1α is only up-regulated in muscle tissue, suggesting HIF-1α expression varies between tissues and exposure time (Rimoldi et al. 2012). Because HIF-1α in the current study remained constant, exposure of largemouth bass to 3.0 mg L^{-1} for 50 days likely did not result in advantageous changes to the genetic function of the red blood cells, and other tissues (e.g., internal organs such as the liver or brain) may be a suitable alternate.

Exposure of largemouth bass to a low DO challenge induced physiological disturbances caused by anaerobic metabolism. Following the low oxygen shock, largemouth bass from both treatment groups experienced an increase in lactate, as well as an increase in the concentration of chloride ions in plasma, relative to control values. When tissue oxygen levels are too low to support aerobic ATP synthesis, the process continues anaerobically, resulting in an accumulation of lactate (Wright et al. 1989). Quantifying lactate in plasma can measure the intensity and duration of the response to hypoxia in fish (Zhou et al. 2000; O'Connor et al. 2011). Exposure of largemouth bass to DO concentrations for similar durations of time resulted in increases of lactate comparable in magnitude to those in the current study (Suski et al. 2006; VanLandeghem et al. 2010). Metabolic demands are typically lower for fish at cooler temperatures (Chabot and Dutil 1999); however, the similar increase in magnitude (>threefold increase) of plasma lactate is believed to show sufficient evidence of anaerobic respiration for the study fish. The increase in plasma Cl^- observed following hypoxia exposure may have occurred due to the increased gill permeability and decreased gas diffusion distance. Sea bass acclimated to a mild hypoxia regime (70–80 % of the saturation value) showed a Cl^- ion loss at the gills when exposed to a hypo-osmotic challenge (Saroglia et al. 2010). These results are similar to previous studies with similar methodology where gas diffusion efficiency increased as a result of an increase in respiratory surface area, a decrease in gas diffusion distance, and an observed change in ion loss at the gills (Saroglia et al. 2000, 2002). This osmo-respiratory compromise may have occurred in the current study where a loss of Cl^- through the gills may have been a more advantageous adjustment than other compensatory responses, e.g., increases in cortisol. Conversely, earlier work has shown, however, that Cl^- does not play a role in increasing or decreasing the affinity for oxygen to bind to hemoglobin during a hypoxic challenge (Wells 2009) and likely did not have a significant role in any acclimation benefits or detriments.

Many physiological parameters previously shown to increase following exposure to low oxygen in fishes did not change significantly for largemouth bass in the current study. Concentrations of plasma cortisol and plasma glucose did not significantly change as a result of the oxygen shock, and fish did not experience a loss of cations from plasma. Cortisol is an important primary stress response released following the onset of a stressor, while glucose is a fuel for aerobic tissues such as gills and the heart (Wendelaar Bonga 1997; Barton 2002). Previous studies examining the response of fishes to hypoxia has shown that exposure to 2.1 mg L^{-1} for 2.5 h can cause a threefold increase in cortisol levels in the spotted wolfish (*Anarhichas minor*; Lays et al. 2009), and exposure of juvenile Atlantic sturgeon (*Acipenser oxyrhynchus*) to a 1 h hypoxia challenge of approximately 1.5 mg L^{-1} (10 mmHg) doubled levels of plasma glucose (Kieffer et al. 2011). Response of largemouth bass to hypoxia of this magnitude generated concentrations of plasma cortisol similar to those in the current study (VanLandeghem et al. 2010). Unlike VanLandeghem et al. (2010), we did not observe a significant increase in plasma glucose following exposure to 2.0 mg L^{-1} DO, likely due to cooler water temperatures [$\sim$15 °C in this study compared to 20 °C for VanLandeghem et al. (2010)]. Additionally, gill permeability typically increases during exposure to low DO, leading to a decrease in plasma ion concentrations (Gonzalez and McDonald 1994). Combined, the results of this study suggest an oxygen shock for high- and low-acclimated fish at the temperatures we examined was not significant to elicit a range of stress responses.

Following a 50 days exposure to hypoxia, largemouth bass in the high oxygen group were 8 % longer and 24 % heavier than fish in the low oxygen group. Fish growth typically decreases in hypoxia (Cech et al. 1984; Pichavant et al. 2001; Brandt et al. 2009), and the size differences seen are likely due to a sampling effect and relatively small sample sizes (8 individuals

per treatment), not an actual increase in growth rates. In addition, length and weight were initially added as covariates in the statistical analyses for all the parameters measured, but were removed in the final models when they were found to have no effect on any results (Engqvist 2005).

In conclusion, we demonstrated hematological differences between largemouth bass acclimated to a low oxygen environment relative to control individuals following a low oxygen shock. These compensatory differences can be attributed to a beneficial acclimation of the low oxygen group to an increase in the number of erythrocytes (i.e., increased substrata for oxygen binding) without changes in the genetic function of the cells. The differences observed may have important implications for an understanding of how fish in the environment respond to chronic changes in environmental variables. Fish exposed to chronic hypoxia in environments such as urban streams may therefore impart beneficial changes to their phenotypes to allow improved oxygen tolerances when behavioral mechanisms are not feasible (e.g., movement away from low oxygen environments). These long-term changes can be of benefit and are advantageous for the animal to survive in these environments.

Acknowledgments The authors would like to thank the Water Environment Research Foundation (project U3R09) for providing the funds for this study, as well as the Illinois-Indiana Sea Grant College Program Grant # NA06OAR4170079 for supplemental funds. D. Kates, Z. Zuckerman, S. Liss, and D. Sutter provided assistance with data collection. Dr. R. Schooley provided valuable insight into earlier drafts of this work.

References

Alp EA (2006) A method to evaluate duration of the storm effects on in-stream water quality. Dissertation, Marquette University

Anderson RO, Neumann RM (1996) Length, weight, and associated structural indices. In: Murphy BR, Willis DW (eds) Fisheries techniques, 2nd edn. American Fisheries Society, Bethesda, pp 447–482

Baker LA (2009) New concepts for managing urban pollution. In: Baker LA (ed) The water environment of cities. Springer, New York

Barton BA (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr Comp Biol 42:517–525

Bell GW, Eggelston DB, Wolcott TG (2003) Behavioral responses of free-ranging blue crabs to episodic hypoxia. I. Movement. Mar Ecol Prog Ser 259:215–225

Bernhardt ES, Band LE, Walsh CJ, Berke PE (2008) Understanding, managing, and minimizing urban impacts on surface water nitrogen loading. Ann NY Acad Sci 1132:61–96

Bracken CP, Whitelaw ML, Peet DJ (2003) The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. Cell Mol Life Sci 60:1376–1393

Brandt SB, Gerken M, Hartman KJ, Demers E (2009) Effects of hypoxia on food consumption and growth of juvenile striped bass (*Morone saxatilis*). J Exp Mar Biol Ecol 381:S143–S149

Burton GA, Pitt BE (2002) Stormwater effects handbook: a toolbox for watershed managers, scientists, and engineers. Lewis Publishers, Washington

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Caraguel CGB, Stryhn H, Gagné N, Dohoo IR, Hammell KL (2011) Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: analytical and epidemiologic approaches. J Vet Diagn Invest 23:2–15

Cech J, Mitchell S, Wragg T (1984) Comparative growth of juvenile white sturgeon and striped bass: effects of temperature and hypoxia. Estuaries Coasts 7:12–18

Chabot D, Dutil JD (1999) Reduced growth of Atlantic cod in non-lethal hypoxic conditions. J Fish Biol 55:472–491

Chapman LJ, Chapman CA, Crisman TL, Nordlie FG (1998) Dissolved oxygen and thermal regimes of a Ugandan crater lake. Hydrobiologia 385:201–211

Deere JA, Chown SL (2006) Testing the beneficial acclimation hypothesis and its alternatives for locomotor performance. Am Nat 168:630–644

Engqvist L (2005) The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. Anim Behav 70:967–971

Fu SJ, Brauner CJ, Cao ZD, Richards JG, Peng JL, Dhillon R, Wang YX (2011) The effect of acclimation to hypoxia and sustained exercise on subsequent hypoxia tolerance and swimming performance in goldfish (*Carassius auratus*). J Exp Biol 214:2080–2088

Geister TL, Fischer K (2007) Testing the beneficial acclimation hypothesis: temperature effects on mating success in a butterfly. Behav Ecol 18:658–664

Gonzalez R, McDonald D (1994) The relationship between oxygen uptake and ion loss in fish from diverse habitats. J Exp Biol 190:95–108

Hasler CT, Suski CD, Hanson CD, Cooke SJ, Tufts BL (2009) The influence of dissolved oxygen on winter habitat selection by largemouth bass: an integration of field biotelemetry studies and laboratory experiments. Physiol Biochem Zool 82:143–152

Houston AH (1990) Blood and circulation. In: Schreck CB, Moyle PB (eds) Methods for fish biology. American Fisheries Society, Bethesda, pp 273–334

Jensen FB, Nikinmaa M, Weber RE (1993) Environmental perturbations of oxygen transport in teleost fishes: causes, consequences and compensations. In: Rankin JC, Jensen FB (eds) Fish ecophysiology. Chapman & Hall, London, pp 162–179

Kieffer JD, Baker DW, Wood AM, Papadopoulos CN (2011) The effects of temperature on the physiological response to low oxygen in Atlantic sturgeon. Fish Physiol Biochem 37:809–819

Kramer DL, Mehegan JP (1981) Aquatic surface respiration, an adaptive response to hypoxia in the guppy *Poecilia reticulate* (Pisces, Poeciliidae). Environ Biol Fishes 6:299–313

Lai JCC, Kakuta I, Mok HOL, Rummer JL, Randall D (2006) Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. J Exp Biol 209:2734–2738

Lays N, Iversen MMT, Frantzen M, Jorgensen EH (2009) Physiological stress responses in spotted wolfish (*Anarhichas minor*) subjected to acute disturbance and progressive hypoxia. Aquaculture 295:126–133

Leroi AM, Bennet AF, Lenski RE (1994) Temperature acclimation and competitive fitness: an experimental test of the beneficial acclimation assumption. Proc Natl Acad Sci USA 91:1917–1921

Lewis JM, Hori TS, Rise ML, Walsh PJ, Currie S (2010) Transcriptome responses to heat stress in the nucleated red blood cells of the rainbow trout (*Oncorhynchus mykiss*). Physiol Genomics 42:361–373

Lowry OH, Passonneau JV (1972) A flexible system of enzymatic analysis. Academic Press, New York

Lurman GJ, Bock CH, Portner HO (2009) Thermal acclimation to 4 or 10°C imparts minimal benefit on swimming performance in Atlantic cod (*Gadus morhua* L.). J Comp Physiol B 179:623–633

Nikinmaa M, Rees BB (2005) Oxygen-dependent gene expression in fishes. Am J Physiol Regul Integr Comp Physiol 288:R1079–R1090

O'Connor EA, Pottinger TG, Sneddon LU (2011) The effects of acute and chronic hypoxia on cortisol, glucose and lactate concentrations in different populations of three-spined stickleback. Fish Physiol Biochem 37:461–469

Officer CB, Biggs RB, Taft JL, Cronin LE, Tyler MA, Boynton WR (1984) Chesapeake Bay anoxia: origin, development, and significance. Science 223:22–27

Paul MJ, Meyer JL (2008) Streams in the urban landscape. Annu Rev Ecol Syst 32:333–365

Petersen LH, Gamperl AK (2010) Cod (*Gadus morhua*) cardiorespiratory physiology and hypoxia tolerance following acclimation to low-oxygen conditions. Physiol Biochem Zool 84:18–31

Petersen JK, Petersen GI (1990) Tolerance, behavior and oxygen consumption in the sand goby, *Pomatoschistus minutus* (Pallas), exposed to hypoxia. J Fish Biol 37:921–933

Pichavant KJ, Person-Le-Ruyet J, Le Bayon N, Severe A, Le Roux A, Boeuf G (2001) Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. J Fish Biol 59:875–883

Pollock MS, Clarke LMJ, Dubé MG (2007) The effects of hypoxia on fishes: from ecological relevance to physiological effects. Environ Rev 15:1–14

Richards JG (2009) Metabolic and molecular responses of fish to hypoxia. In: Richards JG, Farrell AP, Brauner CJ (eds) Fish physiology: hypoxia, 1st edn. Academic Press, New York, pp 443–485

Rimoldi S, Terova G, Ceccuzzi P, Merelli S, Antonini M, Saroglia M (2012) HIF-1α mRNA levels in Eurasian perch (*Perca fluviatilis*) exposed to acute and chronic hypoxia. Mol Biol Rep 39:4009–4015

Saroglia M, Cecchini S, Terova G, Caputo A, De Stradis A (2000) Influence of environmental temperature and water oxygen concentration on gas diffusion distance in sea bass (*Dicentrarchus labrax*, L.). Fish Physiol Biochem 23:55–58

Saroglia M, Terova G, De Stradis A, Caputo A (2002) Morphometric adaptations of sea bass gills to different dissolved oxygen partial pressures. J Fish Biol 60:1423–1430

Saroglia M, Caricato G, Frittella F, Brambilla F, Terova G (2010) Dissolved oxygen regimen (PO_2) may affect osmorespiratory compromise in European sea bass (*Dicentrarchus labrax*, L.). Ital J Anim Sci 9e15:73–78

Scott AL, Rogers WA (1981) Haematological effects of prolonged sublethal hypoxia on channel catfish *Ictalurus punctatus* (Rafineque). J Fish Biol 18:591–601

Sink TD, Lochmann RT, Fecteau KA (2008) Validation, use, and disadvantages of enzyme-linked immunosorbent assay kits for detection of cortisol in channel catfish, largemouth bass, red pacu, and golden shiners. Fish Physiol Biochem 34:95–101

Soitamo AJ, Rabergh CM, Gassmann M, Sistonen L, Nikinmaa M (2001) Characterization of hypoxia-inducible factor (HIF-1α) from rainbow trout. J Biol Chem 276: 19699–19705

Suski CD, Killen SS, Kieffer JD, Tufts BL (2006) The influence of environmental temperature and oxygen concentration on the recovery of largemouth bass from exercise: implications for live-release angling tournaments. J Fish Biol 68:120–136

Terova G, Rimoldi S, Cora S, Bernardini G, Gornati R, Saroglia M (2008) Acute and chronic hypoxia affects HIF-1α mRNA levels in sea bass (*Dicentrarchus labrax*). Aquaculture 279:150–159

Timmerman CM, Chapman LJ (2004) Behavioral and physiological compensation for chronic hypoxia in the sailfin molly (*Poecilia latipinna*). Physiol Biochem Zool 77: 601–610

Tun N, Houston AH (1986) Temperature, oxygen, photoperiod, and the hemoglobin system of the rainbow trout, *Salmo gairdneri*. Can J Zool 64:1883–1888

VanLandeghem MM, Wahl DH, Suski CD (2010) Physiological responses of largemouth bass to acute temperature and oxygen stressors. Fish Manag Ecol 17:414–425

Wells RMG (2009) Gas transport and hemoglobin function: adaptations for functional and environmental hypoxia. In: Richards JG, Farrell AP, Brauner CJ (eds) Fish physiology: hypoxia, 1st edn. Academic Press, New York, pp 255–299

Wendelaar Bonga SE (1997) The stress response in fish. Physiol Rev 77:591–625

Wetzel RG (1983) Limnology. Saunder, Philadelphia

Wilson R, Condon CHL, Johnston IA (2007) Consequences of thermal acclimation for the mating behavior and swimming performance of female mosquito fish. Philos Trans R Soc B 362:2131–2139

Wright PA, Perry SF, Moon TW (1989) Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in

rainbow trout during environmental hypoxia. J Exp Biol 147:169–188

Zhao WW, Pang X, Peng JL, Cao ZD, Fu SJ (2012) The effects of hypoxia acclimation, exercise training and fasting on swimming performance in juvenile qingbo (*Spinibarbus sinensis*). Fish Physiol Biochem 38(5):1367–1377

Zhou BS, Wu RSS, Randall DJ, Lam PKS, Ip YK, Chew SF (2000) Metabolic adjustments in the common carp during prolonged hypoxia. J Fish Biol 57:1160–1171

Zhu W, Graney J, Salvage K (2008) Land-use impact on water pollution: elevated pollutant input and reduced pollutant retention. J Contemp Water Res Educ 139:15–21