

RESEARCH ARTICLE

Mitochondrial physiology and reactive oxygen species production are altered by hypoxia acclimation in killifish (*Fundulus heteroclitus*)

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ABSTRACT

Many fish encounter hypoxia in their native environment, but the role of mitochondrial physiology in hypoxia acclimation and hypoxia tolerance is poorly understood. We investigated the effects of hypoxia acclimation on mitochondrial respiration, O₂ kinetics, emission of reactive oxygen species (ROS), and antioxidant capacity in the estuarine killifish (*Fundulus heteroclitus*). Killifish were acclimated to normoxia, constant hypoxia (5 kPa O₂) or intermittent diel cycles of nocturnal hypoxia (12 h:12 h normoxia: hypoxia) for 28–33 days and mitochondria were isolated from liver. Neither pattern of hypoxia acclimation affected the respiratory capacities for oxidative phosphorylation or electron transport, leak respiration, coupling control or phosphorylation efficiency. Hypoxia acclimation also had no effect on mitochondrial O₂ kinetics, but P₅₀ (the O₂ tension at which hypoxia inhibits respiration by 50%) was lower in the leak state than during maximal respiration, and killifish mitochondria endured anoxia–reoxygenation without any impact on mitochondrial respiration. However, both patterns of hypoxia acclimation reduced the rate of ROS emission from mitochondria when compared at a common O₂ tension. Hypoxia acclimation also increased the levels of protein carbonyls and the activities of superoxide dismutase and catalase in liver tissue (the latter only occurred in constant hypoxia). Our results suggest that hypoxia acclimation is associated with changes in mitochondrial physiology that decrease ROS production and may help improve hypoxia tolerance.

KEY WORDS: Energy metabolism, Free radicals, Oxidative stress, Teleost

INTRODUCTION

Mitochondria are central to many of the cellular effects of hypoxia, and mitochondrial dysfunction is a crucial factor in many diseases of oxygen limitation, but the role of mitochondrial physiology in naturally evolved hypoxia tolerance is poorly understood (Galli and Richards, 2014). Several fish species live in environments with reduced O₂ availability and can tolerate prolonged and severe hypoxia (Stecyk et al., 2004; Scott et al., 2008; Richards et al., 2009; Speers-Roesch et al., 2012; Borowiec et al., 2015). Although mitochondrial abundance and respiratory capacity are known to vary substantially between fish species (Moyes et al., 1992), we are just beginning to appreciate the relationship between mitochondrial physiology and hypoxia tolerance.

Mitochondrial physiology appears to differ between hypoxia-tolerant and -intolerant species. Although the capacity for oxidative phosphorylation in permeabilized heart fibres is reduced by acute hypoxia in the hypoxia-intolerant shovelnose ray (*Aptychotrema rostrata*), it is unaffected by acute hypoxia exposure in the hypoxia-tolerant epaulette shark (*Hemiscyllium ocellatum*) (Hickey et al., 2012). Reactive oxygen species (ROS) emission from heart fibres, much of which is believed to originate from mitochondria, is also lower in the epaulette shark than in the shovelnose ray (Hickey et al., 2012). ROS accumulation, lipid peroxidation and protein carbonylation can increase during hypoxia and/or reoxygenation (Lushchak and Bagnyukova, 2007; Zuo et al., 2013), so the lower rate of ROS emission in the epaulette shark may be important for helping to minimize oxidative stress. Correspondingly, many (Hermes-Lima and Zenteno-Savin, 2002) but not all (Leveelahti et al., 2014) hypoxia-tolerant species also seem to have evolved a high antioxidant capacity.

Hypoxia acclimation has often been shown to improve hypoxia tolerance (Fu et al., 2011; Dan et al., 2014; Borowiec et al., 2015) but the importance of changes in mitochondrial physiology to this process has not been well examined in fish, particularly in hypoxia-tolerant species. Acclimation of mammals to moderate hypoxia (such as at high altitudes) reduces or has no effect on mitochondrial respiratory capacity (Costa et al., 1997; Nouette-Gaulain et al., 2005; Horscroft and Murray, 2014). The O₂ dependence of mitochondrial respiration is also unaffected by hypoxia acclimation in rats (Costa et al., 1997), despite the theoretical expectation that it should influence the ability of mitochondria to synthesize ATP and help match ATP supply and demand in hypoxia (Gnaiger et al., 1998). However, we have a poor appreciation of how hypoxia acclimation alters mitochondrial respiration or O₂ kinetics in fish. This is especially true for the mechanisms of acclimation to intermittent hypoxia, which has received much less attention in the literature than constant hypoxia (Bickler and Buck, 2007; Richards et al., 2009) but imposes unique challenges that likely demand distinct coping strategies (Borowiec et al., 2015). In particular, intermittent hypoxia could differ from constant hypoxia as a stressor if it accentuates ROS production and oxidative stress (Prabhakar and Semenza, 2012). Reductions in mitochondrial ROS production during the course of acclimation could help fish cope by reducing oxidative stress.

The objectives of this study were to examine the effects of hypoxia acclimation on mitochondrial physiology (mitochondrial respiration, O₂ kinetics and ROS emission) and antioxidant capacity in the common killifish [*Fundulus heteroclitus* (Linnaeus 1766)]. Killifish are very tolerant of hypoxia (Richards et al., 2008; Borowiec et al., 2015) and must cope with both seasonal and daily fluctuations in dissolved oxygen in their native estuarine habitat (Tyler et al., 2009). Hypoxia acclimation alters many physiological

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List of symbols and abbreviations

CCCP	carbonyl cyanide <i>m</i> -chlorophenyl hydrazone
E_{PMG}	uncoupled respiration with pyruvate, malate and glutamate
E_{PMGS}	uncoupled respiration with pyruvate, malate, glutamate and succinate
$E_{S(Rot)}$	uncoupled respiration with succinate and rotenone
E_{Tm}	uncoupled respiration with TMPD and ascorbate
J_{max}	maximal respiration
L_N	leak respiration in the absence of ATP
L_T	leak respiration in the presence of ATP
L_{Omy}	leak respiration in the presence of oligomycin
\dot{M}_{O_2}	rate of oxygen consumption
oxphos	oxidative phosphorylation
P_{PM}	oxphos respiration with pyruvate and malate
P_{PMG}	oxphos respiration with pyruvate, malate and glutamate
P_{PMGS}	oxphos respiration with pyruvate, malate, glutamate and succinate
P_{Tm}	oxphos respiration with TMPD and ascorbate
P_{O_2}	partial pressure of oxygen
P_{50}	P_{O_2} at 50% inhibition of maximal respiration
ROS	reactive oxygen species
SOD	superoxide dismutase
TMPD	<i>N,N,N',N'</i> -tetramethyl- <i>p</i> -phenylenediamine

traits involved in O_2 transport and utilization in this species, including the activity of several oxidative and gluconeogenic enzymes in the liver (Borowiec et al., 2015). We therefore examined the adjustments in mitochondrial physiology that occur in liver mitochondria with hypoxia acclimation. This was examined for both constant hypoxia and intermittent diel cycles of nocturnal hypoxia, in order to appreciate the range of mitochondrial responses to the various patterns of hypoxia exposure that occur in estuaries.

MATERIALS AND METHODS**Study animals and experimental hypoxia acclimations**

Wild-caught adult killifish (*F. heteroclitus*) were purchased from a commercial supplier (Aquatic Research Organisms, Hampton, NH, USA) and were held for at least 1 month before experimentation in charcoal-filtered brackish (4 ppt) water at room temperature (~20°C). Water quality (pH, ammonia, etc.) was measured regularly, and was maintained with routine water and charcoal filter changes. Fish were fed commercial flakes (Big Al's Aquarium Supercentres, Mississauga, ON, Canada) six days per week. All animal procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

Killifish were exposed to normoxia, constant hypoxia or nocturnal (intermittent) hypoxia for 28–33 days in 35 litre glass aquaria with the same water chemistry as described above. Normoxia (20 kPa, 8 mg O_2 l⁻¹) was maintained by continuously bubbling the water with air. Constant hypoxia (5 kPa, 2 mg O_2 l⁻¹) was maintained at the appropriate partial pressure of O_2 (P_{O_2}) using a feedback system, wherein a galvanic O_2 sensor automatically controlled the bubbling of water with nitrogen gas using a solenoid valve (Loligo Systems, Tjele, Denmark). The same O_2 controller was used to maintain nocturnal hypoxia (5 kPa, 2 mg O_2 l⁻¹), but gas flow was alternated between nitrogen (19:00 to 07:00 h) and air (07:00 to 19:00 h) with an additional solenoid valve controlled by a photoperiod timer that was synchronized with the light cycle (12 h:12 h light:dark). This level of hypoxia was chosen because it is realistic of what killifish can experience naturally in estuaries (Tyler et al., 2009). Fish were prevented from respiring at the water

surface with a plastic grid barrier. Fish were euthanized by a sharp blow to the head followed by pithing at the end of the exposure period, and the liver was sampled for mitochondrial isolation or was frozen in liquid N_2 for assays.

Mitochondrial isolation

Mitochondria were isolated from liver tissue using methods modified from Fanguie et al. (2009). Chemicals used for isolation and all other protocols are from Sigma-Aldrich (Mississauga, ON, Canada) unless otherwise stated. The livers of two to six fish (totalling a combined body mass of 13 g) were pooled for each mitochondrial isolation, and then finely diced in 10 ml of ice-cold isolation buffer (in mmol l⁻¹ unless otherwise stated: 250 sucrose, 50 KCl, 25 KH_2PO_4 , 10 HEPES, 0.5 EGTA, 1.5% mass:volume fatty-acid free bovine serum albumin; pH 7.4 when measured at 20°C). The tissue was gently homogenized on ice with six passes of a loose-fitting Potter-Elvehjem homogenizer at 30 r.p.m. Homogenate was centrifuged at 600 g for 10 min at 4°C. The supernatant was filtered twice through glass wool and was centrifuged at 6000 g for 10 min at 4°C (the same conditions were used for all subsequent centrifugation). The pellet was washed with isolation buffer to remove all excess fat and was gently re-suspended in 10 ml of fresh isolation buffer. The suspension was centrifuged again, washed in storage buffer (same as isolation buffer, but without bovine serum albumin and with 2 mmol l⁻¹ each of pyruvate and malate), and gently re-suspended in 10 ml of storage buffer. The suspension was centrifuged one final time, and the resulting pellet was re-suspended in 500 μ l of storage buffer. This mitochondrial suspension was assayed for protein content using the Bradford assay (following instructions from the supplier), and was stored on ice until respirometry experiments were completed. The remaining mitochondria were stored at -80°C for later use in antioxidant enzyme assays.

Mitochondrial physiology

The physiology of isolated mitochondria (0.4 mg of mitochondrial protein) was assessed using high-resolution respirometry and fluorometry (Oxygraph-2k with O2k-Fluorescence module, Oroboros Instruments, Innsbruck, Austria) in 2 ml of respiration buffer (in mmol l⁻¹: 110 sucrose, 60 K-lactobionate, 20 taurine, 20 HEPES, 10 KH_2PO_4 , 3 $MgCl_2 \cdot H_2O$, 0.5 EGTA, 1.5% bovine serum albumin; pH 7.4 at 20°C) at 20°C. Two separate experiments were conducted that used different substrate additions and/or fluorophores. Results are expressed per milligram mitochondrial protein (where applicable). DatLab 2 software (Oroboros Instruments) was used to fit respiration rate (rate of oxygen consumption, \dot{M}_{O_2}) data from the transitions into anoxia to the equation $\dot{M}_{O_2} = J_{max} \times P_{O_2} / (P_{50} + P_{O_2})$, where J_{max} is maximal respiration (uninhibited by hypoxia) and P_{50} is the P_{O_2} at which respiration is half of J_{max} . Delay in response time, internal zero drift and background O_2 flux of the O_2 sensor were accounted for as previously described (Gnaiger et al., 1995; Gnaiger and Lassnig, 2010).

In experiment 1, ROS emission from mitochondria was measured in parallel with mitochondrial respiration (sample sizes for each treatment group were as follows: normoxia, $n=8$; intermittent hypoxia, $n=10$; constant hypoxia, $n=8$) (Fig. 1A). Superoxide dismutase (SOD; at 22.5 U ml⁻¹) was added to the chamber to catalyze the reaction of the superoxide produced by mitochondria to hydrogen peroxide, and horseradish peroxidase (3 U ml⁻¹) was added to catalyze the reaction of hydrogen peroxide with Ampliflu

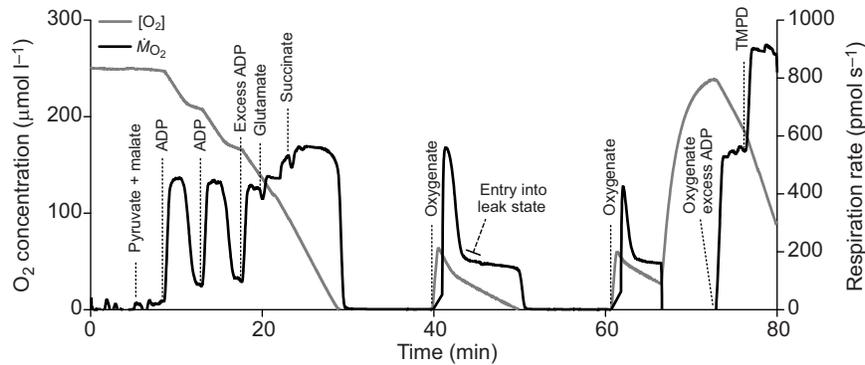


Fig. 1. Representative experiments on mitochondria isolated from killifish liver for measuring respiration and reactive oxygen species (ROS) emission. Anoxia periods were maintained for 10 min in the oxidative phosphorylation (oxphos) state (with maximal ADP stimulation) and then the leak state (no ADP). The transient increase in respiration rate (\dot{M}_{O_2}) in the leak state after anoxia is caused by an accumulation of ADP during anoxia from ATP hydrolysis by the ATP synthase. ROS emission rate was concurrently measured by the fluorescent detection of resorufin, which is produced from mitochondrial superoxide when in the presence of superoxide dismutase, horseradish peroxidase and Ampliflu Red. See Materials and methods for additional details.

Red ($15 \mu\text{mol l}^{-1}$) to produce the fluorescent product resorufin (detected using an excitation wavelength of 525 nm and AmR filter set; Oroboros Instruments). The resorufin signal was calibrated with additions of exogenous hydrogen peroxide. Leak state respiration (without ATP) using complex I substrates was first stimulated with malate (2 mmol l^{-1}) and pyruvate (2 mmol l^{-1}). Flux ratios of ADP to atomic oxygen (P/O ratios) were determined twice by the conventional method (Gnaiger et al., 2000) by adding $125 \mu\text{mol l}^{-1}$ ADP. After the depletion of ADP and re-establishment of leak state respiration (with ATP), saturating ADP ($0.6\text{--}0.9 \text{ mmol l}^{-1}$) was added to stimulate maximal pyruvate oxidation (state 3). The capacities for oxidative phosphorylation (oxphos) via complex I and complexes I+II were then determined by adding 10 mmol l^{-1} glutamate and 10 mmol l^{-1} succinate, respectively. Oxphos state respiration via complexes I+II was maintained until all O_2 was consumed (to determine mitochondrial P_{50}), and anoxia was maintained for 10 min. Oxygen tension was then raised, mitochondria were allowed to consume all ADP to reach leak state respiration (with ATP; state 4) via complexes I+II, and all O_2 was again consumed (to determine mitochondrial P_{50}) and anoxia was maintained for 10 min. Oxygen tension was raised, and ADP (1.25 mmol l^{-1}), ascorbate (2 mmol l^{-1}) and TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine; 0.5 mmol l^{-1}) were added to determine oxphos capacity via complex IV.

In experiment 2, a mitochondrial uncoupler (CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone) was used to determine the capacities for electron transport by the mitochondrial complexes (sample sizes for each treatment group were as follows: normoxia, $n=7$; intermittent hypoxia, $n=10$; constant hypoxia, $n=8$). Malate (2 mmol l^{-1}), pyruvate (2 mmol l^{-1}), glutamate (10 mmol l^{-1}) and ADP (1.25 mmol l^{-1}) were first added to stimulate maximal oxphos respiration via complex I. Addition of cytochrome *c* ($10 \mu\text{mol l}^{-1}$) was then used to assess mitochondrial integrity, which never increased respiration by more than 10% (mean \pm s.d. increase, $3.3\pm 4.3\%$). CCCP was then slowly titrated to $0.5 \mu\text{mol l}^{-1}$ to uncouple the mitochondria and determine the capacity of complex I for electron transport. Succinate (10 mmol l^{-1}) was then added to determine the capacity of complexes I+II for electron transport, followed by the addition of rotenone ($0.5 \mu\text{mol l}^{-1}$) to inhibit complex I and determine the capacity of complex II for electron transport. Finally, ascorbate (2 mmol l^{-1}) and TMPD (0.5 mmol l^{-1}) were added to determine the capacity of complex IV for electron transport.

Antioxidant enzyme activities and protein carbonyls

Assays were performed on homogenates of intact liver tissue and of isolated liver mitochondria. Frozen liver samples were homogenized in a Kontes tissue grinder in 20 volumes of homogenization buffer (20 mmol l^{-1} HEPES, 1 mmol l^{-1} sodium EDTA, 0.1% Triton X-100; pH 7.0 at 20°C) on ice. Protein content of the homogenates was determined using the BCA assay with bovine serum albumin as a standard (following instructions from the supplier, Sigma-Aldrich). Frozen suspensions of mitochondrial isolate were also homogenized in a Kontes tissue grinder before being assayed. Homogenates were frozen and stored at -80°C until assayed. All assays were performed in triplicate at 25°C using a SpectraMax Plus 384 microplate reader with temperature control (Molecular Devices, Sunnyvale, CA, USA), and activity is expressed per milligram protein.

SOD activity and protein carbonyl content were measured using commercially available kits (following the instructions of the manufacturer, Sigma-Aldrich). Catalase activity was determined by monitoring the disappearance of H_2O_2 . Activity was measured in sonicated homogenates as the rate of change in H_2O_2 absorbance at 240 nm, minus the background rates measured in absence of homogenate or H_2O_2 , in an assay solution containing 20 mmol l^{-1} H_2O_2 and 20 mmol l^{-1} KH_2PO_4 (pH 7.0). Each activity was assayed in both tissue and mitochondria homogenates, but protein carbonyls were only assayed in tissue homogenates.

Calculations and statistics

Data are reported as means \pm s.e.m. One- or two-factor ANOVA followed by Bonferroni multiple comparisons *post hoc* tests were used to compare hypoxic treatments with normoxic controls (performed in Prism, GraphPad Software, La Jolla, CA, USA). The statistical results shown throughout are for ANOVA analyses unless otherwise stated. A significance level of $P<0.05$ was used throughout.

RESULTS

Fish were acclimated to normoxia (P_{O_2} in the water $\sim 20 \text{ kPa}$), constant (sustained) hypoxia (5 kPa) or nocturnal hypoxia ('intermittent hypoxia': 12 h at 5 kPa in dark phase, 12 h at $\sim 20 \text{ kPa}$ in light phase) for 28–33 days. There were no differences in body mass between treatment groups acclimated to normoxia ($3.10\pm 0.18 \text{ g}$; $n=58$), constant hypoxia ($3.35\pm 0.14 \text{ g}$; $n=65$) or intermittent hypoxia ($3.20\pm 0.15 \text{ g}$; $n=61$) ($F_{2,181}=0.621$, $P=0.539$).

We used substrate–uncoupler–inhibitor titration and fluorometry protocols to examine the effects of hypoxia acclimation on the function of mitochondria isolated from the liver. Mitochondrial respiration measurements were used to determine oxphos capacities (using substrates of multiple complexes of the electron transport system), phosphorylation efficiency and oxygen kinetics, concurrent with measurements of ROS emission (using Ampliflu Red) (Fig. 1). Electron transport capacities were also determined by measuring respiration in uncoupled mitochondria using substrates of multiple complexes.

Mitochondrial respiration

Respiration of liver mitochondria was largely unaltered by acclimating killifish to hypoxia (Fig. 2). Oxphos capacities were similar between treatment groups when measured with substrates of complex I (P_{PM} and P_{PMG}), both complexes I and II (P_{PMGS}), or complex IV (P_{Tm}) (Fig. 2A). Leak state respiration was similar between treatment groups when measured in the absence (L_N) or presence (L_T) of ATP (Fig. 2A). The capacities for electron transport, measured in uncoupled mitochondria using substrates of complex I (E_{PMG}), complex II ($E_{S(Rot)}$), complexes I and II (E_{PMGS}), or complex IV (E_{Tm}), were also unaffected by treatment (Fig. 2B). The phosphorylation system (e.g. ATP synthase, adenine nucleotide

translocase, inorganic phosphate transporter) appeared to have more control over oxphos respiration by complex I than by complex II, as reflected by a greater effect of uncoupling on respiration with complex I substrates ($E_{PMG}/P_{PMG} \approx 1.24$) than with complex I+II substrates ($E_{PMGS}/P_{PMGS} \approx 1.12$). Phosphorylation efficiency was unaffected by hypoxia acclimation, as reflected by statistically similar P/O ratios for mitochondria from fish acclimated to normoxia (2.49 ± 0.06), intermittent hypoxia (2.25 ± 0.08) and constant hypoxia (2.40 ± 0.15) ($F_{2,25} = 1.52$, $P = 0.24$).

The sensitivity of liver mitochondria to low P_{O_2} was also unaltered by acclimating killifish to hypoxia (Fig. 3). Oxygen kinetics of isolated mitochondria were assessed during both leak state (L_T) and oxphos state (P_{PMGS}) respiration by measuring the P_{50} . P_{50} was strongly influenced by respiration state, but there were no significant differences between treatment groups (Fig. 3A). Catalytic efficiency (J_{max}/P_{50}), a measure that better reflects hypoxia sensitivity because it indicates the magnitude of respiration that can be sustained at low P_{O_2} (Gnaiger et al., 1998), was similar between respiration states and treatment groups (Fig. 3B). We also evaluated the propensity for the ATP synthase to act as an ATP consumer during anoxia by measuring the transient increase in leak state respiration after an anoxia bout (which reflects the amount of ADP accumulated during anoxia by ATP consumption). The magnitude of the transient increase in leak respiration (shown as symbols with error bars in Fig. 3C) was not affected by hypoxia acclimation, nor was the duration over which respiration was elevated above stable leak state values after reoxygenation (normoxia, 203 ± 18 s; intermittent hypoxia, 231 ± 15 s; constant hypoxia, 225 ± 16 s; $F_{2,20} = 0.842$, $P = 0.447$). The first 10 min bout of anoxia (Fig. 1) had no effect on oxphos respiration (P_{PMGS}) in any group, and the second 10 min bout of anoxia had no effect on leak respiration (Fig. 3D).

Mitochondrial ROS emission

Acclimating killifish to hypoxia had a substantial effect on the rate of ROS emission from liver mitochondria (Fig. 4), in contrast to the minor effects of hypoxia acclimation on mitochondrial respiration. ROS emission was measured at ~ 2.5 kPa (to control for potential effects of P_{O_2} on this variable) in oxphos state (P_{PMGS} ; both before and after a 10 min anoxia exposure) and in leak state (L_T). Acclimation to constant hypoxia appeared to have the greatest impact on reducing ROS emission, as rates were significantly lower in this treatment group than in normoxic controls in all three respiration states (Fig. 4). The effects of acclimation to intermittent hypoxia were qualitatively similar to those of acclimation to constant hypoxia, but the effects of the former were only significant in the oxphos state after exposure to anoxia (Fig. 4).

Oxidative stress and antioxidant enzymes

Acclimating killifish to hypoxia increased oxidative stress and the activity of antioxidant enzymes in liver tissue (Fig. 5). The abundance of protein carbonyls increased after acclimation to both intermittent and constant hypoxia, suggesting that hypoxia increased oxidative damage to proteins (Fig. 5A). This was associated with increases in the activity of SOD – which catalyzes the dismutation of O_2^- into H_2O_2 – in liver tissue with acclimation to both patterns of hypoxia exposure (Fig. 5B). Acclimation to constant hypoxia also increased catalase activity – which catalyzes H_2O_2 decomposition to water – in liver tissue (Fig. 5B). In contrast, there were no effects of hypoxia acclimation on antioxidant activity in isolated liver mitochondria, suggesting that the observed changes in liver tissue occurred outside the mitochondria.

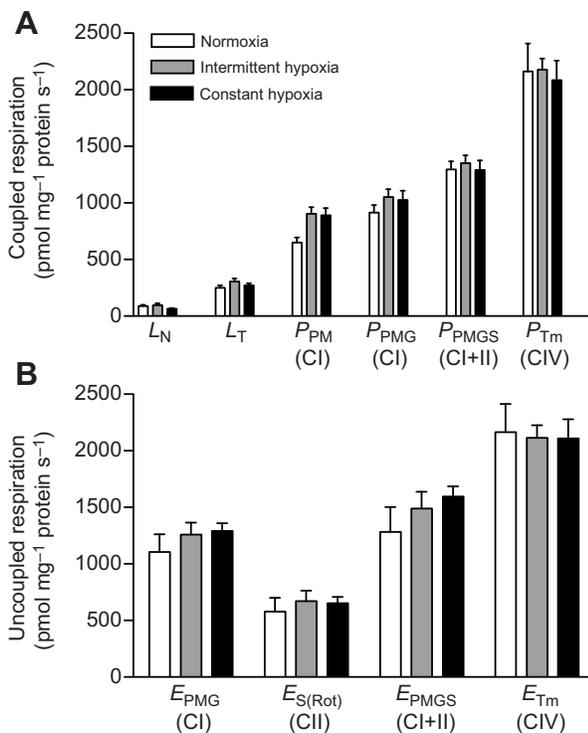


Fig. 2. Mitochondrial respiration was unaltered by hypoxia acclimation. (A) Coupled respiration was measured in the leak state (L) and during oxidative phosphorylation with maximal ADP (oxphos, P). Leak respiration before the first anoxia bout was measured with pyruvate and malate, both without adenylates (L_N) and with ATP (L_T). Oxidative phosphorylation was measured with substrates of complex I (CI) [P_{PM} with pyruvate (P) and malate (M); P_{PMG} with P, M and glutamate (G)], both complexes I and II (CI+II) [P_{PMGS} with P, M, G and succinate (S)], or complex IV (P_{Tm} with ascorbate and TMPD). (B) The capacity for electron transport (E) was measured in mitochondria uncoupled with CCCP using substrates of CI (E_{PMG}), complex II (CII) [$E_{S(Rot)}$ with S and the CI inhibitor rotenone], CI+II (E_{PMGS}) or CIV (E_{Tm}). There were significant main effects of substrate on coupled ($F_{5,138} = 220.1$, $P < 0.001$) and uncoupled ($F_{3,86} = 56.22$, $P < 0.001$) respiration, but no effect of hypoxia acclimation (coupled: $F_{2,138} = 1.585$, $P = 0.209$; uncoupled: $F_{2,86} = 0.868$, $P = 0.424$).

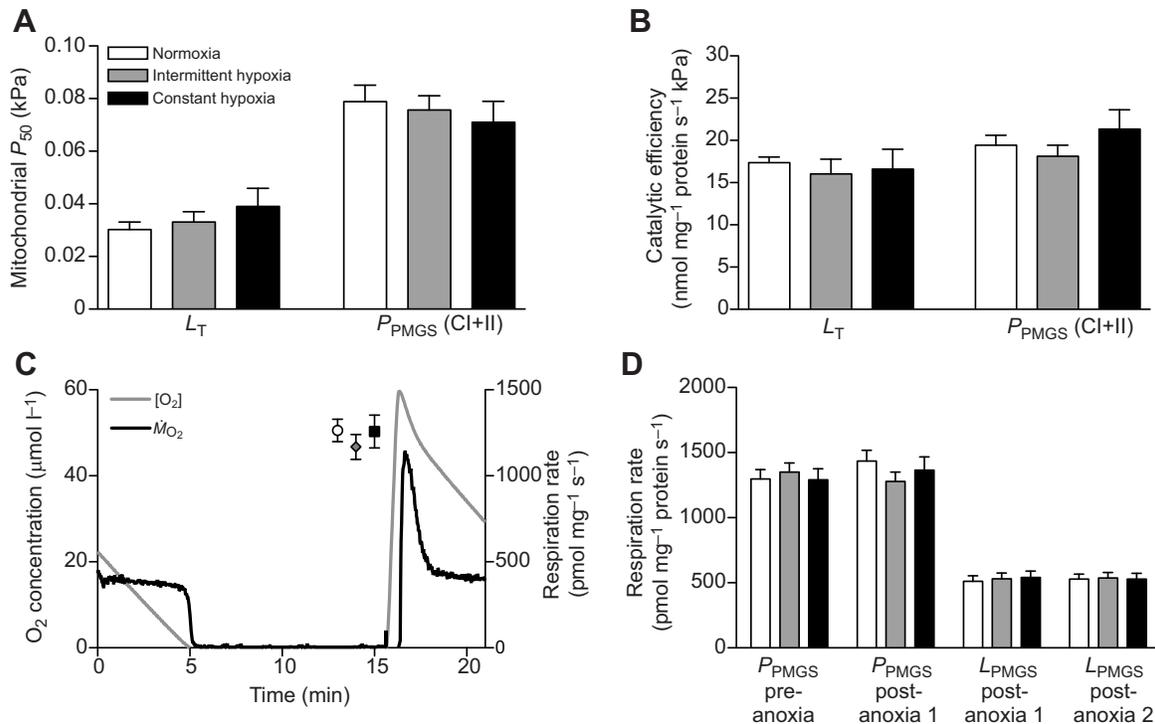


Fig. 3. Oxygen dependence of liver mitochondria was unaltered by hypoxia acclimation. (A) Mitochondrial P_{50} , the P_{O_2} at which the respiration rate is half of normoxic values, was higher in the maximal ADP-stimulated oxphos state (P_{PMGS} , oxphos state respiration with substrates for complex I and II, 'CI+II': pyruvate, malate, glutamate and succinate) than in the leak state (L_T , leak state respiration in the presence of ATP) ($F_{1,51}=50.58$, $P<0.001$), but there was no significant effect of hypoxia acclimation ($F_{2,51}=0.0046$, $P=0.995$). (B) Catalytic efficiency for O_2 (the quotient of maximum O_2 flux, J_{max} , and P_{50}) is not significantly affected by respiration state ($F_{1,51}=3.57$, $P=0.065$) or by hypoxia acclimation ($F_{2,51}=0.505$, $P=0.606$). (C) The transient increase in respiration rate (\dot{M}_{O_2}) in the leak state after anoxia, which is caused by an accumulation of ADP during anoxia from ATP hydrolysis by the ATP synthase, was similar in magnitude ($F_{2,23}=0.544$, $P=0.588$) between acclimation groups. The peak respiration rates after anoxia (plotted on right y-axis as symbols with error bars) are shown overlaid upon a representative trace of O_2 concentration [O_2] and \dot{M}_{O_2} over a cycle of entrance into and exit from anoxia. (D) There was no effect of the first 10 min bout of anoxia on oxphos state respiration with substrates of complexes I and II (P_{PMGS}) in any acclimation group (anoxia effect, $F_{1,46}=0.505$, $P=0.481$; acclimation effect, $F_{2,46}=0.219$, $P=0.804$) and no effect of the second 10 min bout of anoxia on leak state respiration with the same substrates (L_{PMGS}) (anoxia effect, $F_{1,44}=0.011$, $P=0.917$; acclimation effect, $F_{2,44}=0.076$, $P=0.927$).

DISCUSSION

Fish are among the most hypoxia-tolerant vertebrate species, yet very little is known about the plasticity of mitochondrial function in fish in response to hypoxia acclimation. Our current findings in the hypoxia-tolerant killifish show that hypoxia acclimation – either constant hypoxia or diel cycles of intermittent hypoxia – has relatively little effect on mitochondrial respiratory capacities or O_2 kinetics, but that mitochondria from this species are more resilient to bouts of anoxia–reoxygenation than mitochondria from hypoxia-intolerant species. However, hypoxia acclimation causes a pronounced decrease in the rate of ROS emission from liver mitochondria, and increases the activities of antioxidant enzymes in liver tissue. Our results therefore suggest that hypoxia acclimation may alter mitochondrial physiology to help reduce oxidative stress during hypoxia and/or subsequent reoxygenation.

Hypoxia had no effects on respiratory capacities or O_2 kinetics

Respiratory capacities for oxidative phosphorylation and electron transport, as well as leak respiration, were unaltered by hypoxia acclimation in killifish mitochondria (Fig. 2). We previously observed that acclimation to intermittent hypoxia increases the activities of citrate synthase or cytochrome *c* oxidase in the liver, a response that does not occur by a significant magnitude in constant hypoxia (Borowiec et al., 2015). Combined with this previous

result, our findings here (Fig. 2) suggest that acclimation to intermittent hypoxia may increase mitochondrial abundance in the liver. By contrast, acclimation to high-altitude hypoxia often reduces mitochondrial abundance and the mitochondrial capacity for oxidative phosphorylation in skeletal muscle of humans (Horscroft and Murray, 2014). This reduction has been suggested to be either an adaptive trait that helps balance O_2 supply and demand, or a maladaptive loss of normal function that could result from increases in ROS production and oxidative stress (Horscroft and Murray, 2014). Prolonged (2 weeks) exposure of the common frog (*Rana temporaria*) or the pond slider turtle (*Trachemys scripta*) to anoxia (i.e. complete absence of O_2) also decreases oxphos respiration in cardiac fibres and/or isolated mitochondria (St-Pierre et al., 2000a,b; Galli et al., 2013). However, in these species, the decreases are largely mediated by adaptive reductions in complex V activity to help prevent complex V from operating in reverse, which occurs in humans and other anoxia-intolerant animals in anoxia (but not hypoxia) to maintain mitochondrial membrane potential and can lead to a catastrophic consumption of ATP by the mitochondria (Galli and Richards, 2014). Therefore, it is likely that reductions in mitochondrial respiratory capacity are an adaptive response to oxygen limitation in at least some circumstances. Although killifish may be somewhat resistant to a loss of oxidative capacity during hypoxia acclimation, it is possible that they would depress mitochondrial respiratory capacity if the level of hypoxia were more severe. Killifish can tolerate and continue feeding in deeper

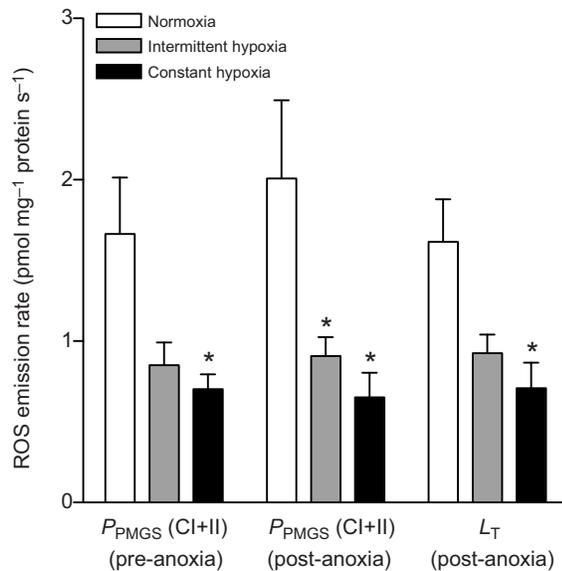


Fig. 4. Reactive oxygen species (ROS) emission from liver mitochondria was reduced by hypoxia acclimation. ROS emission rates were measured during oxphos state respiration using substrates of complex I and II (CI+II) (pyruvate, malate, glutamate and succinate) (P_{PMGS}), both before and after a 10 min period of anoxia, and during leak state respiration (L_T) with the same substrates in the presence of ATP. ROS emission from mitochondria was coupled to H_2O_2 production using exogenous superoxide dismutase and was measured fluorometrically using Ampliflu Red. ROS emission rates are dependent on O_2 tension (P_{O_2}), so the ROS emission rates shown were all measured at $P_{O_2} \sim 2.5$ kPa. ROS emission rates were significantly affected by acclimation treatment ($F_{2,47}=19.09$, $P<0.001$) but not significantly affected by respiration state ($F_{2,47}=0.241$, $P=0.787$). Asterisks indicate a significant pairwise difference from normoxia controls (* $P<0.05$).

levels (2 kPa) of constant or intermittent hypoxia for at least 7 days (Borowiec et al., 2015).

The O_2 dependence of isolated mitochondria was unaltered by hypoxia acclimation in killifish (Fig. 3). Mitochondria can routinely become oxygen limited, even in normoxia, so the O_2 kinetics of mitochondrial respiration has an important bearing on mitochondrial function (Gnaiger et al., 1998; Gnaiger, 2001; Cano et al., 2013). Correspondingly, mitochondrial O_2 affinity has been shown to vary between individuals in association with metabolic rate (Larsen et al., 2011). However, our work in killifish and that of others in rats (Costa et al., 1997) suggest that hypoxia acclimation may not generally alter mitochondrial P_{50} to help minimize cellular O_2 limitation. Mitochondrial O_2 affinity has been shown to increase in response to hypoxic hypometabolism in overwintering frogs, but the reduction in P_{50} was in proportion to a reduction in oxphos and leak respiration (St-Pierre et al., 2000c), suggesting that catalytic efficiency for O_2 was unaltered. P_{50} often varies with respiration rate, as we observed between leak and oxphos respiration states (Fig. 3A), at least in part because the catalytic turnover rate of cytochrome *c* oxidase is greater at higher respiration rates (Gnaiger et al., 1998). There is also evidence in birds that variation in hypoxia tolerance between species is not coupled with variation in mitochondrial O_2 kinetics: mitochondrial P_{50} in the bar-headed goose – a species that must sustain the high O_2 demands of flight during hypoxia during migration across the Himalayas – is similar to that in low-altitude birds (Scott et al., 2009). As mitochondrial P_{50} is quite low across taxa in general, likely to minimize potential O_2 limitation and impairment of mitochondrial ATP synthesis (Cano

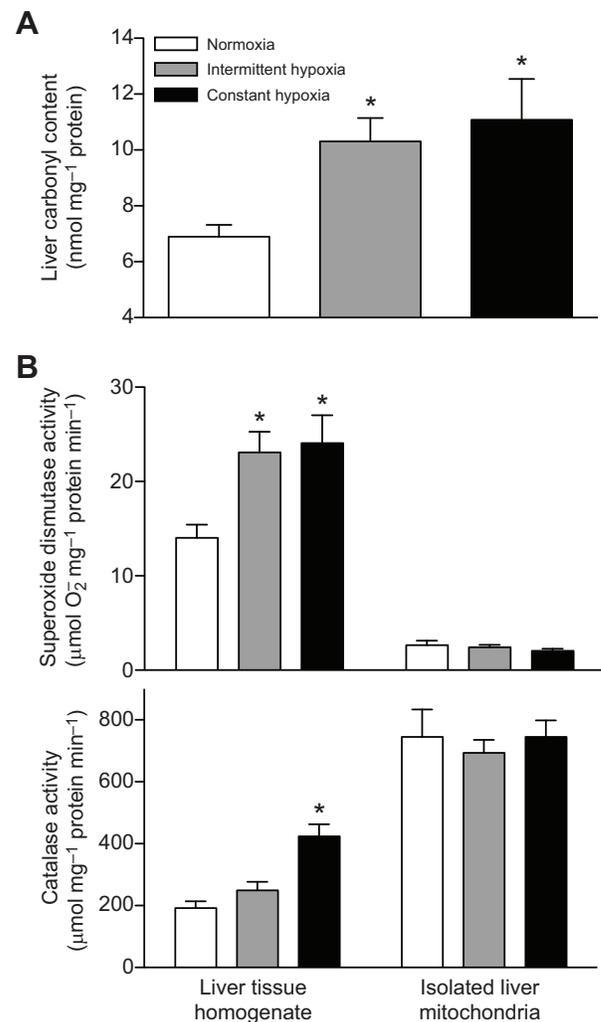


Fig. 5. Hypoxia acclimation increased oxidative stress and the activity of antioxidant enzymes in liver tissue. (A) The abundance of protein carbonyls in liver tissue increased after hypoxia acclimation ($F_{2,23}=6.08$, $P=0.008$). (B) The activity of superoxide dismutase, which catalyzes the dismutation of O_2^- into H_2O_2 , was significantly affected by hypoxia acclimation ($F_{2,45}=4.24$, $P=0.021$) and sample type (tissue or isolated mitochondria; $F_{1,45}=159.4$, $P<0.001$). However, there was a significant interaction between these main effects ($F_{2,45}=5.10$, $P=0.010$), and there were significant pairwise differences between hypoxia treatment groups and normoxia controls in the whole-liver tissue only (* $P<0.05$). There were also significant effects of hypoxia acclimation ($F_{2,45}=4.63$, $P=0.015$), sample type ($F_{1,45}=151.0$, $P<0.001$) and their interaction ($F_{2,45}=3.31$, $P=0.045$) on the apparent activity of catalase, which catalyzes the decomposition of H_2O_2 to water, and there was a significant pairwise difference between constant hypoxia and normoxia treatment groups.

et al., 2013), there may be constraints on the scope for reducing P_{50} to help cope with environmental hypoxia.

Exposing killifish mitochondria to 10 min of anoxia *in vitro* led to ATP consumption by complex V during anoxia (Fig. 3C), but it had no effect on steady-state mitochondrial respiration (Fig. 3D). The latter contrasts many studies of mammalian mitochondria from various tissues, in which mitochondrial dysfunction (as reflected by increases in leak respiration and/or reductions in oxphos respiration and P/O ratio) has been often observed after exposure to 1–20 min or more of anoxia followed by reoxygenation (Du et al., 1998; Ascensão et al., 2006; Galli and Richards, 2014). In this respect, killifish mitochondria are more similar to those from the heart of

pond turtles, which can endure 20 min of anoxia–reoxygenation without any impact on respiration or P/O ratio (Galli et al., 2013). Hypoxia acclimation in killifish had no effect on mitochondrial anoxia sensitivity, and it did not alter the magnitude of ATP consumption by complex V during anoxia.

Hypoxia acclimation reduced mitochondrial ROS emission

Hypoxia acclimation of killifish reduced the rate of ROS release from liver mitochondria, akin to the effects of estivation on skeletal muscle mitochondria in burrowing frogs (Reilly et al., 2014), and we observed qualitatively similar effects of acclimation to constant hypoxia and intermittent hypoxia (Fig. 4). It is possible that acclimation treatments could have differed if fish had been exposed to more severe levels of hypoxia, in which intermittent patterns of hypoxia might intensify oxidative stress during reoxygenation events. Furthermore, mitochondria from other tissues, such as the heart, could foreseeably respond to hypoxia differently than liver mitochondria. Hypoxia and/or reoxygenation likely pose a greater challenge to mitochondria in the heart, as the spongy myocardium receives its O₂ supply from venous blood and may therefore experience greater O₂ deprivation during hypoxic events (Steffensen and Farrell, 1998).

It is possible that the decrease in ROS emission resulted from a decrease in the amount of superoxide produced by mitochondria as a by-product of electron transport. Mitochondrial superoxide production is dictated by P_{O₂}, the abundance of enzymes and proteins containing electron carrying sites, the proportion of these sites that are in a redox form that can react with O₂, and the rate constant for the reaction of the electron carriers with O₂ to form superoxide (Murphy, 2009). The decrease we observed was not caused by biased differences in P_{O₂} in the respirometer, because the measurements of ROS emission we report here were made at a physiologically realistic P_{O₂} (~2.5 kPa) that was constant across groups. The sites of mitochondrial superoxide production under native conditions (without electron transport inhibitors) are not well understood, and although complexes I and III have most often been invoked as the main sites of production, several other sites (e.g. β-oxidation, complex II, several dehydrogenase enzymes, and others) are likely important as well (Murphy, 2009; Brand, 2010; Quinlan et al., 2013, 2014). Nevertheless, superoxide production is increased by a high proton-motive force, a reduced coenzyme Q pool, and a high NADH/NAD⁺ ratio in the matrix (Murphy, 2009). Mitochondrial ROS emission may have been reduced in killifish by adjusting these traits during the course of hypoxia acclimation.

Decreases in ROS emission have been previously suggested to result from reductions in mitochondrial membrane potential. Mild increases in proton leak conductance are believed to avoid conditions that favour high rates of ROS generation by allowing mitochondria to operate at a lower membrane potential (Cunha et al., 2011). For example, ROS emission rates from liver mitochondria of big brown bats (*Eptesicus fuscus*) are 70% lower than the rates from liver mitochondria of domestic mice, concurrent with much higher rates of proton conductance (Brown et al., 2009). However, decreases in mitochondrial membrane potential are expected to be associated with increases in leak respiration and/or decreases in oxfos respiration, responses that we did not observe after hypoxia acclimation in killifish mitochondria (Fig. 2).

It is also possible that the decrease in mitochondrial ROS emission with hypoxia acclimation was caused by changes in ROS elimination within the mitochondria. We measured mitochondrial ROS emission in the presence of exogenous SOD, such that mitochondrial superoxide is converted to H₂O₂ and can thus be detected. Not all of the ROS produced by mitochondria will survive

to leave the mitochondria (and then be measured as H₂O₂) because some is detoxified to water in the matrix (Murphy, 2009; Treberg et al., 2010). If hypoxia acclimation did indeed reduce mitochondrial ROS emission by enhancing ROS degradation, it was not associated with any change in the maximal activities of SOD or catalase in isolated mitochondria (Fig. 5). Hypoxia acclimation could have instead increased the effectiveness of H₂O₂ degradation systems that were not measured here, such as those involving glutathione peroxidase or peroxiredoxin.

The signalling mechanisms that lead to the change in ROS emission with hypoxia acclimation are not known, but could involve hypoxia-inducible factor (HIF) signalling pathways. HIF-α proteins can be stabilized in response to both cellular hypoxia and ROS, and can ultimately lead to a variety of adaptive and maladaptive cellular responses to O₂ limitation (Prabhakar and Semenza, 2012). The HIF-1α and HIF-2α sequences of fish exhibit greater amino-acid divergence at functionally important sites than mammals (Rytkönen et al., 2011), but HIF-1α protein abundance increases as expected in zebrafish exposed to acute hypoxia (Kopp et al., 2011; Egg et al., 2013). Interestingly, genetic knockout of prolyl hydroxylase 1 (PHD1) – the oxygen-sensitive enzyme that hydroxylates HIF-α and targets it for degradation – stabilizes HIF-2α protein, reduces mitochondrial respiration and ROS production, and improves ischemia tolerance in skeletal muscle in mice (Aragonés et al., 2008). If homologous signaling mechanisms are at play in killifish liver, hypoxia acclimation may reduce mitochondrial ROS emission and increase cellular hypoxia tolerance by reducing the expression and/or activity of prolyl hydroxylase and thus activating the HIF-2α signalling pathway.

Hypoxia acclimation increased oxidative stress and antioxidant capacity

Hypoxia acclimation was associated with oxidative stress in the liver in killifish, as reflected by an increase in the abundance of protein carbonyls (Fig. 5A), consistent with previous observations in other fish species (Lushchak and Bagnyukova, 2007). This suggests that the rate of ROS production exceeded the capacity for ROS detoxification, at least transiently, during exposure to either constant hypoxia or intermittent hypoxia. It is unclear whether mitochondria were the sole source of this ROS, but the acclimation-induced decline in mitochondrial ROS emission likely helped curtail the magnitude of oxidative stress. The increased non-mitochondrial activity of SOD and catalase in liver tissue (Fig. 5B) may have also helped minimize the incidence of oxidative stress with prolonged hypoxia exposure. However, only SOD activity increased in both constant hypoxia and intermittent hypoxia, and the activity of this enzyme often increases in response to hypoxia exposure in fish (Lushchak et al., 2005; Lushchak and Bagnyukova, 2007), suggesting that SOD may be an important general defense against superoxide during all patterns of hypoxia exposure. However, unless the H₂O₂ produced by SOD is decomposed, H₂O₂ can still enter the Fenton reaction, produce hydroxyl radicals and lead to oxidative damage. This could be accomplished by catalase, but it has been shown that catalase may play only a minor role in antioxidant defense during hypoxia–reoxygenation in some fish species, as pharmacological inhibition of catalase did not alter the occurrence of oxidative stress in the liver of Nile tilapia exposed to a cycle of severe hypoxia and reoxygenation (Welker et al., 2012).

The lack of variation in SOD and catalase activities in mitochondria with hypoxia acclimation (Fig. 5B) suggests that the abundance of these enzymes is only changing in other sites in the cell. For SOD, this could be explained by differences in how hypoxia regulates the

cytosolic (Cu/Zn-SOD, encoded by the SOD1 gene) and mitochondrial (Mn-SOD, encoded by the SOD2 gene) forms of the enzyme (Yu, 1994). There is a hypoxia response element in the promoter of SOD1 that activates SOD1 expression when HIF is bound (Tuller et al., 2009). There is also a hypoxia response element in the promoter of SOD2, but for this gene HIF acts as a transcriptional repressor (Gao et al., 2013). An increase in HIF signalling could have therefore increased the expression and activity of cytosolic but not mitochondrial SOD in killifish with hypoxia acclimation. Catalase is predominantly a peroxisomal enzyme (Yu, 1994), so although it may be found in mitochondria (Salvi et al., 2007) its activity there does not appear to be regulated by hypoxia.

Hypoxia tolerance and mitochondria

The decrease in ROS emission from mitochondria may be part of the general suite of plastic adjustments that help fish cope with hypoxia. We have previously shown using an equivalent pattern of exposure that hypoxia acclimation improves hypoxia tolerance, as reflected by a reduction in the O₂ tension at which killifish lose equilibrium (Borowiec et al., 2015). It is not yet clear whether these observations are related mechanistically, but a low rate of mitochondrial ROS emission has previously been shown to distinguish hypoxia-tolerant from hypoxia-intolerant species of elasmobranchs (Hickey et al., 2012). It is possible that mechanisms to help avoid oxidative stress are an important feature of hypoxia tolerance, as supported by the high antioxidant capacity of some hypoxia-tolerant fish species (Hermes-Lima and Zenteno-Savin, 2002). It is also possible that variation in mitochondrial ROS production has an important role in hypoxia signalling, as ROS is known to be involved in HIF signalling and in many other signalling pathways (Prabhakar and Semenza, 2012; Holmström and Finkel, 2014). Tolerance of prolonged hypoxia is well known to require that animals continue matching O₂ supply and demand during O₂ deprivation (Boutilier, 2001; Bickler and Buck, 2007; Richards, 2009), but our results suggest that hypoxia tolerance may also be associated with changes in mitochondrial physiology that regulate ROS and oxidative stress.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

G.R.S. designed and supervised the experiments and drafted the manuscript. All authors contributed to experimentation, data collection, analysis and data interpretation, led by S.N.N.D. All authors contributed to the revision of the manuscript, and approve of the final version of the paper.

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