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RESEARCH ARTICLE

Hypoxia acclimation alters reactive oxygen species homeostasis and oxidative status in estuarine killifish (*Fundulus heteroclitus*)

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ABSTRACT

Hypoxia is common in aquatic environments, and exposure to hypoxia followed by re-oxygenation is often believed to induce oxidative stress. However, there have been relatively few studies of reactive oxygen species (ROS) homeostasis and oxidative status in fish that experience natural hypoxia–re-oxygenation cycles. We examined how exposure to acute hypoxia (2 kPa O_2) and subsequent re-oxygenation (to 20 kPa O_2) affects redox status, oxidative damage and anti-oxidant defenses in estuarine killifish (*Fundulus heteroclitus*), and whether these effects were ameliorated or potentiated by prolonged (28 days) acclimation to either constant hypoxia or intermittent cycles of nocturnal hypoxia (12 h:12 h normoxia:hypoxia). Acute hypoxia and re-oxygenation led to some modest and transient changes in redox status, increases in oxidized glutathione, depletion of scavenging capacity and oxidative damage to lipids in skeletal muscle. The liver had greater scavenging capacity, total glutathione concentrations and activities of anti-oxidant enzymes (catalase, glutathione peroxidase) than muscle, and generally experienced less variation in glutathiones and lipid peroxidation. Unexpectedly, acclimation to constant hypoxia or intermittent hypoxia led to a more oxidizing redox status (muscle and liver) and it increased oxidized glutathione (muscle). However, hypoxia-acclimated fish exhibited little to no oxidative damage (as reflected by lipid peroxidation and aconitase activity), in association with improvements in scavenging capacity and catalase activity in muscle. We conclude that hypoxia acclimation leads to adjustments in ROS homeostasis and oxidative status that do not reflect oxidative stress, but may instead be part of the suite of responses that killifish use to cope with chronic hypoxia.

KEY WORDS: Anti-oxidants, Diel cycles, Oxidative stress, Redox, Re-oxygenation, ROS

INTRODUCTION

Hypoxia and hypoxia–re-oxygenation cycles are common in the aquatic environment (Breitburg et al., 2018; Diaz, 2001; Diaz and Breitburg, 2009). In order to effectively cope with such variations in environmental oxygen availability, aerobic organisms must overcome the challenges of energy shortfall (Boutilier, 2001; Richards, 2009) and the potential for oxidative stress (Hermes-Lima and Zenteno-Savín, 2002; Lushchak, 2011; Lushchak and Bagnyukova, 2006). Hypoxia-tolerant fish exhibit a range of well-described mechanisms for avoiding energy shortfall by maintaining the balance between cellular ATP supply and ATP demand (Bickler and Buck, 2007; Richards, 2009), but the challenge that hypoxia poses to oxidative stress in fish is not as well understood despite intensive research effort.

Acute hypoxia and re-oxygenation could induce oxidative stress by upsetting the balance between reactive oxygen species (ROS) production and ROS scavenging by anti-oxidants, thereby producing excess ROS that damages critical cellular components and/or disrupts redox signalling (Costantini, 2019; Hermes-Lima and Zenteno-Savín, 2002; Jones, 2006). For example, ROS generation is well known to increase in response to hypoxia–re-oxygenation in cells in culture (Chandel et al., 2000; Giraud-Billoud et al., 2019; Guzy et al., 2005; Korde et al., 2011), and in response to ischemia–reperfusion in mammalian biomedical models (Bhogal et al., 2010; Chouchani et al., 2014; Kalogeris et al., 2014; Sanderson et al., 2013; Zhang et al., 2007). In fish, exposure to acute hypoxia has been observed to cause oxidative damage (Lushchak and Bagnyukova, 2007) and to increase anti-oxidant capacity (Birnie-Gauvin et al., 2017; Lushchak et al., 2005, 2001). Fish have also been shown to experience oxidative stress during re-oxygenation periods that follow exposure to acute hypoxia, and some species also increase the activity of anti-oxidant enzymes (Hermes-Lima and Zenteno-Savín, 2002; Lushchak et al., 2001). However, there are also many situations in which acute hypoxia and/or re-oxygenation have not been observed to cause oxidative stress, potentially because of interspecific or inter-tissue variation in how redox status is managed over time (Lau et al., 2019), or because the biological meaning of changes in specific markers has been unclear (Costantini, 2019; Halliwell and Whiteman, 2004; Jones, 2006). Understanding these discrepancies are key to understanding how the management of oxidative stress may influence life history traits (Costantini, 2019; Monaghan et al., 2009; Smith et al., 2016; Speakman et al., 2015).

The effects of prolonged periods of sustained hypoxia or of repeated hypoxia–re-oxygenation cycles on oxidative stress in fish are poorly understood. In mammalian biomedical models (e.g. obstructive sleep apnea), repeated cycles of hypoxia–re-oxygenation increase ROS production, and thereby lead to maladaptive changes in respiratory and cardiovascular system function, metabolism and redox-sensitive gene expression (Lavie, 2003; Neubauer, 2001; Prabhakar et al., 2007; Semenza and Prabhakar, 2007). Fish adapted to environments that undergo natural periods of hypoxia or hypoxia–re-oxygenation might be expected to mitigate these effects by better managing ROS levels and protecting tissues against oxidative stress. Indeed, chronic exposure to either constant or intermittent patterns of hypoxia increase anti-oxidant enzyme activities and reduce rates of mitochondrial ROS emission in some fish (Du et al., 2016; Lushchak, 2011; Lushchak and Bagnyukova, 2007). Some animals have also been shown to elevate anti-oxidant capacity during

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periods of metabolic depression, which is believed to be an anticipatory strategy to defend against future over-production of ROS during the surge in O_2 supply that occurs upon resumption of normal metabolic rates (Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015, 1998; Hermes-Lima and Storey, 1993; Moreira et al., 2016; Welker et al., 2013). However, there have been relatively few detailed examinations of the effects of chronic hypoxia and/or hypoxia–re-oxygenation cycles on ROS and redox homeostasis, scavenging capacity and anti-oxidant enzymes, and oxidative stress in hypoxia-tolerant fish.

The mummichog killifish *Fundulus heteroclitus* is a useful species in which to examine the effects of prolonged exposure to constant hypoxia or to cycles of hypoxia–re-oxygenation on ROS homeostasis and oxidative status. This species lives in estuaries on the east coast of North America, where there are dynamic changes in O_2 levels and other environmental variables (Burnett et al., 2007). *Fundulus heteroclitus* is amongst the most hypoxia tolerant of fundulid killifish and can tolerate prolonged periods of low O_2 (e.g. only ∼10% of normoxic air saturation), in association with substantial alterations in respiratory physiology and metabolism that appear to help maintain energy homeostasis and improve hypoxia tolerance (Borowiec et al., 2015, 2020, 2018). Chronic hypoxia also reduces mitochondrial ROS emission in the liver of mummichog killifish (Du et al., 2016), but we otherwise have a poor understanding of how ROS homeostasis and oxidative status in various tissues are affected by hypoxia and/or re-oxygenation. Our objectives were to investigate this issue by examining how mummichog killifish respond to (i) acute exposure to hypoxia followed by re-oxygenation in normoxia and (ii) chronic exposure to constant hypoxia or to repeated hypoxia–normoxia cycles, in tissues with low and high endogenous anti-oxidant capacity (skeletal muscle and liver, respectively).

MATERIALS AND METHODS

Study animals

Adult, wild-caught killifish, *Fundulus heteroclitus* (Linnaeus 1766), of mixed sex (∼2–5 g) were shipped from a commercial supplier (Aquatic Research Organisms, Hampton, NH, USA) to McMaster University, ON, Canada. Prior to acclimation treatments (see below), killifish were held in large (∼300 l) fibreglass tanks filled with brackish (4 ppt) water that was maintained at room temperature (∼22°C) and continuously aerated with an air stone. Fish were fed commercial flakes (Big Al's Aquarium Supercentres, Mississauga, ON, Canada) at least 5 days a week. The photoperiod was maintained at 12 h:12 h light:dark, with the daylight portion occurring from 07:00 h to 19:00 h local time. Water quality (ammonia, pH, nitrates and nitrites) was maintained by cycling water through a charcoal filter, and by routine water changes. Fish were maintained in these conditions for at least 4 weeks before acclimation treatments began. The acclimation treatments and hypoxia–re-oxygenation exposures that are described below were equivalent to and were conducted alongside of another experiment examining the effects of hypoxia acclimation on energy metabolism in killifish (Borowiec et al., 2018). All animal use protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

Acclimation treatments

Fish were subjected to 28-day acclimation to either normoxia, constant hypoxia (2 kPa O_2, ∼0.8 mg O_2 l^{-1}), or nocturnal ('intermittent') hypoxia (12 h normoxia during the light phase and 12 h hypoxia during the dark phase), all of which were carried out in 35-liter glass aquaria. We selected this standardized pattern of intermittent hypoxia because it is a reasonable approximation of the diel patterns of hypoxia that occur naturally in estuaries along the east coast of North America (Tyler et al., 2009). Normoxia (∼20 kPa O_2, ∼8 mg O_2 l^{-1}) was achieved by continuously bubbling aquarium water with air. Hypoxia (2 kPa O_2, 0.8 mg O_2 l^{-1}) exposure was achieved by regulating the O_2 tension in the aquaria using a feedback system controlled by a galvanic O_2 sensor (Loligo Systems, Tjele, Denmark), as described previously (Borowiec et al., 2015, 2018). Transitions between normoxia and hypoxia during acclimation to intermittent hypoxia were typically completed within 45 min. Because killifish sometimes respond to hypoxia with aquatic surface respiration (Stierhoff et al., 2003), we prevented fish from respiring at the water surface during hypoxic periods by placing a plastic grid barrier (made from plastic egg crate) just below the surface of the water and overlaying it with bubble wrap. Each acclimation treatment was replicated at least twice, and animals were split between two tanks for each acclimation treatment.

Hypoxia–re-oxygenation exposures

Fish were sampled during exposure to a hypoxia–re-oxygenation cycle, as described previously (Borowiec et al., 2018). Briefly, fish were transferred at ∼10:00 h local time in groups of three to four fish into custom-built, mesh-sided 2.1 l enclosures that were weighed down to sit at the bottom of a 35 l glass aquarium. Animals recovered from handling for ∼8 h, during which they were exposed to the P_{O_2} appropriate to their acclimation condition at that time of day.

Animals were sampled at the times indicated in Fig. 1 under normoxic or hypoxic conditions beginning with an initial ('0 h') sampling period between 18:30 h and 19:00 h local time (i.e. shortly before lights off at 19:00 h). Subsequent sampling points, relative to 19:00 h local time, were as follows: 1 h (20:00 h), 6 h (01:00 h), 12 h (07:00 h), 13 h (08:00 h) or 18 h (13:00 h). Fish acclimated to normoxia were either (i) sampled in normoxia for all six time points ('normoxia control' treatment group), or (ii) sampled in normoxia at 0 h, and then sampled after 1, 6 or 12 h of acute hypoxia (2 kPa O_2) during the dark phase of the photoperiod, and then after 1 h (13 h) and 6 h (18 h) of re-oxygenation in normoxia during the light phase of the photoperiod ('acute hypoxia–re-oxygenation' treatment group). Fish acclimated to intermittent hypoxia were sampled following their typical acclimation P_{O_2} pattern (i.e. in normoxia at 0 h, after 1, 6 or 12 h of hypoxia, or after 1 or 6 h of re-oxygenation in normoxia). For fish acclimated to constant hypoxia, the first four sampling points were in hypoxia at the same times of day as the other groups (corresponding to 0, 1, 6 or 12 h time points). Fish in this acclimation group were also subjected to re-oxygenation in normoxia (starting at 07:00 h) and were sampled after 1 h and 6 h (corresponding to 13 h and 18 h time points, respectively).

At each sampling time point, two mesh-covered enclosures were gently removed from the aquarium, and excess water was drained out of the mesh sides. Fish in the remaining ∼1 liter reservoir of water were quickly euthanized by adding a concentrated solution of benzocaine (Sigma-Aldrich, Oakville, ON, Canada; dissolved in 95% ethanol to a final concentration 1 g l^{-1}). Fish were removed and weighed. Two transverse sections of the axial muscle were cut from the anterior base of the anal fin. The anterior section was immediately freeze-clamped between two aluminium blocks pre-cooled in liquid nitrogen, and was then stored in liquid N_2. Portions of muscle were punched out of the posterior section using a tissue biopsy punch, and were then transferred unfrozen to ice-cold Tris buffer (100 mmol l^{-1} Tris-HCl, 2 mmol l^{-1} EDTA, 5 mmol l^{-1}

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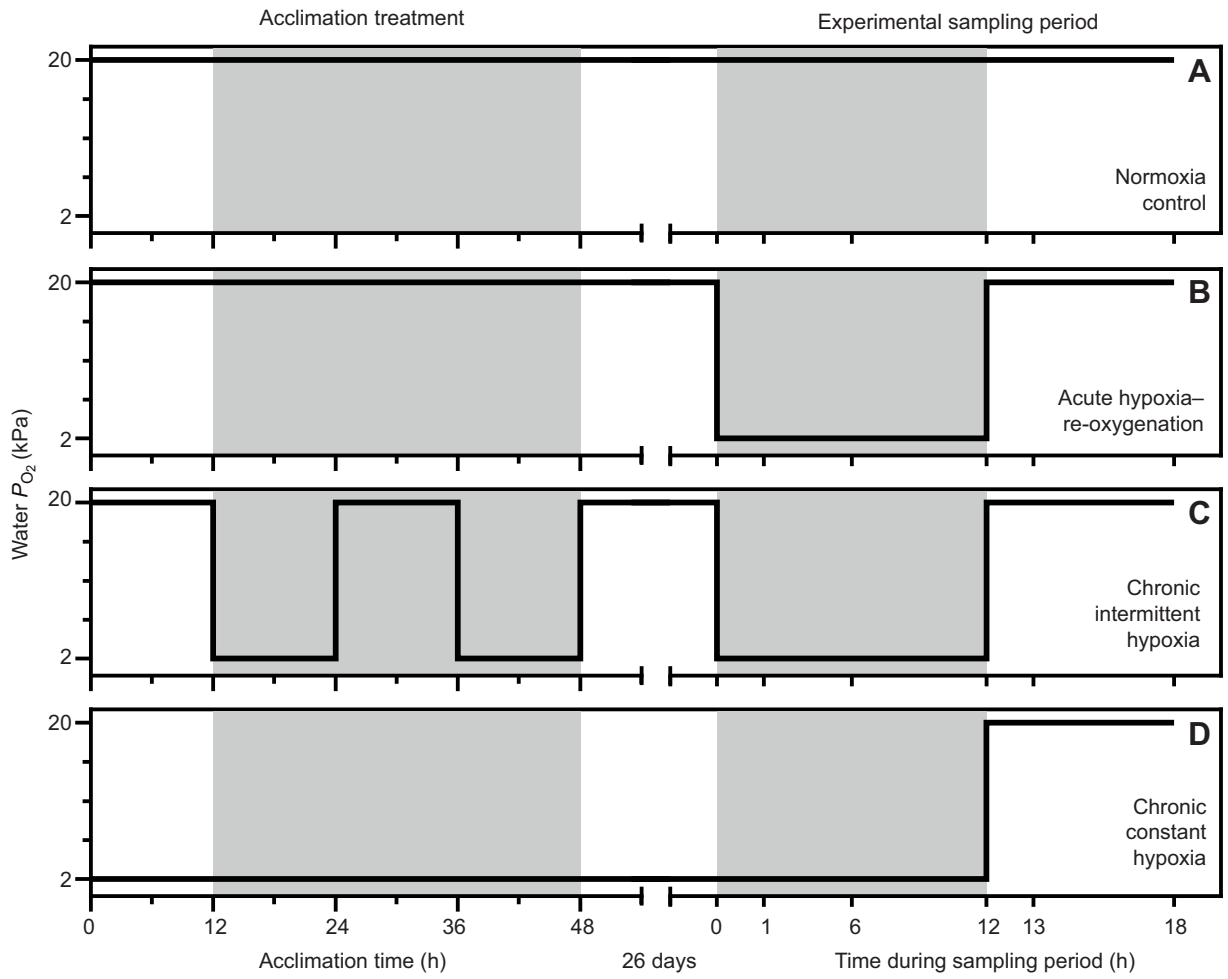

Fig. 1. Experimental groups used in this study. Killifish were first acclimated for 28 days to normoxia (~20 kPa), diel cycles of nocturnal hypoxia ('intermittent hypoxia', 12 h normoxia during the daytime light phase (white; from 07:00 to 19:00 h local time) and 12 h of hypoxia at 2 kPa O_2 during the night-time dark phase (grey), or constant hypoxia at 2 kPa O_2. These acclimation groups are shown to the left of the break in the *x*-axis. Following these 28 day acclimation periods, fish were divided into one of four treatment groups. (A) One batch of normoxia-acclimated fish were held in constant normoxia throughout to act as time-matched normoxia controls ('normoxia control group'). (B) A second batch of normoxia-acclimated fish were exposed to hypoxia (2 kPa O_2) for 12 h and then subsequent re-oxygenation in normoxia ('acute hypoxia–re-oxygenation group'). (C) Fish acclimated to intermittent hypoxia were exposed to a hypoxia–re-oxygenation cycle identical to their acclimation treatment ('chronic intermittent hypoxia group'). (D) Fish acclimated to constant hypoxia continued to be held in hypoxia until they were exposed to re-oxygenation in normoxia ('chronic constant hypoxia group'). Sampling times for fish in each treatment group are indicated right of the break in the *x*-axis (fish at the 0 and 12 h time points were sampled just before the change in partial pressure of O_2 (P_{O_2}) and light/dark phase).

$MgCl_2$; pH 7.75) and placed on ice for assays of oxidation potential (see below). Most of the liver was also freeze-clamped, except for a small piece (~20–50 mg) that was placed in ice-cold Tris buffer for oxidation potential assays. All freeze-clamped samples were stored at −80°C, and were ground into a fine powder under liquid nitrogen before use in glutathione, lipid peroxidation and enzyme activity assays (see below). Sampling was completed for all fish within a few minutes of removing the enclosure from the aquarium, such that fish sampled from hypoxic water were not exposed to normoxia prior to sampling.

Oxidation potential and total oxidant scavenging capacity (TOSC) assays

Muscle and liver portions that had been placed in ice-cold Tris buffer during sampling were processed immediately for measurement of oxidation potential using a fluorometric assay (Amado et al., 2009; Chen et al., 2010; Gomes et al., 2005). This assay is suggested to detect hydrogen peroxide and various one-electron-oxidizing species (e.g. hydroxyl radical, nitrogen dioxide, etc.) (Kalyanaraman et al., 2012). However, because ROS species are extremely short-lived *in vivo*, this assay cannot accurately measure the levels of these oxidizing species that were generated prior to sampling. Rather, the assay measures the production of oxidizing species during the assay, which we call the oxidation potential of the tissue, so this assay is best used as a general indicator of redox status (Jiang et al., 2018; Kalyanaraman et al., 2012). Tissues were homogenized in a glass Tenbroeck tissue grinder containing 10 volumes of ice-cold Tris buffer, and were then centrifuged at 10,000 ***g*** for 20 min at 4°C. The supernatant was retained, its protein content was measured using a standard Bradford assay (according to instructions from the manufacturer; Bio-Rad, Mississauga, ON, Canada), and it was then diluted to 1 mg protein ml^{-1} with Tris buffer. This solution (10 µl) was added to 135 µl of dichlorofluorescein (DCF) assay buffer (200 mmol l^{-1} KCl and 1 mmol l^{-1} $MgCl_2$ in 30 mmol l^{-1} Hepes; pH 7.2) containing 16 µmol l^{-1} of 2′,7′-dichlorodihydrofluorescein diacetate (Sigma-Aldrich) (from a stock solution in 95% ethanol). Measurements were made in triplicate by tracking the accumulated

fluorescence signal over 60 min at 37°C from the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate into the fluorescent product 2′,7′-dichlorofluorescein (excitation: 485 nm, emission: 528 nm). Background conversion was determined using the Tris buffer described above in place of tissue supernatant. Total fluorescence over time was fitted to a polynomial function, and the area under this curve was subtracted from the area under the curve representing the background reaction to calculate the oxidation potential of the tissue, which we report in arbitrary units (AU) g^{-1} tissue. Preliminary assays determined that background fluorescence was minimal and there was full dose–recovery for the amount of homogenate protein that was used in the assay, based on manipulations of homogenate protein around this range. The remaining diluted supernatants were frozen in liquid nitrogen and stored at −80°C for later determination of TOSC.

TOSC was determined by similarly measuring the change in 2′,7′-dichlorofluorescein fluorescence in response to the strong oxidant 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP). Diluted supernatants were thawed on ice and added to the assay buffer described above in sextuplicate. ABAP (final concentration of 20 µmol l^{-1}) was added to half of the wells immediately before the start of the assay. The oxidation signal was then measured as above as the accumulated fluorescence signal of 2′,7′-dichlorofluorescein over 60 min at 37°C. The relative area for the wells containing homogenate but not ABAP (background oxidation signal) was subtracted from the area of wells containing homogenate and ABAP (e.g. oxidant-induced plus background oxidation signal), and then divided by the area produced by wells containing homogenate but not ABAP to produce a relative metric of the oxidation signal that was produced by exogenous ABAP in the presence of tissue homogenate. As a result, this relative metric is larger for tissue homogenate samples that have lower scavenging capacity (e.g. a greater fluorescent signal produced by the inclusion of ABAP), and is expressed in AU g^{-1} tissue.

Glutathione assays

Concentrations of oxidized glutathione (GSSG), reduced glutathione (GSH) and total glutathione (GSH+GSSG) were determined in duplicate using established protocols (Baker et al., 1990; Rahman et al., 2006). Frozen and powdered samples of muscle and liver were sonicated at low power in 20 or 50 volumes, respectively, of potassium phosphate extraction buffer (16 mmol l^{-1} KH_2PO_4, 84 mmol l^{-1} K_2HPO_4, 5 mmol l^{-1} EDTA, 0.6% w/v sulfosalicylic acid, 0.1% Triton X-100; pH 7.5) in two 10-s bursts on ice with a ≥10 s wait between bursts. These sonicated samples were centrifuged for 10 min at 8000 ***g*** at 4°C.The supernatants were then split into two groups: one for measurement of total glutathione, and one for the measurement of GSSG. To prepare the latter samples for measurement of GSSG, 5 µl of 0.35 mol l^{-1} 2-vinylpyridine (which covalently reacts with GSH, but not GSSG, and makes GSH undetectable by the assay) was added to 70 µl of supernatant in a new 0.5 ml Eppendorf tube, which was then incubated for 1 h at room temperature with gentle mixing. Excess 2-vinylpyridine was then neutralized by adding 3 µl of 20% triethanolamine (TEA; diluted from ≥98% stock in 16 mmol l^{-1} KH_2PO_4 and 84 mmol l^{-1} K_2HPO_4), vortexing and incubating for 10 min. Following this incubation, samples were fully neutralized with TEA, and were centrifuged again for 10 min at 8000 ***g***. Samples for measuring both total glutathione and GSSG were incubated at 25°C for 10 min in a 96-well plate under the following initial conditions: 0.15 mmol l^{-1} DTNB, 5 mmol l^{-1} EDTA, glutathione reductase (5.5 U ml^{-1}), 16 mmol l^{-1} KH_2PO_4 and 84 mmol l^{-1} K_2HPO_4; pH 7.5. The reaction was begun by adding NADPH (final concentration 0.3 mmol l^{-1}) to each well, and the rate of change in absorbance at 412 nm was monitored for at least 2 min. A standard curve produced by known concentrations of GSH and GSSG was used to calculate the total glutathione and GSSG concentrations in each sample. The concentration of GSH was determined by subtracting the measured amount of GSSG from total glutathione (GSH+GSSG).

Lipid peroxidation assays

Lipid peroxidation was determined in triplicate using a Xylenol Orange assay (Hermes-Lima et al., 1995) that was modified for use in a standard 96-well plate. Powdered samples of frozen liver and muscle were homogenized in a glass Tenbroeck tissue grinder in 20 volumes of ice-cold methanol, and these homogenized samples were then centrifuged for 5 min at 1000 ***g*** at 4°C. The assay was carried out in solution with the following initial conditions: 0.25 mmol l^{-1} Fe(II)SO_4, 25 mmol l^{-1} H_2SO_4 and 0.1 mmol l^{-1} Xylenol Orange. Absorbance of this solution was first read at 580 nm after 15 min incubation in the dark at 25°C (‘blank reading’), and absorbance at 580 nm was monitored continuously after the rapid addition of homogenate (or distilled water as a control) to each well until a stable plateau was reached (‘plateau reading’, ∼15 min for liver tissue, ∼25 min for muscle tissue). Finally, cumene hydroperoxide (5 µmol l^{-1} final concentration) was added to each well, and the change in absorbance at 580 nm was monitored for another ∼20 min until it stabilized (‘final reading’). Lipid peroxidation was reported in cumene equivalents per gram of tissue by relating the change in absorbance caused by the addition of tissue (i.e. plateau reading–blank reading) to the change in absorbance caused by adding a known concentration of cumene hydroperoxide (i.e. final reading–plateau reading).

Assays of anti-oxidant and metabolic enzyme activities

The maximal activities of the anti-oxidant enzymes glutathione peroxidase and catalase were assayed using standard protocols in duplicate wells (Ahmad and Pardini, 1988; Dawson and Storey, 2016). Frozen, powdered muscle and liver tissues were homogenized on ice in 20 volumes of homogenization buffer (16 mmol l^{-1} KH_2PO_4, 84 mmol l^{-1} K_2HPO_4, 1 mmol l^{-1} EDTA and 0.1% Triton X-100; pH 7.4) using a PowerGen 125 electric homogenizer (Fisher Scientific, Whitby, ON, Canada), with two 10 s bursts at the highest setting. Homogenates were then centrifuged at 10,000 ***g*** for 10 min at 4°C and the supernatant was retained. For catalase only, these supernatants were also then sonicated on ice with three 10 s bursts interspersed by ≥10 s breaks between bursts. These samples were used in assays to determine the maximal activity of glutathione peroxidase (0.2 mmol l^{-1} NADPH, 3 U ml^{-1} glutathione reductase, 1 mmol l^{-1} NaN_3, 1 mmol l^{-1} reduced glutathione, 12 mmol l^{-1} cumene hydroperoxide, 1 mmol l^{-1} EDTA, 8 mmol l^{-1} KH_2PO_4 and 42 mmol l^{-1} K_2HPO_4; pH 7.4) and catalase (20 mmol l^{-1} H_2O_2, 3.2 mmol l^{-1} KH_2PO_4 and 16.8 mmol l^{-1} K_2HPO_4; pH 7.0) by measuring the rate of change in absorbance at 340 nm for 10 min for glutathione peroxidase or at 240 nm for least 2 min for catalase.

The assay for maximal activity of aconitase was adapted from a previous study (Gardner et al., 1994) as follows: samples were sonicated with ten 1-s bursts in 10 volumes of ice-cold isolation buffer (50 mmol l^{-1} Tris-HCl, 0.6 mmol l^{-1} $MnCl_2$, 2 mmol l^{-1} sodium citrate and 0.1% Triton X-100; pH 7.4), centrifuged at 10,000 ***g*** for 10 min at 4°C, and the supernatant was used in assays with the following conditions: 5 mmol l^{-1} sodium citrate, 0.2 mmol l^{-1} $NADP^+$, 0.6 mmol l^{-1} $MnCl_2$, 0.5 U ml^{-1} isocitrate

dehydrogenase and 50 mmol l^{-1} Tris-HCl; pH 7.4. Aconitase activity was measured as the rate of change in absorbance at 340 nm over 10 min.

All enzyme assays were run in duplicate in a 96-well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 25°C with temperature control or (in the case of catalase only) at room temperature (22°C). Preliminary assays determined that substrate concentrations stimulated maximal activity. There was full dose–recovery for the amount of homogenate that was used in the assay for catalase, glutathione peroxidase and aconitase. We used extinction coefficients (ε) of 6.22 (l $mmol^{-1}$ cm^{-1}) for glutathione peroxidase and aconitase (NADPH) and 0.0260 (l $mmol^{-1}$ cm^{-1}) for catalase (H_2O_2).

Statistical analysis

Two-way ANOVA was used to examine the effects of sampling time point, experimental treatment group, and their interaction. When the effects of treatment group or the treatment×time interaction were significant, we carried out Bonferroni *post hoc* tests to compare each hypoxic treatment group with the appropriate time-matched normoxic control group. These *post hoc* tests were used as the primary evidence for an effect of a specific treatment group. We also tested for circadian rhythms in the absence of hypoxia using one-way ANOVA within the normoxia control group (see Table S1). A significance level of $P<0.05$ was used for all statistical analyses. All data are reported as means±s.e.m. Because of the small size of killifish tissues, we were not able to run all assays in all individuals, so sample sizes varied between measurements.

RESULTS

ROS homeostasis and oxidative status were examined during exposure to hypoxia followed by re-oxygenation in killifish acclimated to normoxia, intermittent hypoxia or constant hypoxia (Fig. 1). There were significant main effects of treatment group on nearly all measured variables in both the muscle and the liver (Table 1). To describe the effects of hypoxia, our general approach will be to first describe the effects of exposure to acute hypoxia followed by re-oxygenation in killifish acclimated to normoxia, based on pairwise comparisons between the acute hypoxia–re-oxygenation treatment group compared with time-matched normoxic controls. We then describe the effects of hypoxia acclimation, based on the pairwise comparisons between each of the hypoxia acclimation groups (intermittent hypoxia and constant hypoxia; Fig. 1) and time-matched normoxic controls.

ROS homeostasis and oxidative status in the muscle during hypoxia–re-oxygenation

We first considered how redox status and glutathione levels varied during hypoxia followed by re-oxygenation. Exposure of normoxia-acclimated fish to acute hypoxia and re-oxygenation did not lead to any significant changes in redox status in the muscle, as reflected by the oxidation potential of the tissue measured during the DCF accumulation assay, based on the absence of any significant pairwise differences to time-matched controls (Fig. 2). There was a significant transient increase in GSSG in normoxia-acclimated fish after 1 h of hypoxia compared with time-matched normoxic controls (Fig. 3A), and this increase in GSSG was rapidly corrected by 6 h. The levels of reduced GSH (Fig. 3B) and total glutathione levels (Fig. 3C) exhibited modest transient increases after 6–12 h of acute hypoxia, but were otherwise similar to normoxic controls across time points. These alterations in oxidized and reduced glutathione were not associated with changes in the ratio of GSH and GSSG concentrations (Fig. 3D). Therefore, exposure of normoxia-acclimated killifish to acute hypoxia–re-oxygenation had little influence on redox status in the muscle.

In contrast, redox status was appreciably altered in the muscle of killifish that were chronically exposed to diel cycles of hypoxia (intermittent hypoxia) or to constant hypoxia. Fish acclimated to both patterns of chronic hypoxia exhibited a more oxidizing redox status than normoxic controls (i.e. higher tissue oxidation potential in DCF assays) at several times during hypoxia (Fig. 2). However, there was some variation in the temporal pattern of how each

Table 1. Statistical results of two-way ANOVA in the muscle and liver

	Main effect of treatment group		Main effect of time		Treatment×time effect	
	F	*P*	*F*	*P*	*F*	*P*
Muscle tissue						
Oxidation potential	20.88 (3,264)	<0.0001	1.646 (5,264)	0.1480	2.226 (15,264)	0.0060
[GSH+GSSG]	14.90 (3,241)	<0.0001	0.6568 (5,241)	0.6566	1.382 (15,241)	0.1565
[GSH]	14.90 (3,225)	<0.001	0.5809 (5,225)	0.7146	1.613 (15,225)	0.0715
[GSSG]	40.36 (3,237)	<0.0001	0.7721 (5,237)	0.5707	0.8935 (15,237)	0.5721
[GSH]:[GSSG]	24.58 (3,235)	<0.0001	1.931 (5,235)	0.0900	1.013 (15,235)	0.4419
Lipid peroxidation	8.434 (3,245)	<0.0001	0.9328 (5,245)	0.4603	1.102 (15,245)	0.3545
TOSC	18.68 (3,267)	<0.0001	2.283 (5,267)	0.0468	1.392 (15,267)	0.1506
Aconitase	0.4542 (3,204)	0.7146	0.6243 (5,204)	0.6814	0.6380 (15,204)	0.8413
Catalase	22.78 (3,232)	<0.0001	1.240 (5,232)	0.2912	0.5682 (15,232)	0.8974
GPX	6.696 (3,232)	0.0002	2.659 (5,232)	0.0233	1.277 (15,232)	0.2175
Liver tissue						
Oxidation potential	7.797 (3,306)	<0.0001	1.054 (5,306)	0.3861	1.811 (15,306)	0.0324
[GSH+GSSG]	8.948 (3,200)	<0.0001	0.8161 (5,200)	0.5394	0.9758 (15,200)	0.4821
[GSH]	10.72 (3,187)	<0.001	0.6319 (5,187)	0.6756	0.5613 (15,187)	0.9013
[GSSG]	6.238 (3,187)	0.0005	1.191 (5,200)	0.3150	1.061 (15,187)	0.3957
[GSH]:[GSSG]	2.896 (3,183)	0.0366	1.107 (5,183)	0.3581	0.7136 (15,183)	0.7689
Lipid peroxidation	4.751 (3,202)	0.0032	1.819 (5,202)	0.1107	1.608 (15,202)	0.0738
TOSC	14.58 (3,311)	<0.0001	1.269 (5,311)	0.2770	2.524 (15,311)	0.0015
Catalase	15.12 (3,176)	<0.0001	2.274 (5,176)	0.0493	1.305 (15,176)	0.2032
GPX	1.222 (3,173)	0.3033	1.629 (5,173)	0.1546	0.8129 (15,173)	0.6623

GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH+GSSG, total glutathione; TOSC, total oxidant scavenging capacity. The degrees of freedom for the numerator and the denominator, respectively, for each ANOVA are reported in parentheses.

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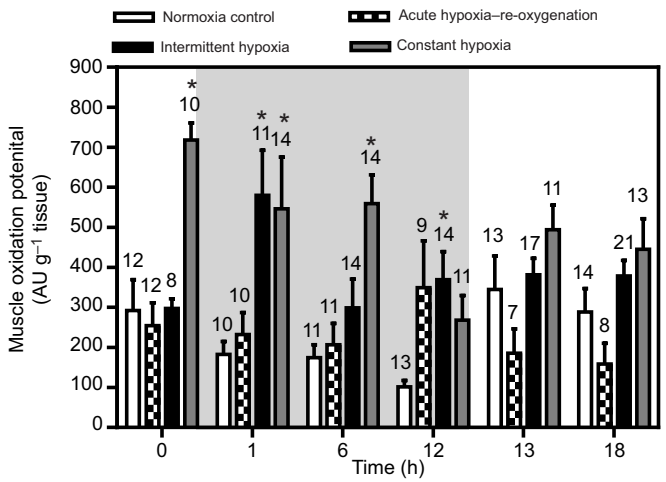

Fig. 2. Hypoxia acclimation altered redox status in the muscle, as reflected by the oxidation potential measured during dichlorofluorescein accumulation assays. The night-time dark phase (19:00 to 07:00 h local time) is indicated by the grey shaded section (see Fig. 1 for definition of treatment groups). Data were analysed by two-way ANOVA (Table 1) followed by Bonferroni *post hoc* tests to test for significant pairwise differences from the normoxic control group within a time point (*P<0.05). Sample sizes for each group are indicated directly above each bar. AU, arbitrary units (see Materials and Methods).

hypoxia-acclimated treatment group differed from normoxic controls. Fish acclimated to intermittent hypoxia had more oxidizing redox status early (1 h) and late (12 h) in the night-time hypoxia exposure, whereas fish acclimated to constant hypoxia had more oxidizing redox status throughout hypoxia except for a transient decline at the 12 h time point at the end of the night time (Fig. 2). These differences probably contributed to the significant interaction between treatment group and time in the two-way ANOVA for oxidation potential (P=0.0060) (Table 1). The hypoxia acclimation groups also had higher GSSG and total glutathione (GSH+GSSG) concentrations, along with lower GSH:GSSG in the muscle at several times during hypoxia and into re-oxygenation compared with normoxic controls (Fig. 3). There was also a transient decrease in reduced GSH levels at 0 h compared with normoxic controls.

We examined whether hypoxia–re-oxygenation induced oxidative stress in the muscle by measuring lipid peroxidation and the activity of aconitase (an enzyme in the tricarboxylic acid cycle that is highly sensitive to inactivation by oxidation of its iron-sulfur core; Bulteau et al., 2003). Exposure of normoxia-acclimated fish to acute hypoxia–re-oxygenation led to significant increases in lipid peroxidation compared with time-matched normoxic controls after 1 h of hypoxia and after 6 h of re-oxygenation in normoxia, and there appeared to be a general elevation in lipid peroxidation in this treatment group (Fig. 4A). Lipid peroxidation in the chronic intermittent hypoxia group did not appear to be as high as in the acute hypoxia–re-oxygenation group on average, but fish acclimated to intermittent hypoxia did show significantly higher levels of lipid peroxidation than normoxic controls at 12 h and 18 h. However, lipid peroxidation in the chronic constant hypoxia group was never elevated above the levels in normoxic controls. In contrast, there was no significant variation in aconitase activity between any treatment group and the normoxic controls, with no significant effects detected by two-way ANOVA (Fig. 4B; Table 1). Therefore, chronic hypoxia exposure was associated with similar or lower levels of oxidative stress than that experienced in fish exposed to acute hypoxia–re-oxygenation.

We measured the total oxidant scavenging capacity of the muscle tissue in response to treatment with the strong oxidant ABAP. Exposure to acute hypoxia–re-oxygenation led to an elevation in the DCF oxidation signal induced by ABAP after 1 and 6 h of hypoxia compared with time-matched normoxic controls (Fig. 5). In contrast, fish acclimated to both hypoxia acclimation groups maintained or in some cases (e.g. at 12 h of hypoxia) improved their scavenging capacity compared with normoxic controls (Fig. 5).

The loss of scavenging capacity in normoxia-acclimated fish exposed to acute hypoxia–re-oxygenation was concurrent with a substantial and more prolonged reduction in the maximal activity of the anti-oxidant enzyme catalase (Fig. 6A). This reduction began after 1 h of hypoxia and was persistent throughout re-oxygenation compared with time-matched normoxic controls. However, normoxic fish exposed to acute hypoxia–re-oxygenation exhibited some modest increases in glutathione peroxidase activity after 12 h of hypoxia and 1 h of re-oxygenation compared with normoxic controls (Fig. 6B).

Hypoxia acclimation appeared to attenuate the changes in anti-oxidant enzyme activity during hypoxia and re-oxygenation. In particular, fish acclimated to chronic hypoxia exhibited partial (intermittent hypoxia group) or full (constant hypoxia group) restoration of catalase activity compared with time-matched normoxic controls (Fig. 6A). The intermittent hypoxia group had increased glutathione peroxidase activity after 1 h of re-oxygenation, but the variation for this enzyme in this acclimation group was less appreciable than the variation in the acute hypoxia–re-oxygenation group, and there was no significant variation for this enzyme in the constant hypoxia group (Fig. 6B).

ROS homeostasis and oxidative status in the liver during hypoxia–re-oxygenation

The patterns of variation in liver redox status (as reflected by the oxidation potential of the tissue measured during DCF assays) during hypoxia–re-oxygenation were qualitatively similar to or greater than those in the muscle. Exposure of normoxia-acclimated fish to hypoxia–re-oxygenation led to a significantly more oxidizing redox status after 1 h of hypoxia, but the variation at other time points was not significant (Fig. 7). Similar to the patterns of variation in the muscle, fish acclimated to intermittent hypoxia and constant hypoxia exhibited much more oxidizing redox status than normoxic controls, which was particularly apparent during hypoxia in the constant hypoxia group and also extended to 1 h of re-oxygenation in the intermittent hypoxia group (Fig. 7). This variation probably contributed to the significant interaction between treatment group and time in the ANOVA for the liver oxidation potential (P=0.032) (Table 1).

Compared with muscle, the liver had a much greater total glutathione pool, but generally had similar GSH:GSSG on average (Table 2). Exposure of normoxic fish to acute hypoxia–re-oxygenation led to some increases in total glutathione (after 6 and 12 h of hypoxia) and GSSG (after 12 h of hypoxia and 1 h of re-oxygenation) compared with normoxic controls, as well as a trend for an increase in reduced GSH, but GSH:GSSG was generally maintained (Table 2). Lipid peroxidation also increased transiently in the acute hypoxia–re-oxygenation group after 1 h re-oxygenation compared with normoxic controls (Table 2). Hypoxia-acclimated fish generally showed very little variation in glutathione redox status or lipid peroxidation during hypoxia or re-oxygenation relative to time-matched normoxic controls, with the exception of a rise in GSSG in the constant hypoxia group after 1 h of re-oxygenation and lower lipid peroxidation in the intermittent hypoxia group after 12 h

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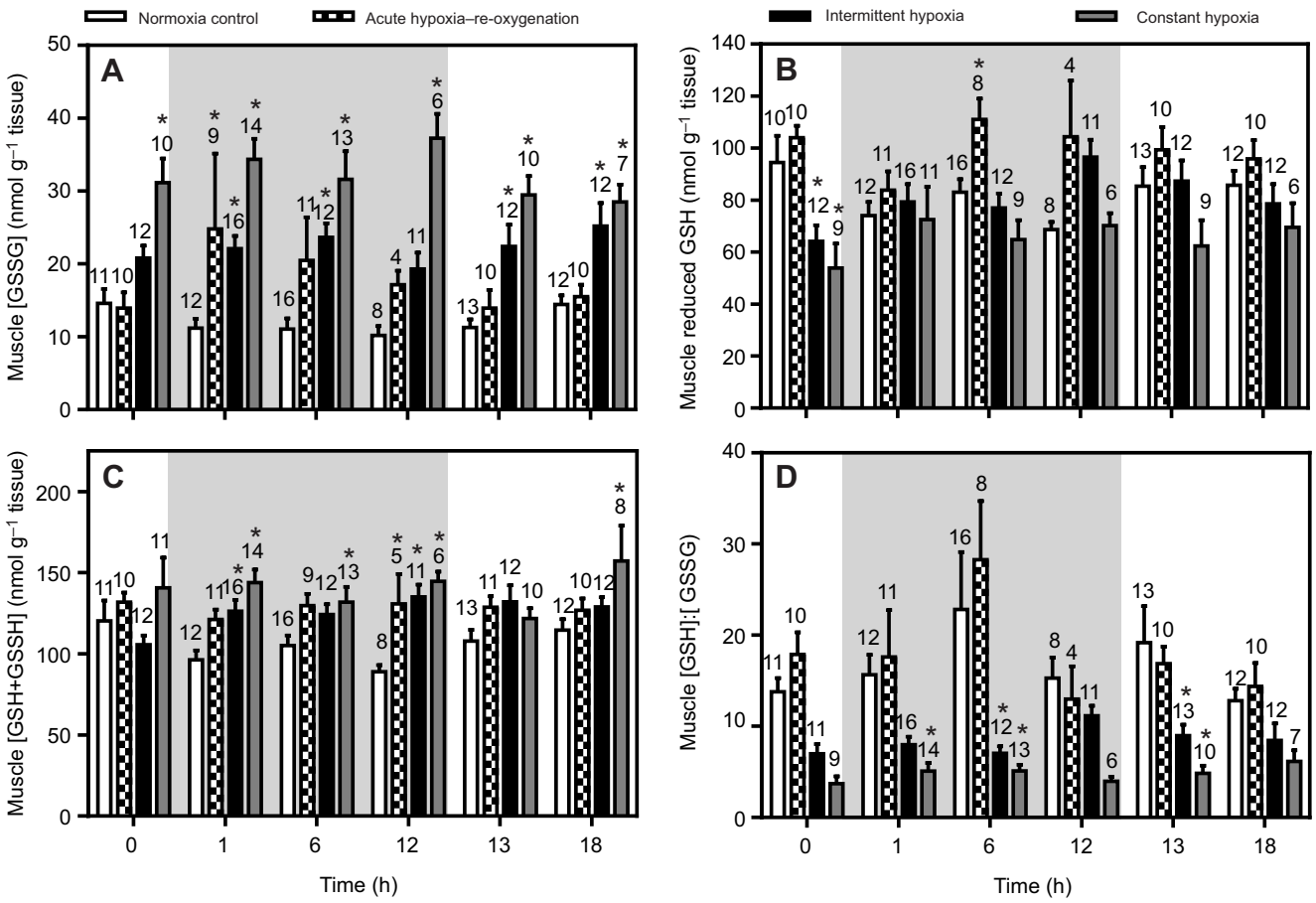

Fig. 3. Hypoxia acclimation altered glutathione redox status in the muscle. (A) Oxidized glutathione (GSSG) concentration. (B) Reduced glutathione (GSH) concentration. (C) Total glutathione (GSH+GSSG) concentration. (D) Ratio of GSH concentration to GSSG concentration. The night-time dark phase (19:00 to 07:00 h local time) is indicated by the grey shaded section (see Fig. 1 for definition of treatment groups). Data were analysed by two-way ANOVA (Table 1) followed by Bonferroni *post hoc* tests to test for significant pairwise differences from the normoxic control group within a time point (*P<0.05). Sample sizes for each group are indicated directly above each bar. AU, arbitrary units (see Materials and Methods).

of normoxia (the 0 h time point) (Table 2). The emergent pattern was that glutathione redox status and lipid peroxidation during hypoxia and re-oxygenation varied less in the liver than in the muscle.

TOSC was also much higher in the liver than in the muscle, as reflected by much smaller responses to the oxidant ABAP, and the liver also had much greater maximal activities of anti-oxidant enzymes (Table 3). Scavenging capacity in the liver was maintained or elevated (i.e. the response to ABAP were maintained or reduced), as was the activity of catalase, in groups exposed to hypoxia–re-oxygenation relative to time-matched normoxic controls (Table 3). However, there was some variation in how often each hypoxia acclimation group differed from normoxic controls, with fish acclimated to intermittent hypoxia generally showing higher catalase activities across several time points compared with normoxic controls (Table 3). In contrast, there was no significant variation in glutathione peroxidase activity (Tables 1 and 3).

Circadian rhythms in ROS homeostasis and oxidative status

We also considered whether any of the measured variables exhibited circadian rhythmicity. There were very few significant main effects of time in two-way ANOVA for measurements in either the muscle or liver (Table 1), but such analyses do not allow for examination of circadian rhythms in the absence of hypoxia. We therefore tested for circadian rhythms in the absence of hypoxia using one-way ANOVA of data for only the normoxia control group. These analyses revealed significant main effects of time for redox status (i.e. oxidation potential of the tissue in DCF accumulation assays) in both the muscle and liver (Table S1). The pattern of variation in both tissues was for redox status to become more oxidizing during the daytime photophase and less oxidizing during the night-time scotophase (Figs 2 and 7). These circadian rhythms were altered by hypoxia, as reflected by the significant interactions between treatment group and time in the two-way ANOVAs including all treatment groups, such that an overall main effect of time was no longer observed (Table 1). In the liver, but not in the muscle, there was also circadian rhythmicity in TOSC, in which a significant main effect of time appeared to be driven by lower TOSC at night (i.e. a larger response to the oxidant ABAP) (Table 3; Table S1). Hypoxia also altered this circadian rhythm in liver TOSC, with a significant treatment×time interaction but no overall main effect of time in the two-way ANOVAs including all treatment groups (Table 1).

DISCUSSION

Prolonged periods of low O_2 and daily cycles of hypoxia–re-oxygenation are common in many aquatic environments, but the implications of these challenging environments to ROS homeostasis and oxidative status in fish remain elusive. Here, we show that acute hypoxia and re-oxygenation led to some modest transient disturbances in redox status that resulted in oxidative damage in

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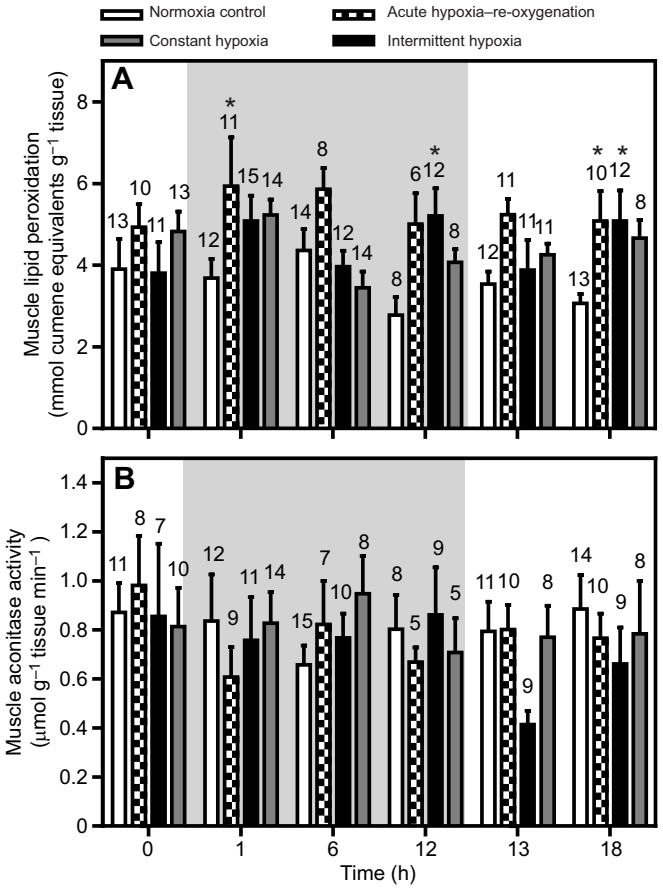

Fig. 4. Effects of hypoxia and re-oxygenation on lipid peroxidation and aconitase activity in the muscle. (A) Lipid peroxidation. (B) Aconitase activity. The night-time dark phase (19:00 to 07:00 h local time) is indicated by the grey shaded section (see Fig. 1 for definition of treatment groups). Data were analysed by two-way ANOVA (Table 1) followed by Bonferroni *post hoc* tests to test for significant pairwise differences from the normoxic control group within a time point (**P*<0.05). Sample sizes for each group are indicated directly above each bar.

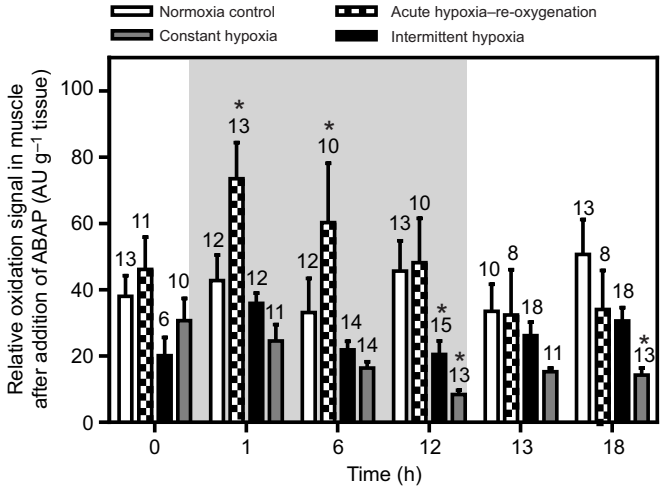

Fig. 5. Acute hypoxia decreased scavenging capacity in the muscle, but hypoxia acclimation restored or even enhanced scavenging capacity. Total oxidant scavenging capacity was measured as the change in relative DCF oxidation signal in response to the strong oxidant 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP), such that a greater oxidation signal is indicative of reduced scavenging capacity (AU, arbitrary units; see Materials and Methods). The night-time dark phase (19:00 to 07:00 h local time) is indicated by the grey shaded section (see Fig. 1 for definition of treatment groups). Data were analysed by two-way ANOVA (Table 1) followed by Bonferroni *post hoc* tests to test for significant pairwise differences from the normoxic control group within a time point (**P*<0.05). Sample sizes for each group are indicated directly above each bar.

killifish. Chronic acclimation to constant hypoxia or to diel cycles of nocturnal hypoxia (intermittent hypoxia) led to much greater alterations in redox status, but with minimal signs of oxidative damage, and this seemed to arise largely from a maintenance or improvement in anti-oxidant capacity. Therefore, rather than representing a sign of oxidative stress, the adjustments in oxidative status after hypoxia acclimation may be part of the suite of beneficial adjustments that killifish use to cope with chronic hypoxia.

Technical considerations for measurements of ROS and oxidative status

Reactive species, including oxygen (ROS), nitrogen (RNS), sulfur (RSS) and others, are products of mitochondrial metabolism and various other biological processes (Giles et al., 2001; Halliwell, 2006; Murphy, 2009; Patel et al., 1999). They can be involved in cellular signalling, but can also lead to oxidative stress if their levels are not effectively managed (D'Autréaux and Toledano, 2007; Mishanina et al., 2015; Patel et al., 1999). Measuring these reactive species is technically challenging due to their short-lived nature (in the order of milliseconds), high reactivity with cellular components, and potential sensitivity to O_2 levels, temperature and other cellular conditions (Degli Esposti, 2002; Passos et al., 2013). Measurements of *in vivo* ROS production can be made using exogenous probes that react with ROS *in vivo* to form a stable measurable product (e.g. mitoB) (Lau et al., 2019; Logan et al., 2014), but these probes must be injected into the animal in advance. Several widely used approaches for measuring reactive species make use of the probe 2′,7′-dichlorodihydrofluorescein diacetate (Halliwell and Whiteman, 2004; Kalyanaraman et al., 2012), which is cleaved by oxidation and produces a fluorescent product (DCF). However, this probe tends to cross-react with RNS, RSS, metals and even some proteins, so it is not a direct measure of ROS alone (Chen et al., 2010; DeLeon et al., 2016; Jakubowski and Bartosz, 2000). Furthermore, because ROS species are extremely short-lived, *in vitro* assays on tissue homogenates using this probe cannot accurately measure *in vivo* ROS levels prior to sampling. Such assays can measure *in vitro* production of oxidants by tissue homogenates during the assay, but this will be sensitive to the scavenging capacity of the tissue (e.g. anti-oxidant enzymes). *In vitro* DCF assays on tissue homogenates better reflect the oxidation potential of the tissue, and these assays are believed to be best suited to providing a general indication of redox status within the tissue (Jiang et al., 2018; Kalyanaraman et al., 2012). Therefore, we have used this assay in the current study to measure tissue oxidation potential as a general indicator of redox status, in combination with other more specific indices of redox status (e.g. glutathione redox status), and have coupled these measurements with various markers of scavenging capacity, anti-oxidant enzyme activity and oxidative damage.

Oxidative status during acute hypoxia and re-oxygenation

Acute hypoxia and re-oxygenation led to relatively modest changes in redox status in killifish. There are multiple possible sources and mechanisms for increased ROS production that could contribute to a change in redox status, during hypoxia and/or re-oxygenation,

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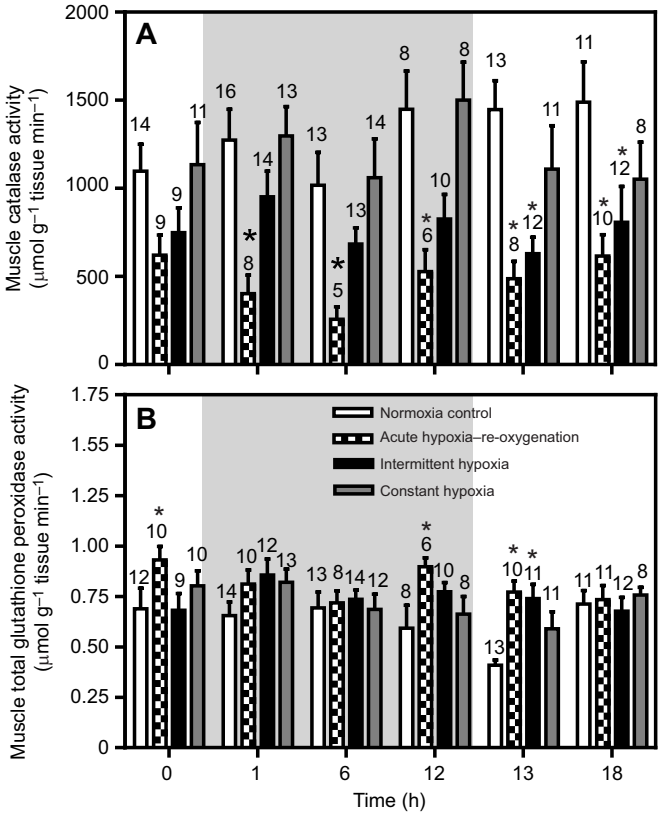

Fig. 6. Effects of hypoxia and re-oxygenation on the activities of anti-oxidant enzymes in the muscle. (A) Catalase activity. (B) Glutathione peroxidase activity. The night-time dark phase (19:00 to 07:00 h local time) is indicated by the grey shaded section (see Fig. 1 for definition of treatment groups). Data were analysed by two-way ANOVA (Table 1) followed by Bonferroni *post hoc* tests to test for significant pairwise differences from the normoxic control group within a time point (*P<0.05). Sample sizes for each group are indicated directly above each bar.

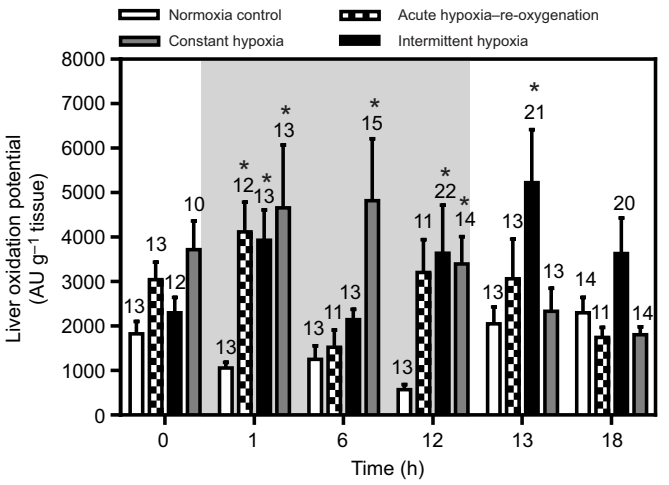

Fig. 7. Hypoxia acclimation altered redox status in the liver, as reflected by the oxidation potential measured during dichlorofluorescein accumulation assays. The night-time dark phase (19:00 to 07:00 h local time) is indicated by the grey shaded section (see Fig. 1 for definition of treatment groups). Data were analysed by two-way ANOVA (Table 1) followed by Bonferroni *post hoc* tests to test for significant pairwise differences from the normoxic control group within a time point (*P<0.05). Sample sizes for each group are indicated directly above each bar. AU, arbitrary units (see Materials and Methods).

including mitochondrial complexes, NADPH oxidases and xanthine oxidase (Brand, 2016; Granger and Kvietys, 2015; Murphy, 2009; Smith et al., 2017). Increases in ROS production might be expected to tip the balance between oxidants and anti-oxidants in the tissue, and thus lead to a more oxidized redox status. However, in the current study, acute hypoxia–re-oxygenation had a relatively minor influence on oxidation potential and redox status in the muscle (Fig. 2). It is possible that ROS production increased during the very early phases of acute hypoxia (<1 h) and were rapidly scavenged thereafter (Chandel et al., 1998; Zuo and Clanton, 2005). This may account for the early transient increase in GSSG and depletion of scavenging capacity in the muscle (Figs 3 and 5), which was later associated with modest but persistent increases in lipid damage (Fig. 4). Although changes in redox status were observed in the liver, changes in other markers of oxidative status were more moderate and delayed until late hypoxia or into re-oxygenation (Tables 2 and 3).

The skeletal muscle experienced more substantial alterations in glutathione redox state, lipid peroxidation and scavenging capacity than the liver (Figs 3–5 and 7; Tables 2 and 3). This observation may originate from the relatively lower scavenging capacity, total glutathione concentrations and activities of anti-oxidant enzymes in the muscle compared with the liver, as reported in previous studies (Ansaldo et al., 2000; Cooper et al., 2002; Hegazi et al., 2010; Leggatt et al., 2007; Lushchak et al., 2001; Otto and Moon, 1996). It is also possible that changes in tissue perfusion (and thus tissue O_2 levels) associated with hypoxia are greater in the skeletal muscle than in the liver, because of preferential redistribution of blood flow away from the muscle to critical organs like the brain (Axelsson and Fritsche, 1991; Hylland et al., 1994; Nilsson et al., 1994).

Killifish in this study generally had anti-oxidant enzyme activities and total glutathione pools that are comparable to many other fishes (Farombi et al., 2007; Lisser et al., 2017; Lushchak et al., 2001; Sinha et al., 2014). However, acute hypoxia led to a surprising reduction in catalase activity in the muscle that may have contributed to the decline in scavenging capacity and the increase in oxidative damage to lipids (Figs 4–6). Indeed, inhibition of catalase leads to increased oxidative damage in both goldfish and frogs in normoxia (Bagnyukova et al., 2005; Barja de Quiroga et al., 1989), as well as in Nile tilapia during periods of re-oxygenation (Welker et al., 2012). Although the killifish in this study showed only modest levels of oxidative stress during acute hypoxia–re-oxygenation, the associated loss of catalase activity may leave these animals especially vulnerable to greater oxidative stress if hypoxia occurs concurrently with other oxidative stressors, such as aquatic pollution (Birnie-Gauvin et al., 2017).

Hypoxia acclimation leads to substantial changes in ROS homeostasis and oxidative status

Chronic exposure to constant hypoxia or to cycles of hypoxia–re-oxygenation amplified changes in oxidation potential in the muscle and liver during hypoxia, and it increased oxidized glutathione levels in the muscle (Figs 2, 3 and 7). The mechanisms for the observed changes in redox status after hypoxia acclimation is not entirely clear, but in theory they could have arisen from reductions in the overall scavenging capacity of the tissue. However, our measurements of total scavenging capacity and anti-oxidant enzyme activities suggest that this was not the case. Instead, changes in redox status after hypoxia acclimation could have resulted from increases in pro-oxidants. ROS production *in vivo* tends to originate

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Table 2. Effects of hypoxia and re-oxygenation on glutathione redox status and lipid peroxidation in the liver

		Treatment group			
	Time	Normoxia control	Acute hypoxia–re-oxygenation	Chronic intermittent hypoxia	Chronic constant hypoxia
[GSH+GSSG] (nmol g^{-1} tissue)	0 h	894.5±82.7 (13)	902.3±172.7 (7)	764.6±78.1 (16)	679.1±192.5 (4)
	1 h	764.0±89.1 (13)	972.6±71.6 (15)	859.2±88.8 (7)	994.5±98.5 (5)
	6 h	812.7±52.3 (15)	1142±64 (9)*	747.8±126.5 (7)	964.3±70.5 (9)
	12 h	802.0±72.1 (11)	1147±214 (11)*	631.4±124.0 (3)	717.5±121.4 (4)
	13 h	793.4±83.1 (11)	1087±57 (12)	709.5±55.8 (7)	866.6±65.0 (10)
	18 h	775.7±65.4 (12)	894.0±140.9 (6)	567.4±46.2 (9)	966.2±118.2 (8)
[GSH] (nmol g^{-1} tissue)	0 h	711.2±73.8 (13)	823.3±158.0 (6)	483.6±87.7 (11)	709.8±122.4 (3)
	1 h	594.5±74.2 (13)	845.7±56.4 (13)	651.2±89.3 (7)	831.4±66.9 (5)
	6 h	641.4±47.9 (14)	875.0±77.7 (9)	567.1±118.1 (7)	765.6±79.6 (9)
	12 h	671.4±66.2 (11)	917.6±183.2 (11)	467.6±145.3 (3)	564.5±141.4 (4)
	13 h	675.3±85.8 (11)	855.5±60.1 (12)	461.4±61.4 (5)	635.5±70.2 (10)
	18 h	636.1±61.9 (12)	733.7±161.0 (6)	390.8±57.8 (9)	740.3±113.9 (7)
[GSSG] (nmol g^{-1} tissue)	0 h	91.67±12.53 (13)	103.5±15.8 (7)	94.01±12.69 (11)	86.10±14.31 (4)
	1 h	84.74±9.75 (13)	95.56±9.97 (14)	104.0±16.8 (7)	81.56±19.82 (5)
	6 h	92.00±11.23 (13)	133.4±14.4 (9)	90.33±9.28 (7)	99.40±11.83 (9)
	12 h	65.33±6.82 (11)	126.1±23.3 (10)*	81.90±10.82 (3)	76.50±27.22 (4)
	13 h	59.06±8.14 (11)	126.4±21.2 (11)*	106.7±7.1 (5)	115.5±12.2 (10)*
	18 h	69.79±11.05 (12)	80.14±13.63 (6)	88.32±13.49 (9)	90.07±24.02 (7)
[GSH]:[GSSG]	0 h	18.65±2.97 (13)	21.90±8.81 (6)	11.28±1.76 (11)	21.05±6.37 (3)
	1 h	14.79±1.42 (13)	27.19±8.86 (13)	16.11±4.59 (7)	22.95±3.15 (5)
	6 h	18.96±4.33 (13)	15.48±3.06 (9)	12.95±2.26 (7)	18.28±3.22 (9)
	12 h	21.77±2.13 (11)	16.98±3.13 (10)	12.77±5.12 (3)	17.70±7.00 (4)
	13 h	22.96±4.66 (10)	16.57±2.43 (11)	8.97±1.63 (5)	12.72±2.35 (10)
	18 h	26.68±5.85 (12)	22.45±5.44 (6)	12.76±3.62 (9)	28.42±9.39 (7)
Lipid peroxidation (mmol l^{-1} cumene equivalents g^{-1} tissue)	0 h	12.71±1.54 (13)	8.50±0.69 (7)	8.04±0.70 (12)*	10.60±1.58 (5)
	1 h	10.75±1.03 (13)	7.81±0.65 (13)	7.16±0.79 (8)	9.64±1.32 (9)
	6 h	10.15±0.63 (15)	7.94±0.77 (10)	8.49±0.86 (7)	11.43±1.18 (10)
	12 h	10.38±1.10 (11)	7.85±1.01 (8)	9.02±0.96 (3)	10.94±1.92 (5)
	13 h	9.03±0.87 (11)	15.00±3.68 (11)*	9.31±1.79 (8)	12.62±0.75 (10)
	18 h	11.14±0.57 (12)	10.05±1.36 (8)	7.31±0.72 (10)	13.16±1.94 (7)

GSH, reduced glutathione; GSSG, oxidized glutathione, GSH+GSSG, total glutathione. Data are reported as means±s.e.m., with sample sizes in parentheses. Data were analysed by two-way ANOVA (Table 1) followed by Bonferroni *post hoc* tests to test for significant pairwise differences from the normoxic control within a time point (*P<0.05).

from mitochondria, NADPH oxidases and/or xanthine oxidoreductase (Brand, 2016; Granger and Kvietys, 2015; Murphy, 2009; Smith et al., 2017). Mitochondria in particular are a significant source of ROS in hypoxia, but it is not clear whether hypoxia increases mitochondrial ROS production as a direct result of the influence of low P_{O_2} on mitochondrial electron transport, or

Table 3. Effects of hypoxia and re-oxygenation on total oxidant scavenging capacity and anti-oxidant enzyme activities in the liver

		Treatment group			
	Time	Normoxia control	Acute hypoxia–re-oxygenation	Chronic intermittent hypoxia	Chronic constant hypoxia
TOSC (AU g^{-1} tissue)	0 h	7.16±1.42 (14)	1.47±0.24 (13)	4.70±0.89 (12)	4.50±0.59 (11)
	1 h	2.72±0.23 (13)	2.74±0.76 (12)	5.12±1.15 (15)	2.68±0.43 (13)
	6 h	4.13±0.76 (14)	3.01±0.56 (13)	5.77±1.00 (13)	2.44±0.29 (15)
	12 h	4.26±0.49 (14)	3.50±0.74 (11)	6.00±0.79 (21)	2.69±0.36 (13)
	13 h	6.10±1.18 (13)	2.61±0.63 (13)*	4.30±0.52 (21)	4.31±0.51 (13)
	18 h	5.56±0.65 (14)	2.82±0.67 (11)*	3.56±0.47 (19)	3.03±0.45 (14)*
Catalase (μmol g^{-1} tissue min^{-1})	0 h	12.04±1.65 (11)	16.54±2.84 (8)	19.00±3.56 (10)	3.11±2.39 (4)
	1 h	8.43±1.93 (12)	12.38±1.25 (14)	22.84±3.70 (8)*	14.08±3.47 (5)
	6 h	9.96±1.52 (15)	8.60±1.75 (7)	17.47±2.62 (5)	11.21±3.57 (8)
	12 h	8.05±1.84 (11)	9.75±2.48 (8)	10.72±1.68 (3)	6.30±3.51 (4)
	13 h	7.58±1.76 (11)	17.41±2.12 (10)*	22.42±3.64 (7)*	10.71±3.67 (6)
	18 h	6.82±2.05 (10)	10.38±3.73 (7)	18.78±2.84 (7)*	8.87±2.39 (9)
Glutathione peroxidase (μmol g^{-1} tissue min^{-1})	0 h	8.15±1.31 (11)	8.77±1.28 (9)	9.21±2.33 (8)	4.54±1.98 (4)
	1 h	7.88±0.82 (13)	9.11±1.31 (14)	7.32±1.76 (8)	10.52±2.60 (5)
	6 h	7.60±0.96 (14)	7.27±0.90 (8)	9.08±2.17 (5)	8.27±0.98 (8)
	12 h	4.89±1.04 (11)	8.56±1.84 (8)	6.03±2.00 (3)	2.47±1.42 (3)
	13 h	7.92±1.12 (10)	10.47±1.72 (10)	9.50±1.60 (6)	7.34±1.73 (7)
	18 h	9.84±1.61 (10)	7.77±1.27 (7)	8.32±1.57 (7)	7.81±2.42 (8)

Total oxidant scavenging capacity (TOSC) is expressed in 10^4 AU g^{-1} tissue. Data are reported as means±s.e.m., with the sample size in parentheses. Data were analysed by two-way ANOVA (Table 1) followed by Bonferroni *post hoc* tests to test for significant pairwise differences from the normoxic control within a time point (*P<0.05).

instead as a result of low P_{O_2} on other factors in the cell (e.g. nitric oxide signalling, other signalling pathways, etc.) that interact with mitochondria to regulate ROS efflux (Murphy, 2009; Smith et al., 2017). The latter possibility is more consistent with some previous mitochondrial studies, which have shown that mitochondrial ROS emission decreases with reductions in P_{O_2} (Treberg et al., 2018) and that hypoxia acclimation further reduces mitochondrial ROS emission in killifish (Du et al., 2016) and epaulette shark (Hickey et al., 2012).

The more oxidizing redox status in muscle and liver of hypoxia acclimation groups was most apparent during night-time hypoxia, at a time of day when normal circadian rhythms in normoxia had the opposite effect and resulted in a less oxidizing redox status. Mitochondrial H_2O_2 signalling (Rhee and Kil, 2016) and energy metabolism (Eckel-Mahan and Sassone-Corsi, 2013; Sahar and Sassone-Corsi, 2012; Yang et al., 2006) normally exhibit some circadian rhythmicity (Putker and O'Neill, 2016), so it is possible that hypoxia could disrupt this normal circadian variation. Indeed, exposure to hypoxia has been shown to dampen circadian oscillations (Egg et al., 2013; Mortola, 2007) and disrupt the circadian clock (Tamaru et al., 2013). Our results here suggest that chronic hypoxia alters normal circadian oscillations of redox status in multiple tissues of killifish.

Although chronic hypoxia altered redox status in the muscle and liver, it did not increase lipid or protein damage (Fig. 4; Table 2). In the muscle, this avoidance of oxidative damage may have been associated with the restoration of catalase activity and increased oxidant scavenging capacity after hypoxia acclimation (Figs 5 and 6). Changes in these parameters were less apparent in the liver after hypoxia acclimation, possibly because the liver is already well protected by its relatively high scavenging capacity, total glutathione concentrations and anti-oxidant enzyme activities (Tables 2 and 3).

Changes in redox status after hypoxia acclimation could be involved in important signalling functions that help killifish to cope with hypoxia. Changes in ROS levels and/or redox status may have important implications for a number of cellular signalling events, and they can do so in localized regions of the cell where they do not induce oxidative damage (Smith et al., 2017; Costantini, 2019; D'Autréaux and Toledano, 2007). For example, ROS has been shown to stabilize hypoxia inducible factor (HIF) 1α during short periods of hypoxia, and thereby contribute to hypoxia signalling (Prabhakar and Semenza, 2012). However, this is unlikely to have persisted for prolonged periods of hypoxia in killifish, as we have previously shown that HIF-1α protein levels are low in the muscle of killifish after chronic exposure to either constant hypoxia or intermittent hypoxia (Borowiec et al., 2018). Changes in ROS levels and/or redox status could instead be involved in various other signalling pathways during chronic hypoxia, through its modulating influence on the reversible oxidation of cysteine thiol groups of proteins (Smith et al., 2017). Efforts to better understand how killifish manage ROS homeostasis, oxidative status and potential signalling by ROS during chronic hypoxia will provide valuable insight into key mechanisms for coping with life in harsh and variable environments.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: B.G.B., G.R.S.; Methodology: B.G.B., G.R.S.; Validation: B.G.B., G.R.S.; Formal analysis: B.G.B., G.R.S.; Investigation: B.G.B.; Resources: G.R.S.; Data curation: B.G.B.; Writing - original draft: B.G.B., G.R.S.; Writing - review & editing: B.G.B., G.R.S.; Visualization: B.G.B.; Supervision: G.R.S.; Project administration: B.G.B., G.R.S.; Funding acquisition: G.R.S.

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Data availability
Raw data have been uploaded to figshare: https://doi.org/10.6084/m9.figshare.12521573.v1.

Supplementary information
Supplementary information available online at
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