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Does hypoxia or different rates of re-oxygenation after hypoxia induce an oxidative stress response in *Cyphocharax abramoides* (Kner 1858), a Characid fish of the Rio Negro?

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ABSTRACT

We examined whether oxidative damage and antioxidant responses are more likely to occur during hypoxia or re-oxygenation in hypoxia-tolerant fish, and whether there is an influence of the rate of re-oxygenation. An hypoxia/re-oxygenation experiment using wild-caught *Cyphocharax abramoides* (Rio Negro, Brazil), was designed to answer these questions. Lipid peroxidation (MDA), a measure of oxidative damage, and antioxidant activities (superoxide dismutase (SOD), glutathione peroxidase (GPx), antioxidant capacity against peroxyl radicals (ACAP)), were measured in brain, gill and liver tissues after normoxia, 3-h hypoxia (2.7 kPa), and 3-h hypoxia followed by 1-h or 3-h re-oxygenation, implemented either immediately or slowly (3.0 kPa·h^{-1}). Critical oxygen tension of routine oxygen consumption rate (Pcrit) (4.1 kPa) and the P_{O2} at loss of equilibrium (LOE) (1.7 kPa) were determined to set the experimental hypoxia exposure. The Regulation Index, a measure of oxyregulation with declining P_{O2}, was 0.32. Oxidative damage occurred during hypoxia: no additional damage was observed during re-oxygenation. Tissues responded differentially. GPx and MDA rose in the brain and gills, and SOD (and likely GPx) in the liver during hypoxia. Antioxidants increased further at LOE. Rate of oxygen increase during re-oxygenation did not affect antioxidant responses. In brain and gills, GPx and MDA decreased or recovered after 1-h re-oxygenation. In liver, SOD remained high and GPx increased. In summary, *C. abramoides* incurred oxidative damage during hypoxic exposure with no additional damage inflicted during re-oxygenation: the rate of re-oxygenation was inconsequential. Literature data support conclusion of greater damage during hypoxia than during re-oxygenation in hypoxia-tolerant fish.

1. Introduction

The world is populated with many low oxygen environments, some chronically low in oxygen and some with oscillating oxygen regimes (Diaz and Breitberg, 2011). Consequently, many organisms, including fish, experience seasonal and/or diel fluctuations in oxygen tension (P_{O2}). More extreme fluctuations, as between hypoxic and normoxic environments, come at a cost physiologically, metabolically and sometimes physically, in terms of cellular damage (Hochachka and Somero, 1984; Hochachka et al., 1996; Perry et al., 2009; Lushchak, 2011; Richards, 2011).

Cellular damage, caused by excess reactive oxygen species (ROS), is associated with oxidative stress which can develop under hypoxic conditions. Oxidative stress is a state of imbalance between oxidants, such as ROS, and antioxidants in the cell in the favour of the oxidants (Jones and Sies, 2007; Sies, 2014, 2015), and is expected to occur in mitochondria under both strong reducing (hypoxia) and strong oxidizing conditions (hyperoxia) (Aon et al., 2010). Cellular damage caused under these conditions includes increases in protein carbonyls and lipid peroxides, as well as alterations in DNA structure (Traystman et al., 1991; Kramer et al., 1994; Liepelt et al., 1995; Mustafa et al., 2011).

The majority of our knowledge of oxidative stress and of the production of excess ROS comes from mammalian studies of mitochondrial function and antioxidant responses (e.g. Ambrosio et al., 1993; Fridovich, 1998; Chandel et al., 1998; Guzzy and Schumacker, 2006;

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Hoffman and Brookes, 2009; Lenaz et al., 2010; Soliani et al., 2010; Kaludercic et al., 2014; Munroe and Treberg, 2017). Normally, the production of ROS, and its control by antioxidants is thought to be a tightly integrated and regulated system (Sena and Chandel, 2012; Munroe and Treberg, 2017) which allows for ROS signalling. With respect to exposure to hypoxia, the mitochondria send a burst of hydrogen peroxide (H_2O_2), a form of ROS, into the cell to signal the onset of hypoxia (Chandel et al., 1998, 2000) and initiate adaptations to hypoxic conditions. These adaptations may be both non-transcriptional and transcriptional, the latter controlled by the hypoxia inducible factor (HIF-1α) (Chandel et al., 1998; Nikinmaa et al., 2004; Hickey et al., 2012; Sena and Chandel, 2012). Similarly a burst of ROS is released on re-oxygenation, though not in such a controlled manner, and often leads to oxidative damage (Ambrosio et al., 1993; Rymsa et al., 1991; Chouchani et al., 2014; Andrienko et al., 2017).

Work on the 'ROS-antioxidant system' in fish is not nearly so developed, and to date, no one has studied whether a burst of ROS occurs either on entering hypoxia or during re-oxygenation in intact fish. A burst of ROS is an important signal in mammals, initiating the appropriate cellular responses to hypoxia through stabilizing HIF-1α. A ROS burst on entering hypoxia is also likely present in fish. Nikinmaa et al. (2004) demonstrated that reduced cellular conditions, which occur during hypoxia, were needed to stabilize HIF-1α in salmonid cell lines and that a supeoxide dismutase (SOD) mimic maintained HIF-1α stability. Further, the genetic structures of HIF-1α of fish and mammals were functionally similar (Nikinmaa and Rees, 2005). These lines of evidence suggest that a burst of ROS is also released in fish when they enter hypoxic conditions. It is not clear whether this burst of ROS, which is controlled and used for signalling, is sufficient to cause oxidative damage. It is even less clear whether ROS is released during re-oxygenation in fish. Thus the relative roles of hypoxia and re-oxygenation on oxidative damage in fish is unclear.

Extrapolating from the mammalian system, we might expect the rate of re-oxygenation to affect the degree of oxidative damage on re-oxygenation, if a ROS burst occurs during re-oxygenation in fish. The reason is as follows. Mitochondria are highly reduced in hypoxic conditions having accumulated electrons in their electron transport system (ETS). These electrons are not equally available to react with oxygen (Murphy, 2009). At each site holding an electron, there is a distinct and specific probability that the electron will be available to interact with an oxygen to produce ROS at any specific time (Pe), and a separate probability that an oxygen will be available for interaction at that time (Murphy, 2009). Thus the rate of interaction will depend on both Pe and oxygen concentration. Oxygen availability is controlled by oxygen gradients which naturally develop within cells and mitochondria based on oxygen supply from the blood and oxygen usage within the mitochondria and cell (e.g. Erecińska and Silver, 2001). Therefore, the reaction rate of an electron is unique and regulated both by gradients in P_{O2} availability and by the probability of reaction. For these reasons, a slow infusion of oxygen into the cell may allow a more ordered set of reactions with oxygen and provide time for greater control of the ROS produced. Thus, slower access to oxygen should lead to lower levels of cellular damage. For this reason, we were also interested in whether the rate of re-oxygenation influenced oxidative damage during re-oxygenation in fish.

The Amazon provides an excellent location for studying questions of hypoxia tolerance, responses and recovery in fish. Through geological history, the region has been subjected to long periods of low oxygen conditions (Almeida-Val et al., 2006). Many fish species evolved under these conditions and developed biochemical, physiological, morphological and/or behavioural mechanisms to survive hypoxia and in some cases, anoxia (Val, 1996). Low oxygen environments are common throughout the Amazon today (Kramer et al., 1978; Almeida-Val et al., 2006).

In the frequently hypoxic black water rivers and back waters of the Amazon, one might expect fish to be well adapted to hypoxic exposure and re-oxygenation. Here fish may make the transition from hypoxic to normoxic conditions on a regular basis. Fish may have developed mechanisms to minimize oxidative damage. One possible mechanism is to control the rate of re-oxygenation, which is often feasible by behavioural choice of environments in nature. We asked whether oxidative stress and anti-oxidant responses are more likely to occur during hypoxia or during re-oxygenation, in these fish, and secondly, whether there is an influence of the rate of re-oxygenation.

We chose *Cyphocharax abramoides* (Kner 1858) from the family Curimatidae, Order Characiformes, for this study. *Cyphocharax abramoides* is one of a group of species known as 'branquinha'. This fish group is important in subsistence and commercial fishing, with large volumes of fish landed in the ports of Manaus and Belém (Soares et al., 2011). The Order Characiformes includes an abundance of hypoxia-tolerant species, many of them found in the Amazon (Almeida-Val et al., 2006). *Cyphocharax* spp. are bottom-dwelling fish, found in the flood plain lakes where oxygen levels can be low for much of the hydrological cycle (Zuanon and Ferreira, 2008). While only a little is known of their general ecology, nothing is directly known of the physiology of *Cyphocharax* spp.

Thus, the purpose of this study was two-fold: first, to characterize the respiratory characteristics of *C. abramoides* for the first time by determining its critical oxygen pressure (Pcrit) and P_{O2} at loss of equilibrium, and secondly, to present the results of an investigation of hypoxia/re-oxygenation oxidative injury and tissue antioxidant responses under conditions of hypoxia and of rapid and slow re-oxygenation. The levels of oxidative damage were assessed by measuring malondialdehyde (MDA), a proxy for lipid peroxidation host antioxidants defensesmeasured were enzymes SOD, glutathione peroxidase (GPx), and a measure of antioxidant capacity against peroxyl radicals (ACAP). Thus, this study contributes both to questions of hypoxia/re-oxygenation injury in a low-oxygen, dwelling fish, and also to knowledge of the physiology of *C. abramoides*.

2. Methods

2.1. Study region

The study was conducted during a research expedition between December 6th and 13th, 2014 within the Archipelago of the Anavilhanas National Park on the Rio Negro, Amazonas, Brazil, approximately 110 km upstream from Manaus (2° 43′ 10.9″ S, 60° 45′18.8″ W) (map available in Johannsson et al., 2017, Fig. 1). The research vessel, Ana Clara, was moored within a side channel of the Archipelago, abutting a floating house. Experiments were conducted on the porch of the house and samples processed on the Ana Clara or appropriately stored (see below) for later analysis at the Brazilian National Institute for Research of the Amazon (INPA) in the Laboratory of Ecophysiology and Molecular Evolution in Manaus.

2.2. Fish

All handling of fish conformed to Brazilian National and The University of British Columbia (Canada) animal care regulations (CEUA authorization # 026/2015), which are similar to the EU 2010/63 animal care guidelines. Fishermen from INPA brought fresh fish to the ship each day, caught by angling from the surrounding regions. On each day, four to seven *Cyphocharax abramoides* were transferred to a darkened, aerated, 500-l plastic tank. Rio Negro water was pumped through the system continuously to maintain oxygen (16.1 kPa) and temperature (31 °C) conditions close to those normally experienced by the fish in its natural environment. *Cyphocharax abramoides* were held without food and generally not kept for more than a day. Their standard length ranged from 12.7 cm to 15.3 cm (13.9 cm, 13.8 cm, 0.7 cm - mean, median and SD respectively), and their wet mass from 53 g to 91 g (71.5 g, 67.2 g, 13.9 g - mean, median and SD respectively). The

fish species was identified by Dr. Jansen Zuanon, (INPA, Biodiversity Co-ordinator (CBIO), Manaus, Brazil).

2.3. Hypoxic range as defined by Pcrit and LOE

As environmental P_{O2} decreases, a P_{O2} is reached at which oxygen consumption decreases sharply indicating a marked increase in dependence on anaerobic metabolism (Pörtner and Grieshaber, 1993). Pcrit was determined to define the upper limit of the hypoxic range of *C. abramoides*. The lower limit of its hypoxic range was considered the P_{O2} at which it could no longer maintain its equilibrium (LOE).

2.3.1. Series 1: Pcrit

Five fish, caught that day and held in aerated, Rio Negro water, were placed in the evening in 1.7 l, individual respirometer chambers, immersed in an aerated water bath, to determine Pcrit. The background oxygen consumption of the water in the chambers was monitored for six hours before the fish were added. In all instances, it was zero: the Rio Negro is a very nutrient poor river with low productivity. The fish were allowed to acclimate overnight. Intermittent-flow respirometry was used to determine routine metabolic rate (Steffensen, 1989). We used an automated apparatus DAQ-M (Loligo Systems, Tjele, Denmark) to measure MO_2, which consists of a recirculating circuit with 3 phases: flush, wait, and measurement. The time phases were 180-s flush, followed by 120-s wait, and 300-s measurement. The routine metabolic rate, MO_2 (mg $O_2 \cdot kg^{-1} \cdot h^{-1}$), was calculated as:

$$MO_2 = -\Delta O \, Vresp \, B^{-1}$$

where, ΔO is the rate of change in oxygen concentration (mg $O_2 \cdot l^{-1} \cdot h^{-1}$), Vresp is the volume of the respirometer chamber, and B is the mass of the individual (kg). To determine critical oxygen tension, fish were exposed to a brief period of progressive hypoxia by omitting the flush phase so that P_{O2} decreased as oxygen was consumed through respiration in the chamber. The oxygen consumption rates were calculated using the above equation. The critical P_{O2} (Pcrit) was determined by plotting the oxygen consumption rate versus the oxygen tension in the chamber, followed by segmented linear regression using the SegReg program (www.waterlog.info) (De Boeck et al., 2013). The slope of the MO_2 vs P_{O2} line above and below Pcrit was calculated for each individual.

As P_{O2} declines, MO_2 may be maintained at a constant level (total or 100% oxyregulation), or may decline gradually until reaching Pcrit, never passing below the line of perfect oxyconformity. Oxyconformity is defined by the straight line on the MO_2 vs P_{O2} plot joining (0,0) to MO_2 at maximum observed P_{O2}. If MO_2 remains above the line of oxyconformity, the organism is exerting some degree of oxyregulation. Many animals, including fish, fall into this region of partial regulation. Mueller and Seymour (2011) developed the Regulation Index (RI) to quantify the extent of oxyregulation, making it easier to compare amongst species. RI equals the area between the observed MO_2 curve and line of oxyconformity, divided by the area between total regulation and oxyconformity, and falls between 0 and 1. RI was calculated for each fish. All calculations were between maximum experimental P_{O2} and the P_{O2} where MO_2 was 0.

2.3.2. Series 2: loss of equilibrium

Five *C. abramoides*, which had been caught in the early morning, were added to a tank of Rio Negro water [20 l, 30.7 °C, 15.4 kPa, (6.08 mg $O_2 \cdot l^{-1}$)] at 7:55 a.m. and allowed to acclimate to the tank conditions for 3.5 h, P_{O2} declining at 1.1 kPa h^{-1}. At that time, the surface of the water was covered with bubble wrap and nitrogen bubbled into the water to lower the oxygen concentration more rapidly. P_{O2} fell nearly linearly for the first 50 min at 11.2 kPa h^{-1}. The rate of decline in P_{O2} slowed rapidly, declining to 0.45 kPa$\cdot h^{-1}$ between +72 min and +203 min, the end of the experiment. As the individual fish lost equilibrium, the time as well as the P_{O2} and temperature of the water were recorded, and the fish were individually euthanized.

In both Series 2 and Series 3 (below) experiments, the fish were euthanized in 1.0 g$\cdot l^{-1}$ neutralized MS222 at the end of the experiment. The brain, gills and liver were harvested. One portion of each tissue was wrapped in aluminum foil and frozen in liquid nitrogen for future analysis of lipid peroxidation, through the quantification of MDA concentration. A second portion, collected for enzyme and ACAP analysis, was added to a 1.5 ml bullet tube containing phosphate buffer [440 ml (50 mM K_2PO_4) + 50 ml (50 mM $NaH_2PO_4 \cdot H_20$) + enough EDTA to give a final concentration of 0.1 mM, pH 7.8] and held on ice. These samples were sonicated on ice with three times 3-s bursts of energy interrupted by 3 s of rest, and then centrifuged for 10 min at 10,000 rpm at 4 °C. The supernatant was transferred in 100-µl aliquots to small bullet tubes and frozen at −20 °C for the duration of the fieldwork. Only one tube was collected per tissue per fish from the LOE experiment: four tubes were collected from the hypoxia/re-oxygenation experiment (Series 3). Both liquid-nitrogen frozen tissue and frozen aliquots of supernatant were transferred to a −80 °C freezer on return to the INPA laboratory in Manaus.

2.4. Series 3: effects of hypoxia and rate of re-oxygenation

The experimental design consisted of 6 treatments: normoxia (N) (16.1 kPa), hypoxia (H) (2.4–3.0 kPa for 3 h), 3 h hypoxia followed by rapid (Fast) exposure to oxygenated waters (16 kPa) for either 1 h (1H F) or 3 h (3H F), and 3 h hypoxia followed by a gradual (Slow) increase in P_{O2} (3.0 kPa$\cdot h^{-1}$) for either 1 h (1H S) or 3 h (3H S). In the latter case, the P_{O2} at sampling at a rate of increase of 3.0 kPa$\cdot h^{-1}$ was 5.7 kPa and 11.7 kPa respectively. Replicate numbers of fish per treatment ranged from 7 to 9, but not all parameters were measured on all fish. Actual replicate numbers for each parameter are given in the Figures.

In the evening, four to seven fish were placed in the aerated, 250-l experimental tank freshly filled with Rio Negro water. The next morning, two thirds of the water was removed and fresh Rio Negro water added to cover the fish by 10 cm for a total volume of approximately 80 l. A sheet of plastic bubble wrap was placed over the entire surface of the water to preserve P_{O2} and prevent aquatic surface respiration (ASR): we did observe one fish using ASR when the plastic cover came away from the side of the tank. Two small, submersible pumps (Eheim compact 600, www.eheim.com) constantly circulated the water to maintain uniform conditions within the tank. The temperature was not controlled but changed slowly with air temperature. Within an experimental run, temperature changed by less than a degree across the experimental period, the treatment average maximum and minimum temperatures were 32.0 °C and 28.5 °C. Treatment mean temperatures, calculated as the average of the individual replicate temperature exposures, were: normoxia (30.0 °C), hypoxia (29.7 °C), and re-oxygenation at 1H F (29.8 °C), 3H F (31.6 °C), 1H S (29.7 °C), and 3H S (29.1 °C).

P_{O2}, measured with an YSI Pro20 dissolved oxygen meter (www.ysi.com/pro20), was lowered by bubbling with nitrogen, reaching target levels of 2.4 kPa to 3.0 kPa (0.9 mg$\cdot l^{-1}$ to 1.1 mg$\cdot l^{-1}$) within 70 min. Levels were kept within this range with short periods of oxygen bubbling. At the end of 3 h of hypoxia, the fish were either euthanized, transferred into Rio Negro water at 16.1 kPa, or remained in situ while the oxygen level was slowly raised (3.0 kPa$\cdot h^{-1}$). At the slow rate of re-oxygenation, P_{O2} rose through Pcrit (4.1 kPa) after +28 min. Initially one treatment was run per day. During the latter days of the experiment, fish from several treatments were run together through the hypoxia treatment. Some were euthanized at this point while others were put into either the fast or slow recovery regime. Therefore, within a treatment, some fish would be considered pseudo replicates, but independent from the remaining fish in the treatment. In defense of the protocol used, fish normally occur together, thus their stress levels were likely reduced when several fish were run together. No fish died during

the experiment. Liquid nitrogen became scarce and tissues from only 36 of the fish were frozen; however, they did cover all treatments.

2.5. Physiological measures of oxidative stress and balance

In each of the three tissues collected, we measured both oxidative damage (MDA) and the activity of antioxidants (SOD, GPx, ACAP) which respond to oxidative stress.

2.5.1. Lipid peroxidation

The frozen tissues were prepared for analysis over several days, always in small batches to minimize thawing before they were immersed in buffer. Approximately 25 mg of frozen tissue was cut from each tissue sample, placed in a bullet tube, weighed immediately and immersed in ice-cold RIPA buffer at a ratio of 1:10 (wt:vol, mg:µl). The RIPA buffer was composed of TRIS-HCl (250 mM), NaCl (750 mM), Triton-X (5%), sodium deoxycholate (2.5% (60.3 mM)), and sodium dodecylsulfate (0.5% (17.3 mM)). EDTA (1 mM) and protease inhibitor (Halt™, EDTA-Free, (100 ×); 500 µl of diluted cocktail. 50 ml^{-1} RIPA buffer) had been added to previously prepared RIPA buffer just before tissue preparation. The samples were sonicated with three times 3-s pulses of energy and of rest, and then centrifuged at 1600 *g*, 4 °C for 10 min. The supernatant was collected and frozen (−80 °C).

The Cayman TCA TBARS kit (www.caymanchem.com) was employed to measure thiobarbituric acid reactive substances, standardized against malondialdehyde (MDA), one of the end products of lipid peroxidation which interacts with thiobarbituric acid. The samples and standards were acidified (10% trichloroacetic acid), boiled for an hour, immersed in ice water for 10 min, centrifuged at 1600 *g*, 4 °C for 10 min and then read spectrophotometrically at 535 nm. All data are expressed as nM MDA·g wet wt^{-1}.

2.5.2. Enzymes

We measured ACAP, SOD and GPx activities in the gill, brain and liver supernatants. These assays measure the total capacity for enzyme activity under optimal conditions, not the actual rates of activity in the tissues. The tissue supernatants were normalized to a protein content of 1 mg protein·ml^{-1} which was used in all assays. The protein content of the supernatants was measured at 595 nm according to the Bradford (1976) method using bovine serum albumin as the standard.

2.5.2.1. ACAP. The total scavenging (antioxidant) capacity within the cell against peroxyl radicals, a species of ROS, (ACAP) was measured according to Amado et al. (2008). Six replicates were prepared from each supernatant in a black 96-well plate. 127.5 µl of reaction buffer [30 mM HEPES, 200 mM KCl, and 1 mM $MgCl_2$ (pH 7.2)] and 10 µl of fluorescent dye 2′, 7′ dichlorofluorescein diacetate (H_2DCF-DA) [240 µM in 90% ethanol] were added to 10 µl of the supernatants with 7.5 µl of MilliQ water and held in the spectrophotometer at 37 °C. Subsequently, 7.5 µl of ABAP (2, 2′ –azobis-2 2 methylpropionamidine dihydrochloride, 4 mM) were added to half of the replicates, MilliQ water was added to the others. ABAP produces peroxyl radicals, a species of ROS, and is used to challenge the antioxidant defenses of the tissue. Readings were taken every 5 min during 30 min at 488 nm (excitation) and 525 nm (emission). Intracellular esterases, present in the samples, cleaved the acetate groups of H_2DCF-DA. After that, the non-fluorescent compound H_2DCF was oxidized by ROS to the fluorescent compound, DCF, which was detected by the spectrophotometer. Total fluorescence produced was calculated by integrating the fluorescence units (FU) over the measurement time and expressed as the area of fluorescence. The results were expressed as area difference of FU in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). The relative difference between ROS area with and without ABAP was considered an inverse measure of total antioxidant capacity against peroxyl radicals (ACAP); that is, the higher the number, the lower the antioxidant capacity.

2.5.2.2. SOD. Total cellular SOD activities were determined according to the method of McCord and Fridovich (1969). Inhibition of the reduction rate of cytochrome *c* by the superoxide radical at 550 nm and 25 °C is the basis of this method. The supernatants were pipetted (5 µl) into plastic cuvettes and 1 ml of reaction media was added. The reaction media consisted of 47.5 ml of phosphate buffer (50 mM NaH_2PO_4, 50 mM K_2HPO_4, 0.1 mM EDTA, pH 7.8, 25 °C), 2.5 ml of 1 mM xanthine (diluted in 1 mM NaOH) plus 0.01235 g cytochrome *c*. The reaction was initiated by the addition of 20 µl of 0.2 U·ml^{-1} xanthine oxidase to 990 µl of the reaction media and 5 µl of supernatant. Spectrophotometric readings were taken every 15 s for 1 min at 550 nm. SOD activity is expressed in U SOD·mg of $protein^{-1}$, assuming one U of SOD as the quantity of enzyme that promotes a 50% change in the rate of reduction of cytochrome *c*.

2.5.2.3. GPx. Total cellular activities of selenium-dependent glutathione peroxidase (Se-GPx) were determined using the method of Hopkins and Tudhope (1973). This method is based on NADPH oxidation in the presence of GSH and H_2O_2 at 340 nm. The supernatants were pipetted (10 µl) into quartz cuvettes and 1 ml of reaction media was added to each. The reaction media consists of 0.1 M phosphate buffer (equal volumes of 100 mM NaH_2PO_4 and 100 mM K_2HPO_4, 2 mM EDTA, pH 7.0, 25 °C), plus 0.2 mM of NADPH, 1 mM NaN_3, and 1 mM of GSH. The reaction was initiated by the addition of 20 µl of 1 U·ml^{-1} glutathione reductase and 20 µl of 20 mM H_2O_2 in the cuvette. GPx is expressed in µmol of NADPH oxidized per min.mg of $protein^{-1}$ using a molar extinction coefficient of 6.22 mM cm^{-1}.

2.6. Statistics

In Series 2 and Series 3, data were first tested for normality of distribution (Shapiro-Wilk normality test). In the analyses listed below, if the data from all treatments were normally distributed,1 a parametric *t*-test or Analysis of Variance was applied, the latter followed by Tukeys' post-hoc multiple comparisons test. If any of the treatment data were not normally distributed, the non-parametric t-test, the Mann-Whitney *U* test or Kruskal-Wallis ANOVA was employed followed by Dunn's multiple comparison test or the Mann-Whitney U test. Statistical tests were performed in Graph Pad Prism 7 (GraphPad Software, Inc., La Jolla, CA, www.graphpad.com/scientific-software/prism). A significance level of 0.05 was accepted. Data are presented as means +/-1 SEM (N).

2.6.1. Effects of the rate of re-oxygenation – hypoxia and recovery

In the hypoxia/re-oxygenation experiment (Series 3), sample sizes were sometimes low; however, they could not be increased because the study was undertaken in the field under a fixed timeline. Therefore, the data were analyzed judiciously, asking specific questions, to avoid multiple comparisons using the same data set. The following tests were performed sequentially:

A) Effects of the rate of re-oxygenation on oxidative damage and antioxidant activities were examined between the fast and slow re-oxygenation treatments at 1 h and at 3 h (*t*-tests). There were no significant differences in any of the variables tested. The 1 h and 3 h data were combined. The combined fast rate of re-oxygenation data were then compared with the combined slow rate of re-oxygenation data (*t*-tests).
B) Do oxidative damage or antioxidant activities increase during hypoxia and/or re-oxygenation? For these analyses, all fast and slow re-oxygenation data were combined for each variable because no significant differences were detected between the fast and slow re-oxygenation treatments. MDA and antioxidant activities in the normoxic, hypoxic, and re-oxygenation (recovery) treatments were compared using ANOVA.

2.6.2. Oxidative responses: LOE vs hypoxia

MDA, ACAP and SOD responses of the fish in the LOE experiment were compared with those in the 3-h hypoxic treatment (1-tailed *t*-tests as SOD and MDA were expected to increase, and ACAP to decrease, in LOE): no other response variables were measured in the LOE fish (Series 2 and 3).

2.6.3. Oxidative status within individuals

A) The status of MDA, SOD, ACAP and GPx were compared across tissues within normoxic fish using ANOVA (Series 3).
B) Within tissues, possible correlations were assessed between oxidative damage (MDA) and antioxidant responses (SOD and GPx) using Pearson's correlation coefficient. As MDA did not differ between the LOE and hypoxia treated fish, the LOE data were included (Series 2 and 3).
C) Correspondence of antioxidant responses across tissues was examined to assess similarity of oxidative responses within individuals. Regression relationships were sought between GPx and SOD in the different tissues (e.g. GPx in tissue 1 vs GPx in tissue 2 or SOD in tissue 2). Normoxia, hypoxia and, when it was available, LOE treatment data were included in these analyses (Series 2 and 3).

3. Results

3.1. Series 1 and series 2: hypoxic range as defined by Pcrit and LOE

3.1.1. Series 1: Pcrit

Cyphocharax abramoides was closer to an oxyconformer than to an oxyregulator below 14.3 kPa at 26 °C, the experimental temperature, decreasing oxygen consumption gradually as P_{O2} declined: RI averaged 0.32 ± 0.05 (n = 5) (Supplemental data Fig. 1, Supplemental data Table 1). The change to a much steeper rate of decline, Pcrit, occurred at 4.1 ± 0.31 kPa (n = 5) (Fig. 1). The average rate of decline in MO_2 between 14.3 kPa and Pcrit was 7.63 ± 1.08 MO_2/P_{O2} (mg $O_2 \cdot kg^{-1} \cdot h^{-1}$/kPa) (n = 5), and the average rate of decline below Pcrit was 30.00 ± 6.76 MO_2/P_{O2} (mg $O_2 \cdot kg^{-1}\, h^{-1}$/kPa) (n = 5), a rate of aerobic metabolic suppression with declining P_{O2}, 4-fold higher than that above Pcrit, (Supplemental Data, Table 1).

3.1.2. Series 2: LOE

LOE defined the lower functional limits of hypoxia tolerance of *C. abramoides*. The fish were acclimated to the experimental chamber for 3.5 h. P_{O2} slowly decreased from 16.5 kPa to 12.6 kPa during this time. At this point, nitrogen gas was bubbled near the bottom of the container. P_{O2} decreased in an exponential fashion (Fig. 1B). Fish were not allowed to access the surface of the water; therefore, responses reported here are solely reflective of the capacity to extract O_2 from the O_2-depleted water.

Fish lost equilibrium over a range of oxygen concentrations from 0.7 kPa to 1.7 kPa (0.26 mg $O_2 \cdot l^{-1}$ to 0.62 mg $O_2 \cdot l^{-1}$), with a mean ± 1SE of 1.1 ± 0.16 kPa (0.42 ± 0.06 mg $O_2 \cdot l^{-1}$) (n = 5). All fish were in hypoxia for at least 25 min before the first fish lost equilibrium. The last fish lost equilibrium approximately 160 min into hypoxia, or 131 min after the first fish lost equilibrium (Fig. 1B).

The Pcrit and LOE data defined the hypoxic range of *C. abramoides* (Fig. 1, B). Based on these data, the hypoxic exposure of the hypoxia/reoxygenation experiment (Series 3) was allowed to vary between 2.4 kPa–3.0 kPa (0.9 mg $O_2 \cdot l^{-1}$ and 1.1 mg $O_2 \cdot l^{-1}$).

3.2. Series 3: physiological measures of oxidative stress

3.2.1. Oxidative damage

3.2.1.1. Lipid peroxidation (MDA). Sample sizes per treatment for MDA were low and no significant differences were observed between the reoxygenation treatments either at 1 h or 3 h or when they were grouped into fast vs slow recovery treatments, within any tissue (Fig. 2A, B and C). Therefore, the re-oxygenation data were combined. ANOVA was used to test for differences amongst the normoxic, hypoxic and reoxygenation groups. Significant increases were observed from the normoxic to the hypoxic MDA levels in the brain and gills (brain, $F_{(2,23)} = 4.776$, p = 0.018, n = 10; gills, $F_{(2,24)} = 4.243$, p = 0.027, n = 10), but not in the liver (Fig. 2D, E and F). The MDA levels in the liver during hypoxia were 50% higher than those in normoxia: An ANOVA of normoxic, 3-h hypoxic and LOE data showed a significant difference between MDA concentrations at normoxia and at LOE with hypoxic concentrations intermediate and not significantly different from either other treatment (Kruskal-Wallis$_{(3,\ 15)} = 7.22$, p = 0.019, n = 15). These data suggest an increasing oxidative stress and damage as P_{O2} levels fell.

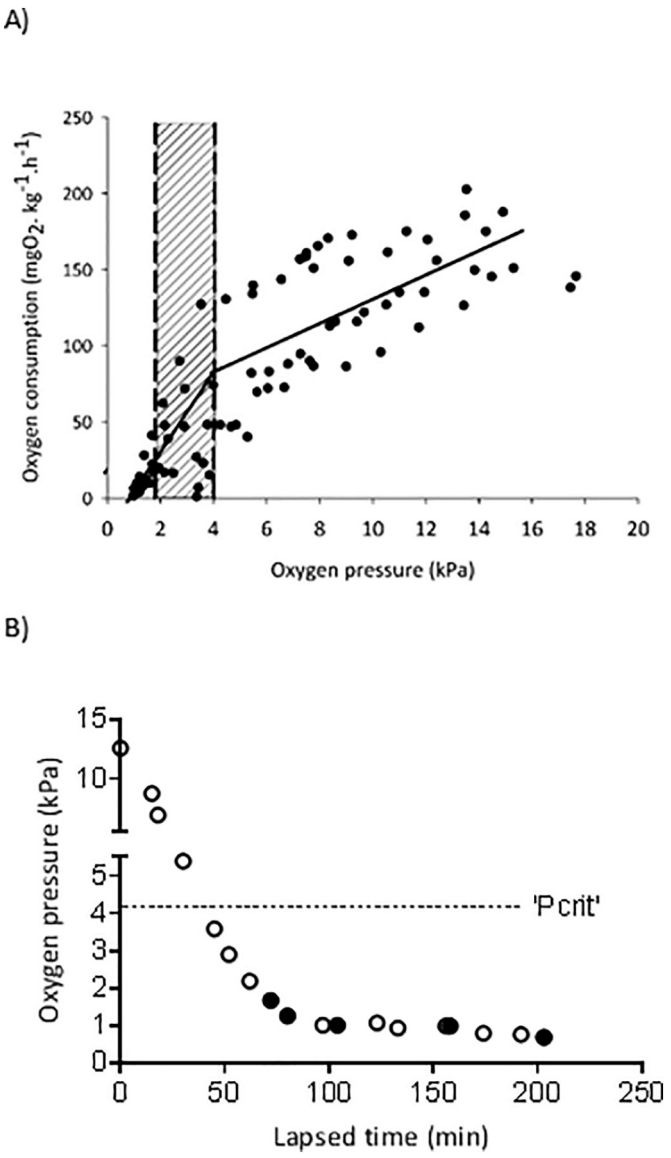

Fig. 1. A: Oxygen consumption (MO_2) of *Cyphocharax abramoides* with decreasing P_{O2} (●) at 26 °C, n = 5. A segmented linear regression program was employed to determine the critical P_{O2} (Pcrit) which set the upper edge of the hypoxic range for the fish. The hypoxic range is represented by the hatched region. The lower edge was defined by the P_{O2} of the first fish to lose its equilibrium as P_{O2} decreased. B: Timeline of the decrease in P_{O2} (○), and the P_{O2} (●) at which each fish lost equilibrium, against time since the commencement of nitrogen bubbling: This was preceded by 3.5 h of acclimation. n = 5. Temperature was 30.7 °C.

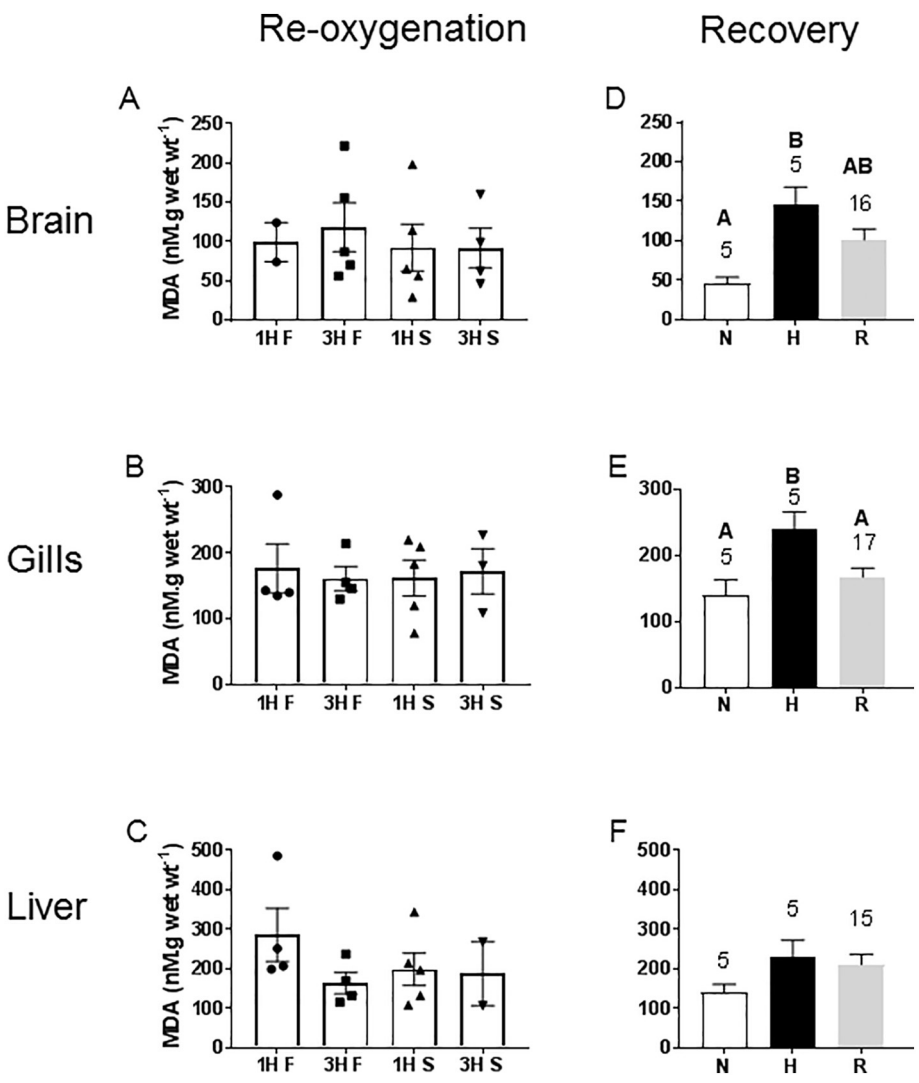

Fig. 2. Concentrations of MDA (nM MDA·gwetwt^{-1}), a measure of lipid peroxidation, in the brain, gills and liver of *Cyphocharax abramoides* from the hypoxia/re-oxygenation experiment (Series 3). Fig. A–C present the mean, SEM and distribution of the data in the four re-oxygenation treatments: 1H F and 3H F (sampled 1 h or 3 h after immediate transfer to normoxic waters after 3 h of hypoxia); 1H S and 3H S (sampled at 1 h or 3 h during slow re-oxygenation [3.0 kPa·h^{-1}] after 3 h of hypoxia). Fig. D–F, under the column 'Recovery', present the mean, SEM and sample size of the normoxia (N), hypoxia (H) and collated re-oxygenation (R) treatments. The letters denote bars which are similar (same letter) or significantly different (different letters) according to one-way Analysis of Variance. If no letters are posted in a figure, no significant differences were observed.

The gill MDA levels on re-oxygenation had returned to normoxic concentrations. In the brain, the re-oxygenation MDA levels were intermediate and not significantly different from those in normoxia or hypoxia showing some but not total recovery. These two sets of data indicate that no further oxidative stress was induced by re-oxygenation.

3.2.2. Antioxidant activities

3.2.2.1. Brain. In the brain, no differences occurred amongst the re-oxygenation treatments (Fig. 3A, B, and C) or between the combined fast vs slow rates of re-oxygenation comparisons. The treatments were combined to form a single re-oxygenation group to compare normoxic, hypoxic and re-oxygenation responses. SOD (Fig. 3D) and ACAP activities (Fig. 3F) did not change during hypoxia or re-oxygenation; however, GPx activity more than doubled during hypoxia (Fig. 3E) ($F_{(2,39)}$ = 6.646, p = 0.005, n = 42). GPx activities in re-oxygenation fish were not significantly different from those of normoxic fish, suggesting complete recovery.

3.2.2.2. Gills. In gill tissue, no differences occurred amongst the re-oxygenation treatments (Fig. 4A, B, and C) nor between the grouped fast and slow rates of re-oxygenation treatments. All treatments were combined to form a single re-oxygenation group in the next analysis comparing normoxic, hypoxic and re-oxygenation responses. No SOD response was observed in the gills in the hypoxia/re-oxygenation experiment (Fig. 4D). GPx activities were significantly higher in fish from the hypoxia treatment than from the normoxia or re-oxygenation treatments ($F_{(2,39)}$ = 11.66, p = 0.001, n = 42) (Fig. 4E). GPx activity of the re-oxygenation and normoxia groups were not significantly different. ACAP activity increased slightly in hypoxia (Kruskal-Wallis$_{(3,28)}$ = 6.521, p = 0.0384, n = 28: Dunn's multiple comparison test p = 0.05), and was intermediate between hypoxic and normoxic levels in the re-oxygenaiton group (Fig. 4F). Increases in the ACAP index indicate a lower antioxidant capacity against peroxyl radicals.

3.2.2.3. Liver. In the liver, no differences occurred amongst the re-oxygenation treatments (Fig. 5A, B, and C). They were combined to form a single re-oxygenation group in the next analysis comparing normoxic, hypoxic and re-oxygenation responses. SOD activity increased in response to oxidative stress during hypoxia, though the increase was not significant (Fig. 5D). During re-oxygenation, SOD activity remained at this increased level: it was now significantly higher than in the normoxia treatment - the larger sample size increased the statistical power (Kruskal-Wallis$_{(3,41)}$ = 7.380, p = 0.025, n = 41). Therefore, the increase during hypoxia was also considered significant. GPx activity increased significantly in the re-oxygenation treatment over the normoxic activity levels (Kruskal-Wallis$_{(3,16)}$ = 7.725, p = 0.021, n = 36) (Fig. 5E). Activity during hypoxia was intermediate and not significantly different from either the normoxic or re-oxygenation treatment activties suggesting a small increase during hypoxia and further increase during re-oxygenation (Fig. 5F).

It should be noted that the fish moved directly from hypoxic into

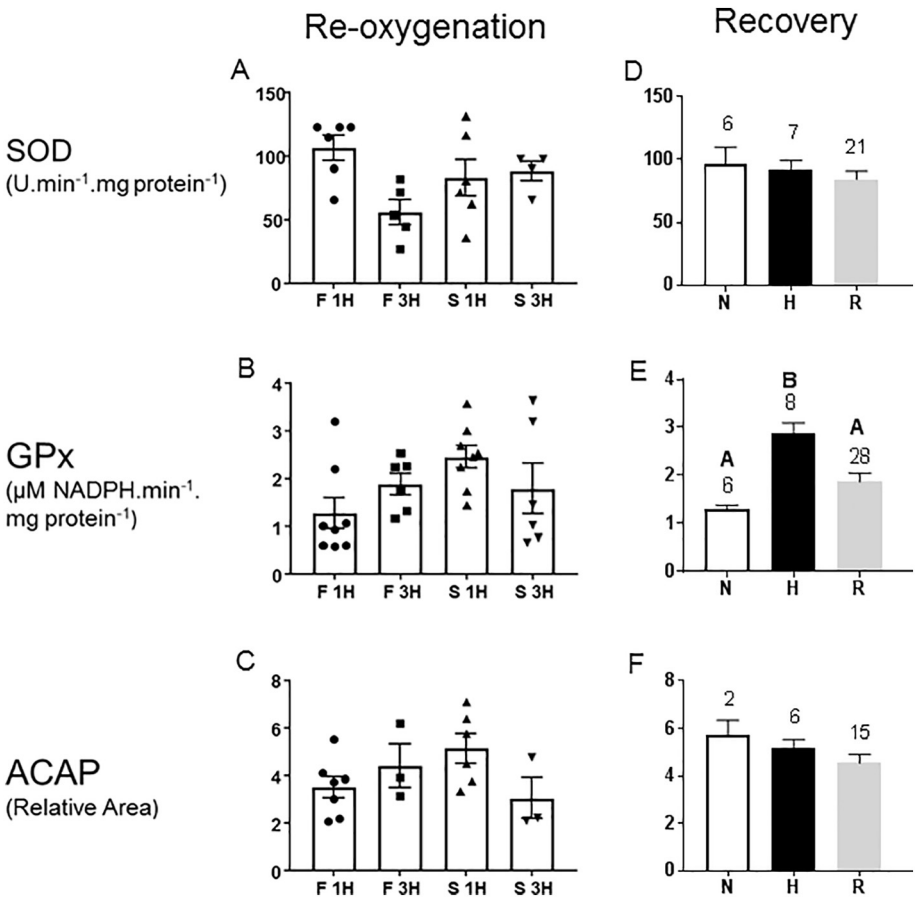

Fig. 3. Brain: Activities of common antioxidants. (A) superoxide dismutase (SOD), (B) glutathione peroxidase (GPx), and (C) the antioxidant capacity against peroxyl radicals (ACAP) in brain tissue of *Cyphocharax abramoides* from the hypoxia/re-oxygenation experiment (Series 3). Treatments, symbols and information as per Fig. 2.

normoxic waters (fast re-oxygenation) incurred additional handling stress than the fish in other treatments. The results across all treatments indicate that this did not significantly affect the responses: no differences were observed between fast and slow re-oxygenated fish.

3.2.3. Oxidative status: hypoxia vs LOE

Brain, gill and liver MDA levels did not differ between fish from the LOE and the 3-h hypoxia treatments (Fig. 6A). In the brain, no significant differences were observed in the activities of SOD or ACAP between the two treatments (Fig. 6B and C). In the gills, SOD activity in the LOE experiment was significantly higher than SOD activity in the 3-h hypoxic treatment ($t = 2.899$, $p = 0.018$, $n = 11$) (Fig. 6B). In both the gills and liver, ACAP activities were lower (and thus antioxidant capacity was higher) in the LOE fish than in the hypoxia fish ($p = 0.001$, $n = 10$; $p = 0.047$, $n = 11$, respectively) (Fig. 6C). There was insufficient tissue from LOE fish to measure GPx activity in any tissue, or SOD activity in the liver.

3.2.4. Oxidative status within individuals under normoxia

Under normoxia, SOD and GPx activity were significantly higher in the brain than in the gills or liver and the concentration of MDA was lower (Table 1A). The liver and gills were similar in enzyme activity and levels of MDA. No differences were observed in ACAP amongst the tissues.

3.3. Series 3: correlation of responses within tissues

In both the brain and gills, the levels of tissue lipid peroxidation (MDA) were positively correlated with GPx activity when assessed across all treatments (normoxia, hypoxia, re-oxygenation) [gills ($r = 0.509$, $p < 0.01$, $n = 25$), brain ($r = 0.550$, $p < 0.001$, $n = 24$)] (Fig. 7A and B). Liver MDA was not correlated with SOD, GPx or ACAP activity.

3.4. Series 3: correlation of enzyme activities within fish across tissues

Of the six possible across-tissue comparisons of SOD and GPx activities, three showed significant, positive, linear relationships. Within individuals, SOD activity in the gill increased with SOD activity in the brain ($Y = 0.5483\ X - 7.61$, $p = 0.0018$, $R^2 = 0.6014$, $n = 11$) (Fig. 8A) even though SOD did not respond to hypoxia in these tissues. Both GPx and SOD activities in the liver rose with GPx activity in the gill of the same individual (liver GPx activity: $Y = 0.4504\ X + 0.22$, $p = 0.0158$, $R^2 = 0.5887$, $n = 8$), (liver SOD activity $Y = 41.25\ X + 2.51$, $p = 0.0003$, $R^2 = 0.8281$, $n = 10$) (Fig. 8B and C). These enzyme activities had all increased to some degree in hypoxia. The latter relationship was significant with or without inclusion of the one outlier, denoted by ▲ in Fig. 8C. No significant relationships occurred between brain and liver enzyme activities. Although GPx activities rose in the brain and gill during hypoxia, they were not correlated within an individual.

4. Discussion

The present study on the physiology of *C. abramoides* has fulfilled two purposes, one to determine the capacity of this species to handle decreasing oxygen tensions in the environment, and two, to investigate whether hypoxia or the subsequent re-oxygenation is more likely to cause oxidative damage in this species and whether a slow, gradual reintroduction of higher oxygen tensions at the end of a bout of hypoxia

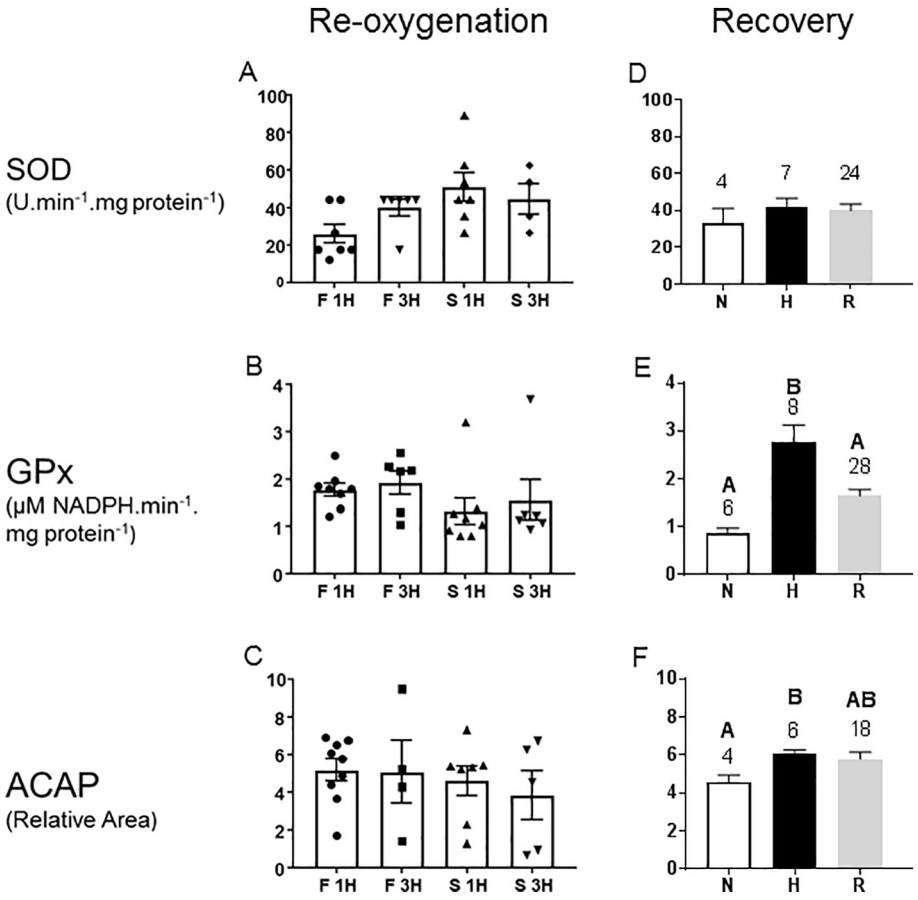

Fig. 4. Gills: Activities of common antioxidants. (A) superoxide dismutase (SOD), (B) glutathione peroxidase (GPx), and (C) the antioxidant capacity against peroxyl radicals (ACAP) in gill tissue of *Cyphocharax abramoides* from the hypoxia/re-oxygenation experiment (Series 3). Treatments, symbols and information as per Fig. 2.

enables the fish to decrease any oxidative damage incurred during re-oxygenation. *Cyphocharax abramoides* was found to be moderately hypoxia tolerant. Oxidative damage and antioxidant responses occurred during hypoxia: no further oxidative damage occurred on re-oxygenation although antioxidant activity increased in the liver. Thus, for this species, the rate of re-oxygenation had no impact. As expected, each tissue responded individually to the hypoxic stress. The correlation between the degree of damage (MDA) in gill and brain tissues and their antioxidant responses (GPx activity) suggests a linked/common cause of these changes, putatively, increases in ROS upon hypoxic exposure. Within the normoxic state, the brain was better protected by antioxidants (SOD and GPx) and incurred lower levels of oxidative damage, at least in terms of MDA.

4.1. Tolerance of hypoxia

Tolerance of *C. abramoides* to hypoxia was assessed by two parameters: loss of equilibrium (LOE) and Pcrit.

4.1.1. LOE

Cyphocharax abramoides demonstrated a moderate degree of hypoxia tolerance: from a group of 5 fish, the least hypoxia tolerant lasted 0.4 h, reaching 1.7 kPa, while the most hypoxia tolerant lasted 1.3 h, reaching 0.7 kPa. Several different protocols exist to determine time to and P_{O2} at LOE. Consequently, it is difficult to compare the P_{O2} at LOE of other Amazonian fish directly with that of *C. abramoides*. The lower limit of the experimental hypoxic range of *C. abramoides* was set by the highest P_{O2} at LOE, that is, 1.7 kPa, although the time to LOE at this P_{O2} suggests that even this value would be too low for the fish to survive for the length of the hypoxia/re-oxygenation experiment. In both the LOE and hypoxia/re-oxygenation experiment, the P_{O2} was lowered continuously until the desired P_{O2} (hypoxia/re-oxygenation experiment) or result (LOE) was reached. Therefore the data from the LOE experiment are appropriate for informing the lower bound of the hypoxia/re-oxygenation experiment.

4.1.2. Pcrit

The P_{O2} at Pcrit in *C. abramoides* supports the conclusion, that *C. abramoides* has moderate hypoxia tolerance. Pcrit increases with temperature (e.g. Schurmann and Steffensen, 1997; McBryan et al., 2013) and can alter with body size (Sloman et al., 2006; Heinrich et al., 2014). Thus, Pcrits are not directly comparable across all circumstances, but expected to be higher in tropical areas. The Pcrit of *C. abramoides* (4.1 kPa, 26 °C) was higher than those of other well-known hypoxia-tolerant fish from warm waters, such as, the African mouth-brooding cichlid *Pseudocrenilabrus multicolor victoriae* (1.02 kPa, 25 °C (Reardon and Chapman, 2010)) and considerably higher than the hypoxia tolerant, cool water sculpins *Blepsias cirrhosis*, *Leptocottus armatusave* and *Oligocottus maculosus* (1.76, 1.48 and 1.03 kPa, respectively, 12 °C (Speers-Roesch et al., 2013)). However, *C. abramoides* presents relatively close values to those of moderately hypoxia-tolerant, warm-water fish, such as, the Atlantic killifish *Fundulus heteroclitus* (4.5 kPa, 20 °C, southern population (McBryan et al., 2016)), the epaulette shark *Hemiscyllium ocellatum* (5.1 kPa, 28 °C (Speers-Roesch et al., 2012) or 4.7 kPa, 28.7 °C Heinrich et al., 2014) and the cardinal tetra *Paracheirodon axelrodi* (4.05 kPa, 26 °C (Campos et al., 2017)).

In spite of its general use, Pcrit is not always a good measure of hypoxia tolerance. For instance, the hypoxia tolerant Amazonian species, the tambaqui (*Colossoma macropomum*) and oscar (*Astronotus ocellatus*) have similar Pcrit values to those of *C. abramoides*, that is,

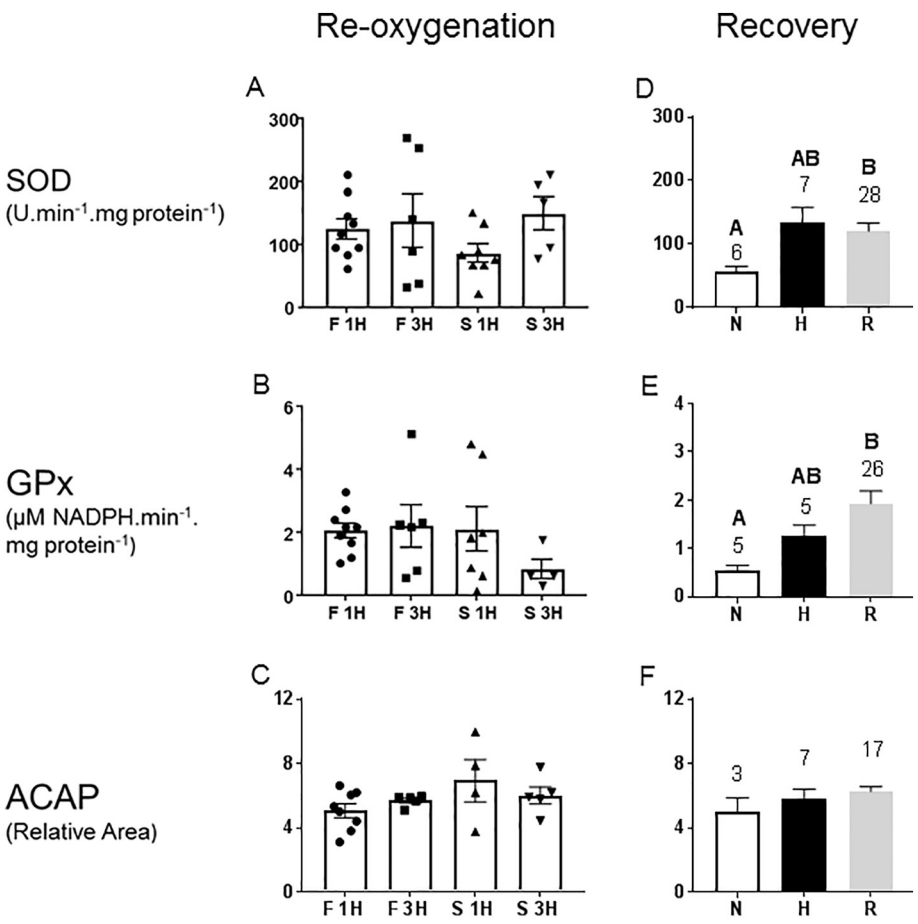

Fig. 5. Liver: Activities of common antioxidants. (A) superoxide dismutase (SOD), (B) glutathione peroxidase (GPx), and (C) the antioxidant capacity against peroxyl radicals (ACAP) in liver tissue of *Cyphocharax abramoides* from the hypoxia/re-oxygenation experiment (Series 3). Treatments, symbols and information as per Fig. 2.

5 kPa (Saint-Paul, 1984), and 4.2 kPa–4.5 kPa (Scott et al., 2008; De Boeck et al., 2013), respectively. Scott et al. (2008) have shown that oscar survive severe hypoxia and anoxia by strongly supressing aerobic metabolism and by possessing a high tolerance of anaerobic bi-products which compensate for a higher Pcrit. Pcrit measurements for the same species can also be variable. Robertson et al. (2015), when summarizing Pcrit data from the literature for several species, found values that ranged from 3.6 kPa to 15.3 kPa for bluegill sunfish (*Lepomis macrochirus*) and 3.6 kPa to 15.7 kPa for rainbow trout (*Oncorhyncus mykiss*), to list just two. The fish's condition, body size and life history, as much as the experimental technique used to measure oxygen uptake, can greatly influence the rates and measurements of metabolism. The Pcrit in the present study was determined for fish of the same body size range, caught from the same population, in the same manner, by the same fisherman, at the same time and held in the same manner for similar periods of time as the individuals used in the hypoxia/re-oxygenation experiment (Series 3). Therefore, for *C. abramoides*, they provide the best estimates of Pcrit with which to set the upper limit of the hypoxic range for the hypoxia/re-oxygenation experiment.

Amazonian fish typically have a suppressed aerobic capacity as seen in lower metabolic rates compared with temperate species of fish of the same size at the same temperatures (Almeida-Val and Hochachka, 1995; Driedzic and Almeida-Val, 1996; West et al., 1999; Campos et al., 2017). These lower rates of routine metabolism are thought to aid survival in the warmer, hypoxic waters of tropical environments (Almeida-Val et al., 2006). *Cyphocharax abramoides* also maintains lower metabolic rates, similar to or lower than other Amazonian fish. The routine metabolism of *C. abramoides* (4.27–6.41 mmol·kg^{-1}·h^{-1}, 93–96 g, 26 °C, Supplemental Table 1) at 70% of normoxic P_{O2} (highest P_{O2} values measured) was higher than that of 50–300 g oscar (Sloman et al., 2006) [2.10 mmol·kg^{-1}·h^{-1}, 28 °C, Fig. 1], similar to that of small tambaqui, (4.64 mmol·kg^{-1}·h^{-1}, 120 g, 30–32 °C) and lower than that of the herbivore, pacu (*Myleus lobatus*) (8.31 mmol·kg^{-1}·h^{-1}, 96 g, 30–32 °C) (Pelster et al., 2015). In the same study, 540-g black piranha (*Serrasalmus rhombeus*) at 30–32 °C had a routine metabolic rate of 5.60 mmol·kg^{-1}·h^{-1}, similar to that of *C. abramoides*; however, the rate for smaller fish of the same size as *C. abramoides* would be higher, and thus, also likely higher than that of *C. abramoides*.

4.2. Oxidative stress under hypoxia

Under hypoxia, *C. abramoides* showed signs of oxidative stress. Levels of lipid peroxidation end products (MDA) and the activities of antioxidant enzymes were higher after 3 h of hypoxia than under normoxia: GPx activities and MDA in the gill and brain, and SOD, and perhaps a small rise in GPx, activities in the liver. We did not examine other indices of oxidative damage, namely, protein carbonyls and DNA damage.

Other studies have also reported increases in antioxidant activity and/or oxidative damage in hypoxia-tolerant fish during both short- and long-term exposure to hypoxia (Table 2). A strong SOD response in the liver is consistent across the seven studies in which it was measured (Table 2). Sometimes increased SOD activity was accompanied by an increased antioxidant response against H_2O_2 (CAT or GPx in killifish, piapara and perhaps *C. abramoides*) and oxidative damage which was observed in the liver in 55% of these experiments. The exception was the common carp (*Cyprinus carpio*) held at a higher P_{O2} where increased SOD was not observed [c.f. Víg and Nemcshók, 1989 vs Lushchak et al.,

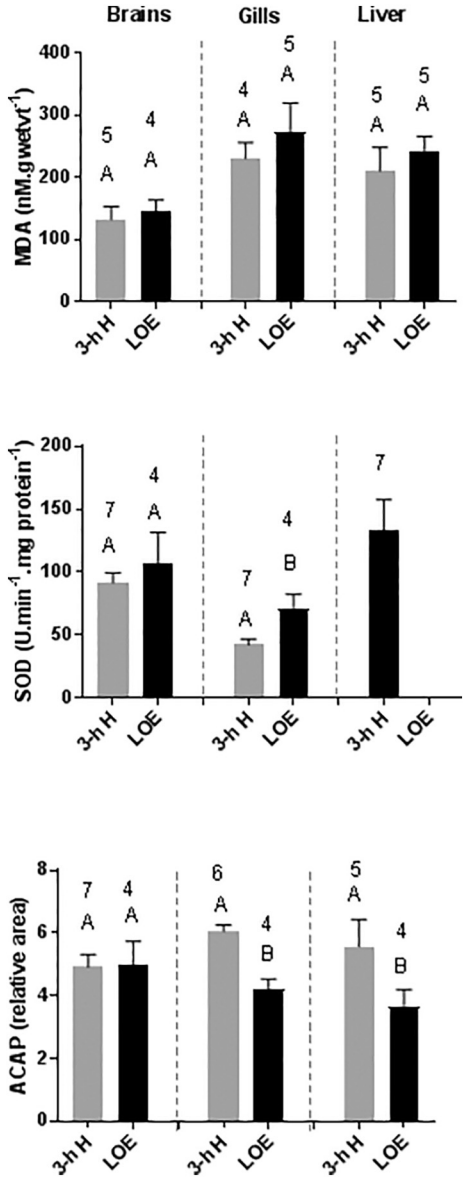

Fig. 6. Comparison of the concentration of lipid peroxidation (MDA) and antioxidant activities (ACAP and SOD) in brain, gills and liver tissues of *Cyphocharax abramoides* after three hours of hypoxia (H) and at 'loss of equilibrium' (LOE). Data are expressed as means ± 1 SEM. Sample size is given above the bar. Different letters above the bars indicate significant difference, as determined by *t*-tests.

Table 1
Levels of antioxidant activity [SOD (U min^{-1}·mg protein^{-1}), GPx (μM NADPH oxidized·min^{-1}·mg protein^{-1}), ACAP (relative area)] and lipid peroxidation (nM MDA·gwetwt^{-1}) in tissues of normoxia (A) and 3-h hypoxia, and (B) exposed fish: mean ± 1 SEM (n).

Conditions	Brain	Gills	Liver
A. Normoxia			
SOD	96.99 ± 12.60 (6)[a]	33.48 ± 7.62 (4)	55.89 ± 8.76 (6)
GPx	1.288 ± 0.092 (6)[a]	0.899 ± 0.130 (4)	0.547 ± 0.109 (5)
ACAP	5.879 ± 0.400 (3)	4.583 ± 0.355 (4)	4.996 ± 0.866 (3)
MDA	45.4 ± 8.7 (5)[a]	139.0 ± 24.2 (5)	141.8 ± 19.0 (5)
B. Hypoxia			
SOD	91.47 ± 7.73 (7)	42.09 ± 4.66 (7)[a]	133.10 ± 24.56 (5)
GPx	2.871 ± 0.228 (8)	2.765 ± 0.365 (8)	1.270 ± 0.219 (5)[a]
ACAP	4.915 ± 0.399 (7)	6.055 ± 0.202 (6)	5.796 ± 0.618 (7)
MDA	145.2 ± 23.0 (5)	246.0 ± 25.5 (5)	231.1 ± 41.6 (5)

[a] Marks the tissue which is significantly different for each variable.

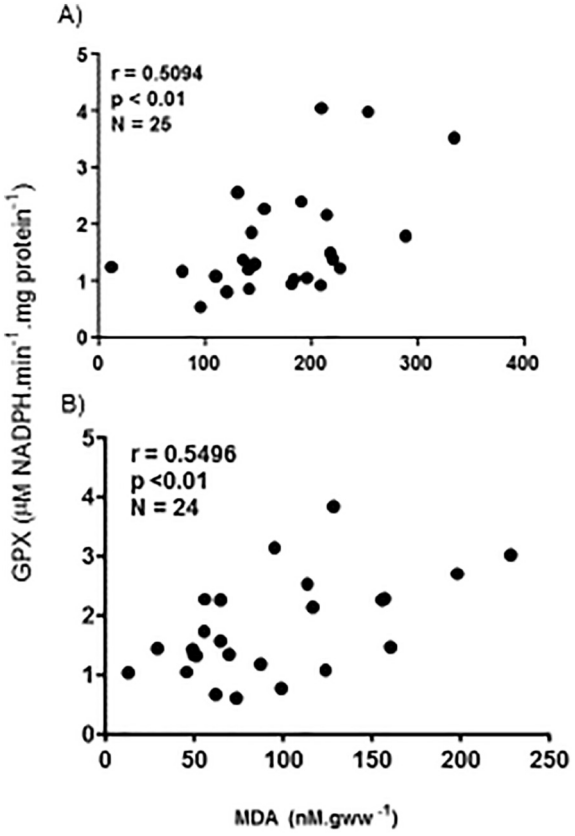

Fig. 7. The relationships between lipid peroxidation (nM MDA·gwetwt^{-1}) and the activity of the antioxidant enzyme GPx (uM NADPH·min^{-1}·mg protein^{-1}) in (A) gill and (B) brain tissue from the hypoxia/re-oxygenation experiment (Series 3) – the normoxia, hypoxia and re-oxygenation data were included in the analyses. Pearson correlations are significant and given for each tissue on the figure.

2005Table 2]. Across the studies (Table 2), brain suffered oxidative damage in four of the six experiments in which oxidative damage was assessed. The damage was accompanied by increased antioxidant activity against H_2O_2 in two of these studies. Antioxidant activity against H_2O_2 (GPx and CAT) could also increase in the brain in the absence of oxidative damage. Only in cabezon sculpin was there no response to hypoxia in the brain. Gills were examined for oxidative damage in *C. abramoides* and the two sculpin species: oxidative damage was only observed in the gills of *C. abramoides* and it was accompanied by increase in GPx.

Although limited, the comparisons from Table 2 confirm that oxidative damage occurs frequently in hypoxia-tolerant fish exposed to hypoxia. They also suggest that different patterns of response occur amongst tissues and that the degree of hypoxic exposure may influence the response as seen in common carp and tidepool sculpins. The degree of hypoxic exposure likely encompass a suite of variables, such as, the rate of entering hypoxia, the intensity of hypoxia (as compared with Pcrit), and the length of exposure. Previous exposure to hypoxia may also modify the response as some fish have exhibited the ability to acclimate to hypoxic conditions (Dowd et al., 2010; Fu et al., 2011; Leveelahti et al., 2011; Du et al., 2016).

Our understanding of ROS production and fate during hypoxia is incomplete, especially in non-mammalian systems. We do know that the ROS produced on entering hypoxia are essential as signalling molecules, particularly to initiate the hypoxic response through HIF-1α, that increased ROS production has been observed during hypoxia at least in some mammalian cell cultures, and that ROS are normally

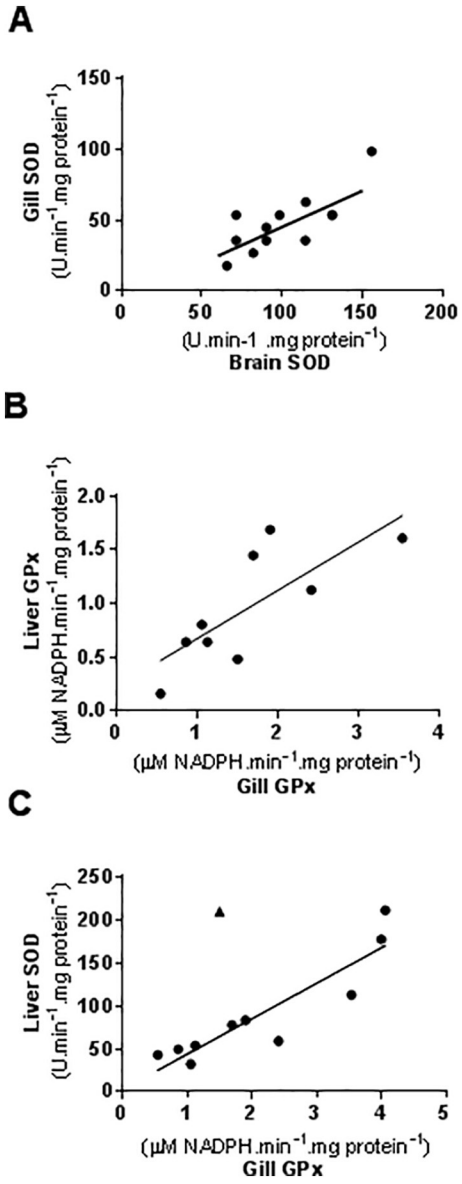

Fig. 8. Regression relationships between antioxidant activities in different tissues. Data from normoxic, hypoxic and, when available, LOE treatments. A). SOD activity in the gill vs that in the Brain – Y = 0.5483 X – 7.61, p = 0.0018, R^2 = 0.6014, n = 11; B). GPx in the liver vs that in the gill - Y = 0.4504 X + 0.22, p = 0.0158, R^2 = 0.5887, n = 8; and C). SOD in the liver vs GPx in the gill - Y = 41.25 X + 2.51, p = 0.0003, R^2 = 0.8281, n = 10, with the outlier (▲) removed. The relationship was still significant when the outlier was included (p = 0.0208). No significant relationships occurred between brain and liver antioxidant activities.

tightly controlled by the antioxidant system (Chandel et al., 1998; Chandel et al., 2000; Sena and Chandel, 2012; Munroe and Treberg, 2017). Given this information, the increases in activity of GPx in the brain and gills, and SOD, and perhaps GPx, in the liver in the present study, together with evidence of oxidative damage (increased MDA levels) in gill and brain tissues, suggest an increase in superoxide and H_2O_2 production in these tissues at some point during the 3-h hypoxia exposure. Interestingly, each tissue has its own antioxidant response pattern in the face of hypoxic exposure which was not related to the relative activities of the antioxidant enzymes amongst tissues during normoxia (Table 1). For instance, gills had the lowest SOD activity amongst the three tissues during normoxia, yet SOD activity, which is the first line of defense against superoxide radicals, was not increased during hypoxia. Defenses (GPx) were raised against H_2O_2. On the other hand, the liver showed the more expected response. SOD and GPx, activities were moderate/low during normoxia and the activities of SOD, and perhaps GPx, increased during hypoxia. In normoxia, the brain appeared to be the best protected against oxidative stress of the three tissues: it had the highest activity levels of SOD and GPx, and the lowest MDA levels. The brain is a very fatty tissue and damage to its lipids may impede its function. The brain increased only the activity of GPx when exposed to hypoxia. Despite the generally high levels of antioxidant activity in the brain under normoxia, and this increased GPx activity in hypoxia, the brain sustained some lipid damage (increased MDA levels) during hypoxia.

Despite the different antioxidant responses of the three tissues, the lipid damage incurred in each was very similar – from 90 nM MDA·g wet wt^{-1} to 107 nM MDA·g wet wt^{-1}. It should be noted that while those increases were significant in the brain and gill, they were not significant in the liver. Each strategy presumably minimized the oxidative impact within its own tissue. Although we know the functions of the different antioxidant enzymes (of which we have only measured three key ones), the present study emphasizes that we do not understand the strategies and trade-offs occurring in the different tissue types in response to ROS production during hypoxia.

4.3. Oxidative stress at LOE

Cyphocharax abramoides was capable of further antioxidant responses to the more extreme hypoxic conditions at LOE: no further oxidative damage was observed. The liver and gills, but not the brain, expressed increased antioxidant activity, beyond that observed after the 3-h hypoxia treatment (Series 3). SOD activity increased in the gills (no SOD data were acquired for the liver at LOE) and activity against peroxyl radicals increased in the gills and liver (decreased ACAP value), as the fish reached the point of LOE. The increase in activity against peroxyl radicals at LOE, but not hypoxia, indicates that the body's defenses against peroxyl radicals were not being brought to bear at 2.4–3.0 kPa but reserved for the greater stress of even lower P_{O2} conditions. The response to oxidative stress appears to be in proportion to the degree of oxidative stress. This was further confirmed by the positive correlations between GPx activity in the brain and gill and the degree of oxidative damage (MDA) in those tissues (Fig. 7).

4.4. Response to rate of re-oxygenation

We observed no evidence that the rate at which P_{O2} was raised after 3 h of hypoxia altered the degree of oxidative damage in *C. abramoides*. In fact, we did not observe any increase in tissue damage in any re-oxygenation treatment as assessed by the lipid peroxidation end product, MDA. Protein carbonyls and DNA damage were not assessed. We did observe increases in GPx activity in the liver at +1 and +3 h after the start of re-oxygenation; however, it was not related to the rate of re-oxygenation. We are aware of only a few hypoxia/re-oxygenation studies with fish that examined the oxidative response: (a) the common carp (Lushchak et al., 2005), (b) the goby, *P. glenii* (Lushchak and Bagnyukova, 2007), (c) Nile tilapia (*Oreochromis niloticus*) (Welker et al., 2012), and (d) intertidal sculpins, *Scorpaenichthys marmoratus* and *Oligocottus maculatus* (Lau, 2018) (Table 3). None examined the effect of the rate of re-oxygenation; however, they provide some insight on the conditions provoking the most oxidative damage for these hypoxia-tolerant fish species. Specific similarities in responses to hypoxia/re-oxygenation were rare amongst the species (carp, goby, Nile tilapia, intertidal sculpin and *C. abramoides*). Of the 17 assessments of oxidative damage and antioxidant activities across gill, brain and liver tissues across Tables 2 to Table 3, only one exhibited new oxidative damage on re-oxygenation, which occurred in the liver of the Nile tilapia. In three cases, oxidative damage incurred during hypoxia was carried over into the re-oxygenation period and was accompanied by increases in

Table 2
Oxidative damage and antioxidant activities which increased in brain, gill and liver tissue of hypoxia tolerant fish species in short and long term hypoxia exposure. All four antioxidants listed (SOD [superoxide dismutase], GPx [glutathione peroxidase, CAT [catalase], and TOSC-H2O2 [total oxidative scavenging capacity against hydrogen peroxide]] and the two measures of oxidative damage (lipid peroxidation (MDA) and protein carbonyls (PC)) were not measured in all studies. Tissues not examined are coloured light grey (Wilhelm Filho et al., 2005).

Species	Variables measured	Tissue Examined			Duration & P_{O2} of Treatment	Reference
		Brain	Gill	Liver		
Cyprinus carpio common carp	SOD	SOD	SOD	SOD	6 hours 0.9 kPa	Víg and Nemcshók, 1989
Cyprinus carpio common carp	SOD GPx CAT ***MDA PC***	GPx & CAT		***MDA***	5.5 hours 1.9 kPa	Lushchak et al., 2005
Leporinus elongates piapara	SOD GPx CAT ***MDA***			SOD GPx	14 days 4.8 kPa	Wilhelm Filho et al., 2005
Percottus glenii goby	SOD GPx CAT ***MDA PC***	***MDA***		SOD ***PC***	2 to 10 hours 0.9 kPa	Lushchak & Bagnyuova, 2007
Fundulus heteroclitus killifish	SOD CAT ***PC***			SOD CAT ***PC***	28 days 5 kPa	Du et al., 2016
Hemiscyllium ocellatum Epaulette shark	SOD GPx CAT				2 h 0.34	Leveelahti et al., 2011
Oligocottus maculosus tidepool sculpin	TOSC_H_2O_2 ***MDA***	TOSC ***MDA***		***MDA***	6 h hypoxia 3.5 kPa (at Pcrit)	Lau, 2018
Oligocottus maculosus tidepool sculpin	TOSC_H_2O_2 ***MDA***	***MDA***			6 h hypoxia 2.5 kPa (65% of Pcrit)	Lau, 2018
Scorpaenichthys marmoratus cabezon sculpin	TOSC_H_2O_2 ***MDA***				6 h hypoxia 3.5 kPa (65% of Pcrit)	Lau, 2018
Oreochromis niloticus nile tilapia	SOD, CAT, GPx ***PC MDA***			SOD	3 h hypoxia 0.66 kPa	Welker et al., 2012
Cyphocharx abramoides branquina	SOD GPx ***MDA***	GPx ***MDA***	GPx ***MDA***	SOD & GPx ***MDA****	3 hours 2.4-2.9 kPa	present study

Table 3
Oxidative damage and antioxidant activities which increased within 2 h of re-oxygenation after hypoxia exposure in brain, gill and liver tissue of hypoxia tolerant fish species. All four antioxidants listed (SOD [superoxide dismutase], GPx [glutathione peroxidase, CAT [catalase], and TOSC-H_2O_2 [total oxidative scavenging capacity against hydrogen peroxide]] and the two measures of oxidative damage (lipid peroxidation (MDA) and protein carbonyls (PC)) were not measured in all studies. Tissues not examined are coloured light grey.

Species	Variables measured	Tissue Examined			Duration & P_{O2} of Treatment	Reference
		Brain	Gill	Liver		
Cyprinus carpio common carp	SOD GPx CAT ***MDA PC***	SOD CAT*			5.5 hours h 1.9 kPa	Lushchak et al., 2005
Percottus glenii goby	SOD GPx CAT ***MDA PC***	GPx CAT ***MDA****		SOD* GPx ***PC****	2 to 10 hours 0.9 kPa	Lushchak & Bagnyuova, 2007
Oligocottus maculosus tidepool sculpin	TOSC_H_2O_2 ***MDA***				6 h hypoxia 3.5 kPa (at Pcrit)	Lau, 2018
Oligocottus maculosus tidepool sculpin	TOSC_H_2O_2 ***MDA***				6 h hypoxia 2.5 kPa (65% of Pcrit)	Lau, 2018
Scorpaenichthys marmoratus cabezon sculpin	TOSC_H_2O_2 ***MDA***				6 h hypoxia 3.5 kPa (65% of Pcrit)	Lau, 2018
Oreochromis niloticus nile tilapia	SOD, CAT, GPx ***PC, MDA***			SOD ***PC***	3 h hypoxia 0.66 kPa	Welker et al., 2012
Cyphocharx abramoides branquina	SOD GPx MDA			SOD* GPx ***MDA****	3 hours 2.4-2.9 kPa	present study

antioxidant activity. In six cases, the tissue had recovered from oxidative damage and lowered its antioxidant responses. Thus, in these hypoxia-tolerant species, of which *C. abramoides* is one, re-oxygenation does not usually incur new oxidative damage. In fact, stasis or rapid repair of damage and a return to "normal" antioxidant activity levels occurs during the first couple of hours of re-oxygenation.

4.5. Integrity of individual oxidative state

Different tissues in the body experience different oxygen tensions, both relative to each other and associated with metabolic demands. During hypoxia, blood is preferentially shunted to critical organs with high oxygen demand, such as the brain and heart (Perry et al., 2009). Our data show that in spite of these differences, the body (at least the three tissues studied here) has a coherent oxidative state. Within individuals, the activity of SOD in the gills was positively correlated to the level of SOD activity in the brain (Fig. 8A): note that SOD did not respond to hypoxic exposure in these tissues, so these are baseline activities. The body also showed some co-ordinated responses to hypoxia, at least between the gills and liver. Within individual fish, as GPx activity increased in the gills, so did the activities of SOD and GPx in the liver (Fig. 8B and C). The hypoxic antioxidant response of the brain,

however, was independent of these other two tissues. Brain GPx activity, which increased on average in hypoxia, was not correlated within individuals with increases in antioxidant activities in the gills or liver, suggesting that oxidative responses in the brain were not closely linked to those in the gill and liver. This may be due to variation in vascular shunting to the brain amongst individuals. Alternately, other antioxidants may be important in the brain which modify any relationship with the gill and liver antioxidants measured here.

4.6. Perspective

Addressing our question on the relative importance of hypoxia and re-oxygenation to oxidative damage in hypoxia-tolerant fish, the data on *C. abramoides*, supported by literature information, indicate that the majority of oxidative damage occurs during hypoxia not during re-oxygenation. The data from *C. abramoides* further indicate that the rate of re-oxygenation does not influence the level of oxidative damage on re-oxygenation. The lack of oxidative damage on re-oxygenation and lack of effect of the rate of re-oxygenation observed in the present study, may mean that: (1) the rate does not matter, (2) the fish were already acclimated to re-oxygenation after hypoxic exposure from their daily experience in the river, and/or (3) hypoxic exposure was sufficient to trigger protective mechanisms, such as, higher antioxidant activity to prevent (or lessen) further damage on re-oxygenation, as suggested by Hermes-Lima et al. (2015) in their 'Protection against Oxidative Stress' (POS) hypothesis. Many fish species do not have elevated antioxidant enzyme activities with hypoxia exposure (Leveelahti et al., 2014). Thus, the hypothesis that the rate of re-oxygenation may affect the level of oxidative damage, still needs to be tested in a less hypoxia-tolerant species which does not increase its antioxidant activity in hypoxic conditions.

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