

## RESEARCH ARTICLE

# Distinct metabolic adjustments arise from acclimation to constant hypoxia and intermittent hypoxia in estuarine killifish (*Fundulus heteroclitus*)

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## ABSTRACT

Many fish experience daily cycles of hypoxia in the wild, but the physiological strategies for coping with intermittent hypoxia are poorly understood. We examined how killifish adjust O<sub>2</sub> supply and demand during acute hypoxia, and how these responses are altered after prolonged acclimation to constant or intermittent patterns of hypoxia exposure. We acclimated killifish to normoxia (~20 kPa O<sub>2</sub>), constant hypoxia (2 kPa) or intermittent cycles of nocturnal hypoxia (12 h:12 h normoxia: hypoxia) for 28 days, and then compared whole-animal O<sub>2</sub> consumption rates ( $\dot{M}_{O_2}$ ) and tissue metabolites during exposure to 12 h of hypoxia followed by reoxygenation in normoxia. Normoxia-acclimated fish experienced a pronounced 27% drop in  $\dot{M}_{O_2}$  during acute hypoxia, and modestly increased  $\dot{M}_{O_2}$  upon reoxygenation. They strongly recruited anaerobic metabolism during acute hypoxia, indicated by lactate accumulation in plasma, muscle, liver, brain, heart and digestive tract, as well as a transient drop in intracellular pH, and they increased hypoxia inducible factor (HIF)-1 $\alpha$  protein abundance in muscle. Glycogen, glucose and glucose-6-phosphate levels suggested that glycogen supported brain metabolism in hypoxia, while the muscle used circulating glucose. Acclimation to constant hypoxia caused a stable ~50% decrease in  $\dot{M}_{O_2}$  that persisted after reoxygenation, with minimal recruitment of anaerobic metabolism, suggestive of metabolic depression. By contrast, fish acclimated to intermittent hypoxia maintained sufficient O<sub>2</sub> transport to support normoxic  $\dot{M}_{O_2}$ , modestly recruited lactate metabolism and increased  $\dot{M}_{O_2}$  dramatically upon reoxygenation. Both groups of hypoxia-acclimated fish had similar glycogen, ATP, intracellular pH and HIF-1 $\alpha$  levels as normoxic controls. We conclude that different patterns of hypoxia exposure favour distinct strategies for matching O<sub>2</sub> supply and O<sub>2</sub> demand.

**KEY WORDS:** **Respirometry, Hypoxia tolerance, Metabolic depression, Excess post-hypoxic oxygen consumption**

## INTRODUCTION

Hypoxia (low O<sub>2</sub> availability) is widespread in aquatic environments (Breitburg et al., 2018; Diaz and Breitburg, 2009; Ficke et al., 2007). Animals can be exposed to low O<sub>2</sub> for prolonged periods in ice-covered ponds or in deep and/or stratified waters, particularly when

hypoxic zones are difficult to avoid or escape (e.g. ponds isolated from other waterways, large dead zones stretching hundreds of kilometres) (Diaz, 2001; Diaz and Rosenberg, 2008). Animals can also be exposed to intermittent cycles of hypoxia, in which bouts of low O<sub>2</sub> are separated by periods of normoxia (or even hyperoxia), as is common in tide pools, estuaries, coral reefs and heavily vegetated systems (Andersen et al., 2017; Breitburg, 1992; Diaz, 2001; Nilsson and Renshaw, 2004; Richards, 2011; Tyler et al., 2009). Instances of both constant hypoxia and intermittent hypoxia are well documented in aquatic environments, but the biological effects of the latter have received comparably little attention. Studies that have investigated these effects in fish suggest that intermittent hypoxia can affect whole-animal O<sub>2</sub> consumption rates ( $\dot{M}_{O_2}$ ) (Taylor and Miller, 2001), growth rates (Cheek, 2011; Stierhoff et al., 2009), behaviour (Brady and Targett, 2010; Brady et al., 2009), gene expression and protein abundance in tissues (Dowd et al., 2010; Rytönen et al., 2012), and some other aspects of physiology that underlie hypoxia tolerance (Borowiec et al., 2015; Du et al., 2016; Routley et al., 2002; Taylor and Miller, 2001; Yang et al., 2013). With only sporadic investigation of the physiological effects of intermittent hypoxia, it is unclear whether these two patterns of hypoxia exposure represent similar or distinct stressors to aquatic animals.

Hypoxia challenges animals by disrupting the balance between O<sub>2</sub> supply and O<sub>2</sub> demand, and thus constraining cellular ATP supply (Bickler and Buck, 2007; Boutilier, 2001; Hochachka et al., 1996). Animals encountering environmental hypoxia may compensate for decreased O<sub>2</sub> supply to tissues through an assortment of cardiorespiratory responses, including amplified gill ventilation and greater gill surface area, increased haematocrit, and enhanced haemoglobin–O<sub>2</sub> binding affinity (Hughes, 1973; Nikinmaa and Soivio, 1982; Perry et al., 2009; Scott et al., 2008; Sollid et al., 2003), to help sustain routine aerobic metabolism. Alternatively, a decrease in aerobic ATP production can be offset by relying more heavily on O<sub>2</sub>-independent (anaerobic) energy pathways or by decreasing ATP demands (i.e. metabolic depression). Both strategies can lengthen survival in hypoxia, but they have different underlying mechanisms, costs and benefits (Bickler and Buck, 2007; Gorr et al., 2010; Hopkins and Powell, 2001; Richards, 2009). Increased use of anaerobic glycolysis is a common response of fish to hypoxia (Bickler and Buck, 2007; Richards, 2009; Routley et al., 2002; Virani and Rees, 2000; Vornanen et al., 2009). Increases in flux through anaerobic pathways can occur rapidly (Richards et al., 2008; van Ginneken et al., 1995; Wallimann et al., 1992) and can then be reinforced over time by modifications in gene expression and/or enzyme activities (Almeida-Val et al., 2011; Greaney et al., 1980; Martinez et al., 2006). However, chronic use of anaerobic metabolism is unsustainable in most species due to the depletion of carbohydrate fuel stores, the accumulation of end products (e.g. lactate, protons), and other detrimental repercussions such as the development of

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metabolic acidosis (Dunn and Hochachka, 1986; Richards, 2009; Richards et al., 2007; Speers-Roesch et al., 2012, 2013; Thomas et al., 1986). During normoxic recovery, repayment of the 'O<sub>2</sub> debt' accrued during hypoxia exposure requires that glucose/glycogen be resynthesized and anaerobic end products be cleared, potentially associated with various transcriptomic and proteomic adjustments (Dowd et al., 2010; Rytönen et al., 2012), all of which require additional energy and may contribute to the excess post-hypoxic O<sub>2</sub> consumption (EPHOC) (Lewis et al., 2007; Plambech et al., 2013; van den Thillart and Verbeek, 1991).

The use of metabolic depression when O<sub>2</sub> supply is limited is a less common response to hypoxia that is only exhibited in some hypoxia-tolerant species (Guppy and Withers, 1999; Hochachka et al., 1996; Lutz and Nilsson, 2004; Nilsson and Renshaw, 2004). In these species, ATP demands are greatly decreased by regulated cutbacks in many energetically costly processes (Bickler and Buck, 2007; Guppy and Withers, 1999; Hochachka et al., 1996; Richards, 2009). While this approach avoids the pitfalls of accelerated anaerobic metabolism, the so-called 'turning down the pilot light' (Hochachka et al., 1996) has other physiological and ecological costs. Metabolically depressed animals may have little energy to invest in important fitness-relevant functions beyond those that maintain survival (e.g. locomotion, reproduction, growth, immune defence, etc.), they may have diminished cognitive ability (Johansson et al., 1997), and they may be more susceptible to disease and predation (Burton and Reichman, 1999; Nilsson et al., 1993; Prendergast et al., 2002). It is therefore likely that the use of metabolic depression is restricted to bouts of hypoxia that are especially prolonged or severe (Regan et al., 2017a).

Intermittent cycles of hypoxia are likely to pose challenges that require physiological coping responses that are distinct from the responses to constant hypoxia. Routine hypoxia–reoxygenation cycles may lessen the costs of prolonged reliance on anaerobic metabolism by providing regular periods of recovery in normoxia. Moreover, if hypoxia occurs rapidly, then physiological responses to hypoxia that are relatively slow to induce or reverse may not be possible. For example, gradual induction of hypoxia leads to significantly lower critical O<sub>2</sub> tension in goldfish (*Carassius auratus*), suggesting that a minimum amount of time is needed for some hypoxia responses to take place (Regan and Richards, 2017). Intermittent hypoxia may also introduce further, unique challenges associated with recurrent reoxygenation or the costs of repeated metabolic transitions (Ivanina et al., 2016; Prabhakar et al., 2001; Semenza and Prabhakar, 2007). The responses to such challenges of cyclical hypoxia may contribute a natural hypoxic preconditioning that improves tolerance of subsequent hypoxia exposure (Nilsson and Renshaw, 2004).

Our recent work on the mummichog (*Fundulus heteroclitus*), a well-studied estuarine fish (Burnett et al., 2007), suggests that nocturnal intermittent hypoxia and constant hypoxia are indeed distinct stressors (Borowiec et al., 2015; Du et al., 2016). Fish exhibited different acclimation responses to each pattern of hypoxia exposure to improve hypoxia tolerance, including changes in routine daytime  $\dot{M}_{O_2}$ , the activity of metabolic enzymes involved in glycolysis and recovery, the O<sub>2</sub> carrying capacity of the blood, gill morphology, and muscle phenotype. However, the implications of these distinct acclimation responses to the strategy used by fishes to balance cellular O<sub>2</sub> supply and O<sub>2</sub> demand, and thus to cope with hypoxia, remain unclear. For example, it is unclear how  $\dot{M}_{O_2}$  may vary across a hypoxia–normoxia cycle in fish acclimated to intermittent hypoxia, especially during night-time hypoxia exposure, and how this might compare to fish acclimated to

constant hypoxia. Our earlier work suggested that there are considerable differences in metabolic enzyme activities in the liver between fish acclimated to intermittent and constant patterns of hypoxia exposure (Borowiec et al., 2015; Du et al., 2016), but it remains unclear how these changes affect tissue energy metabolism. Our objective here was to investigate this issue by contrasting the whole-animal and tissue-level metabolic responses to hypoxia in fish acclimated to constant hypoxia versus intermittent diel cycles of nocturnal hypoxia. To do this, we determined the responses to acute hypoxia in normoxia-acclimated killifish, and then examined how these responses are altered by chronic exposure to intermittent hypoxia or constant hypoxia.

## MATERIALS AND METHODS

### Study animals

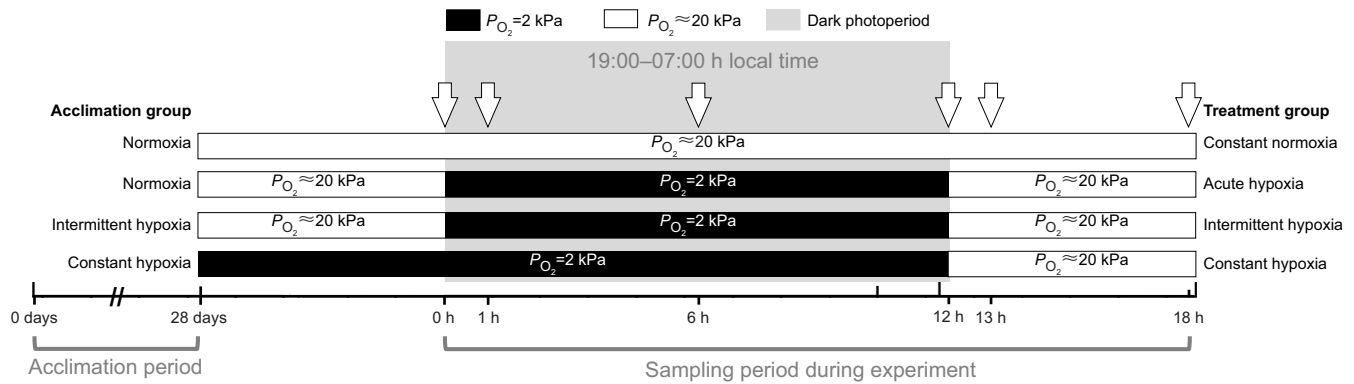
Adult, wild-caught *Fundulus heteroclitus* (Linnaeus 1766) of mixed sex (~2–5 g) were purchased and shipped from a commercial supplier (Aquatic Research Organisms, NH, USA) to McMaster University, Ontario, Canada. Fish were held at room temperature (~22°C) in well-aerated brackish (4 ppt) water on a photoperiod of 12 h light (07:00 to 19:00 h local time) to 12 h dark. Fish were fed by hand between ~09:00 and 11:00 h local time to satiation with commercial flakes (Big Al's Aquarium Supercentres, Mississauga, ON, Canada) 5 days per week. Water quality (ammonia, pH, nitrates and nitrites) was maintained by cycling water through a charcoal filter, and by routine water changes. Fish were held in well-aerated normoxic conditions for at least 4 weeks before acclimation treatments (see below). All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

### Hypoxia acclimations

Fish were subjected to one of three 28 day acclimation treatments: (i) normoxia (~20 kPa O<sub>2</sub>, ~8 mg O<sub>2</sub> l<sup>-1</sup>), (ii) constant hypoxia (2 kPa O<sub>2</sub>, ~0.8 O<sub>2</sub> l<sup>-1</sup>), or (iii) nocturnal ('intermittent') hypoxia (12 h normoxia during the daytime light phase: 12 h of hypoxia during the night-time dark phase). We chose a 12 h:12 h pattern of intermittent hypoxia, in which hypoxia occurred during the dark phase of the daily cycle, because it represents a reasonable representation of the diel patterns of hypoxia experienced in estuaries along the east coast of North America (Tyler et al., 2009). Acclimations were carried out in 35 l glass aquaria under the same salinity, photoperiod, temperature and feeding conditions as are described above. Normoxia was achieved by continuously bubbling aquarium water with air. Hypoxia exposure was achieved by regulating the O<sub>2</sub> tension in the aquaria using a galvanic O<sub>2</sub> sensor that automatically controlled the flow of nitrogen with a solenoid valve (Loligo Systems, Tjele, Denmark), as described previously (Borowiec et al., 2015). Fish were prevented from respiring at the water surface during hypoxic periods with a plastic grid barrier and bubble wrap.

### Respirometry measurements

We used stop-flow respirometry to measure the changes in  $\dot{M}_{O_2}$  during a 24 h cycle of normoxia–hypoxia–reoxygenation for each acclimation group (Fig. 1). As a normoxic control, we also measured  $\dot{M}_{O_2}$  during a 24 h cycle of normoxia in an additional set of fish from the normoxia acclimation group, so that diurnal changes in  $\dot{M}_{O_2}$  could be measured across the same times of day (Fig. 1). Measurements were made in respirometry chambers (90 ml cylindrical glass) that were situated in a buffer tank (with its sides covered in dark plastic to minimize visual disturbance to the fish)



**Fig. 1. Experimental groups used in this study.** Killifish were first acclimated for 28 days to normoxia ( $\sim 20$  kPa), intermittent cycles of nocturnal hypoxia (12 h normoxia during the daytime light phase: 12 h of 2 kPa  $O_2$  during the night-time dark phase), or constant hypoxia at 2 kPa  $O_2$ . Whole-animal  $O_2$  consumption was measured throughout a normoxia–hypoxia–reoxygenation cycle in each acclimation group, with the exception that fish acclimated to constant hypoxia were held in hypoxia until transitioning to normoxia at the same time as the acute hypoxia and intermittent hypoxia groups. A second treatment group of normoxia-acclimated fish were held in constant normoxia throughout to act as time-matched normoxia controls. The grey shading represents the dark phase of the daily cycle, which lasted from 07:00 to 19:00 h local time in all groups, with the light phase making up the remainder of the daily cycle. In a different set of fish, each treatment group was sampled for measurements of tissue metabolites at the times indicated by white arrows (fish at the 0 and 12 h time points were sampled immediately before the change in  $P_{O_2}$  and/or light phase took effect).

and connected to two water circulation circuits. One of these circuits flushed the respirometry chamber with water from the surrounding buffer tank ('flushing circuit'). The second circuit continuously pumped water from the respirometry chamber across a fibre-optic  $O_2$  sensor (PreSens, Regensburg, Germany) in a closed loop ('recirculation circuit'). The  $O_2$  level in the respirometry chambers could thus be set by controlling the  $O_2$  levels in the buffer tank, which was accomplished by bubbling with air or by using the same galvanic  $O_2$  sensor system that was used for acclimations (see above). Water temperature and salinity were matched to the acclimation conditions.

To obtain respirometry measurements, fish were transferred into a respirometry chamber immediately following their 28 day acclimation at approximately 09:00 h local time. During this period of habituation to the chambers,  $O_2$  levels were maintained at those that the fish would normally experience at that time of day in acclimation (i.e. hypoxia for the constant hypoxia group, normoxia for all other acclimation groups).  $\dot{M}_{O_2}$  was then measured every  $\sim 14$  min by alternating between 10 min flush periods, when the flushing circuit was on and the respirometry chamber was maintained at the conditions in the buffer tank, and 3 min measurement periods, when the flushing circuit was off, and the chamber was thus a closed system, allowing  $\dot{M}_{O_2}$  to be measured as the rate of change in oxygen concentration in the water. The effects of handling stress subsided and fish achieved a stable resting  $\dot{M}_{O_2}$  within 8 h of being placed in the chamber. Animals were held at their acclimation  $O_2$  level until shortly before 19:00 h local time. Fish in the normoxia and intermittent hypoxia acclimation groups were then transitioned to hypoxia (2 kPa  $O_2$ , which took  $\sim 25$  min), while fish acclimated to constant hypoxia were maintained at the 2 kPa hypoxia in which they were held during acclimation, and  $\dot{M}_{O_2}$  was measured in hypoxia for 12 h for all three acclimation groups. Each of these groups (including the constant hypoxia group) was then transitioned to normoxia (which again took  $\sim 25$  min), and  $\dot{M}_{O_2}$  was measured for an additional  $\sim 6$  h. The normoxic controls were treated similarly, except that they were held in normoxia for the full duration of respirometry measurements. The changes in  $\dot{M}_{O_2}$  within each treatment group were analyzed using one-way repeated-measures ANOVA (see 'Statistical analysis' below).

We compared the  $\dot{M}_{O_2}$  data, processed into five distinct measurements, between each treatment group using one-way ANOVA (see 'Statistical analysis' below). Resting daytime  $\dot{M}_{O_2}$  in normoxia was determined as the average of the last 10 stable measurements before lights off at 19:00 h local time. The minimum  $\dot{M}_{O_2}$  during night-time (the single lowest value recorded between 19:00 and 07:00 h local time, 0 h and 12 h in our study) was also determined for each individual fish, as was the average  $\dot{M}_{O_2}$  over the entire 12 h night-time dark phase. EPHOC was determined during reoxygenation in normoxia by calculating the area under the curve of measured  $\dot{M}_{O_2}$  above resting  $\dot{M}_{O_2}$  over time. EPHOC was calculated for the initial 90 min of normoxia (12–13.5 h) and for the entire 6 h recovery period (12–18 h). For comparison purposes, measurements analogous to EPHOC (referred to as daytime excess  $O_2$  consumption) were also made for the constant normoxia group, although these animals were not exposed to hypoxia during the experiment.

### Metabolite measurements

We investigated the tissue-level metabolic changes that occurred over the hypoxia–reoxygenation cycle by sampling fish in each treatment group over a time course of the daily cycle (Fig. 1). The day before sampling, at  $\sim 10:00$  h local time following the 28 day acclimation, fish were transferred into a series of small plastic enclosures (up to 4 fish per enclosure) held within the same glass aquaria as was used for acclimations (and weighed down to sit at the bottom of the tank with large air stones), to facilitate rapid euthanasia and freeze-clamping of tissues – an approach that has previously been used with success in this species (Richards et al., 2008). Enclosures were 2.1 l in volume and were constructed with a mesh lid and sides to allow water to flow through the submerged enclosure. The galvanic  $O_2$  sensor system that is described above (Loligo Systems) continued to control  $O_2$  levels in the water to those appropriate for each acclimation group. A submersible filter and water pumps ensured adequate water circulation and uniform  $O_2$  tensions within the enclosures and throughout the aquarium. Fish were held in these enclosures in their acclimation condition until the following day, and were then sampled at times during the daily cycle depicted by arrows in Fig. 1. One set of fish in the normoxia acclimation group were sampled in normoxia at 18:00 h (0 h), after 1, 6 or 12 h of acute hypoxia (2 kPa  $O_2$ ) during the night-time dark

phase (at 20:00, 01:00 or 07:00 h, respectively), or after 1 or 6 h of reoxygenation in normoxia during the daytime light phase (at 08:00 or 13:00 h, respectively). A second set of fish in the normoxia acclimation group acted as time-matched normoxia controls, held in normoxia and sampled at the same times of day but not exposed to low oxygen. Fish in the intermittent hypoxia acclimation group were also sampled at the same times of day, during the normal hypoxia–reoxygenation cycle that they experienced in acclimation (i.e. in normoxia at 18:00 h, after 1, 6 or 12 h of hypoxia, or after 1 or 6 h of reoxygenation in normoxia). Fish acclimated to constant hypoxia were sampled in hypoxia (2 kPa  $O_2$ ) at 18:00, 20:00, 01:00 or 07:00 h, or were transitioned to normoxia at 07:00 h and sampled after 1 h (08:00 h) or 6 h (13:00 h) of daytime reoxygenation. A two-way ANOVA was used to assess the effects of treatment group, sampling time and their interaction on metabolite levels (see ‘Statistical analysis’ below).

To sample fish at each time point, the mesh-covered enclosures were gently removed from the aquaria and excess water was allowed to drain out of the mesh sides, leaving the fish in a  $\sim 1$  l reservoir of water. Fish were quickly anaesthetized by adding a concentrated solution of benzocaine (Sigma-Aldrich, Oakville, ON, Canada) dissolved in 95% ethanol into the enclosure (final concentration  $1 \text{ g l}^{-1}$ ) to the reservoir. Because the water in the reservoir was from the aquarium, fish were held in the partial pressure of  $O_2$  ( $P_{O_2}$ ) of their current treatment during euthanasia, such that fish sampled from hypoxic water were not exposed to normoxia prior to sampling. Fish were removed in pairs, weighed and quickly dissected. A transverse section of the axial muscle (containing both red and white muscle fibres) was cut at the anterior base of the anal fin and immediately freeze clamped between two aluminium blocks pre-cooled in liquid nitrogen. Blood was collected from the caudal blood vessels in a heparinized capillary tube, spun at  $12,700 \text{ g}$  for 3 min, and the plasma was isolated and frozen in liquid nitrogen. The liver and the whole brain were dissected and freeze clamped. Freeze clamping occurred rapidly (within 1 min of euthanasia) to minimize changes in metabolite levels. The heart and the full length of the digestive tract (cleared of its contents) were then dissected and frozen in liquid nitrogen. All samples were stored at  $-80^\circ\text{C}$  until analyzed.

### Intracellular pH and tissue metabolite analysis

For all analyses, frozen samples of muscle, brain, liver and digestive tract were first ground into a fine powder using an insulated mortar and pestle that was pre-cooled with liquid nitrogen, and then returned to storage at  $-80^\circ\text{C}$  until the subsequent homogenization and analysis of metabolites (see ‘Statistical analysis’ below). The only exception was the heart, which was not ground into a powder but was instead homogenized whole for analysis.

ATP, phosphocreatine and carbohydrate metabolites were measured in approximately 20–30 mg of powdered muscle, liver and brain samples. Tissue was homogenized for 20 s in 300  $\mu\text{l}$  of ice-cold 6%  $\text{HClO}_4$  using the highest setting of a PowerGen 125 electric homogenizer (Fisher Scientific, Whitby, ON, Canada). Homogenates were vortexed, and 100  $\mu\text{l}$  of the acidified extract was immediately frozen in liquid nitrogen for later analysis of glycogen, glucose and glucose-6-phosphate content. The remaining homogenate was centrifuged at  $4^\circ\text{C}$  for 10 min at  $10,000 \text{ g}$ . The supernatant was transferred to a new microcentrifuge tube and neutralized ( $6.8 \leq \text{pH} \leq 7.2$ ) with  $3 \text{ mol l}^{-1} \text{ K}_2\text{CO}_3$ . Neutralized extracts were centrifuged at  $10,000 \text{ g}$  for 10 min at  $4^\circ\text{C}$ , and the resulting supernatant was immediately used for quantification of ATP, phosphocreatine and lactate content by standard methods adapted

for a 96-well plate format (Bergmeyer, 1983). Initial assay conditions were as follows: ATP,  $5 \text{ mmol l}^{-1}$  glucose,  $2 \text{ mmol l}^{-1} \text{ NADP}^+$ ,  $5 \text{ mmol l}^{-1} \text{ MgCl}_2$  and excess coupling enzyme ( $1 \text{ U ml}^{-1}$  glucose-6-phosphate dehydrogenase) in  $20 \text{ mmol l}^{-1}$  Tris (pH 8.0); lactate,  $2.5 \text{ mmol l}^{-1} \text{ NAD}^+$ , in glycine buffer ( $0.6 \text{ mol l}^{-1}$  glycine,  $0.5 \text{ mol l}^{-1}$  hydrazine sulphate, pH 9.4); phosphocreatine, conditions as listed for ATP, plus  $1 \text{ mmol l}^{-1}$  ADP. Assays were then conducted by adding an excess amount ( $5 \text{ U ml}^{-1}$ ) of the appropriate coupling enzyme (hexokinase for ATP, lactate dehydrogenase for lactate and creatine kinase for phosphocreatine). All assays were coupled to a change in NADH or NADPH concentration in the well, such that the absolute change in absorbance at 340 nm following the addition of the final coupling enzyme was indicative of the metabolite content of the extract. All metabolite assays were run in duplicate at  $37^\circ\text{C}$  on a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Lactate content was also assayed in the digestive tract ( $\sim 10 \text{ mg}$  of powdered tissue) and heart (the whole heart, typically  $\sim 5 \text{ mg}$ ) using the same methods that are described above for lactate.

Glycogen, glucose and glucose-6-phosphate content were measured in the acidified homogenates of muscle, liver and brain tissues. Aliquots of the acidified homogenate were thawed on ice, and 50  $\mu\text{l}$  of  $1 \text{ mol l}^{-1} \text{ K}_2\text{HCO}_3$  and 100  $\mu\text{l}$  of  $400 \text{ mmol l}^{-1}$  acetate buffer (pH 4.8) were added. Half of this solution was used to determine total glycogen content, by digesting the glycogen in the solution with 7  $\mu\text{l}$  of amyloglucosidase ( $4 \text{ U l}^{-1}$ , suspended in  $300 \text{ mmol l}^{-1}$  Tris-HCl,  $4.05 \text{ mmol l}^{-1} \text{ MgSO}_4$ , pH 7.5) for 2 h at  $40^\circ\text{C}$ . The other half of this solution was used to determine free glucose and glucose-6-phosphate, by simply incubating the solution without amyloglucosidase at  $4^\circ\text{C}$  for 2 h. All samples were neutralized with  $1 \text{ mol l}^{-1} \text{ K}_2\text{HCO}_3$  following the 2 h incubation. Glucose-6-phosphate content was first determined in undigested samples by measuring the difference in absorbance after the addition of excess of the coupling enzyme glucose-6-phosphate dehydrogenase ( $3 \text{ U ml}^{-1}$ ) under the following conditions:  $1 \text{ mmol l}^{-1}$  ATP,  $0.5 \text{ mmol l}^{-1} \text{ NADP}^+$  and  $5 \text{ mmol l}^{-1} \text{ MgCl}_2$  in  $20 \text{ mmol l}^{-1}$  imidazole (pH 7.4). Glucose content was then assayed in both undigested samples (containing only endogenous free glucose) and digested samples (containing both endogenous free glucose and glucose originating from the enzymatic breakdown of glycogen) by measuring the change in absorbance with the addition of excess of the coupling enzyme hexokinase ( $5 \text{ U ml}^{-1}$ ) under the same conditions as for glucose-6-phosphate plus excess coupling enzyme (glucose-6-phosphate dehydrogenase). The difference in glucose content detected between the digested and undigested samples was used to calculate sample glycogen content.

Intracellular pH ( $\text{pH}_i$ ) was measured in a separate portion of powdered muscle and liver ( $\sim 10$ – $30 \text{ mg}$ ) samples, using a method similar to those that have been described previously (Baker et al., 2009; Pörtner, 1990; Pörtner et al., 1990). Tissue was briefly homogenized in 0.5 ml Pellet Pestle Microtubes (Fisher Scientific) containing five volumes of ice-cold  $150 \text{ mmol l}^{-1} \text{ KCl}$  and  $8 \text{ mmol l}^{-1}$  of the metabolic inhibitor nitrilotriacetic acid. The microtube was quickly capped to minimize loss of  $\text{CO}_2$  and incubated on ice for 10 min. The homogenate was then vortexed and its pH was measured within 15–20 s with a glass microelectrode (Sartorius, Bohemia, NY, USA) that was preconditioned to the appropriate temperature in ice-cold homogenization solution.

### Protein abundance of HIF1 $\alpha$

We measured hypoxia inducible factor (HIF)-1 $\alpha$  protein abundance in skeletal muscle tissue by immunoprecipitation followed by western

blotting. Powdered muscle tissue (~50 mg) was homogenized on ice with 30 passes of a glass Tenbroeck tissue grinder in 20 volumes of immunoprecipitation (IP) buffer, which was composed of 1% IGEPAL CA 630 (octylphenol ethoxylate), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol l<sup>-1</sup> EDTA, 1 mmol l<sup>-1</sup> orthovanadate, 1% protease inhibitor cocktail (Sigma P-8340) and 50 µg ml<sup>-1</sup> MG 132 (Z-Leu-Leu-Leu-aldehyde) in phosphate buffered saline (137 mmol l<sup>-1</sup> NaCl, 2.7 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 1.76 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, pH 7.6). Homogenates were centrifuged at 10,000 g for 10 min at 4°C, and supernatant protein content was determined using the bicinchoninic acid assay (Thermo Fisher Scientific), in which interfering substances were first removed by precipitation with trichloroacetic acid and deoxycholate (Brown et al., 1989). Tissue supernatants were diluted to 2 mg protein ml<sup>-1</sup> in IP buffer and HIF-1α was immunoprecipitated from 1 ml samples using a chicken polyclonal antibody generated against a recombinant fragment of HIF-1α from *F. heteroclitus* (Townley et al., 2017). We first eliminated non-specific binding by pre-incubating the samples with 20 µl of secondary antibody (goat anti-chicken IgY bound to agarose beads; Aves Labs, Tigard, OR, USA) for 30 min at 4°C, followed by centrifugation at 1500 g for 10 min at 4°C, and then reserving the supernatant for use in the following steps. Primary antibody (4 µl of 0.92 mg ml<sup>-1</sup> solution) was added to each 1 ml of supernatant, which was then incubated for 1 h on ice with gentle rocking. This was followed by addition of 20 µl of secondary antibody and incubation overnight at 4°C with gentle rocking. The following morning, samples were centrifuged for 5 min at 1500 g at 4°C, and the supernatant was discarded. The agarose beads (bound to HIF-1α) were washed by resuspension in TBST (20 mmol l<sup>-1</sup> Tris, 150 mmol l<sup>-1</sup> NaCl, 0.05% Tween-20, pH 7.5), incubated for 5 min on ice with gentle rocking, centrifuged as above, and the supernatant was discarded. The agarose beads were washed once more in TBST, followed by two washes in TBS (10 mmol l<sup>-1</sup> Tris, 150 mmol l<sup>-1</sup> NaCl, 0.02% NaN<sub>3</sub>, pH 8.0) and two washes in 50 mmol l<sup>-1</sup> Tris (pH 6.8). The final pellet (containing the washed agarose beads bound to HIF-1α) was re-suspended in 26 µl of distilled H<sub>2</sub>O, 10 µl of LDS 4× sample buffer (Invitrogen) and 4 µl of 0.5 mol l<sup>-1</sup> dithiothreitol (DTT). The solution was briefly centrifuged (~15 s) and heated at 70°C for 10 min to denature the protein and to release HIF-1α from the immunoprecipitation beads. The solution was cooled to room temperature, spun at 1500 g for 5 min, and the supernatant was transferred to a new microcentrifuge tube. The pellet was then centrifuged again at 15,000 g for 5 min to crush the immunoprecipitation beads, and the small volume of resulting supernatant was combined with the first supernatant.

The final solution from the above immunoprecipitation of HIF-1α protein was used for gel electrophoresis. Solutions (~25 µl total) were loaded into a SDS-polyacrylamide gel (pre-cast 4–12% gradient NuPage Bis-tris gels; Invitrogen), and run at 150 V for 65 min in MOPS-SDS running buffer (50 mmol l<sup>-1</sup> MOPS, 50 mmol l<sup>-1</sup> Tris base, 0.1% SDS, 1 mmol l<sup>-1</sup> EDTA, pH 7.7). Proteins were wet transferred at 10°C onto a PVDF membrane for 2 h at 100 V using Invitrogen NuPage Transfer Buffer (25 mmol l<sup>-1</sup> Bicine, 25 mmol l<sup>-1</sup> Bis-Tris, 1 mmol l<sup>-1</sup> EDTA, pH 7.2; Fisher Scientific) containing 20% methanol and 0.05% SDS. After transfer, blots were blocked in 5% skim milk powder in TBST for 1 h at room temperature. The blot was then incubated in primary anti-HIF-1α antibody, diluted 1:500 in blocking buffer, and incubated for 1 h at room temperature and then overnight at 4°C. The blot was washed 3 times for 5 min each in TBST, and then incubated for 1 h at room temperature with a donkey anti-chicken

horseradish peroxidase secondary antibody (2 µl of 32 µg ml<sup>-1</sup> solution), diluted 1:5000 in blocking buffer. Blots were washed 5 times for 5 min each in TBST. Bound secondary antibody was detected by incubating the blot in ECL reagent (1.3 mmol l<sup>-1</sup> luminol, 0.2 mmol l<sup>-1</sup> p-coumaric acid, 0.01% H<sub>2</sub>O<sub>2</sub> in 100 mmol l<sup>-1</sup> Tris, pH 8.5) and imaging the resulting bands by chemiluminescence on a ChemiDoc XRS imager. The band intensity of each sample, relative to the intensity of the IgY band as a loading control, was analyzed using Image Lab software (Bio-Rad) as an indication of protein abundance. We only quantified HIF-1α protein in the skeletal muscle due to the large quantity of tissue required to perform the immunoprecipitation (at 12 h, 07:00 h sampling time only), and we compared each treatment group using one-way ANOVA (see ‘Statistical analysis’ below). HIF-1α protein abundance is reported in arbitrary units (band intensity of HIF-1α/band intensity of IgY) and is expressed relative to the abundance of the constant normoxia group.

### Statistical analysis

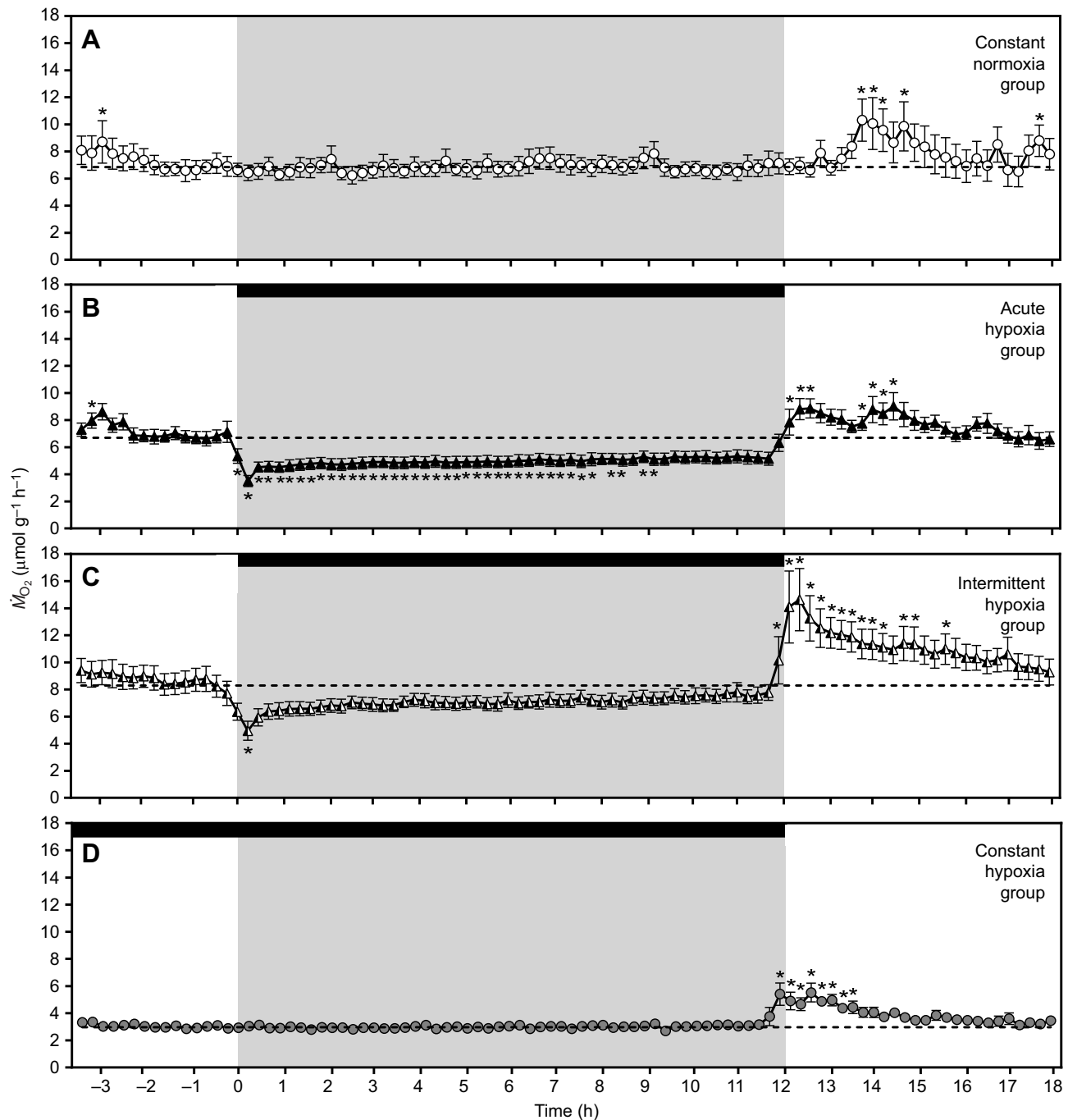
One-way or two-way ANOVA accompanied by *post hoc* Bonferroni tests were used to examine the effects of hypoxia exposure and treatment group. A significance level of  $P < 0.05$  was used for all statistical analyses. All data are reported as means ± s.e.m.

## RESULTS

### Whole-animal metabolism during the hypoxia–reoxygenation cycle

We examined the changes in  $\dot{M}_{O_2}$  during exposure to hypoxia followed by reoxygenation in normoxia in killifish that were acclimated for 28 days to normoxia, intermittent hypoxia or constant hypoxia (Fig. 1). Control fish held in normoxia (‘constant normoxia’ treatment group) had a relatively stable  $\dot{M}_{O_2}$ , but exhibited some circadian variation in  $\dot{M}_{O_2}$ , primarily reflected by modest increases in  $\dot{M}_{O_2}$  during the daytime from ~1.5 to 3 h after the lights were turned on, the typical time of day at which the fish were fed (Fig. 2A). Normoxia-acclimated fish that were exposed to their first bout of hypoxia (‘acute hypoxia’ treatment group) exhibited an initial ~50% decrease in  $\dot{M}_{O_2}$  after ~20 min of exposure (Fig. 2B). This decrease in  $\dot{M}_{O_2}$  recovered slightly by ~45 min of exposure, but  $\dot{M}_{O_2}$  remained low throughout much of the night-time hypoxia exposure. Reoxygenation in normoxia led to a moderate increase in  $\dot{M}_{O_2}$  that lasted ~5 h during recovery in normoxia. The changes in  $\dot{M}_{O_2}$  during hypoxia–reoxygenation were similar in fish that had been acclimated to this hypoxia–reoxygenation cycle on a daily basis for several weeks (‘intermittent hypoxia’ treatment group) (Fig. 2C). However, the decrease in  $\dot{M}_{O_2}$  in hypoxia was smaller and was completely recovered within ~2 h of exposure, and the increase in  $\dot{M}_{O_2}$  during reoxygenation appeared to be greater and to last longer relative to fish experiencing their first cycle of acute hypoxia. By contrast, fish acclimated to constant hypoxia (‘constant hypoxia’ treatment group) exhibited a low and stable  $\dot{M}_{O_2}$ , which increased modestly and transiently during reoxygenation in normoxia (Fig. 2D).

Hypoxia acclimation altered metabolic rate during hypoxia–reoxygenation compared to normoxia-acclimated fish that were experiencing their first bout of hypoxia (Fig. 3). Acclimation to constant hypoxia decreased  $\dot{M}_{O_2}$  both during hypoxia and after reoxygenation in normoxia. This appeared to be caused by general, active decreases in O<sub>2</sub> demands, because fish acclimated to constant hypoxia showed minimal EPHOC during reoxygenation in normoxia beyond a small increase in  $\dot{M}_{O_2}$  shortly after reoxygenation (Fig. 3B, 12–13.5 h of the daily cycle). The initial 90 min (12–13.5 h) of excess O<sub>2</sub> consumption of fish acclimated to constant hypoxia

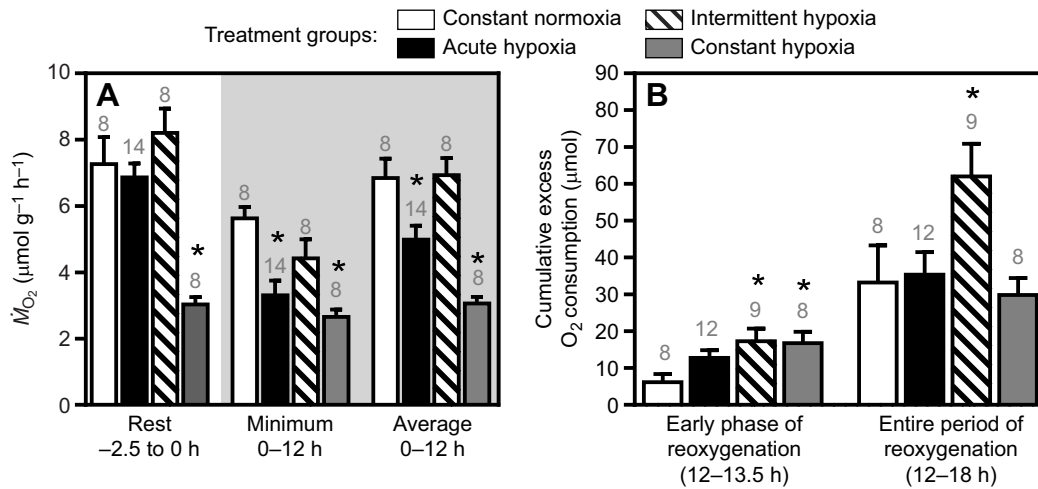


**Fig. 2.** Whole-animal oxygen consumption rate ( $\dot{M}_{O_2}$ ) of killifish acclimated to normoxia, intermittent hypoxia or constant hypoxia. (A) Fish acclimated to and held in constant normoxia (main effect of time on  $\dot{M}_{O_2}$ :  $F_{94,658}=2.543$ ,  $P<0.0001$ ). (B) Fish acclimated to constant normoxia, but exposed to 12 h of acute hypoxia at 2 kPa  $O_2$  during the night (0–12 h) followed by 6 h of reoxygenation in normoxia during the day ( $F_{94,1222}=19.59$ ,  $P<0.0001$ ). (C) Fish acclimated to intermittent cycles of nocturnal hypoxia (12 h normoxia:12 h hypoxia at 2 kPa  $O_2$ ) and measured during the same cycle ( $F_{94,658}=10.75$ ,  $P<0.0001$ ). (D) Fish acclimated to and measured in constant hypoxia at 2 kPa  $O_2$  until 12 h, followed by reoxygenation in normoxia ( $F_{98,686}=5.982$ ,  $P<0.0001$ ). The dark phase of the photoperiod (19:00 to 07:00 h local time) is indicated by the grey section of the background, and the timing of hypoxia exposures are indicated by black bars along the top of each panel. Dashed lines represent average resting  $\dot{M}_{O_2}$ . \*Significant pairwise difference from resting  $\dot{M}_{O_2}$ . Sample sizes were as follows: constant normoxia and constant hypoxia, 8; acute hypoxia, 14; intermittent hypoxia, 8.

represented ~50% of the total excess  $O_2$  consumption over the 6 h reoxygenation period, which far exceed that of the constant normoxia (~18%), acute hypoxia (~36%) and intermittent hypoxia (~30%) groups (Fig. 3B). In contrast, fish acclimated to intermittent hypoxia maintained  $\dot{M}_{O_2}$  in hypoxia at levels that were statistically indistinguishable from fish held in normoxia (Fig. 3A) and exhibited much greater EPHOC during reoxygenation than other

acclimation groups (Fig. 3B). This increase in EPHOC appeared to be due to increases in both the peak  $\dot{M}_{O_2}$  reached during reoxygenation as well as in total duration of the increase in  $\dot{M}_{O_2}$  (Fig. 2B).

Interestingly, the timing and pattern of the excess  $O_2$  consumption also appeared to change with hypoxia acclimation. The increase in  $\dot{M}_{O_2}$  during the day in the constant normoxia group did not occur until ~1.5–3 h after lights on and peaked at ~14 h of



**Fig. 3. Effects of hypoxia acclimation on  $\dot{M}_{O_2}$  during a hypoxia–reoxygenation cycle.** (A) Resting  $\dot{M}_{O_2}$  (measured from  $-2.5$  to  $0$  h of the daily cycle, which was in normoxia for all groups except the constant hypoxia group; main effect of treatment group,  $F_{3,34}=13.96$ ,  $P<0.0001$ ), minimum overnight  $\dot{M}_{O_2}$  (measured between  $0$  and  $12$  h of the daily cycle, which was in hypoxia for all groups except normoxic controls;  $F_{3,34}=7.771$ ,  $P=0.0004$ ) and average overnight  $\dot{M}_{O_2}$  (also measured from  $0$  to  $12$  h of the daily cycle;  $F_{3,34}=13.62$ ,  $P<0.0001$ ). (B) Cumulative increase in  $O_2$  consumption above resting  $\dot{M}_{O_2}$  in the early phase of reoxygenation in normoxia ( $12$ – $13.5$  h of the daily cycle;  $F_{3,32}=3.200$ ,  $P=0.0359$ ) and the entire normoxic reoxygenation period ( $12$ – $18$  h of the daily cycle;  $F_{3,33}=3.653$ ,  $P=0.0223$ ). \*Significant pairwise difference from the constant normoxia group ( $P<0.05$ ). Sample sizes for each group for each measurement are indicated directly above the bar.

the daily cycle. By contrast, normoxia-acclimated fish that were exposed to acute hypoxia exhibited a biphasic EPHOC response, with separate peaks in  $\dot{M}_{O_2}$  apparent at approximately  $12.5$  h and  $14$  h (Fig. 2B). The pattern of these daytime increases in  $\dot{M}_{O_2}$  in both normoxia-acclimated groups differed from fish acclimated to intermittent hypoxia and constant hypoxia, which showed single, early peaks at  $\sim 12.5$  h that declined thereafter, with no obvious second peak (Fig. 2C,D).

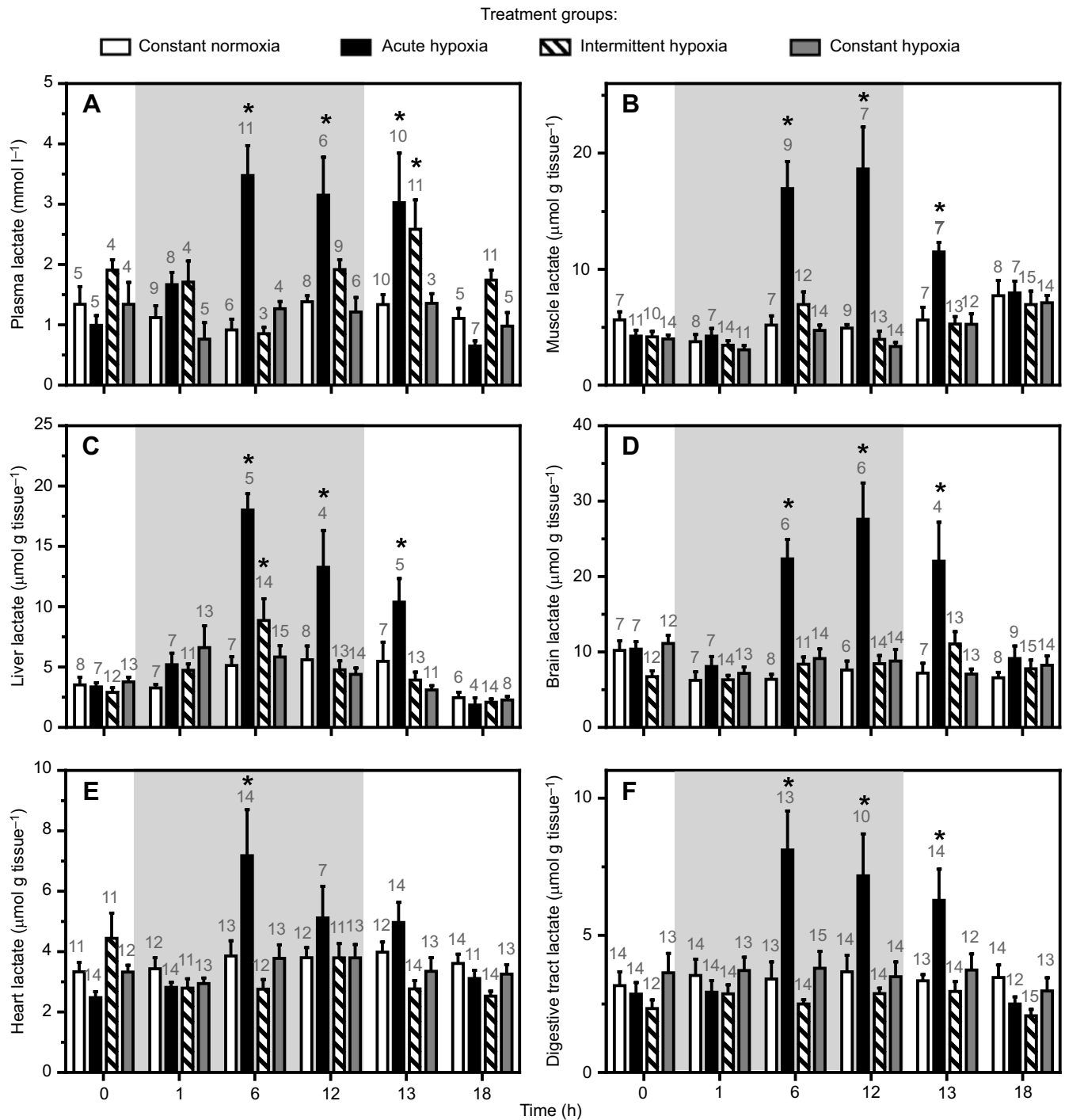
#### Tissue metabolites during a hypoxia–reoxygenation cycle

We tracked changes in the concentration of lactate, the end-product of anaerobic glycolysis, in a variety of tissues during hypoxia–reoxygenation. There was a significant time $\times$ treatment interaction on the lactate content of a number of tissues (Fig. 4, Table 1), because lactate concentration changed appreciably over time in the acute hypoxia group but not in the other groups. Fish experiencing their first cycle of hypoxia showed substantial 3.5-, 4.4-, 3.1-, 2.7- and 2.8-fold increases in lactate in the plasma, muscle, liver, brain and digestive tract, respectively, that peaked at 6–12 h of hypoxia and remained elevated after 1 h of recovery (13 h) in normoxia. Lactate content in the heart also increased at 6 h of hypoxia in the acute hypoxia group, but recovered to levels typical of normoxia before the end of the hypoxia exposure. These large increases in lactate content in the acute hypoxia group drove the significant main effects of time and treatment group in muscle, brain, digestive tract and heart (Table 1): there were no statistically significant changes in lactate content in these tissues in fish acclimated to constant hypoxia or to intermittent hypoxia. Lactate levels were also stable in the plasma and liver of fish acclimated to constant hypoxia (Fig. 4). However, fish acclimated to intermittent hypoxia accumulated a small amount of lactate in the liver at 6 h hypoxia (Fig. 4C) and released some lactate into the plasma at 1 h of recovery in normoxia (Fig. 4A).

We also examined  $\text{pH}_i$  in muscle and liver during hypoxia–reoxygenation. There was a significant time $\times$ treatment interaction on  $\text{pH}_i$  in both tissues (Fig. 5, Table 1), again caused by changes in only the acute hypoxia group, which also drove the significant main effects of time and treatment (Table 1). In the muscle, these effects

were mainly attributed to a pronounced but transient decline of  $0.32$  pH units in the acute hypoxia group at 6 h of hypoxia that was absent in all other groups (Fig. 5A). For normoxic-acclimated fish exposed to acute hypoxia, there was a less severe but still significant decrease in  $\text{pH}_i$  of  $0.17$  units at 6 h of hypoxia in liver, as well as a  $0.04$  unit rise in  $\text{pH}_i$  at 1 h of recovery in normoxia (Fig. 5B). Despite our observation that fish acclimated to intermittent hypoxia accumulated lactate in the liver during hypoxia exposure (Fig. 4C), they maintained a stable  $\text{pH}_i$  in this tissue and in the muscle throughout the time course.

The concentrations of glycogen, free glucose and glucose-6-phosphate were also examined in muscle and liver during hypoxia–reoxygenation. There appeared to be some diel variation in muscle glycogen levels, reflected by a significant main effect of time without significant effects of treatment group of the time $\times$ treatment interaction, although these statistical results seemed to be driven largely by lower glycogen levels in the late afternoon ( $0$  h) in most groups (Fig. 6A, Table 1). However, there were significant time $\times$ treatment interactions for free glucose and glucose-6-phosphate content in the muscle that resulted from changes in only the acute hypoxia group, and that also drove significant or marginally significant main effects of treatment and time (Table 1). These effects were primarily driven by large, 2.6- and 14.0-fold, increases at 6 h in free glucose and glucose-6-phosphate content, respectively, in the acute hypoxia treatment group, and these increased levels continued after reoxygenation in normoxia (Fig. 6B,C). There were also significant time $\times$ treatment interactions and main effect of time for glycogen content of the liver (Table 1), which seemed to be primarily driven by decreased liver glycogen at 12 h of hypoxia and after 1 h of reoxygenation in fish from the acute hypoxia treatment group (although the post-tests did not reach statistical significance) (Fig. 6B). This variation in the liver of the acute hypoxia group was associated with a 3.0-fold increase in free glucose content at 12 h, which was associated with a significant time $\times$ treatment interaction and a significant main effect of treatment (Fig. 6C, Table 1). Glucose-6-phosphate levels were somewhat variable in the liver, but there was a significant



**Fig. 4.** Fish exposed to acute hypoxia accumulated lactate in several tissues during hypoxia. (A–F) Dark phase of the photoperiod (19:00–07:00 h local time) is indicated by the grey section of the background. \*Significant pairwise difference from the constant normoxia group at the same time point ( $P < 0.05$ ). Sample sizes for each group at each time point are indicated directly above the bar.

time  $\times$  treatment interaction resulting from noticeable variation across time between acclimation groups (e.g. glucose-6-phosphate levels in the acute hypoxia group tended to be very low after 12 h of hypoxia) (Fig. 6F, Table 1). Overall, there appeared to be much more variation in these metabolites in fish from the acute hypoxia group that were experiencing their first cycle of hypoxia than in fish acclimated to constant hypoxia or intermittent hypoxia.

There was a pronounced depletion of brain glycogen stores in normoxia-acclimated fish experiencing acute hypoxia (Fig. 7). This

was reflected by a strong decrease in glycogen content in the acute hypoxia group at 6 h of hypoxia, which did not worsen by 12 h but persisted into the reoxygenation period in normoxia. Glycogen depletion did not occur during hypoxia in other treatment groups, such that there was a significant time  $\times$  treatment interaction on brain glycogen levels along with the significant main effects of time and of treatment (Table 1). The decreases in brain glycogen in the acute hypoxia group occurred in conjunction with increases in free glucose content, for which there was also a significant



**Table 1. Statistical analysis of data presented in Figs 4–7 and Table 2**

	Main effect of acclimation		Main effect of time		Interaction	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
<b>Brain</b>						
ATP	$F_{3,194}=1.331$	0.2654	$F_{5,194}=3.103$	0.0102	$F_{15,194}=1.697$	0.0541
Glucose	$F_{3,208}=11.63$	<0.0001	$F_{5,208}=2.100$	0.0667	$F_{15,208}=2.257$	0.0058
G6P	$F_{3,157}=1.282$	0.2826	$F_{5,157}=2.721$	0.0218	$F_{15,157}=1.136$	0.3284
Glycogen	$F_{3,202}=7.255$	0.0001	$F_{5,202}=3.400$	0.0057	$F_{15,202}=3.236$	<0.0001
Lactate	$F_{3,217}=37.59$	<0.0001	$F_{5,217}=10.42$	<0.0001	$F_{15,217}=6.741$	<0.0001
<b>Heart</b>						
Lactate	$F_{3,274}=4.574$	0.0038	$F_{5,274}=4.426$	0.0007	$F_{15,274}=3.452$	<0.0001
<b>Digestive tract</b>						
Lactate	$F_{3,298}=14.8$	<0.0001	$F_{5,298}=5.491$	<0.0001	$F_{15,298}=3.669$	<0.0001
<b>Liver</b>						
ATP	$F_{3,199}=2.177$	0.0919	$F_{5,199}=4.798$	0.0004	$F_{15,199}=1.309$	0.1992
Glucose	$F_{3,211}=4.103$	0.0072	$F_{5,211}=1.166$	0.0327	$F_{15,211}=1.163$	0.0722
G6P	$F_{3,167}=0.823$	0.4832	$F_{5,167}=2.099$	0.0679	$F_{15,167}=1.794$	0.0391
Glycogen	$F_{3,209}=2.076$	0.1044	$F_{5,209}=3.558$	0.0048	$F_{15,209}=2.344$	0.0040
Intracellular pH	$F_{3,185}=6.043$	0.0006	$F_{5,185}=6.594$	<0.0001	$F_{15,185}=3.821$	<0.0001
Lactate	$F_{3,207}=16.19$	<0.0001	$F_{5,207}=20.90$	<0.0001	$F_{15,207}=5.123$	<0.0001
<b>Muscle</b>						
ATP	$F_{3,226}=0.2029$	0.8943	$F_{5,226}=8.169$	<0.0001	$F_{15,226}=1.465$	0.1199
Glucose	$F_{3,212}=19.65$	<0.0001	$F_{5,212}=2.218$	0.0537	$F_{15,212}=3.815$	<0.0001
G6P	$F_{3,208}=5.772$	0.0008	$F_{5,208}=7.372$	<0.0001	$F_{15,208}=2.114$	0.0105
Glycogen	$F_{3,214}=1.196$	0.3121	$F_{5,214}=5.796$	<0.0001	$F_{15,214}=0.985$	0.4721
Intracellular pH	$F_{3,221}=8.181$	<0.0001	$F_{5,221}=7.072$	<0.0001	$F_{15,221}=6.159$	<0.0001
Lactate	$F_{3,226}=41.62$	<0.0001	$F_{5,226}=14.71$	<0.0001	$F_{15,226}=8.933$	<0.0001
Phosphocreatine	$F_{3,230}=4.184$	0.0066	$F_{5,230}=3.217$	0.0079	$F_{15,230}=1.443$	0.1285
<b>Plasma</b>						
Lactate	$F_{3,135}=7.830$	0.0001	$F_{5,135}=3.070$	0.0117	$F_{15,135}=2.511$	0.0027

G6P, glucose-6-phosphate.

time×treatment interaction and a significant main effect of treatment (Table 1). In contrast, fish acclimated to intermittent hypoxia were able to protect brain glycogen content and experienced no significant variation in free glucose throughout the hypoxia–normoxia cycle. Fish acclimated to constant hypoxia also had stable brain glycogen levels during hypoxia, but they experienced significantly lower brain glycogen content during reoxygenation compared with the constant normoxia group. Glucose-6-phosphate content in the brain was variable and showed a significant main effect of time, but was not affected by treatment or a time×treatment interaction (Table 1).

There was diel variation in the ATP content of all tissues, as reflected by significant main effects of time (Tables 1 and 2). This variation appeared to be most strongly attributed to decreases in ATP levels in the liver and brain, and to decreases in phosphocreatine content in the muscle in fish exposed to their first bout of hypoxia (and in some cases to some more modest variation in ATP in the intermittent hypoxia group). There was a significant main effect of treatment and of the time×treatment interaction on phosphocreatine in the muscle, but neither the main effects of treatment nor the time×treatment interactions were significant for ATP levels in any tissue (Table 1).

### Expression of HIF1 $\alpha$

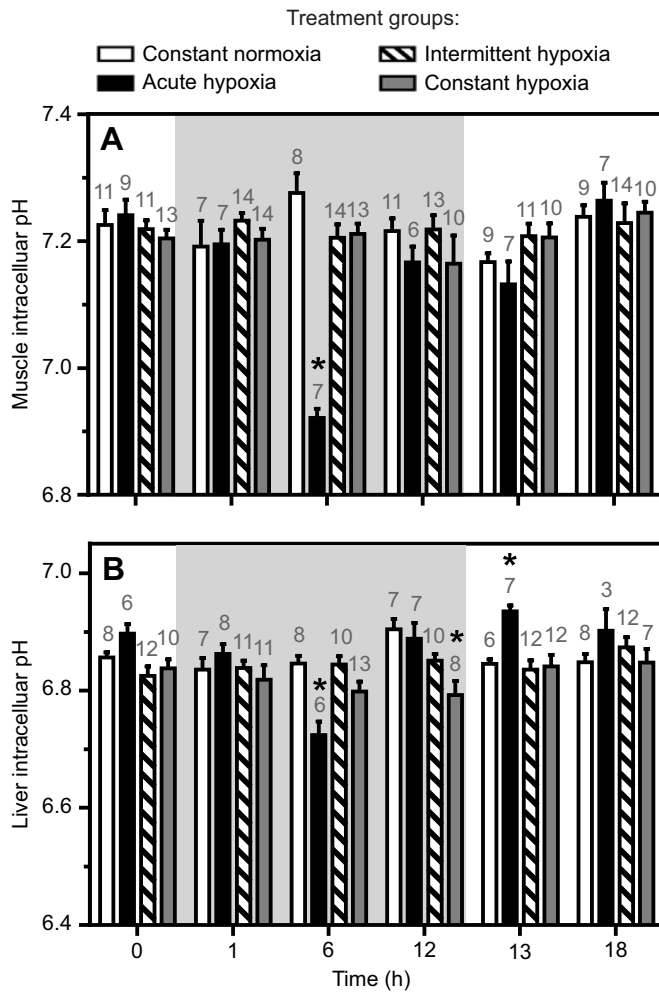
Hypoxia acclimation attenuated the increase in HIF-1 $\alpha$  protein levels in the muscle in both the constant hypoxia and intermittent hypoxia groups (Fig. 8). Fish experiencing their first bout of hypoxia (acute hypoxia group) showed a ~3-fold increase in HIF-1 $\alpha$  protein abundance in the muscle after 12 h of hypoxia exposure. In contrast, HIF-1 $\alpha$  protein levels were similar between fish acclimated to either intermittent hypoxia or constant hypoxia and those held in normoxia.

### DISCUSSION

Hypoxia in the aquatic environment comes in many forms and results from various biotic and abiotic causes. Here, we show that chronic exposure of the estuarine killifish to different patterns of hypoxia exposure can lead to divergent changes in physiology, each of which improve metabolic homeostasis compared with normoxic fish encountering their first bout of acute hypoxia. Acclimation to constant hypoxia appeared to induce a pronounced metabolic depression that helped decrease O<sub>2</sub> demands, avoid the recruitment of anaerobic metabolism, and thus safeguard intracellular pH, carbohydrate stores and cellular ATP levels. In contrast, acclimation to intermittent diel cycles of nocturnal hypoxia helped fish maintain sufficient O<sub>2</sub> transport to support routine O<sub>2</sub> demands, and may have modestly recruited lactate metabolism, but without any apparent metabolic acidosis or glycogen depletion. Therefore, our results suggest that both intermittent hypoxia and constant hypoxia induced robust acclimation responses that differed considerably from the response to acute hypoxia, but that these different patterns of exposure can result in distinct strategies for coping with hypoxia.

### Responses of killifish to acute hypoxia

Killifish exposed to their first bout of acute hypoxia were unable to maintain  $\dot{M}_{O_2}$  at resting normoxic levels (Figs 2 and 3) and appeared to recruit anaerobic metabolism (Figs 4 and 5). Increased use of anaerobic glycolysis is common in fish exposed to acute hypoxia, particularly when they are near or below their critical O<sub>2</sub> tension as they likely were during this experiment (Borowiec et al., 2015), and this is often associated with accumulation of lactate in plasma and tissues, decreases in intracellular pH, and depletion of glycogen stores (Chippari-Gomes et al., 2005; Richards, 2009; Richards et al., 2007; Speers-Roesch et al., 2012). Lactate production in hypoxia generally results from feedback inactivation of pyruvate



**Fig. 5. Fish exposed to acute hypoxia experienced a transient acidosis in the muscle and liver during hypoxia.** (A,B) Dark phase of the photoperiod (19:00–07:00 h local time) is indicated by the grey section of the background. \*Significant pairwise difference from constant normoxia group at the same time point ( $P < 0.05$ ). Sample sizes for each group at each time point are indicated directly above the bar.

dehydrogenase (PDH), which is partly caused by accumulation and signalling by HIF-1 $\alpha$  (Kim et al., 2006; Richards et al., 2008; Seagroves et al., 2001). This may have occurred in killifish, based on our observation that HIF-1 $\alpha$  protein levels were elevated in the muscle after 12 h of acute hypoxia (Fig. 8). This recruitment of anaerobic metabolism likely augmented cellular ATP supply during hypoxia, and may have helped avoid any statistically significant changes in tissue ATP levels over the 12 h exposure to acute hypoxia (Table 2). These results are consistent with a previous study on *F. heteroclitus*, in which ATP was stable in the white muscle over 15 h of severe hypoxia (Richards et al., 2008).

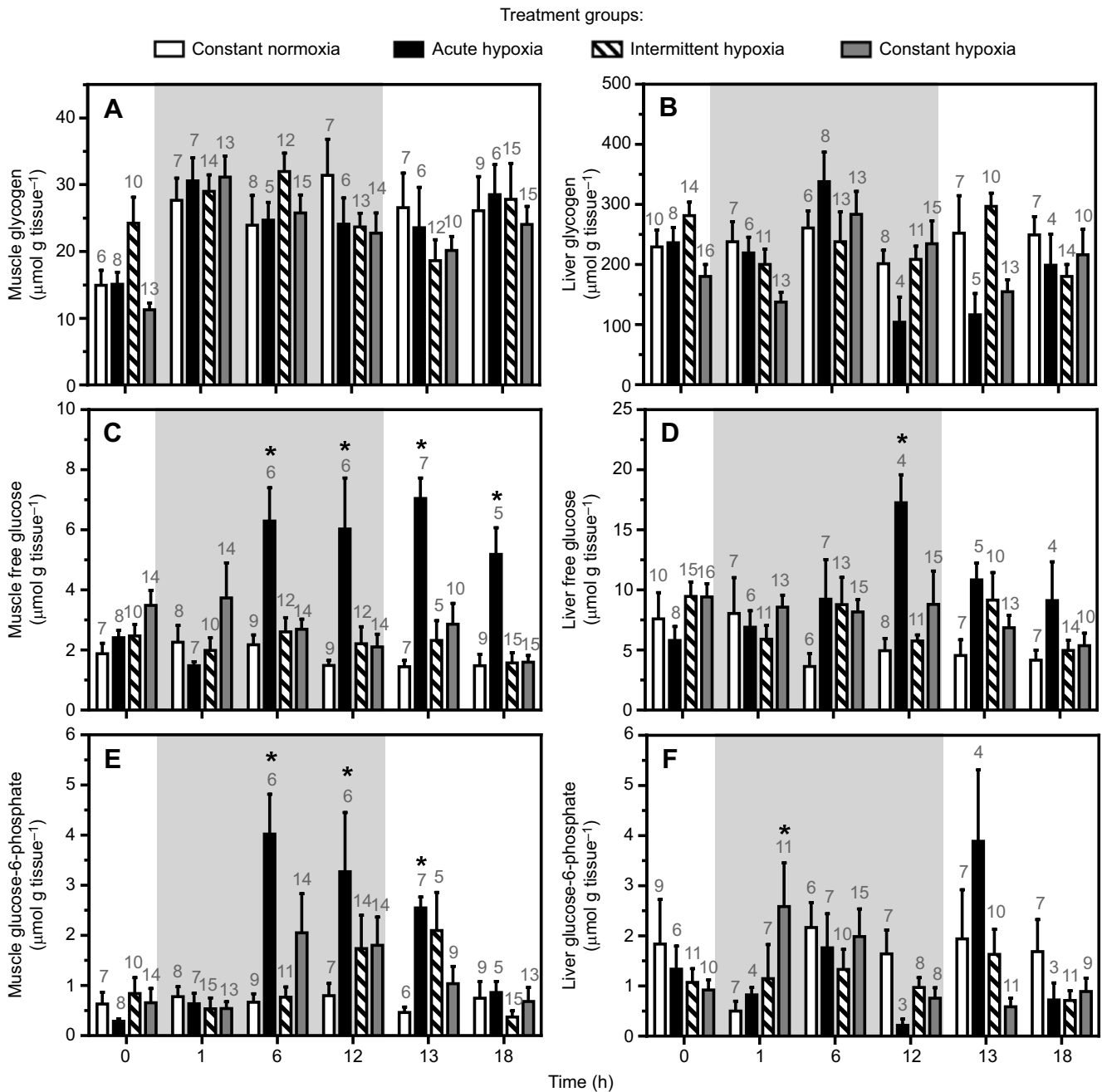
Killifish exposed to acute hypoxia showed some evidence that they accrued an ‘O<sub>2</sub> debt’ that was repaid upon reoxygenation (Figs 2 and 3). The task of correcting tissue homeostasis during recovery from hypoxia exposure – which can involve lactate oxidation, glycogen synthesis, gluconeogenesis, acid-base regulation, etc. – is considered to be energetically expensive and to contribute to the increase in metabolic rate that is often observed upon return to well-oxygenated conditions (EPHOC) (Johansson et al., 1995; Nonnotte et al., 1993; Plambech et al., 2013; Svendsen et al., 2011; van den Thillart and Verbeek, 1991). The total increase

in O<sub>2</sub> consumption observed in the acute hypoxia group was similar in magnitude, but occurred earlier in the day than the circadian cycling of  $\dot{M}_{O_2}$  in normoxic controls (Figs 2 and 3), suggesting that the diurnal changes in  $\dot{M}_{O_2}$  in these groups may have had different underlying causes. For example, the biphasic nature of the change in O<sub>2</sub> consumption in the acute hypoxia group may represent a combination of (i) the effects of recovering from hypoxia, largely represented by the peak at 12.5 h and (ii) circadian cycling in  $\dot{M}_{O_2}$ , largely represented by the peak at 14 h. The lack of a further increase in  $\dot{M}_{O_2}$  at ~14 h in the hypoxia-acclimation groups may be due to either the exaggerated effects of hypoxia on  $\dot{M}_{O_2}$  that masked normal circadian oscillations (such as with intermittent hypoxia) and/or a blunting of circadian oscillations in  $\dot{M}_{O_2}$ , as observed previously (Egg et al., 2013; Mortola, 2007; Pelster and Egg, 2015, 2018). Related to the potential dampening effects of hypoxia on circadian rhythms, our anecdotal observations suggest that fish are less active in the early period of reoxygenation after acute hypoxia, suggesting that increases in  $\dot{M}_{O_2}$  to repay a hypoxic O<sub>2</sub> debt may have also been offset by decreases in the O<sub>2</sub> demands of activity, highlighting the complex nature of  $\dot{M}_{O_2}$  as a metric of whole-animal metabolism.

Different patterns of variation in metabolite concentrations suggested that acute hypoxia had distinct effects between tissues. The decrease in brain glycogen stores in acute hypoxia suggested that this tissue relied at least partly on endogenous carbohydrate stores to fuel energy metabolism (Fig. 7). In contrast, the muscle experienced no decline in glycogen stores in hypoxia, but there were significant increases in free glucose and glucose-6-phosphate levels (Fig. 6), perhaps to support lactate production. Increases in glycogen breakdown products without a significant decrease in glycogen levels may be indicative of glucose entering the muscle from the circulation and/or of an accumulation of metabolites upstream of the potential downstream regulatory sites of glycolysis (e.g. phosphofructokinase) (Nascimben et al., 2004; Özand and Narahara, 1964). It is possible that this glucose, if imported from the blood, originated from glycogenolysis in the liver, at least after ~12 h of hypoxia, based on the declines in glycogen and glucose-6-phosphate levels and the increase in free glucose at this time (Fig. 6). It is intriguing to consider whether the Cori cycle – the shuttling of muscle lactate to the liver to support gluconeogenesis and the return of glucose to the muscle (Cori and Cori, 1929; Milligan and McDonald, 1988) – might have been active in killifish during hypoxia. However, this possibility is uncertain given that, in some fish species, most produced lactate tends to be retained in the muscle rather than entering the circulation, and Cori cycle and liver gluconeogenesis activities after exercise are low (Walsh, 1989; Weber et al., 1986; Wood, 1991).

#### Acclimation to constant hypoxia

Fish acclimated to constant hypoxia appeared to rely on metabolic depression to decrease O<sub>2</sub> demands, as indicated by a ~50% decrease in resting  $\dot{M}_{O_2}$  compared with other groups that was not recovered within several hours of reoxygenation (Figs 2 and 3). This presumed that a decrease in routine energy demands likely helped these animals to maintain stable ATP levels (Tables 1 and 2), and to avoid the recruitment of anaerobic metabolism (Fig. 4), metabolic acidosis (Fig. 5) and depletion of glycogen stores (Figs 6 and 7). Metabolic depression may be a response that only some hypoxia-tolerant fish can use to match O<sub>2</sub> supply and demand and thus cope with oxygen limitation (Nilsson and Renshaw, 2004; Regan et al., 2017b; Richards, 2009; Scott et al., 2008; Vornanen et al., 2009), particularly in response to severe or prolonged bouts of hypoxia

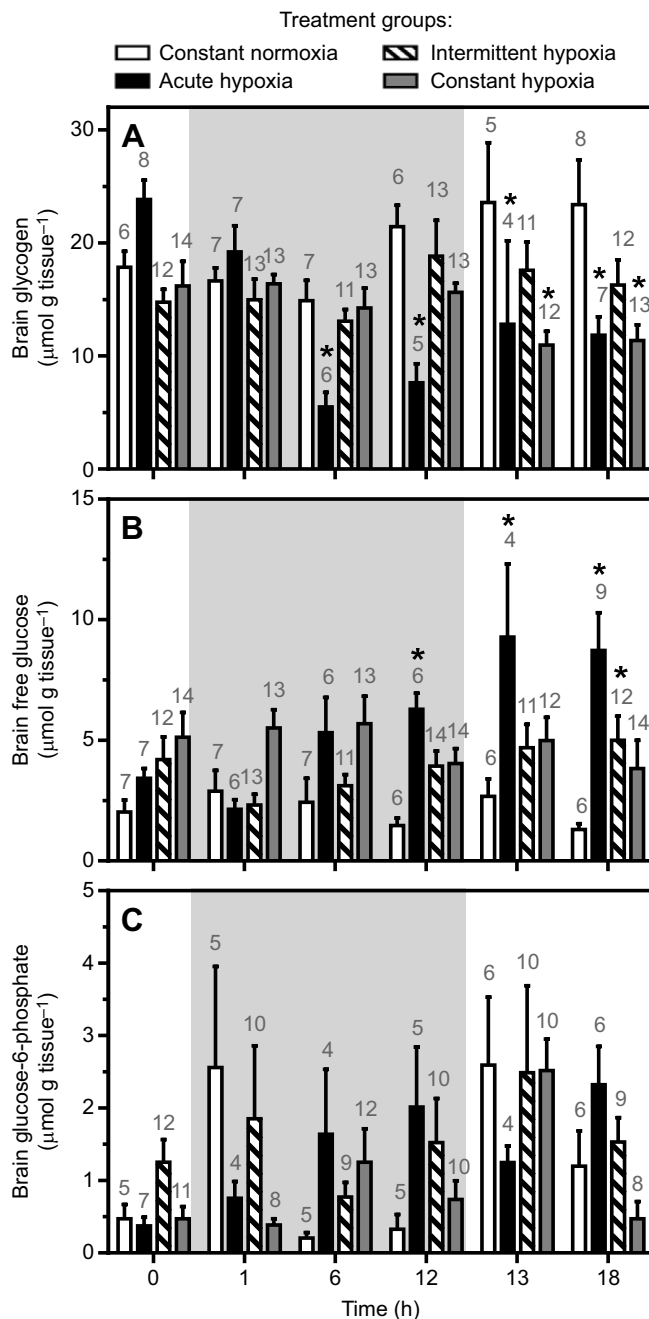


**Fig. 6. Glycogen, glucose and glucose-6-phosphate content of skeletal muscle and liver during hypoxia–reoxygenation.** (A–F) Dark phase of the photoperiod (19:00–07:00 h local time) is indicated by the grey section of the background. \*Significant pairwise difference from constant normoxia group at the same time point ( $P < 0.05$ ). Sample sizes for each group at each time point are indicated directly above the bar.

(Regan et al., 2017a), and our results suggest that killifish are also capable of a similar regulated depression of aerobic metabolism. This appears to be associated with morphological changes in the gills that limit the costs of ionoregulation, and a transition from an oxidative to a glycolytic phenotype in the swimming muscle (Borowiec et al., 2015).

Unlike fish exposed to their first bout of acute hypoxia, fish acclimated to constant hypoxia showed no increase in HIF-1 $\alpha$  protein abundance in the muscle compared with the control levels typical of fish in normoxia (Fig. 8). HIF-1 $\alpha$  is a critical regulator of the cellular and systems-level responses to hypoxia (Iyer et al., 1998; Nikinmaa and Rees, 2005; Richards, 2009; Semenza, 2000; Wang et al., 1995), and HIF signalling regulates many processes that

influence O<sub>2</sub> supply and demand, such as angiogenesis, energy metabolism and the hypoxic chemoreflex (Nikinmaa et al., 2004; Robertson et al., 2014; Semenza, 2000, 2006; Semenza and Prabhakar, 2007). This decrease in HIF-1 $\alpha$  abundance with prolonged acclimation could have resulted from an improvement in tissue O<sub>2</sub> supply or a change in the O<sub>2</sub> sensitivity of HIF degradation (e.g. changes in prolyl hydroxylase activity or O<sub>2</sub> kinetics) (Kopp et al., 2011; Wenger, 2002). Whatever the mechanism, a decrease in HIF-1 $\alpha$  protein abundance in chronic hypoxia has been suggested to decrease lactate production by alleviating the inactivation of PDH via PDH kinase, thus restoring pyruvate oxidation potential (Le Moine et al., 2011), which may have contributed to the low lactate loads in killifish acclimated to hypoxia.



**Fig. 7. Glycogen, glucose and glucose-6-phosphate content of the brain during hypoxia–normoxia.** (A–C) Dark phase of the photoperiod (19:00–07:00 h local time) is indicated by the grey section of the background. \*Significant pairwise difference from constant normoxia group at the same time point ( $P < 0.05$ ). Sample sizes for each group at each time point are indicated directly above the bar.

There was an unexpected decrease in brain glycogen stores during reoxygenation in fish that had been acclimated to constant hypoxia (Fig. 7). The reason for this decrease is unclear, but perhaps the energetic demands of the brain increased upon reoxygenation to an extent that was not fully supported by circulatory fuel supplies. Such an increase in energetic demands may have contributed to the small rise in  $\dot{M}_{O_2}$  upon reoxygenation (Figs 2 and 3). Considering the extreme and rapid increase in  $P_{O_2}$  that these animals experienced after a prolonged 28 day period of continuous hypoxia, it is possible that brain glycogen depletion occurred as animals fuelled the

metabolic costs of repairing damage, activating transcription and translation, or reversing some neuroplasticity that was accrued during hypoxia exposure (Rytkönen et al., 2012). For example, prolonged anoxia is known to decrease cognitive ability in crucian carp (Johansson et al., 1997). Alternatively, it might have been associated with energetic stresses of reoxygenation, which can, in some cases, induce signs of oxidative stress, which has been suggested to be a serious challenge for animals emerging from prolonged periods of metabolic depression (Hermes-Lima et al., 2015, 1998; Hermes-Lima and Zenteno-Savín, 2002; Lushchak et al., 2001).

#### Acclimation to intermittent hypoxia

Fish acclimated to intermittent hypoxia were uniquely able to maintain routine  $\dot{M}_{O_2}$  throughout most of the night-time hypoxia exposure (Fig. 2). This may have resulted from an augmentation of  $O_2$  transport capacity in hypoxia relative to other treatment groups. Acclimation to moderate levels of constant hypoxia has been shown to increase gill surface area, haematocrit and blood haemoglobin content (Claireaux et al., 1988; Hughes, 1973; Richards, 2011; Sollid et al., 2003; Wells, 2009). These responses did not occur in killifish after acclimation to intermittent hypoxia at a higher  $P_{O_2}$  of  $\sim 5$  kPa (Borowiec et al., 2015), but could have occurred in the fish acclimated to the more severe level of intermittent hypoxia used here. Two daily cycles of hypoxia–reoxygenation have been shown to decrease erythrocyte GTP content and thus increase haemoglobin– $O_2$  affinity (without any effect on blood haemoglobin content) in carp (*Cyprinus carpio*) (Lykkeboe and Weber, 1978) and, if a similar change occurred in killifish, it might have helped sustain metabolic rate by increasing arterial  $O_2$  saturation and tissue  $O_2$  delivery. In some mammalian species, chronic exposure to intermittent hypoxia enhances the hypoxic ventilatory response (Garcia et al., 2000; MacFarlane and Mitchell, 2008; Prabhakar and Kline, 2002) and, if a similar response exists in killifish, it could have helped further augment branchial gas exchange in hypoxia.

Fish acclimated to intermittent hypoxia also expressed an appreciable increase in  $\dot{M}_{O_2}$  during reoxygenation (Figs 2 and 3). One possible explanation for this finding is that these fish accrued an exaggerated  $O_2$  debt during hypoxia due to a significant recruitment of anaerobic metabolism. Consistent with this possibility, we have shown previously that acclimation to intermittent hypoxia increases the activities of lactate dehydrogenase, the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, and the mitochondrial enzymes citrate synthase and cytochrome *c* oxidase in the liver (Borowiec et al., 2015), which could have increased the capacity of this tissue to use and recover from anaerobic metabolism. Furthermore, lactate content increased in the liver during hypoxia and appeared to be released into the plasma upon reoxygenation (Fig. 4). However, the magnitude of these changes in lactate levels were relatively modest compared with those in normoxia-acclimated fish exposed to acute hypoxia, and fish acclimated to intermittent hypoxia otherwise avoided metabolic acidosis (Fig. 5) and did not accumulate lactate in a number of other tissues (Fig. 4). This may reflect the sometimes poor relationship between post-hypoxia increases in  $\dot{M}_{O_2}$  and lactate production during hypoxia (Genz et al., 2013; Lewis et al., 2007). It is possible that there were increases in lactate production in some tissues that did not result in lactate accumulation, if that lactate entered the circulation and was oxidized or otherwise disposed of in other tissues (Milligan and Wood, 1986). Alternatively, post-hypoxic increases in  $\dot{M}_{O_2}$  may reflect the cost of correcting ionic and/or osmotic disturbances that arise during hypoxia exposure. For example, in some freshwater species, hypoxia exposure

**Table 2. ATP and phosphocreatine in muscle, liver and brain**

Metabolite	Time (h) <sup>a</sup>	Treatment group			
		Constant normoxia (20 kPa O <sub>2</sub> )	Acute hypoxia (2 kPa O <sub>2</sub> )	Intermittent hypoxia (2 kPa O <sub>2</sub> )	Constant hypoxia (2 kPa O <sub>2</sub> )
<b>Muscle</b>					
ATP	0	5.96±1.17 (7)	3.97±0.34 (10)	4.54±0.60 (10)	4.37±0.43 (14)
	1	6.58±0.91 (8)	6.82±0.91 (7)	5.52±0.42 (12)	6.45±0.53 (14)
	6	4.15±0.72 (7)	2.84±0.40 (10)	3.62±0.48 (13)	4.65±0.74 (14)
	12	3.77±0.63 (8)	4.42±0.63 (10)	4.16±0.56 (11)	4.40±0.53 (13)
	13	4.09±0.42 (7)	3.56±0.43 (7)	4.52±0.83 (13)	2.94±0.35 (12)
	18	3.97±0.57 (8)	6.46±1.07 (7)	4.56±0.67 (14)	4.35±0.67 (14)
Phosphocreatine	0	12.48±1.00 (6)	10.98±0.85 (11)	17.47±2.44 (10)	13.54±1.50 (14)
	1	17.10±4.76 (8)	11.58±1.56 (7)	11.60±1.74 (14)	13.24±1.25 (14)
	6	9.99±1.03 (7)	5.53±0.94 (10)	10.75±0.82 (13)	11.30±0.94 (14)
	12	7.21±1.03 (9)	7.39±0.93 (10)	16.80±4.27 (12)*	14.44±3.34 (13)*
	13	12.38±0.71 (7)	14.94±1.27 (7)	17.53±3.26 (13)	15.09±1.02 (12)
	18	9.52±1.16 (8)	12.04±1.65 (7)	14.03±2.48 (14)	8.51±1.49 (14)
<b>Liver</b>					
ATP	0	2.73±0.54 (7)	3.98±0.87 (6)	2.35±0.47 (11)	2.35±0.44 (10)
	1	3.80±0.41 (8)	2.51±0.45 (7)	3.24±0.46 (11)	3.21±0.48 (13)
	6	2.17±0.52 (8)	1.25±0.26 (6)	1.42±0.17 (13)	1.91±0.23 (14)
	12	2.11±0.47 (8)	1.11±0.22 (6)	2.56±0.39 (11)	2.96±0.73 (12)
	13	3.75±0.86 (7)	1.84±0.45 (7)	3.12±0.63 (12)	2.44±0.28 (13)
	18	3.30±0.50 (8)	2.02±0.66 (4)	3.05±0.42 (12)	2.86±0.34 (9)
<b>Brain</b>					
ATP	0	0.93±0.27 (6)	1.15±0.10 (7)	0.92±0.34 (10)	0.54±0.11 (12)
	1	0.71±0.16 (6)	0.13±0.04 (6)	0.30±0.05 (12)	0.60±0.17 (12)
	6	0.88±0.07 (7)	0.34±0.09 (5)	0.80±0.12 (11)	0.83±0.06 (12)
	12	0.77±0.01 (6)	0.33±0.09 (5)	0.81±0.13 (14)	0.82±0.08 (13)
	13	0.62±0.12 (7)	1.07±0.60 (4)	0.66±0.26 (12)	0.75±0.11 (13)
	18	0.90±0.14 (8)	0.41±0.12 (9)	0.56±0.05 (11)	0.59±0.14 (10)

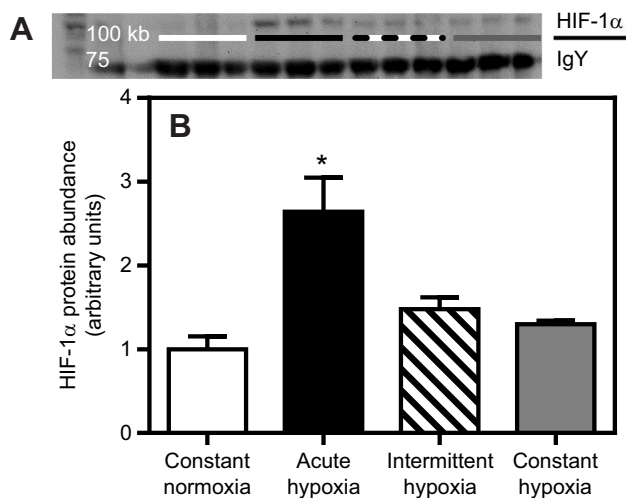
<sup>a</sup>Time refers to hours from the start of the treatment.

Metabolite contents are expressed in  $\mu\text{mol g tissue}^{-1}$ . Data are reported as means±s.e.m., with the sample size in brackets. \*Significant pairwise difference from constant normoxia at the same time point ( $P<0.05$ ), tested via a two-way ANOVA followed by a Bonferroni *post hoc* comparison. Further statistical information is detailed in Table 1.

increases passive ion losses across the gills due to the well-known trade-off between the requirements for respiration and ionoregulation at the gills, termed the osmorepiratory compromise (Iftikar et al.,

2010; Robertson et al., 2015). This does not appear to occur in normoxic killifish exposed to acute hypoxia (Robertson et al., 2015), but could arise after acclimation to intermittent hypoxia if other physiological adjustments (e.g. increases in ventilation, or in gill surface area or permeability) intensify the osmorepiratory compromise, and thus increase the metabolic demands of re-establishing ionic homeostasis upon reoxygenation. Reoxygenation-induced increases in transcription and translation could also contribute to the post-hypoxic increases in  $\dot{M}_{O_2}$  (Dowd et al., 2010; Rytönen et al., 2012).

An alternative and intriguing potential explanation for the large post-hypoxic increases in  $\dot{M}_{O_2}$  in killifish acclimated to intermittent hypoxia is that they amplified and/or hastened some normal circadian processes so they could be completed faster during the normoxic period in the daytime. We synchronized the photoperiod and oxygen cycles in this study such that night-time hypoxia was predictable in fish that were acclimated to intermittent hypoxia. Furthermore, feeding occurred predictably ~2 h after lights on and the onset of normoxia. Therefore, fish could have anticipated feeding (although they were not fed for ~8 h before onset of the experiment, nor during the sampling period), potentially ramping up energetically expensive digestive processes before the predicted feeding time (López-Olmeda et al., 2012; Montoya et al., 2010; Vera et al., 2007). Fish acclimated to intermittent hypoxia may have exaggerated such anticipatory digestive processes to ensure that ingested food could be rapidly digested before the next bout of night-time hypoxia. Whatever the cause of this distinct metabolic



**Fig. 8. Fish exposed to acute hypoxia had increased HIF-1 $\alpha$  protein in skeletal muscle at 12 h of the hypoxia–reoxygenation cycle.** (A) Western blot of HIF-1 $\alpha$  and IgY in the different treatment groups shown in B. (B) HIF-1 $\alpha$  protein abundance in the different treatment groups. There was a significant main effect of treatment group ( $F_{3,8}=9.818$ ,  $P=0.0047$ ;  $n=3$  for each acclimation group). \*Significant pairwise difference from constant normoxia group ( $P<0.05$ ).

phenotype, it is clear that killifish acclimate to intermittent hypoxia in a very different way than they acclimate to constant hypoxia, and thus acquire a distinct strategy for coping with O<sub>2</sub> limitation.

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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.B.M., B.B.R., G.R.S.; Validation: B.G.B.; Formal analysis: B.G.B., G.B.M., B.B.R., G.R.S.; Investigation: B.G.B.; Resources: G.B.M., B.B.R., G.R.S.; Writing - original draft: B.G.B.; Writing - review & editing: B.G.B., G.B.M., B.B.R., G.R.S.; Visualization: B.G.B., G.R.S.; Supervision: G.R.S.; Funding acquisition: G.R.S.

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