

## RESEARCH ARTICLE

# Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*)

Brittney G. Borowiec\*, Kimberly L. Darcy, Danielle M. Gillette and Graham R. Scott

## ABSTRACT

Many fish encounter hypoxia on a daily cycle, but the physiological effects of intermittent hypoxia are poorly understood. We investigated whether acclimation to constant (sustained) hypoxia or to intermittent diel cycles of nocturnal hypoxia (12 h normoxia:12 h hypoxia) had distinct effects on hypoxia tolerance or on several determinants of  $O_2$  transport and  $O_2$  utilization in estuarine killifish. Adult killifish were acclimated to normoxia, constant hypoxia, or intermittent hypoxia for 7 or 28 days in brackish water (4 ppt). Acclimation to both hypoxia patterns led to comparable reductions in critical  $O_2$  tension and resting  $O_2$  consumption rate, but only constant hypoxia reduced the  $O_2$  tension at loss of equilibrium. Constant (but not intermittent) hypoxia decreased filament length and the proportion of seawater-type mitochondrion-rich cells in the gills (which may reduce ion loss and the associated costs of active ion uptake), increased blood haemoglobin content, and reduced the abundance of oxidative fibres in the swimming muscle. In contrast, only intermittent hypoxia augmented the oxidative and gluconeogenic enzyme activities in the liver and increased the capillarity of glycolytic muscle, each of which should facilitate recovery between hypoxia bouts. Neither exposure pattern affected muscle myoglobin content or the activities of metabolic enzymes in the brain or heart, but intermittent hypoxia increased brain mass. We conclude that the pattern of hypoxia exposure has an important influence on the mechanisms of acclimation, and that the optimal strategies used to cope with intermittent hypoxia may be distinct from those for coping with constant hypoxia.

**KEY WORDS:** Hypoxia tolerance, Respiration, Energy metabolism, Gill morphology, Haematology, Muscle histology

## INTRODUCTION

Variations in oxygen availability influence the quantity and quality of habitat available for fish (Graham, 1990; Burnett, 1997; Breitburg et al., 2009). Relatively stable, constant hypoxia can develop in ice-covered or stratified lakes, or following eutrophication events (Diaz, 2001; Diaz and Rosenberg, 2008). Intermittent patterns of hypoxia exposure are common in tide pools and estuaries due to a variety of factors, including daily cycles of respiration and photosynthesis (Breitburg, 1992; Diaz, 2001; Tyler et al., 2009). Overall, the incidence of aquatic hypoxia is expected to rise due to global climate change, urbanization, pollution, and other anthropogenic causes (Diaz, 2001; Ficke et al., 2007).

The major challenge presented by hypoxia is the potential development of a cellular ATP supply–demand imbalance

(Hochachka et al., 1996; Boutilier, 2001). Fish encountering hypoxia often attempt to maintain cellular ATP supply either by increasing  $O_2$  transport to support aerobic respiration, or by increasing the use of anaerobic energy metabolism. Tissue  $O_2$  supply can be improved by increasing branchial  $O_2$  uptake (e.g. increasing ventilation, lamellar perfusion) or the rate of circulatory  $O_2$  transport (e.g. increasing haemoglobin content or blood flow, changes in the concentration of allosteric modifiers) to counteract the effects of  $O_2$  limitation on aerobic metabolism in hypoxia (Holeton and Randall, 1967; Hughes, 1973; Greaney and Powers, 1977, 1978; Nikinmaa and Soivio, 1982; Claireaux et al., 1988; Weber and Jensen, 1988; Perry et al., 2009). At any oxygen tension ( $P_{O_2}$ ) above the critical oxygen tension ( $P_{crit}$ ), tissue  $O_2$  supply is sufficient to meet metabolic demands, and support some aerobic scope for activity (Burton and Heath, 1980; Pörtner and Grieshaber, 1993; Lefrancois et al., 2005; Pörtner, 2010). Below  $P_{crit}$ , aerobic metabolism becomes dependent upon and decreases with environmental  $P_{O_2}$ , and anaerobic metabolism is often used to help supplement ATP supply (Dunn and Hochachka, 1986; Pörtner and Grieshaber, 1993; Scott et al., 2008; Richards, 2009). Anaerobic metabolism can be favoured by increasing the activity and gene expression of glycolytic enzymes (e.g. lactate dehydrogenase), or by reducing carbohydrate flux into the tricarboxylic acid cycle and decreasing aerobic enzyme activity (e.g. cytochrome c oxidase, citrate synthase) (van den Thillart et al., 1980, 1994; Almeida-Val et al., 1995; Martínez et al., 2006; Richards et al., 2008). In addition to mechanisms that help maintain ATP supply, some tolerant organisms can also reduce ATP demands through active depression of resting metabolic rate (van Waversveld et al., 1989; van Ginneken et al., 1997). This is associated with reductions in energetically costly processes, such as ion transport, protein synthesis and mitochondrial proton leak (Bickler and Buck, 2007; Wood et al., 2007, 2009; De Boeck et al., 2013). The reliance on each of these strategies to balance ATP supply and demand varies between species, and could foreseeably be altered by the pattern or severity of hypoxia exposure.

The effects of intermittent hypoxia on fish physiology are poorly understood. This is starkly contrasted by the extensive literature on intermittent hypoxia in mammals, which has uncovered widespread physiological, developmental and genomic consequences that are distinct from continuous hypoxia exposure (Neubauer, 2001; Douglas et al., 2007; Farahani et al., 2008). There is evidence that exposure to repeated bouts of hypoxia compromises growth in some fish species (Atlantic salmon, *Salmo salar*, and southern catfish, *Silurus meridionalis*) but not others (spot, *Leiostomus xanthurus*, and killifish, *Fundulus heteroclitus*) (Stierhoff et al., 2003; McNatt and Rice, 2004; Burt et al., 2013; Yang et al., 2013). Exposure to daily oxygen cycles has been observed to increase hypoxia tolerance and aerobic swimming performance in hypoxia in southern catfish (Yang et al., 2013), to increase resting metabolism measured in normoxia in summer flounder (*Paralichthys dentatus*) (Taylor and Miller, 2001),

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1.

\*Author for correspondence (borowibg@mcmaster.ca)

**List of symbols and abbreviations**

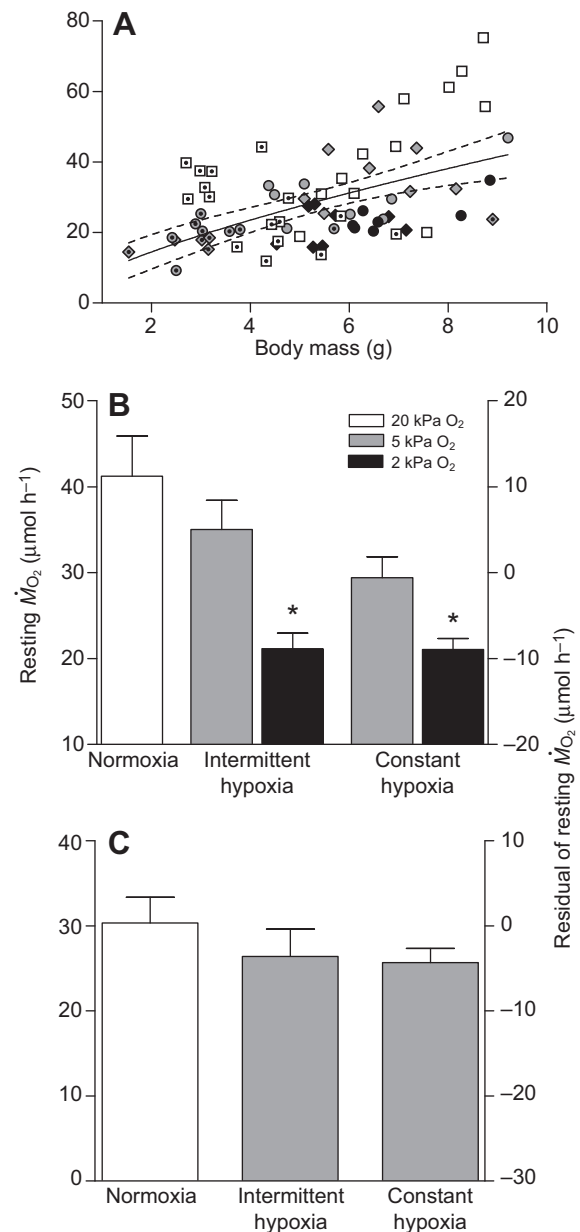
COX	cytochrome c oxidase
CS	citrate synthase
HOAD	3-hydroxyacyl-CoA dehydrogenase
LDH	lactate dehydrogenase
LOE	loss of equilibrium
$\dot{M}_{O_2}$	rate of oxygen consumption
MRC	mitochondrion-rich cell
$P_{crit}$	critical oxygen tension
PEPCK	phosphoenolpyruvate carboxykinase
PK	pyruvate kinase
$P_{O_2}$	partial pressure of oxygen
PVC	pavement cell
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SEM	scanning electron micrograph

and to reduce red blood cell GTP concentration and increase plasma bicarbonate concentration in carp (*Cyprinus carpio*) (Lykkeboe and Weber, 1978). Repeated 2 h bouts of hypoxia have also been shown to amplify some of the transcriptional responses to hypoxia compared with a single bout in the epaulette shark (*Hemiscyllium ocellatum*), including some genes in oxygen and energy homeostasis pathways (Rytkönen et al., 2012). However, the extent to which the physiological effects of intermittent hypoxia differ from those of constant hypoxia is unclear. Intermittent hypoxia differs from constant hypoxia as a stressor because it potentiates the production of reactive oxygen species (ROS), which may cause oxidative stress, but it also provides opportunities for recovery during the oxygenated periods between hypoxia bouts – distinctions that could favour divergent coping mechanisms between these two patterns of hypoxia exposure.

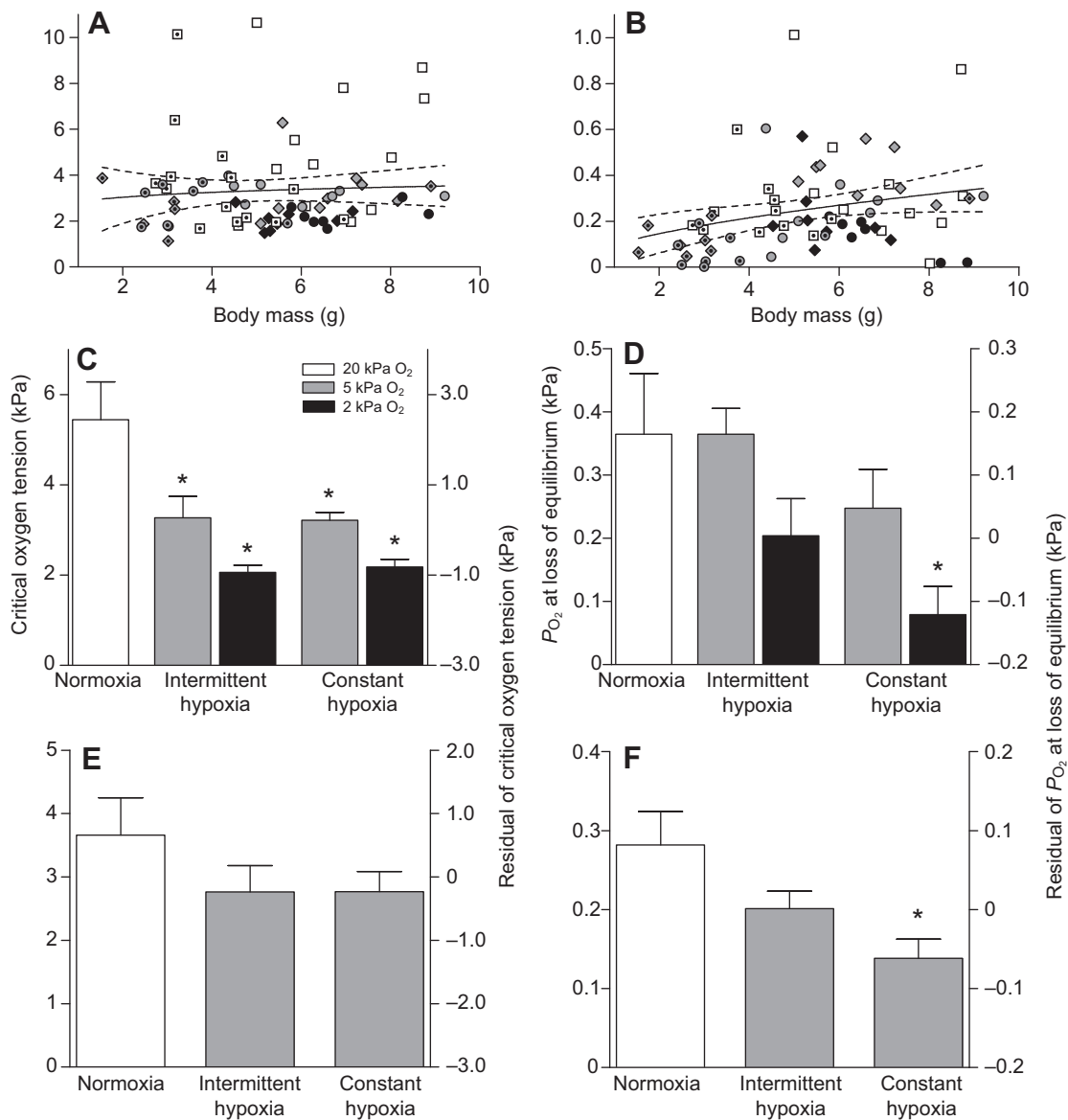
The objectives of this study were (i) to compare the effects on hypoxia tolerance of acclimation to constant hypoxia versus intermittent diel cycles of nocturnal hypoxia, and (ii) to investigate the physiological mechanisms underlying the acclimation response to each pattern of hypoxia exposure. We examined the killifish *F. heteroclitus*, an estuarine species that copes with both seasonal and daily fluctuations in dissolved oxygen content in its native habitat (Stierhoff et al., 2003; Burnett et al., 2007; Tyler et al., 2009). These fluctuations can be sudden, severe, and are mediated by factors such as the daily interplay between photosynthesis and cellular respiration, tidal movements, temperature, and wind patterns (Tyler et al., 2009). We integrated whole-animal respirometry with measurements of subordinate physiological traits dictating oxygen transport and utilization, including gill morphology, haematology, capillarity and fibre composition of skeletal muscle, and the activities of metabolic enzymes in the skeletal muscle, liver, heart and brain.

**RESULTS****Effects of hypoxia acclimation on hypoxia tolerance**

We exposed killifish to constant hypoxia or diel cycles of nocturnal hypoxia (12 h hypoxia at night, 12 h normoxia during the day) for 7 or 28 days at a moderate  $O_2$  tension ( $P_{O_2}=5$  kPa) or for 7 days at a severe  $P_{O_2}$  (2 kPa). Hypoxia acclimation tended to reduce resting rates of oxygen consumption ( $\dot{M}_{O_2}$ ). Because  $\dot{M}_{O_2}$  did not scale isometrically ( $\dot{M}_{O_2}=12.061M^{0.491}$ , where  $M$  is body mass), as previously observed in the closely related *F. grandis* (Everett and Crawford, 2010), we corrected  $\dot{M}_{O_2}$  for body mass using the residuals from an allometric regression (Fig. 1A). Exposure to 7 days of either pattern of severe hypoxia substantially reduced  $\dot{M}_{O_2}$  compared to normoxic controls (Fig. 1B). Though not significant, 7 days of



**Fig. 1. The effects of hypoxia acclimation on resting metabolic rate in *Fundulus heteroclitus*.** (A) The resting rate of oxygen consumption ( $\dot{M}_{O_2}$ ) measured in normoxia was regressed to body mass ( $M$ ) with an allometric equation generated using all data ( $\dot{M}_{O_2}=12.061M^{0.491}$ ). Dashed lines represent the 95% confidence intervals of the regression. Symbols are as follows: white squares, 7 days normoxia acclimation at 20 kPa; grey diamonds, 7 days intermittent hypoxia acclimation at 5 kPa; black diamonds, 7 days intermittent hypoxia acclimation at 2 kPa; grey circles, 7 days constant hypoxia acclimation at 5 kPa; black circles, 7 days constant hypoxia acclimation at 2 kPa; dotted white squares, 28 days normoxia acclimation; dotted grey diamonds, 28 days intermittent hypoxia acclimation at 5 kPa; dotted grey circles, 28 days constant hypoxia acclimation at 5 kPa. Acclimation to 7 days of severe (2 kPa) intermittent or constant hypoxia reduced the resting  $\dot{M}_{O_2}$  measured in normoxia (B), but there were no significant differences between the 28 days acclimation groups (C). The right axes in B and C represent the residuals from the regression in A for each treatment group. The left axes in B and C are the  $\dot{M}_{O_2}$  calculated for average-sized 5.39 g killifish, determined by adding the residual to the  $\dot{M}_{O_2}$  value predicted at 5.39 g by the regression. \*Significant difference from normoxia ( $P<0.05$ ). Sample sizes were as follows:  $N=8$  for 7 days groups, except normoxia ( $N=12$ ) and 2 kPa constant hypoxia ( $N=7$ ). For the 28 days groups,  $N=16$  for normoxia,  $N=7$  for 5 kPa intermittent hypoxia and  $N=8$  for 5 kPa constant hypoxia.



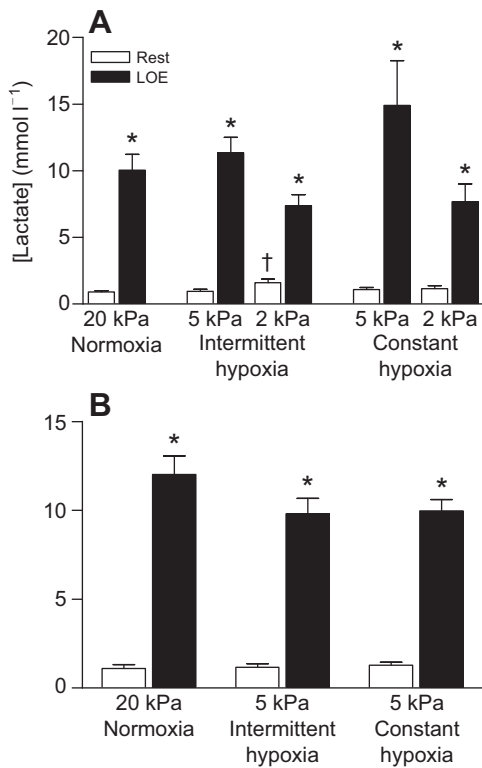
**Fig. 2. The effects of hypoxia acclimation on critical oxygen tension ( $P_{crit}$ ) and the oxygen tension ( $P_{O_2}$ ) at loss of equilibrium (LOE).** (A)  $P_{crit}$  was regressed to body mass ( $M$ ) with an allometric equation ( $P_{crit}=2.527M^{0.096}$ ). (B)  $P_{O_2}$  at LOE was also regressed to  $M$  with an allometric equation ( $P_{O_2}$  at LOE= $0.039M^{0.930}$ ). The 95% confidence intervals of each regression are represented by dashed lines. Symbols are as described in Fig. 1. The right axes in C–F represent the residuals from the regression using all data for  $P_{crit}$  (C,E) or  $P_{O_2}$  at LOE (D,F). The left axes are the  $P_{crit}$  (C,E) or  $P_{O_2}$  at LOE (D,F) calculated for average-sized 5.31 g killifish, determined by adding the residual to the  $P_{crit}$  or  $P_{O_2}$  at LOE value predicted at 5.31 g by the regression. \*Significant difference from normoxia-acclimated controls ( $P<0.05$ ). Sample sizes for 7 days exposures (C,D) were as follows:  $N=11$  for normoxia,  $N=7$  for 2 kPa constant hypoxia,  $N=8$  for all other groups. Sample sizes for 28 days exposures (E,F) were as follows:  $N=15$  for normoxia,  $N=6$  for 5 kPa intermittent hypoxia,  $N=8$  for 2 kPa constant hypoxia.

moderate hypoxia also tended to reduce  $\dot{M}_{O_2}$  (Fig. 1B). There was no effect of moderate hypoxia on  $\dot{M}_{O_2}$  after 28 days of exposure (Fig. 1C), but the  $\dot{M}_{O_2}$  across all 28 days groups appeared to be lower on average than the same 7 days treatment groups.

Hypoxia acclimation increased hypoxia tolerance, indicated by a lower  $P_{crit}$  and in some acclimation groups, a lower  $P_{O_2}$  at loss of equilibrium (LOE) during progressive hypoxia, relative to normoxia-acclimated controls (Fig. 2). Exposure severity, but not pattern, influenced the magnitude of the effect of 7 days of hypoxia acclimation on  $P_{crit}$ , which was lowest after acclimation to severe hypoxia but was also reduced by moderate hypoxia (Fig. 2C). In contrast, only constant hypoxia significantly reduced  $P_{O_2}$  at LOE, after acclimation to either 7 days of severe hypoxia or 28 days of moderate hypoxia (Fig. 2D,F). We corrected  $P_{crit}$  and  $P_{O_2}$  at LOE for body mass using the same residual

approach that we used for  $\dot{M}_{O_2}$  (Fig. 2A,B), but very similar results were obtained without using this mass correction (data not shown).

Hypoxia acclimation also affected blood lactate concentration (Fig. 3). Unlike other treatment groups, resting blood [lactate] was higher after acclimation to severe intermittent hypoxia, relative to normoxic controls. Blood [lactate] rose substantially at LOE across all treatments compared with levels in each group at rest in their acclimation condition ( $P<0.001$  for both the 7 and 28 days data for the main effect of sampling point, i.e. rest versus LOE, in two-factor ANOVA) (Fig. 3). Furthermore, there was a significant interaction between sampling point and severity of hypoxia during acclimation in the 7 days group ( $P=0.042$ ), and a similar trend was seen in the 28 days groups ( $P=0.090$ ). This implies that acclimation to severe hypoxia for 7 days, whether constant or intermittent, blunts the rise

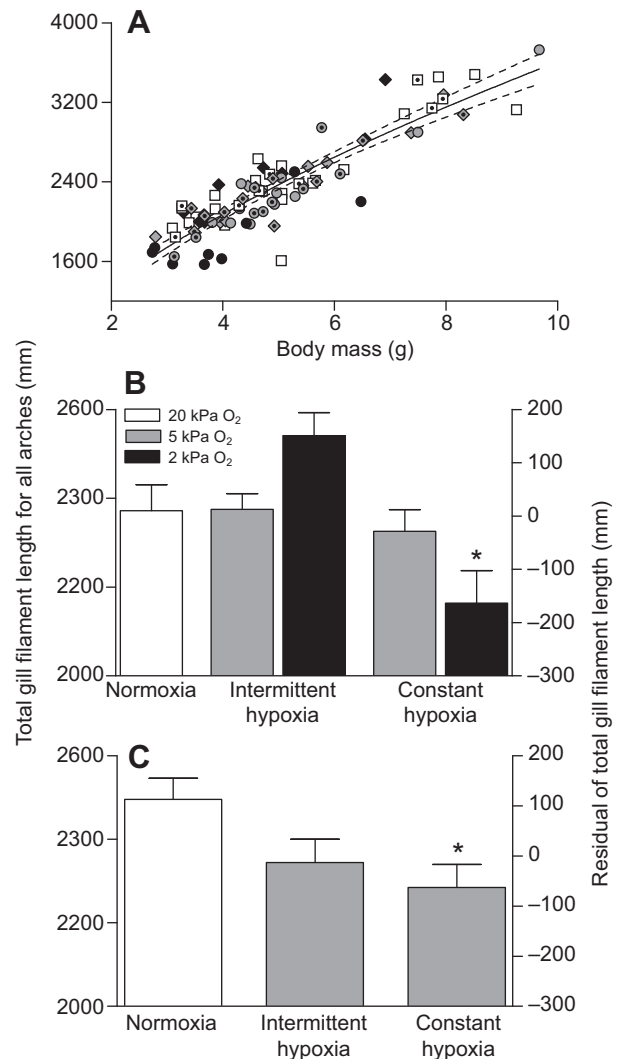


**Fig. 3. Hypoxia acclimation reduced the blood lactate concentration at loss of equilibrium.** Plasma lactate measured at LOE was significantly higher than lactate measured in whole blood at rest in both the 7 days (A) and 28 days (B) acclimation conditions. There was also a significant effect of hypoxia acclimation on the rise in blood lactate (see text for details). \*Significant difference from the resting fish within each acclimation group ( $P < 0.05$ ). †Significant difference from resting normoxic controls ( $P < 0.05$ ). Sample sizes for fish at rest were as follows:  $N = 10$  for 7 days normoxia;  $N = 9$  for 7 days 5 kPa intermittent hypoxia, 7 days 2 kPa constant hypoxia and 28 days 5 kPa constant hypoxia;  $N = 8$  for 28 days normoxia and 28 days 5 kPa intermittent hypoxia;  $N = 7$  for 7 days 5 kPa constant hypoxia;  $N = 6$  for 7 days 2 kPa intermittent hypoxia. Sample sizes for fish at LOE were as follows:  $N = 12$  for 7 days normoxia;  $N = 8$  for 7 days 5 kPa constant hypoxia;  $N = 7$  for 7 days 5 kPa intermittent hypoxia, 7 days 2 kPa constant hypoxia, 28 days normoxia and 28 days 5 kPa constant hypoxia;  $N = 6$  for 7 days 2 kPa intermittent hypoxia and 28 days 5 kPa intermittent hypoxia.

in blood [lactate] at LOE. Indeed, the fold increase in blood [lactate] at LOE was lower on average in fish acclimated to severe hypoxia (approximately 6.6- and 4.6-fold above resting levels in the constant and intermittent groups, respectively) compared with that in normoxic controls ( $\sim 11.1$ -fold). Considering the concurrent differences in the  $P_{O_2}$  at LOE, it suggests that hypoxia acclimation changed the relationship between  $P_{O_2}$  and blood lactate accumulation.

#### Effects of hypoxia acclimation on gill morphology

Constant hypoxia had numerous effects on gill morphology. Because body mass has a significant effect on overall gill size, we again used the residuals from an allometric regression of gill morphometric measurements to compare these variables statistically (Fig. 4). Constant but not intermittent hypoxia acclimation led to a minor ( $< 10\%$ ) but significant decrease in the total length of gill filaments relative to normoxia, after both 7 days of severe hypoxia or 28 days of moderate hypoxia (Fig. 4). Both 28 days hypoxia acclimations also decreased the average length of the gill filaments slightly ( $< 8\%$ ) (Table 1). Clearly, hypoxia acclimation does not lead



**Fig. 4. Hypoxia acclimation reduced the total length of gill filaments.** (A) The total length ( $L$ ) of gill filaments across all four pairs of gill arches was regressed to body mass ( $M$ ) with an allometric equation generated using all data ( $L = 930.79M^{0.5785}$ ). Dashed lines represent the 95% confidence intervals of the regression, and symbols are as described in Fig. 1. Acclimation to 7 days of severe (2 kPa) constant hypoxia (B) or 28 days of moderate (5 kPa) constant hypoxia (C) reduced total gill filament length. The right axes in B and C represent the residuals from the regression in A for each treatment group. The left axes in B and C are the total gill filament lengths calculated for average-sized 4.97 g killifish, determined by adding the residual to the value predicted at 4.97 g by the regression. \*Significant difference from normoxia ( $P < 0.05$ ). Sample size was  $N = 10$  for each group, except 7 days normoxia ( $N = 20$ ).

to morphometric changes that increase the surface area for branchial  $O_2$  diffusion in this species.

The cell composition of the gill epithelium also changed following 28 days acclimation to moderate constant hypoxia (Fig. 5). In the 4 ppt brackish water in which we held the killifish, most of the mitochondrion-rich cells (MRCs) of normoxia-acclimated animals exhibited a typical seawater morphology with deep apical crypts. The proportion of freshwater-type MRCs increased after acclimation to constant hypoxia, as many more MRCs exhibited a transitional (wide and shallow apical crypts) or typical freshwater (convex surface) morphology (Fig. 5D). There were no differences in pavement cell (PVC) surface area or MRC density on the trailing edge of the filaments (Fig. 5C; Table 1).

**Table 1. Gill morphometrics**

Exposure duration		Normoxia 20 kPa O <sub>2</sub>	Intermittent hypoxia		Constant hypoxia	
			5 kPa O <sub>2</sub>	2 kPa O <sub>2</sub>	5 kPa O <sub>2</sub>	2 kPa O <sub>2</sub>
7 days	Average filament length (mm)	3.15±0.10	3.07±0.15	3.08±0.09	3.12±0.14	2.57±0.11*
	Total number of filaments	769±14	789±21	779±23	767±18	727±18
28 days	Average filament length (mm)	3.21±0.14	2.99±0.11*		2.94±0.09*	
	Total number of filaments	781±23	771±15		751±21*	
	Depth of filaments (µm)	163±15	176±10		190±7	
	Depth of lamellae (µm)	122±7	127±3		126±7	
	Height of lamellae (µm)	25.1±1.1	24.9±1.4		26.5±2.5	
	Lamellar area (µm <sup>2</sup> )	3066±216	3140±163		3391±426	
	PVC surface area (µm <sup>2</sup> )	27.2±1.8	31.5±2.1		30.2±2.2	
	MRC surface area (µm <sup>2</sup> )	1.21±0.13	1.69±0.38		2.43±0.34*	

PVC, pavement cell; MRC, mitochondrion-rich cell. Data are reported as means±s.e.m. \*Significant difference from normoxia ( $P<0.05$ ). Sample size of  $N=20$  for normoxia,  $N=10$  for other groups for average filament length and total number of gill filament measurements. Sample size  $N=6$  per group for all other measurements.

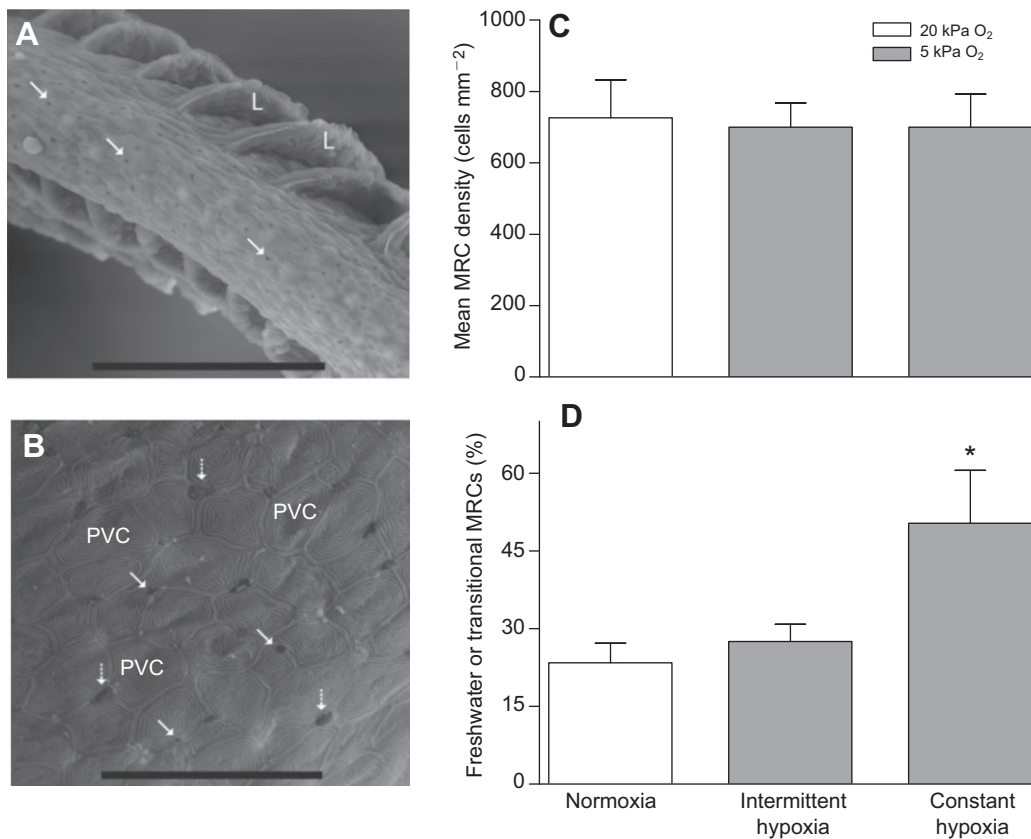
### Effects of hypoxia acclimation on haematology

There were substantial differences in the O<sub>2</sub> carrying capacity of the blood between fish acclimated to constant and intermittent hypoxia. Fish acclimated to either duration of constant hypoxia significantly increased haematocrit and whole-blood haemoglobin concentration, whereas fish acclimated to intermittent hypoxia did not differ from normoxic controls (Fig. 6). The increases in constant hypoxia acclimation were greater with severe hypoxia (~1.9-fold) than with moderate hypoxia (~1.4-fold). There were no changes in mean

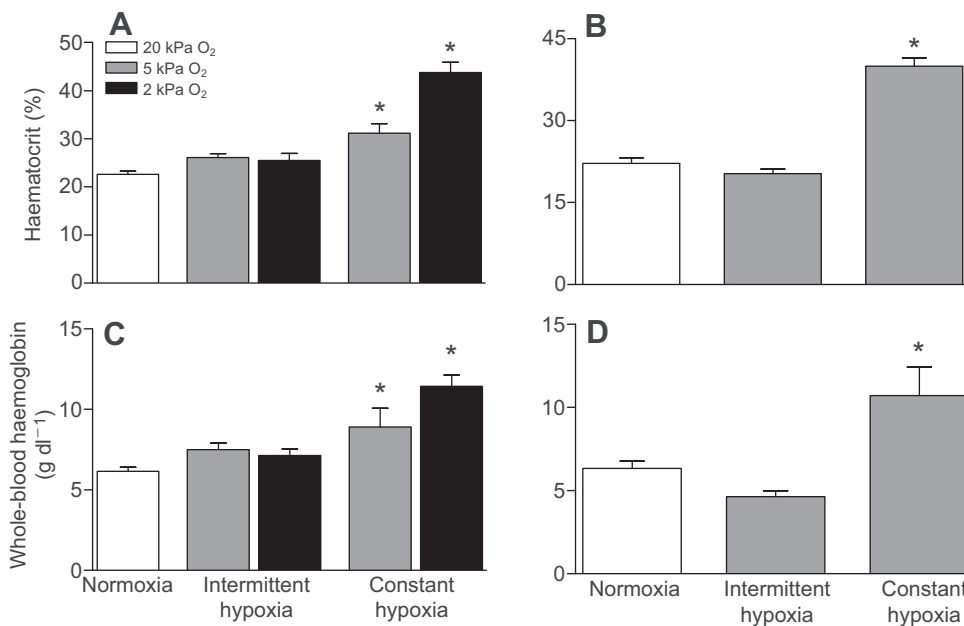
cellular haemoglobin concentration ( $P=0.876$  for 7 days groups, and  $P=0.371$  for 28 days groups; data not shown).

### Effects of hypoxia acclimation on the swimming muscle

Hypoxia acclimation influenced the oxidative phenotype of the axial (swimming) muscle. Relative to normoxia, killifish acclimated to 28 days of constant hypoxia had significantly less total oxidative muscle area [as reflected by succinate dehydrogenase (SDH) positive staining], fewer oxidative fibres, and a 27% lower density



**Fig. 5. Acclimation to 28 days constant hypoxia affected mitochondrion-rich cell (MRC) morphology on the trailing edge of the gill filament.** (A) SEM at  $\times 500$  magnification of the gill filament, with lamellae (L) and apical crypts (arrows) visible. Scale bar is 100 µm. (B) SEM of the trailing edge of the gill filament at  $\times 2000$  magnification with pavement cells (PVC) and two morphologies of MRCs. Freshwater- or transitional-type MRCs have wide and shallow or absent apical crypts and are indicated by dashed, vertical arrows. Seawater-type MRCs have the typical narrow and deep apical crypts and are indicated by continuous, diagonal arrows. Scale bar, 25 µm. There was no significant effect of hypoxia acclimation on the total MRC density on the trailing edge of the gill filament (C), but acclimation to constant hypoxia for 28 days increased the proportion of MRCs with a freshwater- or transitional-type morphology compared with normoxic controls (D). \*Significant difference from normoxia ( $P<0.05$ ). Sample size of  $N=6$  for each group.



**Fig. 6. Acclimation to constant hypoxia increased the haematocrit (A,B) and haemoglobin concentration (C,D) of the blood relative to normoxia.** There were no significant differences in mean cell haemoglobin concentration (data not shown). \*Significant difference from normoxia ( $P < 0.05$ ). Sample sizes for 7 days exposures (A,C) were as follows:  $N=18$  for normoxia,  $N=8$  (A) or  $N=10$  (C) for 5 kPa intermittent hypoxia,  $N=10$  for 2 kPa intermittent hypoxia,  $N=10$  (A) or  $N=8$  (C) for 5 kPa constant hypoxia,  $N=10$  (A) or  $N=7$  (C) for 2 kPa constant hypoxia. Sample sizes for 28 days exposures (B,D) were as follows:  $N=10$  for normoxia and 5 kPa constant hypoxia,  $N=9$  (B) or  $N=6$  (D) for 5 kPa intermittent hypoxia.

of oxidative muscle as a proportion of the entire axial musculature (Fig. 7; Table 2). The muscle phenotype of fish acclimated to intermittent hypoxia did not differ significantly from normoxia-acclimated animals. Oxidative muscle area declined exclusively due to a decrease in the number of modestly oxidative fibres (MOx) at the interface between the oxidative and glycolytic regions (Table 2), with no change in the number of highly oxidative (HOx) fibres or in the average size of either fibre type (see Materials and methods for details). There were no significant differences in the myoglobin content of the entire axial musculature, but the non-significant pattern of variation after 28 days of acclimation mirrored the variation in muscle oxidative phenotype (Tables 3 and 4). There was also a significant 15% decrease in cytochrome c oxidase (COX) activity in the entire axial musculature (sampled to include all fibre types) following 28 days acclimation to both hypoxia patterns. However, there were no other differences in the activities of metabolic enzymes in the muscle (Tables 3 and 4).

Only intermittent hypoxia significantly altered muscle capillarity. Capillary density and capillary-to-fibre ratio in the glycolytic (SDH-negative) muscle increased 30% following acclimation to 28 days of intermittent hypoxia (Fig. 7; Table 2). In contrast, there were no changes in the capillarity of the oxidative muscle with intermittent hypoxia, or in capillarity for either muscle fibre type in fish acclimated to constant hypoxia.

#### Effect of hypoxia acclimation on enzyme activities in the liver, heart and brain

Intermittent hypoxia, but not constant hypoxia, increased the biochemical capacities for oxidative energy metabolism and gluconeogenesis in the liver. Acclimation to severe intermittent hypoxia for 7 days increased the maximal activities of COX, citrate synthase (CS), lactate dehydrogenase (LDH) and phosphoenolpyruvate carboxykinase (PEPCK), without affecting pyruvate kinase (PK) or hydroxyacyl-coA dehydrogenase (HOAD) activities (Fig. 8). In contrast, acclimation to severe constant hypoxia for 7 days increased only LDH activity. There was no significant variation in liver mass or protein contents in the 7 days groups. Acclimation to moderate intermittent hypoxia for 28 days also increased liver COX activity and

liver protein content, and acclimation to either of the hypoxia patterns for 28 days reduced liver mass (Table 4).

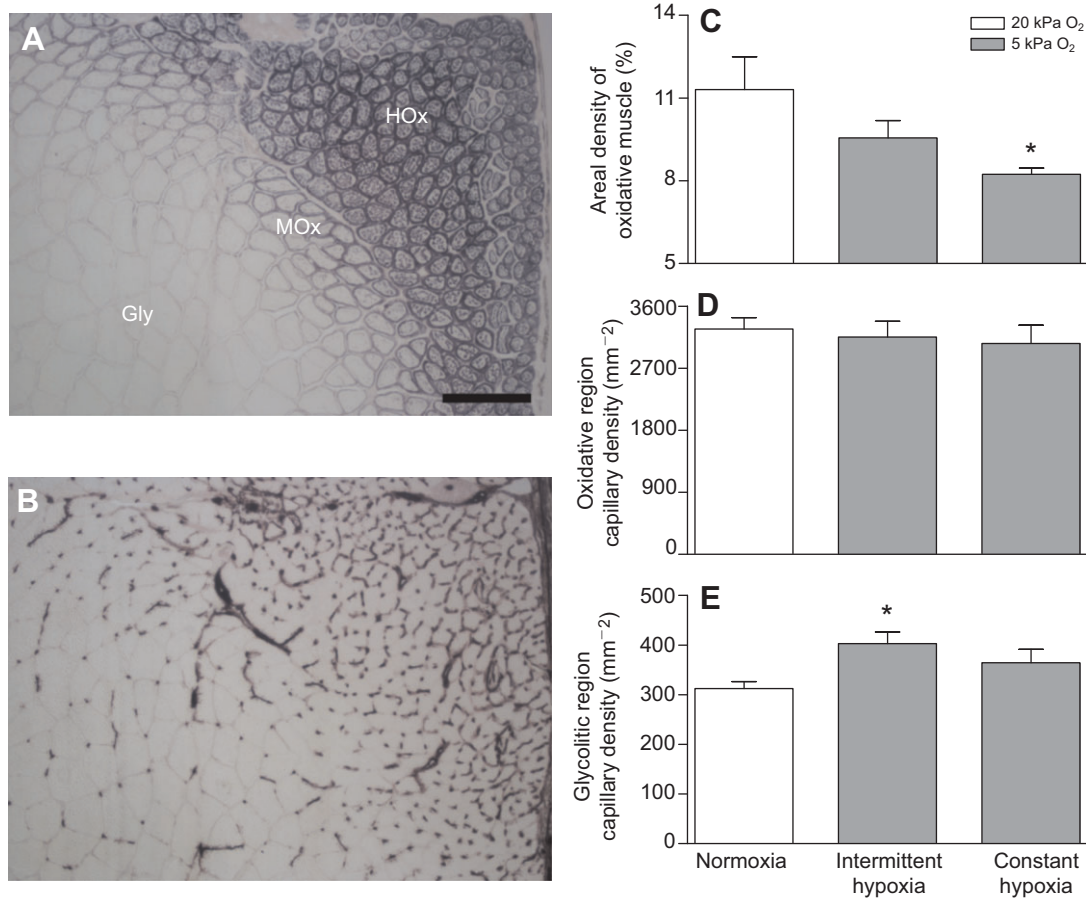
Capacities for oxidative phosphorylation and substrate oxidation appeared to remain unchanged by hypoxia acclimation in the heart and brain, as there were no differences in the activities of COX, CS, LDH, PK or HOAD (the latter two measured in heart only) (Tables 3 and 4). PEPCK activity was not detected in muscle, heart or brain. Interestingly, brain mass was larger in fish acclimated to 7 days of severe intermittent hypoxia, but heart mass did not vary between treatments (Tables 3 and 4).

#### DISCUSSION

Killifish are routinely exposed to fluctuating conditions in their native estuarine environment. Our results show that the responses of killifish to intermittent hypoxia during the course of acclimation are distinct from those to constant hypoxia. Although acclimation to both patterns of hypoxia exposure reduced resting  $\dot{M}_{O_2}$  and  $P_{crit}$ , there were considerable differences between patterns in several physiological traits that dictate oxygen transport and utilization. Constant hypoxia reduced gill surface area, increased blood haemoglobin content, and led to greater reductions in muscle oxidative capacity. These changes did not occur in response to intermittent hypoxia, which was alone in amplifying the oxidative and gluconeogenic capacities of the liver and in increasing the capillarity of glycolytic fibres in the swimming muscle, both of which should improve recovery and lactate clearance between hypoxia bouts. Our results suggest that there are different mechanisms of acclimation that depend upon the pattern of exposure, and that the strategies for coping with constant and intermittent hypoxia may differ.

#### Responses that occur for both patterns of hypoxia exposure

Acclimation to the same duration and magnitude of constant or intermittent hypoxia had similar effects on  $P_{crit}$  (Fig. 2C,E), suggesting that both patterns of exposure somehow improve the extraction and transport of  $O_2$  in hypoxia, and thus broaden the functional  $P_{O_2}$  range for sustaining resting metabolism (Chapman et al., 2002; Søllid et al., 2003; Fu et al., 2011). This was at least partly explained by reductions in the resting  $\dot{M}_{O_2}$  measured in



**Fig. 7. Hypoxia acclimation for 28 days affected the phenotype of the axial swimming muscle.** (A) Representative image of a transverse muscle section of a fish acclimated to 5 kPa intermittent hypoxia, stained for succinate dehydrogenase activity to indicate oxidative capacity. Highly oxidative (HOx), modestly oxidative (MOx) and glycolytic (Gly) fibres are indicated. Scale bar, 0.1 mm. (B) A serial muscle section from the same individual as in A, stained for alkaline phosphatase activity to identify capillaries. (C) Acclimation to constant hypoxia reduced the areal density of oxidative muscle as a percentage of total muscle area. (D) There were no significant differences in capillary density in the oxidative muscle after hypoxia acclimation, but (E) intermittent hypoxia increased capillary density in the glycolytic (SDH-negative) muscle. \*Significant difference from normoxia ( $P < 0.05$ ). Sample sizes were as follows: (A)  $N = 10$  for normoxia and intermittent hypoxia,  $N = 7$  for constant hypoxia; (B)  $N = 9$  for normoxia,  $N = 8$  for intermittent hypoxia and  $N = 4$  for constant hypoxia; (C)  $N = 10$  for normoxia,  $N = 8$  for intermittent hypoxia, and  $N = 6$  for constant hypoxia.

normoxia (Fig. 1), which has been shown to be related to  $P_{crit}$  in many previous studies (van Ginneken et al., 1997; Mandic et al., 2009; Speers-Roesch et al., 2010). This supports the notion that low routine O<sub>2</sub> demands are associated with hypoxia tolerance, but there are clearly other changes in physiology with hypoxia acclimation that are also important (including those observed here).

Although constant and intermittent hypoxia led to comparable reductions in  $P_{crit}$  and resting  $\dot{M}_{O_2}$ , there is only modest evidence that this is caused by similar underlying physiological mechanisms. Increases in plasma lactate concentration with acute hypoxia are well documented in several fish species (Dunn and Hochachka, 1986; Cochran and Burnett, 1996; Virani and Rees, 2000; Scott et al., 2008). However, severe hypoxia acclimation, whether constant or intermittent, partially blunted this rise in blood lactate concentration at LOE (Fig. 3). This suggests that severe hypoxia acclimation reduces lactate accumulation at a given level of hypoxia, in association with lower rates of metabolism and ATP demand. Nevertheless, the capacity for lactate production by LDH is either similarly unaffected (heart, brain and muscle) or elevated (liver) (Fig. 8) by acclimation to both patterns of hypoxia. The general absence of any changes in metabolic capacity in the heart or brain with hypoxia acclimation, and the increases in LDH in the liver, are

similar to previous observations in killifish (Greaney et al., 1980; Martinez et al., 2006). As the organs that are most sensitive to oxygen limitation, the heart and brain are probably protected from hypoxia by a preferential redistribution of blood flow (Axelsson and Fritsche, 1991; Gamperl et al., 1995; Soengas and Aldegunde, 2002). Otherwise, the physiological responses to constant and intermittent hypoxia were largely distinct.

#### Unique responses to constant hypoxia

Acclimation to constant hypoxia leads to the greatest reduction in the absolute lower  $P_{O_2}$  limit for acute survival, as reflected by a significant decrease in  $P_{O_2}$  at LOE, relative to normoxic controls (Fig. 2D,F). The potential causes of LOE in hypoxia are numerous, and could include metabolic acidosis or the cascade of cellular events that result from ATP supply–demand imbalance (Boutilier, 2001; Bickler and Buck, 2007). Variation in the time to or  $P_{O_2}$  at LOE has been suggested to arise from differences in total glycogen stores, the capacity for anaerobic metabolism, tolerance of metabolic acidosis, and the capacity for metabolic depression (Almeida-Val et al., 2000; Nilsson and Östlund-Nilsson, 2008; Mandic et al., 2013). As discussed above, anaerobic capacity probably did not distinguish constant hypoxia from intermittent

**Table 2. Muscle phenotype after 28 days of hypoxia acclimation**

	Normoxia 20 kPa O <sub>2</sub>	Intermittent hypoxia 5 kPa O <sub>2</sub>	Constant hypoxia 5 kPa O <sub>2</sub>
<b>Oxidative fibres</b>			
Total stained area (mm <sup>2</sup> )	3.07±0.23	2.53±0.19	2.25±0.17*
Total number of oxidative fibres	3762±139	3371±156	3200±154*
Number of highly oxidative fibres	1926±84	1805±78	1806±89
Number of modestly oxidative fibres	1836±76	1566±129	1394±85*
Fibre size (µm <sup>2</sup> )	816±47	752±51	708±55
Capillary-to-fibre ratio	2.83±0.21	2.39±0.19	2.44±0.23
<b>Glycolytic fibres</b>			
Fibre size (µm <sup>2</sup> )	1737±122	1756±89	1687±90
Capillary-to-fibre ratio	0.54±0.03	0.70±0.04*	0.61±0.03

Oxidative fibres were identified based upon relative succinate dehydrogenase activity. Capillaries were identified by staining for alkaline phosphatase activity. Data are reported as means±s.e.m. \*Significant difference from normoxia ( $P<0.05$ ). Sample size  $N=9$  for normoxia and intermittent hypoxia,  $N=7$  for constant hypoxia, except for fibre size and capillary-to-fibre ratio measurements, where  $N=10$  for normoxia,  $N=8$  for intermittent hypoxia,  $N=4$  (oxidative fibre size) or  $N=6$  (glycolytic fibre size) for constant hypoxia.

hypoxia because LDH activities were generally similar between groups (Fig. 8; Tables 3 and 4). However, it is possible that fish acclimated to constant hypoxia have a greater capacity for depressing metabolism or tolerating metabolic acidosis in acute hypoxia than those acclimated to normoxia or intermittent hypoxia.

The reductions in gill filament length and the changes in cell composition on the gill epithelium in response to constant hypoxia may be mechanisms for depressing the metabolic costs of ion transport. As the first step of the O<sub>2</sub> transport cascade, the gills are crucial for O<sub>2</sub> uptake, and gill surface area often increases in water-breathing fishes with hypoxia acclimation (Hughes, 1966; Chapman et al., 1999; Søllid et al., 2003; Evans et al., 2005). However, the large gas exchange area of the gills facilitates passive ion loss in a hypo-osmotic environment, which necessitates active ion pumping to maintain ionic homeostasis. This underlies the ‘osmorepiratory compromise’ that leads to trade-offs between respiratory gas exchange and osmoregulation (Randall et al., 1972; Nilsson, 2007). Correspondingly, some species reduce gill surface area and/or ion permeability in response to hypoxia exposure, presumably to minimize ionic disruption rather than facilitate O<sub>2</sub> uptake (McDonald and McMahon, 1977; Wood et al., 2007, 2009; Matey et al., 2011; De Boeck et al., 2013). The killifish acclimated to constant hypoxia in this study decreased gill surface area and shifted their gill epithelium towards a freshwater morphology (Figs 4 and 5; Table 1). Because the freshwater gill is less permeable to ions than the seawater gill due to the presence of deep tight junctions between cells (Chasiotis et al., 2012), this transition should have reduced passive ion loss to the surrounding hypo-osmotic brackish water (Sardet et al., 1979; Scott et al., 2004; Chasiotis et al., 2012). The structural changes in the gills with constant hypoxia acclimation may then act to minimize the costs of ion transport and facilitate metabolic depression. This probably reduced O<sub>2</sub> diffusion capacity across the gill epithelium, and may have limited the ability of killifish acclimated to constant hypoxia to support high metabolic rates (e.g. exercise). However, as the fish in this study were inactive and their oxygen demands were quite low

**Table 3. Metabolic enzyme activities in the muscle, heart and brain for 7 days acclimation groups**

	Normoxia 20 kPa O <sub>2</sub>	Intermittent hypoxia		Constant hypoxia	
		5 kPa O <sub>2</sub>	2 kPa O <sub>2</sub>	5 kPa O <sub>2</sub>	2 kPa O <sub>2</sub>
<b>Muscle</b>					
Protein content [Mb]	72.0±4.7	64.8±2.7	69.5±2.5	76.7±7.0	65.9±2.8
COX	1.85±0.22	1.77±0.39	1.76±0.22	1.78±0.38	2.09±0.28
CS	11.1±0.8	12.5±1.3	11.7±0.7	10.9±1.3	11.1±1.1
PK	5.27±0.28	4.59±0.34	5.45±0.30	4.96±0.36	6.11±0.65
HOAD	234±13	201±15	265±22	252±24	299±51
LDH	0.82±0.08	0.67±0.11	0.90±0.11	0.84±0.10	0.87±0.11
	500±21	533±44	598±56	572±47	575±46
<b>Heart</b>					
Organ mass	1.51±0.15	1.56±0.07	1.52±0.06	1.48±0.10	1.54±0.13
Protein content	54.1±2.8	57.4±5.2	46.2±3.7	50.5±3.3	50.8±5.1
COX	10.2±1.7	12.0±2.2	13.0±1.3	10.7±2.2	12.6±2.7
CS	10.8±0.6	9.35±0.41	9.75±0.95	9.53±0.33	10.8±0.6
PK	89.9±8.3	88.9±3.8	114±13	93.9±9.5	90.1±6.7
HOAD	2.99±0.25	2.93±0.25	2.10±0.27	3.09±0.42	3.10±0.34
LDH	338±26	328±31	328±32	376±28	359±50
<b>Brain</b>					
Organ mass	5.44±0.39	6.97±0.35	7.62±0.48*	5.94±0.88	5.97±0.62
Protein content	105±7.4	117±5.6	103±4.5	117±3.9	98.2±3.9
COX	4.32±0.45	3.17±0.27	3.75±0.22	4.11±0.30	3.87±0.33
CS	5.11±0.13	5.10±0.13	5.08±0.17	4.97±0.35	5.13±0.17
LDH	300±13	315±27	302±22	342±8	280±19

[Mb], myoglobin content; COX, cytochrome c oxidase; CS, citrate synthase; PK, pyruvate kinase; HOAD, hydroxyacyl-coA dehydrogenase; LDH, lactate dehydrogenase. Organ masses are expressed relative to body mass (mg g<sup>-1</sup>). Tissue protein and whole-muscle myoglobin contents are reported per unit tissue mass (mg g<sup>-1</sup>). Enzyme activities are expressed relative to tissue mass (µmol g tissue<sup>-1</sup> min<sup>-1</sup>). Data are reported as means±standard error. \*Significant difference from normoxia ( $P<0.05$ ). For heart and muscle enzyme activities,  $N=20$  for normoxia, and  $N=10$  for all other groups, except for myoglobin content ( $N=17$  for normoxia,  $N=9$  for 5 kPa intermittent hypoxia and  $N=10$  for all other groups). For brain enzyme activities,  $N=12$  for normoxia and  $N=8$  for all other groups, except for CS ( $N=11$  for normoxia) and LDH ( $N=7$  for 2 kPa intermittent hypoxia and 5 kPa constant hypoxia).



**Table 4. Metabolic enzyme activities in the muscle, liver, heart and brain for 28 days acclimation groups**

	Normoxia 20 kPa O <sub>2</sub>	Intermittent hypoxia 5 kPa O <sub>2</sub>	Constant hypoxia 5 kPa O <sub>2</sub>
<b>Muscle</b>			
Protein content	60.7±2.7	60.6±2.2	60.9±2.0
[Mb]	1.90±0.36	1.70±0.37	1.64±0.32
COX	15.9±0.7	13.6±0.6*	13.4±0.6*
CS	5.36±0.54	5.61±0.53	5.61±0.30
PK	278±22	285±27	285±21
HOAD	2.41±0.23	2.17±0.37	2.20±0.27
LDH	410±19	435±23	446±20
<b>Liver</b>			
Organ mass	21.8±1.5	16.1±1.3*	13.9±1.1*
Protein content	82.5±2.5	99.3±7.1*	83.7±3.4
COX	27.3±2.1	41.3±5.4*	37.1±3.3
CS	4.25±0.19	5.78±0.53	5.17±0.77
PK	8.39±1.82	8.54±1.45	8.50±0.56
HOAD	2.41±0.23	2.17±0.37	2.20±0.27
LDH	393±22	465±43	476±22
PEPCK	3.59±0.22	2.49±0.34	3.12±0.34
<b>Heart</b>			
Organ mass	1.44±0.10	1.59±0.22	1.74±0.18
Protein content	44.0±2.9	44.9±2.0	42.7±1.0
COX	10.2±1.3	10.5±1.5	8.6±0.8
CS	10.2±0.7	9.17±1.11	8.26±0.55
PK	155±13	156±13	150±7
HOAD	2.56±0.17	2.10±0.35	1.82±0.24
LDH	340±31	395±47	325±20
<b>Brain</b>			
Organ mass	7.45±0.62	9.60±0.64	8.96±0.64
Protein content	90.7±8.3	74.0±16	76.1±9.6
COX	6.12±1.10	7.75±0.97	6.63±0.84
CS	5.12±0.24	4.82±0.09	4.54±0.28
LDH	323±21	311±25	288±23

[Mb], myoglobin content; COX, cytochrome c oxidase; CS, citrate synthase; PK, pyruvate kinase; HOAD, hydroxyacyl-coA dehydrogenase; LDH, lactate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase. Organ masses are expressed relative to body mass (mg g<sup>-1</sup>). Tissue protein and whole-muscle myoglobin contents are reported per unit tissue mass (mg g<sup>-1</sup>). Enzyme activities are expressed relative to tissue mass (μmol g tissue<sup>-1</sup> min<sup>-1</sup>). Data are reported as means±standard error.

\*Significant difference from normoxia ( $P<0.05$ ). For heart and muscle enzyme activities,  $N=10$  per group, except for myoglobin content ( $N=9$  for normoxia and 5 kPa intermittent hypoxia,  $N=8$  for 5 kPa constant hypoxia). For brain enzyme activities,  $N=15$  for normoxia and  $N=8$  for all other groups, except for COX ( $N=16$  for normoxia).

(Fig. 1), there may have been excess gill surface area and uptake capacity that could be done without in hypoxic fish.

Fish acclimated to constant hypoxia increased whole-blood haemoglobin content and haematocrit (Fig. 6). A similar response has been observed in many species following hypoxia acclimation, and is often accompanied by increases in haemoglobin–O<sub>2</sub> affinity that are mediated by changes in allosteric effectors (Wood and Johansen, 1972; Greaney and Powers, 1977, 1978; Claireaux et al., 1988; Weber and Jensen, 1988; Chapman et al., 2002; Silkin and Silkina, 2005). Circulatory O<sub>2</sub> carrying capacity would have been enhanced in killifish acclimated to constant hypoxia if the measured increase in haemoglobin content was reflective of the entire blood volume. It is also possible that changes in peripheral vasoconstriction and blood flow, which are known to occur in some species in hypoxia (Axelsson and Fritsche, 1991), reduced the entrance of erythrocytes into the capillaries, thus reducing capillary haematocrit and concentrating the erythrocytes in the major vessels (from which

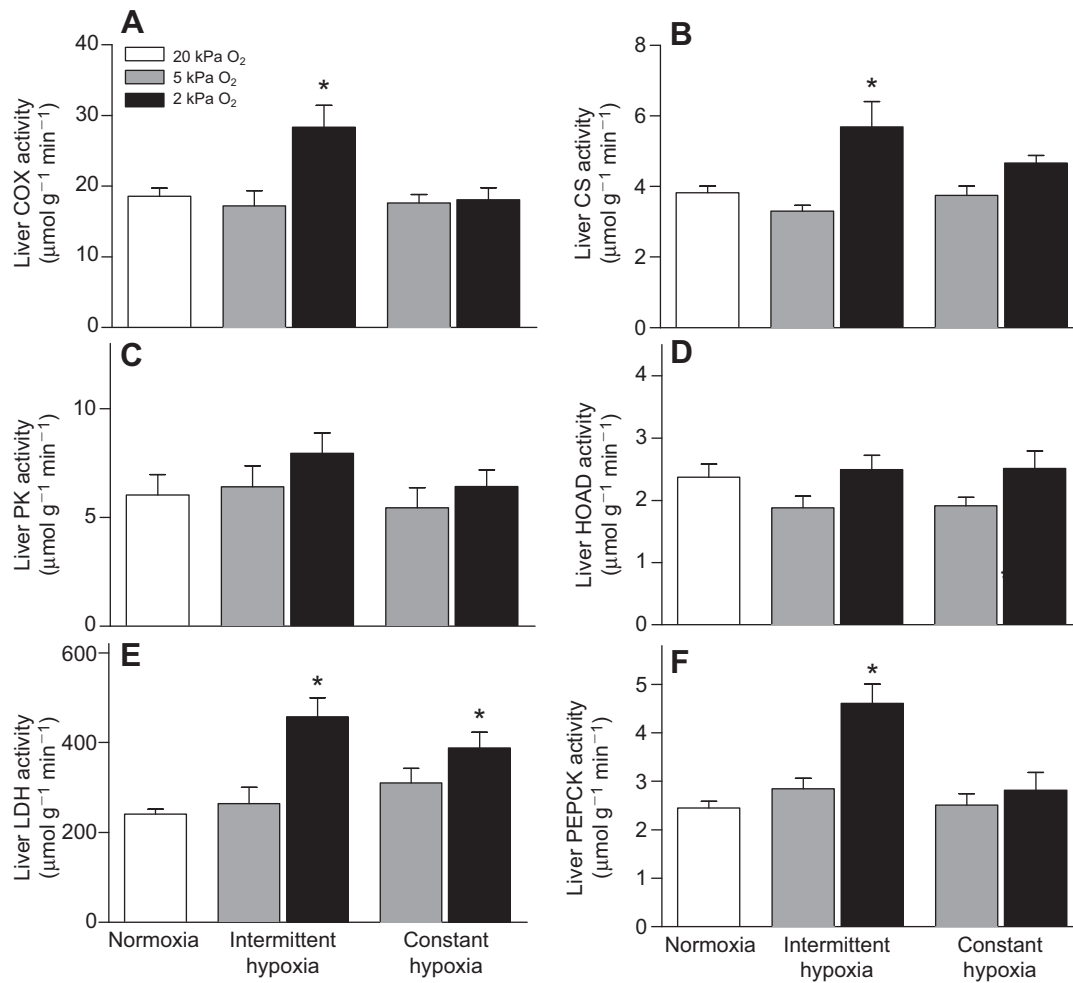
haemoglobin content and haematocrit were measured). Interestingly, we and others (Lykkeboe and Weber, 1978; Taylor and Miller, 2001) have shown that the same haemoglobin and haematocrit responses do not occur with intermittent hypoxia acclimation. This suggests that cumulative exposure duration, and not only absolute  $P_{O_2}$  during each hypoxia bout, may control haemoglobin content by promoting erythropoiesis and erythropoietin release by the kidneys, or by changing blood volume and/or the proportion of plasma and erythrocytes in the capillaries and secondary circulation (Lai et al., 2006; Rummer et al., 2014).

Constant hypoxia reduced the abundance of oxidative fibres in the muscle, due to a reduction in the number of modestly oxidative fibres at the interface between the oxidative and glycolytic regions, where fast oxidative muscle fibres are normally situated (Fig. 7; Table 2) (Scott and Johnston, 2012; McClelland and Scott, 2014). Because muscle recruitment proceeds from slow oxidative, to fast oxidative, to fast glycolytic as swimming intensity increases (Rome et al., 1984), a general reduction in swimming activity with hypoxia could have reduced the neural stimulation of fast oxidative fibres. Reduced neural activation is a key stimulus initiating the transition of fast fibres from an oxidative to a glycolytic phenotype (Bassel-Duby and Olson, 2006), so it is foreseeable that this process was induced in the fast oxidative muscle region due to a reduction in activity levels during hypoxia acclimation. Hypoxia could also regulate mitochondrial abundance within individual fibres, as hypoxia inducible factor stimulates mitochondrial autophagy in mammals (Zhang et al., 2008), and thus reduce overall muscle oxidative capacity even further. However, there is some interspecific variability in the effects of hypoxia on muscle phenotype, as tench (*Tinca tinca*), but not crucian carp (*Carassius carassius*), have been observed to reduce muscle oxidative capacity by decreasing mitochondrial content with hypoxia acclimation (Johnston and Bernard, 1982, 1984).

### Unique responses to intermittent hypoxia

Acclimation to intermittent hypoxia appears to improve the capacity to recover from each hypoxia bout during the intervening periods of normoxia. The use of anaerobic metabolism during hypoxia, reflected by increases in lactate production (Fig. 3) and metabolic acidosis (Johnston, 1975; Dunn and Hochachka, 1986; Scott et al., 2008), incurs an oxygen debt that must later be repaid (Heath and Pritchard, 1965). Increases in the activity of several enzymes occurred in the liver in response to intermittent hypoxia acclimation, including COX, CS, LDH and PEPCK (Fig. 8). These changes should have augmented the capacity for gluconeogenesis and lactate oxidation in the liver, possibly to increase this organ's capacity for metabolizing the lactate produced during each hypoxia bout. Constant hypoxia did not affect PEPCK activity (Fig. 8), consistent with previous observations (Martínez et al., 2006).

The capillarity of the glycolytic (but not oxidative) muscle also increased in fish acclimated to intermittent hypoxia (Fig. 7), which should increase the capacity for lactate clearance from the muscle (the largest tissue in the body) during the daily normoxic periods between hypoxia bouts. Capillarity did not increase in response to constant hypoxia in this study (Fig. 7) or in previous studies of other fish species (Johnston and Bernard, 1982; Jaspers et al., 2014). As fibre size and number were unaffected by intermittent hypoxia, the increased capillarity appears to be caused by angiogenesis, and not a reduction in fibre size due to muscle atrophy. Angiogenesis could have been stimulated by an increase in muscle lactate (Constant et al., 2000; Gladden, 2004) or by high blood flows during recovery from hypoxia that may be needed to clear a lactate load (Egginton, 2011). Angiogenesis could have



**Fig. 8. Acclimation to severe intermittent hypoxia for 7 days increased the oxidative and gluconeogenic capacities of the liver.** Cytochrome c oxidase (COX), citrate synthase (CS), lactate dehydrogenase (LDH) and phosphoenolpyruvate carboxykinase (PEPCCK) activities were elevated after 7 days of severe intermittent hypoxia, without any change in pyruvate kinase (PK) or hydroxyacyl-coA dehydrogenase (HOAD). Neither liver masses (relative to body mass, in  $\text{mg g}^{-1}$ : normoxia,  $17.3 \pm 1.5$ ; 5 kPa intermittent hypoxia,  $17.2 \pm 2.1$ ; 2 kPa intermittent hypoxia,  $15.6 \pm 1.2$ ; 5 kPa constant hypoxia,  $13.7 \pm 1.9$  and 2 kPa constant hypoxia,  $22.6 \pm 2.1$ ), nor liver protein contents (relative to tissue mass, in  $\text{mg g}^{-1}$ : normoxia,  $117 \pm 6$ ; 5 kPa intermittent hypoxia,  $128 \pm 9$ ; 2 kPa intermittent hypoxia,  $99.2 \pm 4.5$ ; 5 kPa constant hypoxia,  $110 \pm 14$  and 2 kPa constant hypoxia,  $121 \pm 12$ ) varied between 7 days acclimation groups. \*Significant difference from normoxia ( $P < 0.05$ ). Sample sizes were  $N=10$ , except for the normoxia-acclimated group ( $N=20$ ).

also occurred as a response to a decline in intracellular  $P_{\text{O}_2}$  (Mathieu-Costello, 1993; Hoppeler and Vogt, 2001). However, we did not observe any significant variation in the myoglobin content of the muscle, which would have increased cellular  $\text{O}_2$  supply and has been observed to occur in response to

hypoxia (Fraser et al., 2006). Regardless of the cause of this increase in capillarity, it is possible that it represents part of a general strategy to enhance the overall capacity for lactate turnover. This could even involve an increased use of the Cori cycle, in which the liver resynthesizes glucose from lactate

**Table 5. Body masses and lengths of fish used in each series of experiments**

Exposure duration	Normoxia 20 kPa $\text{O}_2$	Intermittent hypoxia		Constant hypoxia		
		5 kPa $\text{O}_2$	2 kPa $\text{O}_2$	5 kPa $\text{O}_2$	2 kPa $\text{O}_2$	
Respirometry experiments						
7 days	Body mass (g)	$7.00 \pm 0.37$	$6.49 \pm 0.38$	$5.68 \pm 0.31$	$5.93 \pm 0.58$	$6.80 \pm 0.40$
28 days	Body mass (g)	$4.17 \pm 0.30$	$3.33 \pm 0.83$		$3.36 \pm 0.37$	
Sampling experiments						
7 days	Body mass (g)	$5.31 \pm 0.39$	$5.21 \pm 0.50$	$4.60 \pm 0.40$	$5.36 \pm 0.58$	$4.05 \pm 0.37$
	Standard length (mm)	$61.0 \pm 1.3$	$61.7 \pm 2.2$	$60.4 \pm 1.9$	$62.1 \pm 2.2$	$56.1 \pm 1.5$
28 days	Body mass (g)	$5.2 \pm 0.60$	$4.93 \pm 0.49$		$4.76 \pm 0.29$	
	Standard length (mm)	$60.9 \pm 2.2$	$61.7 \pm 1.9$		$61.1 \pm 1.3$	

Data are means  $\pm$  standard error, and there were no significant differences between treatments within an exposure duration for each experiment. Fish were measured at the time of sampling either after loss of equilibrium in the respirometry experiments, or at rest in the acclimation condition in the sampling experiments (standard body length was only measured in the latter experiments).

through gluconeogenesis and then returns glucose to the muscle via the circulation, although the existence of the Cori cycle in fish is uncertain (Milligan and Girard, 1993).

Acclimation to intermittent hypoxia increases hypoxia tolerance in killifish, and the mechanisms involved appear to be distinct from those for constant hypoxia. Intermittent hypoxia is also known to have different effects than constant hypoxia on the control of ventilation and circulation in mammals (MacFarlane et al., 2008; Prabhakar and Semenza, 2012). On the one hand, it is possible that comparable changes occur in fish and mammals in response to intermittent hypoxia. On the other hand, fish that are routinely exposed to intermittent hypoxia in their native environment, such as estuarine killifish, might employ uniquely evolved strategies for coping with intermittent hypoxia. As the occurrence of hypoxia increases worldwide, it will be important to better appreciate how the pattern of exposure influences the impacts of hypoxia on aquatic organisms.

## MATERIALS AND METHODS

### Study animals and experimental hypoxia acclimations

Adult, wild-caught *Fundulus heteroclitus* Linnaeus 1766 of both sexes were purchased from a commercial supplier (Aquatic Research Organisms, NH, USA), shipped to McMaster University, and held for at least one month in brackish (4 ppt) water at room temperature ( $\sim 21^\circ\text{C}$ ) before experimentation. Water quality (pH, nitrates, nitrites and ammonia) was maintained with regular water changes. Fish were fed commercial flakes (Big Al's Aquarium Supercentres, Mississauga, ON, Canada) 6 days per week, and were not fed for 24 h prior to respirometry or sampling.

Exposures were carried out in 35 l glass aquaria with the same water chemistry as described above, in which normoxia, constant hypoxia, or nocturnal (intermittent) hypoxia was sustained for 7 or 28 days (Table 5). Constant normoxia (20 kPa,  $8\text{ mg O}_2\text{ l}^{-1}$ ) was maintained by continuously bubbling the water with air. Constant hypoxia was maintained by bubbling the water with nitrogen gas, and the appropriate  $P_{\text{O}_2}$  was maintained by a feedback loop using a galvanic oxygen sensor that automatically controlled the flow of nitrogen with a solenoid valve (Loligo Systems, Tjele, Denmark). Nocturnal ('intermittent') hypoxia was maintained using the same  $\text{O}_2$  controller as for constant hypoxia, but gas flow was alternated between air (08:00 h to 20:00 h local time) and nitrogen (20:00 h to 08:00 h) with an additional solenoid valve controlled by a photoperiod timer that was synchronized with the light cycle (12 h:12 h light:dark). During the hypoxic periods, the set-point (i.e. 5 or 2 kPa) was tightly regulated with a hysteresis of 0.02 kPa. Larger deviations from set-point were infrequent and never exceeded 0.4 kPa. Two levels of hypoxia were used for 7 days exposures, either moderate (5 kPa,  $2\text{ mg O}_2\text{ l}^{-1}$ ) or severe (2 kPa,  $0.8\text{ mg O}_2\text{ l}^{-1}$ ), but only moderate hypoxia was used for 28 days exposures. Fish were prevented from respiring at the water surface with a plastic grid barrier. The body masses and standard body lengths of fish used for each series of experiments are shown in Table 5.

### Respirometry experiments

Stop-flow respirometry was used to determine  $\dot{M}_{\text{O}_2}$ ,  $P_{\text{crit}}$  and the  $P_{\text{O}_2}$  at LOE. Fish were held overnight in normoxia in a respirometry chamber (90 ml cylindrical glass) that was situated in a darkened buffer tank and continuously flushed with normoxic water (flushing circuit). The chamber was also connected to a recirculating circuit that flowed past a fibre-optic oxygen sensor (PreSens, Regensburg, Germany). Both circuits were driven by pumps controlled by AutoResp software (Loligo Systems).

Oxygen consumption measurements began the following morning in normoxia, with two sequential flush and measurement periods. During flush periods, both the flush and recirculating pumps were active, and the chamber received a steady flow of water from the buffer tank (i.e. the chamber and buffer tank were equilibrated). During measurement periods, the flush pump was turned off, isolating the chamber from the buffer tank, and the change in oxygen concentration due to fish respiration was measured. Fish were then subjected to a progressive hypoxia protocol, in which buffer tank  $P_{\text{O}_2}$  was reduced in 2 kPa steps using the  $\text{O}_2$  control system described above. Each

level of hypoxia was maintained for 10 min, and oxygen consumption rate was measured as in normoxia. After measurement at an ambient  $P_{\text{O}_2}$  of 2 kPa, the chamber was closed such that the fish consumed the remaining oxygen until it lost equilibrium. Oxygen consumption was calculated from the change in chamber oxygen concentration over time as previously recommended (Clark et al., 2013).  $P_{\text{crit}}$  was calculated using a program developed by Yeager and Ultsch (1989).

Immediately after losing equilibrium, fish were euthanized by a blow to the head followed by pithing. The tail was bisected at the base of the anal fin, and blood was collected from the caudal blood vessels in a heparinized capillary tube. Whole blood was used to measure haemoglobin content (using Drabkin's reagent following manufacturer's instructions; Sigma-Aldrich, Oakville, ON, Canada) or was frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for later determination of lactate concentration. Some blood was also centrifuged for 5 min at 12,700 g to measure haematocrit.

### Sampling

A separate set of fish from those used for respirometry were acclimated and sampled at rest (rather than at LOE). Sampling was done at a consistent time of day (between 13:00 h and 16:00 h) to minimize the effect of circadian rhythms and other diurnal variations on our results. Sampling was therefore during the normoxic period for the intermittent hypoxia acclimation groups. Fish were euthanized and blood was collected and analysed as described above. A transverse steak of the trunk was cut at the anterior base of the anal fin, coated in embedding medium (Fisher Scientific Company, Ottawa, ON, Canada), frozen in liquid  $\text{N}_2$ -cooled isopentane, and stored at  $-80^\circ\text{C}$  until use for muscle histology. An adjacent hemi-section of the axial muscle (containing the entirety of the red and white fibres) and the entire intact liver, heart and brain were removed, frozen immediately in liquid nitrogen, and stored at  $-80^\circ\text{C}$  for later measurement of enzyme activities. The gill baskets were removed intact and fixed (2% paraformaldehyde, 2% glutaraldehyde) at  $4^\circ\text{C}$ .

### Gill morphometrics

After fixing, the four arches on one side of the gill basket were isolated and cleaned of excess tissue such that individual filaments and lamellae were visible. Images at  $\times 10$  magnification were taken on each side of the arches using a stereomicroscope to determine the length and number of filaments. Total gill filament length for an entire fish was calculated by doubling the sum of all individual filament lengths measured, and then multiplying the result by 1.15 to account for the approximate effects of curling, as recommended previously (Hughes, 1966).

Six fish from each 28 days treatment group that had a similar body mass (3–5 g) were selected for scanning electron microscopy. Filaments from the middle portion of one side of the second gill arch were removed to enable viewing of both the leading and trailing edges of the remaining filaments. The gills were post-fixed in 1%  $\text{OsO}_4$  for 1 h and then dehydrated in progressively higher concentrations of ethanol (from 50 to 100%). After critical point drying with liquid  $\text{CO}_2$ , samples were sputter-coated and viewed in an ESEM 2020 scanning electron microscope (Electroscan Corporation, Wilmington, MA, USA). Images were taken at  $\times 350$  and  $\times 500$  magnification to determine gill filament depth (parallel to water flow), maximum lamellar height (perpendicular to the filament and to water flow), and lamellar depth (parallel to filament depth). High-magnification images were taken at  $\times 2000$  and  $\times 5000$  to quantify MRC density, MRC and PVC size, and MRC phenotype (seawater-type apical crypts or freshwater and transitional-type MRCs for which the cell surface is visible) (Scott et al., 2004; Laurent et al., 2006). PVC and MRC measurements were restricted to the trailing edge. All gill morphometric images were analysed using ImageJ software (Rasband, 2014).

### Muscle histology

Muscle blocks were cut into  $10\text{ }\mu\text{m}$  sections at  $-20^\circ\text{C}$  with a cryostat (Leica Microsystems, Wetzlar, Germany), mounted on Superfrost Plus slides (Fisher Scientific Company), air dried, and stored at  $-80^\circ\text{C}$  until staining. Staining of sections was performed using standard methods that have been previously described (Egginton, 1990; Scott and Johnston, 2012). Sections were stained for SDH activity to identify oxidative muscle fibres, using the following assay conditions:  $41.7\text{ mmol l}^{-1}\text{ Na}_2\text{HPO}_4$ ,  $8.3\text{ mmol l}^{-1}\text{ NaH}_2\text{PO}_4$ ,  $80\text{ mmol l}^{-1}$  sodium succinate, 0.1% NBT, pH 7.6. Alkaline

phosphatase activity was used to stain for capillaries, using the following assay conditions (in mmol l<sup>-1</sup>): 28 NaBO<sub>2</sub>, 7 MgSO<sub>4</sub>, 1 NBT, 0.5 BCIP, pH 9.3. Sections were stained for 1 h at room temperature in each protocol, after which slides were mounted with Aquamount (Fisher Scientific Company) and stored at 2°C until they were imaged with a Nikon Eclipse E800 light microscope (Nikon Instruments, Melville, NY, USA).

The total transverse area and number of SDH-positive (oxidative) muscle fibres were determined for each fish from the SDH activity stains. Average oxidative fibre size was calculated by dividing the area by the number of fibres within that area. We observed two distinct patterns of SDH-positive staining intensity – one indicative of high oxidative capacity (HOx; dark SDH staining throughout the fibre) and one indicative of modest oxidative capacity (MOx; less intense but still positive SDH staining, usually near the fibre periphery) (Fig. 7) – so we also quantified the total number of fibres exhibiting each staining pattern. We quantified from only one lateral side of the fish, but we multiplied the results by two to calculate the total oxidative fibre area and number of fibres in the entire trunk. Based on their location in the axial muscle, the fibres we characterized as having a high oxidative capacity included both slow oxidative and a subset of fast oxidative fibre types (Scott and Johnston, 2012; McClelland and Scott, 2014).

Average glycolytic fibre size, capillary density, and capillary-to-fibre ratios were determined from the alkaline phosphatase stains. Average glycolytic (SDH-negative) fibre size was determined from six to eight images taken throughout the white muscle of each fish, by dividing the known area of the image by the unbiased number of fibres within the image (Egginton, 1990). Capillary density (number of capillaries counted per unit of area) and capillary-to-fibre ratio (the number of capillaries relative to the number of fibres counted in sections) were quantified in the entirety of the highly oxidative region on one lateral side of the fish. The same capillary indices were determined from six to eight images taken throughout the glycolytic region. Preliminary assessments verified that a sufficient number of images were analysed to account for heterogeneity across the axial musculature (determined by the number of replicates necessary to yield a stable mean value). For all histological measurements, the average value for each fish was calculated and used for statistical comparison between treatment groups. All muscle histology images were analysed using ImageJ by an observer that was blind to treatment group.

## Assays

The maximal activities of several enzymes were assayed using standard methods (Bears et al., 2006; Schnurr et al., 2014). The frozen muscle, liver, heart and brain samples were weighed and homogenized on ice in 20 volumes of homogenization buffer (20 mmol l<sup>-1</sup> Hepes, 1 mmol l<sup>-1</sup> sodium EDTA and 0.1% Triton X-100) at pH 7.0. Assays were performed to determine the maximal activity of each enzyme in the tissue homogenate at 25°C, by measuring the rate of change in absorbance at 550 nm (COX), 340 nm (HOAD, LDH, PEPCK, PK), or 412 nm (CS) for at least 5 min. The COX assay was performed immediately following homogenization, after which the homogenate was stored at -80°C. Other enzymes were assayed after a consistent number of freeze-thaw cycles for each enzyme. Assay conditions were as follows: COX, 100 μmol l<sup>-1</sup> (brain, liver and heart) or 50 μmol l<sup>-1</sup> (muscle) of fully reduced cytochrome c in 50 mmol l<sup>-1</sup> Tris containing 0.5% Tween-20 at pH 8.0; CS, 1.0 mmol l<sup>-1</sup> oxaloacetate and 0.15 mmol l<sup>-1</sup> acetyl-CoA (brain), or 0.5 mmol l<sup>-1</sup> oxaloacetate and 0.3 mmol l<sup>-1</sup> acetyl-CoA (other tissues), each in 50 mmol l<sup>-1</sup> Tris containing 0.1 mmol l<sup>-1</sup> DTNB at pH 8.0; HOAD, 0.1 mmol l<sup>-1</sup> acetoacetyl-CoA and 0.3 mmol l<sup>-1</sup> NADH in 50 mmol l<sup>-1</sup> imidazole at pH 7.2; LDH, 0.3 mmol l<sup>-1</sup> NADH and 0.5 mmol l<sup>-1</sup> pyruvate (heart, liver and muscle) or 0.15 mmol l<sup>-1</sup> NADH and 1 mmol l<sup>-1</sup> pyruvate (brain), each in 50 mmol l<sup>-1</sup> Hepes at pH 7.4; PEPCK, 1.1 mmol l<sup>-1</sup> PEP, 0.15 mmol l<sup>-1</sup> NADH, 0.5 mmol l<sup>-1</sup> dGDP, 20 mmol l<sup>-1</sup> NaHCO<sub>3</sub>, 1 mmol l<sup>-1</sup> MnCl<sub>2</sub>·4H<sub>2</sub>O, and excess coupling enzyme (malate dehydrogenase) in 50 mmol l<sup>-1</sup> imidazole at pH 7.4; PK, 10 mmol l<sup>-1</sup> PEP, 0.15 mmol l<sup>-1</sup> NADH, 5 mmol l<sup>-1</sup> ADP, 100 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 μmol l<sup>-1</sup> fructose-1,6-bisphosphate, and excess coupling enzyme (LDH) in 50 mmol l<sup>-1</sup> Mops at pH 7.4. Preliminary assays determined the lowest possible substrate concentrations that would stimulate maximal activity. All enzyme assays were run in triplicate in a 96-well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) with

temperature control. Activities were determined by subtracting the background reaction rate without a key substrate (COX, cytochrome c; CS, oxaloacetate; HOAD, acetoacetyl-CoA; LDH, pyruvate; PK, phosphoenolpyruvate; PEPCK, dGDP) from the rates measured in the presence of all substrates. We used extinction coefficients (ε) of 28.5 and 13.6 optical density (mmol l<sup>-1</sup>) cm<sup>-1</sup> for COX and CS assays, respectively. We calculated ε empirically for the remaining assays by constructing standard curves of absorbance versus NADH concentration in the buffers appropriate for each assay.

Undiluted skeletal muscle homogenate was assayed for myoglobin concentration using a modification of the method described by Reynafarje (Reynafarje, 1963). Homogenates were centrifuged at 13,700 g for 100 min at 4°C (Eppendorf, Hamburg, Germany). The supernatant was completely reduced by rotating for 8 min in a tonometer containing pure carbon monoxide gas, followed by addition of sodium dithionite and a further 2 min of rotation in CO. Reduced samples were diluted and absorbance was read at 538 and 568 nm, and tissue myoglobin content was calculated as described (Reynafarje, 1963).

Whole-blood lactate concentrations were measured by thawing the frozen samples and acidifying them using an excess of 8% HClO<sub>4</sub> solution. Acidified extracts were incubated for 40 min at 37°C in assay buffer (0.6 mol l<sup>-1</sup> glycine, 0.5 mol l<sup>-1</sup> hydrazine sulphate with excess β-NAD<sup>+</sup> and LDH), and then absorbance was read at 340 nm in duplicate.

## Calculations and statistics

A residual approach accounted for the influence of body mass on O<sub>2</sub> consumption rate (Fig. 1), critical oxygen tension and oxygen tension at loss of equilibrium (Fig. 2) and total gill filament length (Fig. 4). Data were first regressed to body mass (*M*) using the general allometric equation  $Y=aM^b$  (where *a* and *b* are constants), and residuals from the regression were then calculated for each individual. The calculated residuals were used for statistical comparisons (see below). Data are reported graphically as both residuals and as the sum of the residual and the expected value for an average-sized killifish (see Figs 1, 2 and 4 for details).

Data are reported as means±standard error (except where data for individual fish are shown). For most data, hypoxic acclimation treatments were compared with normoxic controls with a one-way ANOVA. A two-way ANOVA was used to assess the effects of hypoxic acclimation treatment, sampling point (i.e. rest versus LOE), and their interaction on blood [lactate]. Bonferroni multiple comparisons post-tests were used for paired comparisons. A significance level of *P*<0.05 was used throughout.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

B.G.B. wrote the paper and led the majority of the experimentation, data collection and analysis. K.L.D., D.M.G. and G.R.S. contributed to data collection and analysis. G.R.S. designed and supervised the experiments. All authors contributed to the interpretation of data and to revising the manuscript. All authors approve the manuscript.

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