

## RESEARCH ARTICLE

# The effect of acclimation to hypoxia and sustained exercise on subsequent hypoxia tolerance and swimming performance in goldfish (*Carassius auratus*)

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### SUMMARY

The objective of this study was to determine whether acclimation to hypoxia and sustained exercise would increase hypoxia tolerance (as indicated by a decrease in critical oxygen tension,  $P_{crit}$ ) and swimming performance in goldfish (*Carassius auratus*), and to investigate the relationship between changes in performance and gill remodelling and tissue metabolic capacity. Goldfish were acclimated to either hypoxia (48 h at 0.3 mg O<sub>2</sub> l<sup>-1</sup>) or sustained exercise (48 h at 70% of critical swimming speed,  $U_{crit}$ ) and then  $P_{crit}$  and  $U_{crit}$  were determined in normoxia (10 mg O<sub>2</sub> l<sup>-1</sup>) and hypoxia (1 mg O<sub>2</sub> l<sup>-1</sup>) and compared with values from control fish. Acclimation to both hypoxia and sustained exercise improved hypoxia tolerance ( $P_{crit}$  was reduced by 49% and 39%, respectively), which was associated with an increase in lamellar surface area (71% and 43%, respectively) and an increase in blood [Hb] (26% in both groups). Exercise acclimation also resulted in a decrease in routine  $\dot{M}_{O_2}$  ( $\dot{M}_{O_2, rout}$ ). Acclimation to both hypoxia and sustained exercise resulted in a significant increase in  $U_{crit}$  in hypoxia (18% and 17%, respectively), which was associated with an increase in maximal O<sub>2</sub> consumption rate at  $U_{crit}$  ( $\dot{M}_{O_2, active}$ ; 35% and 39%, respectively). While hypoxia acclimation resulted in an increase in  $U_{crit}$  in normoxia, acclimation to sustained exercise did not improve subsequent swimming performance in normoxia. This lack of improvement was possibly due to depleted oxidizable substrates during exercise acclimation.

Key words: hypoxia, exercise, critical swimming speed, oxygen consumption.

### INTRODUCTION

Aerobic metabolism may ultimately be limited by oxygen uptake at the gills, O<sub>2</sub> transport by the blood, and oxygen delivery and metabolism at the level of the tissue. Acclimation at any of these levels may occur during a physiological challenge, indicating a possible rate-limiting step in the overall process (Randall and Brauner, 1991), but acclimation may also increase performance in a subsequent physiological challenge. In fish, hypoxia and exercise are environmentally relevant physiological challenges that can play an important role in dictating a species' ecological distribution (Hughes, 1973). In this study, we investigated the effects of acclimation to hypoxia and sustained exercise in goldfish on hypoxia tolerance and exercise performance. We selected goldfish (*Carassius auratus*) as an experimental model because of the species' plastic cardio-respiratory system and muscle metabolic capacity (Davison and Goldspink, 1978; Gamperl and Farrell, 2004; Sollid et al., 2005; Mitrovic et al., 2009).

Critical O<sub>2</sub> tension ( $P_{crit}$ ) defines the environmental O<sub>2</sub> tension at which an organism's routine oxygen consumption rate ( $\dot{M}_{O_2, rout}$ ) transitions from being independent of, to dependent on, environmental O<sub>2</sub>. As such,  $P_{crit}$  is considered a viable indicator of an animal's hypoxia tolerance (Ultsch et al., 1978; Mandic et al., 2008). Recently, Mandic and colleagues (Mandic et al., 2008) showed, in a group of closely related fish species, that a low  $P_{crit}$  (hypoxia tolerance) was phylogenetically independently related to

a low  $\dot{M}_{O_2, rout}$ , larger gill surface area and higher blood haemoglobin O<sub>2</sub> binding affinity.

In several species of cyprinid, including crucian carp (*Carassius carassius*) (Sollid et al., 2003), goldfish (Sollid et al., 2005; Mitrovic et al., 2009) and scaleless carp (*Gymnocypris przewalskii*) (Matey et al., 2008), hypoxia exposure has been found to result in dramatic changes in gill morphology that, in some cases, increase gill surface area by over 7-fold (Sollid et al., 2003) and reduce the water–blood diffusion distance (Matey et al., 2008). Several other species of carp (grass carp, *Ctenopharyngodon idellus*; silver carp, *Hypophthalmichthys molitrix*; and bighead carp, *Aristichthys nobilis*) have also recently been shown to exhibit extensive gill remodelling following exposure to hypoxia (J.G.R., R.D., V. Matey, C.J.B., Y.-X.W. and S.-J.F., unpublished data). These changes in gill morphology presumably enhance oxygen uptake from the water and in the crucian carp, gill remodelling is associated with a reduction in  $P_{crit}$  (Sollid et al., 2003). Extensive gill remodelling has also been shown to occur during exposure to an elevation in temperature in crucian carp (Sollid and Nilsson, 2006; Nilsson, 2007), goldfish (Sollid and Nilsson, 2006; Nilsson, 2007), grass carp (experimental unpublished data), silver carp (unpublished data) and bighead carp (unpublished data). Because both hypoxia and elevated temperature induce gill remodelling, it has been suggested that the signal for gill remodelling may be related to changes in the ambient oxygen supply or oxygen demand of the fish. An increase in

sustained exercise may represent the greatest increase in oxygen demand of a fish, and acclimation to sustained swimming for 48 h has been shown to induce gill remodelling in crucian carp (C.J.B., V. Matey, W. Zhang, J.G.R., R.D., Z.-D.C., Y.-X.W. and S.-J.F., unpublished).

Exposure to hypoxia also results in an increase in haemoglobin concentration ([Hb]) and blood oxygen-carrying capacity (Wood and Johansen, 1972; Tetens and Lykkeboe, 1981; Randall, 1982; Saint-Paul, 1984; Wells et al., 1989; Val et al., 1995; Silkin and Silkina, 2005), an increase in the number of muscle mitochondria and muscle myoglobin concentration, and a higher capillarization of muscle, which improves the extraction and utilization of circulating oxygen stores at low  $P_{O_2}$  (Johnston and Bernard, 1984; Sänger, 1993). At the same time, fish may down-regulate energy turnover and improve the efficiency of ATP production (Hochachka et al., 1996), resulting in a lower maintenance metabolism. Thus, adjustments at the level of the gills, blood and tissues during acclimation to hypoxia may potentially increase subsequent hypoxia tolerance in goldfish.

In many fish species, swimming performance is thought to be a central determinant of Darwinian fitness (Brett, 1964; Plaut, 2001; Blake, 2004). In fish, the determination of maximum sustainable swimming speed or critical swimming speed ( $U_{crit}$ ) is widely used to evaluate aerobic swimming performance (Gregory and Wood, 1998; Plaut, 2001; Lee et al., 2003; MacNutt et al., 2004). Aerobic swimming performance may be limited by  $O_2$  uptake and delivery or aerobic metabolic capacity of the muscle.

As mentioned above, changes in gill morphology during sustained swimming have not previously been investigated, but if swimming performance is limited by gill diffusion capacity and acclimation to sustained swimming is associated with changes in gill morphology, this may influence subsequent swimming performance. At swimming velocities approaching  $U_{crit}$ , the metabolic demand for oxygen may be greater than that which can be provided by the cardio-respiratory system, just as would occur in hypoxic conditions (Jones and Randall, 1978). Likewise, exercise training in fish may also alter components of the cardio-respiratory system, for example, resulting in larger hearts or higher pumping performance (Hochachka, 1961; Farrell et al., 1990; Gamperl and Farrell, 2004), higher Hb and myoglobin concentration (Holk and Lykkeboe, 1998), and increased skeletal muscle capillarity and tissue  $O_2$  extraction (Love et al., 1977; Gamperl and Farrell, 2004). An increase in  $\dot{M}_{O_2,max}$  and  $U_{crit}$  has been observed after exercise training in rainbow trout (*Oncorhynchus mykiss*) (Holk and Lykkeboe, 1998), darkbarbel catfish (*Peltebagnrus vachelli*) (Liu et al., 2009; Li et al., 2010) and striped bass (*Morone saxatilis*) (Young and Cech, 1993; Young and Cech, 1994); however, they have not previously been investigated in goldfish.

In this study we hypothesized that hypoxia acclimation will enhance hypoxia tolerance, and that acclimation to exercise may enhance exercise performance in goldfish. Furthermore, we investigated whether there was evidence for cross-tolerance between hypoxia- and exercise-acclimated fish. To address our hypothesis, goldfish were acclimated to either hypoxia (48 h at  $0.3 \text{ mg } O_2 \text{ l}^{-1}$ ) or sustained exercise (48 h at 70% of  $U_{crit}$ ), and then  $P_{crit}$  and  $U_{crit}$  were determined in normoxia ( $10 \text{ mg } O_2 \text{ l}^{-1}$ ) and hypoxia ( $1 \text{ mg } O_2 \text{ l}^{-1}$ ) for comparison with values from control fish. Furthermore, the physiological basis for the hypothesized increase in tolerance and cross-tolerance was investigated by measuring changes in gill morphology, metabolic rate ( $\dot{M}_{O_2}$ ), blood  $O_2$  transport capacity (Hb level) and concentrations of oxidizable substrates and products (plasma and muscle lactate, glucose and/or glycogen) at rest or immediately after a  $U_{crit}$  test.

## MATERIALS AND METHODS

### Experimental fish and holding conditions

Juvenile goldfish (*C. auratus* L.) ( $N=200$ ,  $4.29-7.87 \text{ g}$ ) were obtained from a local market in Chongqing, China. The fish were maintained in a re-circulating water tank system at Chongqing Normal University for 2 weeks prior to experimentation. During this time, the temperature of the de-chlorinated freshwater was maintained at  $12.0 \pm 0.5^\circ\text{C}$  and the oxygen level was kept above  $10 \text{ mg l}^{-1}$ . Fish were fed to satiation daily at 21:00 h with a commercial diet.

### Acclimation to hypoxia and sustained exercise

After the 2 week housing period, fish were starved for 48 h and 150 fish of similar size ( $6.28 \pm 0.09 \text{ g}$ ) were randomly selected and divided into three groups of 50 fish: (i) hypoxia acclimation group, (ii) sustained exercise acclimation group and (iii) control group, where water  $[O_2]$  was maintained at  $10 \text{ mg l}^{-1}$  (with the exception of the hypoxic treatment as described below). Water temperature was maintained at  $12^\circ\text{C}$  and fish were fed to satiation daily at 21:00 h.

#### Acclimation to hypoxia

Fifty fish were transferred to a 120 l exposure chamber where hypoxia was achieved by covering the surface of the water with translucent plastic and bubbling nitrogen into the water (Matey et al., 2008). Water  $[O_2]$  was reduced from aerated values of  $10 \text{ mg l}^{-1}$  to  $0.3 \text{ mg l}^{-1}$  over 1 h and was then maintained at  $0.3 \text{ mg l}^{-1}$  ( $0.2-0.4 \text{ mg l}^{-1}$  regulated by hand) for 48 h, during which time water  $[O_2]$  level was continuously monitored using a dissolved oxygen probe (HQ20, Hach Company, Loveland, CO, USA).

#### Acclimation to sustained exercise

Fifty fish were simultaneously transferred to an experimental exercise chamber [for details, see Li et al. (Li et al., 2010)]. Water velocity within the exercise chamber was gradually increased by  $3 \text{ cm s}^{-1} \text{ h}^{-1}$  to  $21 \text{ cm s}^{-1}$ , which represented  $\sim 70\%$  of  $U_{crit}$  (where  $U_{crit}$  was determined in a pilot experiment). Fish were then maintained at this water velocity for 48 h.

#### Control group

Fish in the control group were maintained in an identical chamber to that used for exercise training for 48 h prior to experimentation, with a flow rate of  $3 \text{ cm s}^{-1}$ , which was sufficient to replenish the water without inducing apparent swimming activity.

### $\dot{M}_{O_2,rout}$ and $P_{crit}$ determination

After 48 h acclimation to hypoxia, sustained exercise or control conditions, feeding was withheld for 48 h and 10 fish were randomly selected from each acclimation group and placed in a 160 ml volume respirometer for measurement of  $\dot{M}_{O_2}$  and  $P_{crit}$  (Zhang et al., 2010). Fish were allowed to recover from transfer to the respirometer for 1 h (to minimize reversible acclimation effects of hypoxia and exercise as described in the Discussion), during which time there was continuous aerated water flow through the respirometer. Subsequently, the respirometer was closed and  $\dot{M}_{O_2}$  was measured over a range of water  $[O_2]$  as the fish depleted the oxygen within the closed respirometer, starting from about 95% saturation down to 1% saturation. If the fish did not remain calm over the entire duration, data from that trial were discarded. For measurement of dissolved oxygen, the circulating water from the respirometer was drawn from the respirometer by a peristaltic pump, forced past a dissolved oxygen probe (HQ20) housed in a sealed thermostatically regulated chamber, and then returned to the respirometer. The system

temperature was maintained at  $12 \pm 0.2^\circ\text{C}$  using a thermostatically regulated water bath.

The following formula was used to calculate the  $\dot{M}_{\text{O}_2}$  ( $\text{mg kg}^{-1} \text{h}^{-1}$ ) of individual fish:

$$\dot{M}_{\text{O}_2} = ([\text{O}_2]_k - [\text{O}_2]_{k+1}) v / tm, \quad (1)$$

where  $[\text{O}_2]_k$  refers to the oxygen concentration ( $\text{mg l}^{-1}$ ) at time point  $k$ ,  $[\text{O}_2]_{k+1}$  is that at the next time point (these values were calculated according to the  $\text{O}_2$  solubility coefficient in water under corresponding temperature and pressure),  $v$  ( $\text{l}$ ) is the total volume of the respirometer minus the volume of the fish,  $t$  ( $\text{h}$ ) is the interval between time points  $k$  and  $k+1$ , and  $m$  ( $\text{kg}$ ) is the body mass of the fish.

$P_{\text{crit}}$ , the point at which  $\dot{M}_{\text{O}_2, \text{rout}}$  could no longer be maintained with a further reduction in water  $\text{O}_2$  tension, was estimated for individual fish by the two-segment linear model described by Yeager and Ultsch (Yeager and Ultsch, 1989).

#### $U_{\text{crit}}$ and swimming $\dot{M}_{\text{O}_2}$

After 48 h acclimation to hypoxia, sustained exercise or control conditions, feeding was withheld for 48 h and 16 fish were randomly selected from each group and subjected to a  $U_{\text{crit}}$  test in either normoxic water ( $10 \text{ mg l}^{-1}$ ,  $N=8$ ) or hypoxic water ( $1 \text{ mg l}^{-1}$ ,  $N=8$ ). The water  $[\text{O}_2]$  ranged from  $10.20$  to  $10.40 \text{ mg l}^{-1}$  for normoxic swimming and from  $1.10$  to  $1.30 \text{ mg l}^{-1}$  for hypoxic swimming.

A Brett-type swimming tunnel respirometer with a swim chamber of  $19.87 \text{ cm}^2$  cross-sectional area was used to measure the fish's  $U_{\text{crit}}$  [total volume  $3.5 \text{ l}$ ; for details see Li et al. (Li et al., 2010) and Pang et al. (Pang et al., 2010)]. Fish were individually transferred into the swim tunnel and allowed to recover for 1 h, during which time there was continuous aerated water flow through the respirometer. Water temperature in the swimming chamber was controlled at  $12 \pm 0.2^\circ\text{C}$  using a waterbath connected to a stainless steel heat exchanger. Water velocity was increased in  $3 \text{ cm s}^{-1}$  increments ( $\sim 0.5 \text{ BL s}^{-1}$ , where BL is body lengths) every 30 min until the fish fatigued. Fatigue was defined as the point at which the fish failed to move off the rear honeycomb screen of the swimming chamber after 20 s (Lee et al., 2003).  $U_{\text{crit}}$  was calculated for individual fish using Brett's equation (Brett, 1964):

$$U_{\text{crit}} = V + (t_s / T) \Delta V, \quad (2)$$

where  $V$  is the highest speed at which the fish swam for the full time period ( $\text{cm s}^{-1}$ ),  $\Delta V$  is the velocity increment ( $3 \text{ cm s}^{-1}$ ),  $T$  is the prescribed period of swimming per speed (30 min) and  $t_s$  is the length of time that the fish swam at the final speed (min). The swim tunnel was designed to switch between a closed mode and an open mode, the former for respirometry, the latter to replenish the oxygen levels.

In open mode, the respirometer was supplied with  $12^\circ\text{C}$  water from a 350 l reservoir tank at a flow rate of  $500 \text{ ml min}^{-1}$ . In normoxia, the water in the reservoir tank was fully aerated ( $10 \text{ mg l}^{-1}$ ); in hypoxia, the surface of the reservoir tank was covered with translucent plastic and bubbled with nitrogen to achieve a nominal water  $[\text{O}_2]$  of  $1.0 \text{ mg l}^{-1}$ .

In closed mode, the tunnel was isolated from the reservoir tank and water was recirculated within the system. A small volume of water was drawn from the sealed respirometer by a peristaltic pump, forced past a dissolved oxygen probe housed in a sealed temperature-controlled chamber, and then returned to the respirometer. Oxygen concentration ( $\text{mg l}^{-1}$ ) was recorded once every 2 min. The  $\dot{M}_{\text{O}_2}$  ( $\text{mg kg}^{-1} \text{h}^{-1}$ ) of individual fish while swimming was calculated from the depletion of oxygen according to the equation:

$$\dot{M}_{\text{O}_2} = 60S v / m, \quad (3)$$

where slope  $S$  ( $\text{mg l}^{-1} \text{min}^{-1}$ ) is the decrease in the water's dissolved oxygen content per minute,  $v$  is the total volume of the respirometer ( $3.5 \text{ l}$ ) minus the volume of the fish, and  $m$  is the body mass ( $\text{kg}$ ) of the fish. The slope ( $S$ ) was obtained through linear regression between time (min) and the water's dissolved oxygen content ( $\text{mg l}^{-1}$ ); only slopes with an  $r^2 > 0.95$  were considered in the analysis. The decrease in water  $[\text{O}_2]$  in the respirometer during each  $\dot{M}_{\text{O}_2}$  measurement was never allowed to drop by more than  $0.25 \text{ mg l}^{-1}$  in either the normoxic or hypoxic  $U_{\text{crit}}$  determination. The maximal  $\dot{M}_{\text{O}_2}$  during the  $U_{\text{crit}}$  test was defined as active  $\dot{M}_{\text{O}_2}$  ( $\dot{M}_{\text{O}_2, \text{active}}$ ) and the difference between  $\dot{M}_{\text{O}_2, \text{active}}$  and  $\dot{M}_{\text{O}_2, \text{rout}}$  (calculated from the  $P_{\text{crit}}$  trials described above) was used to calculate metabolic scope. In normoxia,  $\dot{M}_{\text{O}_2, \text{rout}}$  was determined from the  $P_{\text{crit}}$  trials as the measured  $\dot{M}_{\text{O}_2}$  at  $P_{\text{crit}}$ ; in hypoxia,  $\dot{M}_{\text{O}_2, \text{rout}}$  was also calculated from the  $P_{\text{crit}}$  trials, but over an  $[\text{O}_2]$  range of  $0.95$ – $1.05 \text{ mg O}_2 \text{ l}^{-1}$ .

#### Gill morphology

After 48 h acclimation to hypoxia, sustained exercise or control conditions, feeding was withheld for 48 h and fish were allowed to recover for 1 h in normoxia. Four fish from each group were immediately killed using neutralized tricaine methanesulphonate (MS-222,  $50 \text{ mg l}^{-1}$ ). The second gill arch from the right-hand side of each fish was dissected out, rinsed and immediately fixed in cold Karnovsky's fixative for later scanning electron microscopy (SEM) (in the Third Military Medical University, Chongqing, China).

The middle part of each fixed gill arch ( $5 \text{ mm}$  long) bearing up to 20 filaments in both anterior and posterior rows was used for SEM. All fixed gill tissue was rinsed in phosphate-buffered saline (PBS) and post-fixed in 1% osmium tetroxide for 1 h. Gill tissue was dehydrated in ascending concentrations of ethanol from 30% to 100%, critical-point dried with liquid  $\text{CO}_2$ , mounted on stubs, sputter-coated with gold-palladium, and examined with a Hitachi S 3400 scanning electron microscope at the accelerating voltage of  $15 \text{ kV}$ .

Protruding lamellar height in contact with ambient water, lamellar thickness, and protruding lamellar basal length were measured by SEM in control, hypoxia-acclimated and exercise-acclimated fish. A total of 20 measurements (in five randomly selected lamellae in four fish, and five randomly selected filaments in four fish) of each of these parameters were performed in each acclimation group. The surface area of protruding lamellae ( $a$ ) was approximated to a half-ellipse and calculated according to the formula proposed by Sollid et al. (Sollid et al., 2003):

$$a = \pi l [(r^2 + h^2) / 2]^{0.5}, \quad (4)$$

where  $l$  is the basal length of the protruding part of the lamellae,  $h$  is the height of the protruding part of the lamellae and  $r$  is the half-value of the thickness of lamellae.

#### Biochemistry

After 48 h acclimation to hypoxia, sustained exercise or control conditions, feeding was withheld for 48 h. Fish were allowed to recover from each treatment for 1 h in normoxia and six fish from each group were immediately killed using neutralized MS-222 ( $50 \text{ mg l}^{-1}$ ) and samples were taken for measurement of metabolites in resting fish. Six out of eight fish subjected to the  $U_{\text{crit}}$  test were also sampled immediately following fatigue for measurement of post-exercise values of metabolites. A dorsal white muscle sample was excised posterior to the dorsal fin with a scalpel. Blood was drawn from the caudal vein using a heparinized syringe and [Hb] was measured by spectrophotometric analysis after conversion to

cyanmethaemoglobin (Lin and Liu, 2006). The remaining blood was transferred to a 1.5 ml centrifuge tube, spun at 11,000 g, and the plasma was separated and frozen for later analysis. Plasma glucose concentration was measured by the *o*-toluidine method, and plasma and muscle lactate levels were measured by the *p*-phenylphenol method. The white muscle glycogen content was measured by the anthrone method [for details, see Li et al. (Li et al., 2007)]. All concentrations were determined using a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) and appropriate standards.

#### Data analysis

Statistica 4.5 (StatSoft Inc., www.statsoft.com) was used for data analysis. The effects of acclimation group (hypoxia, sustained exercise, control) and water [O<sub>2</sub>] levels on  $U_{crit}$ ,  $\dot{M}_{O_2,active}$ , muscle lactate and glycogen levels, and plasma glucose and lactate levels were determined by a two-way analysis of variance (ANOVA). The effects of acclimation group and water [O<sub>2</sub>] levels on swimming  $\dot{M}_{O_2}$  at different swimming speeds were determined by a two-way repeated-measures analysis of variance (ANOVA). The effects of acclimation group on [Hb], gill morphological parameters,  $P_{crit}$  and  $\dot{M}_{O_2,rout}$  were determined by a one-way ANOVA. The ANOVA was followed by a least significant difference multiple comparison test or a *t*-test if it was necessary to determine where significant differences occurred.  $P < 0.05$  was considered statistically significant and all data are presented as means  $\pm$  s.e.m.

## RESULTS

### $\dot{M}_{O_2,rout}$ and $P_{crit}$

As expected, in all acclimation groups (hypoxia, exercise and control)  $\dot{M}_{O_2,rout}$  remained constant over a broad range of water [O<sub>2</sub>], but declined at [O<sub>2</sub>] below  $P_{crit}$  (Fig. 1). The  $P_{crit}$  values of the hypoxia- and exercise-acclimated groups were significantly lower than those of the control group (Table 1).  $\dot{M}_{O_2,rout}$  at  $P_{crit}$  of the exercise-acclimated group was significantly lower than that of the control fish.

### $U_{crit}$ , $\dot{M}_{O_2,active}$ and metabolic scope

In all acclimation groups,  $U_{crit}$ ,  $\dot{M}_{O_2,active}$  and metabolic scope for fish swum in hypoxia were all significantly lower than those determined for fish swum in normoxia (Table 2).

#### Hypoxia acclimation effect

Fish acclimated to hypoxia for 48 h had significantly higher  $U_{crit}$ ,  $\dot{M}_{O_2,active}$  and metabolic scope relative to control fish, when fish were swum in hypoxia. While  $U_{crit}$  was also elevated in fish that were swum in normoxia, no differences in  $\dot{M}_{O_2,active}$  or metabolic scope were observed (Table 2).

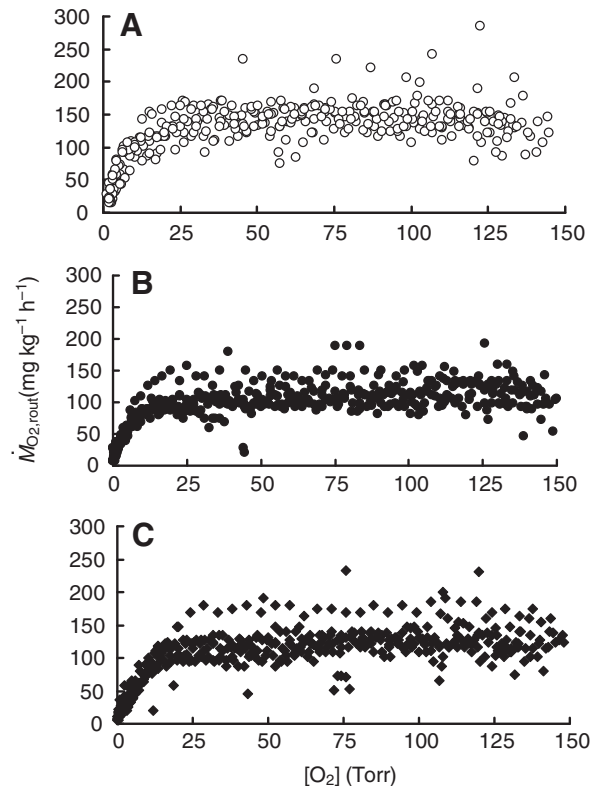


Fig. 1. The routine O<sub>2</sub> consumption rate ( $\dot{M}_{O_2,rout}$ ) of control (A), hypoxia-acclimated (B) and exercise-acclimated (C) goldfish relative to water [O<sub>2</sub>] during closed respirometry. See Table 1 for sample size and calculated critical O<sub>2</sub> tension ( $P_{crit}$ ) values.

#### Exercise acclimation effect

For exercise-acclimated fish,  $U_{crit}$ ,  $\dot{M}_{O_2,active}$  and metabolic scope were all significantly higher than those of control fish when swum in hypoxia, but no differences were observed when fish were swum in normoxia (Table 2).

### $\dot{M}_{O_2}$ vs swimming speed

In general,  $\dot{M}_{O_2}$  increased with swimming speed in all acclimation groups as expected (Fig. 2). Within each acclimation group, the  $\dot{M}_{O_2}$  values of fish swum in hypoxia were significantly lower than those of fish swum in normoxia at any given swimming speed (Fig. 2).

#### Hypoxia acclimation effect

When fish were swum in hypoxia,  $\dot{M}_{O_2}$  of hypoxia-acclimated fish was significantly higher than that of control fish at the highest

Table 1. Effect of control conditions and acclimation to hypoxia or sustained exercise on subsequent critical oxygen tension ( $P_{crit}$ ) and oxygen consumption rate ( $\dot{M}_{O_2}$ ) at  $P_{crit}$  in goldfish

	Control	Hypoxia acclimated	Exercise acclimated
<i>N</i>	7	7	6
Body mass (g)	4.88 $\pm$ 0.16 <sup>a</sup>	5.10 $\pm$ 0.20 <sup>a</sup>	4.83 $\pm$ 0.14 <sup>a</sup>
Body length (cm)	5.44 $\pm$ 0.11 <sup>a</sup>	5.47 $\pm$ 0.10 <sup>a</sup>	5.42 $\pm$ 0.08 <sup>a</sup>
$P_{crit}$ (Torr)	22.97 $\pm$ 3.01 <sup>a</sup>	11.63 $\pm$ 0.86 <sup>b</sup>	14.08 $\pm$ 1.58 <sup>b</sup>
$\dot{M}_{O_2}$ at $P_{crit}$ (mg kg <sup>-1</sup> h <sup>-1</sup> )	145.7 $\pm$ 7.8 <sup>a</sup>	120.4 $\pm$ 10.1 <sup>a,b</sup>	101.5 $\pm$ 8.9 <sup>b</sup>

Fish were acclimated to hypoxia (48 h at 0.3 mg O<sub>2</sub> l<sup>-1</sup>) or sustained exercise (48 h at 70% of critical swimming speed,  $U_{crit}$ ), or maintained under control conditions.

Superscript letters that differ within a row indicate statistically significant differences among acclimation groups ( $P < 0.05$ ). Values are reported as means  $\pm$  s.e.m.

Table 2. Effect of control conditions and acclimation to hypoxia or sustained exercise on subsequent swimming performance in goldfish in either normoxia or hypoxia

	Control		Hypoxia acclimated		Exercise acclimated	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
<i>N</i>	8	8	8	8	8	8
Body mass (g)	6.45±0.81	6.52±0.86	6.31±0.27	6.09±0.21	6.47±1.37	6.04±1.03
Body length (cm)	5.85±0.36	5.85±0.26	5.94±0.08	5.89±0.11	5.90±0.42	5.73±0.24
Mean [O <sub>2</sub> ] during <i>U</i> <sub>crit</sub> test (mg l <sup>-1</sup> )	10.26±0.02	1.09±0.02	10.12±0.02	1.14±0.02	10.15±0.02	1.12±0.02
<i>U</i> <sub>crit</sub> (BL s <sup>-1</sup> )	5.35±0.09	3.29±0.14 <sup>†</sup>	5.79±0.16*	3.88±0.19* <sup>†</sup>	4.99±0.24	3.84±0.18* <sup>†</sup>
$\dot{M}_{O_2,active}$ (mg kg <sup>-1</sup> h <sup>-1</sup> )	543.9±35.1	180.5±12.8 <sup>†</sup>	577.4±24.9	243.7±6.3* <sup>†</sup>	506.4±29.5	250.3±13.2* <sup>†</sup>
$\dot{M}_{O_2,rout}$ (mg kg <sup>-1</sup> h <sup>-1</sup> ) <sup>‡</sup>	145.7±7.8	113.7±6.0 <sup>†</sup>	120.4±10.1	104.5±3.4 <sup>†</sup>	101.5±8.9*	95.4±4.6*
Metabolic scope (mg kg <sup>-1</sup> h <sup>-1</sup> )	398.2±24.8	68.9±9.8 <sup>†</sup>	457.0±24.9	139.2±6.5* <sup>†</sup>	406.7±29.1	154.9±19.1* <sup>†</sup>

Fish were acclimated to hypoxia (48 h at 0.3 mg O<sub>2</sub> l<sup>-1</sup>) or sustained exercise (48 h at 70% of critical swimming speed, *U*<sub>crit</sub>), or maintained under control conditions. Fish swam in normoxia (10 mg O<sub>2</sub> l<sup>-1</sup>) or hypoxia (1 mg O<sub>2</sub> l<sup>-1</sup>). Routine oxygen consumption rate ( $\dot{M}_{O_2,rout}$ ) and maximum  $\dot{M}_{O_2}$  during *U*<sub>crit</sub> ( $\dot{M}_{O_2,active}$ ) are given.

\*Statistically significant difference relative to the control group at the respective water [O<sub>2</sub>] (i.e. normoxia or hypoxia; *P*<0.05). <sup>†</sup>Statistically significant difference relative to the normoxic group within a given acclimation group (*P*<0.05). Values are reported as means ± s.e.m.

<sup>‡</sup>In normoxia, the  $\dot{M}_{O_2,rout}$  values used to calculate metabolic scope were the  $\dot{M}_{O_2,rout}$  values measured at *P*<sub>crit</sub>. For hypoxia,  $\dot{M}_{O_2,rout}$  values were also calculated from the *P*<sub>crit</sub> determination, but when [O<sub>2</sub>] was in the range 0.95–1.05 mg O<sub>2</sub> l<sup>-1</sup>.

swimming speed tested (18 cm s<sup>-1</sup>; Fig. 2). There was no significant difference in  $\dot{M}_{O_2}$  between hypoxia-acclimated and control fish at any swimming speed when fish were swum in normoxia.

#### Exercise acclimation effect

When fish were swum in hypoxia, exercise acclimation resulted in a significantly higher  $\dot{M}_{O_2}$  at the fastest swimming speed (18 cm s<sup>-1</sup>) compared with control fish. There was no significant difference in  $\dot{M}_{O_2}$  between exercise-acclimated and control fish at any given swimming speed when fish were swum in normoxia.

#### Gill morphology

Hypoxia acclimation induced changes in gill morphology including a significant increase in protruding lamellar height, lamellar thickness, protruding lamellar basal length and lamellar surface area (Table 3). Exercise acclimation also induced a significant increase in protruding lamellar basal length and lamellar surface area, but there was no significant change in protruding lamellar height or lamellar thickness (Table 3).

#### Biochemistry

##### Haemoglobin

Blood [Hb] was significantly higher in both the hypoxia-acclimated (12.45±0.41 g 100 ml<sup>-1</sup>) and exercise-acclimated (12.54±0.26 g 100 ml<sup>-1</sup>) fish compared with control fish (9.92±0.51 g 100 ml<sup>-1</sup>).

##### Muscle glycogen content

In fish sampled at rest, hypoxia and exercise acclimation resulted in a 24% and 73% decrease in the muscle glycogen content, respectively (Fig. 3A). In all three acclimation groups, muscle glycogen content significantly decreased following *U*<sub>crit</sub> in both normoxia and hypoxia. Relative to control fish, muscle glycogen content following *U*<sub>crit</sub> was significantly lower in exercise-acclimated fish swum in normoxia and higher in hypoxia-acclimated fish swum in hypoxia.

##### Muscle lactate content

In fish sampled at rest, hypoxia acclimation resulted in an 84% increase in white muscle lactate content while exercise acclimation had no significant effect (Fig. 3B). Relative to resting levels, *U*<sub>crit</sub> resulted in a significant increase in muscle lactate content in the control fish (swum in both normoxia and hypoxia) and the hypoxia-

acclimated fish (swum in hypoxia) but no differences were observed in the exercise-acclimated fish.

Hypoxia acclimation resulted in significantly greater muscle lactate content relative to that of control fish following *U*<sub>crit</sub> in normoxia, but a lower muscle lactate content following *U*<sub>crit</sub> in hypoxia. In exercise-acclimated fish, muscle lactate content was significantly lower than that in control fish following *U*<sub>crit</sub> in normoxia and hypoxia.

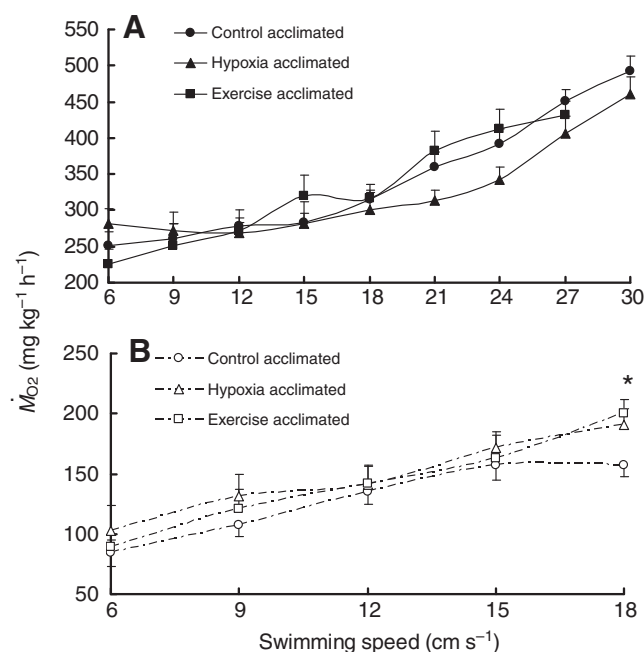


Fig. 2. Effect of acclimation to hypoxia (48 h at 0.3 mg O<sub>2</sub> l<sup>-1</sup>), sustained exercise (48 h at 70% critical swimming speed, *U*<sub>crit</sub>) and control conditions on oxygen consumption rate ( $\dot{M}_{O_2}$ ) at progressively increasing water velocity for goldfish swum in normoxia (10 mg O<sub>2</sub> l<sup>-1</sup>; A) or hypoxia (1 mg O<sub>2</sub> l<sup>-1</sup>; B). \*Significant difference in  $\dot{M}_{O_2}$  (*P*<0.05). Within each acclimation group, the  $\dot{M}_{O_2}$  values of fish swum in hypoxia were significantly lower than those of fish swum in normoxia at any given swimming speed (two-factor ANOVA, *P*<0.05).

Table 3. Effect of control conditions and acclimation to hypoxia or sustained exercise on gill morphology in goldfish

	Control	Hypoxia acclimated	Exercise acclimated
<i>N</i>	4	4	4
Body mass (g)	6.20±0.29 <sup>a</sup>	6.26±0.18 <sup>a</sup>	6.14±0.21 <sup>a</sup>
Body length (cm)	5.98±0.14 <sup>a</sup>	5.85±0.08 <sup>a</sup>	5.75±0.09 <sup>a</sup>
Protruding lamellar height (µm)	41.85±0.12 <sup>b</sup>	56.59±2.25 <sup>a</sup>	42.47±4.02 <sup>b</sup>
Lamellar thickness (µm)	7.51±0.44 <sup>b</sup>	10.71±0.73 <sup>a</sup>	8.52±0.48 <sup>b</sup>
Protruding lamellar basal length (µm)	75.26±4.98 <sup>b</sup>	95.93±8.92 <sup>a</sup>	105.88±6.21 <sup>a</sup>
Lamellar surface area (mm <sup>2</sup> )	0.0070±0.0005 <sup>b</sup>	0.0120±0.0010 <sup>a</sup>	0.0099±0.0005 <sup>a</sup>

Fish were acclimated to hypoxia (48 h at 0.3 mg O<sub>2</sub> l<sup>-1</sup>) or sustained exercise (48 h at 70% of critical swimming speed,  $U_{crit}$ ), or maintained under control conditions.

Superscript letters that differ within a row indicate statistically significant differences among acclimation groups ( $P < 0.05$ ). Values are reported as means ± s.e.m.

#### Plasma [glucose]

In fish sampled at rest, there was no effect of any acclimation condition on plasma [glucose] (Fig. 3C), but plasma [glucose] significantly increased following  $U_{crit}$  conducted in normoxia and hypoxia except in the exercise-acclimated fish swum in normoxia. In both hypoxia- and exercise-acclimated fish, plasma [glucose] following  $U_{crit}$  was lower than that of control fish when fish were swum in hypoxia.

#### Plasma [lactate]

There were no significant changes in the plasma [lactate] (Fig. 3D) with the exception of hypoxia-acclimated fish, where plasma [lactate] increased significantly relative to resting values following  $U_{crit}$  conducted in normoxia and hypoxia.

### DISCUSSION

This study found that following acclimation to hypoxia (48 h at 0.3 mg O<sub>2</sub> l<sup>-1</sup>) or sustained exercise (48 h at 70%  $U_{crit}$ ), hypoxia tolerance, evaluated as  $P_{crit}$ , dramatically increased. The effects of hypoxia acclimation on hypoxia tolerance observed here were similar to those seen in the crucian carp (*C. carassius*) (Sollid et al., 2003); however, increased hypoxia tolerance following acclimation to sustained exercise has not previously been documented. This study also found that acclimation to hypoxia or sustained exercise resulted in increases in  $U_{crit}$  and  $MO_{2,active}$  when fish were swum in hypoxia, but only hypoxia acclimation resulted in an increase in  $U_{crit}$  in normoxia. In general, both hypoxia tolerance and swimming performance were improved following acclimation to hypoxia and sustained exercise in goldfish; however, the mechanism(s) of acclimation involved differed as described below.

Many of the acclimation responses to hypoxia and exercise are expected to be rapidly reversed following return to normoxia or when exercise stops. Thus, in order to evaluate the effects of acclimation on  $P_{crit}$  and  $U_{crit}$  the standard protocol of an overnight recovery period in normoxia respirometers or swim tunnels prior to analysis was not an option. In order to minimize the potential loss of acclimation responses to hypoxia or exercise, fish were only allowed 1 h recovery from transfer to the respirometer or swim tunnel in hypoxia-acclimated, exercise-acclimated and control goldfish. This short recovery period after transfer would be expected to induce a similar level of stress in each group and it is assumed that, if anything, it would reduce differences associated with the respective treatments. Thus, the findings here may underestimate the degree to which acclimation to hypoxia and exercise result in enhanced performance.

#### The effect of acclimation to hypoxia on hypoxia tolerance and swimming performance

Organisms increase their hypoxia tolerance by enhancing their respiratory capacity (Wood and Johansen, 1972; Randall, 1982; Saint-Paul, 1984; Woo and Wu, 1984) and/or through metabolic rate depression (Hochachka, 1997; Seidl et al., 2005; Almeida-Val et al., 2000). In the present study, hypoxia tolerance in goldfish improved after 48 h acclimation to hypoxia as indicated by a 49% reduction in  $P_{crit}$  (Table 1). This is consistent with a previous study that found a 50% reduction in  $P_{crit}$  after hypoxia acclimation (0.75 mg O<sub>2</sub> l<sup>-1</sup> at 8°C for 7 days) in crucian carp and the decrease in  $P_{crit}$  was primarily ascribed to extensive gill remodelling and an increase in gill surface area (Sollid et al., 2003). While in the current study a 71% increase in lamellar surface area was observed following acclimation to hypoxia, there was also a 26% increase in blood [Hb], which also probably contributed to increased hypoxia tolerance. Fish that are not known to exhibit gill remodelling have also been shown to increase hypoxia tolerance following hypoxia acclimation; however, the degree of change is not as large as that observed in this study. After 6 weeks of acclimation to 1.0 mg O<sub>2</sub> l<sup>-1</sup> at 10°C in the sailfin molly (*Poecilia latipinna*), a 20% decrease in  $P_{crit}$  was observed compared with normoxia-acclimated fish (Timmerman and Chapman, 2004). Similarly, long-term (>6 weeks) hypoxia acclimation to 4.5 mg O<sub>2</sub> l<sup>-1</sup> at 25°C led to a 20% decrease in  $P_{crit}$  in Atlantic cod (*Gadus morhua*) (L. H. Petersen and A. K. Gamperl, unpublished data), which was associated with increased blood [Hb], Hb-O<sub>2</sub> binding capacity and O<sub>2</sub> extraction efficiency (Petersen and Gamperl, 2010; Lamarche et al., 2009). Thus, it appears that the large improvement in hypoxia tolerance associated with hypoxia acclimation in cyprinids compared with other groups of fish is primarily due to the well-described gill remodelling (Sollid et al., 2003) and improved O<sub>2</sub> uptake; however, cyprinids are very hypoxia tolerant to start with largely because of their very high affinity haemoglobins (Sollid et al., 2005).

It has long been known that fish can improve their swimming performance following acclimation that results in improved muscle and/or cardio-respiratory performance (Davison, 1997; Sanger, 1993; Nilsson, 2007; Gamperl and Farrell, 2004). In this study, the increased swimming performance in hypoxia-acclimated goldfish was clearly demonstrated by an 8% increase in  $U_{crit}$  when fish were swum in normoxia and an 18% increase when they were swum in hypoxia (Table 2). The latter is somewhat surprising given that hypoxia acclimation resulted in lower muscle glycogen content and higher muscle lactate content in resting fish (Fig. 3A,B). In rainbow trout, hypoxia acclimation has been shown to result in increased oxygen uptake efficiency (Bushnell et al., 1984), but hypoxia acclimation has not previously been shown to have any significant

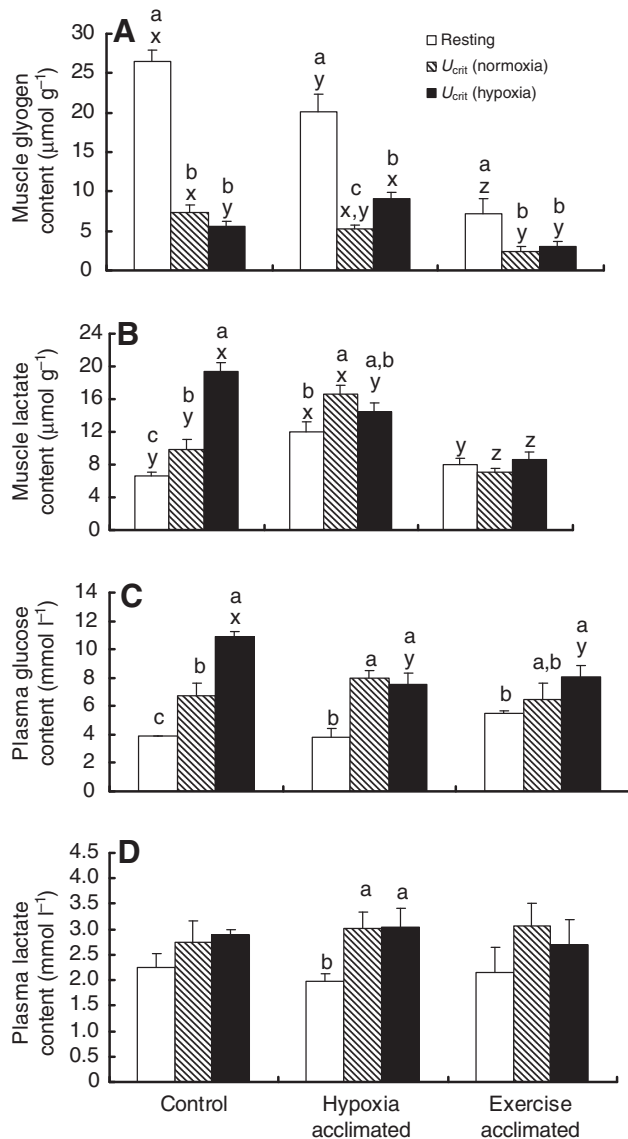


Fig. 3. Effect of acclimation to hypoxia (48 h at  $0.3 \text{ mg O}_2 \text{ l}^{-1}$ ), sustained exercise (48 h at  $70\% U_{crit}$ ) and control conditions on white muscle glycogen (A) and lactate (B) content, and plasma glucose (C) and lactate (D) concentration in goldfish sampled at different exercise states: rest, following  $U_{crit}$  in normoxia and following  $U_{crit}$  in hypoxia. Data are means  $\pm$  s.e.m.,  $N=6$ . Different letters indicate statistically significant differences in exercise state (rest,  $U_{crit}$  normoxia and  $U_{crit}$  hypoxia) within an acclimation group (control, hypoxia acclimated and normoxia acclimated: a,b,c) or differences between treatment groups in a given exercise state (x,y,z).

effect on swimming performance in rainbow trout (Bushnell et al., 1984), cod (Peterson and Gamperl, 2010) or zebrafish (*Danio rerio*) (Widmer et al., 2006). The improved swimming performance observed in hypoxia-acclimated goldfish may be partly due to gill remodelling and the associated increase in aerobic scope as well as possible changes at the level of the muscle.

Aerobic metabolic scope has long been considered an important determinant of aerobic swimming performance in fish (Fry, 1971; Claireaux et al., 2006; Chabot and Claireaux, 2008). Thus, factors either limiting maximum  $\dot{M}O_2$  or increasing maintenance metabolism would reduce metabolic scope and negatively impact swimming performance. In this study, hypoxia acclimation increased  $U_{crit}$  in

hypoxia and normoxia, and these increases in  $U_{crit}$  were accompanied by a 102% and 15% increase in metabolic scope when compared with goldfish assayed under the same conditions from the control group (Table 2). The increased metabolic scope was largely accounted for by increased  $\dot{M}O_{2,active}$ , which was elevated by 35% in hypoxia and 6% in normoxia (Table 2). Down-regulation of maintenance metabolism appears to play a relatively minor role. The increased swimming performance of hypoxia-acclimated fish swum in hypoxia was in part due to increased aerobic metabolic capacity and aerobic energy production for swimming, as suggested by lower post-exercise muscle lactate levels (74% lower than that of the control group) and a lower lactate production:glycogen depletion ratio (L/G ratio, 64% that of control group). However, the higher post-exercise muscle lactate concentration (167% of control group level) and L/G ratio (162% of control group level) in hypoxia-acclimated fish swum in normoxia suggests that the anaerobic metabolic capacity may also have increased.

#### The effect of acclimation to sustained exercise on hypoxia tolerance and swimming performance

It was interesting to note that acclimation to sustained exercise increased hypoxia tolerance by roughly the same degree as that seen in response to hypoxia acclimation (Table 1). Exercise acclimation resulted in a 39% decrease in  $P_{crit}$  compared with control goldfish and this increased hypoxia tolerance was associated with an increase in lamellar surface area (43%) and blood Hb level (26%), and a decrease (17%) in  $\dot{M}O_{2,rout}$  (Table 2). To our knowledge, this is the first example of exercise training improving hypoxia tolerance. Previous studies in other cyprinids, such as the tench (*Tinca tinca*) and common carp (*Cyprinus carpio*), have demonstrated that exercise induces an increase in arterial  $P_{O_2}$  during exercise (Jensen et al., 1983; Knudsen and Jensen, 1998); thus, exercise is known to enhance  $O_2$  uptake in fish. We did not measure arterial  $P_{O_2}$  in this study, but the direct effect of exercise (the fish underwent 48 h swimming with only 1 h recovery) in conjunction with gill remodelling probably increased respiratory capacity and the lower  $P_{crit}$  was probably associated with this enhanced respiratory capacity and possibly a down-regulation of maintenance metabolism.

The effect of exercise training (or acclimation) on  $U_{crit}$  is known to be dependent on fish species, training intensity and training duration (Liu et al., 2009). In this study, 48 h swimming at  $70\% U_{crit}$  resulted in increases in both  $U_{crit}$  and  $\dot{M}O_{2,active}$ , but only when fish were swum under hypoxia conditions (Table 2). These results are similar to the effects of hypoxia acclimation on  $U_{crit}$  (Table 2). However, the biochemical data indicate that the mechanism was somewhat different from that of hypoxia acclimation. Post-exercise muscle lactate content was not different from resting values in exercise-acclimated goldfish and the post-exercise muscle glycogen content decreased to the lowest level (32% that of control group) (Table 2), suggesting exercise-acclimated fish may have had much higher muscle aerobic capacity in comparison to hypoxia-acclimated fish. Long-term swimming acclimation (17 weeks) in cyprinids is well known to result in increases in red muscle cell diameter and fibre number, muscle type proportions, capillarization and red muscle mitochondrial and lipid volume densities (reviewed in Sanger, 1993), but it is unknown whether any changes to muscle morphology could occur within the 48 h acclimation period used here. Previous studies have found that the physiological factors limiting swimming performance are species specific and vary with environmental conditions such as temperature (Pang et al., 2010), dissolved oxygen (Jourdan-Pineau et al., 2010) and even artificial manipulation such as exercise training (Li et al., 2010). Recent work by Fu and colleagues (Fu et al., 2009) and

Jourdan-Pineau and colleagues (Jourdan-Pineau et al., 2010) suggests that, at least on some occasions,  $U_{crit}$  is limited by the functional properties of muscle rather than by those of the gills and heart. Clearly, further investigations are needed to determine whether the short acclimation protocol used here could result in changes in muscle morphology and aerobic capacity.

It must be noted that the effects of swimming acclimation and the associated increased respiratory capacity (as suggested by the increased lamellar surface area and blood Hb level) and metabolic capacity (as suggested by the lower post-exercise muscle lactate and glycogen content) had no positive effect on  $U_{crit}$  and  $\dot{M}_{O_2,active}$  when the exercise-acclimated fish were made to swim under normoxic conditions (Table 2). We only observed beneficial effects of swimming acclimation on fish swum under hypoxic conditions (Table 2). The lack of increase in  $U_{crit}$  and  $\dot{M}_{O_2,active}$  under normoxic conditions may be associated with depletion of oxidizable substrate associated with exercise acclimation as muscle glycogen content was only 27% that of control fish.

### CONCLUSIONS

In conclusion,  $P_{crit}$  decreased significantly after both hypoxia acclimation and exercise training. In hypoxia-acclimated fish, the change was concurrent with a substantial (71%) increase in lamellar surface area and a 26% increase in [Hb]. In exercise-acclimated fish, it was concurrent with a modest down-regulation of maintenance metabolism (assessed in hypoxia), a 43% increase in gill surface area and a 26% increase in [Hb]. When made to swim under hypoxic conditions, hypoxia- and exercise-acclimated fish showed a similar increase in  $U_{crit}$  (17–18%) and  $\dot{M}_{O_2,active}$  (35–39%) because of the increased oxygen uptake capacity and aerobic metabolic capacity. However, for exercise-acclimated fish, the increased respiratory and metabolic capacities had no positive effect on  $U_{crit}$  and  $\dot{M}_{O_2,active}$  when the fish were made to swim under normoxic conditions, possibly because of depleted oxidizable substrate during experimental treatment.

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