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# Excess post-hypoxic oxygen consumption is independent from lactate accumulation in two cyprinid fishes

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## ABSTRACT

*Carassius carassius* responds to hypoxic conditions by conversion of lactate into ethanol, which is excreted over the gills. However, a closely related species, *Cyprinus carpio*, does not possess the ability to produce ethanol and would be expected to accumulate lactate during hypoxic exposure. While the increase in oxygen consumption in fish required following strenuous exercise or low environmental oxygen availability has been frequently considered, the primary contributing mechanism remains unknown. This study utilized the close relationship but strongly divergent physiology between *C. carpio* and *C. carassius* to examine the possible correlation between excess post-hypoxic oxygen consumption (EPHOC) and lactate accumulation. No difference in the EPHOC:O<sub>2</sub> deficit ratio was observed between the two species after 2.5 h anoxia, with ratios of 2.0 ± 0.6 (*C. carpio*) and 1.3 ± 0.3 (*C. carassius*). As predicted, lactate accumulation dynamics did significantly differ between the species in both plasma and white muscle following anoxic exposure. Significant lactate accumulation was seen in both plasma and muscle in *C. carpio*, but there was no accumulation of lactate in white muscle tissue of *C. carassius*. These findings indicate that lactate accumulated as a consequence of 2.5 h anoxic exposure is not a major determinant of the resulting EPHOC.

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## 1. Introduction

Three cyprinid teleosts, the crucian carp (*Carassius carassius* (L)), goldfish (*Carassius auratus* (L)), and bitterling (*Cyprinus amarus* (Bloch)), are unique among vertebrates for their ability to convert lactate into ethanol as the end product of anaerobic metabolism (Shoubridge and Hochachka, 1980; Johnston and Bernard, 1983; Wissing and Zebe, 1988). The produced ethanol is freely diffusible over the cell membrane and is excreted from the fish via the gills (Shoubridge and Hochachka, 1980; van den Thillart et al., 1983; Stecyk et al., 2004). This rare adaptation is instrumental in a greatly enhanced tolerance to hypoxic conditions. Indeed, *C. carassius* can survive more than 24 h of anoxia at room temperature, and at least 4.5 months

at near-zero temperatures (Holopainen and Hyvärinen, 1985; Piironen and Holopainen, 1986; Nilsson and Renshaw, 2004). In contrast, the common carp (*Cyprinus carpio*), a cyprinid species closely related to *C. carassius*, does not possess the ability to produce ethanol (Nilsson, 1988), yet is regarded as a good anaerobe tolerating anoxic exposure of at least 1 h at 20 °C (van Waarde et al., 1990; van Raaij et al., 1996), and surviving less severe hypoxia (0.5 mg O<sub>2</sub> L<sup>-1</sup>) for at least 7 days at 22–23 °C (Zhou et al., 2000).

The comparison of the hypoxia tolerance strategies between these two species is based on the distinct differences in metabolic responses to oxygen limitation each species employs. Standard metabolic rate (MO<sub>2std</sub>) is the minimum oxygen requirement for the maintenance of unimpaired physiological reactions in postprandial unstressed animals at rest. When the oxygen saturation (O<sub>2sat</sub> (%)) in the water is too low to support these basal requirements by aerobic metabolism, phosphocreatine (PCr) acts as an “energy buffer”, stabilizing the [ATP] by rapidly regenerating ATP from ADP. The capacity to maintain the [ATP] by PCr hydrolysis is limited (van Ginneken et al., 1995; Dalla Via et al., 1997) and anaerobic glycolysis is therefore the principal ATP-generating pathway that can function during long periods of anoxia (Bickler and Buck, 2007). Due to the low ATP yield from anaerobic glycolysis, cells compensate for the diminished aerobic energy production by a substantial rise in glucose consumption rates resulting in lactate accumulation (Hochachka, 1986). For every mole of glucosyl-units used to support

**Abbreviations/symbols:** ADP, adenosine diphosphate; AMS, aerobic metabolic scope (MO<sub>2max</sub>/MO<sub>2std</sub>); ATP, adenosine triphosphate; EPHOC, excess post-hypoxic oxygen consumption (mg O<sub>2</sub> kg<sup>-1</sup>); MO<sub>2max</sub>, maximal oxygen consumption rate (mg O<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>); MO<sub>2post-anoxia</sub>, oxygen consumption rate (mg O<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>) following anoxic exposure; MO<sub>2std</sub>, standard metabolic rate (mg O<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>); O<sub>2sat</sub>, oxygen saturation (%); PCr, phosphocreatine; S<sub>crit</sub>, critical oxygen saturation.

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anaerobic glycolysis, 2 mol of lactate is produced; deviations from a 2:1 ratio therefore indicate sources additional to glycogen depletion. This is the response to low oxygen saturation observed in most teleost fish species, including *C. carpio*. In conjunction with this process, *C. carassius* converts lactate into ethanol, which addresses the problem of acidification by ATP hydrolysis associated with lactate production (Hochachka and Mommsen, 1983) and, combined with one of the largest known glycogen stores of any vertebrate (Hyvärinen et al., 1985) allows *C. carassius* to maintain a relatively high glycolytic rate for extended periods (Nilsson, 1990). The conversion of lactate to ethanol in *C. carassius* happens exclusively in muscle tissue and lactate produced in tissues other than the muscle is transported via the blood to the muscle tissue for fermentation (Johnston and Bernard, 1983; Nilsson, 1988).

Fish can increase their oxygen consumption rate by several folds compared to  $MO_{2std}$  until reaching their maximum capacity ( $MO_{2max}$ ) wherein all aerobic activities are undertaken. During recovery from anoxia, oxygen consumption increases above  $MO_{2std}$  for an extended period of time, but studies that quantify the total metabolic cost of recovery from severe hypoxia are rare (van den Thillart and Verbeek, 1991; Maxime et al., 2000; Svendsen et al., 2012). The accumulated excess post hypoxic oxygen consumption (EPHOC,  $mg\ O_2\ kg^{-1}$ ) has classically been attributed to the lactate load, but evidence suggests that EPHOC is only partially related to the lactate load, and that resynthesis of glycogen from lactate during recovery is not the major component of the increased  $O_2$  consumption. Instead, the EPHOC has been attributed to re-synthesis of ATP and PCr in addition to glycogen, and also the buffering of protons generated from ATP utilization (van den Thillart and Verbeek, 1991; Virani and Rees, 2000; Mandic et al., 2008). However, the relative contributions of these processes to EPHOC in fish, and in particular the role of lactate, remain an area of ongoing investigation.

The present study examines the hypothesis that EPHOC associated with acute exposure to anoxia ( $\leq 1\% O_{2sat}$ ) is positively correlated to lactate accumulation. Utilizing the close phylogenetic relationship, yet distinct difference in hypoxia tolerance physiology between *C. carpio* and *C. carassius* this study investigates the link between lactate load and EPHOC. Because lactate is converted to ethanol in *C. carassius*, but not in *C. carpio*, it was hypothesized that 1) acute exposure to anoxia would cause substantial lactate accumulation in *C. carpio*, while it would be limited in *C. carassius*; and 2) the lactate accumulation would result in greater EPHOC relative to the produced  $O_2$  deficit in *C. carpio*, compared to *C. carassius*. In this study, we therefore quantified 1) concentration of lactate in muscle and plasma during exposure to anoxia in juvenile *C. carassius* and *C. carpio*, and 2) EPHOC ( $mg\ O_2\ kg^{-1}$ ) after exposure to 2.5 h anoxia.

## 2. Materials and methods

### 2.1. Experimental animals

A total of 34 juvenile *C. carpio* and 33 *C. carassius* (110–130 mm) were collected from a pond near Slagelse, Denmark ( $55^{\circ}17'58\ N$   $11^{\circ}27'47\ E$ ) in April 2009. At capture water temperature was 12.5–14.0 °C. Fish were transferred to the University of Copenhagen, Marine Biological Laboratory, Helsingør, Denmark and kept indoors in a 400 L tank supplied with a continuous flow of unchlorinated tap water. Water was filtered using a mechanical filter pump ( $1100\ L\ h^{-1}$ ) connected to the tank, and water temperature was kept at  $15 \pm 0.1\ ^{\circ}C$  and continually aerated to maintain normoxic conditions. The fish were kept in a 12L:12D light regime and were fed to satiation 2–4 times per week with commercial fish pellets (Ecolife 3 mm, Biomar, Denmark). Prior to experimentation, fish were acclimated to these conditions for 4 months. No fish was used more than once. All methods applied in the present study were in agreement with current Danish regulations for the treatment and welfare of experimental animals.

### 2.2. Respirometry

#### 2.2.1. Equipment setup

The setup consisted of a static respirometer and a mixing pump submerged in a 50 L opaque tank on a wet table, filled with unchlorinated tap water maintained at  $15 \pm 0.1\ ^{\circ}C$ . The respirometer was made of transparent Perspex tubing and was fitted with two outlet and two inlet ports. The mouth of the outlet tube, through which water left the respirometer, was elevated slightly above the water surface level to prevent the ambient water from entering the respirometer. Inside the respirometer, a plate positioned 5 mm from the ports propagated water mixing and prevented the fish from disturbing the inflow and outflow. A perforated tube was inserted into the respirometer to minimize spontaneous activity associated with exposure to decreased  $O_{2sat}$  levels, a behavior that has been previously observed in *C. carpio* (Vianen et al., 2001). The tank was positioned behind a black curtain to minimize stressful stimuli.

Measurements of  $O_2$  consumption rate ( $MO_2$ ;  $mg\ O_2\ kg^{-1}\ h^{-1}$ ) were carried out every 7 min 50 s using computerized intermittent-flow respirometry allowing long term ( $> 48\ h$ ) repeated measurements as previously described (Steffensen et al., 1984; Steffensen, 1989). The repeated respirometric loops consisted of a 3 min 20 s flushing phase during which a pump flushed the respirometer with ambient water through one set of ports. The second set of ports and a pump allowed the water in the respirometer to be re-circulated in a closed circuit phase for 4 min 30 s, divided into a waiting period (2 min) and a measurement period (2 min 30 s).

Oxygen partial pressure was measured at  $1\ s^{-1}$  by a fiber optic sensor (Fibox 3 connected to a dipping probe; PreSens, Regensburg, Germany) located in the recirculated loop. The flush pump was controlled by AutoResp software (Loligo Systems Aps, Tjele, Denmark) that also calculated the oxygen consumption rate in the measuring phase using the oxygen partial pressure and standard equations (Schurmann and Steffensen, 1997). Preliminary testing demonstrated that the duration of the measurement period (2 min 30 s) in combination with the mass of the experimental fish ( $19.5 \pm 0.7\ g$ ) and the volume of the respirometer and re-circulated loop (0.335 L) ensured that the coefficient of determination ( $r^2$ ) associated with the  $MO_2$  measurements was always  $> 0.90$  as in previous studies (Behrens and Steffensen, 2007; Campbell et al., 2008). Moreover, in normoxia the respiration of the fish never reduced the  $O_{2sat}$  to less than 84% (approx. 17.5 kPa).

Water for the flush pump was supplied from one of two different tanks containing either normoxic or hypoxic water maintained at  $15 \pm 0.1\ ^{\circ}C$ . Adequate water quality in the system was maintained by an internal filter pump and an ultraviolet light sterilizer running continually. Prior to initiation of an experiment the adjustable tank was reduced to  $\leq 2.5\% O_{2sat}$  (approx. 0.5 kPa) by circulating water from the tank through a vertical cylinder (0.25 m in diameter, 1 m high) where the water was exposed to a stream of nitrogen bubbles (Behrens and Steffensen, 2007). To minimize diffusion of  $O_2$  from the ambient air, water surfaces were covered by floating bubblewrap. The  $O_{2sat}$  in the adjustable tank was measured using a Mini DO probe (Loligo Systems Aps., Tjele, Denmark) connected to a relay that controlled the  $O_{2sat}$  in the tank via a solenoid valve regulating nitrogen gas delivery to the cylinder similar to the procedure described by (Jordan and Steffensen, 2007). The  $O_{2sat}$  in the normoxic tank was maintained at a constant high normoxic level ( $\geq 95\% O_{2sat}$ , approx. 19.8 kPa) using air stones. The desired  $O_{2sat}$  in the hypoxic tank was adjusted and stabilized before the flush pump started supplying water from this tank. In this way, the experiment was not influenced by any delays caused by the time required to reduce the  $O_{2sat}$  in the hypoxic tank. The shift from normoxic to hypoxic water was made by manually closing the valve regulating outflow from the normoxic tank and opening the valve from the adjustable tank, which had been previously brought to  $\leq 2.5\% O_{2sat}$  as described above. Both

204 valves were situated outside the tank to eliminate disturbance of the  
 205 fish, and preliminary tests confirmed that the procedure did not influ-  
 206 ence the metabolic rate of the fish. During the flush phase, the flush  
 207 pump exchanged greater than 8 times the volume of water in the respi-  
 208 rometer, which is sufficient to replace >99% of the water  
 209 (Steffensen, 1989). Using this arrangement, the  $O_{2sat}$  inside the respi-  
 210 rometer reached the designated  $O_{2sat}$  level in <3.5 min and was im-  
 211 mediately followed by  $MO_2$  measurements.

### 2.2.2. Experimental protocol of $MO_2$ measurements

212 EPHOC following anoxia was determined in two size-matched  
 213 groups of 9 *C. carpio* ( $19.5 \pm 1.1$  g) and 8 *C. carassius* ( $19.5 \pm 0.7$  g).  
 214 Fish were fasted for 24 h prior to experimentation. Individual  
 215  $MO_{2max}$  was tested in normoxia by transfer of the fish from the hold-  
 216 ing tank to a bucket and chasing to exhaustion, according to Richards  
 217 et al. (2002). This protocol has been used to induce  $MO_{2max}$  in several  
 218 teleost species as an alternative to swimming the fish in the respi-  
 219 rometer (Peake and Farrell, 2006; Jordan and Steffensen, 2007;  
 220 Killen et al., 2007). Upon exhaustion, identified by no further re-  
 221 sponse to manual stimulation (after 5–6 min), fish were transferred  
 222 to the respirometer where  $MO_2$  measurements were started immedi-  
 223 ately. After the  $MO_{2max}$  measurements, fish were acclimated to the  
 224 respirometer for 24–48 h.

225 Preliminary testing confirmed previous work that indicated the  
 226 maximum survival time for *C. carpio* exposed to anoxia at  $15^\circ\text{C}$  was  
 227 approx. 2.5 h (Stecyk and Farrell, 2002), and 2.5 h was consequently  
 228 used as the duration of anoxic exposure. Tests with the two different  
 229 species were carried out in random order. It was not possible to reduce  
 230 the  $O_{2sat}$  in the hypoxic tank to less than 2.5% (0.5 kPa). Therefore, to in-  
 231 duce anoxia in the respirometer, the flush pump was turned off after the  
 232 first flush period of the experiment. Shutting off the water exchange  
 233 caused the fish to induce anoxia ( $\leq 1\%$   $O_{2sat}$ , approx. 0.2 kPa) in the respi-  
 234 rometer in  $\leq 15$  min. After the anoxic exposure the flush pump was  
 235 engaged and the respirometer flushed with normoxic water. Due to the  
 236 lag time of the fiber optic sensor adjusting from  $\leq 0.1$  to  $>95\%$   
 237  $O_{2sat}$ , the flush period of the first respirometric loop was extended by  
 238 3 min and the measurement discarded. Collection of  $MO_2$  data every  
 239 7 min and 50 s continued for  $>12$  h after the exposure to anoxia.

### 2.2.3. Acquisition and analysis of respirometry data

241 Because of the rapid turnover of water, both the exact rate of change  
 242 of the  $O_{2sat}$  and the response time of the  $O_2$  consumption rate of the fish  
 243 were unknown during the flush periods; because of these uncertainties  
 244 the flush periods used to modify the  $O_{2sat}$  inside the respirometer were  
 245 not included in the calculations.  $MO_{2std}$  was defined as the mean of the  
 246 last seven measurements (54 min 50 s) (Fig. 1) before onset of hypoxia,  
 247 similar to previously employed procedures (Scarabello et al., 1991;  
 248 Svendsen et al., 2010). The EPHOC protocol involved rapid changes of

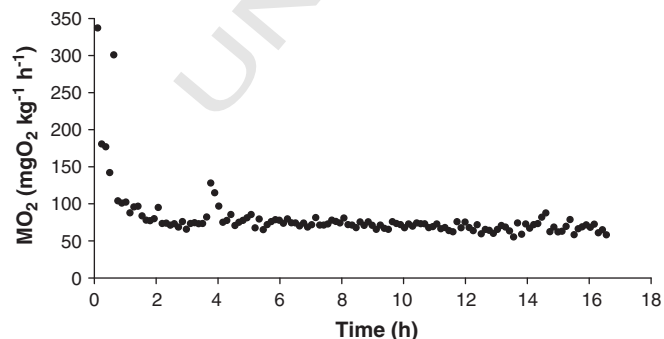


Fig. 1. Representative trace of the time course of  $MO_2$  measurements ( $\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) during acclimation in a static respirometer. Data were collected using a 23.7 g common carp (*Cyprinus carpio*) at  $15^\circ\text{C}$ . Each datum represents a 7 min 50 s period.  $MO_2$  is corrected for background respiration.

250 the  $O_{2sat}$  inside the respirometer during single flush periods (from  
 251 normoxia to anoxia and vice versa). The oxygen deficit ( $\text{mg O}_2 \text{ kg}^{-1}$ )  
 252 accumulated during the anoxic period was quantified as the  $MO_{2std}$  dur-  
 253 ing the 2.5 h. Individual recovery periods were regarded as completed  
 254 when the first  $MO_2$  datum in the post anoxia recovery period ( $MO_{2-}$   
 255  $_{post-anoxia}$ ) was within a 95% confidence interval (CI) of the  $MO_{2std}$   
 256 (Fig. 2) as previously described (Bushnell et al., 1994; Svendsen et al.,  
 257 2010). The metabolic cost of recovery ( $\text{mg O}_2 \text{ kg}^{-1}$ ) was determined  
 258 by subtracting the  $MO_{2std}$  from  $MO_{2-}$   $_{post-anoxia}$ , following Jordan and  
 259 Steffensen (2007). Aerobic metabolic scope was calculated as the differ-  
 260 ence between  $MO_{2max}$  and  $MO_{2std}$ , following Farrell and Richards  
 261 (2009).

### 2.3. Measurements of plasma and muscle lactate

#### 2.3.1. Equipment setup

263 Two groups of 25 size matched *C. carpio* and *C. carassius* ( $20.9 \pm 0.5$  g)  
 264 were used for the time series measurements of lactate development in  
 265 plasma and white muscle. A 180 L aquarium was fitted with black plastic  
 266 on all sides to prevent visual disturbance, filled with unchlorinated tap  
 267 water, and fitted with an internal filter pump to ensure adequate mixing.  
 268 The temperature was kept at  $15 \pm 0.1^\circ\text{C}$  and the water was maintained  
 269 normoxic by continuous aeration by air stones. The  $O_{2sat}$  was monitored  
 270 using a Mini DO probe (Oxyguard International, Birkerød, Denmark)  
 271 connected to a relay controlling the  $O_{2sat}$  in the tank via a solenoid  
 272 valve that regulated nitrogen gas delivery to multiple air stones on the  
 273 bottom of the aquarium. All holes around tubes and cables into the  
 274 aquarium were covered with plastic film. The sealed container facilitated  
 275 precise regulation of  $O_{2sat}$  from  $\geq 95\%$  to 1%. To allow individual sampling  
 276 with a minimum of disturbance of the remaining fish in the aquarium,  
 277 each fish was inserted in a small cage made from plastic mesh tube  
 278 (40 mm diameter). A nylon string was fitted to each cage and a small  
 279 weight kept the cage on the bottom and made it impossible for the fish  
 280 to move the cage.

#### 2.3.2. Experimental protocol of lactate sampling

281 Fish were starved for 24 h before being transferred from the hold-  
 282 ing tank to the aquarium and inserted in the cages. Acclimation to the  
 283 aquarium under normoxia lasted for 36 h, and fish were not fed dur-  
 284 ing this time. Five fish of each species were sampled immediately be-  
 285 fore the onset of hypoxia as a normoxic baseline. Within 1 h anoxia  
 286 was reached ( $1 \pm 0.2\%$   $O_{2sat}$ , approx. 0.2 kPa) by nitrogen bubbling.  
 287 Subsequently, a fish was sampled every 4 min. Alternating between

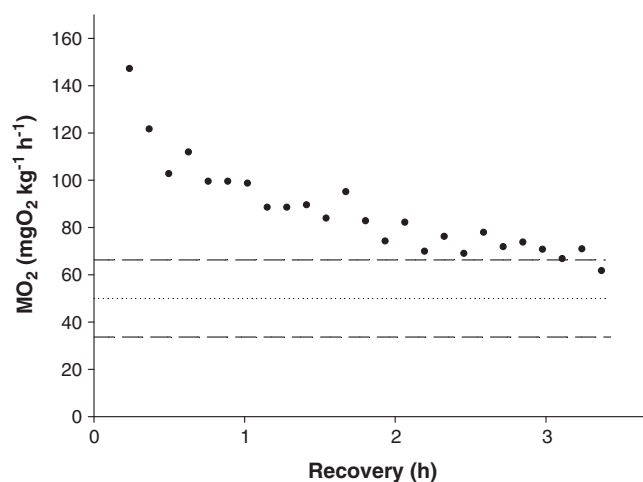


Fig. 2. Representative trace of  $MO_{2post-anoxia}$  in a 19.2 g crucian carp (*Carassius carassius*) after 2.5 h anoxia exposure.  $MO_{2std} \pm 95\%$  CI are illustrated using a dotted and two dashed lines, respectively.  $MO_2$  is corrected for background respiration. The first  $MO_2$  datum in the post anoxia recovery period within the 95% confidence interval (CI) of the  $MO_{2std}$  was used as the marker indicating completion of the recovery period.



each species, a total of 20 *C. carpio* and 20 *C. carassius* were sampled, giving a total anoxic exposure period of 2 h 40 min.

At sampling, the lid was lifted slightly and a cage retracted from the tank by the nylon string. The cage was immediately transferred to a 2.5% benzocaine solution (Sigma-Aldrich Chemicals, USA) made from a 4% ethanol stock solution. At complete anesthesia ( $\leq 1$  min), the fish were removed from the cages, patted dry and weighed to the nearest 0.1 g. Blood samples were collected by severing the tail from the body and collecting the blood flowing from the caudal vein with a heparinized 1 mL syringe (LEO Pharma A/S, Ballerup, Denmark). The blood sample was transferred to a 0.5 mL centrifuge tube and centrifuged at 2000 g for 30 s to isolate the plasma. A tissue sample was taken as a cross section of the trunk musculature posterior to the dorsal fin and wrapped in an aluminum foil. Both the tissue and plasma samples were flash frozen in liquid N<sub>2</sub> and stored at  $-80$  °C until analysis.

2.3.3. Determination of lactate concentration

Extraction of lactate from the tissue samples was carried out following procedures previously described (Viant et al., 2003; Lin et al., 2007). The frozen muscle samples were ground to a fine powder in a N<sub>2</sub>-cooled mortar. The frozen, powdered tissue (100 mg) was weighed in a N<sub>2</sub>-cooled 1.5 mL centrifuge tube and extracted using 5 mL g<sup>-1</sup> (wet mass) ice cold 6% perchloric acid. Samples were kept on ice throughout the extraction procedure. Samples were vortexed for 15 s three times, centrifuged (10,000 g, 10 min, 4 °C), and the supernatant was removed and neutralized to pH 7.5 with 2 M K<sub>2</sub>CO<sub>3</sub>, testing pH using small drops of sample on pH paper (pH paper range: 5.5–9.0). Samples were kept on ice for an additional 30 min to facilitate complete precipitation. Following centrifugation (10,000 g, 10 min, 4 °C), the supernatant was removed and stored at  $-80$  °C. Muscle extracts and plasma were analyzed for lactate using a commercial kit (Biomedical Research Service, NY, USA). The measurements were corrected using internal lactate standards in samples from fish of both species sampled in normoxic conditions.

2.4. Statistical analysis

All values are reported as mean  $\pm$  standard error of the mean (SEM). Means were compared using Student's *t*-test (two-tailed) after testing the assumptions of normal distribution of data and homogeneity of variance. Means of data found not to be normally distributed were compared using the Mann–Whitney test. Least square linear regression analysis was performed using SigmaPlot 10.0 (Systat Software Inc. San Jose, CA, USA), and regression line slopes were compared using analysis of covariance. Statistical analyses were carried out using SPSS 15.0 (IBM SPSS, Armonk, NY, USA). Means were considered significantly different when  $P < 0.05$ .

3. Results

3.1. Oxygen consumption rates

MO<sub>2 std</sub> differed significantly between *C. carassius* and *C. carpio* (Table 1,  $P < 0.02$ ). Similarly, MO<sub>2 max</sub> in *C. carassius* was significantly lower than the MO<sub>2 max</sub> measured in *C. carpio* ( $P < 0.0001$ ). Despite the overall greater oxygen consumption seen in *C. carpio*, aerobic metabolic scope (AMS, MO<sub>2 max</sub>/MO<sub>2 std</sub>) did not differ between these two species ( $P > 0.8$ ). As a consequence of the different MO<sub>2 std</sub> the accumulated O<sub>2</sub> deficit during the anoxic period was greater in *C. carpio* than *C. carassius* ( $P < 0.02$ ), and there was also a significant difference in EPHOC ( $P < 0.02$ ). Although both EPHOC and O<sub>2</sub> deficit were lower in *C. carassius* than *C. carpio*, the ratio of EPHOC:O<sub>2</sub> deficit did not differ between species ( $P > 0.48$ ). The average time to complete metabolic recovery was longer for *C. carpio* (7.0  $\pm$  1.4 h) than for *C. carassius* (3.8  $\pm$  0.7 h) ( $P < 0.034$ ). As was the case with the

**Table 1** Observations of metabolic parameters in normoxia and during recovery from 2.5 h acute anoxic exposure in crucian carp (*Carassius carassius*, n=8, 19.5  $\pm$  0.6 g) and common carp (*Cyprinus carpio*, n=9, 19.5  $\pm$  1.1 g) at 15 °C. Asterisks indicate significant differences between species using two tailed Student's *t*-test, \*  $P < 0.05$ ; and \*\*\*  $P < 0.0001$ ; NS, not significant.

	<i>C. carassius</i>	<i>C. carpio</i>	P
MO <sub>2 standard</sub> (mg O <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	43.7 $\pm$ 5.3	66.5 $\pm$ 6.2	*
MO <sub>2 max</sub> (mg O <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	213.7 $\pm$ 7.3	329.5 $\pm$ 10.3	***
AMS (MO <sub>2 max</sub> /MO <sub>2 std</sub> )	5.2 $\pm$ 0.4	5.4 $\pm$ 0.6	NS
O <sub>2</sub> deficit (mg O <sub>2</sub> kg <sup>-1</sup> )	108.5 $\pm$ 13.1	164.9 $\pm$ 15.5	*
EPHOC (mg O <sub>2</sub> kg <sup>-1</sup> )	124.4 $\pm$ 18.9	281.1 $\pm$ 53.5	*
EPHOC:O <sub>2</sub> deficit	1.3 $\pm$ 0.3	2.0 $\pm$ 0.6	NS
Time to recovery (h)	3.8 $\pm$ 0.7	7.0 $\pm$ 1.4	*
% of MO <sub>2 max</sub>	65.4 $\pm$ 8.3	61.6 $\pm$ 7.6	NS

AMS, the utilized metabolic scope was similar for the two species, and during the recovery phase neither of the species utilized their full metabolic scope, with the highest measurements of MO<sub>2</sub> representing 65.4  $\pm$  8.3% of MO<sub>2 max</sub> in *C. carassius* and 61.6  $\pm$  7.6% in *C. carpio* ( $P > 0.7$ ).

3.2. Lactate accumulation

Parameters describing the production and accumulation of lactate during anoxic exposure are summarized in Table 2. Concentrations of lactate prior to anoxic exposure did not differ between species in plasma (3.8  $\pm$  0.5 in *C. carassius* vs. 3.3  $\pm$  0.4 mM in *C. carpio*,  $P > 0.48$ ) nor muscle (2.1  $\pm$  0.1 vs. 1.7  $\pm$  0.3  $\mu$ mol g<sup>-1</sup>;  $P > 0.26$ ). While the lactate concentration in the plasma rose significantly in both species, in the muscle tissue the concentration of lactate increased only in *C. carpio* (Fig. 3). In consequence the accumulation of lactate in *C. carassius* was significantly higher in plasma than in muscle ( $P < 0.0001$ ), with plasma [lactate] increasing 3 fold to 12 mmol L<sup>-1</sup> (Table 2, Fig. 3A). *C. carpio* plasma [lactate] increased 6 fold to 21 mmol L<sup>-1</sup>, and in contrast to *C. carassius* the lactate accumulation in plasma was significantly higher than in muscle ( $P < 0.0001$ ; Table 2), with muscle [lactate] increasing 5 fold to a final concentration of 8.97  $\mu$ mol g<sup>-1</sup> (Table 2, Fig. 3B). The lactate accumulation was faster in *C. carpio* than in *C. carassius* in both plasma (mmol L<sup>-1</sup> h<sup>-1</sup>,  $P < 0.01$ ) and muscle ( $\mu$ mol g<sup>-1</sup> h<sup>-1</sup>,  $P < 0.01$ ).

4. Discussion

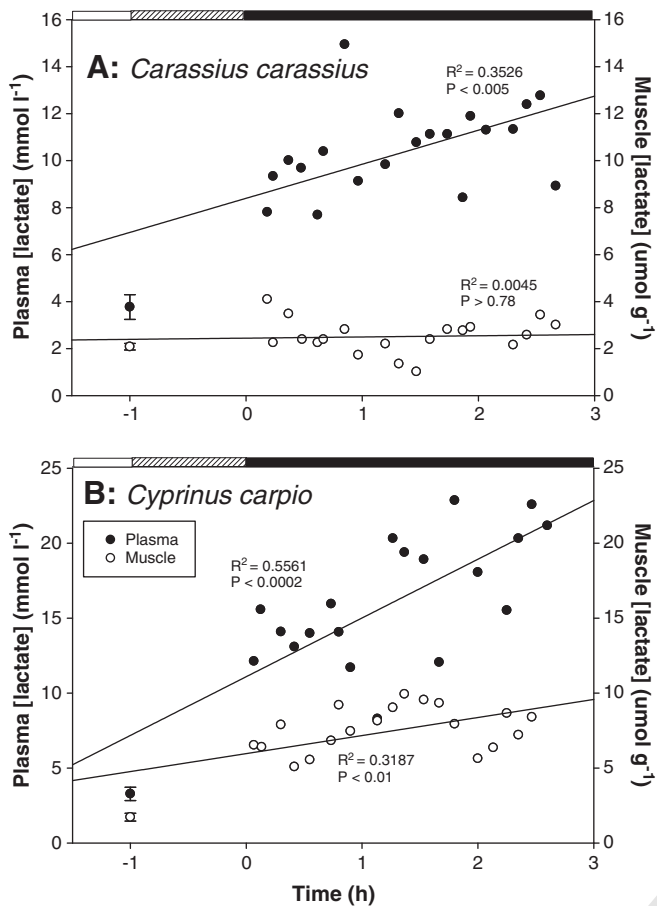
4.1. Respirometry

4.1.1. Extent of EPHOC in various species

To our knowledge, EPHOC following exposure to oxygen levels below S<sub>crit</sub> has been quantified for only three other fish species: *C. auratus* (van den Thillart and Verbeek, 1991) *Scophthalmus maximus* (Maxime et al., 2000), and *Oncorhynchus mykiss* (Svendsen et al., 2012). Several species of flatfish are moderately hypoxia tolerant (Dalla Via et al., 1994; Pichavant et al., 2002), and in hypoxia trials on *S. maximus*, a benthic flatfish found in temperate seas, the EPHOC:O<sub>2</sub> deficit ratio was 16:1 (Maxime et al., 2000), which is only half of the ratio of up to 35:1 observed in the hypoxia intolerant rainbow trout (*O. mykiss*) (Svendsen et

**Table 2** Lactate development during 2.7 h acute anoxic exposure at 15 °C in crucian carp (*Carassius carassius*) and common carp (*Cyprinus carpio*) (20.8  $\pm$  0.5 g combined mean body mass). Concentrations at 2.5 h anoxia were calculated from the linear regression (see Fig. 3). Asterisks (\*) indicate statistical differences between species ( $P < 0.01$ ).

Lactate parameters	Plasma (mmol L <sup>-1</sup> )			Muscle ( $\mu$ mol g <sup>-1</sup> )		
	<i>C. carassius</i>	<i>C. carpio</i>	P	<i>C. carassius</i>	<i>C. carpio</i>	P
Normoxia	3.8 $\pm$ 0.5	3.3 $\pm$ 0.4	NS	2.1 $\pm$ 0.1	1.7 $\pm$ 0.3	NS
2.5 h anoxia	12.0	20.9	-	2.6	9.0	-
Increase (fold)	3.16	6.33	-	1.23	5.28	-
Slope (h <sup>-1</sup> )	1.45 $\pm$ 0.45	3.92 $\pm$ 0.82	*	0.05 $\pm$ 0.18	1.20 $\pm$ 0.41	*



**Fig. 3.** Linear accumulation of lactate in plasma (mmol L<sup>-1</sup>, black) and muscle (μmol g<sup>-1</sup>, white) in A: crucian carp (*Carassius carassius*, 20.3 ± 0.6 g) and B: common carp (*Cyprinus carpio*, 21.4 ± 0.8 g) prior to and during 2.5 h of anoxia. The white bar indicates the normoxia (> 95% O<sub>2sat</sub>) period, the hatched bar indicates the period with decreasing O<sub>2sat</sub>, and the black bar indicates the anoxia period (≤ 1% O<sub>2sat</sub>), beginning at 0 h. Each datum represents a measurement on one fish in the anoxia period, except at t = -1, which is the mean ± SEM of fish sampled in normoxia (n = 5). Linear regressions are represented by the following equations: *C. carassius* plasma y = 1.4499x + 8.3973, and muscle y = 0.0511x + 2.4467; *C. carpio* plasma y = 3.9176x + 11.0967, and muscle y = 1.2026x + 5.9680.

al., 2012), but still an EPHOC:O<sub>2</sub> deficit ratio far greater than the ratios determined for both *C. carassius* and *C. carpio* in the present study (1.3:1 and 2.0:1, respectively), and of approximately 1.5:1 observed in *C. auratus* (van den Thillart and Verbeek, 1991). Of the fish species so far investigated for EPHOC, it is interesting to note that the species that accumulate only minimal oxygen debt are all members of the Cyprinidae. Of even greater interest, this capability does not seem to depend entirely on the ability to produce ethanol, as *C. carpio* demonstrates substantially lower EPHOC:O<sub>2</sub> deficit than other non-ethanol-producing species, but an approximately equivalent deficit to ethanol-producing *C. carassius* and *C. auratus*.

The observed EPHOC:O<sub>2</sub> debt ratio in *C. carassius* of 1.3:1 following 2.5 h anoxia at 15 °C (Table 1) is quite similar to the results obtained for the closely related species *C. auratus* (van den Thillart and Verbeek, 1991), which showed an EPHOC:O<sub>2</sub> deficit ratio of 1.5:1 after 12 h of anoxia at 20 °C. However EPHOC in the goldfish (*C. auratus*) was not observed following 3 h of anoxia, (van den Thillart and Verbeek, 1991) requiring greater time and temperature than those needed for EPHOC to be recorded in *C. carassius*. The fact that we did observe an EPHOC in *C. carassius* after the relatively short 2.5 h anoxia exposure could be attributed to the following factors: 1) species-specific physiological differences, despite the fish belonging to the same genus, 2) difference in the timescale of changing O<sub>2sat</sub> levels and hence time for adjusting ventilatory and cardiac response as well as for the initiation of metabolic

depression, 3) an overestimate of the resting metabolic rate in the previous study, thereby “hiding” the EPHOC, or 4) metabolic suppression continuing after reestablishment of normoxia. In addition to the conversion of lactate to ethanol, *C. carassius*, unlike *C. carpio*, can also depress its metabolism; van Ginneken and van den Thillart (2009) demonstrated that metabolic depression in *C. auratus* was initiated within 20–30 min after reduction of environmental O<sub>2sat</sub>, and additionally that 1–2 h was needed to accomplish the full metabolic depression (by approximately 70% from MO<sub>2std</sub>). In van den Thillart and Verbeek’s (1991) study discussed above, in which EPHOC did not occur following 3 h anoxia, complete anoxia was not reached until after approximately 2.5 h, giving the goldfish sufficient time to reach full metabolic depression before anoxia was reached. In the present study, anoxia was reached in < 30 min, and in consequence, *C. carassius* would only have been able to take full advantage of the ability to depress metabolism for approximately the last hour of the exposure. Regardless of the reason for this difference between our results and the observations by van den Thillart and Verbeek (1991), a very small EPHOC in *C. carassius* was observed in the present study, indicating comparatively higher hypoxia tolerance than is observed in *C. carpio* and other fish species.

#### 4.1.2. Small and uniform EPHOC in Cyprinidae

The oxygen deprivation utilized in this study was at a near lethal level for *C. carpio* (Johnston and Bernard, 1983; van der Linden et al., 2001; Stecyk and Farrell, 2002) but should be easily tolerated by *C. carassius*, yet no difference in the ratio of EPHOC:O<sub>2</sub> was found between the two species (Table 1). Interestingly, both species only increased metabolic rate to approximately 60% of their MO<sub>2max</sub> in the recovery period and for a relatively short period of time (4–7 h), given the length of the exposure.

During anoxia, ATP levels in the brain of *C. carpio* slowly decrease (van Ginneken et al., 1996) and a significant swelling of the brain is seen over time due to the inactivation of the ATP dependent pumps regulating cell volume (Nilsson, 2001; van der Linden et al., 2001). These physiological responses to anoxia cause *C. carpio* to in essence slowly die during anoxia, while *C. carassius* is protected from such effects. Hallman et al. (2008) showed that *C. carpio* have a fairly large capacity for maintaining ATP levels using PCr as a buffer during O<sub>2</sub> levels below S<sub>crit</sub> (approx. 13% O<sub>2sat</sub> or 2.7 kPa). During this exposure it took approximately 2 h to reduce the [PCr] by half. Over the same timespan only a minor rise in plasma lactate took place in white muscle, indicating that *C. carpio* preferentially uses its PCr reserves before initiating the fermentation pathway for ATP resynthesis, presumably as an attempt to reduce metabolic acidification (Hochachka and Mommsen, 1983; van den Thillart and van Waarde, 1993).

In *C. carpio* (Hallman et al., 2008) as well as *C. auratus* (Mandic et al., 2008) both pH and PCr are completely recovered before lactate recovers. Despite high lactate loads remaining during recovery from exercise, fish can perform strenuous exercise at pre-fatigue levels when excess post-exercise oxygen consumption is repaid (Brett, 1964). This suggests that the acidification from the fermentation of glucose is likely of greater importance for the EPHOC than the lactate load itself. Indeed, in *C. auratus* (van den Thillart and Verbeek, 1991) and *C. carassius* (present study) the accumulation of lactate per se does not appear to burden the fish, and seems only to have a limited impact on the EPHOC in the two species at shorter timescales. A lactate-independent EPHOC could also indicate that *C. carpio* may have evolved to be able to cope with high lactate loads through residence in eutrophic habitats that experience regular hypoxic events (e.g. during the night). High amounts of stored lactate could subsequently be converted to glucose for aerobic respiration. Lactate is an excellent substrate for oxidation, and lactate in the blood can be metabolized by the heart, kidney and gills during period of high oxygen levels, or used for glyconeogenesis in situ. By not having to produce glycogen from the accumulated lactate, *C. carpio* would only have to

472 repay an EPHOC corresponding to the required regeneration of ATP, PCr  
473 and internal O<sub>2</sub> stores (Scarabello et al., 1991).

474 Unique to ethanol producing species is the extensive loss of carbonic  
475 molecules due to anaerobic metabolism. *C. auratus* excrete 80% of the  
476 ethanol produced (van den Thillart and Verbeek, 1991) during anoxia,  
477 and continues to excrete significant amounts of ethanol for several  
478 hours after return to normoxic conditions (Mandic et al., 2008), indicat-  
479 ing that lactate is preferentially converted to ethanol, even under  
480 normoxic conditions. If similar processes occur in *C. carassius*, accumulat-  
481 ed lactate would have a minor influence on the EPHOC and it follows that  
482 the observed EPHOC from the duration of anoxia examined here (2.5 h)  
483 would mainly consist of regeneration of ATP, PCr and internal O<sub>2</sub> stores  
484 in a similar way as *C. carpio*, with limited remaining substrate for either  
485 Cori cycle or in situ glyconeogenesis. This may, at least in part, explain  
486 the observed similarity of the EPHOC despite significantly different lactate  
487 loads and diverse physiology between *C. carpio* and *C. carassius*. Further  
488 investigation of pH, lactate, ethanol, PCr and ATP dynamics during  
489 anoxia and recovery is needed, in combination with MO<sub>2</sub> measurements,  
490 to shed light on the cause of this unexpected observation of small and  
491 uniform EPHOC:O<sub>2</sub> deficit in *C. carassius* and *C. carpio*.

## 492 4.2. Lactate

### 493 4.2.1. Diverse lactate accumulation

494 As predicted, there was a difference in the pattern of lactate accumu-  
495 lation between *C. carpio* and *C. carassius*. Both plasma and muscle [lactate]  
496 rose significantly in *C. carpio*, but in *C. carassius* only plasma [lactate] in-  
497 creased (Table 2). Our measurements in muscle of *C. carassius* (Table 2)  
498 indicate no accumulation over normoxic values, which can be attributed  
499 to the short duration of anoxic exposure. In this species [lactate] the mus-  
500 cle increases approximately 4 fold following 6 h anoxia, yet no accumula-  
501 tion is seen following 3 h progressive hypoxia (Johnston and Bernard,  
502 1983). The magnitude of lactate accumulation in the plasma also differed  
503 between species, with *C. carpio* accumulating almost twice as much lac-  
504 tate in plasma (Table 2), indicating a larger glycolytic flux in *C. carpio*.

### 505 4.2.2. High plasma lactate concentrations

506 *C. carpio* exerting moderate levels of exercise maintain levels of  
507 plasma [lactate] of approximately 1.5 mmol L<sup>-1</sup> (van Ginneken et al.,  
508 2004a), which is similar to that measured in the present study  
509 (Table 2). However, both *C. carpio* and *C. carassius* completely at rest  
510 in normoxia have only 0.2–0.5 mmol L<sup>-1</sup> lactate in the plasma  
511 (Holopainen et al., 1986; Vianen et al., 2001) at 20 °C and 18 °C, respec-  
512 tively, indicating that the fish in this study (at 15 °C) were most likely  
513 exhibiting some spontaneous activity prior to sacrifice for lactate quanti-  
514 fication, despite efforts to limit this activity. Following anoxic exposure,  
515 the accumulated plasma [lactate] in *C. carpio* (20.9 mmol L<sup>-1</sup>) is also  
516 higher than reported in other studies of carp exposed to hypoxia.  
517 Vianen et al. (2001) measured 6–13 mmol L<sup>-1</sup> in plasma of cannulated  
518 *C. carpio* after 6 h progressive severe hypoxia. In *C. carassius*, plasma [lac-  
519 tate] increased approximately 3-fold to 12 mmol L<sup>-1</sup>, demonstrating a  
520 similar qualitative response to anoxia as in previous studies where plas-  
521 ma [lactate] doubled following anoxic exposure (Holopainen et al., 1986).

522 There are two probable explanations for the high plasma [lactate]  
523 after exposure to anoxia. Firstly, the quick entry into anoxia (~1 h) di-  
524 rectly from normoxia, compared to a gradual transition that allows  
525 for metabolic depression before entry into hypoxia. Change in O<sub>2sat</sub>  
526 over only 1 h might be too fast to ensure sufficient time to initiate  
527 metabolic depression (van Ginneken and van den Thillart, 2009) or  
528 adequate ventilatory and cardiac responses (van Ginneken et al.,  
529 2004b; Wilkie et al., 2008), creating a higher S<sub>crit</sub>, and forcing initia-  
530 tion of anaerobic metabolism earlier than if extraction capacity was  
531 able to be adjusted during the O<sub>2sat</sub> decrease. Second, the metabolic  
532 stress during anoxia caused by relying exclusively on anaerobic me-  
533 tabolism may produce additional lactate accumulation, compared to  
534 the scenario in hypoxia where some aerobic metabolism can be

535 maintained. In an Amazonian cichlid, *Astronotus ocellatus*, the lactate  
536 accumulation was 5 fold higher at 6% O<sub>2sat</sub> than at 10% O<sub>2sat</sub>  
537 (Muusze et al., 1998) and in *Solea solea* a 4–5 fold higher accumula-  
538 tion at 6% O<sub>2sat</sub> than at 12% O<sub>2sat</sub> was observed (Dalla Via et al.,  
539 1994). This illustrates how the shift to complete reliance on anaerobic  
540 metabolism happens relatively swiftly when anoxia is approached,  
541 and why data obtained in different levels of hypoxia remain difficult  
542 to compare.

543 Both species considered in this study demonstrated higher [lac-  
544 tate] in plasma compared to muscle. This may be a distinguishing fac-  
545 tor for lactate accumulation due to hypoxia. For example the response  
546 of *S. solea* to severe hypoxia is qualitatively similar to our observa-  
547 tions in *C. carpio* (Dalla Via et al., 1994), however, during exercise in  
548 *S. solea* the pattern is quite different, with the majority of lactate  
549 being produced and subsequently retained in the working muscles,  
550 resulting in lactate concentrations in muscle that are several folds  
551 higher in muscle than in plasma (Dalla Via et al., 1997). This is an ad-  
552 vantage in normoxia due to the higher buffer capacity of the muscle  
553 tissue and because any acidification of the blood will lead to lowering  
554 of the hemoglobin binding affinity reducing O<sub>2</sub> extraction capacity,  
555 which is likely to prolong the duration of recovery. Indeed, accumula-  
556 tion of lactate in both the plasma and muscle tissue of *C. carpio*, but  
557 not *C. carassius*, coincides with significantly longer metabolic recov-  
558 ery (Table 1).

### 559 4.2.3. Impact of ethanol production on lactate accumulation

560 The ethanol production in *C. carassius* is well described (Johnston  
561 and Bernard, 1983) and is evident in the present study by the complete  
562 absence of accumulation of lactate in muscle tissue of *C. carassius*. Un-  
563 like in *C. carpio*, ATP levels in *C. carassius* are not primarily maintained  
564 by PCr stores. Mandic et al. (2008) measured a significant excretion of  
565 ethanol by *C. auratus* to the surrounding water within 2 h of initiation  
566 of anoxia but found only a 50% reduction in [PCr] after 10 h of anoxia  
567 at 15 °C. These results, considering the time needed for lactate produc-  
568 tion, conversion to ethanol and diffusion into the water, and the ab-  
569 sence of any initial rise in lactate concentration, suggest an immediate  
570 activation of ethanol production.

571 When *C. carassius* is exposed to anoxia, lactate is shuttled to the mus-  
572 cles for conversion to ethanol. The continuous rise in plasma [lactate] but  
573 constant low muscle concentration indicates either that 1) the lactate  
574 shuttling from blood to muscle is quite slow, or 2) that the lactate shuttle  
575 is tightly regulated in a way that no more than the lactate that can be in-  
576 stantly converted to ethanol is transported into the tissue. The first op-  
577 tion seems most plausible since Mandic et al. (2008) measured 7 μmol  
578 lactate g<sup>-1</sup> in white muscle of *C. auratus* after 10 h anoxia, indicating  
579 higher transport of lactate into the tissue than can be quickly converted.  
580 The presence of lactate accumulation in the study by Mandic et al.  
581 (2008) but not in the present study is potentially a species-specific dif-  
582 ference, but a slow shuttling mechanism combined with the relatively  
583 short exposure period may have prevented detection of any lactate accu-  
584 mulation in the muscle tissue in the present study.

## 585 5. Conclusions

586 Despite the significant difference in lactate accumulation, no differ-  
587 ence in EPHOC:O<sub>2</sub> deficit ratio could be detected between *C. carassius*  
588 and *C. carpio*. As discussed above, the measured EPHOC for *C. carassius*  
589 is in agreement with previous studies by being small compared to less  
590 hypoxia tolerant and non-ethanol-producing species, but how *C. carpio*  
591 achieves such a small EPHOC after near lethal anoxia exposure, without  
592 depressing its metabolism or converting lactate into ethanol, is not eas-  
593 ily explained. Despite its inability to produce ethanol in response to ox-  
594 ygen deprivation, the metabolic profile of *C. carpio* is more similar to the  
595 ethanol-producing members of Cyprinidae than other taxa that cannot  
596 produce ethanol. *C. carpio* accumulates a greater EPHOC and requires  
597 longer recovery time than *C. carassius*, but this is likely related to the



598 severity of the anoxic exposure used in this study relative to the overall  
599 anoxia survival capability of each species. No lactate accumulation in  
600 white muscle of *C. carassius* and less severe accumulation in plasma,  
601 in comparison to *C. carpio*, probably indicates rapid implementation of  
602 the ethanol production pathway upon exposure to anoxia, but a slow  
603 shuttling mechanism from plasma to muscle. The results of the present  
604 study emphasize the importance of metabolic depression to *C. carassius*  
605 and PCr buffering capacity to *C. carpio*, and thus factors other than abil-  
606 ity to produce ethanol are suggested to contribute in large part to  
607 EPHOC development in fishes.

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