© 2022. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2022) 225, jeb243194. doi:10.1242/jeb.243194

RESEARCH ARTICLE

Different patterns of chronic hypoxia lead to hierarchical adaptive mechanisms in goldfish metabolism

Elisa Thoral[1,*], Elie Farhat[2], Damien Roussel[1], Hang Cheng[2], Ludovic Guillard[1], Matthew E. Pamenter[2,3], Jean-Michel Weber[2] and Loïc Teulier[1]

ABSTRACT

Some hypoxia-tolerant species, such as goldfish, experience intermittent and severe hypoxia in their natural habitat, causing them to develop multiple physiological adaptations. However, in fish, the metabolic impact of regular hypoxic exposure on swimming performance in normoxia is less well understood. Therefore, we experimentally tested whether chronic exposure to constant (30 days at 10% air saturation) or intermittent hypoxia (3 h in normoxia and 21 h in hypoxia, 5 days a week) would result in similar metabolic and swimming performance benefits after reoxygenation. Moreover, half of the normoxic and intermittent hypoxic fish were put on a 20-day normoxic training regime. After these treatments, metabolic rate (standard and maximum metabolic rates: SMR and MMR) and swimming performance [critical swimming speed (U_{crit}) and cost of transport (COT)] were assessed. In addition, enzyme activities [citrate synthase (CS), cytochrome *c* oxidase (COX) and lactate dehydrogenase (LDH)] and mitochondrial respiration were examined in red muscle fibres. We found that acclimation to constant hypoxia resulted in (1) metabolic suppression (−45% SMR and −27% MMR), (2) increased anaerobic capacity (+117% LDH), (3) improved swimming performance (+80% U_{crit}, −71% COT) and (4) no changes at the mitochondrial level. Conversely, the enhancement of swimming performance was reduced following acclimation to intermittent hypoxia (+45% U_{crit}, −41% COT), with a 55% decrease in aerobic scope, despite a significant increase in oxidative metabolism (+201% COX, +49% CS). This study demonstrates that constant hypoxia leads to the greatest benefit in swimming performance and that mitochondrial metabolic adjustments only provide minor help in coping with hypoxia.

KEY WORDS: Mitochondrial respiration, Red muscle, Swimming performance, Anaerobic metabolism, Exercise

INTRODUCTION

To improve physical performance, humans often combine high-altitude hypoxic stays and sea-level normoxic training to induce beneficial physiological and metabolic acclimation to low oxygen. This 'live high–train low' strategy supports improvements upon sea-level endurance, notably by increasing maximal oxygen uptake capacity (Stray-Gundersen and Levine, 2008).

In natura, multiple species must cope with hypoxic environments during their daily life. Indeed, variations of oxygen concentration in water owing to biotic and abiotic factors are commonly encountered by aquatic organisms (Mandic and Regan, 2018). Cyprinids and especially *Carassius* sp. are well known hypoxia-tolerant champions (Weber, 2016). Goldfish (*Carassius auratus*) exposed for 2 days to severe hypoxia (3% air saturation) or sustained exercise (70% of the critical swimming speed, U_{crit}) exhibited improved swimming performance, notably by increasing U_{crit} (Fu et al., 2011, 2014). Furthermore, their maximum metabolic rate (MMR) was not different from normoxic fish under normoxic conditions, indicating that they had preserved their aerobic scope (defined as the capacity to increase aerobic metabolism; Fu et al., 2011, 2014) to support digestive or locomotive activities (Halsey et al., 2018). Goldfish therefore likely increase their locomotor efficiency by lowering the cost of transport (COT). COT is defined as the amount of energy (or oxygen) needed to move one unit of body mass by one unit of distance (Schmidt-Nielsen, 1972), and is influenced by muscle efficiency, which is expected to increase in response to limited oxygen availability.

Limiting oxygen consumption ($\dot{M}_{O_2}$) also occurs at the cellular level, because hypoxia exposure can upregulate glycolysis and downregulate aerobic metabolism (Farhat et al., 2021). Moreover, the frequency of hypoxia, often described as either constant (sustained) or intermittent, can be a key driver of divergent metabolic consequences in fish. For example, in killifish (*Fundulus heteroclitus*), 28 days of acclimation to constant hypoxia causes a decrease in metabolic rate, whereas intermittent hypoxia does the opposite and increases both glycolytic and oxidative capacity after reoxygenation (Borowiec et al., 2015, 2018).

Oxidative metabolism mainly occurs in the mitochondria, where various substrates (carbohydrate, lipid, protein) are oxidized to phosphorylate ADP to ATP (Brand, 2005). The efficiency of mitochondrial ATP production is strongly correlated with exercise activity in humans (Conley, 2016). Therefore, endurance training could stimulate the capacity for aerobic metabolism of goldfish, particularly in red muscle, which is responsible for sustained swimming in fish (McKenzie, 2011). Unfortunately, very little information on the combined effects of hypoxia and normoxic endurance training on swimming performance and energy metabolism is presently available.

Therefore, our aims were to investigate: (1) whether different patterns of chronic hypoxia acclimation would elicit metabolic adjustments that improve swimming performance in fish after returning to normoxic conditions, and (2) whether these adaptive metabolic mechanisms would occur simultaneously at the organismal and cellular levels. We hypothesized that intermittent and constant hypoxia would lead to a lower overall oxidative capacity both *in vivo* and *in vitro*, with a shift towards carbohydrate oxidation (Pamenter, 2014), to limit the oxygen cost of energy

[1]Univ. Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR 5023 LEHNA, Villeurbanne 69622, France. [2]Biology Department, University of Ottawa, Ottawa, ON, Canada, K1N 6N5. [3]University of Ottawa Brain and Mind Research Institute, Ottawa, ON, Canada, K1N 6N5.

*Author for correspondence (elisathoral@gmail.com)

E.T., 0000-0001-5218-9907; D.R., 0000-0002-8865-5428; H.C., 0000-0002-5251-640X; M.E.P., 0000-0003-4035-9555; J.-M.W., 0000-0003-2218-2952; L.T., 0000-0001-7779-7634

Received 20 July 2021; Accepted 2 December 2021

Journal of Experimental Biology (2022) 225, jeb243194. doi:10.1242/jeb.243194

List of symbols and abbreviations

AAS	absolute aerobic scope
AUC	area under the curve
CAS	cellular aerobic scope
COT	cost of transport
COX	cytochrome *c* oxidase
CS	citrate synthase
ETS	electron transport system
FAS	factorial aerobic scope
LDH	lactate dehydrogenase
LEAK respiration	basal respiration rate
MMR	maximum metabolic rate
$\dot{M}_{O_2}$	oxygen consumption rate
OXPHOS	oxidative phosphorylation
PM	pyruvate and malate
P_{O_2}	partial pressure of oxygen
RCR	respiratory control ratio
SCH	sedentary fish in constant hypoxia
SIH	sedentary fish in intermittent hypoxia
SN	sedentary fish in normoxia
SMR	standard metabolic rate
TIH	trained fish in intermittent hypoxia
TN	trained fish in normoxia
U_{crit}	critical swimming speed

production (Ferrannini, 1988). Thus, normoxic training would classically counteract the detrimental effect of hypoxia, by enhancing the aerobic capacity, associated with a metabolic preference for lipids (Davison and Goldspink, 1978). To obtain an integrative view of the hypoxia-driven metabolic changes, *in vivo* swimming performance (U_{crit}, COT) and $\dot{M}_{O_2}$, as well as *in vitro* bioenergetics [red muscle fibre respiration and activity of main metabolic enzymes of aerobic metabolism (cytochrome *c* oxidase, COX; citrate synthase, CS) and anaerobic metabolism (lactate dehydrogenase, LDH)] were assessed in goldfish red muscle after reoxygenation.

MATERIALS AND METHODS

Experimental animals

Adult goldfish [*Carassius auratus* (Linnaeus 1758)] were obtained from a commercial breeder (Anthias Aquariologie, Les Chères, France). Shortly after their arrival, all fish were anaesthetized with MS-222 (100 mg l^{-1}) and marked with an RFID tag implanted in dorsal muscle (Biolog-id, Bernay, France, 0.03 g, i.e. <0.3% of lowest goldfish body mass). Fish were fed a commercial diet (Tetra granules goldfish) once a day to satiety, 6 days a week. Animals were randomly split into four 200 litre tanks filled with aerated water (12 h light: 12 h dark cycle; 8 mg O_2 l^{-1}) with temperature maintained at 20°C (Teco TC 5 Aquarium Cooler, White Corals, Germany) for at least 2 weeks prior to experimentation. One-third of the water of each aquarium was replaced every 2 days and water quality was monitored throughout the experiment with test strips (JBL Easy Test 6 in 1, JBL GMBH; www.jbl.de). All experimental procedures were approved by the French ethical committee (no. 12,354-2017112814209289_v5).

Hypoxia acclimation and swimming activity

Goldfish were separated into five groups (n=10–13 in each group; see Fig. 1), including three sedentary groups – (1) sedentary normoxic (SN; 100% air saturation; 8 mg O_2 l^{-1}), (2) sedentary constant hypoxia (SCH; 10% air saturation, 0.8 mg O_2 l^{-1}) and (3) sedentary intermittent hypoxia (SIH; 5 days a week: 21 h at 10% air saturation/3 h at 100% air saturation) – and two trained groups: (4) trained normoxia (TN; 100% air saturation) and (5) trained intermittent hypoxia (TIH; same hypoxic conditions as the SIH group).

Hypoxic fish were held in two 200 litre tanks, with a Plexiglas lid to limit air–water gas exchange, and oxygen concentration was gradually lowered from 100% to 10% air saturation over 5 days, adapted from a previous protocol (Farhat et al., 2019). Briefly, oxygen was decreased from 100% on day 1 to 50% on day 2 and thereafter decreased 10% every day until reaching 10% air saturation after 5 days. Animals were then held at this level for 30 days. In each tank, oxygen concentration was continuously monitored by an optode system (Robust Oxprobes and Firesting O_2, Pyroscience, Germany), wired to a data logger (CR1000, Campbell Scientific Ltd, Vincennes, France), which controlled two solenoids that regulated the bubbling of ambient air or nitrogen to stabilize water partial pressure of oxygen (P_{O_2}) to the desired level. Feeding and water cleaning did not affect the water P_{O_2} in the hypoxic tanks (data not shown).

Trained fish were forced to swim 3 h day^{-1}, 5 days a week, for 20 days. The swimming activity was performed in normoxia for all trained fish. Animals were placed in a continuously aerated training circular arena (diameter=80 cm, volume=100 dm^3) with a water current (sustained by water pump 4000S, 3400 l h^{-1}, Aquavie France) of approximately 1.5 body lengths per second (BL s^{-1}).

SN and SIH fish were placed in normoxia 3 h day^{-1}, 5 days a week, in an identical arena but without current, to mimic the same conditions as trained fish. SCH fish remained in their housing tanks during the 30-day experimental period. The acute reoxygenation undergone by the IH groups did not lead to stress, mortality or deleterious physiological consequences (see pain signs if fish listed in Sneddon, 2009).

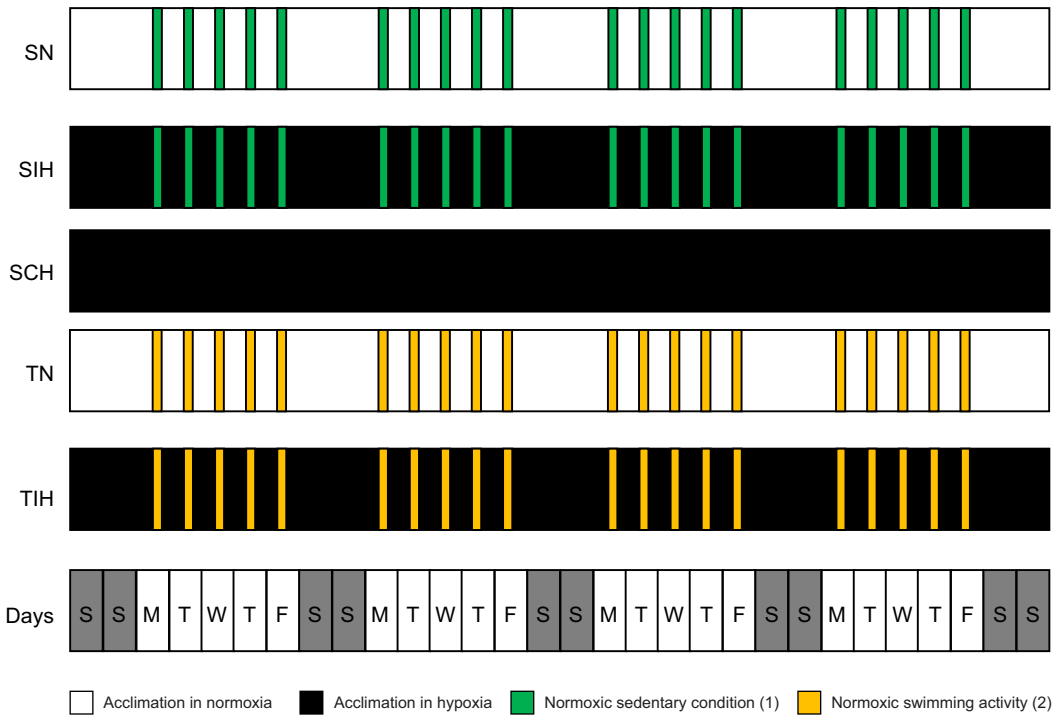
Swim tunnel and U_{crit} protocol

After 30 days of hypoxia acclimation, fish were anaesthetized with MS-222 (100 mg l^{-1}) and then measured, weighed, and placed individually in a 5 litre swim tunnel respirometer (Loligo Systems, Tjele, Denmark). Fish were allowed to recover overnight (minimum 15 h) at a water velocity of 0.5 BL s^{-1} before starting the U_{crit} protocol. MO_2 was monitored during the recovery period and fish were fasting for 24 h before the U_{crit} protocol. All the experiments were conducted at 20°C (Teco TC 5 Aquarium Cooler, White Corals) and in normoxic conditions. Oxygen consumption and temperature were continuously recorded using an optode device (Firesting O_2, Pyroscience, Germany) in the swim tunnel. The standard metabolic rate (SMR) was measured as the average of the 10% lowest oxygen consumption values measured during the night (Chabot et al., 2016). During the U_{crit} protocol, water velocity was increased by 0.5 BL s^{-1} every 30 min until exhaustion (defined as resting continuously for more than 10 s on the grid at the back of the swimming area). The MMR was estimated as the maximum oxygen consumption recorded during the U_{crit} protocol. After exhaustion, the water velocity was reduced to 0.5 BL s^{-1} and the fish recovered for 2 h before being returned to its holding tank. Absolute aerobic scope (AAS) was calculated as the difference between MMR and SMR, and factorial aerobic scope (FAS) by dividing MMR by the SMR ratio (Halsey et al., 2018).

U_{crit} was calculated using Brett's equation (Brett, 1964):

$$U_{crit} = V_f + \left(\frac{T_f}{T_i}\right) V_i, \quad (1)$$

Journal of Experimental Biology (2022) 225, jeb243194. doi:10.1242/jeb.243194



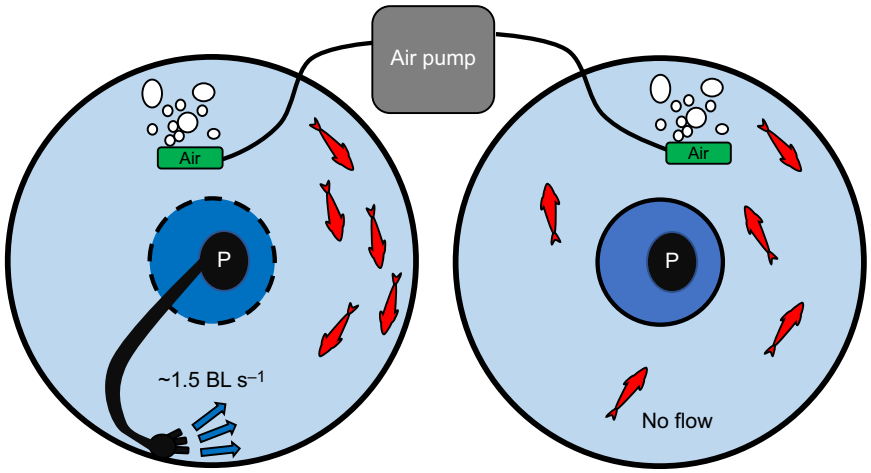


Fig. 1. Description of experimental groups in this study. Goldfish were acclimated for 30 days to normoxia (N, 100% air saturation), constant hypoxia (CH, 10% air saturation) or intermittent hypoxia (IH, 3 h in normoxia: 21 h in hypoxia, 5 days a week). Some fish acclimated in intermittent hypoxia (TIH) and normoxia (TN) were trained in normoxia 3 h per day, 5 days a week for 20 days (bottom left). The sedentary fish acclimated in intermittent hypoxia (SIH) and normoxia (SN) were placed 3 h per day, 5 days a week in normoxia in a training tank (bottom right) equivalent to the trained fish but without current. P, pump.

where V_f is the speed reached by the fish for the last full period (cm s^{-1}), T_f is the time held at the last speed step reached (min), T_i is the full period of a speed step (30 min), and V_i is the speed increment between steps (0.5 BL s^{-1}).

The COT obtained at the maximum aerobic capacity was calculated as follows:

$$\mathrm{COT} = \frac{\mathrm{MMR}}{U_{\mathrm{aerobic}}}, \quad (2)$$

where MMR is expressed in mg O_2 s^{-1} kg^{-1} and U_{aerobic} is the water velocity at which the MMR was measured in m s^{-1}.

To estimate the effect of acute reoxygenation, the area under the curve of $\dot{M}_{O_2}$ was calculated using trapezoidal integration minus the individual SMR during the first 3 h of reoxygenation.

Mitochondrial respiration

Approximately 2 weeks after the U_{crit} protocol, fish were anaesthetized with MS-222 (100 mg l^{-1}) and then euthanized by cervical dislocation (American Veterinary Medical Association, 2020). Body mass, body length and red muscle mass were then recorded. The red muscle proportion was calculated by dividing red muscle mass by body mass and expressed in percentage. Red muscle samples were removed at the level of the dorsal fin and stored at 4°C in a respiration buffer (MiR05: 0.5 mmol l^{-1} EGTA,

3 mmol l^{-1} $MgCl_2$, 60 mmol l^{-1} K-lactobionate, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} HEPES, 110 mmol l^{-1} sucrose, 1 g l^{-1} free-fatty-acid bovine serum albumin, pH 7.1; Teulier et al., 2019) to be dissected to separate muscle fibres. The red muscle not used to measure mitochondrial respiration was frozen at −80°C.

Mitochondrial respiration was measured directly from the red muscle fibres without any permeabilization using high-resolution respirometers (Oxygraphs 2k high-resolution respirometers, Oroboros Instruments, Innsbruck, Austria). Two different protocols adapted from Thoral et al., 2021a and Teulier et al., 2019 were used to measure mitochondrial respiratory capacities after the injection of a glycolytic or a lipidic substrate. Initial oxygen consumption occurring before the addition of substrates was measured at the beginning of each protocol, and this measurement was subtracted from all others. Note that this respiratory rate was similar to that measured in the presence of antimycin A, which was systematically added at the end of the experiment (see Table S1). For the glycolytic protocol, basal oxygen consumption not linked to ATP production (i.e. LEAK respiration) was obtained after the injection of pyruvate (5 mmol l^{-1}) and malate (2.5 mmol l^{-1}). The phosphorylation respiration rate (i.e. oxygen consumption linked to ATP production; OXPHOS respiration) was then obtained by injecting ADP (1 mmol l^{-1}).

For the lipidic protocol, palmitoyl-carnitine (40 µmol l^{-1}) and malate (2.5 mmol l^{-1}) were added to obtain LEAK respiration. ADP (1 mmol l^{-1}) was then injected to obtain OXPHOS respiration.

For both protocols, cytochrome *c* (10 µmol l^{-1}) was injected to evaluate the outer mitochondrial membrane integrity (Gnaiger, 2014). As the effect of cytochrome *c* was homogeneous among individuals and groups, no mitochondrial data were removed due to this test. The respiratory control ratio (RCR) was calculated by dividing OXPHOS respiration by the LEAK respiration to assess the level of coupling of the electron transport system (ETS; Salin et al., 2018). Finally, the cellular aerobic scope (CAS) was obtained by subtracting LEAK respiration from OXPHOS respiration.

Enzyme activities

Red muscle samples were thawed *a posteriori* and then homogenized using a glass piston-glass Potter in 500 µl in a phosphate buffer (100 mmol l^{-1}). The homogenates were then kept at −80°C. After a thawing cycle, samples were vortexed and then centrifuged at 1000 ***g*** for 10 min, and the supernatants were used for LDH and CS enzyme assays. A COX enzyme assay was performed on the homogenate samples. All enzyme activities were measured at 26.1±1.9°C.

LDH activity was measured in a reaction medium composed of 40 mmol l^{-1} imidazole (pH 7), 0.04% bovine serum albumin, 300 µmol l^{-1} NADH, and supplemented with 16.5±12.3 µg ml^{-1} of red muscle sample. After 3 min of incubation, the reaction was started by adding 5 mmol l^{-1} pyruvate. Then, the optical density was recorded at 340 nm for 3 min. Enzyme activity was quantified using an extinction coefficient of 6.22 l mol^{-1} cm^{-1}.

CS activity was measured in a reaction medium composed of 100 mmol l^{-1} Tris Buffer, 100 µmol l^{-1} 5,5′-dithiobis (2-nitrobenzoic acid), and 300 µmol l^{-1} acetyl-CoA, supplemented with 74.9±57.1 µg ml^{-1} of red muscle sample (pH 8). After 3 min of incubation, 10 mmol l^{-1} oxaloacetate was added to start the reaction and the optical density was recorded for 3 min at 412 nm. Enzyme activity was quantified using an extinction coefficient of 13.6 l mol^{-1} cm^{-1}.

COX activity was measured in a reaction medium composed of 50 mmol l^{-1} phosphate buffer (pH 7.4) and 100 µmol l^{-1} reduced cytochrome *c*. After 3 min of incubation, 0.53 mg ml^{-1} of red muscle sample was added to start the reaction and the optical density was recorded for 3 min at 550 nm. Enzyme activity was quantified using an extinction coefficient of 18.5 l mol^{-1} cm^{-1}.

The LDH/CS ratio was calculated to estimate the contribution of anaerobic metabolism relative to aerobic metabolism. OXPHOS respiration obtained with a glycolytic substrate (OXPHOS PM, where PM refers to pyruvate and malate) was divided by CS activity to estimate the intrinsic activity of mitochondria in red muscle fibres.

Statistical analysis

The effects of hypoxia (IH or CH) and training were evaluated for all parameters of the five experimental groups using linear models (LMs) or linear mixed models (LMMs) performed using R v. 4.0.3. *In vitro* parameters were normalized to body mass. When the assumption of normality was not met, the data were transformed using a Box–Cox transformation. When the results of the analyses on the transformed data were equivalent to those obtained on the original data, the analyses performed on the original data were kept. A *post hoc* test, using the emmeans function, was performed on all groups when the interaction between hypoxia and training was significant ($P<0.05$). For $\dot{M}_{O_2}$, a *post hoc* test was performed using the glht function when the interaction between time and groups was significant ($P<0.05$). Moreover, statistical analysis was refined by considering only the sedentary groups (SN, SIH and SCH) to measure the effect of constant and intermittent hypoxia. Similarly, analysis was refined by considering only the groups in normoxia or intermittent hypoxia (SN, SIH, TN and TIH) to measure intermittent hypoxia and training effects. Results of statistical analyses are presented in Table S2.

RESULTS

Fish characteristics

There was no interaction between hypoxia and training on all fish characteristics presented in Table 1 ($P>0.050$). However, red muscle mass was increased in the trained fish ($F_{1,35}=4.77$, $P=0.036$), and red muscle proportion was higher in the SCH fish compared with SN and SIH fish ($P=0.039$ and $P=0.013$, respectively).

Whole-organism metabolism

$\dot{M}_{O_2}$ was measured during the first 15 h of reoxygenation before the U_{crit} protocol (Fig. 2A). For the SN, SIH and TIH groups, $\dot{M}_{O_2}$ started to decrease after the second hour in normoxia ($P<0.01$). However, the $\dot{M}_{O_2}$ of the TN fish started to decrease after 4 h ($P<0.01$), and the $\dot{M}_{O_2}$ of the SCH fish was decreased between 6 and 13 h, corresponding to the night period ($P<0.01$). During the first hour of reoxygenation, the $\dot{M}_{O_2}$ of TIH fish was higher relative to TN fish ($P=0.045$). The area under the curve during the first 3 h of reoxygenation was lower in SCH compared with SIH fish ($P=0.025$). The area under the curve was not affected by training ($F_{1,30}=3.55$, $P=0.069$; Fig. 2B).

In vivo parameters measured before and during the U_{crit} protocol are presented in Table 1. There was no interaction between training and hypoxia ($P<0.05$) and there was no effect of training ($P>0.05$) on SMR, MMR, AAS, U_{crit} or COT. SMR was lower in SCH fish compared with SN ($P=0.011$) and SIH fish ($P<0.001$). MMR was also lower in SCH fish compared with SN fish ($P=0.030$). AAS was not different between SCH and SN fish ($P>0.05$), but it was decreased in SIH fish compared with SN ($P=0.009$) and SCH fish ($P=0.046$). FAS was increased in SCH fish compared with SIH fish ($P=0.002$). There was an interaction between intermittent hypoxia

Journal of Experimental Biology (2022) 225, jeb243194. doi:10.1242/jeb.243194

Table 1. Fish characteristics measured before mitochondrial respiration experiment with body length (cm), body mass (g), red muscle mass (mg) and proportion of red muscle (%), and the *in vivo* parameters measured during the U_{crit} protocol

	SN	SIH	SCH	TN	TIH
Fish characteristics					
Number of fish	8	10	10	9	10
Body length (cm)	9.31±0.13[a]	9.33±0.30[a]	9.41±0.20[a]	9.56±0.40	10.08±0.27
Body mass (g)	13.71±1.12[a]	14.64±1.89[a]	15.87±1.26[a]	16.09±2.06	17.77±1.53
Red muscle mass (mg)	116.95±25.99[a]	115.59±28.44[a]	154.76±14.28[a]	**193.34±44.38***	**179.58±29.52***
Red muscle proportion (% body mass)	0.84±0.17[a]	0.76±0.14[a]	1.49±0.20[b]	1.14±0.24	0.99±0.15
In vivo parameters					
Number of fish	7	7-8	7	9	10
SMR (mg O_2 h^{-1} kg^{-1} fish)	123.33±14.70[a]	151.64±10.04[a]	68.06±10.20[b]	129.07±10.82	123.00±8.49
MMR (mg O_2 h^{-1} kg^{-1} fish)	269.92±23.91[a]	217.08±12.11[a,b]	196.11±17.75[b]	258.47±22.63	245.37±19.62
AAS (mg O_2 h^{-1} kg^{-1} fish)	146.58±19.79[a]	65.37±9.56[b]	128.05±19.85[a]	129.40±19.91	122.37±20.39
FAS (MMR/SMR)	2.31±0.26[a,b]	1.28±0.20[a,$]	3.30±0.56[b]	2.07±0.21	2.08±0.23
U_{crit} (BL s^{-1})	2.70±0.46[a]	3.93±0.48[a,b,$]	4.89±0.35[b]	3.29±0.42	4.27±0.48[$]
COT (mg O_2 kg^{-1} fish m^{-1})	0.48±0.06[a]	0.28±0.05[b,$]	0.14±0.01[b]	0.42±0.06	0.33±0.06[$]

All groups are represented: sedentary normoxic (SN, n=7–8), sedentary intermittent hypoxic (SIH, n=7–10), sedentary constant hypoxic (SCH, n=7–10), trained normoxic (TN, n=9) and trained intermittent hypoxic (TIH, n=10). Values are means±s.e.m. Different lowercase letters indicate significant differences between sedentary groups (P<0.05). Bold text with an asterisk indicates a significant global effect of training (P<0.05) and a $ indicates a significant intermittent hypoxia effect within the same treatment (sedentary and/or trained fish, P<0.05).

and training on FAS ($F_{1,30}$=5.26, P=0.029), with a higher FAS in SN compared with SIH fish (P=0.017). U_{crit} was increased in SCH fish compared with SN fish (P=0.008), and it was globally higher in intermittent hypoxic fish (SIH and TIH) compared with normoxic fish (SN and TN, $F_{1,32}$=5.63, P=0.024). Finally, the COT was higher in SN fish compared with SCH (P<0.001) and SIH fish (P=0.024), and it was globally lower in intermittent hypoxic fish (SIH and TIH) compared with normoxic fish (SN and TN; $F_{1,32}$=5.50, P=0.025).

Mitochondrial respiration

LEAK and OXPHOS respiration obtained with a glycolytic substrate (pyruvate) are presented in Fig. 3A, and the respiration values obtained with a lipidic substrate (palmitoyl-carnitine) are presented in Fig. 3B. LEAK respiration obtained with a glycolytic substrate was not affected by hypoxia or training, and there was no interaction between these variables (P>0.05). However, OXPHOS respiration was higher in SIH fish compared with SCH fish (P=0.006), and it was globally higher in intermittent hypoxic fish (SIH and TIH) compared with normoxic fish (SN and TN, $F_{1,34}$=4.43, P=0.043). Thus, the resulting CAS was higher in SIH fish compared with SCH fish (P=0.007). LEAK and OXPHOS respiration and the resulting CAS with a lipidic substrate were not affected by hypoxia and training (P>0.05), and there was no interaction between these variables (P>0.05). The RCR with a glycolytic substrate was unchanged between groups with a glycolytic substrate (P>0.05; Fig. 3C), but it was higher in SCH fish compared with SIH fish with a lipidic substrate (P=0.037; Fig. 3D). For all these parameters, those obtained with a glycolytic substrate were higher than those obtained with a lipidic substrate (P<0.05). Finally, the ratio of the OXPHOS respiration obtained with a glycolytic substrate divided by the maximum activity of CS (OXPHOS PM/CS ratio; Table 2) was higher in SCH fish compared with SIH fish (P=0.015), and was increased in trained fish (P=0.006). This suggests that the intrinsic activity of mitochondria was improved by both the training protocol and the constant hypoxia condition.

Enzymes

CS activity was higher in SIH fish compared with SN (P=0.033) and SCH fish (P<0.001), and it was higher in SN fish compared with SCH fish (P=0.047). Moreover, CS activity was higher in trained fish compared with sedentary fish ($F_{1,32}$=7.18, P=0.012; Table 2) and it was globally higher in intermittent hypoxic fish (SIH and TIH) compared with normoxic fish (SN and TN, $F_{1,32}$=9.94, P=0.004). The interaction between hypoxia and training was significant for COX activity ($F_{1,40}$=11.79, P=0.001). This activity was not different between SCH and SN fish (P>0.05), but it was higher in SIH fish compared with the other sedentary fish (P<0.001). Moreover, COX activity was higher in SIH fish compared with TIH fish (P=0.003, Table 2). LDH activity was not affected by training (P>0.05) but it was higher in SIH fish compared with SCH fish (P=0.047) and SN fish (P<0.001), and it was higher in SCH compared with SN fish (P=0.002, Table 2). Moreover, it was globally higher in intermittent hypoxic fish (SIH and TIH) compared with normoxic fish (SN and TN, $F_{1,35}$=57.28, P<0.001). Finally, the anaerobic contribution to metabolism, represented by the ratio of LDH activity to CS activity, was higher in SCH fish compared with SN (P<0.001) and SIH fish (P=0.005; Table 2). It was also globally higher in intermittent hypoxic fish (SIH and TIH) compared with normoxic fish (SN and TN, $F_{1,32}$=18.42, P<0.001).

DISCUSSION

This study reveals the onset of adaptive metabolic mechanisms induced by long-term severe constant or intermittent hypoxia in untrained or trained goldfish. Through a comprehensive panel of *in vivo* parameters (metabolic rate, swimming performance) and cellular bioenergetics (red muscle mitochondrial metabolism and enzymatic activities), our results demonstrate that goldfish acclimated to constant hypoxia have markedly improved swimming performance in normoxia, with a higher U_{crit} and a lower COT compared with normoxic fish. Moreover, they maintained metabolic rate suppression in normoxia while simultaneously increasing aerobic capacity dedicated to locomotion represented by FAS. Interestingly, this enhanced *in vivo* performance occurs with minimal change in mitochondrial metabolism, suggesting that mitochondrial plasticity is preferentially used for rapid variations in environmental parameters rather than long-term acclimation. However, intermittent daily return to normoxia for a few hours per day seems to suppress most of the enhancement in swimming performance, most notably with an increase in maintenance costs. Intermittent hypoxia also leads to an increase in enzymatic activities

Journal of Experimental Biology (2022) 225, jeb243194. doi:10.1242/jeb.243194

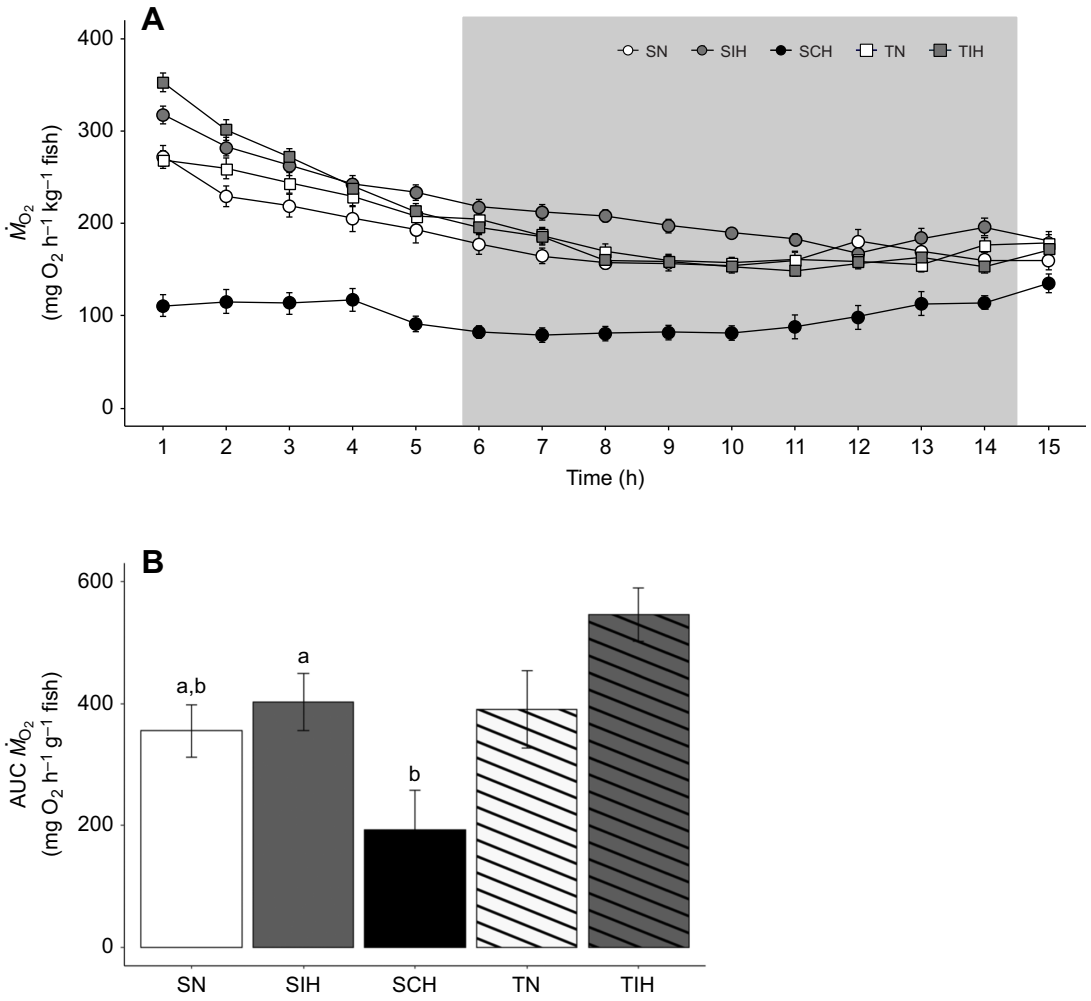


Fig. 2. Metabolic rate of goldfish during the first 15 h of reoxygenation. (A) Oxygen consumption rate ($\dot{M}_{O_2}$) in the first 15 h after reoxygenation and (B) the area under the curve (AUC) of $\dot{M}_{O_2}$ during the first 3 h of reoxygenation. $\dot{M}_{O_2}$ is expressed in mg O_2 h^{-1} kg^{-1} of fish. The AUC of $\dot{M}_{O_2}$ and above the SMR is expressed in mg O_2 h^{-2} kg^{-1} of fish. All groups are represented: sedentary normoxic (SN, white dots, *n*=7), sedentary intermittent hypoxic (SIH, grey dots, *n*=9), sedentary constant hypoxic (SCH, black dots, *n*=10), active normoxic (TN, white squares and white bar with diagonal lines, *n*=8) and active intermittent hypoxic (TIH, grey squares and grey bar with diagonal lines, *n*=10. The grey bar symbolizes the night period. The different letters indicate an effect of hypoxia in the sedentary groups (P<0.05). Value are mean±s.e.m.

in red muscle that does not lead to an increase in aerobic capacity. Finally, under our experimental conditions, training does not appear to improve the swimming performance of fish acclimated to intermittent hypoxia.

Thrifty metabolism induced by constant hypoxia is persistent in normoxia

Chronic exposure to 10% air saturation (SCH) induces *in vivo* metabolic depression. The reduction of both SMR and MMR is in line with previous research demonstrating this metabolic consequence of constant hypoxia acclimation in fish (reviewed in Domenici et al., 2013; Pollock et al., 2007; Weber, 2016). However, it is notable that this metabolic depression persists after returning to normoxic conditions, as observed in killifish (Borowiec et al., 2018). Indeed, goldfish acclimated to chronic hypoxia exhibit a similar $\dot{M}_{O_2}$ under hypoxic (Farhat et al., 2019) or normoxic conditions (present study). This metabolic suppression is induced through multiple adaptive mechanisms (i.e. gill remodelling, lower digestive activity, decreased ion pumping, changes in muscle fibre phenotype), the reversal of which may be both costly and slow (reviewed in Richards, 2011). In support of this, the aerobic metabolic scope (Fry and Hart, 1948; Domenici et al., 2013) is equivalent between fish in normoxia or constant hypoxia.

In addition, by lowering their maintenance costs, fish acclimated to constant hypoxia improve their aerobic capacity dedicated to locomotion with an increase in FAS compared with normoxic and intermittent hypoxic fish (+43% and +158%, respectively), indicating that hypoxia constrains MMR to a lesser extent than the SMR (Halsey et al., 2018). The maintenance of AAS is also an index of the preservation of aerobic capacity in fish after their return to normoxia following acclimation in constant hypoxia (Norin et al., 2016). Their higher U_{crit} (+80%) along with a lower oxygen consumption result in a tremendous decrease of the COT (–71%) in fish acclimated in constant hypoxia compared with normoxic fish. This is in accordance with previous experiments showing beneficial consequences of a pre-exposure to hypoxia on these parameters in goldfish (Fu et al., 2011). These enhancements could arise through multiple physiological adjustments. First, to maximize the limited oxygen available, assimilation and transport of oxygen is improved via gill remodelling (Sollid and Nilsson, 2006), increasing

Journal of Experimental Biology (2022) 225, jeb243194. doi:10.1242/jeb.243194

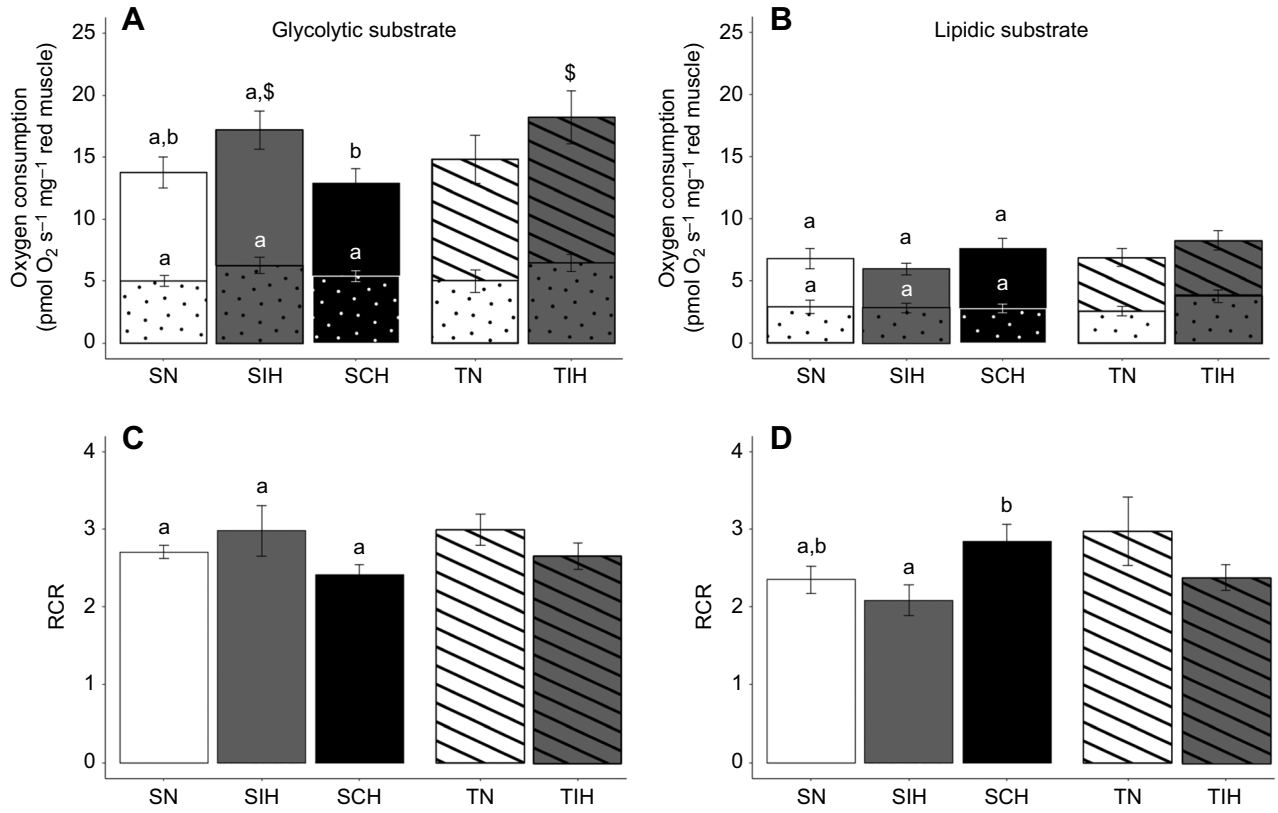


Fig. 3. Mitochondrial metabolism as a function of housing conditions and training. The SN (white bars, *n*=7-8), SIH (grey bars, *n*=7–10), SCH (black bars, *n*=7–10), TN (white bars with diagonal lines, *n*=8–9) and TIH groups (grey bars with diagonal lines, *n*=9–10) are represented. OXPHOS respiration (solid or striped bars) and LEAK respiration (dotted bars) obtained with (A) a glycolytic substrate or (B) a lipidic substrate are expressed in pmol O_2 s^{-1} mg^{-1} red muscle. The difference between OXPHOS and LEAK respiration is the cellular aerobic scope (CAS). The respiratory control ratio (RCR) is the ratio of OXPHOS respiration to LEAK respiration obtained with (C) a glycolytic substrate or (D) a lipidic substrate. Different letters indicate a significant effect of hypoxia in the sedentary groups (*P*<0.05) and a $ indicates a significant intermittent hypoxia effect within the same treatment (sedentary and/or trained fish, *P*<0.05).Values are means±s.e.m.

haemoglobin blood content (Borowiec et al., 2015) or by a shift in haemoglobin isoforms (Pan et al., 2017). Second, LDH activity was increased in fish acclimated in constant hypoxia, which could be due to a prioritization of the anaerobic metabolic pathway (Shoubridge and Hochachka, 1980; Pastoris et al., 1995; Omlin and Weber, 2010).

Exposure frequency is a key determinant of hypoxia acclimation

The duration (chronic versus acute) and frequency (constant or intermittent) of hypoxia exposure are both important in determining the severity of the hypoxic stress, and fish display different adaptations to varied hypoxic conditions (Farrell and Richards, 2009; Borowiec et al., 2015). Intermittent hypoxia in fish is mainly studied using protocols mimicking nocturnal environmental hypoxia, with a cycle of 12 h normoxia and 12 h hypoxia inducing contrasting results relative to constant hypoxia (Borowiec et al., 2015, 2018). In our study, only 3 h per day and 5 days a week of normoxic reoxygenation are sufficient to abolish the improvements in swimming performance observed following constant hypoxia. As in constant hypoxia, MMR is lower, owing to the limited O_2 supply during the 21 h per day in which animals experience hypoxia in this protocol. In parallel, SMR remains at the same level of normoxic fish and is much higher than fish acclimated in constant hypoxia (+123%), implying an increase in energy cost for maintaining vital processes (Norin and Metcalfe, 2019). This inability to lower the cost of maintenance could be due to the necessity of maintaining essential cellular functions such as ion

Table 2. Maximal enzymatic activities and mitochondrial indices of red muscle measured for all groups: sedentary in normoxia (SN, *n*=8–9), sedentary in intermittent hypoxia (SIH, *n*=8–10), sedentary in constant hypoxia (SCH, *n*=9–10), trained in normoxia (TN, *n*=9) and trained in intermittent hypoxia (TIH, *n*=7–9)

Enzyme activity	SN	SIH	SCH	TN	TIH
Citrate synthase (CS)	11.51±2.32^{a}	17.17±1.04$^{b,\$}$	6.29±0.70^{c}	**7.90±0.77***	**9.83±1.28$^{\$,*}$**
Cytochrome *c* oxidase (COX)	3.07±0.50^{a}	9.25±1.51^{b}	3.05±0.39^{a}	4.45±1.13	3.67±0.69*
Lactate dehydrogenase (LDH)	92.45±16.11^{a}	254.67±20.45$^{b,\$}$	200.31±15.47^{c}	70.48±10.55	215.61±26.67$^{\$}$
LDH/CS	10.29±2.56^{a}	15.27±1.21$^{a,\$}$	34.48±3.84^{b}	9.81±1.72	24.80±3.19$^{\$}$
OXPHOS PM/CS	1.47±0.29^{a,b}	0.98±0.08^{a}	2.30±0.41^{b}	**2.14±0.41***	**2.13±0.34***

Enzyme activities are expressed in µmol min^{-1} g^{-1} red muscle. Values are means±s.e.m. Different lowercase letters indicate significant differences between sedentary groups (*P*<0.05). Bold text with an asterisk indicates a significant global effect of training (*P*<0.05). An asterisk alone indicates a significant effect of training for the same acclimation condition (*P*<0.05). $ indicates a significant intermittent hypoxia effect within the same treatment (sedentary and/or trained fish, *P*<0.05). PM, pyruvate and malate.

pumping or replenishment of substrates stocks in tissues (Sokolova, 2018).

In parallel with the increases in COX, CS and LDH activities observed in our study, OXPHOS respiration obtained with a glycolytic substrate increases in fish acclimated in intermittent hypoxia compared with fish acclimated in constant hypoxia. This global increase in cellular metabolism could explain the maintenance of U_{crit}, despite a drastic decrease in the aerobic scope (~55%) resulting from the decrease in MMR associated with a higher SMR. But this increase in metabolism represents a high maintenance cost, such that SMR of fish acclimated in intermittent hypoxia is equivalent to that of normoxic fish. Moreover, higher OXPHOS respiration after acclimation in intermittent hypoxia could be explained by an increase in carbohydrate oxidation, which is in agreement with the existing literature. In addition, both constant and intermittent hypoxia result in an increase in LDH activity, which could lead either to an increase in lactate production (Omlin and Weber, 2010; Borowiec et al., 2015), or to improving the capacity to metabolize lactate back to pyruvate (Van Hall, 2000).

One of the main goals of this study was to measure the combined effects of normoxic training with acclimation to intermittent hypoxia on aerobic performance in normoxia. Even if the higher red muscle mass of trained fish (Table 1) seems intuitively connected to a higher swimming activity, it cannot be directly ascribed to our training protocol. Moreover, it did not lead to benefits in swimming performance in normoxia. This lack of training effect on swimming performance could be explained by difficulty in translating the benefits of training into improved swimming performance in a relatively sedentary species not adapted to sustained exercise (Fu et al., 2014). In addition, fish are able to find the region with the lowest flow to minimize swimming effort. Therefore, heterogeneity of current in the training pool could also partially explain this lack of training effect. However, even if the training effect had been less efficient as expected, trained fish responded homogeneously to this constraint. Thus, even with underestimated consequences, this daily exercise did help to mitigate some of the negative effects observed following acclimation to intermittent hypoxia, including limiting the decrease in AAS (Table 1). Taken together, these results demonstrate that a daily swimming activity is sufficient to reduce the negative effects of daily oxygen fluctuations, although this is not sufficient to have as much effect as acclimation in constant hypoxia on swimming performance.

Level-dependent adaptive mechanisms: differences between *in vivo* and cellular metabolism

Chronic exposure to constant hypoxic conditions leads to major metabolic adaptations that persist after reoxygenation. However, these metabolic adjustments appear at different levels of biological organization. Indeed, despite a massive *in vivo* metabolic depression, the effects of hypoxia were mitigated at the cellular level. Contrary to previous results obtained in brain, liver and white muscle from similarly treated goldfish (Farhat et al., 2021), proxies of anaerobic metabolism, such as LDH activity and the ratio of LDH/CS (Pette and Dölken, 1975), significantly increase compared with SN fish (+117% and 235%, respectively), and CS activity, which is representative of oxidative metabolism, is decreased (−45%).

By contrast, COX activity and mitochondrial metabolism remain unchanged after acclimation to constant hypoxia, consistent with previous observations (Farhat et al., 2021). Several hypotheses could explain the lack of effect of hypoxia on mitochondrial parameters in our experiment. The level of hypoxia and the duration of exposure used in our experiment may have been insufficient to induce mitochondrial changes in red muscle fibres, which were in line with results already observed in goldfish after 30 days in constant hypoxia at 10% air saturation in the same tissue (Farhat et al., 2021). Moreover, *in vitro* responses of mitochondrial metabolism could differ from *in vivo* responses. Indeed, enzyme activities and microrespirometry measurements of mitochondrial respiration are optimized to quantify maximal capacities that are rarely observed *in vivo* (Kuznetsov et al., 2008). In addition, *in vitro* measurements ignore the link that mitochondria may have with the whole organism, such as modulating effects of hormones (Goglia et al., 2002; Tormos and Chandel, 2010). Thus, further studies should be conducted to compare mitochondrial metabolism using complementary *in vitro* and *in situ* measurements. However, studies with *in vitro* measurements of mitochondrial metabolism have already shown differences of metabolism depending on oxygen levels (Thoral et al., 2021b), indicating that variations in environmental parameters can lead to variations in mitochondrial metabolism, even when measured *in vitro*.

The lack of effect could also be explained by variations in mitochondrial content (Du et al., 2016). Exposure to hypoxia could lead to an increase (Guderley, 1990; Sänger, 1993) or a decrease in mitochondrial content (Johnston and Bernard, 1982). In the present study, mitochondrial content seems also to be lowered by constant hypoxia, because CS activity, which is an index of mitochondrial volume within a tissue, is significantly decreased compared with the fish reared in normoxia (Table 2). Despite the lowering in mitochondrial content, the OXPHOS/CS ratio is similar, which could partially explain why hypoxic fish are able to maintain high aerobic capacity when swimming in normoxia.

Finally, the last hypothesis is that 30 days in constant hypoxia may be sufficient for mitochondria to return to their optimal level if offsetting physiological mechanisms occur [e.g. increase in affinity of haemoglobin for oxygen (Regan and Richards, 2017) or gill remodelling (Sollid and Nilsson, 2006)]. Indeed, because of their versatile functionality, mitochondria are expected to be one of the first physiological components to be modified when individuals are exposed to stressful energetic conditions, such as hypoxia. Such a relationship is particularly intuitive in skeletal muscle, which can modulate its energetic demand tremendously, depending on the conditions (Guderley and Johnston, 1996; Seebacher et al., 2010). Thus, after 2 days in hypoxia (10% air saturation), OXPHOS respiration is decreased in zebrafish after reoxygenation (Cadiz et al., 2019), and a few hours in hypoxia (<0.1% air saturation) results in decreases in mitochondrial substrate oxidation in scallops (Ivanina et al., 2016). However, in our experiment, mitochondrial respiration of goldfish red muscle did not change after either 10 or 30 days at 10% air saturation (see Figs S1 and S2). Mitochondria from hypoxia-tolerant species are resilient, and can rapidly recover after a long-term hypoxia exposure (Sokolova, 2018). Further studies with more frequent measurements of mitochondrial function are warranted to investigate the sequential setup of these adaptive metabolic processes.

Conclusions

Chronic exposure to constant hypoxia in goldfish leads to a metabolic rate suppression that is maintained after reoxygenation and to an increase in aerobic capacity, which supports improvement in swimming performance with a higher U_{crit} and a lower COT. However, a few hours per day in normoxia appear to be sufficient to minimize the benefits of acclimation to constant hypoxia on

swimming performance after reoxygenation. Despite an increase in the enzymatic activities related to aerobic and anaerobic metabolisms, *in vivo* aerobic capacity is decreased in fish acclimated in intermittent hypoxia. Nevertheless, a daily exercise carried out during 3 h in normoxia appears to be sufficient to limit the decline in aerobic capacity with intermittent hypoxia, although this training does not appear to be sufficiently intense to improve swimming performance in fish. Finally, mitochondria do not seem to be the source of the changes in metabolic rate observed *in vivo*, indicating that this organelle was not constrained enough by a chronic hypoxia or may instead play a predominant role during acute variations in environmental parameters rather than chronic changes in oxygen availability. The metabolic changes caused by severe hypoxic condition shown in this study contribute to explaining why the goldfish is capable of inhabiting widespread environments with fluctuating oxygen availability.

Acknowledgements
We thank Marine Hoareau for her help with experimentations, and Morgane Touzot for her help with animal housing. We thank Yann Voituron for his constructive advice about the design of the study.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.T., D.R., J.-M.W., L.T.; Formal analysis: E.T., L.T.; Investigation: E.T., E.F., D.R., H.C., L.G., L.T.; Resources: D.R., L.G., L.T.; Data curation: E.T., L.T., D.R.; Writing - original draft: E.T., L.T.; Writing - review & editing: E.T., E.F., D.R., H.C., L.G., M.E.P., J.-M.W., L.T.; Visualization: E.T.; Supervision: D.R., M.E.P., J.-M.W., L.T.; Project administration: E.T., D.R., L.T.; Funding acquisition: E.T., M.E.P., J.-M.W.

Funding
This work was supported by the French Government (to E.T., PhD grant 2018–2021) and by the France Canada Research Fund attributed to J.-M.W. and M.E.P. (FCRF, 149716-2018 grant for E.F. and H.C. to travel and work in France). This work was also supported by Natural Sciences and Engineering Research Council of Canada Discovery Grants to J.-M.W. (05955-2017) and M.E.P.

Data availability
Data from the present study are available from figshare at: https://figshare.com/articles/dataset/Data_Goldfish_Hypoxia/17122217.

References
American Veterinary Medical Association (2020). Guidelines for the Euthanasia of Animals. Schaumburg, IL: American Veterinary Medical Association.

Borowiec, B. G., Darcy, K. L., Gillette, D. M. and Scott, G. R. (2015). Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **218**, 1198-1211. doi:10.1242/jeb.114579

Borowiec, B. G., McClelland, G. B., Rees, B. B. and Scott, G. R. (2018). Distinct metabolic adjustments arise from acclimation to constant hypoxia and intermittent hypoxia in estuarine killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **221**, jeb190900. doi:10.1242/jeb.190900

Brand, M. (2005). The efficiency and plasticity of mitochondrial energy transduction. *Biochem. Soc. Trans.* **33**, 897-904. doi:10.1042/BST0330897

Brett, J. R. (1964). The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Board Can.* **21**, 1183-1226.

Cadiz, L., Bundgaard, A., Malte, H. and Fago, A. (2019). Hypoxia enhances blood O_2 affinity and depresses skeletal muscle O_2 consumption in zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **234**, 18-25. doi:10.1016/j.cbpb.2019.05.003

Chabot, D., Steffensen, J. F. and Farrell, A. P. (2016). The determination of standard metabolic rate in fishes. *J. Fish Biol.* **88**, 81-121. doi:10.1111/jfb.12845

Conley, K. E. (2016). Mitochondria to motion: optimizing oxidative phosphorylation to improve exercise performance. *J. Exp. Biol.* **219**, 243-249. doi:10.1242/jeb.126623

Davison, W. and Goldspink, G. (1978). The effect of training on the swimming muscles of the goldfish (*Carassius auratus*). *J. Exp. Biol.* **74**, 115-122. doi:10.1242/jeb.74.1.115

Domenici, P., Herbert, N. A., Lefrançois, C., Steffensen, J. F., McKenzie, D. J. (2013). The effect of hypoxia on fish swimming performance and behaviour. In *Swimming Physiology of Fish* (ed. A. Palstra and J. Planas), pp. 129-159 . Berlin, Heidelberg: Springer. doi:10.1007/978-3-642-31049-2_6

Du, S. N. N., Mahalingam, S., Borowiec, B. G., Scott, G. R. (2016). Mitochondrial physiology and reactive oxygen species production are altered by hypoxia acclimation in killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **219**, 1130-1138. doi:10.1242/jeb.132860

Farhat, E., Turenne, E. D., Choi, K. and Weber, J. M. (2019). Hypoxia-induced remodelling of goldfish membranes. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **237**, 110326. doi:10.1016/j.cbpb.2019.110326

Farhat, E., Cheng, H., Romestaing, C., Pamenter, M. and Weber, J.-M. (2021). Goldfish response to chronic hypoxia: mitochondrial respiration, fuel preference and energy metabolism. *Metabolites* **11**, 187. doi:10.3390/metabo11030187

Farrell, A. P. and Richards, J. G. (2009). *Defining hypoxia: an integrative synthesis of the responses of fish to hypoxia*. In *Fish Physiology, Vol. 27* (ed. J. G. Richards, A. P. Farrell and C. J. Brauner), pp. 487-503. Elsevier. doi:10.1016/S1546-5098(08)00011-3

Ferrannini, E. (1988). The theoretical bases of indirect calorimetry: a review. *Metabolism* **37**, 287-301. doi:10.1016/0026-0495(88)90110-2

Fry, F. E. J. and Hart, J. S. (1948). The Relation of temperature to oxygen consumption in the goldfish. *Biol. Bull.* **94**, 66-77. doi:10.2307/1538211

Fu, S. J., Brauner, C. J., Cao, Z. D., Richards, J. G., Peng, J. L., Dhillon, R. and Wang, Y. X. (2011). The effect of acclimation to hypoxia and sustained exercise on subsequent hypoxia tolerance and swimming performance in goldfish (*Carassius auratus*). *J. Exp. Biol.* **214**, 2080-2088. doi:10.1242/jeb.053132

Fu, S. J., Fu, C., Yan, G. J., Cao, Z. D., Zhang, A. J. and Pang, X. (2014). Interspecific variation in hypoxia tolerance, swimming performance and plasticity in cyprinids that prefer different habitats. *J. Exp. Biol.* **217**, 590-597. doi:10.1242/jeb.089268

Gnaiger, E. (2014). *Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis*. 5th edn. Bioenerg Commun 2020.2. https://doi.org/10.26124/bec:2020-0002

Goglia, F., Silvestri, E. and Lanni, A. (2002). Thyroid hormones and mitochondria. *Biosci. Rep.* **22**, 17-32. doi:10.1023/A:1016056905347

Guderley, H. (1990). Functional significance of metabolic responses to thermal acclimation in fish muscle. *Am. J. Physiol.* **259**, R245-R252. doi:10.1152/ajpregu.1990.259.2.R245

Guderley, H. and Johnston, I. A. (1996). Plasticity of fish muscle mitochondria with thermal acclimation. *J. Exp. Biol.* **199**, 1311-1317. doi:10.1242/jeb.199.6.1311

Halsey, L. G., Killen, S. S., Clark, T. D. and Norin, T. (2018). Exploring key issues of aerobic scope interpretation in ectotherms: absolute versus factorial. *Rev. Fish Biol. Fish.* **28**, 405-415. doi:10.1007/s11160-018-9516-3

Ivanina, A. V., Nesmelova, I., Leamy, L., Sokolov, E. P. and Sokolova, I. M. (2016). Intermittent hypoxia leads to functional reorganization of mitochondria and affects cellular bioenergetics in marine molluscs. *J. Exp. Biol.* **219**, 1659-1674. doi:10.1242/jeb.134700

Johnston, I. A. and Bernard, L. M. (1982). Routine oxygen consumption and characteristics of the myotomal muscle in tench: effects of long-term acclimation to hypoxia. *Cell Tissue Res.* **227**, 161-177. doi:10.1007/BF00206339

Kuznetsov, A. V., Veksler, V., Gellerich, F. N., Saks, V., Margreiter, R. and Kunz, W. S. (2008). Analysis of mitochondrial function *in situ* in permeabilized muscle fibers, tissues and cells. *Nat. Protoc.* **3**, 965-976. doi:10.1038/nprot.2008.61

Mandic, M. and Regan, M. D. (2018). Can variation among hypoxic environments explain why different fish species use different hypoxic survival strategies? *J. Exp. Biol.* **221**, jeb161349. doi:10.1242/jeb.161349

McKenzie, D. J. (2011). *Swimming and Other Activities Energetics of Fish Swimming*. Elsevier Inc.

Norin, T. and Metcalfe, N. B. (2019). Ecological and evolutionary consequences of metabolic rate plasticity in response to environmental change. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20180180. doi:10.1098/rstb.2018.0180

Norin, T., Malte, H. and Clark, T. D. (2016). Differential plasticity of metabolic rate phenotypes in a tropical fish facing environmental change. *Funct. Ecol.* **30**, 369-378. doi:10.1111/1365-2435.12503

Omlin, T. and Weber, J.-M. (2010). Hypoxia stimulates lactate disposal in rainbow trout. *J. Exp. Biol.* **213**, 3802-3809. doi:10.1242/jeb.048512

Pamenter, M. E. (2014). Mitochondria: a multimodal hub of hypoxia tolerance1. *Can. J. Zool.* **92**, 569-589. doi:10.1139/cjz-2013-0247

Pan, Y. K., Ern, R., Morrison, P. R., Brauner, C. J. and Esbaugh, A. J. (2017). Acclimation to prolonged hypoxia alters hemoglobin isoform expression and increases hemoglobin oxygen affinity and aerobic performance in a marine fish. *Sci. Rep.* **7**, 1-11. doi:10.1038/s41598-016-0028-x

Pastoris, O., Dossena, M., Foppa, P., Arnaboldi, R., Gorini, A., Villa, R. F. and Benzi, G. (1995). Modifications by chronic intermittent hypoxia and drug treatment on skeletal muscle metabolism. *Neurochem. Res.* **20**, 143-150. doi:10.1007/BF00970538

Pette, D. and Dölken, G. D. (1975). Some aspects of regulation of enzyme levels in muscle energy-supplying metabolism. *Adv. Enzyme Regul.* **13**, 355-377. doi:10.1016/0065-2571(75)90025-4

Pollock, M. S., Clarke, L. M. J. and Dubé, M. G. (2007). The effects of hypoxia on fishes: from ecological relevance to physiological effects. *Environ. Rev.* **15**, 1-14. doi:10.1139/a06-006

Regan, M. D. and Richards, J. G. (2017). Rates of hypoxia induction alter mechanisms of O_2 uptake and the critical O_2 tension of goldfish. *J. Exp. Biol.* **220**, 2536-2544. doi:10.1242/jeb.154948

Richards, J. G. (2011). Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. *J. Exp. Biol.* **214**, 191-199. doi:10.1242/jeb.047951

Salin, K., Villasevil, E. M., Anderson, G. J., Selman, C., Chinopoulos, C. and Metcalfe, N. B. (2018). The RCR and ATP/O indices can give contradictory messages about mitochondrial efficiency. *Integr. Comp. Biol.* **58**, 486-494. doi:10.1093/icb/icy085

Sänger, A. M. (1993). Limits to the acclimation of fish muscle. *Rev. Fish Biol. Fish.* **3**, 1-15. doi:10.1007/BF00043295

Schmidt-Nielsen, K. (1972). Locomotion: energy cost of swimming, flying, and running. *Science* **177**, 222-228. doi:10.1126/science.177.4045.222

Seebacher, F., Brand, M. D., Else, P. L., Guderley, H., Hulbert, A. J. and Moyes, C. D. (2010). Plasticity of oxidative metabolism in variable climates: molecular mechanisms. *Physiol. Biochem. Zool.* **83**, 721-732. doi:10.1086/649964

Shoubridge, E. A. and Hochachka, P. W. (1980). Ethanol: novel end product of vertebrate anaerobic metabolism. *Science* **209**, 308-309. doi:10.1126/science.7384807

Sneddon, L. U. (2009). Pain perception in fish: Indicators and endpoints. *ILAR J.* **50**, 338-342. doi:10.1093/ilar.50.4.338

Sokolova, I. (2018). Mitochondrial adaptations to variable environments and their role in animals' stress tolerance. *Integr. Comp. Biol.* **58**, 519-531. doi:10.1093/icb/icy017

Sollid, J. and Nilsson, G. E. (2006). Plasticity of respiratory structures - Adaptive remodeling of fish gills induced by ambient oxygen and temperature. *Respir. Physiol. Neurobiol.* **154**, 241-251. doi:10.1016/j.resp.2006.02.006

Stray-Gundersen, J. and Levine, B. D. (2008). Live high, train low at natural altitude. *Scand. J. Med. Sci. Sports* **18** Suppl. 1, 21-28. doi:10.1111/j.1600-0838.2008.00829.x

Teulier, L., Thoral, E., Queiros, Q., McKenzie, D. J., Roussel, D., Dutto, G., Gasset, E., Bourjea, J. and Saraux, C. (2019). Muscle bioenergetics of two emblematic Mediterranean fish species: *Sardina pilchardus* and *Sparus aurata*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **235**, 174-179. doi:10.1016/j.cbpa.2019.06.008

Thoral, E., Queiros, Q., Roussel, D., Dutto, G., Gasset, E., McKenzie, D. J., Romestaing, C., Fromentin, J. M., Saraux, C. and Teulier, L. (2021a). Changes in foraging mode caused by a decline in prey size have major bioenergetic consequences for a small pelagic fish. *J. Anim. Ecol.* **90**, 2289-2301. doi:10.1111/1365-2656.13535

Thoral, E., Roussel, D., Chinopoulos, C., Teulier, L. and Salin, K. (2021b). Low oxygen levels can help to prevent the detrimental effect of acute warming on mitochondrial efficiency in fish. *Biol. Lett.* **17**, 20200759. doi:10.1098/rsbl.2020.0759

Tormos, K. V. and Chandel, N. S. (2010). Inter-connection between mitochondria and HIFs. *J. Cell. Mol. Med.* **14**, 795-804. doi:10.1111/j.1582-4934.2010.01031.x

Van Hall, G. (2000). Lactate as a fuel for mitochondrial respiration. *Acta Physiol. Scand.* **168**, 643-656. doi:10.1046/j.1365-201x.2000.00716.x

Weber, J. M. (2016). Revealing how goldfish defy anoxia. *J. Exp. Biol.* **219**, 1422-1423. doi:10.1242/jeb.128884

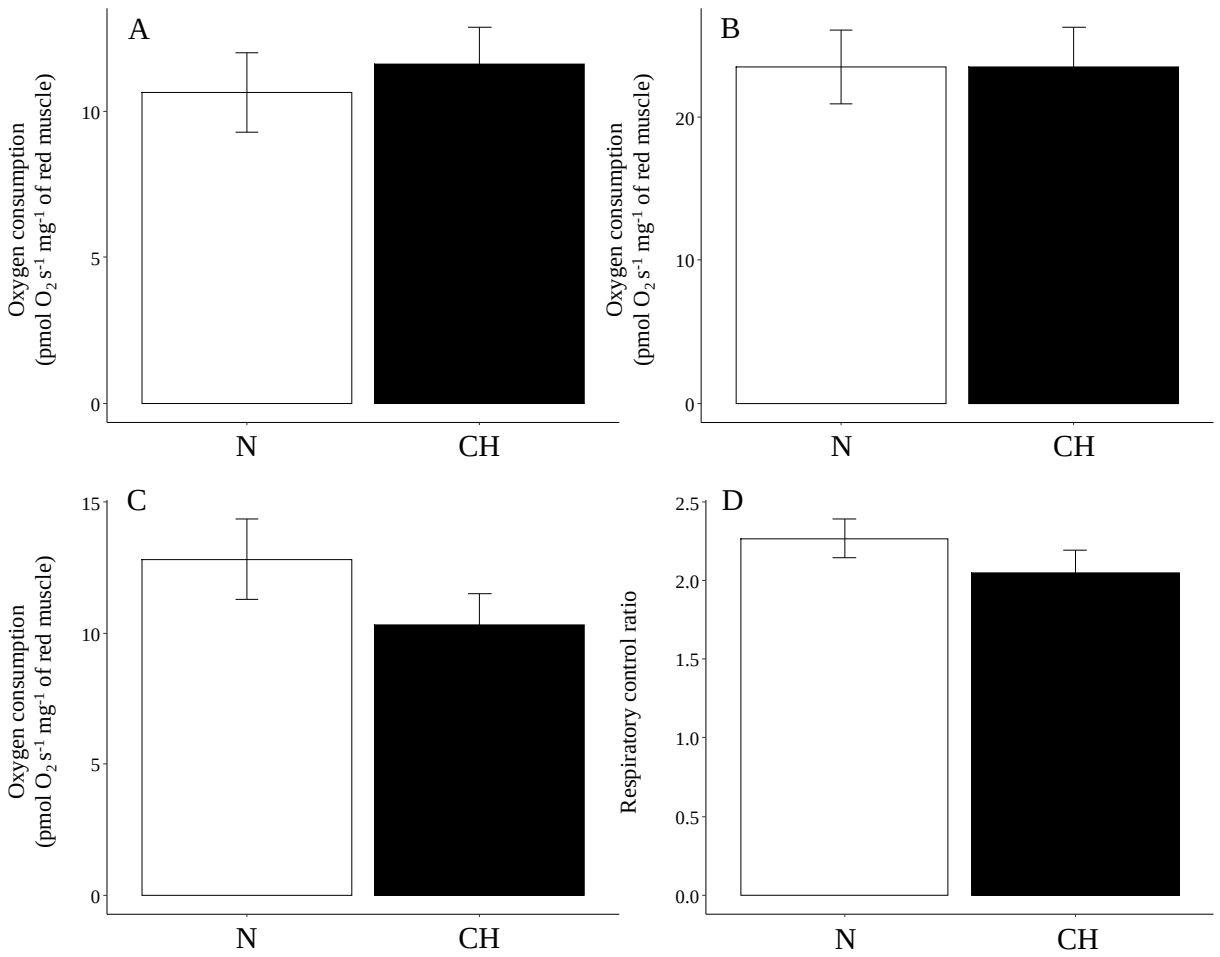


Fig. S1. Mitochondrial parameters after a full activation of the ETS assayed in fish acclimated to normoxia (N; n=8) or constant hypoxia (CH; n=10; 10% air saturation) for 30 days. Different parameters are represented : LEAK respiration (A) obtained after inhibiting the electron transport system with oligomycin; OXPHOS respiration (B) after having added pyruvate, malate, glutamate, ADP and succinate; cellular aerobic scope (CAS; C) by removing the LEAK respiration from the OXPHOS respiration and the respiratory control ratio (RCR; D) by dividing the OXPHOS respiration by the LEAK respiration. No effect of treatment was found on any parameter ($P > 0.05$).

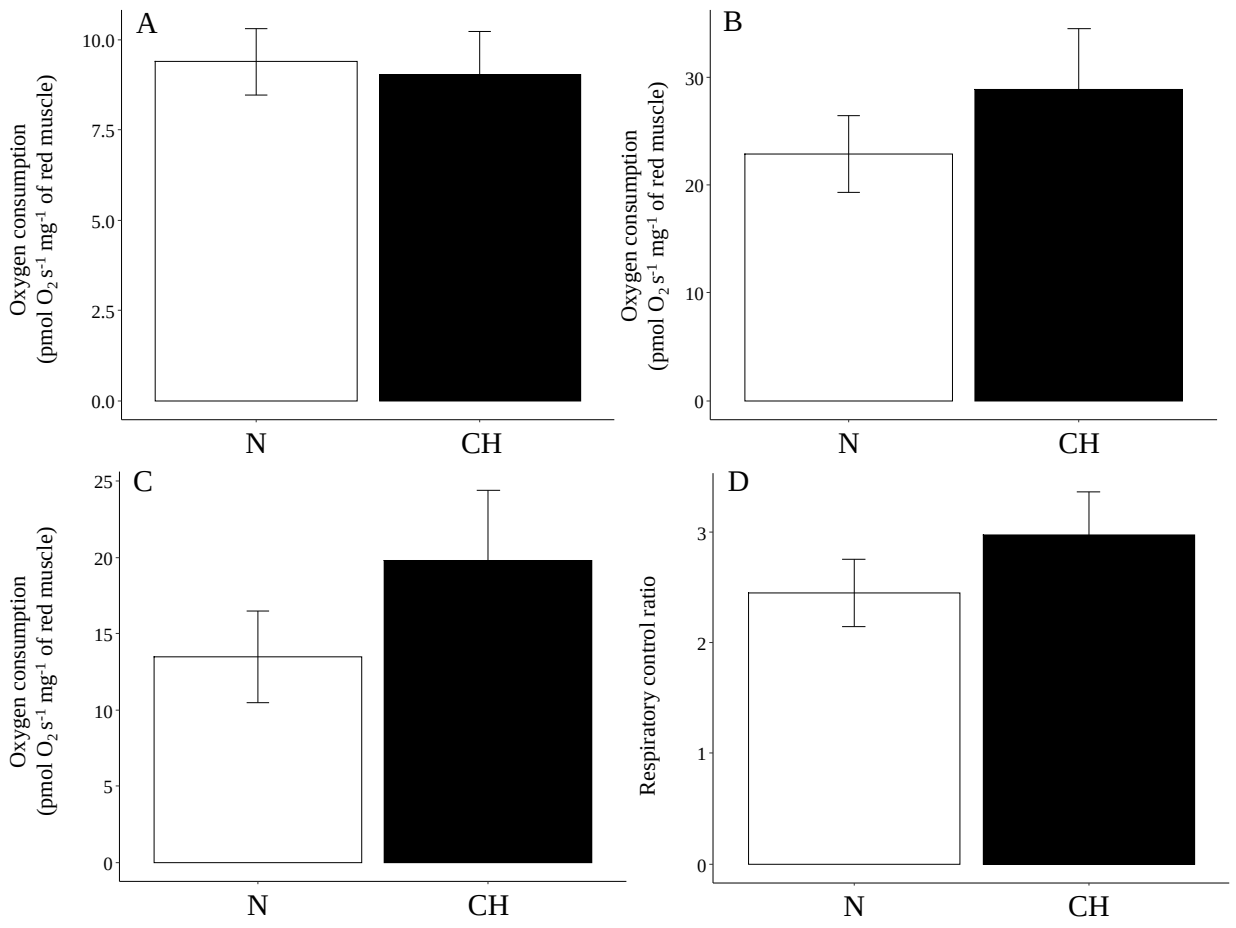


Fig. S2. **Mitochondrial parameters assayed in fish (N = 10 per treatment) acclimated to normoxia (N) or constant hypoxia (CH; 10% air saturation) for 10 days before the assays**. Different parameters are represented: LEAK respiration (A) obtained after inhibiting the electron transport system with oligomycin; OXPHOS respiration (B) after having added pyruvate, malate, glutamate, ADP and succinate; cellular aerobic scope (CAS; C) by removing the LEAK respiration to the OXPHOS respiration and the respiratory control ratio (RCR; D) by dividing the OXPHOS respiration by the LEAK respiration. No effect of treatment was found on any parameter ($P > 0.05$).

Table S1. Mitochondrial respiration measurements of red muscle fibres without substrates ("Fibre"), after adding antimycin A and the statistical results of their comparison (n = 7 – 10).

	SN		SIH		SCH		TN		TIH	
Substrate	Glycolytic	Lipidic	Glycolytic	Lipidic	Glycolytic	Lipidic	Glycolytic	Lipidic	Glycolytic	Lipidic
Fibre	0.71 ± 0.36	0.72 ± 0.70	1.29 ± 0.43	1.30 ± 0.45	0.84 ± 0.43	1.54 0.37	1.14 ± 0.40	1.36 ± 0.36	0.35 ± 0.41	0.40 ± 0.29
Antimycin A	2.55 ± 0.47	0.75 ± 0.37	2.16 ± 0.55	0.93 ± 0.25	1.22 ± 0.56	0.01 ± 0.37	2.25 ± 0.30	0.66 ± 0.39	1.29 ± 0.54	0.30 ± 0.28
Statistical analysis	P = 0.012	n.s.	n.s.	n.s.	n.s.	n.s.	P = 0.045	n.s.	n.s.	n.s.

Table S2. Parameters of the linear mixed models used in the study. Except for the MO_2 for which groups and hours were considered as fixed effect, the rest of the results are those of the analyses having as fixed effects the treatment (training or not) and the acclimation condition (normoxia, intermittent or constant hypoxia). When the interaction between treatment and acclimation condition was not significant, the analysis was refined to look at the effect of acclimation condition on the sedentary groups (SN, SIH and SCH) on the one hand and the effect of training and intermittent hypoxia on the SN, SIH, TN and TIH groups on the other. The effects shown are those of the first model, and then of the most simplified models.

Click here to download Table S2