Comparative Biochemistry and Physiology, Part A 297 (2024) 111734

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Hypoxia-acclimation adjusts skeletal muscle anaerobic metabolism and burst swim performance in a marine fish

Benjamin Negrete Jr [a,b,*], Kerri Lynn Ackerly [a], Andrew J. Esbaugh [a]

[a] Marine Science Institute, The University of Texas at Austin, Port Aransas, TX 78373, USA
[b] Department of Zoology, The University of British Columbia, Vancouver, BC V6T 1Z4, Canada

ARTICLE INFO

Edited by Michael Hedrick

Keywords:
Respiratory physiology
Red muscle
White muscle
Glycolysis
Swim tunnels

ABSTRACT

Red drum, *Sciaenops ocellatus*, are a marine teleost native to the Gulf of Mexico that routinely experiences periods of low oxygen (hypoxia). Recent work has demonstrated this species has the capacity to improve aerobic performance in hypoxia through respiratory acclimation. However, it remains unknown how hypoxia acclimation impacts anaerobic metabolism in red drum, and the consequences of exhaustive exercise and recovery. Juvenile fish were acclimated to normoxia ($n = 15$, DO 90.4 ± 6.42 %) or hypoxia (n = 15, DO 33.6 ± 7.2 %) for 8 days then sampled at three time points: at rest, after exercise, and after a 3 h recovery period. The resting time point was used to characterize the acclimated phenotype, while the remaining time points demonstrate how this phenotype responds to exhaustive exercise. Whole blood, red muscle, white muscle, and heart tissues were sampled for metabolites and enzyme activity. The resting phenotype was characterized by lower pH_e and changes to skeletal muscle ATP. Exhaustive exercise increased muscle lactate, and decreased phosphocreatine and ATP with no effect of acclimation. Interestingly, hypoxia-acclimated fish had higher pH_e and pH_i than control in all exercise time points. Red muscle ATP was lower in hypoxia-acclimated fish versus control at each sample period. Moreover, acclimated fish increased lactate dehydrogenase activity in the red muscle. Hypoxia acclimation increased white muscle ATP and hexokinase activity, a glycolytic enzyme. In a gait-transition swim test, hypoxia-acclimated fish recruited anaerobic-powered burst swimming at lower speeds in normoxia compared to control fish. These data suggest that acclimation increases reliance on anaerobic metabolism, and does not benefit recovery from exhaustive exercise.

1. Introduction

Hypoxia, or low oxygen (O_2), is a critical stressor impacting marine and aquatic environments that has many downstream implications for marine vertebrates (Breitburg et al., 2018). Hypoxia is argued as the most threatening of "the big trio" (including acidification and warming) because it affects more life processes by limiting the generation of energy as ATP (Sampaio et al., 2021). Hypoxic events can even cause changes in population-level fish assemblages to favor those that have mechanisms to counteract hypoxic limitations (Deutsch et al., 2015; Kang et al., 2021). Fishes mitigate the impacts of hypoxia through respiratory adjustments that counteract aerobic limitations by upregulating O_2 binding and delivery mechanisms (Gamperl and Farrell, 2004; Rummer and Brauner, 2011; Claireaux and Chabot, 2016; Negrete Jr et al., 2022). Constraints on aerobic ATP may also be mitigated by concurrent changes in anaerobic metabolism to supplement ATP production. Understanding the balance of ATP production the metabolic processes that make up the ATP pool, and how organisms like fish use ATP at the whole animal level is becoming a more important focus of research as chronic hypoxic events become more commonplace in the world's ocean and impact more species (Breitburg et al., 2018).

The aerobic pathway for efficiently generating ATP involves the breakdown of carbohydrates, amino acids, or fatty acid substrates by the TCA cycle for subsequent oxidative phosphorylation. Both carbohydrate and fatty acid substrates enter the TCA as acetyl-CoA and are substrates for citrate synthase in the TCA cycle, while amino acids can enter the TCA cycle as α-keto acids following deamination. In the absence of O_2, hypoxia tolerant fish can decrease aerobic ATP demand, or upregulate ATP-generating pathways through substrate level phosphorylation (i.e. anaerobic pathways; Mandic et al., 2013). One key anaerobic pathway is

* Corresponding author at: Department of Zoology, The University of British Columbia, Vancouver, BC V6T 1Z4, Canada.
E-mail address: bnegrete@zoology.ubc.ca (B. Negrete).
@postalben (B. Negrete), @KerriAckerlyPhD (K.L. Ackerly)

https://doi.org/10.1016/j.cbpa.2024.111734
Received 17 June 2024; Received in revised form 26 August 2024; Accepted 28 August 2024
Available online 30 August 2024
1095-6433/© 2024 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

glycolysis, the breakdown of glycogen/glucose. Glycolysis is initiated either by the irreversible phosphorylation of glucose via the enzyme hexokinase or the hydrolyzation of glycogen to produce glucose 6-phosphate. The end product of glycolysis is pyruvate, which can be converted to acetyl-CoA and enter the aerobic pathway or it can enter an anaerobic pathway where it is transformed to lactate via lactate dehydrogenase. A final pathway to generate ATP is via the hydrolysis of phosphocreatine (PCr) via creatine kinase, which passes a high energy phosphate from PCr to ADP to quickly generate ATP during bouts of exercise (Wood, 1991; Van den Thillart and Verbeek, 1991; Dalla Via et al., 1997; Hallman et al., 2008; Genz et al., 2013).

Fish exposed to acute hypoxia experience limitations on aerobic ATP production and thus supplement ATP demand by increasing the contribution of anaerobic metabolism. These shifts can be detected by increases in anaerobic substrates such as tissue lactate and glucose (Omlin and Weber, 2010). Severe, chronic hypoxia also stimulates lactate generation but to a lesser degree (Dalla Via et al., 1997), suggesting that as fish acclimate to low O_2 they also upregulate pathways to mitigate the lactate burden. Any extra lactate load must be cleared through the recycling of lactate to restore glycogen (Moyes et al., 1992; Richards et al., 2002; Suski et al., 2006) or oxidized via the reversible reaction catalyzed by lactate dehydrogenase to generate pyruvate (Van den Thillart and Verbeek, 1991; Wood, 1991; Kam and Milligan, 2006). The recycling of lactate to pyruvate can serve as a useful oxidative substrate to fuel energetic costs in some aerobic tissues (i.e., the heart or red muscle) or power low-speed swimming (Van den Thillart and Verbeek, 1991; Weber et al., 2016).

Metabolic adjustments following environmental or exercise stress are tissue-specific and vary with the magnitude and duration of stressful events (e.g. acute or chronic) (van Ginneken et al., 1995; Lushchak et al., 1998; van Ginneken et al., 1999; Speers-Roesch et al., 2013; Borowiec et al., 2018). These tissue-specific responses between aerobic and anaerobic metabolism can particularly be studied in the distinct skeletal muscle types (white and red muscle) of some bony fish. White muscle is powered by anaerobic metabolism because it lacks sufficient capillarization throughout its fibers to deliver O_2 (Richards et al., 2002) and is generally used to power quick, burst swimming movements. Red muscle is dominated by aerobic metabolism because it is rich in mitochondria, and has access to oxygenated red blood cells. Thus, red muscle is prioritized in routine movement (He et al., 2013). Acclimation to chronic hypoxia can alter the size of skeletal muscle fibers (Rossi et al., 2018), increase capillary contact (Johnston and Bernard, 1982), and change metabolic enzyme content (Chen et al., 2013). At the whole-animal level, changes in these distinct muscle types can alter the burst-and-coast swimming pattern seen in most fish, where coasting is prioritized at low speeds and burst activity is recruited to achieve higher speeds, or sudden responses such as ambush feeding and escape responses (Richards et al., 2002; Hechter and Hasler, 2019). Because these muscles have differences in their reliance on O_2, they may be differentially affected by O_2 limitations (Forgan and Forster, 2010) with the potential to affect the dominant mode of swimming (Johnson et al., 1994; van Ginneken et al., 1999; Dutil et al., 2007; Johansen and Esbaugh, 2017).

Red drum, *Sciaenops ocellatus*, are a marine teleost endemic to habitats known to experience prolonged hypoxia in the Gulf of Mexico. Importantly, red drum have demonstrated a hypoxic phenotype when acclimated to oxygen saturations near their critical oxygen tension, P_{crit} (~30 % dissolved oxygen; Pan et al., 2017). This phenotype shows beneficial respiratory adjustments that increase hemoglobin-O_2 binding at the gill, red muscle mitochondrial efficiency, maximum metabolic rate in hypoxia, critical swim speed in normoxia, and decreases in hypoxia vulnerability (Pan et al., 2017; Dichiera et al., 2022; Negrete Jr et al., 2022; Ackerly et al., 2023). We were interested in further exploring this phenotype by characterizing how chronic hypoxia acclimation impacts the anaerobic metabolic processes that supplement ATP production, and are intrinsic to exhaustive exercise and recovery. Specifically, we sought to test the hypotheses that the respiratory plasticity exhibited by red drum following hypoxia acclimation would be augmented by improved anaerobic processes. Further, we hypothesized that increases in anaerobic processes would benefit the whole-animal as indicated by exercise metabolite profiles following exhaustive exercise and recovery, and that hypoxia-acclimated fish would exhibit a greater capacity for anaerobic performance as determined by burst swim tests. For this study we acclimated juvenile red drum to hypoxia similar to previous studies (Negrete Jr et al., 2022; Dichiera et al., 2022; Martin et al., 2023) which characterized the aerobic phenotype, exercise performance, swim performance, and recovery in the juvenile life stage of these fish.

2. Methods

2.1. Husbandry

Fish were obtained from a local aquaculture company (Ekstrom Aquaculture LL, Palacios, TX) and held at the University of Texas at Austin Marine Science Institute in Port Aransas, TX for at least two months prior to acclimations. Holding tanks consisted of a recirculating system with biofiltration, aeration and temperature control. Water quality was monitored and fish were fed daily ad libitum (Purina Animal Nutrition, LLC, USA). Protocols and procedures were carried out in accordance with the UT Institutional Animal Care and Use Committee (AUP-2018-00231).

2.2. Hypoxia acclimation

Juvenile fish were acclimated to normoxia (n = 15, mass: 171 ± 47.4 g, dissolved O_2, DO: 90.4 ± 6.42 %) or hypoxia (n = 15, mass: 161 ± 56.8, DO: 33.6 ± 7.2 %) for 8-days, a level near P_{crit} (~30 % DO) that has been previously been validated to stimulate an acclimated phenotype (Pan et al., 2017; Dichiera et al., 2022; Negrete Jr et al., 2022; Ackerly et al., 2023). Water quality was checked twice daily (24.1 ± 1.2 °C, 30 ppt) and ammonia every other day. Hypoxia was induced and maintained by gently bubbling N_2 through an automated gas controller, Oxy-Reg (Loligo Systems, Viborg, Denmark), which was set to the desired O_2 level and bubbled either air or N_2 to maintain hypoxia. All tanks were covered with a thin layer of plastic to reduce surface O_2 mixing. Fish were fed daily ad libitum, except for 24 h prior to sampling.

2.3. Series I - phenotype determination and response to exhaustive exercise

2.3.1. Sampling

We sampled fish at three time points: at rest, immediately following exhaustive exercise, and 3 h post-exercise (recovery). The exhaustive exercise regime followed a standard chase protocol in hypoxia (DO 37.2 ± 10.2 %, range 26 %–48 %). The DO variability in chasing did not have an impact on any parameters measured, and remained within the range to aerobically challenge and exhaust both control and hypoxia-acclimated fish equally (Ackerly and Esbaugh, 2021; Negrete Jr et al., 2022). The day before sampling, fish used to characterize the resting phenotype were moved into isolated boxes on a wet table that circulated the same water (control or hypoxic) as their respective acclimation tanks. Prior to sampling, buffered MS-222 anesthetic (250 mg L^{-1}; 500 mg L^{-1} $NaHCO_3$) was poured into the boxes and fish were removed when gill movement ceased. Recovery fish were placed in these same boxes for 3 h immediately following exhaustive exercise and similarly sampled. All fish recovered in hypoxic waters to specifically explore the potential benefits of acclimation on exhaustive exercise recovery in hypoxia. Exercise fish were exposed to an overdose of buffered MS-222 (500 mg L^{-1}; 500 mg L^{-1} $NaHCO_3$) immediately following the exhaustive exercise protocol.

During blood sampling anesthetized fish had gills irrigated with

water dosed with anesthetic which has shown to minimize sampling stress and produce metabolite and blood chemistry similar to catheterized fish (Montgomery et al., 2019; Negrete Jr et al., 2022; Davison et al., 2023). Blood was collected from the caudal vein into heparinized syringes and then fish were euthanized in an overdose of buffered MS-222 (500 mg L^{-1}; 500 mg L^{-1} $NaHCO_3$) followed by spinal transection. Whole-blood was collected and a subsample was centrifuged at 12,000 x*g* for 2 min to determine hematocrit. The remaining whole-blood was centrifuged at 10,000 x*g* for 3 min to separate red blood cells (RBC) and plasma. Immediately after separation plasma was measured for extracellular pH (pH_e) using a micro-pH meter (Accumet), and then snap-frozen in liquid N_2 for later analyses. RBC were aliquoted for measurements of hemoglobin (Hb), organophosphates, and intracellular pH (pH_i). pH_i, was measured following a freeze-thaw of the RBC (Zeidler and Kim, 1977) using a micro-pH meter. White muscle, red muscle, and the heart were simultaneously sampled and snap-frozen for metabolite and enzyme measurements. All sampling was completed within 5 min and consistent across groups.

2.3.2. Blood parameters

For Hb concentration, [Hb], and mean corpuscular Hb concentration (MCHC, the amount of Hb per RBC), we used a standard Drabkin's reagent and measured absorbance at 540 nm and applied an extinction coefficient of 11 mM cm^{-1}. Organophosphates (i.e., ATP, GTP; collectively NTP) for RBC were measured using a spectrophotometric assay (Bergmeyer and Bergmeyer, 1985). RBC were deproteinized with 3 % $HClO_4$ then centrifuged at 20,000 x*g* for 5 min at 4 °C. The supernatant was neutralized with 3 M Tris Base before being spun again. The supernatant was placed in a 96-well plate in assay buffer (57 mM triethanolamine hydrochloride, 14 mM K_2CO_3, 2.31 mM $MgSO_4$, 0.61 mM EDTA, 0.18 mM phosphoglyceric acid) and incubated for 15 min before initial absorbance. 3-phosphoglycerate kinase (PGK) solution was added (0.61 mM EDTA, 0.18 mM phosphoglyceric acid, 0.03 U PGK, 1.94 mM NADH, 1 U GAPDH) and the reaction incubated for 45 min and before measuring the final absorbance. The oxidation of NADH to NAD^+ was measured by the change in absorbance at 340 nm and is proportional to the amount of NTP. PGK is non-selective and reacts with ATP, ITP, and GTP. NTP concentration, [NTP], is presented in mM and is normalized to [Hb] (Weber and Lykkeboe, 1978; Val, 2000), as it is an allosteric modulator of Hb that negatively impacts Hb-O_2 affinity (Nikinmaa, 2001). Absorbances of [NTP] were compared to a standard curve on each plate.

Plasma osmolarity was measured in triplicate using an osmometer (VAPRO, Wescor). For plasma lactate concentration ([lactate]), plasma was deproteinized using 6 % $HClO_4$, mixed, and centrifuged at 10,000 x*g* for 10 min. The supernatant was neutralized using 2.5 M K_2CO_3 and re-centrifuged. The [lactate] was determined using an endpoint spectrophotometric method for detecting the reduction of NAD^+ to NADH in a glycine buffer (0.2 M glycine, 0.07 M hydrazine sulphate, 2.5 mM NAD^+, and 25 U LDH, pH 9.2). Reactions were incubated for 1 h to completion at 37 °C (Lowry and Passonneau, 1972; Gutmann and Wahlfefeld, 1974). The final absorbance of the supernatant was read in triplicate at 340 nm, and each plate contained a standard curve for normalizing absorbances.

2.3.3. Tissue metabolites

Frozen tissue was ground to powder using a mortar and pestle over liquid nitrogen. Ground, frozen tissue was weighed and deproteinized using 70 % $HClO_4$. Subsequent slurries were centrifuged for 5 min at 1000 x*g* and the supernatant moved to a new tube and weighed. A base neutralizing solution (2 M KOH, 0.4 M KCl, 0.3 M Imidazole) was added to the supernatant and 1 μL was tested on a pH strip to ensure neutral pH (pH 7–8). Samples were vortexed, and centrifuged as above to obtain a final supernatant that was aliquoted and frozen at −80 °C for tissue [lactate], [ATP], and [PCr]. All samples for metabolite concentrations were run on a Spectra Max spectrophotometer, and each plate contained a corresponding standard curve to normalize well values.

Tissue [lactate] was determined by the reduction of NAD^+ to NADH in a hydrazine solution with NAD^+ (190 mM hydrazine, 0.95 mM NAD^+). Background absorbance was measured before adding 1 U lactate dehydrogenase. The reaction was incubated at room temperature for 4 h to completion and read at 340 nm in triplicate (Lowry and Passonneau, 1972; Gutmann and Wahlfefeld, 1974).

[ATP] was measured as the breakdown of glucose-6-phosphate in the presence of hexokinase and glucose-6-phosphate dehydrogenase (G6PDH). An aliquot was pipetted onto a 96-well plate in a buffer (1 mM dextrose, 0.5 mM NAD^+, and 3 mM $MgCl_2$) for initial absorbance. Hexokinase (0.15 U) was quickly added to each well and incubated for 30 min at room temperature before final measurements at 340 nm. [PCr] was measured in the same assay by adding additional buffer to each well and reading a new initial absorbance at 340 nm. A 0.25 M glycine buffer with 3 mM ADP and 87.5 U mL^{-1} of creatine kinase was added. The reaction was incubated for 1 h at room temperature before final measurements in triplicate (final well concentrations: 0.86 mM dextrose, 0.43 mM NAD^+, 2.58 mM $MgCl_2$, 2.58 mM ADP, 0.09 U creatine kinase). Concentrations for ATP, PCr, and lactate are presented as μmol per gram of wet tissue (μmol g^{-1}) (Lowry and Passonneau, 1972).

Glycogen was measured following the methods of (Hassid and Abraham, 1957). Glycogen concentration, [glycogen], was determined on a separate sample of frozen tissue (500–2000 mg). The tissue was dissolved in 30 % KOH in a hot water bath before the addition of saturated Na_2SO_4 and 95 % ethanol. The solution was brought to a boil, and cooled to room temperature before centrifugation at 1600 x*g* for 10 min. The supernatant was discarded, and the pellet was returned to the water bath to evaporate excess ethanol. The pellet was resuspended in distilled water, and then 95 % ethanol was added. The solution was boiled and cooled again, followed by centrifugation for 10 min at 1600 x*g*. The supernatant was discarded, and the pellet returned to the water bath to evaporate excess ethanol. The pellet was resuspended in distilled water and used for the spectrophotometric assay. The aliquot was incubated for 2 h at 37 °C in acetate buffer (119 mM acetate, 83.5 mM glacial acetic acid, pH 4.8) and amyloglucosidase (Sigma). The reaction was stopped by adding 70 % $HClO_4$, and neutralized with 3 M K_2CO_3. The resulting solution was centrifuged, and the supernatant assayed for free glucose.

Supernatants were read at 340 nm in an enzyme buffer (final well concentration: 250 mM triethanolamine hydrochloride, 5.12 mM magnesium sulfate, 145 U glucose-6-phosphate dehydrogenase, 2.5 mM NAD^+, and 11 mM ATP). 0.2 U hexokinase was quickly added and reactions were incubated for 15 min at room temperature before final absorbance. Samples were run in triplicate, and [glycogen] normalized to mg of tissue (nmol mg^{-1}).

2.3.4. Enzyme activity

Tissue samples for enzyme activity were powdered over liquid nitrogen with a mortar and pestle, and 15–20 mg of tissue was homogenized in 500 μL of homogenization buffer (5 mM EDTA, 50 mM HEPES, 0.1 % Triton X-100, pH 7.4). Slurries were centrifuged at 4 °C at 10,000 x*g* for 2 min, and to prevent multiple freeze-thaw cycles and minimize loss of activity the supernatant was pipetted into separate aliquots for lactate dehydrogenase (LDH), hexokinase (HK), and citrate synthase (CS) activity. Enzyme assays were performed following previously established protocols (Mandic et al., 2013; Galli et al., 2013) and originally described in Bergmeyer (1983).

LDH was measured in an assay buffer of 0.8 mM NADH and 50 mM Tris, pH 7.4. Background absorption at 340 nm was measured for 10 min before the addition of 2.5 mM pyruvate in each well. Final absorption was measured for 10 min and corrected for background absorbance. HK was measured in a buffer of 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM glucose, 0.16 mM NAD^+, 2 U G6PDH. Background absorption at 340 nm was measured for 10 min before the addition of 1 mM ATP in each well. Final absorption was measured for 10 min and corrected for background absorbance. Both LDH and HK activity were calculated using an extinction coefficient of 6.22 mM cm^{-1}.

CS activity was measured in a buffer of 0.30 mM acetyl-CoA, 0.15 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 50 mM Tris, pH 7.4. The reaction was initiated by quickly adding 0.5 mM oxaloacetate. Final absorption was measured for 10 min and corrected for background absorbance. Activity was measured as the change in absorbance at 412 nm and calculated with an extinction coefficient of 13.6 mM cm^{-1}.

All enzyme activity was measured in triplicate, and all assays were run at 25 °C. Protein abundance of respective samples was determined using a standard Bradford assay and albumin standards (ThermoFisher) following enzyme assays. All enzyme activities were normalized to mg of protein per g of tissue (μmol min^{-1} mg^{-1} protein).

2.4. Series II - burst activity

A second group of juvenile drum ($N = 30$, mass 55.0 ± 1.29 g) were acclimated to either control normoxia ($n = 15$, mass 54.0 ± 1.39 g, DO: 99.9 ± 0.3 %) or hypoxia treatment (n = 15, mass 56.0 ± 2.20 g, DO: 31.8 ± 0.3 %) as in *Series I*. Fish of both treatments went through a gait-transition burst swim trial in a 30 L swim tunnel respirometer (Loligo Systems, Viborg, Denmark). We used a cross design wherein fish from both treatments were swam in either normoxic (96.7 ± 0.45 %) or hypoxic (32.2 ± 0.28 %) water. This resulted in the following sample sizes: control swam in normoxia, $n = 7$; control swam in hypoxia, $n = 8$; hypoxia swam in normoxia, n = 7; and hypoxia swam in hypoxia, $n = 8$. All trials were performed at the same temperature and salinity as the acclimation conditions (23.8 ± 0.06 °C, 31.7 ± 0.48 ppt). Fish were moved from their acclimation tanks into the swim tunnels where they rested overnight in their respective acclimation DO at a speed of 0.5–0.75 body lengths per second (BL s^{-1}). For fish that swam in the opposite DO of their acclimation, the DO was changed over a 1 h period before the burst trial. Normoxia was maintained by bubbling air through an air stone, and hypoxia was established and maintained using an automated gas controller, Oxy-Reg (Loligo Systems, Viborg, Denmark). The swim tunnels were covered with a layer of plastic except for a window directly above the working area (46x14x14 cm) for video recording.

The flow velocity of two swim tunnels were calibrated using a digital flow meter (Hontzsch, Germany), and solid blocking effects from the fish's cross-sectional area were corrected according to Bell and Terhune (1970). Fish swam for 25 s at increasing increments of 0.25 BL s^{-1}. Trials concluded when the fish could no longer maintain its position in the swim tunnel, or could not swim off the back gate. Videos for the swim trials were blinded, and bursts manually counted at each swim interval. The total number of bursts for each fish was counted, as well as the final speed interval fish completed (U_{max}). An estimation of the transition from aerobic to anaerobic swimming was designated when fish would perform at least 4 bursts in one interval (Killen et al., 2015), indicating a reliance on anaerobic, burst swimming (observed U_{burst}). This threshold documented in Killen et al., 2015 was used in swimming increments of 1 min as opposed to the shorter intervals used here (25 s). Thus, to get a precise measurement of the speed at which 4 bursts (modeled U_{burst}) would appear we objectively modeled bursts using the swimming equation typically used for maximum swim speed, U_{crit}. This is an exponential regression equation fitted by least squares to the bursts versus swimming speed for individual fish:

$$Bursts = a + bU^{c}$$

where U is the swim speed (BL s^{-1}) and a, b, and c are estimated unitless constants from the fitted model (Claireaux et al., 2006). Fitted lines were used to calculate U where 4 bursts occur.

2.5. Statistical analyses

All data were tested in R for homogeneity of variance using Levene's tests, and residuals tested for normality using a Shapiro-Wilkes test. Data were log transformed (plasma lactate, heart PCr, red muscle glycogen, white muscle PCr and CS), square root transformed (red muscle lactate). Or square transformed (white muscle lactate) as needed, and any data that failed these assumptions was tested using a non-parametric Wilcox test ([NTP], and NTP:MCHC ratio). The anaerobic phenotype induced by hypoxia acclimation in *Series I* was tested using a two-tailed Student's *t*-test ($\alpha = 0.05$) between fish at rest. Cohen's D effect size for *t*-tests were calculated where 0.2, 0.5, and 0.8 indicate small, medium, and large effect sizes, respectively.

Exercise and recovery differences were tested and analyzed separately, since all fish went through the exercise regime and recovery in hypoxic waters. Thus, the exercise and recovery time points were analyzed using a two-way ANOVA ($\alpha = 0.05$) with acclimation and time point as main effects using the packages *afex* (Singmann et al., 2015) and *emmeans* (Lenth et al., 2018). ANOVA effect sizes (η^2) were calculated where 0.02, 0.13, and 0.26 indicate small, medium and large effect sizes, respectively.

For *Series II,* burst activity was analyzed using a two-way ANOVA ($\alpha = 0.05$) with acclimation and swim tunnel DO as main effects. When an interaction was detected, we investigated differences using Tukey post-hoc tests.

3. Results

Detailed statistical outputs for all tests are presented in Tables S1 (blood and metabolites), S2 (enzymes), and S3 (swim trials).

3.1. Series I – hypoxic phenotype

Hct was ~30 % higher in hypoxia-acclimated fish, and was accompanied by an increase in [Hb] (Table 1, $p < 0.01$ and $p = 0.02$, respectively). pH_e was lower in hypoxia-acclimated fish (Fig. 1, $p = 0.04$), but pH_i was unaffected by treatment (Fig. 1, $p = 0.11$). There were no differences in other blood parameters (plasma lactate, osmolarity, MCHC, [NTP], or NTP:MCHC ratio) ($p > 0.05$ for all, Table 1).

There was no effect of acclimation on any metabolites in the heart ($p > 0.05$ for all, Fig. 2). Hypoxia-acclimated fish had less red muscle [ATP] ($p = 0.02$), and [PCr] ($p = 0.05$) compared to control fish (Fig. 3B-C). There was higher white muscle [ATP] in hypoxia-acclimated fish compared to control ($p = 0.03$, Fig. 4B). All other metabolites in both red and white muscle were similar regardless of acclimation ($p > 0.05$, Fig. 3–4).

Hypoxia-acclimation increased LDH activity in red muscle ($p = 0.03$, Fig. 5A), and HK activity in white muscle ($p = 0.02$, Fig. 5B), but decreased HK activity in the heart ($p = 0.04$, Fig. 5C). LDH activity did not differ between treatments in the white muscle or heart. There was no change in HK activity between treatments in red muscle, and CS activity did not differ as a result of acclimation in any tissue ($p > 0.05$ in all cases, Fig. 5C).

3.2. Acclimation response to exhaustive exercise

After exhaustive exercise both control and treatment fish had similar osmolarity ($p = 0.11$, Table 1). Control fish increased osmolarity during recovery compared to exercise, while hypoxia-acclimated fish decreased osmolarity during the recovery period ($p < 0.01$, Tukey's post-hoc; Table 1). Hb, MCHC, [NTP], and NTP:MCHC were higher following exercise compared to the recovery time point ($p < 0.05$, for all) but did not differ between control or hypoxia-acclimated fish ($p > 0.05$, Table 1). Hct showed no difference between acclimation, or time point ($p = 0.76$, and $p = 0.44$, respectively). Plasma lactate was similar between acclimation treatments following exhaustive exercise ($p = 0.43$), and in both treatments plasma lactate increased during recovery ($p < 0.01$, Table 1). pH_e was lower following exhaustive exercise than during recovery ($p < 0.01$, Fig. 1) for both groups of fish, while pH_i was similar across time points ($p = 0.65$). Hypoxia-acclimated fish maintained

Table 1
Blood parameters for fish acclimated to 8-day normoxic control or hypoxia. Data are mean ± s.e.m. A *t*-test was used for comparisons between resting phenotypes and differences noted with (*). Fish were exhaustively exercised and recovered in hypoxia. These data were analyzed using a two-way ANOVA. (†) indicates a main effect of acclimation, and (‡) a main effect of time point. Different letters indicate differences from Tukey's post-hoc test following a detection of an interaction. NTP = organophosphates, Hb = hemoglobin, MCHC = mean corpuscular hemoglobin concentration.

Condition	Acclimation	Plasma Osmolarity (mOsm)	Hematocrit (% RBC)	[NTP]‡ (mM)	MCHC‡	$Hb_4^‡$	NTP:MCHC‡	Plasma [Lactate]‡ (mM)
Rest	Control	346 ± 3.51	23.0 ± 1.13	0.72 ± 0.39	7.45 ± 1.08	0.43 ± 0.07	0.12 ± 0.07	0.48 ± 0.12
	Hypoxia	353 ± 7.45	29.8 ± 1.11*	0.77 ± 0.41	9.70 ± 0.69	0.73 ± 0.07*	0.08 ± 0.04	1.07 ± 0.41
Exercise	Control	388 ± 3.81[a]	33.5 ± 1.06	1.95 ± 0.34[a]	9.70 ± 0.78	0.81 ± 0.07	0.21 ± 0.04[a]	15.2 ± 1.06
	Hypoxia	395 ± 3.96[a]	34.2 ± 0.60	2.61 ± 0.25[a]	9.88 ± 0.81	0.85 ± 0.08	0.27 ± 0.03[a]	14.9 ± 0.59
Recovery	Control	420 ± 8.09[b]	33.6 ± 1.96	0.88 ± 0.15[b]	8.01 ± 0.57	0.68 ± 0.09	0.11 ± 0.02[b]	36.2 ± 2.53
	Hypoxia	388 ± 12.8[a]	32.2 ± 0.86	0.40 ± 0.06[b]	7.91 ± 1.01	0.64 ± 0.09	0.06 ± 0.01[b]	32.7 ± 4.41

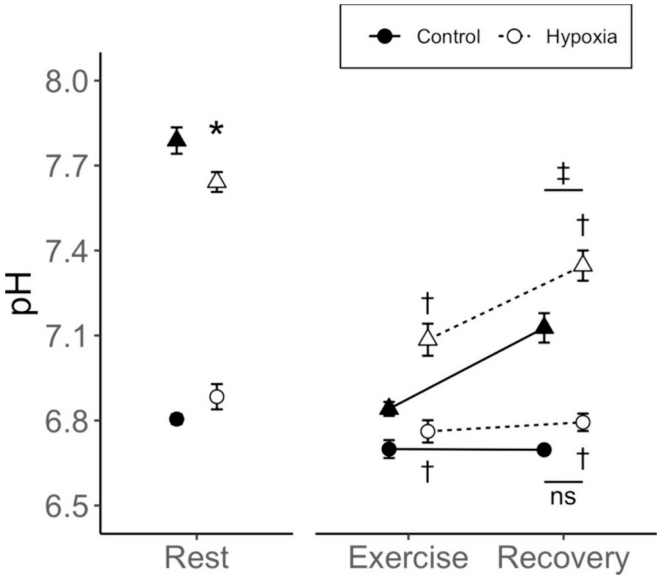


Fig. 1. Measures of pH_e (triangles) and pH_i (circles) in fish acclimated to control normoxia (dark shapes) or hypoxia treatment (open shapes). Data are mean ± s.e.m. (n = 5–6 each group). Fish at rest were sampled from respective acclimation conditions, and tested using *t*-test. Hypoxia acclimated fish had lower pH_e (*), but both groups had similar pH_i ($p > 0.05$) at rest. Fish underwent exhaustive exercise followed by 3 h of recovery in hypoxia, and these data were analyzed independent of rest using a two-way ANOVA. Hypoxia acclimated fish had higher pH_e and pH_i throughout the exercise and recovery regime (†). Both control and hypoxia acclimated fish had higher pH_e during recovery from exercise (‡), but pH_i was similar across exercise and recovery (ns = not significant, $p > 0.05$).

higher pH_e and pH_i than control fish during both exercise and recovery time points ($p < 0.01$ and $p = 0.02$, respectively, Fig. 1).

In the heart, lactate was similar between acclimation treatments ($p = 0.43$), but did show an increase from exercise to recovery ($p = 0.04$, Fig. 2A). Similarly, PCr and ATP were lower following exercise compared to recovery ($p = 0.04$ for both, Fig. 2B-C), but there was no effect of acclimation at any time point ($p = 0.06$, and $p = 0.43$, respectively). There was no difference in heart glycogen due to exercise time point ($p = 0.19$) nor acclimation ($p = 0.62$, Fig. 2D).

Red muscle lactate was higher following exhaustive exercise then decreased during recovery ($p = 0.01$) for both acclimation treatments. ATP was similar after exercise in both control and hypoxia-acclimated fish ($p < 0.01$). Control fish increased ATP during recovery, while hypoxia-acclimated fish maintained low ATP over the same recovery time ($p < 0.01$, Tukey's post-hoc). PCr had no effect of time point ($p = 0.17$) nor acclimation ($p = 0.06$, Fig. 3C). Red muscle glycogen, similarly, did not change as a result of exercise time point nor acclimation ($p = 0.89$ and $p = 0.11$, respectively, Fig. 3D).

White muscle lactate was higher following exercise than during recovery ($p < 0.01$), but was similar between acclimations ($p = 0.39$, Fig. 4A). ATP and PCr were both lower after fish were exercised and increased during recovery ($p < 0.01$ for both) with no effect of acclimation ($p = 0.31$ and $p = 0.12$, respectively, Fig. 4B-C). Interestingly, glycogen did not show statistical differences between time point ($p = 0.47$) nor acclimation treatment ($p = 0.13$, Fig. 4D).

3.3. Series II - burst activity

Fish from both acclimation treatments swam for longer periods in normoxia than in hypoxia ($p < 0.01$, Table 2). The longer normoxic swim trials meant all fish reached higher U_{max} and performed more bursts ($p < 0.01$ and $p = 0.05$, respectively). Hypoxia-acclimated fish performed more bursts compared to control fish in both normoxic and hypoxic trials ($p = 0.01$, Table 2). In hypoxic trials both control and hypoxia-acclimated fish reached an observed U_{burst} at similar speeds (3.78 ± 39 and 3.53 ± 0.28 BL s^{-1}, respectively; Fig. 6, Table 2). In normoxic trials control fish reached an observed U_{burst} at higher speeds (4.32 ± 0.40 BL s^{-1}) compared to hypoxia-acclimated fish, which burst at the same speed as in hypoxic trials (3.43 ± 0.35 BL s^{-1}). The observed U_{burst} was not different between acclimations ($p = 0.12$), nor the DO of the swim trial ($p = 0.55$, Table 2; Fig. 6). However, the modeled U_{burst} found that hypoxia-acclimated fish reached U_{burst} at significantly lower speeds than control ($p = 0.01$, Table 2).

4. Discussion

We sought to understand the anaerobic adjustments made by fish acclimated to chronic sub-lethal hypoxia, and how these fish may use these energy sources during exhaustive exercise and recovery. Red drum have shown evidence of respiratory plasticity to increase their capacity for aerobic metabolism under hypoxia (Pan et al., 2017; Negrete Jr et al., 2022), and the present study shows that anaerobic metabolism is also plastic and augmented to contribute to ATP demand. While the observed plasticity changed the dynamics of anaerobic performance during exercise, there was little evidence that plasticity benefited recovery from exhaustive exercise.

4.1. Hypoxic phenotype

Hypoxia-acclimated fish showed an increase in Hct and Hb but no change in MCHC indicating an overall increase in circulating RBC, a common response in chronic hypoxia (Borowiec et al., 2015; Negrete Jr et al., 2022). Hypoxia-acclimated fish also exhibited modestly lower pH_e, although this did not extend to RBC pH_i, likely owing to red cell β-adrenergic Na^+/H^+ exchange (β-NHE) activity (Nikinmaa, 1982; Salama and Nikinmaa, 1988). It seems likely that the reduction in plasma pH_e is associated with a greater reliance on anaerobic metabolism that results in a shift in the ADP:ATP ratios that would result in a greater proton concentration (Robergs et al., 2004; Robergs, 2019). This

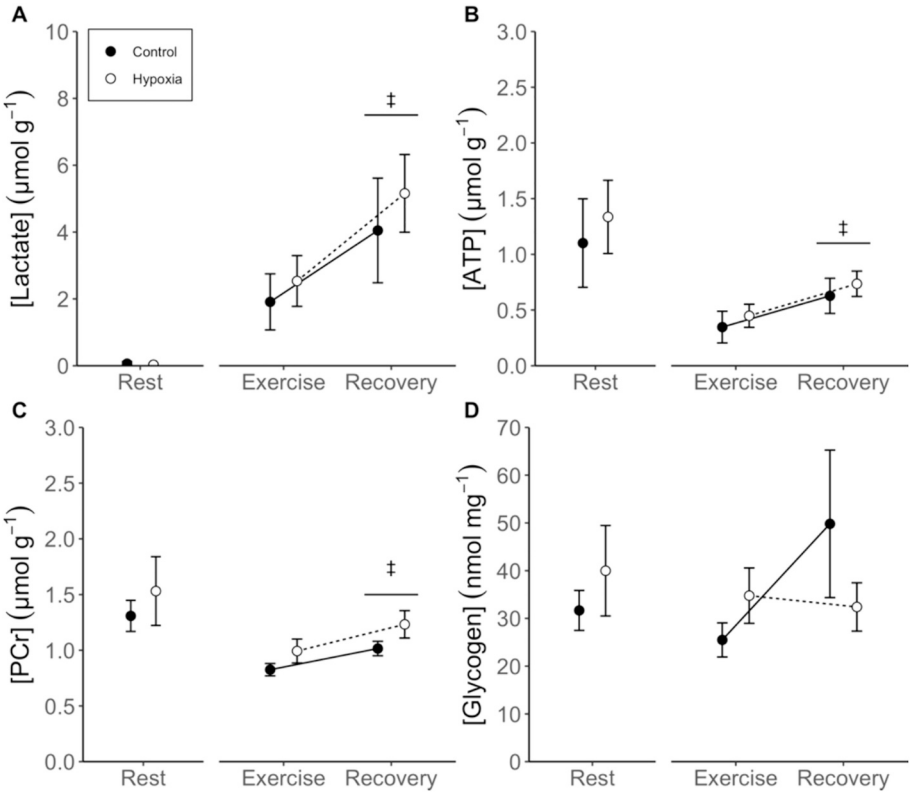


Fig. 2. Metabolite concentrations in the heart for normoxic control (dark circles), and hypoxia-acclimated (open circles). Metabolites include (A) lactate, (B) ATP, (C) phosphocreatine (PCr), and (D) glycogen. Data are mean ± s.e.m. (n = 5–6 each group). Fish at rest are compared with a t-test and sampled from their respective acclimation conditions. Fish then went through exhaustive exercise followed by 3 h recovery in hypoxia. These data were analyzed separately using a two-way ANOVA with exercise regime and acclimation as main effects. During recovery, both control and hypoxia-acclimated fish increased lactate, ATP, and PCr with no difference due to acclimation (‡, $p > 0.05$).

hypothesis is further reinforced by the observations of the red muscle, which is a tissue known to exhibit change in response to environmental O_2 (Johnston and Bernard, 1982; Forgan and Forster, 2010; Rossi et al., 2018). At rest, there was a significantly lower ATP and PCr concentration in the hypoxia-acclimated fish. It seems likely that the lower concentrations of ATP and PCr are the result of a mis-match between consumption rate relative to the replenishment rate owing to the reduced O_2 availability in hypoxia. But the response in ATP and PCr observed here differs somewhat from prior work on acute hypoxia in teleosts, which shows red muscle ATP levels are maintained while PCr concentrations decline (Dalla Via et al., 1997; van Ginneken et al., 1999). This likely indicates that PCr is depleted first in an attempt to supplement ATP before ATP levels decline. Importantly, the glycogen load of the red muscle remained unchanged, which demonstrates that the changes in ATP and PCr are not related to substrate availability.

Red drum red muscle also showed a significant increase in LDH activity following hypoxia acclimation, which may be indicative of increased lactate utilization by the cell. This could either serve the formation of lactate production from pyruvate for the purposes of enhancing glycolytic ATP production (Omlin and Weber, 2010), or an increase in the reverse reaction that would use lactate to generate pyruvate that would be oxidized via aerobic processes (Van Hall, 2000). Given that lactate concentration did not differ between acclimation treatments, our data seem to provide support for the premise that the red muscle is scavenging plasma lactate to use as an oxidative fuel source. Importantly, such a process would still require oxygen. Nonetheless, the overall changes in the red muscle indicate a decrease in the energy available for cellular function (i.e. ATP and PCr), while also indicating a subtle shift in substrate utilization that would capitalize on the anaerobic production of lactate.

Hypoxia acclimation also resulted in subtle changes in the anaerobic capacity of the heart in the form of a significant reduction in HK activity. This enzyme is responsible for phosphorylating glucose as it enters the cell from the blood stream, effectively trapping it in the cell and making it available for glycolysis or glycogenesis. Unfortunately, we did not measure blood glucose in the current study, but it seems unlikely that hypoxia would result in a major change in the availability of glucose that would alter glucose metabolism. Although, acute hypoxia exposure in rainbow trout results in a significant increase in plasma glucose (Haman et al., 1997) that may be scavenged by the heart during circulation, and the armored catfish (*Liposarcus pardalis*) can increase heart glucose accompanied with an increase in heart lactate (Treberg et al., 2007). The current data suggest that the heart is reducing its reliance on glucose for oxidative metabolism, which could relate to an increased reliance on glycogen or plasma lactate as the initial substrates. We did not observe a decline in heart glycogen stores, an increase in heart lactate concentration, nor did we observe an increase in heart LDH activity. However, it is important to note that bulk concentrations should not be confused with substrate flux, and it is possible that that heart is significantly improving the capacity for lactate uptake and that existing levels of LDH are sufficient for lactate turnover. For example, several monocarboxylate transporter isoforms are up-regulated in zebrafish hearts following 48 h to 96 h exposure to hypoxia (Ngan and Wang, 2009). It would be interesting to explore whether similar changes in lactate transporter abundance may occur in red drum acclimated to hypoxia.

The white muscle showed the opposite response to the heart, with a significant increase in HK activity. The increase in HK activity in hypoxia-acclimated red drum is similar to the increases seen in muscle of goldfish (*Carassius auratus*; Farhat et al., 2021) following hypoxia exposure. Increased HK activity can facilitate a glucose gradient that benefits glucose uptake into the muscle (MacCormack and Driedzic, 2007; Wasserman et al., 2011). Indeed, hypoxia tolerant fish can

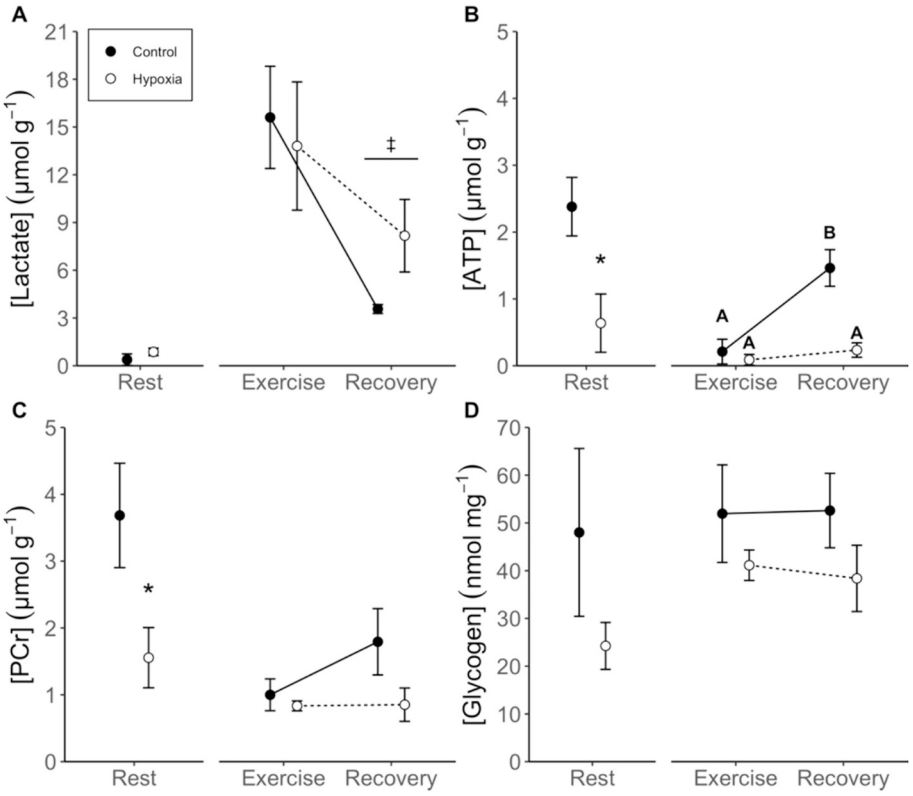


Fig. 3. Metabolite concentrations in the red muscle for control (dark circles), and hypoxia-acclimated (open circles) fish at rest, during exercise, and after three hours of recovery. Metabolites include (A) lactate, (B) ATP, (C) phosphocreatine (PCr), and (D) glycogen. Data are mean ± s.e.m. (n = 5–6 each group). Fish at rest are compared with a t-test and sampled from their respective acclimation conditions. Fish then went through exhaustive exercise followed by 3 h recovery in hypoxia. These data were analyzed separately using a two-way ANOVA with exercise regime and acclimation as main effects. There was lower ATP and PCr in hypoxia-acclimated fish at rest (*; $p < 0.05$). There was an interaction between exercise condition and acclimation on ATP in the red muscle ($p < 0.05$). During exercise, ATP was depleted to near zero, and control fish increased ATP after recovery while hypoxia-acclimated fish had consistently low ATP ($p < 0.05$, Tukey's post-hoc). Different letters indicate statistical differences. Lactate for both groups of fish was higher immediately after exercise than during the 3 h recovery (‡, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increase glucose concentrations accompanied by increases in HK activity in tissues like the heart during hypoxia (Treberg et al., 2007). Changes to tissue glucose are important because physiological concentration and transport of glucose can determine the capacity for glucose metabolism (Clow et al., 2004; Clow et al., 2017). Interestingly, the acclimated white muscle also showed higher concentrations of ATP, which suggests that red drum may be modulating the flux of glucose into the white muscle and increasing energetic reserves. Although, as above, it is important to note that our data should not be confused with direct measurements of glucose flux, which is an intriguing area of future research. Particularly considering that the hypoxia stimulated white muscle glucose uptake does not appear to be a universal trend among fishes (MacCormack et al., 2006; Hall et al., 2009; Farhat et al., 2021).

4.2. Response to exhaustive exercise

We detected a typical exercise-induced stress response in the circulatory system of fish with an increase in osmolarity and decrease in pH compared to recovery. RBC swelling, another common response to exercise, was not directly measured, however, swelling can be indirectly detected by an increase in hct and concomitant decrease in MCHC and hemoglobin (Nikinmaa, 2001). In the present study, we did not find evidence of cell swelling as there was no change in hct or MCHC between rest and recovery. The control fish experienced lower pH_e and pH_i compared to hypoxia-acclimated fish throughout the exercise and recovery regime, indicating that hypoxia-acclimation led to a smaller acid-base disturbance in the blood (Fig. 1). These disturbances may be mitigated in hypoxia-acclimated fish by upregulating protection mechanisms such as β-NHE. β-NHE is also O_2 sensitive and increasingly activated in low pO_2 which results in lower pH_e (Jensen, 2004; Aboagye and Allen, 2018). Alternatively, the smaller pH disturbance could also be partially alleviated by the increase in hemoglobin in hypoxia-acclimated fish (Table 1) as Hb can act as a buffer for excess H^+ at the expense of O_2 binding.

Interestingly, the hypoxia-acclimated fish decreased osmolarity between exercise and recovery, while the control fish showed a continued increase during recovery (Table 1). The changes in osmolarity cannot be fully explained by lactate as both control and hypoxic fish showed continued increase in plasma lactate during recovery. Interestingly, in other fishes increases in plasma lactate levels are attributed to hypoxic conditions (Suski et al., 2006; Borowiec et al., 2015). For example, largemouth bass (*Micropterus salmoides*) recovering from exhaustive exercise in normoxia show plasma and muscle lactate decrease after 2–4 h; however, when these fish recover in hypoxia, they maintain high plasma lactate and osmolarity (Suski et al., 2006). The marked increase in plasma lactate during recovery in red drum is similar to that found by Martin et al. (2023). In that study, plasma lactate peaked at 1 h post-exercise, remained elevated, and did not decrease until 6 h post-exercise. The elevation of plasma lactate during recovery is thus unsurprising in the current study, and is not conclusively attributed to the fact that these fish recovered in hypoxia. The elevated plasma lactate can be used for metabolism in the red blood cells (Wells and Baldwin, 2006), heart, kidney, or gills (Genz et al., 2013), or even supply lactate to the red muscle to work as an oxidative substrate (Kam and Milligan, 2006) which may benefit the hypoxia-acclimated fish that show increased LDH activity (Fig. 5).

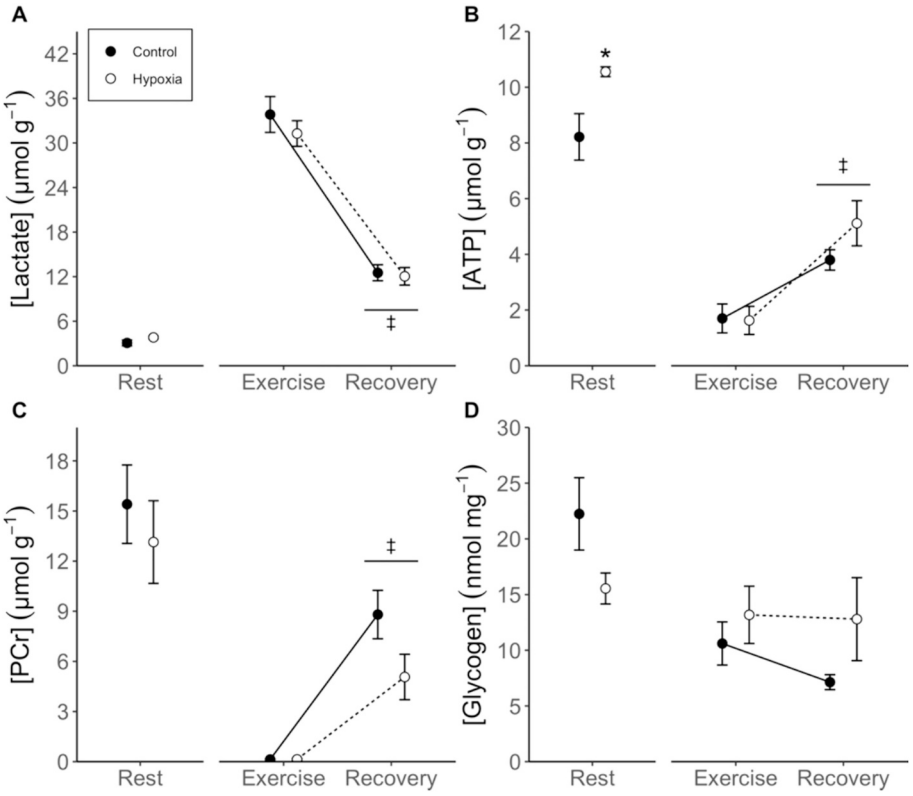


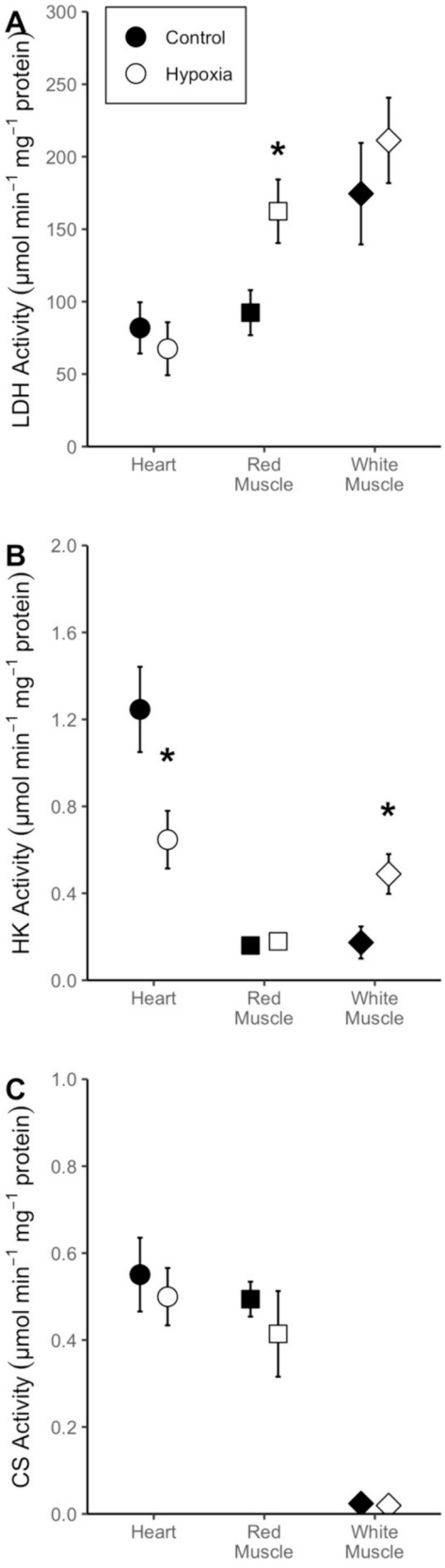
Fig. 4. Metabolite concentrations in the white muscle for normoxic control (dark circles), and hypoxia-acclimated fish (open circles). Metabolites include (A) lactate, (B) ATP, (C) phosphocreatine (PCr), and (D) glycogen. Data are mean ± s.e.m. (n = 5–6 each group). Fish at rest are compared with a t-test and sampled from their respective acclimation conditions. Fish were exhaustively exercised and recovered for 3 h in hypoxia. These data were analyzed separately using a two-way ANOVA with exercise regime and acclimation as main effects. Both groups of fish had higher lactate during exercise than during the 3 h recovery (‡, $p < 0.05$). Hypoxia-acclimated fish had higher ATP at rest compared to control (*, $p < 0.05$). Exercise depleted both ATP and PCr to near zero, which increased during recovery (‡, $p < 0.05$).

The heart showed no metabolite changes due to acclimation, but did follow a typical exercise trend of lowered energetic stores that increased with recovery (Fig. 2). The lack of differences is not surprising as the heart, a primary aerobic organ, prioritizes maintaining function and ATP in sub-lethal stress (Speers-Roesch et al., 2010; Lague et al., 2012; Borowiec et al., 2018; Ackerly et al., 2023). The large continued increase in heart lactate may be a result of circulating lactate from the plasma which shows a similar pattern, or the heart is increasing lactate concentrations for oxidation (Milligan and Girard, 1993).

Both red and white muscle showed low ATP and PCr after exhaustive exercise compared to recovery as expected (Richards et al., 2002; Suski et al., 2006), but the red muscle differed in the recovery profile. Red muscle ATP in control fish increased after exercise, but in hypoxia-acclimated fish concentrations remained low similar to post-exercise (Fig. 3B) indicating a decreased capacity to replenish ATP to higher concentrations. This illustrates an inhibition of the red muscle to power activity such as sustained swimming, particularly if the increase in LDH indicates an increase in anaerobic activity. While we did not find a decrease in CS activity, a common marker of aerobic potential, the subtle shift in LDH and decrease in these key metabolites suggest a potential compromise of red muscle activity and contribution to whole-animal swimming. Interestingly, we did not detect decreases in glycogen in any skeletal muscle typified by swimming fish (Speers-Roesch et al., 2013). This may be due to the type of exercise used in this study – exhaustive exercise via chasing and sprint trials, both powered by anaerobic metabolism. Indeed, during burst sprinting exercise rainbow trout have shown to deplete PCr during bursting exercise and glycogen during sustained swimming (Richards et al., 2002).

4.3. Anaerobic swim performance

The enzyme and metabolite data paint an interesting picture whereby red drum acclimated to hypoxia seem to prioritize glycolytic white muscle over oxidative red muscle, despite the fact that the exposure levels are above the P_{crit} of the species. To assess this more directly, we subjected normoxia and hypoxia-acclimated fish to a gait-transition U_{burst} test, which could quantify the relative contributions of anaerobic and aerobic swim styles. In general, sustained swimming is thought to be dominated by red muscle while burst and coast swimming is dominated by white muscle, and accounts for most swimming at higher speeds (van Ginneken et al., 1999). In hypoxic swim trials, fish from both acclimation treatments transitioned to burst swimming at the same speed, and fish from both acclimations exhibited statistically similar U_{max} values. More importantly, when fish from the normoxic acclimation was subjected to swim trials in normoxia, they conformed to expectations and recruited burst swimming at higher speeds. This is likely the by-product of O_2 availability in hypoxic swim trials impeding sustained swimming via red muscle (Johnson et al., 1994; van Ginneken et al., 1995). In contrast, hypoxia acclimated fish began relying on burst swimming at the same speed regardless of oxygen availability (Table 2, Fig. 6 and S1), and performed more bursts compared to control in both swimming conditions. In short, these results suggest that the phenotypic changes in the red and white muscle following hypoxia acclimation work independent of oxygen availability, whereby hypoxia-acclimated fish have a greater reliance on white muscle for activity. These data also support prior work in red drum that explored aerobic swim performance (U_{crit}) following hypoxia acclimation, and concluded that the observed improvements in U_{crit} were the result of anaerobic contributions, not aerobic contributions (Dichiera et al., 2022). Similar improvements to U_{crit}



(caption on next column)

Fig. 5. Enzyme activity for normoxic control (dark shapes), and hypoxia-acclimated fish (open shapes) in the heart (circles), red muscle (squares), and white muscle (diamonds). Enzymes include (A) lactate dehydrogenase (LDH), (B) hexokinase (HK), and (C) citrate synthase (CS). Fish were sampled during rest from their respective acclimation conditions (n = 5–6 each group). Data are mean ± s.e.m., and tested using *t*-test. Hypoxia acclimated fish had higher LDH activity in red muscle. They also showed decreased HK activity in the heart, and increased in white muscle (*, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

following hypoxia acclimation were observed in goldfish and linked to red muscle function; however, no attempts were made to delineate anaerobic and aerobic swimming (Thoral et al., 2022).

The earlier switch to burst swimming in the hypoxic phenotype may help conserve aerobic energy for vital functions that rely exclusively on oxidative metabolism (Richards et al., 2002). To our knowledge only one study (Atlantic cod, *Gadus morhua*) has investigated gait transition in decreasing O_2 concentrations (Dutil et al., 2007), and only the present study has looked at how hypoxia acclimation impacts U_{burst}. An important aspect of the response in red drum in the context of the current hypoxia acclimation is that there is no evidence of metabolic suppression, as studies have consistently shown no effect of acclimation on standard metabolic rate (Pan et al., 2017; Negrete Jr et al., 2022; Dichiera et al., 2022). This would suggest that the energetic costs of vital functions are not changing; however, it should be noted that metabolic suppression responses can be tissue dependent (van Ginneken et al., 1999; Farhat et al., 2021). Regardless, it seems reasonable to conclude that hypoxia-acclimated red drum have remodeled the respective importance of red and white muscle for exercise with the intent of allowing other tissues to use the limited oxygen that is available.

5. Conclusions

The data provided here provide evidence that red drum exposed to an 8-day hypoxia exposure undertake significant plasticity within their red and white muscle, and to a much lesser degree in the heart, that significantly alters the relative importance of anaerobic vs. aerobic swimming. This was evidenced by the modeled U_{burst} suggesting that hypoxia acclimated fish rely on anaerobic swimming at lower speeds, an overall perform more bursts throughout. The ecological significance of these data are somewhat challenging to ascribe, as observed U_{burst} and U_{max} between treatment groups was not different when the fish were tested in hypoxia. However, the hypoxia acclimated fish seemed to reach higher swim speeds with more bursts, which raises important questions regarding anaerobic swimming efficiency that should be the focus of future work. More importantly, this work contributes to an expanding series of data that suggest that estuarine fishes, like the red drum, are capable of significantly physiological plasticity in response to hypoxia (Pan et al., 2017; Dichiera et al., 2022; Negrete Jr et al., 2022; Ackerly et al., 2023), which must be accounted for when attempting to assess the long-term outlook of species in response to ocean deoxygenation and climate change.

Funding

This material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program under Grant #1610403 for BNJ and Grant #2002549 for AJE. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

CRediT authorship contribution statement

Benjamin Negrete: Writing – original draft, Visualization, Project

Table 2
Results of burst swim trials for normoxic control, and hypoxia-acclimated fish swam in normoxia or hypoxia. Data are mean ± s.e.m., and n = 8 for each group. Data are analyzed using a two-way ANOVA where (†) indicates a main effect of acclimation, and (‡) a main effect of swim trial dissolved oxygen (DO). Differences are significant when $p < 0.05$. U_{max} = maximum speed reached, U_{burst} = speed at which fish rely on burst swimming.

Acclimation	Swim trial DO	Length of trial‡ (s)	Total Bursts†‡	$U^{\ddagger}_{max}$ (BL s^{-1})	Observed U_{burst} (BL s^{-1})	Modeled $U^{\dagger}_{burst}$ (BL s^{-1})
Control	Normoxia	572 ± 40.4	177 ± 20.1	6.50 ± 0.38	4.32 ± 0.40	3.91 ± 0.15
	Hypoxia	401 ± 27.5	118 ± 28.5	4.88 ± 0.26	3.78 ± 0.39	3.27 ± 0.43
Hypoxia	Normoxia	518 ± 31.8	268 ± 46.4	6.00 ± 0.36	3.43 ± 0.35	2.69 ± 0.30
	Hypoxia	470 ± 31.7	198 ± 22.7	5.31 ± 0.28	3.53 ± 0.28	2.74 ± 0.23

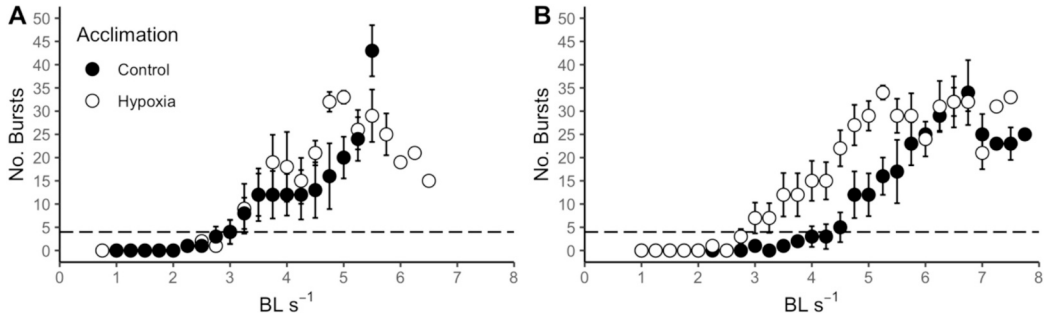


Fig. 6. Burst counts for fish acclimated to normoxic control (dark circles) or hypoxia (open circles). Fish from both groups were swam in either hypoxia (A) or normoxia (B) in increasing speeds in body lengths per second (BL s^{-1}). Data are mean ± s.e.m. (n = 7–8 each group). The horizontal dotted line crosses at 4 bursts as the threshold for when fish increase the proportion of anaerobic burst swimming to aerobic swimming (U_{burst}; Killen et al., 2015). Data above this line indicate reliance on burst swimming, and data below the line indicate swimming predominantly driven by aerobic, red muscle. Hypoxia-acclimated fish hit U_{burst} at the same speed in both swim trials, while control fish hit U_{burst} at higher speeds in normoxia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kerri Lynn Ackerly:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Andrew J. Esbaugh:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We thank Dr. Angelina M Dichiera for fish care help during the hypoxia acclimation, swim tunnel set-up, and providing illustrations for the graphical abstract.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111734.

References

Aboagye, D.L., Allen, P.J., 2018. Effects of acute and chronic hypoxia on acid-base regulation, hematology, ion, and osmoregulation of juvenile American paddlefish. J. Comp. Physiol. B. 188, 77–88.
Ackerly, K.L., Esbaugh, A.J., 2021. The effects of temperature on oil-induced respiratory impairment in red drum (Sciaenops ocellatus). Aquat. Toxicol. 233, 105773.
Ackerly, K.L., Negrete Jr., B., Dichiera, A.M., Esbaugh, A.J., 2023. Hypoxia acclimation improves mitochondrial efficiency in the aerobic swimming muscle of red drum (*Sciaenops ocellatus*). Comp. Biochem. Physiol. A 282, 111443.
Bell, W.H., Terhune, L.D.B., 1970. Water tunnel design for fisheries research. In: Fisheries Research Board of Canada, Biological Station.
Bergmeyer, H.U., 1983. Methods of Enzymatic Analysis. Academic Press, New York, NY.
Bergmeyer, H.U., Bergmeyer, J., 1985. Metabolites. VCH.
Borowiec, B.G., Darcy, K.L., Gillette, D.M., 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.
Borowiec, B.G., McClelland, G.B., Rees, B.B., 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.
Breitburg, D., Levin, L.A., Oschlies, A., Grégoire, M., Chavez, F.P., Conley, D.J., Garçon, V., Gilbert, D., Gutiérrez, D., Isensee, K., et al., 2018. Declining oxygen in the global ocean and coastal waters. Science 359, 46.
Chen, K., Cole, R.B., Rees, B.B., 2013. Hypoxia-induced changes in the zebrafish (Danio rerio) skeletal muscle proteome. J. Proteome 78, 477–485.
Claireaux, G., Chabot, D., 2016. Responses by fishes to environmental hypoxia: integration through Fry's concept of aerobic metabolic scope. J. Fish Biol. 88, 232–251.
Claireaux, G., Couturier, C., Groison, A.-L., 2006. Effect of temperature on maximum swimming speed and cost of transport in juvenile European sea bass (*Dicentrarchus labrax*). J. Exp. Biol. 209, 3420–3428.
Clow, K.A., Rodnick, K.J., MacCormack, T.J., Driedzic, W.R., 2004. The regulation and importance of glucose uptake in the isolated Atlantic cod heart: rate-limiting steps and effects of hypoxia. J. Exp. Biol. 207, 1865–1874.
Clow, K.A., Short, C.E., Driedzic, W.R., 2017. Low levels of extracellular glucose limit cardiac anaerobic metabolism in some species of fish. J. Exp. Biol. 220, 2970–2979.
Dalla Via, J., van den Thillart, G., Cattani, O., Cortesi, P., 1997. Environmental versus functional hypoxia/anoxia in sole Solea solea:the lactate paradox revisited. Mar. Ecol. Prog. Ser. 154, 79–90.
Davison, W.G., Cooper, C.A., Sloman, K.A., Wilson, R.W., 2023. A method for measuring meaningful physiological variables in fish blood without surgical cannulation. Sci. Rep. 13, 899.
Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.-O., Huey, R.B., 2015. Ecophysiology. Climate change tightens a metabolic constraint on marine habitats. Science 348, 1132–1135.
Dichiera, A.M., Negrete Jr., B., Ackerly, K.L., Esbaugh, A.J., 2022. The role of carbonic anhydrase-mediated tissue oxygen extraction in a marine teleost acclimated to hypoxia. J. Exp. Biol. 225, jeb244474.
Dutil, J.-D., Sylvestre, E.-L., Gamache, L., Larocque, R., Guderley, H., 2007. Burst and coast use, swimming performance and metabolism of Atlantic cod Gadus morhua in sub-lethal hypoxic conditions. J. Fish Biol. 71, 363–375.

Farhat, E., Cheng, H., Romestaing, C., Pamenter, M., Weber, J.-M., 2021. Goldfish response to chronic hypoxia: mitochondrial respiration, fuel preference and energy metabolism. Metabolites 11, 187.
Forgan, L.G., Forster, M.E., 2010. Oxygen-dependence of metabolic rate in the muscles of craniates. J. Comp. Physiol. B. 180, 715–729.
Galli, G.L.J., Lau, G.Y., Richards, J.G., 2013. Beating oxygen: chronic anoxia exposure reduces mitochondrial F1F0-ATPase activity in turtle (*Trachemys scripta*) heart. J. Exp. Biol. 216, 3283–3293.
Gamperl, A.K., Farrell, A.P., 2004. Cardiac plasticity in fishes: environmental influences and intraspecific differences. J. Exp. Biol. 207, 2539–2550.
Genz, J., Jyde, M.B., Svendsen, J.C., Steffensen, J.F., Ramløv, H., 2013. Excess post-hypoxic oxygen consumption is independent from lactate accumulation in two cyprinid fishes. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 165, 54–60.
Gutmann, I., Wahlfefeld, A.W., 1974. L-(+)-Lactate determination with lactate dehydrogenase and NAD. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis Volume 3. Academic Press Inc., USA, pp. 1464–1469.
Hall, J.R., Short, C.E., Petersen, L.H., Stacey, J., Gamperl, A.K., Driedzic, W.R., 2009. Expression levels of genes associated with oxygen utilization, glucose transport, glucose phosphorylation in hypoxia exposed Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. D 4, 128–138.
Hallman, T.M., Rojas-Vargas, A.C., Jones, D.R., Richards, J.G., 2008. Differential recovery from exercise and hypoxia exposure measured using ^{31}P and ^{1}H NMR in white muscle of the common carp Cyprinus carpio. J. Exp. Biol. 211, 3237–3248.
Haman, F., Zwingelstein, G., Weber, J.-M., 1997. Effects of hypoxia and low temperature on substrate fluxes in fish: plasma metabolite concentrations are misleading. Am. J. Physiol.-Reg. I. Comp. Physiol. 273, R2046–R2054.
Hassid, W.Z., Abraham, S., 1957. [7] Chemical procedures for analysis of polysaccharides. In: Methods in Enzymology. Academic Press, pp. 34–50.
He, W., Xia, W., Cao, Z.-D., Fu, S.-J., 2013. The effect of prolonged exercise training on swimming performance and the underlying biochemical mechanisms in juvenile common carp (Cyprinus carpio). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 166, 308–315.
Hechter, D.T., Hasler, C.T., 2019. Repeatability of burst swimming performance in medaka (Oryzias latipes). Fish Physiol. Biochem. 45, 1299–1307.
Jensen, F.B., 2004. Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O2 and CO2 transport. Acta Physiol. Scand. 182, 215–227.
Johansen, J.L., Esbaugh, A.J., 2017. Sustained impairment of respiratory function and swim performance following acute oil exposure in a coastal marine fish. Aquat. Toxicol. 187, 82–89.
Johnson, T.P., Syme, D.A., Jayne, B.C., Lauder, G.V., Bennett, A.F., 1994. Modeling red muscle power output during steady and unsteady swimming in largemouth bass. Am. J. Phys. 267, R481–R488.
Johnston, I.A., 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.
Kam, J.C., Milligan, C.L., 2006. Fuel use during glycogenesis in rainbow trout (Oncorhynchus mykiss Walbaum) white muscle studied in vitro. J. Exp. Biol. 209, 871–880.
Kang, B., Bakun, A., Lin, L., Pauly, D., 2021. Increase of a hypoxia-tolerant fish, Harpadon nehereus (Synodontidae), as a result of ocean deoxygenation off southwestern China. Environ. Biol. Fish. 105, 1399–1403.
Killen, S.S., Nati, J.J.H., Suski, C.D., 2015. Vulnerability of individual fish to capture by trawling is influenced by capacity for anaerobic metabolism. Proc. Biol. Sci. 282, 20150603.
Lague, S.L., Speers-Roesch, B., Richards, J.G., Farrell, A.P., 2012. Exceptional cardiac anoxia tolerance in tilapia (Oreochromis hybrid). J. Exp. Biol. 215, 1354–1365.
Lenth, R., Singmann, H., Love, J., Buerkner, P., Herve, M., 2018. Emmeans: Estimated marginal means, aka least-squares means. R package version 1, 3.
Lowry, O.H., Passonneau, J.V., 1972. A Flexible System of Enzymatic Analysis. Academic Press Inc., USA, pp. 199–202.
Lushchak, V.I., Bahnjukova, T.V., Storey, K.B., 1998. Effect of hypoxia on the activity and binding of glycolytic and associated enzymes in sea scorpion tissues. Braz. J. Med. Biol. Res. 31, 1059–1067.
MacCormack, T.J., Driedzic, W.R., 2007. The impact of hypoxia on in vivo glucose uptake in a hypoglycemic fish, Myoxocephalus scorpius. Am. J. Phys. Regul. Integr. Comp. Phys. 292, R1033–R1042.
MacCormack, T.J., Lewis, J.M., Almeida-Val, V.M.F., Val, A.L., Driedzic, W.R., 2006. Carbohydrate management, anaerobic metabolism, and adenosine levels in the armored catfish, *Liposarcus pardalis* (Castelnau), during hypoxia. J. Exp. Zool. 305 A, 363–375.
Mandic, M., Speers-Roesch, B., Richards, J.G., 2013. Hypoxia tolerance in sculpins is associated with high anaerobic enzyme activity in brain but not in liver or muscle. Physiol. Biochem. Zool. 86, 92–105.
Martin, L., Negrete Jr., B., Esbaugh, A.J., 2023. The effects of size on exhaustive exercise and recovery in a marine sportfish, the red drum *(Sciaenops ocellatus)*. Comp. Biochem. Physiol. B. 266, 110844.
Milligan, C.L., Girard, S.S., 1993. Lactate metabolism in rainbow trout. J. Exp. 180, 175–193.
Montgomery, D.W., Simpson, S.D., Engelhard, G.H., Birchenough, S.N.R., Wilson, R.W., 2019. Rising CO2 enhances hypoxia tolerance in a marine fish. Sci. Rep. 9, 15152.
Moyes, C.D., Schulte, P.M., Hochachka, P.W., 1992. Recovery metabolism of trout white muscle: role of mitochondria. Am. J. Phys. 262, R295–R304.
Negrete Jr., B., Ackerly, K.L., Dichiera, A.M., Esbaugh, A.J., 2022. Respiratory plasticity improves aerobic performance in hypoxia in a marine teleost. Sci. Total Environ. 849, 157880.
Ngan, A.K., Wang, Y.S., 2009. Tissue-specific transcriptional regulation of monocarboxylate transporters (MCTs) during short-term hypoxia in zebrafish (Danio rerio). Comp. Biochem. Physiol. B 154, 396–405.
Nikinmaa, M., 1982. Effect of adrenaline on red cell volume and concentration gradient of protons across the red cell membrane in the rainbow trout, *Salmo gairdneri*. Mol. Phys. 2, 287–297.
Nikinmaa, M., 2001. Hemoglobin function in vertebrates: evolutionary changes in cellular regulation in hypoxia. Respir. Physiol. 128, 317–329.
Omlin, T., Weber, J.-M., 2010. Hypoxia stimulates lactate disposal in rainbow trout. J. Exp. Biol. 213, 3802–3809.
Pan, Y.K., Ern, R., Morrison, P.R., Brauner, C.J., 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, 7834.
Richards, J.G., Heigenhauser, G.J.F., Wood, C.M., 2002. Glycogen phosphorylase and pyruvate dehydrogenase transformation in white muscle of trout during high-intensity exercise. Am. J. Phys. Regul. Integr. Comp. Phys. 282, R828–R836.
Robergs, R.A., 2019. Invited review: Quantifying proton exchange from chemical reactions - Implications for the biochemistry of metabolic acidosis. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 235, 29–45.
Robergs, R.A., Ghiasvand, F., Parker, D., 2004. Biochemistry of exercise-induced metabolic acidosis. Am. J. Phys. Regul. Integr. Comp. Phys. 287, R502–R516.
Rossi, G.S., Turko, A.J., Wright, P.A., 2018. Oxygen drives skeletal muscle remodeling in an amphibious fish out of water. J. Exp. Biol. 221, jeb180257.
Rummer, J.L., Brauner, C.J., 2011. Plasma-accessible carbonic anhydrase at the tissue of a teleost fish may greatly enhance oxygen delivery: in vitro evidence in rainbow trout, Oncorhynchus mykiss. J. Exp. Biol. 214, 2319–2328.
Salama, A., Nikinmaa, M., 1988. The adrenergic responses of carp (*Cyprinus carpio*) red cells: effects of pO2 and pH. J. Exp. Biol. 136, 405–416.
Sampaio, E., Santos, C., Rosa, I.C., Ferreira, V., Pörtner, H.-O., Duarte, C.M., Levin, L.A., Rosa, R., 2021. Impacts of hypoxic events surpass those of future ocean warming and acidification. Nat. Ecol. Evol. 5, 311–321.
Singmann, H., Bolker, B., Westfall, J., Aust, F., 2015. afex: Analysis of factorial experiments. R package version 0.13–145.
Speers-Roesch, B., Sandblom, E., Lau, G.Y., Farrell, A.P., Richards, J.G., 2010. Effects of environmental hypoxia on cardiac energy metabolism and performance in tilapia. Am. J. Phys. Regul. Integr. Comp. Phys. 298, R104–R119.
Speers-Roesch, B., Mandic, M., Groom, D.J.E., Richards, J.G., 2013. Critical oxygen tensions as predictors of hypoxia tolerance and tissue metabolic responses during hypoxia exposure in fishes. J. Exp. Mar. Biol. Ecol. 449, 239–249.
Suski, C.D., Killen, S.S., Kieffer, J.D., Tufts, B.L., 2006. The influence of environmental temperature and oxygen concentration on the recovery of largemouth bass from exercise: implications for live-release angling tournaments. J. Fish Biol. 68, 120–136.
Thoral, E., Farhat, E., Roussel, D., Cheng, H., Guillard, L., Pamenter, M.E., Weber, J.-M., Teulier, L., 2022. Different patterns of chronic hypoxia lead to hieracrical adaptive mechanisms in goldfish metabolism. J. Exp. Biol. 225, jeb243194.
Treberg, J.R., MacCormack, T.J., Lewis, J.M., Almeida-Val, V.M.F., Val, A.L., Driedzic, W.R., 2007. Intracellular glucose and binding of hexokinase and phosphofructokinase to particulate fractions increase under hypoxia in heart of the amazonian armored catfish (Liposarcus pardalis). Physiol. Biochem. Zool. 80, 542–550.
Val, A.L., 2000. Organic phosphates in the red blood cells of fish. Comp. Biochem. Physiol. A 125, 417–435.
Van den Thillart, G., Verbeek, R., 1991. Anoxia-induced oxygen debt of goldfish (Carassius auratus L.). Physiol. Zool. 64, 525–540.
van Ginneken, V., van den Thillart, G., Addink, A., Erkelens, C., 1995. Fish muscle energy metabolism measured during hypoxia and recovery: an in vivo ^{31}P-NMR study. Am. J. Phys. Regul. Integr. Comp. Phys. 268, R1178–R1187.
van Ginneken, V.J., van Den Thillart, G.E., Muller, H.J., van Deursen, S., Onderwater, M., Visée, J., Hopmans, V., van Vliet, G., Nicolay, K., 1999. Phosphorylation state of red and white muscle in tilapia during graded hypoxia: an in vivo (31)P-NMR study. Am. J. Phys. 277, R1501–R1512.
Van Hall, G., 2000. Lactate as a fuel for mitochondrial respiration. Acta Physiol. Scand. 168, 643–656.
Wasserman, D.H., Kang, L., Ayala, J.E., Fueger, P.T., Lee-Young, R.S., 2011. The physiological regulation of glucose flux into muscle in vivo. J. Exp. Biol. 214, 254–262.
Weber, R.E., Lykkeboe, G., 1978. Respiratory adaptations in carp blood influences of hypoxia, red cell organic phosphates, divalent cations and CO_2 on hemoglobin-oxygen affinity. J. Comp. Physiol. 128, 127–137.
Weber, J.-M., Choi, K., Gonzalez, A., Omlin, T., 2016. Metabolic fuel kinetics in fish: swimming, hypoxia and muscle membranes. J. Exp. Biol. 219, 250–258.
Wells, R.M.G., Baldwin, J., 2006. Plasma lactate and glucose flushes following burst swimming in silver trevally (Pseudocaranx dentex: Carangidae) support the "releaser" hypothesis. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 143, 347–352.
Wood, C.M., 1991. Acid-base and ion balance, metabolism, and their interactions, after exhaustive exercise in fish. J. Exp. Biol. 160, 285–308.
Zeidler, R., Kim, H.D., 1977. Preferential hemolysis of postnatal calf red cells induced by internal alkalinization. J. Gen. Physiol. 70, 385–401.