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Respiratory plasticity improves aerobic performance in hypoxia in a marine teleost

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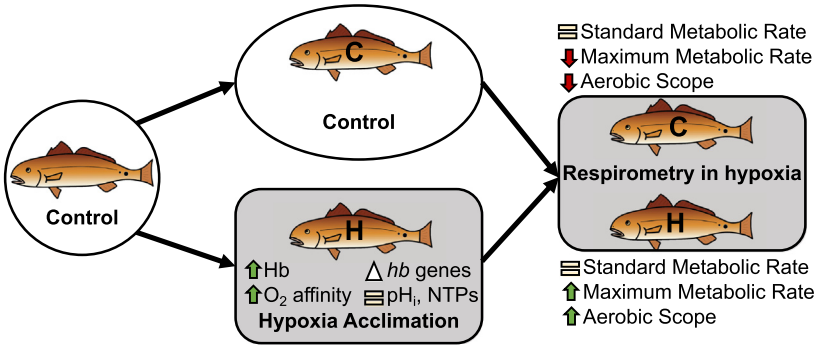
HIGHLIGHTS

- Red drum acclimated to hypoxia show increased maximum metabolic rate in hypoxia.
- Gene expression patterns of hemoglobin are changed with acclimation.
- Fish increase hematocrit while maintaining intracellular pH and allosteric modulators.
- Acclimation also showed increases to hemoglobin-oxygen binding.

GRAPHICAL ABSTRACT



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ABSTRACT

Ocean deoxygenation is a pressing concern in the face of climate change. In response to prolonged hypoxia, fishes have demonstrated the ability to dynamically regulate hemoglobin (Hb) expression to enhance oxygen (O_2) uptake. Here, we examined hypoxia-inducible Hb expression in red drum (*Sciaenops ocellatus*) and the subsequent implications on Hb-O_2 binding affinity and aerobic scope. Fish were acclimated to 30 % air saturation for 1 d, 4 d, 8 d, 2 w, or 6 w, and red blood cells were collected for gene expression and biochemical profiling. Hypoxia acclimation induced significant up-regulation of one Hb subunit isoform (*hbα 2*) relative to control by 4 d with consistent upregulation thereafter. Hematocrit increased in hypoxia, with no changes in the allosteric modulator [NTP] at any time point. Changes in Hb expression co-occurred with a reduced Root effect (~26 % in normoxia, ~14 % in hypoxia) at a physiologically relevant pH while increasing O_2 binding affinity (i.e., lower P_{50}). These changes correlated with increased maximum metabolic rate and aerobic scope relative to controls when fish were tested in hypoxia. These results demonstrate an important role for Hb multiplicity in improving O_2 affinity and maximizing respiratory performance in hypoxia.

1. Introduction

Ocean deoxygenation (hypoxia) is emerging as an important factor impacting global oceans in association with climate change (Breitburg et al., 2018). Severe oxygen (O_2) limitations can force animals to fuel vital metabolic processes through anaerobic pathways. Mild O_2 limitations can constrain aerobic processes, thus reducing the energy available to fuel non-vital functions (Claireaux and Chabot, 2016). Metabolic index theory hypothesizes that equatorial biogeographical distributions of marine organisms are defined by the balance between O_2 supply and demand (Deutsch et al., 2015), and the combined effects of warming and hypoxia on O_2

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balance during routine activity (Deutsch et al., 2020). Overall, these studies suggest that deoxygenation will drive aerobic organisms poleward and constrain habitats vertically (Deutsch et al., 2020, 2015).

The effects of acute hypoxia on fish are relatively well established. Over short exposure timescales fish utilize numerous strategies to balance O_2 supply and demand including altering cardiovascular performance (Gamperl and Farrell, 2004), suppressing baseline metabolism (Borowiec et al., 2018; Gamperl and Farrell, 2004), increasing aquatic surface respiration (Timmerman and Chapman, 2004), increasing gill surface area (Sollid et al., 2003), increasing ventilation (Ern and Esbaugh, 2018, 2016), elevating hematocrit (Hct) (Aboagye and Allen, 2018; Silkin and Silkina, 2005; van den Thillart et al., 2018), and protecting hemoglobin (Hb)-O_2 affinity via allosteric modulators (Bonaventura et al., 2005; Weber and Lykkeboe, 1978). The potential long-term strategies used in response to prolonged hypoxia are less apparent, particularly scenarios that are not severe enough to drive fish to rely on unsustainable anaerobic metabolism. Fish express multiple Hb genes that vary in their ability to bind O_2 and in their sensitivity to allosteric modulators (Rutjes et al., 2007; van den Thillart et al., 2018). These represent an intriguing target for respiratory flexibility that may improve aerobic performance in prolonged hypoxia.

Respiratory flexibility is an increasingly studied subject (Ackerly and Esbaugh, 2021; Borowiec et al., 2018; Cook et al., 2013; Crans et al., 2015; Ern et al., 2016), particularly as it pertains to chronic environmental stress. Studies investigating the effects of hypoxia show metabolic adjustments, as detected by changes in aerobic scope (ASc), which represents the total aerobic capacity a fish can exploit for ecophysiological purposes. Acute hypoxia can inhibit MMR (Ackerly and Esbaugh, 2020; Crans et al., 2015; Ern et al., 2016), but acclimation to chronic hypoxia can lower routine metabolic rate (RMR) and lower the critical O_2 threshold, P_{crit}, the latter of which is a measure of hypoxia tolerance (Borowiec et al., 2018; Pan et al., 2017). Interestingly, one study found that acclimation did not increase MMR when tested in normoxia, however the same study showed that acclimation did lower the hypoxia avoidance threshold in snapper (Cook et al., 2013). Together, these studies indicate that mechanisms of respiratory plasticity used in surviving chronic hypoxia may partially mitigate the effects of hypoxia-induced O_2 supply constraints, thereby improving performance in a hypoxic environment.

Some potential targets for respiratory plasticity lay in the red blood cell (RBC), at the start of the O_2 cascade. Hb-O_2 affinity describes the ability of blood to bind O_2 at the gills, with higher affinity being characteristic of hypoxia-tolerant species (Storz, 2018; Weber and Lykkeboe, 1978). Organic phosphates (e.g. ATP, GTP; collectively NTP) decrease Hb-O_2 affinity by binding to deoxygenated Hb (Nikinmaa, 2001; Russo et al., 2017). Excess protons (H^+) that are metabolically or environmentally derived destabilize oxygenated Hb and enhance O_2 offloading at the tissues, a trait known as the Bohr effect. Many teleost Hb have an exacerbated pH sensitivity, the Root effect, that decreases Hb-O_2 affinity and decreases O_2 binding capacity at high O_2 tensions (Bonaventura et al., 2005). Recent evidence suggests that an increased Root effect is important for O_2 delivery in highly aerobic tissues (Harter et al., 2019; Rummer et al., 2010; Rummer et al., 2013; Rummer and Brauner, 2011) in addition to the traditional role found in tissues with rete, such as the swim bladder and eye (Berenbrink et al., 2005; Damsgaard et al., 2020). The proposed role of the Root effect in mediating O_2 delivery also requires plasma-accessible carbonic anhydrase, intracellular RBC carbonic anhydrase (Dichiera and Esbaugh, 2020), and RBC sodium-proton exchanger, which collectively act to acidify the RBC during capillary transit (Rummer and Brauner, 2011). It is therefore possible that a Root effect Hb may prove advantageous during hypoxia exposure by improving O_2 delivery at aerobic tissues beyond the retina and swim bladder (Bonaventura et al., 2005; Harter et al., 2019; Rummer et al., 2013).

On this background, our objective was to explore the respiratory flexibility of a marine teleost, the red drum (*Sciaenops ocellatus*), in response to prolonged environmental hypoxia. Red drum are common to the Gulf of Mexico, which is home to one of the largest recurring O_2 minimum zones in the world (Breitburg et al., 2018). We hypothesize that prolonged hypoxia exposure would shift the expressed RBC Hb mRNA profile, coincident with increased Hb-O_2 binding affinity, and an increased Root effect. Furthermore, we hypothesize that these biochemical changes will coincide with an improved O_2 supply capacity and elevated ASc under hypoxia.

2. Methods

2.1. Fish

Juvenile red drum were purchased from Ekstrom Aquaculture LLC (Palacios, TX) and held at the Fisheries and Mariculture Laboratory at The University of Texas at Austin Marine Science Institute (Port Aransas, TX). Fish were fed daily except for a 48-h fasting period prior to sampling or respirometry trials. All experimental protocols and procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (AUP-2018-00231).

2.2. Hypoxia acclimation

Red drum were exposed to either normoxia (n = 33, mass: 64.8 ± 2.7 g, DO: 100 ± 0.14 %, pH: 8.02 ± 0.01) or hypoxia (n = 35, mass: 61.9 ± 2.0 g, DO: 33.3 ± 0.22 %, pH: 8.10 ± 0.01). This dissolved oxygen (DO) level for hypoxia (30 %, 6.4 kPa, 2.18 mg L^{-1}) level was chosen because it is above red drum critical O_2 threshold (P_{crit}) (Ackerly and Esbaugh, 2020; Negrete and Esbaugh, 2019; Pan et al., 2017). Tanks were ~250 L (25 ppt; 24.7 ± 0.03 °C) with a recirculation biofilter. Hypoxia was regulated using automated Oxy-Reg (Loligo Systems, Viborg, Denmark) systems that bubbled N_2 or air when O_2 levels went above or below a setpoint. To limit surface O_2 mixing, tanks were partially covered with a layer of plastic bubble wrap. Salinity and ammonia were measured daily, and partial water changes performed as needed. DO and pH were measured twice daily.

2.3. Series I – red blood cell phenotype

The day before sampling, fish were moved to covered, isolated boxes on a wet table with the same recirculating water as acclimation tanks. Fish (n = 6–8) were sampled from control and hypoxia at 1 d, 4 d, 8 d, 2 w, and 6 w. Fish were anesthetized using a solution of buffered MS-222 (250 mg L^{-1}; 500 mg L^{-1} $NaHCO_3$) added to the isolation boxes. Once opercular movement ceased, fish were immediately moved to an irrigation bath (with acclimation water dosed with anesthetic) where a small pump moved water over the gills. Blood was sampled from the caudal vein with heparinized syringes. This method of sampling at rest has produced blood chemistry conditions similar to cannulation (Montgomery et al., 2019). Fish were then euthanized with an overdose of buffered MS-222 followed by spinal transection.

Hct was determined by centrifugation of micro-capillary tubes at 12,000*g* for 2 min. Remaining blood was similarly centrifuged, and RBCs were washed thrice with isotonic saline. RBC were then aliquoted for the following: gene expression (qPCR), mean corpuscular hemoglobin concentration (MCHC), NTP concentration ([NTP]), and O_2 equilibrium curve (OEC) determination. All samples were immediately frozen in −80 °C.

RBC mRNA was isolated using the GeneJET RNA purification kit (Thermo Scientific) and quantified using an ND-1000 spectrophotometer (Thermo Scientific). Following isolation, mRNA was DNase treated (1 U μL^{-1}) and first strand cDNA was synthesized using 0.5 μg of mRNA and the RevertAid Reverse Transcriptase kit (Thermo Scientific). No RT control cDNA was synthesized concurrently to evaluate DNA contamination. Samples were stored at −20 °C. Real-time PCR (MX3000P; Stratagene) was performed using Maxima SYBR green/ROX qPCR Master Mix (Thermo Scientific). Reactions were held at 95 °C for a 10 min hot start, followed by 40 cycles of amplification: 15 s denaturation at 95 °C, 30 s annealing at primer-specific temperatures (58–60 °C), and 30 s elongation at 72 °C. Expression was normalized using the house-keeping gene *ef1α*. Note that the raw ct values for *ef1α* were not affected by hypoxia treatment,

or the duration of exposure (data not shown), and averages of raw ct values were within 0.5 cycles between treatments. Standard curves to evaluate reaction efficiency were prepared for each target gene using a two-fold dilution series of pooled control cDNA. Primer sequences and PCR efficiencies are presented in Table S1. All calculations were performed according to the modified delta-delta Ct method (Pfaffl, 2001).

MCHC and [Hb] were measured using standard Drabkin's methods by measuring the absorbance at 540 nm and using an extinction coefficient of 11 mmol L^{-1} cm^{-1}. [NTP] was determined using established assay methods (Bergmeyer and Bergmeyer, 1985). Plasma lactate was determined using standard established protocols (Gutmann, 1974; Passonneau and Lowry, 1993).

Based on gene expression data, we performed phenotypic analysis on Hb biochemical properties after 8 days of hypoxia exposure. Sample pH_i was measured prior to OEC determination using a micro-sample combination pH electrode (Accumet). OEC determinations were performed on unstripped RBC lysates equivalent to 25 % Hct in buffers (100 mM HEPES, 40 mM KCl, 10 mM Ascorbic acid) at two pH levels (6.95 and 8.2). Note that unstripped lysates were used since there was no statistical change in [NTP], and [NTP]:Hb ratios (see Results) as a consequence of hypoxia. The pH 8.2 hemolysate was only used to define the maximum Hb-O_2 saturation state at 100 % O_2 (i.e. no N_2 balance), while 6.95 was used for the full OEC, and subsequent P_{50} and Root effect determinations. A pH of 6.95 was chosen as representative of resting pH_i in red drum, which was based on a preliminary pH_i to pH_e relationship developed via tonometry at various pCO_2 levels, according to the methods of (Rummer et al., 2010). The defined relationship was $pH_i = 0.313 * pH_e + 4.46$ ($r^2 = 0.96$; data not shown), which resulted in a pH of 6.89 at an extracellular pH of 7.75 (Ern and Esbaugh, 2016; Esbaugh et al., 2016). While lower than pH_i of salmonids, this pH_i is in-line with the pH_i of several other teleost species (Jensen, 2004; Rummer et al., 2010). To reduce methemoglobin (metHb) 10 mM ascorbic acid was included in all hemolysates, which reduced metHb to <18 % after 16 h (data not shown), a value generally in line with previously accepted values (10–15 %) (Barlow et al., 2017; Dichiera and Esbaugh, 2020; Mandic et al., 2009). MetHb was measured in triplicate prior to each OEC determination using standard spectrophotometric methods (Benesch et al., 1973).

OECs were developed using a thin-layer gas equilibration technique as performed using a commercially available blood oxygen binding system (BOBS; Loligo Systems). 1 μL of hemolysate was equilibrated at 24 °C in the following order: 100 % O_2, 100 % N_2 (i.e. 0 % O_2), increments of O_2 (1 %, 2 %, 3 %, 5 %, 15 %, 21 %; balanced with N_2), 100 % O_2 and 100 % N_2. Any changes in sample performance over time were corrected using the initial and final 100 % and 0 % O_2 absorbance. Gas flow was controlled via gas mass flow controllers and was humidified prior to being exposed to the sample. Gas flows were calibrated twice daily using a high-resolution gas flow meter (Definer 220, Mesa Labs). Each hemolysate was tested in triplicate. Absorbance at each O_2 increment was recorded as Hb-O_2 saturation using the wavelength of 436 nm against an isosbestic point of 390 nm (Clark et al., 2008; Russo et al., 2017). Replicates of Hb-O_2 saturation for each lysate were averaged and used to create Hill plots of Hb-O_2 saturations. The P_{50} and Hill coefficients (n_{50}; an indicator of Hb cooperativity) were determined across the environmentally realistic pO_2 range (i.e. 0–21 %) with observed Hb-O_2 saturations at 21 % defined as 100 % saturated (Weber and Lykkeboe, 1978). Calculations were performed using Hill plots of $\log[S/1\text{-}S]$ vs $\log(pO_2)$, where S is Hb-O_2 saturation, between saturations of 0.1–0.9 for pH 6.95. The magnitude of the Root effect was determined using the difference in Hb-O_2 saturation at 100 % O_2 for pH 6.95 compared to 100 % O_2 at pH 8.2. To determine the effect of the Root effect on P_{50}, each OEC was redrawn by parameterizing the equation $\text{Hb-}O_2 = pO_2^{n50} / (P_{50}^{n50} + pO_2^{n50})$ while correcting full saturation using the sample specific Root effect (Barlow et al., 2017; Russo et al., 2017; Storz, 2018).

2.4. Series II – aerobic performance

Owing to the temporal patterns in gene expression and Hb-O_2 binding properties demonstrated in *Series I*, we chose to perform organismal level respirometry following 8 days of acclimation (see Results). Red drum were acclimated as described in *Series I* to control ($n = 16$, mass: 128 ± 8 g) or hypoxia (n = 16, mass: 138 ± 9 g) for 8 d. Fish of both treatments ($n = 8$ each) were exercised following a standard chase protocol for red drum (Ackerly and Esbaugh, 2020) in either normoxia (100 %) or hypoxia (40 %; See Fig. S1). Immediately following exercise fish were placed in acrylic respirometers (5105.3 mL; Loligo Systems) in hypoxia (50 %) to measure mass-specific O_2 consumption (MO_2, $mgO_2\ h^{-1}\ kg^{-1}$). Respirometry trials were performed at a higher pO_2 than the 30 % air saturation used for hypoxia acclimations owing to the fact that the acclimations were close to the reported P_{crit} for red drum (Negrete and Esbaugh, 2019; Pan et al., 2017, 2016) and pilot respirometry experiments (data not shown) demonstrated that a starting pO_2 of approximately 50 % air saturation was required in order for the pO_2 in the chamber to remain above P_{crit} throughout the measurement interval. Importantly, 50 % air saturation still represents a significant constraint on MMR in red drum, as previously demonstrated (Ackerly and Esbaugh, 2020; Ern et al., 2016).

MMR and exercise recovery were defined within the first 3 h post-exercise, and standard metabolic rate (SMR) was determined over the subsequent 24 h. RMR was calculated as the average of all MO_2 points following the 3 h window. MMR is known to be constrained in hypoxia, thus the measurements obtained in this study will hereafter be referred to as MMR_{cons}, and ASc_{cons}. Respirometry cycles were 180 s flush, 30 s wait, and 180 s measure, and measure periods were used only if the drop in O_2 had a slope $r^2 \geq 0.95$. The highest MO_2 within the first five measures was defined as MMR_{cons} (Ackerly and Esbaugh, 2020) and SMR was defined as the lowest 10th percentile (Negrete and Esbaugh, 2019). ASc_{cons} was the difference between MMR_{cons} and SMR. Background measurements were taken for each respirometer before the introduction of fish, and at the end after fish were removed. Background correction was made assuming a linear relationship over time. The % relative inhibition of hypoxia on ASc_{cons} was represented by comparing the measured values against idealized MMR and ASc values as defined by calculating the O_2 supply capacity of control acclimated fish only at 50 % air sat. (MMR/pO_2) and using this to calculate the MMR at 100 % air sat. (Esbaugh et al., 2021; Seibel and Deutsch, 2020). The new MMR_{ideal} was used to find ASc_{ideal} using the average SMR of all fish, as there was no statistical difference between acclimation, or chase DO (see Results). The idealized measures are labeled as MMR_{ideal} and ASc_{ideal} (i.e., where these measures would be in normoxia for a fish of this size).

We assessed recovery from exhaustive exercise in three ways. First, the general pattern of post-exercise O_2 consumption was explored as the % of ASc using the formula $(MO_2\text{-SMR})/ASc * 100\ \%$ where MO_2 is the observed O_2 consumption at each measure period. Values from this equation give an indication as to the percentage of ASc a fish is consuming (i.e. 100 % at MMR, and 0 % at SMR) (Ackerly and Esbaugh, 2020; Johansen and Esbaugh, 2017). Second, we calculated the time to RMR in minutes. Finally, excess post-exercise O_2 consumption (EPOC) was calculated as the total observed O_2 consumption until the fish recovered to RMR using the *trapz* function from the *pracma* package in R (Borchers, 2022). Note that RMR was used for the latter two calculations since red drum rarely recover to SMR before resuming intermittent activity within the chamber.

2.5. Statistical analyses

For *Series I*, type III sum-of-squares two-way ANOVAs were performed with treatment and time as factors for all variables. Gene expression, and P_{50} data were log transformed prior to analyses. We used a *t*-test for the OEC parameters (log P_{50}, Root effect, and Hill coefficient) between treatments. For *Series II*, a type III sum-of-squares two-way ANOVA was performed with treatment and chase DO as factors. Given that chase DO had no effect on aerobic measures (see Results) the differences in RMR and EPOC were pooled by acclimation only and tested using a Student's *t*-test. All data were tested for homogeneity of variance using Levene tests, and normality using Shapiro-Wilkes tests. Data that failed one of these assumptions were square-root transformed prior to analyses. Tukey post-

hoc tests were conducted following ANOVAs with interactions ($\alpha = 0.05$). ANOVA effect sizes (η^2) were calculated where 0.02, 0.13, and 0.26 indicate small, medium and large effect sizes, respectively. Analyses were conducted in R using the packages *afex* (Singmann et al., 2015) and *emmeans* (Lenth et al., 2018).

3. Results

3.1. Series I

Hct was significantly elevated in hypoxia acclimated fish relative to controls (Table 1) and showed an effect of treatment ($F_{1,54} = 10.14$, $p < 0.05$, $\eta^2 = 0.16$), but no interaction with time ($F_{4,54} = 0.95$, $p = 0.44$, $\eta^2 = 0.07$). There was no main effect of hypoxia on MCHC (Table 1; $F_{1,48} = 1.03$, $p = 0.32$, $\eta^2 = 0.02$) nor an interaction with time ($F_{4,48} = 1.08$, $p = 0.38$, $\eta^2 = 0.08$). [Hb] showed no effect of hypoxia ($F_{1,48} = 0.16$, $p = 0.69$, $\eta^2 < 0.01$), nor an interaction ($F_{4,48} = 1.16$, $p = 0.15$, $\eta^2 = 0.13$). Similarly, [NTP] showed no main effect of hypoxia (Table 1; $F_{1,53} = 1.16$, $p = 0.29$, $\eta^2 = 0.02$) nor an interaction with time ($F_{4,53} = 0.70$, $p = 0.60$, $\eta^2 = 0.05$). When [NTP] was normalized to [Hb], there was still no effect of treatment (Table 1; $F_{1,46} = 0.01$, $p = 0.91$, $\eta^2 = 0.00$), nor an interaction with time ($F_{4,46} = 0.48$, $p = 0.75$, $\eta^2 = 0.04$). Plasma lactate showed no effect of hypoxia ($F_{1,48} = 1.08$, $p = 0.30$, $\eta^2 = 0.02$), nor an interaction ($F_{4,48} = 0.59$, $p = 0.67$, $\eta^2 = 0.05$), indicating that these fish did not overly rely on anaerobic metabolism. Hct ($F_{4,48} = 5.43$, $p < 0.01$, $\eta^2 = 0.29$), [Hb] ($F_{4,48} = 3.57$, $p = 0.01$, $\eta^2 = 0.23$), MCHC ($F_{4,48} = 4.96$, $p < 0.01$, $\eta^2 = 0.29$), [NTP] ($F_{4,53} = 5.37$, $p < 0.01$, $\eta^2 = 0.29$), and plasma lactate ($F_{4,48} = 3.09$, $p = 0.02$, $\eta^2 = 0.21$) showed effects of time (Table 1).

Four Hb genes (*hbα 6.1*, *hbβ 3.2*, *hbβ 3.1*, and *hbα 2*) showed an effect of hypoxia ($p < 0.05$), with *hbα 2* showing an effect of time ($p < 0.01$, $\eta^2 = 0.26$) and interaction ($p < 0.01$, $\eta^2 = 0.26$). Detailed outputs are in Table S2. Post-hoc analyses on genes with interactions (*hbα 2*, *hbβ 4*, *hbα short*, *hbβ 1*) showed an up-regulation of *hbα 2* beginning at 4-days in hypoxia (Fig. 1; $p < 0.01$), which was maintained throughout the time course. *Hbβ 4* showed upregulation at 4 d (Fig. 1B, $p < 0.05$), but returned to control values at subsequent time points. Two genes (*hbα short* and *hbβ 1*) showed down-regulation at 1 d relative to control (Fig. 1, $p < 0.05$). *Hbα 3.1* and *hbα 3.2* showed no response to treatment, time, or interaction ($p > 0.05$).

For 8 d acclimated fish, pH_i at rest did not differ between control or hypoxia (Table 2; $p = 0.94$, Student's *t*-test), and metHb values for hemolysates from control and hypoxia acclimated fish were not significantly different ($p = 0.69$, Student's *t*-test). Hemolysates from 8 d hypoxia acclimated fish showed a decreased Root effect (Fig. 2. and Table 2; $p = 0.03$). The pH-dependent P_{50} showed a significant decrease with hypoxia acclimation at pH 6.95 (Fig. 2 and Table 2; $p = 0.01$). The cooperativity (n_{50}) of the Hb did not differ between hypoxia and control fish (Table 2; $p = 0.44$).

3.2. Series II

There was no difference in SMR between control and 8 d hypoxia acclimated fish when tested in hypoxia (Fig. 3; $F_{1,28} = 0.85$, $p = 0.36$, $\eta^2 = 0.03$). However, hypoxia acclimated fish had a significantly increased MMR_{cons} (Fig. 3; $F_{1,28} = 8.88$, $p = 0.01$, $\eta^2 = 0.24$) and ASc_{cons} (Fig. 3; $F_{1,28} = 5.97$, $p = 0.02$, $\eta^2 = 0.18$) when tested in hypoxia. Chase DO did not have an effect on SMR ($F_{1,28} = 0.30$, $p = 0.59$, $\eta^2 = 0.1$), MMR_{cons} ($F_{1,28} = 1.07$, $p = 0.31$, $\eta^2 = 0.04$), or ASc_{cons} ($F_{1,28} = 0.42$, $p = 0.52$, $\eta^2 = 0.01$), nor was any interaction detected for SMR ($F_{1,28} = 0.76$, $p = 0.39$, $\eta^2 = 0.03$), MMR_{cons} ($F_{1,28} = 0.06$, $p = 0.81$, $\eta^2 < 0.01$), or ASc_{cons} ($F_{1,28} = 1.07$, $p = 0.31$, $\eta^2 = 0.04$). When contextualized and compared to a calculated, idealized MMR and ASc (i.e. predicted MMR and ASc based on O_2 supply capacity), these data suggest that hypoxia reduced MMR by ~60.8 % and ASc by ~62.1 % (Fig. 3) in control fish, but ~56.2 % and ~50.6 % in hypoxia acclimated fish, respectively (Fig. 3). This relative inhibition, or difference in ASc_{cons} from ASc_{ideal}, had a significant effect of acclimation (Fig. 3; $F_{1,28} = 5.97$, $p = 0.02$, $\eta^2 = 0.18$) but not chase DO ($F_{1,28} = 0.42$, $p = 0.52$, $\eta^2 = 0.01$) nor an interaction ($F_{1,28} = 1.07$, $p = 0.31$, $\eta^2 = 0.04$).

O_2 consumption following exhaustive exercise under hypoxia was pooled by acclimation group only ($n = 16$ ea.) since there was no effect of chase DO (Fig. 4). MO_2 as a percentage of ASc declined quickly post-exercise in both treatments with the hypoxia-acclimated fish recovering to a lower % ASc asymptote (Fig. 4). Nonetheless, there was no significant difference between treatments for the time to recover to RMR or EPOC (Table 3).

4. Discussion

Prolonged exposure to hypoxia resulted in a shift in hemodynamics within the first 4–8 d of exposure. These changes included a small, but significant increase in Hct, a finding previously demonstrated for several species (Aboagye and Allen, 2018; Silkin and Silkina, 2005; van den Thillart et al., 2018), and an increase in *hbα 2* mRNA. While small transient increases were observed for three other Hb subunits, *hbα 2* showed an increased response magnitude that was maintained throughout the time course. These changes are reminiscent of Hb plasticity exhibited by turtles following hypoxia exposure (Damsgaard et al., 2013), as well as the rigid developmental plasticity observed in freshwater cichlids (Rutjes et al., 2007). Red drum exhibit this hypoxic RBC phenotype relatively quickly as compared to the available information from other fishes, which describe changes in Hb isoforms or Hct between 6 d and 26 d in hypoxia (Taylor and Miller, 2001; Tun and Houston, 1986; Weber and Lykkeboe, 1978). The increase in Hct is likely attributable to an increase in the quantity of RBC, rather than RBC swelling as we did not detect the typical decrease in MCHC or [Hb] associated with swelling (Table 1) (Nikinmaa, 2001).

The observed changes in Hb mRNA profiles following hypoxia exposure also coincided with biochemical changes that are consistent with a hypoxia-

Table 1
Body mass and red blood cell characteristics for fish acclimated to control or hypoxia over 6 weeks. Data are mean ± S.E.M. (n = 6–8 each group), A ‡ denotes main effect of time, and an asterisk (*) denotes main effect of acclimation to hypoxia ($p < 0.05$, Two-way ANOVA). There was no interaction detected between factors for any variable.

Time point	Treatment	n	Mass	Hematocrit*‡	MCHC‡	[Hb]‡	[NTP]‡	NTP:[Hb]	Plasma lactate‡
			(g)	(%)	(mM)	(mM)	(mM)		(mM)
1 day	Control	6	51.0 ± 1.66	24.5 ± 1.5	2.24 ± 0.28	0.55 ± 0.07	1.69 ± 0.13	3.63 ± 0.91	0.65 ± 0.24
	Hypoxia	6	56.9 ± 4.03	26.0 ± 2.1	1.83 ± 0.25	0.47 ± 0.06	1.83 ± 0.11	4.26 ± 0.63	1.13 ± 0.44
4 days	Control	6	58.0 ± 4.30	26.2 ± 0.6	2.67 ± 0.29	0.67 ± 0.06	2.06 ± 0.28	3.56 ± 0.25	0.20 ± 0.11
	Hypoxia	6	58.3 ± 5.03	29.2 ± 1.0	2.61 ± 0.72	0.62 ± 0.07	1.98 ± 0.32	3.45 ± 0.61	0.14 ± 0.04
8 days	Control	8	65.7 ± 4.77	19.9 ± 1.3	2.56 ± 0.24	0.48 ± 0.05	2.43 ± 0.27	5.62 ± 1.04	0.29 ± 0.23
	Hypoxia	8	65.2 ± 5.23	24.9 ± 1.0	2.11 ± 0.21	0.71 ± 0.08	3.16 ± 0.23	4.40 ± 0.63	0.20 ± 0.06
2 weeks	Control	7	75.7 ± 7.61	21.3 ± 1.6	3.27 ± 0.13	0.61 ± 0.06	2.69 ± 0.33	3.90 ± 0.64	0.19 ± 0.08
	Hypoxia	8	60.7 ± 3.37	21.8 ± 2.2	2.94 ± 0.27	0.55 ± 0.12	2.72 ± 0.47	4.48 ± 0.10	0.50 ± 0.20
6 weeks	Control	6	71.5 ± 4.97	22.7 ± 1.6	2.47 ± 0.21	0.74 ± 0.08	2.47 ± 0.26	3.50 ± 0.71	0.50 ± 0.34
	Hypoxia	7	66.9 ± 4.82	27.4 ± 1.1	2.83 ± 0.23	0.82 ± 0.09	2.60 ± 0.22	3.43 ± 0.48	0.59 ± 0.20

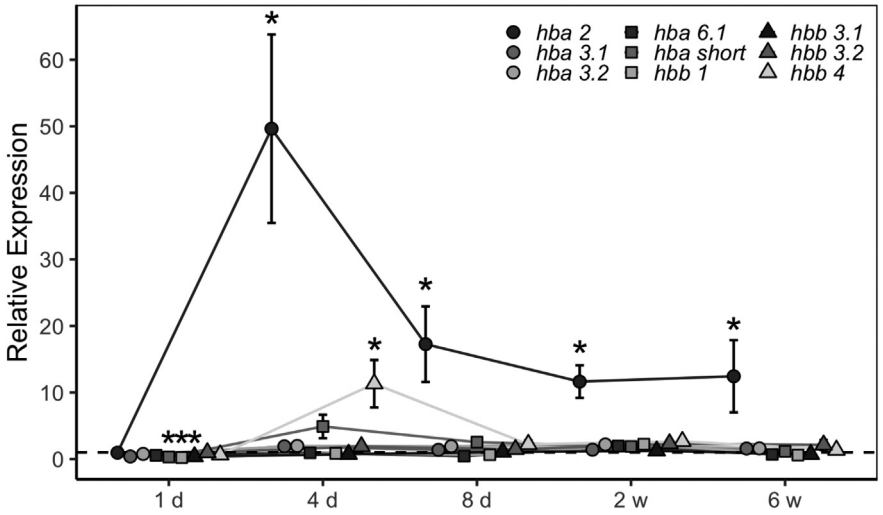

Fig. 1. Changes in relative mRNA for red blood cell transcripts following hypoxia-acclimation in red drum. Note that only data from hypoxia acclimated fish are shown for clarity. Control expression levels are indicated by the dotted line at 1, and asterisks (*) denote differences relative to control within each time point ($p < 0.05$, two-way ANOVA). Note that for clarity only significant differences between treatments within a time point are denoted. In all cases where significant differences are noted there was a significant interaction between time and treatment. Data are mean ± S.E.M. (n = 6–8), and data are expressed relative to the house-keeping gene *ef1α*.

Table 2
Differences in Hb-O_2 affinity of non-stripped hemolysates of red drum acclimated to control or hypoxia for 8-days. Data are mean ± S.E.M. (n = 8). An asterisk (*) denotes differences due to acclimation to hypoxia ($p < 0.05$, *t*-test).

Treatment	pH_i	Buffer	Root effect* (%)	P_{50}* (mm Hg)	Log P_{50}*	n_{50}
Control	6.69 ± 0.04	6.95	25.0 ± 4.86	77.7 ± 13.8	1.85 ± 0.06	1.15 ± 0.07
Hypoxia	6.70 ± 0.08	6.95	12.0 ± 2.09	44.7 ± 4.12	1.64 ± 0.04	1.25 ± 0.10

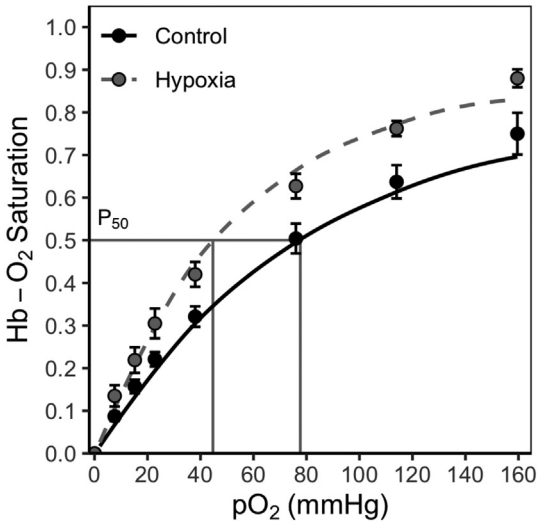

Fig. 2. (A) Oxygen equilibrium curves for non-stripped hemolysates of red drum acclimated to 8 days of hypoxia (grey circles, dotted line, $n = 8$) or control (black circles, solid line, $n = 8$). Data are mean ± standard error. The solid light grey line extends from 50 % Hb-O_2 saturation to indicate half saturation (P_{50}) for each curve. There was a significant decrease (i.e. left shift) in P_{50} and decrease of the Root effect (i.e. elevated Hb-O_2 saturation at 160 mm Hg) due to hypoxia ($p < 0.05$, *t*-test). Points are measured data, and lines are drawn using the Hb-O_2 equation (see Methods).

acclimated phenotype. Hypoxia-acclimated fish demonstrated higher O_2 affinity with a left-ward shift of the OEC curve (Fig. 2), typical of hypoxia tolerant fish (Nikinmaa, 2001; Storz, 2018). Contrary to our hypothesis, hypoxia-acclimated fish decreased the Root effect to half that of normoxia-acclimated fish (~12 % and ~25 %, respectively; Figs. 2, 3; Table 2). The Root effect had significant effects on Hb-O_2 saturation at physiologically relevant pH even at high O_2 tensions, consistent with other fishes (e.g. Verhille and Farrell, 2012), which drop capacity by 13 %–40 % (Clark et al., 2008; Farmer et al., 1979; Rummer et al., 2010). To date, the majority of work exploring hypoxia-induced Hb changes has been performed in freshwater fishes (Bianchini and Wright, 2013; Frey et al., 1998; Houston and Gingras-Bedard, 1994; Rutjes et al., 2007; van den Thillart et al., 2018) with the few marine studies not documenting changes to Hb-O_2 binding affinity (Cook et al., 2013). This work supports the hypothesis that Hb multiplicity can provide a tangible physiological benefit to Hb-O_2 binding affinity in select fishes, likely those pre-adapted to survival in hypoxic environments (Esbaugh et al., 2021; Seibel and Deutsch, 2020).

It is important to note that the P_{50} values are somewhat higher than typically reported in fishes. This is likely because we prioritized in vivo conditions for the determination of the OECs. Prior studies commonly used stripped hemolysates, which remove allosteric modulators and lower P_{50} values. In these studies allosteric modulators are often reintroduced to assess the specific binding characteristics of isoHb pools (Cook et al., 2013; Nelson et al., 2019; Storz et al., 2020; van den Thillart et al., 2018; Weber and Lykkeboe, 1978). Importantly, some of these same studies also found that allosteric modulators are required to detect effects on P_{50} due to hypoxia (van den Thillart et al., 2018; Weber and Lykkeboe, 1978). The second major difference between our study and prior work is the low test pH value, which was selected based on a combination of preliminary

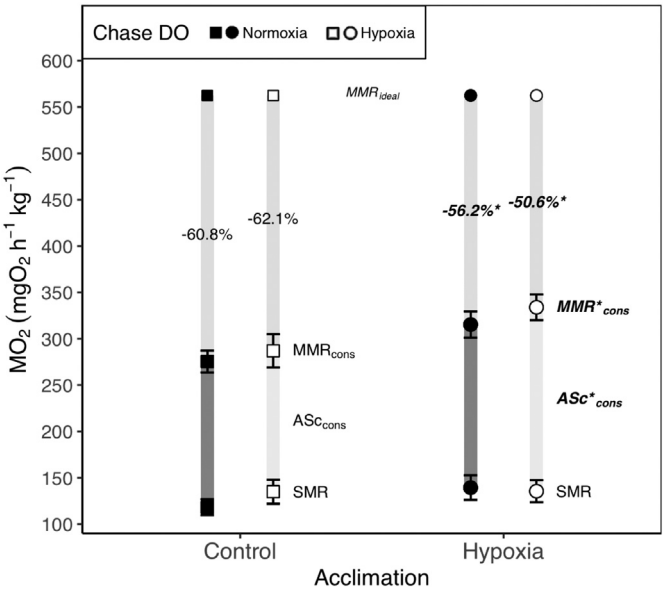

Fig. 3. Standard metabolic rate (SMR), maximum metabolic rate (MMR_{cons}), and aerobic scope (ASc_{cons}, shaded region between MMR_{cons} and SMR) from red drum acclimated to 8-days of hypoxia (circles) or control and recovered in hypoxia. MMR_{cons} and ASc_{cons} are noted as constrained due to the limitation on recovery in hypoxia. The color of the shapes denotes the dissolved oxygen fish were chased in: normoxia (black shapes) or hypoxia (white shapes). There was no effect of chase dissolved oxygen on SMR, MMR_{cons}, or ASc_{cons} ($p > 0.05$, two-way ANOVA). Points above MMR_{cons} are idealized MMR (MMR_{ideal}) for a red drum of this size if recovered in normoxia, calculated using the average MMR_{cons} for control fish and oxygen supply capacity at 50 % and correcting to 100 %. The shaded regions and percentages between MMR_{ideal} and MMR_{cons} demonstrate the relative inhibition of ASc due to hypoxia. Bold text, and (*) indicates significant effects of hypoxia acclimation on MMR_{cons}, ASc_{cons}, and inhibition (two-way ANOVA, $p < 0.05$). Data are mean ± S.E.M. ($n = 8$ per treatment).

investigations using blood gas tonometry, prior studies on the hypoxia acclimation effects on Hb function in red drum (Pan et al., 2017), and a suite of concurrent blood sampling work performed in our lab. Unpublished data using the same sampling methods described here (Montgomery et al., 2019) supports the premise that red drum have surprisingly low RBC pH_i at the experimental temperature ($n = 34$; pH_i 6.74 ± 0.03; pH_e 7.65 ± 0.03; ΔpH −0.92 ± 0.03; mean ± S.E.M.; personal communication Martin and Esbaugh). Similarly low pH_i values have been reported for other species (e.g. Rummer et al., 2010), as have similarly high ΔpH values (Jensen, 1986). As such, it is important to consider the P_{50} values reported here in the in vivo context of our study design, as opposed to a direct comparison to the literature.

Surprisingly, red drum in the current study did not alkalinize RBC pH during hypoxia exposure, nor did they reduce the endogenous levels of NTPs (Tables 1 & 2). These are both common responses of fishes exposed to acute hypoxia exposure (e.g. Jensen, 1986; Nikinmaa, 2001), which act to protect blood O_2 uptake by reducing P_{50}. Previous work has shown that red drum possess the capacity for RBC β-adrenergic Na^+/H^+ exchange (Dichiera and Esbaugh, 2020). It seems likely that the hypoxia levels used here, which were specifically selected to constrain MMR without forcing fish to rely on anaerobic metabolism (i.e. above the P_{crit}) may not have been sufficient to initiate the pH or NTP based protective mechanisms. More importantly for the purposes of this study, the similar pH and NTP values in hypoxia acclimated fish indicate that the differences in P_{50} values and OECs is likely due to the shifting isoHb pool.

At the organismal level, hypoxia exposure is known to constrain MMR and ASc (e.g. Crans et al., 2015; Ern et al., 2016). This is often studied in the context of P_{crit}, the pO_2 at which ASc equals zero (Fry, 1971). Hb-O_2 binding affinity has been previously correlated to P_{crit} across species (Mandic et al., 2009; Speers-Roesch et al., 2013, 2012), and hypoxia-induced shifts in Hb isoforms were related to improved P_{crit} in acclimated red drum (Pan et al., 2017). To our knowledge, this is the first demonstration that hypoxia-acclimation also improves ASc_{cons} when O_2 supply is partly constrained (i.e. pO_2 between normoxia and P_{crit}). Previous work exploring a similar hypoxia acclimation in this range tested acclimated fish in normoxia (Cook et al., 2013). Here, we show that hypoxia acclimation, through inducing respiratory plasticity, can improve O_2 supply capacity to alleviate constraints of hypoxia on ASc_{cons} modestly, yet significantly, by ~8.12 % between acclimations (Fig. 3). Interestingly, these results suggest that while acclimation may not improve aerobic capacity when O_2 is abundant (e.g. Cook et al., 2013), the benefits of respiratory plasticity on O_2 affinity may be specifically suited for performance in hypoxia. These benefits also modestly extend to recovery. O_2 consumption (calculated as % ASc) of hypoxia-acclimated fish stabilized to 10 % lower of their ASc than normoxia fish (Fig. 4). However, no changes were detected in time

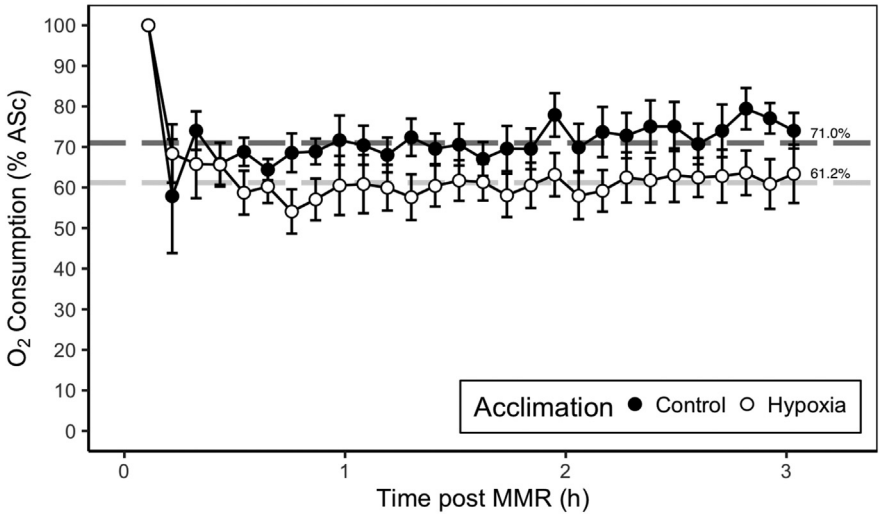

Fig. 4. Oxygen (O_2) consumption following exhaustive exercise of red drum acclimated to hypoxia or normoxia for 8-days. Data (mean ± S.E.M.) are presented as percentage of aerobic scope (ASc), where 100 % is equivalent to maximum metabolic rate (MMR), and 0 % is standard metabolic rate (SMR). Fish are pooled by acclimation ($n = 16$ each treatment) for clarity as there was no effect of dissolved oxygen of the chase tank on aerobic measures (see Fig. 3). Dotted lines indicate asymptote of oxygen consumption during recovery for each treatment.

Table 3
Routine metabolic rate (RMR), time to RMR, and total excess post-exercise oxygen consumption (EPOC) for fish acclimated to control or hypoxia for 8 days. There were no effects of acclimation on any measure ($p > 0.05$, *t*-test). Data are mean ± S.E.M. (n = 16 per treatment).

Treatment	RMR	Time to RMR	EPOC
	($mgO_2\ kg^{-1}\ h^{-1}$)	(min)	($mgO_2\ kg^{-1}\ h^{-1}$)
Control	175 ± 9.93	303 ± 36.3	2498 ± 451
Hypoxia	188 ± 9.43	269 ± 34.7	2533 ± 405

to recover to RMR or total EPOC prior to recovery to RMR (Table 3). While there was no effect of acclimation, it is interesting to note that recovery in hypoxia dramatically increased time to RMR (~5 h for control, and ~4.5 h for hypoxia acclimated) compared to other studies of red drum recovery in normoxia (Ackerly and Esbaugh, 2021; Johansen and Esbaugh, 2017). The benefits to ASc_{cons} occurred with no change in SMR, which agrees with previous work (Cook et al., 2013; Pan et al., 2017; Petersen and Petersen, 1990). It remains unclear whether routine activity and swimming patterns are affected by hypoxia or acclimation in red drum.

Presumably the improved ASc_{cons}, MMR_{cons} and O_2 supply capacity following hypoxia acclimation is driven, in part, by the observed changes in Hb-O_2 binding affinity, but one clear caveat is that other physiological adjustments may have been made that improve respiratory performance. Our work showed no role for NTP or pH_i and a marginal increase in Hct as a consequence of hypoxia exposure. Prior work on red drum has also shown no effect of 14 days of hypoxia exposure on branchial surface area and relative ventricular mass (Pan et al., 2017). So, while we acknowledge that we cannot definitely state that the changes in Hb-O_2 binding characteristics are solely responsible for the improved MMR_{cons}, the data are nonetheless consistent with the hypothesis that hypoxia-induced shifts in Hb will improve respiratory performance in hypoxia.

In theory, the ideal Hb to compensate for hypoxia would have high O_2 affinity for branchial uptake and a marked Root effect to facilitate offloading in aerobic and metabolically active tissues (Damsgaard et al., 2020; Harter et al., 2019; Rummer et al., 2013). While an admitted simplification, as work on trout Hb suggests that possessing a mixture of pH insensitive and sensitive Hb is advantageous when compared to one "ideal" type (Bonaventura et al., 2005; Decker and Nadja, 2007; Farmer et al., 1979), our data demonstrate that the Hb pool in red drum shifts to prioritize O_2-binding at the possible expense of Root effect-induced offloading. Additionally, these data raise questions about the proposed role of the Root effect in O_2 delivery to cardiac muscle (Alderman et al., 2016; Harter et al., 2019), which is thought to be a driver of MMR in fishes. Additional changes may occur in metabolic tissues that can overcome a reduced Root effect, such as increased acidification or increased respiratory carbonic anhydrase (Alderman et al., 2016; Damsgaard et al., 2020; Harter et al., 2019; Rummer et al., 2013). Such changes at the tissue to overcome the reduced Root effect and maintain suitable tissue O_2 extraction would represent a "best-case scenario" for red drum (i.e. protected branchial uptake and maintained tissue extraction), and should be the focus of future studies.

In conclusion, we show that red drum are capable of respiratory plasticity that increases Hb-O_2 binding and reduces the Root effect. This coincides with an ability to increase ASc_{cons} under hypoxia and improve O_2 supply capacity. The ability of fishes to maintain MMR under decreasing pO_2 levels is a significant factor when considering long-term implications of ocean deoxygenation on marine species (Breitburg et al., 2018; Seibel and Deutsch, 2020). This is most apparent in the context of metabolic index (Φ) theory, which is a temperature-dependent index of the ability of the environment to meet an animal's O_2 demand (Deutsch et al., 2015). Studies suggest that equatorial biogeographic ranges of marine species are bound by a minimum Φ between 2 and 5 (Deutsch et al., 2015), and this limitation is defined by the combined hypoxia and thermal sensitivity of the O_2 supply cascade relative to routine activity (Deutsch et al., 2020). Here, we demonstrate that ASc and O_2 supply capacity are flexible traits that respond dynamically to hypoxia-induced constraints on O_2 supply. This would have the benefit of providing some level of resilience against climate change induced poleward population shifts.

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Credit authorship contribution statement

BNJ, KLA, AD, and AJE designed the study. BNJ, KLA, and AD collected samples. BNJ ran all assays, and respirometry. BNJ conducted all statistical analyses, and KLA and AJE verified results. BNJ wrote the manuscript, while KLA, AD, and AJE reviewed and edited the manuscript. BNJ and AJE provided funding.

Data availability

Data will be made available on request.

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.

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