Rapid and reversible modulation of blood haemoglobin content during diel cycles of hypoxia in killifish (*Fundulus heteroclitus*)

Brittney G. Borowiec[1†*] and Graham R. Scott[1]

[1]Department of Biology, McMaster University, Hamilton, Ontario, Canada

*Corresponding author

[†]Current address: Department of Biology, Wilfrid Laurier University, Waterloo, Ontario, Canada

Phone: (519) 884-1970, x3955

Email: bborowiec@wlu.ca

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Dynamics of blood haemoglobin during intermittent hypoxia

Keywords

Nocturnal hypoxia, metabolism, O_2 demands, haemoglobin affinity, red blood cells, hypoxia acclimation, respirometry

Highlights

- Effects of diel cycles of hypoxia-reoxygenation on fish are understudied
- We examined haematological and metabolic adjustments of killifish to such cycles
- Erythrocytes were reversibly released and re-sequestered by the spleen
- Acclimation to hypoxia cycles increased resting but not maximum metabolic rate
- Dynamic modulation of blood haemoglobin helps fish cope with intermittent hypoxia

Abstract

We investigated whether fish can make dynamic haematological adjustments to support aerobic metabolism during repeated cycles of hypoxia-reoxygenation. Killifish were acclimated to normoxia, constant hypoxia (2 kPa O_2), or intermittent cycles of nocturnal hypoxia (12 h of normoxia: 12 h of 2 kPa O_2 hypoxia) for 28 days. Normoxia-acclimated fish were sampled in the daytime in normoxia and after exposure to a single bout of nocturnal hypoxia. Each hypoxia acclimation group were sampled at the PO_2 experienced during acclimation during both the day and night. All acclimation groups had increased blood haemoglobin content and haematocrit and reduced spleen mass during nocturnal hypoxia compared to normoxic controls. Blood haemoglobin content was negatively correlated with spleen mass at both the individual and group level. Fish acclimated to intermittent hypoxia rapidly reversed these changes during diurnal reoxygenation. The concentrations of haemoglobin, ATP, and GTP within erythrocytes did not vary substantially between groups. We also measured resting O_2 consumption rate (MO_2) and maximum MO_2 (induced by an exhaustive chase) in hypoxia in each acclimation group. Fish acclimated to intermittent hypoxia maintained higher resting MO_2 than other groups in hypoxia, comparable to the resting MO_2 of normoxia-acclimated controls measured in normoxia. Differences in resting MO_2 in hypoxia did not result from variation in O_2 transport capacity, because maximal MO_2 in hypoxia always exceeded resting MO_2. Therefore, reversible modulation of blood haemoglobin content along with metabolic adjustments help killifish cope with intermittent cycles of hypoxia in the estuarine environment.

Introduction

Hypoxia occurs in various patterns in the aquatic environment, including periods of stable constant hypoxia or repeated cycles of hypoxia followed by reoxygenation (Diaz, 2001; Diaz and Breitburg, 2009; Richards, 2011; Tyler et al., 2009). The occurrence of hypoxic events is increasing as a result of climate change and other anthropogenic causes (Diaz and Breitburg, 2009; Hasler et al., 2009; Tyler et al., 2009). Coping with hypoxia relies upon several physiological strategies to avoid the development of an ATP supply-demand imbalance (Boutilier, 2001; Hochachka et al., 1996; Richards, 2009), including cardiorespiratory responses that help protect O_2 transport from the environment to mitochondria within tissues (Armelin et al., 2019; Perry et al., 2009; Wells, 2009). However, the extent to which cardiorespiratory physiology of fishes can be dynamically regulated during diel cycles of hypoxia and other intermittent patterns of hypoxia exposure remains unresolved.

Fish exposed acutely to hypoxia can rapidly increase blood haemoglobin content and/or haematocrit through several mechanisms (Hughes, 1973; Wells, 2009). Splenic contraction can release erythrocytes into the circulation and thus augment blood O_2 carrying capacity (Fänge and Nilsson, 1985; Lai et al., 2006; Yamamoto, 1987). Alternatively, red blood cell swelling and haemoconcentration due to a contraction of plasma volume can also occur in hypoxia (Jensen and Weber, 1985; Swift and Lloyd, 1974; Yamamoto et al., 1983). All of these mechanisms could occur concurrently, but the relative contribution of each mechanism to haematological responses to environmental change is unclear and has been the subject of recent debate (Brijs et al., 2020a, b; Hedrick et al., 2020). Longer exposure to constant hypoxia for several weeks can

increase blood haemoglobin content and O_2 carrying capacity further (Borowiec et al., 2015; Greaney et al., 1980), as a result of erythropoiesis by the kidneys that mobilizes erythrocytes into the circulation (Lai et al., 2006). In addition, fish can increase haemoglobin-O_2 binding affinity during hypoxia by reducing the concentrations of negative allosteric modifiers (ATP and GTP) within erythrocytes (Nikinmaa, 1990; Wells, 2009), or by inducing the expression of higher affinity haemoglobin isoforms (Pan et al., 2017; Rutjes et al., 2007; van den Thillart et al., 2018). However, very little is known about the use of these adjustments during intermittent cycles of hypoxia in fishes. Carp exposed to two diel cycles of hypoxia have been observed to reduce erythrocyte GTP, increase blood pH, and thus increase haemoglobin-O_2 affinity (Lykkeboe and Weber, 1978), but little else is known about the haematological responses of fish to repeated cycles of hypoxia and reoxygenation.

Our previous work has found that fishes of the family Fundulidae undergo substantial alterations in metabolism after prolonged acclimation to diel cycles of nocturnal hypoxia (Borowiec et al., 2015; Borowiec et al., 2020; Borowiec et al., 2018), and are capable of maintaining high O_2 consumption rates (MO_2) at rest during nocturnal hypoxia (Borowiec et al., 2018). These previous studies did not examine haematological changes during nocturnal hypoxia, but fish acclimated to diel cycles of hypoxia had normal blood haemoglobin content and haematocrit during daytime normoxia, similar to levels in normoxic controls (Borowiec et al., 2015). We hypothesize that fish exposed to such diel cycles of hypoxia (which we term ‘intermittent hypoxia’) dynamically regulate blood haemoglobin content, such that haemoglobin content is only elevated during nighttime hypoxia periods, and that this contributed to increasing resting MO_2 in hypoxia. We tested this hypothesis by examining haematology along with resting and

maximal MO_2 during a hypoxia-reoxygenation cycle in killifish that were acclimated to intermittent hypoxia, and by comparing them to killifish acclimated to normoxia or constant hypoxia.

Materials and Methods

Animals & hypoxia acclimations

Adult wild-caught *Fundulus heteroclitus* of mixed sex were shipped from a commercial supplier (Aquatic Research Organisms, NH, USA) to McMaster University in Hamilton, Canada. Killifish were initially held in ~300 L fiberglass tanks filled with aerated brackish water (4 ppt) at room temperature (~20°C) for one to two months before acclimation to experimental treatments was initiated. Fish were fed commercial feed (EWOS Canada, Ltd.) to satiation at least four days a week. A 12 h: 12 h photoperiod was maintained with the nighttime portion occurring from 1900 (7 pm) to 0700 (7 am) local time. All animal protocols were approved by the McMaster University Animal Research Ethics Board.

Fish were acclimated for 28 d to normoxia, constant hypoxia (24 h per day at 2 kPa O_2, ~0.8 mg O_2 l^{-1}), or nocturnal ('intermittent') hypoxia (12 h normoxia during the light phase : 12 h at 2 kPa O_2 during the dark phase). We term these acclimation groups 'normoxia control group' (N), 'constant hypoxia group' (CH), and 'intermittent hypoxia group' (IH). Exposures occurred in a multi-stressor exposure system (Aquabiotech, Coaticook, QC, Canada) that modulates water PO_2 with controlled bubbling of oxygen and nitrogen gas based on feedback from a galvanic oxygen

probe. Transitions between normoxia and hypoxia occurred over 1 h starting at 0700 (reoxygenation) or 1900 (deoxygenation) local time. Killifish were either sampled for tissues (which occurred either during the day or night) or put through an exhaustive chase protocol for respirometry experiments (night only) (see Fig.1 for a graphical representation of the experimental design and treatment groups).

Sampling

Following acclimation, we sampled killifish directly from their acclimation conditions at 0100 and 1300 local time in the N, CH, and IH acclimation groups (Fig. 1). In the IH group, the 0100 and 1300 sampling times represented the 6 h midpoint of the normoxia and hypoxia periods of the daily cycle. We also exposed a second group of normoxia acclimated fish to 2 kPa O_2 hypoxia starting at 1900 local time and sampled them after 6 h of night-time hypoxia exposure, which we term the 'acute hypoxia group'. Fish were netted and euthanized with an overdose of benzocaine (final concentration ~1 g l^{-1} in 95% ethanol). The tail was bisected, and blood was collected in a heparinized capillary tube. A 6 µl portion of this blood was quickly removed and used to measure haemoglobin content (using Drabkin's reagent, following manufacturer's instructions; Sigma-Aldrich, Oakville, ON, Canada). The remaining blood was immediately spun for 3 min at 12,700 g to measure haematocrit, and packed erythrocytes were immediately frozen in liquid nitrogen and stored at −80°C. The whole spleen and whole heart (which was composed of the atrium, the ventricle, and bulbus arteriosus) were carefully removed, and their wet masses were recorded. The body masses (mean ± SEM) of each group of sampled fish were as follows:

N at 1300, 5.48 g ± 0.56; N at 0100, 5.28 g ± 0.66; AH at 0100, 5.40 g ± 0.71; IH at 1300, 7.28 g ± 0.89; IH at 0100, 8.00 g ± 0.82; CH at 1300, 4.39 g ± 0.59; CH at 0100, 6.26 g ± 1.21.

Erythrocyte ATP and GTP

Frozen erythrocytes were lysed in 5 volumes of 10 mmol l^{-1} Tris-HCl (pH 7.4), vortexed, and spun at 15,000 g for 10 min at 4°C. A portion of this supernatant (20 µl) was acidified with 3% $HClO_3$ (80 µl) and spun at 10,000 g for 10 min at 4°C. Extracts were neutralized with 16 µl of 3 mol l^{-1} Tris (pH 12.0), and immediately used to quantify ATP and GTP content in triplicate with a Synergy H1 microplate reader at 37°C (BioTek Instruments, Inc., VT, USA). Assay conditions for ATP were as follows: 65 mmol l^{-1} $MgCl_2$, 7.2 mmol l^{-1} glucose, 7.4 mmol l^{-1} EDTA, 6.67 mmol l^{-1} β-mercaptoethanol, 12.7 mmol l^{-1} β-$NADP^+$, 2.5 U ml^{-1} of glucose-6-phosphate dehydrogenase, and excess hexokinase (15 U $m1^{-1}$) in 0.67 mol l^{-1} Tris (pH 8.0). Assay conditions for GTP were the same as for ATP, plus excess ADP (11.8 mmol l^{-1}) and nucleoside diphosphate kinase (15 U $m1^{-1}$). Standard curves were constructed to relate the coupled production of β-NADPH to known concentrations of ATP and GTP. Erythrocyte ATP and GTP concentrations were expressed relative to the concentration of haemoglobin within the sample of lysed erythrocytes (measured as described for whole blood).

Respirometry experiments

In a separate set of fish, we measured resting O_2 consumption rate (resting MO_2) and maximal MO_2 elicited by exhaustive exercise ($MO_{2,max}$). Average body masses (mean ± SEM) were as

follows: N, 4.66 g ± 0.44; AH, 4.46 g ± 0.50; IH, 5.57 g ± 0.36; CH, 4.49 g ± 0.56. There was no significant main effect of body mass across our groups ($F_{[3, 30]} = 1.581$, $p = 0.2145$). MO_2 was measured at ~ 0100 local time for all groups, at a PO_2 of 20 kPa in the N group and at 2 kPa O_2 in the AH, CH, and IH groups (Fig. 1). Killifish were introduced and habituated to 160 ml respirometry chambers starting at ~15 h prior to measurements. These chambers were situated in a buffer tank containing water and were connected to two circulation circuits. One circuit flushed the chamber with water from the surrounding buffer tank (the flushing circuit), and the other circuit continuously pumped water from the respirometry chamber across a fibre-optic O_2 sensor (PreSens, Regensburg, Germany) in a closed loop (the recirculation circuit). The PO_2 in the buffer tank was regulated to match the acclimation period, such that fish in the N group were held at 20 kPa O_2, fish in the CH group were held at 2 kPa O_2, and fish in the AH and IH groups were initially held at 20 kPa O_2 and were then exposed to 2 kPa O_2 hypoxia starting at 1900 local time. MO_2 was measured throughout the habituation period and during subsequent $MO_{2,max}$ trials using intermittent-flow respirometry (Borowiec et al., 2015; Borowiec et al., 2020; Borowiec et al., 2018). We used alternating cycles of 370 s flush periods (when the flushing circuit was active) and 330 s measurement periods (when the flushing circuit was off and the decline in PO_2 was recorded). MO_2 was measured during each measurement period as the overall rate of O_2 consumption from the water, ignoring the first 30 s of the measurement period (the ‘wait’ period) due to unsteady state conditions. We calculated resting MO_2 as the average of the values recorded in the last ten measurement periods before 0100. These times of day correspond to daytime and overnight periods when resting MO_2 is stable, and the resting MO_2 values reported here are comparable to previous measurements in killifish (Borowiec et al., 2018).

Beginning at 0100 local time, killifish were individually removed from the respirometry chamber and chased in a bucket with a net for 2 min (after which they were exhausted and did not respond to gentle tapping), and then exposed to air for 30 s, as previously recommended for inducing maximum rates of oxygen consumption in fish (Roche et al., 2013). Fish were chased in water with the same PO_2 as the appropriate acclimation condition at that time of day, and that matched the PO_2 in the respirometry chamber from which they were removed. Fish were then returned to the respirometry chamber and MO_2 was measured as described above for 6 h, starting with a measurement period in order to determine MO_2 as soon as possible after reintroduction to the chamber. $MO_{2,max}$ was the highest MO_2 measurement recorded during the ~6 h period that fish were monitored after the chase, and was typically observed within the first few measurement periods following the chase. MO_2 typically remained elevated for several hours following the exhaustive chase period. Reported values of MO_2 were corrected for background rates of O_2 consumption in the empty chamber, which were measured daily and were always found to be very low.

Analysis & Statistics

Data are reported as individual values and/or means ± standard error. We checked data for normality with a Shapiro-Wilk test. We used one-way ANOVA (on ranks when appropriate using the Kruskal-Wallis test) followed by either Sidak's (normal data) or Dunn's (non-normal data) multiple comparison tests between each hypoxia group and the normoxic control group. We also conducted simple linear regressions of whole blood haemoglobin content and spleen mass (both for individual values and group means) using the function embedded in GraphPad

Prism (La Jolla, CA) graphing and statistical software. $P<0.05$ was considered significant throughout.

Results

Haematological adjustments during diel cycles of hypoxia

We measured blood haemoglobin content and haematocrit to examine the potential role for adjustments in blood O_2 carrying capacity in killifish during hypoxia. Whole-blood haemoglobin content was sensitive to changes in PO_2, and varied appreciably across treatment groups (main effect of treatment, $F_{[6,67]} = 8.689$, $p < 0.0001$) (Fig. 2A). Normoxia acclimated fish increased haemoglobin content by 57% in response to acute hypoxia exposure (i.e., AH versus N groups during nighttime). Fish acclimated to intermittent hypoxia exhibited a dynamic response to daily cycles of hypoxia: blood haemoglobin content was elevated during nighttime hypoxia bouts and was reduced to levels that were comparable to controls during daytime normoxia (i.e., nighttime versus daytime within IH group). Blood haemoglobin content of fish acclimated to constant hypoxia was elevated during both nighttime and daytime, at slightly lower levels on average comparable to fish exposed to acute hypoxia. The variation in whole-blood haemoglobin content was mirrored by very similar changes in haematocrit (Fig. 2B) ($F_{[6,67]} = 9.610$, $p < 0.0001$), with little variation in mean-cell haemoglobin content (MCHC) across treatment groups ($F_{[6,67]} = 1.232$, $p = 0.3011$) (Table 1). Though chronic elevation of haematocrit could have foreseeably increased blood viscosity and cardiac workload, we did not observed any significant variation in heart mass across groups ($H_{[7]} = 8.631$, $p = 0.1954$) (Table 1).

We also measured the concentrations of allosteric modifiers of haemoglobin-O_2 binding affinity in erythrocytes to examine whether dynamic modulation of O_2 affinity might be involved in the response of killifish to acute or chronic hypoxia. We found no significant variation in erythrocyte ATP ($H_{[7]} = 7.399$, $p = 0.2855$) or GTP ($H_{[7]} = 3.431$, $p = 0.7531$) concentration across groups (Table 1).

Response of the spleen

We measured spleen mass to gain insight into whether splenic contraction and re-sequestration of erythrocytes contributes to the dynamic regulation of blood haemoglobin content during hypoxia. Fish exposed to acute hypoxia had smaller spleens than normoxic controls ($H_{[7]} = 19.81$, $p = 0.0030$) (Fig. 3A), and there was a strong negative correlation between spleen mass and blood haemoglobin content across all groups, and this correlation was significant regardless of whether we used individual data points (Fig. 3B) or group means (Fig. 3C).

We calculated an estimate of the potential contribution of the spleen using a conservation of mass approach (Hedrick et al., 2020). The reduction in spleen mass of 0.28% of body weight in the acute hypoxia treatment would correspond to 0.015 g in a typical killifish with a body mass of 5.4 g. Assuming that total blood volume was 3% of body mass (Conte et al., 1963), we expect the same sized animal to have 0.16 g of whole blood (assuming a blood density of 1 g ml^{-1}). This would correspond to a total blood erythrocyte mass of 0.038 g in normoxia and 0.061 g in acute hypoxia, respectively – a difference of 0.023 g – based on the haematocrit measurements of 24%

and 38% (Fig. 2B). Therefore, the 0.015 g decrease in spleen mass in acute hypoxia could account for 65% of the 0.023 g increase in erythrocyte mass if there were no associated changes in total blood volume. Similar, calculations in the intermittent hypoxia group suggest that the spleen could account for 50% of the reversible changes in erythrocyte mass and blood haemoglobin between day and night in these fish.

Resting O_2 consumption rate (MO_2) and maximum O_2 consumption rate ($MO_{2,max}$) in hypoxia

In a separate group of animals, we examined the ability of killifish to maintain resting O_2 consumption rates and aerobic capacity in hypoxia. There were significant effects of hypoxia treatment on both resting MO_2 ($H_{[4]} = 18.60$, $p = 0.0003$) (Fig. 4A) and $MO_{2,max}$ ($F_{[3,30]} = 16.79$, $p < 0.0001$) (Fig. 4B). Acute hypoxia exposure decreased both resting MO_2 and $MO_{2,max}$ by 50% relative to values exhibited by normoxic controls. Chronic exposure to constant hypoxia was also associated with low resting MO_2 and $MO_{2,max}$, both of which appeared to be depressed lower on average than the values observed in the acute hypoxia group.

Discussion

Role of the spleen in regulating blood haemoglobin content during hypoxia bouts

Taken together, our results for blood haemoglobin content and haematocrit (Fig. 2), spleen mass, and the robust relationship between spleen mass and haemoglobin content (Fig. 3) all strongly suggest that rapid release of erythrocytes from the spleen contributed to increasing blood

haemoglobin content in hypoxia. Contraction of the spleen and a concomitant increase in haematocrit during periods of hypoxia are well-known phenomena in vertebrates, including fishes (Fänge and Nilsson, 1985; Lai et al., 2006; Yamamoto, 1987). This process appears to result from the release of catecholamines into the circulation (Reid et al., 1998) and the subsequent stimulation of α-adrenergic receptors (Nilsson and Grove, 1974). The similarities observed here for the AH and IH acclimation groups in hypoxia show how dynamic these haematological changes can be. Killifish acclimated to daily bouts of nocturnal hypoxia are able to rapidly reduce haematocrit upon return to normoxia during the day, which may be advantageous for reducing blood viscosity and cardiac workload when tissue O_2 supply is less constrained, and then mount anew a haematological response to hypoxia each night that is typical of normoxic fish exposed to their first bout of acute hypoxia. Rats also exhibit reversible increases in blood hemoglobin content via splenic contraction during intermittent hypoxia (Kuwahira et al., 1999), suggesting that rapid splenic modulation of blood haemoglobin content may be a common mechanism to cope with cycles of oxygen limitation in mammals and fish.

We estimated that red blood cells released from the spleen contributed no more than 65% of the total increase in erythrocyte mass in the blood, so other mechanisms likely also contributed to modulating blood haemoglobin content in hypoxia. Indeed, a previous study of rainbow trout subjected to air exposure showed that the spleen released nearly all of its erythrocyte store within 8 min, and contributing to a minority (~31%) of the observed increase in blood haemoglobin content (Pearson and Stevens, 1991). Two other possible mechanisms include haemoconcentration resulting from reductions in plasma volume and erythrocyte swelling (Pearson and Stevens, 1991). It is unlikely that erythrocyte swelling contributed to the

haematological changes in killifish reported here because there were no observed alterations in mean-cell haemoglobin content (Table 1). Reductions in plasma volume could result from increases in blood pressure in hypoxia that increase capillary filtration (Hedrick et al., 2020; Pearson and Stevens, 1991), but this effect would likely be opposed by osmotic water gains that killifish experience during hypoxia exposure in hypo-osmotic environments and would tend to increase extracellular fluid volume (Wood et al., 2019). If reductions in plasma volume occur despite these potential increases in extracellular fluid volume, reductions in plasma volume might have contributed to some of the increase in blood haemoglobin content that remained unexplained by splenic contraction. Unaccounted for alterations in blood volume might have also impacted our estimates based on conservation of mass for the contribution of the spleen, which assumes that total blood volume does not change.

Another mechanism by which vertebrates can increase blood haemoglobin content and haematocrit, albeit over more prolonged periods, is erythropoiesis (Lai et al., 2006). In the hypoxia-sensitive rainbow trout, erythropoietin levels begin to increase between 4 h and 8 h of hypoxia exposure (Lai et al., 2006). However, the process of proliferation and differentiation of progenitor cells that is necessary for the production of new erythrocytes takes time (Kulkeaw and Sugiyama, 2012; Nikinmaa, 2020), so this process is probably too slow to have contributed to the rapid haematological adjustments in response to acute hypoxia but it may have played a role in the responses to chronic hypoxia.

Other potential haematological adjustments

Aside from increasing blood haemoglobin content, animals can increase arterial O_2 content in hypoxia by increasing the affinity of haemoglobin for O_2. This can occur by decreasing the concentration of negative allosteric modifiers such as ATP and GTP within red blood cells (Lykkeboe and Weber, 1978; Nikinmaa, 2001; Pelster and Weber, 1990; Salama and Nikinmaa, 1988; Val, 2000; Wells, 2009), but we found no substantial variation in erythrocyte ATP or GTP concentrations (Table 1). Killifish could foreseeably rely on other mechanisms to modulate haemoglobin-O_2 binding affinity in chronic hypoxia, such as alterations in intracellular pH (Jensen, 2004; Lykkeboe and Weber, 1978; Nikinmaa, 1990), although pH homeostasis tends to be preserved in other tissues during chronic hypoxia (Borowiec et al., 2018). Killifish could have also increased the expression of higher affinity haemoglobin isoforms in chronic hypoxia (Pan et al., 2017; Rutjes et al., 2007; van den Thillart et al., 2018), but it is unlikely that such changes would occur quickly enough to support dynamic adjustments to daily cycles of hypoxia, as fish erythrocytes can persist in the circulation for 80 to 500 days (Avery et al., 1992; Götting and Nikinmaa, 2017). Notwithstanding these possibilities, our results suggest that adjustments in blood hemoglobin content may be more important than potential allosteric modulation of haemoglobin-O_2 affinity in the response of killifish to intermittent hypoxia.

Aerobic capacity during diel cycles of hypoxia

We also examined variation in resting MO_2 and maximal MO_2 in hypoxia. This was motivated by our previous observation that fish acclimated to intermittent hypoxia, but not other hypoxic treatment groups, maintain resting MO_2 during night-time hypoxia at levels that are typical of normoxic controls (Borowiec et al., 2018). We confirmed this finding in the present study, but

we found that maximal MO_2 (i.e. aerobic capacity) was similarly depressed in hypoxia across all treatment groups (Fig. 4). The observed relationships between resting MO_2 and $MO_{2,max}$ have several implications. Firstly, resting MO_2 in acute hypoxia was clearly not limited by O_2 transport capacity, because $MO_{2,max}$ in hypoxia in the acute hypoxia group would have been sufficient to maintain resting MO_2 at the levels exhibited by normoxic controls. This raises in intriguing possibility that reductions in resting MO_2 in acute hypoxia were facultative. Indeed, when considering that maximal MO_2 measured after an exhaustive chase can be slightly lower than the maximal MO_2 measured during maximum sustainable exercise (Raby et al., 2020; Roche et al., 2013), the estimated differences between $MO_{2,max}$ and resting MO_2 observed here are likely conservative. Secondly, the higher resting MO_2 in hypoxia of the intermittent hypoxia group relative to other treatment groups was also unlikely to have resulted from increased O_2 transport capacity, in association with a similar lack of variation in blood haemoglobin content between acclimation groups in hypoxia. The ability of fish in this group to maintain resting MO_2 at a higher proportion of $MO_{2,max}$ may instead reflect a change in metabolic regulation during hypoxia to reactivate processes that were initially down-regulated when fish were first exposed to hypoxia, such as protein synthesis (Cassidy et al., 2018; Cassidy and Lamarre, 2019). Such changes in metabolic regulation may therefore be important for maintaining important fitness-related functions such as growth and reproduction in fish that are chronically exposed to daily bouts of hypoxia (Bera et al., 2017; Cheek, 2011; Cheek et al., 2009).

Distinct responses to constant hypoxia and intermittent hypoxia in killifish

Overall, our results suggest that dynamic modulation of blood haemoglobin content and O_2 carrying capacity, mediated in part by repeated release and re-sequestration of erythrocytes by the spleen, may help estuarine killifish cope with diel cycles of intermittent hypoxia. Interestingly, fish acclimated to intermittent hypoxia maintained higher resting MO_2 during hypoxia than other groups, despite similar $MO_{2,max}$, suggesting that the variation in metabolic rate did not result from alterations in O_2 transport capacity. *Fundulus* killifish are well known for living in highly variable environments and for having a significant capacity for phenotypic plasticity (Burnett et al., 2007), and our work here and elsewhere (Borowiec et al., 2015; Borowiec et al., 2020; Borowiec et al., 2018; Borowiec and Scott, 2020; Du et al., 2016) contributes to the growing evidence that this species can make rapid and reversible adjustments in cardiorespiratory physiology and metabolism in order to cope with the challenges of intermittent hypoxia.

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Competing Interests

We declare no competing or financial interests.

Author Contributions

B.G.B. performed experiments, analyzed data, and drafted the manuscript; B.G.B. and G.R.S. designed experiments, interpreted results, and edited the manuscript.

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Figure Legends

Fig. 1 Experimental design and groups used in this study. Killifish were first acclimated for 28 d to normoxia (~20 kPa), diel cycles of nocturnal hypoxia ('intermittent hypoxia', 12 h normoxia during the daytime light phase [white; from 0700 to 1900 local time] and 12 h of hypoxia at 2 kPa O_2 during the night-time dark phase [gray]), or constant hypoxia at 2 kPa O_2 for 24 h per day. These acclimation periods are shown to the left of the break in the x-axis. Following these 28 d acclimation periods, these fish were divided in the experimental period into one of four treatment groups. (A) One batch of normoxia-acclimated fish were held in constant normoxia throughout to act as time-matched normoxia controls ('N' group), and were examined during the night and/or day. (B) A second batch of normoxia-acclimated fish were exposed to acute hypoxia for 6 h at night and then examined ('AH' group). (C) Fish acclimated to intermittent hypoxia were exposed to a hypoxia-reoxygenation cycle identical to their acclimation treatment ('IH' group), and examined during the night and/or day. (D) Fish acclimated to constant hypoxia continued to be held in hypoxia ('CH' group) and were examined during the night and/or day. Sampling and respirometry occurred at the midpoint of the phase of the cycle (e.g. 6 h before or after hypoxia induction), with solid arrows indicating time points of both tissue sampling and respirometry, and dashed arrows indicating time points of sampling only.

Fig. 2. Dynamic regulation of the blood haemoglobin content during bouts of hypoxia. (A) Whole-blood haemoglobin content and (B) haematocrit during day and night sampling periods. Error bars indicate means ± SEM, and white circle symbols represent individual values.

Measurements were made in normoxia (20 kPa O_2; white bars) or hypoxia (2 kPa O_2; black bars), and the time of day of measurement is indicated by white (1300 in daytime) or grey (0100 in night-time) backgrounds, respectively. * Significant pairwise difference from time-matched normoxic controls. Groups are designated as described in Fig. 1.

Fig. 3. Dynamic changes in spleen size during hypoxia exposure, and it's relationship with blood haemoglobin content. (A) Spleen mass as a proportion of fish wet mass. (B) Linear regression (grey line) and 95% confidence interval (grey shading) between spleen mass and haemoglobin content with each individual represented. (C) Using the same data as the above panel, a linear regression (grey line) and 95% confidence interval (grey shading) between spleen mass and haemoglobin content (means ± SEM). * Significant pairwise difference from time-matched normoxic controls. Groups are designated as described in Fig. 1.

Fig. 4. Resting O_2 consumption rate (MO_2) in hypoxia was augmented in fish acclimated to intermittent hypoxia, without any variation in maximal O_2 consumption rate ($MO_{2,max}$) in hypoxia. (A) Resting MO_2, (B) $MO_{2,max}$ following an exhaustive chase, and (C) resting MO_2 as a percentage of $MO_{2,max}$, with bars indicating means ± SEM and symbols representing individual values. Measurements were made in normoxia (20 kPa O_2; white bars) or hypoxia (2 kPa O_2; black bars) at 0100 h. * Significant pairwise difference from normoxic controls. Groups are designated as described in Fig. 1.

583 **Table**

584

585 **Table 1: Other responses of the circulatory system in killifish sampled at rest**

586

Parameter	Sampled at 1300 h local time			Sampled at 0100 h local time			
	ND (20 kPa)	ID (20 kPa)	CD (2 kPa)	NN (20 kPa)	AH (2 kPa)	IN (2 kPa)	CN (2 kPa)
Heart mass (% wet weight)	0.151 ± 0.007 (10)	0.152 ± 0.014 (7)	0.168 ± 0.011 (9)	0.141 ± 0.011 (11)	0.151 ± 0.014 (8)	0.163 ± 0.007 (11)	0.132 ± 0.007 (7)
MCHC (g dl^{-1})	29.51 ± 1.09 (15)	30.22 ± 1.70 (11)	32.11 ± 2.07 (11)	33.71 ± 0.96 (11)	29.53 ± 0.85 (8)	29.87 ± 1.27 (11)	31.90 ± 1.46 (7)
[ATP] per Hb_4	0.983 ± 0.104 (15)	0.973 ± 0.110 (11)	0.971 ± 0.078 (11)	1.190 ± 0.176 (11)	1.261 ± 0.047 (8)	0.976 ± 0.091 (11)	1.042 ± 0.157 (7)
[GTP] per Hb_4	1.445 ± 0.268 (14)	1.363 ± 0.353 (11)	0.937 ± 0.161 (11)	1.043 ± 0.222 (11)	1.397 ± 0.348 (8)	1.093 ± 0.199 (11)	1.102 ± 0.294 (7)

587 See Fig.1 for treatment group designations.

588 Hb_4, tetrametric haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

589 Data are presented as means ± SEM. Sample sizes are presented in brackets.

590

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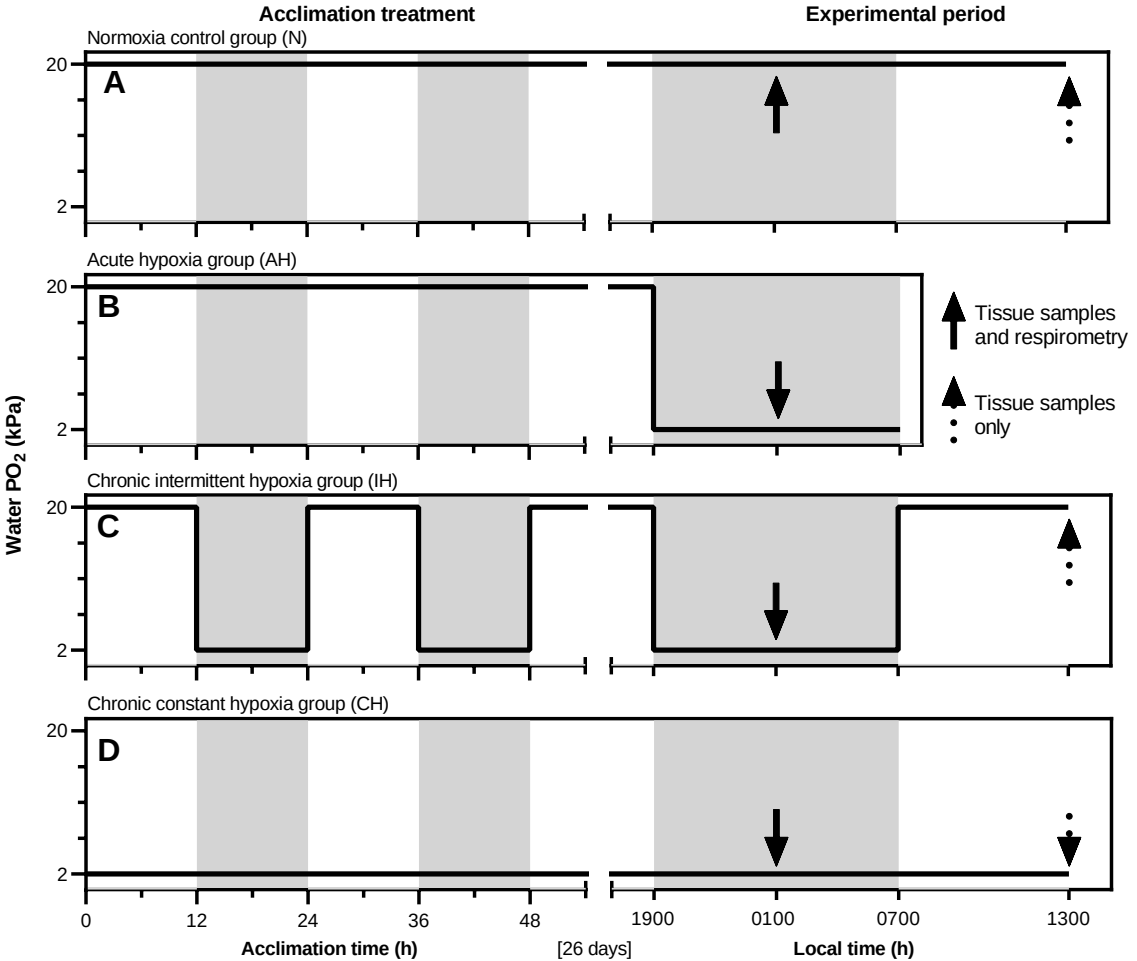
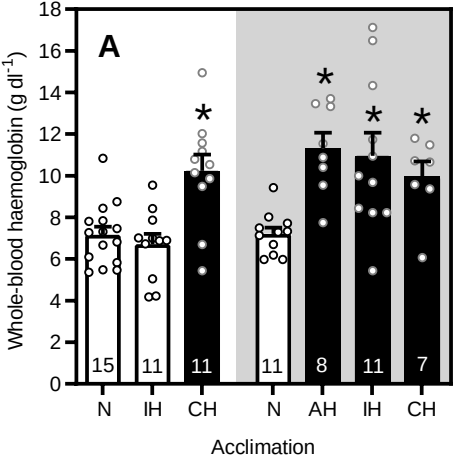
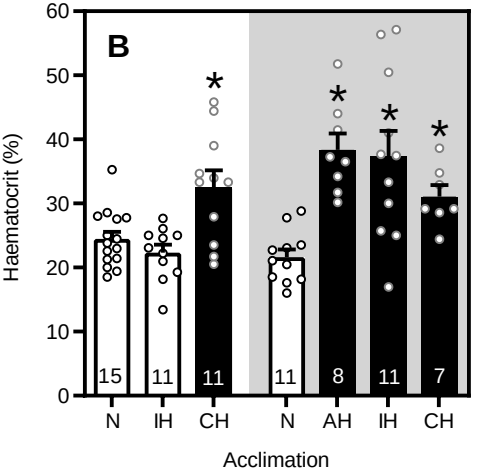
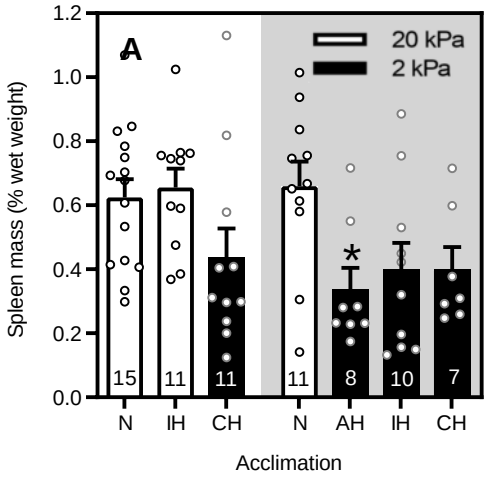
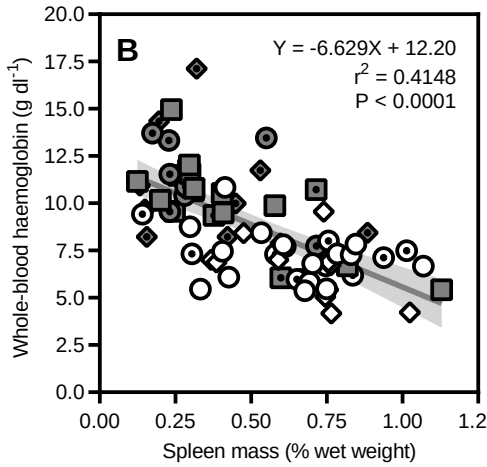
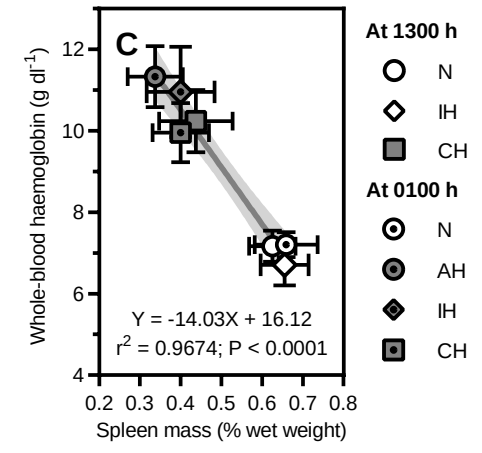

Acclimation treatment
Experimental period
Normoxia control group (N)
A
Acute hypoxia group (AH)
B
Chronic intermittent hypoxia group (IH)
C
Chronic constant hypoxia group (CH)
D
20
2
Water PO2 (kPa)
Tissue samples and respirometry
Tissue samples only
0
12
24
36
48
1900
0100
0700
1300
Acclimation time (h)
[26 days]
Local time (h)

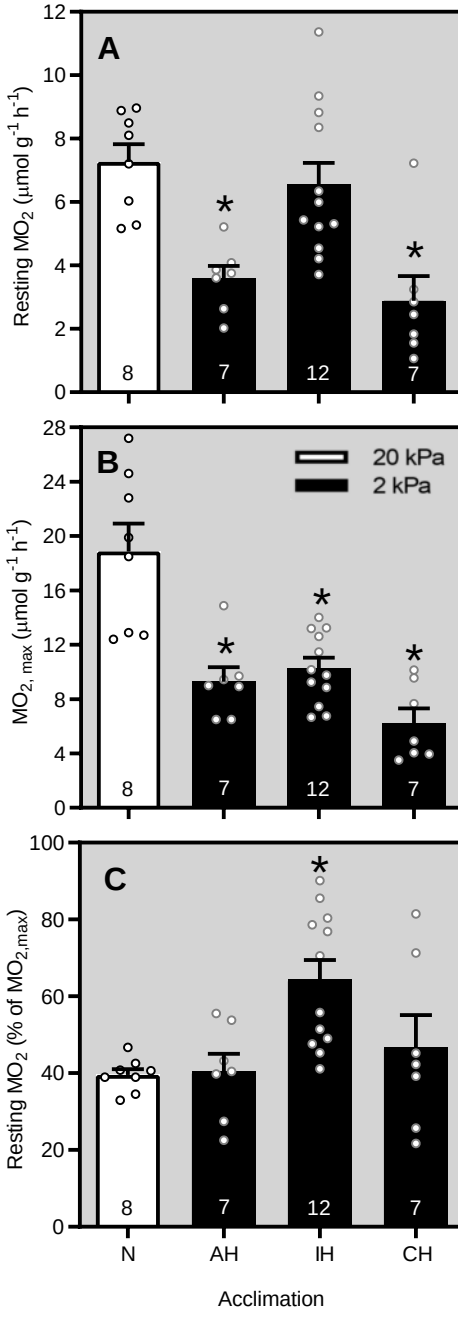

A
Whole-blood haemoglobin (g dl-1)
18
16
14
12
10
8
6
4
2
0
15
11
11
11
8
11
7
N
IH
CH
N
AH
IH
CH
Acclimation


B
Haematocrit (%)
60
50
40
30
20
10
0
15
11
11
11
8
11
7
N
IH
CH
N
AH
IH
CH
Acclimation


A
20 kPa
2 kPa
Spleen mass (% wet weight)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15
11
11
11
8
10
7
*
N
IH
CH
N
AH
IH
CH
Acclimation


B
Y = -6.629X + 12.20
r2 = 0.4148
P < 0.0001
Whole-blood haemoglobin (g dl-1)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0.00
0.25
0.50
0.75
1.00
1.25
Spleen mass (% wet weight)


C
Whole-blood haemoglobin (g dl-1)
12
10
8
6
4
Y = -14.03X + 16.12
r2 = 0.9674; P < 0.0001
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Spleen mass (% wet weight)
At 1300 h
N
IH
CH
At 0100 h
N
AH
IH
CH


A
Resting MO_2 (μmol g^{-1} h^{-1})
12
10
8
6
4
2
0
8
7
12
7
B
20 kPa
2 kPa
$MO_{2,max}$ (μmol g^{-1} h^{-1})
28
24
20
16
12
8
4
0
C
Resting MO_2 (% of $MO_{2,max}$)
100
80
60
40
20
0
N
AH
IH
CH
Acclimation