© 2023. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2023) 226, jeb245425. doi:10.1242/jeb.245425

RESEARCH ARTICLE

Constant temperature and fluctuating temperature have distinct effects on hypoxia tolerance in killifish (*Fundulus heteroclitus*)

Megan R. Ridgway and Graham R. Scott*

ABSTRACT

Climate change is leading to rapid change in aquatic environments, increasing the mean and variability of temperatures, and increasing the incidence of hypoxia. We investigated how acclimation to constant temperatures or to diel temperature fluctuations affects hypoxia tolerance in mummichog killifish (*Fundulus heteroclitus*). Killifish were acclimated to constant cool (15°C), constant warm (25°C) or a diel temperature cycle (15°C at night, 25°C during day) for 6 weeks. We then measured hypoxia tolerance (time to loss of equilibrium in severe hypoxia, t_{LOE}; critical O_2 tension, P_{crit}), whole-animal metabolism, gill morphology, haematology and tissue metabolites at 15°C and 25°C in a full factorial design. Among constant temperature groups, t_{LOE} was highest and P_{crit} was lowest in fish tested at their acclimation temperature. Warm-acclimated fish had lower metabolic rate at 25°C and greater gill surface area (less coverage of lamellae by interlamellar cell mass, ILCM), but cool-acclimated fish had greater brain glycogen stores. Therefore, effects of constant temperature acclimation on hypoxia tolerance were temperature specific and not exhibited broadly across test temperatures, and they were associated with different underlying mechanisms. Hypoxia tolerance was less sensitive to test temperature in fish acclimated to fluctuating temperatures compared with fish acclimated to constant temperature. Acclimation to fluctuating temperatures also increased haemoglobin–O_2 affinity of the blood (decreased P_{50}) compared with constant temperature groups. Therefore, acclimation to fluctuating temperatures helps maintain hypoxia tolerance across a broader range of temperatures, and leads to some distinct physiological adjustments that are not exhibited by fish acclimated to constant temperatures.

KEY WORDS: Teleost, Thermal breadth, Respiration, O_2 transport, Gill remodelling, Anaerobic metabolism

INTRODUCTION

As global climate change continues, it is becoming increasingly critical to understand how the interaction between rising temperatures and other anthropogenic stressors impacts the natural world. The mean and variability of environmental temperatures are increasing in many aquatic ecosystems, which is having profound negative effects on species abundance and distribution (Deutsch et al., 2015; Parmesan, 2006; Parmesan and Yohe, 2003; Sunday et al., 2012). Environmental hypoxia occurs naturally in some aquatic ecosystems, but it is also becoming increasingly prevalent across the globe as a result of rising temperatures and anthropogenic disturbance (Ficke et al., 2007). Thus, understanding the interactive impacts of temperature and hypoxia will be critical for predicting how aquatic organisms, such as fish, will respond to future global change. However, relatively few past studies on this issue have incorporated environmental variability akin to what exists in natural ecosystems. We have a relatively poor understanding of how ecologically relevant fluctuations in temperature contribute to variation in an organism's capacity to withstand other environmental challenges such as hypoxia.

Environmental temperature has pervasive impacts on fish physiology (Schulte, 2015). Acute increases in water temperature tend to increase metabolic rate and influence performance traits owing to thermal effects on biochemical reaction rates, but excessive increases in temperature can lead to declines in performance and induce cellular damage (Schulte et al., 2011). These effects can be altered by thermal acclimation, in which prolonged exposure to elevated temperatures can lead to plastic adjustments of respiratory and metabolic traits and can improve function and performance at warmer temperatures (Lagerspetz, 2006). In general, effects of temperature may be subject to a generalist–specialist trade-off, in which maximum performance at an optimal temperature decreases with an increase in thermal breadth (Seebacher et al., 2015). Acclimation to fluctuating temperatures might be expected to induce plastic adjustments that increase thermal breadth and lead to a more generalist strategy, but this possibility has received relatively little previous attention.

Environmental temperature and thermal acclimation are known to affect hypoxia tolerance. The ability of fish to survive hypoxia is strongly influenced by the ability to maintain sufficient rates of tissue O_2 supply to meet metabolic O_2 demands (Hughes, 1973; Mandic and Regan, 2018). Increases in temperature might therefore be expected to reduce hypoxia tolerance by increasing metabolic rate and reducing aquatic oxygen solubility. Indeed, acute warming (i.e. short-term exposure to a warm test temperature) has been shown to reduce hypoxia tolerance, as reflected by decreased time to loss of equilibrium in severe hypoxia, in several species (Borowiec et al., 2016; Jung et al., 2020; McBryan et al., 2016). However, evidence in several species suggests that warm acclimation can offset such effects of warm temperature on hypoxia tolerance. In mummichog killifish (*Fundulus heteroclitus*), for example, warm acclimation improved hypoxia tolerance compared with fish acclimated to cooler temperature when each was tested and compared at a common warm temperature, and this improvement was associated with an increase in gill surface area (McBryan et al., 2016). Similarly, in the triplefin fish (*Bellapiscis lesleyae*), warm acclimation reduced critical oxygen tension (P_{crit}) compared with cool-acclimated fish when tested at a common warm temperature (Hilton et al., 2008). These and other findings have led to suggestions that warm acclimation leads to general improvements in hypoxia tolerance (Anttila et al., 2015; Hilton et al., 2008;

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON, Canada, L8S 4K1.

*Author for correspondence (scottg2@mcmaster.ca)

M.R.R., 0000-0003-3519-5066; G.R.S., 0000-0002-4225-7475

Received 19 December 2022; Accepted 11 April 2023

Journal of Experimental Biology (2023) 226, jeb245425. doi:10.1242/jeb.245425

McBryan et al., 2016; Sollid et al., 2005a). However, relatively few studies have comprehensively examined the impacts of test temperature and acclimation temperature in a full factorial design, and it remains unclear whether the benefits of warm acclimation are restricted to warm temperatures or apply broadly across cool and warm temperatures (Collins et al., 2021). Furthermore, very little is known about how acclimation to realistic diel cycles of temperature impact hypoxia tolerance, even though diel temperature fluctuations are often more relevant to what is experienced in the wild.

The objectives of this study were to: (i) compare the effects of acclimation to constant cool, constant warm and fluctuating temperatures on hypoxia tolerance, using a full-factorial design at both cool and warm test temperatures, and (ii) investigate the mechanisms that underlie variation in hypoxia tolerance. We used the mummichog killifish (*F. heteroclitus*), an estuarine species that routinely experiences fluctuations of many abiotic factors in their native environment, including temperature and oxygen levels, and is generally appreciated to have a broad range of environmental tolerance (Borowiec et al., 2015; Burnett et al., 2007). On a temporal scale, killifish experience significant seasonal and daily fluctuations in temperature, which, at its extreme, can result in a 10°C change over a matter of hours (Schulte, 2007). Killifish can also experience periods of significant hypoxia in their estuarine habitat (Tyler et al., 2009). We tested the general hypothesis that thermal history alters hypoxia tolerance through thermal plasticity of various respiratory and metabolic traits. We predicted that acclimation to a constant temperature would lead to greatest hypoxia tolerance at that acclimation temperature, but poor hypoxia tolerance at a different test temperature. We also predicted that acclimation to fluctuating temperatures would induce a more generalist strategy, in which hypoxia tolerance is better maintained over a broader range of cool and warm temperatures when compared with fish acclimated to constant temperature.

MATERIALS AND METHODS

Study animals

Adult mummichog killifish [*Fundulus heteroclitus* (Linnaeus 1766)] were wild caught by a commercial supplier (Aquatic Research Organisms, Hampton, NH, USA) and shipped to McMaster University in Hamilton, Ontario, Canada. Fish were kept in 300 liter fibreglass tanks containing well-aerated and charcoal-filtered brackish water (4 ppt) at room temperature (~20°C), and maintained on a 12 h:12 h light:dark photoperiod until they were used in the temperature acclimations described below. Fish were fed to satiation 5 days per week with commercial pellets (AgloNorse Complete Fish Feed, 0.6–0.9 mm, Tromsø, Norway). All animal protocols were developed in accordance with guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

Temperature acclimations

Fish were acclimated for 6 weeks to each of three acclimation treatment groups (Fig. 1): constant water temperature of 15°C ('cool acclimation group'), constant water temperature of 25°C ('warm acclimation group'), or a diel temperature cycle of 15°C during the night-time dark phase and 25°C during the daytime light phase ('fluctuating acclimation group'). Other husbandry conditions (salinity, photoperiod and feeding) were the same as described above. Acclimations were carried out in a multi-stressor exposure system (Aquabiotech, Coaticook, QC, Canada) that constantly monitored and adjusted temperature using water chillers and heaters based on feedback from salinity-resistant temperature probes. Transitions between daytime and night-time temperatures occurred over 4 h beginning at 06:00 h (10°C increase) and 18:00 h (10°C decrease) local time. We chose this pattern of temperature fluctuation because it represents a reasonable estimation of the diel patterns of temperature experienced by this species in estuaries along the east coast of North America (Schulte, 2007). Each acclimation group had 8–9 tank replicates with 9–12 fish of approximately equal sex ratio per exposure tank. Following 6 weeks of acclimation, 3–4 fish from each tank replicate were used to measure time to loss of equilibrium in severe hypoxia, 3–4 separate fish were used for respirometry and P_{crit} measurements (8–9 tank replicates), and many of the remaining fish from each tank replicate were sampled for tissues (8–9 tank replicates) (see details below). These *in vivo* measurements as well as tissue sampling were carried out at 25°C or 15°C in a full factorial design (see Fig. 1 for a graphical representation of the experimental design and treatment groups).

Hypoxia tolerance and respirometry

Time to loss of equilibrium in severe hypoxia (t_{LOE}) was measured as a metric of hypoxia tolerance. Fish were first fasted for 48 h, then transferred before 18:00 h local time to a 40 liter glass aquarium containing well-aerated water at 4 ppt. Fish were placed in a single aquarium in batches of 3–4 fish but each individual was held in a separate plastic chamber, each of which had mesh sides to allow for water flow from the surrounding aquarium and to also prevent aquatic surface respiration. Bubble wrap was placed on the water surface to prevent O_2 diffusion from the air. Fish were first maintained at the overnight temperature appropriate for their acclimation group. The following morning at 06:00 h, water temperature was set to the desired test temperature (either 15°C or 25°C). At 11:00 h, after ~1 h of exposure to the test temperature, the O_2 pressure (P_{O_2}) in the aquarium was rapidly reduced to 0.35 kPa over the course of ~30 min. Fish were continuously monitored using a camera positioned underneath the glass tank while minimizing visual disturbance by covering sides with dark plastic. Time to loss of equilibrium was determined as the duration of time at 0.35 kPa until fish were unable to maintain an upright position and were unresponsive to stimuli.

Resting metabolic rate (RMR) and P_{crit} were measured using stop-flow respirometry. Fish were first fasted for 48 h and then transferred before 18:00 h local time to individual 90 ml cylindrical acrylic chambers held within a darkened buffer tank containing well-aerated water (4 ppt) at the appropriate overnight temperature for their acclimation group. Each of these respirometry chambers were connected to a flush pump, which pumped water from the buffer tank through the chamber, making a 'flushing circuit'. A second 'measurement circuit' used a circulating pump to continuously pump water from the chamber across a fibre-optic O_2 sensor (FireSting FSO2-4, PyroScience GmbH) in a closed loop. Both the flushing and recirculating circuits were active during the overnight adjustment period in the respirometry chambers. The following morning at 06:00 h, water temperature was set to the desired test temperature (either 15°C or 25°C). At 11:00 h, after ~1 h of exposure to the test temperature, sequential activation and deactivation of the flush pumps allowed for repeated measurements of O_2 consumption rate ($\dot{M}_{O_2}$). During 5 min measurement periods, the flush pumps were deactivated, and the circulating pump continued to pass water across the O_2 sensor to measure the decline in P_{O_2} over time. Measurement periods were interspersed with 5 min flush periods, during which both pumps were activated to allow oxygenated water from the buffer tank to enter the chamber.

Journal of Experimental Biology (2023) 226, jeb245425. doi:10.1242/jeb.245425

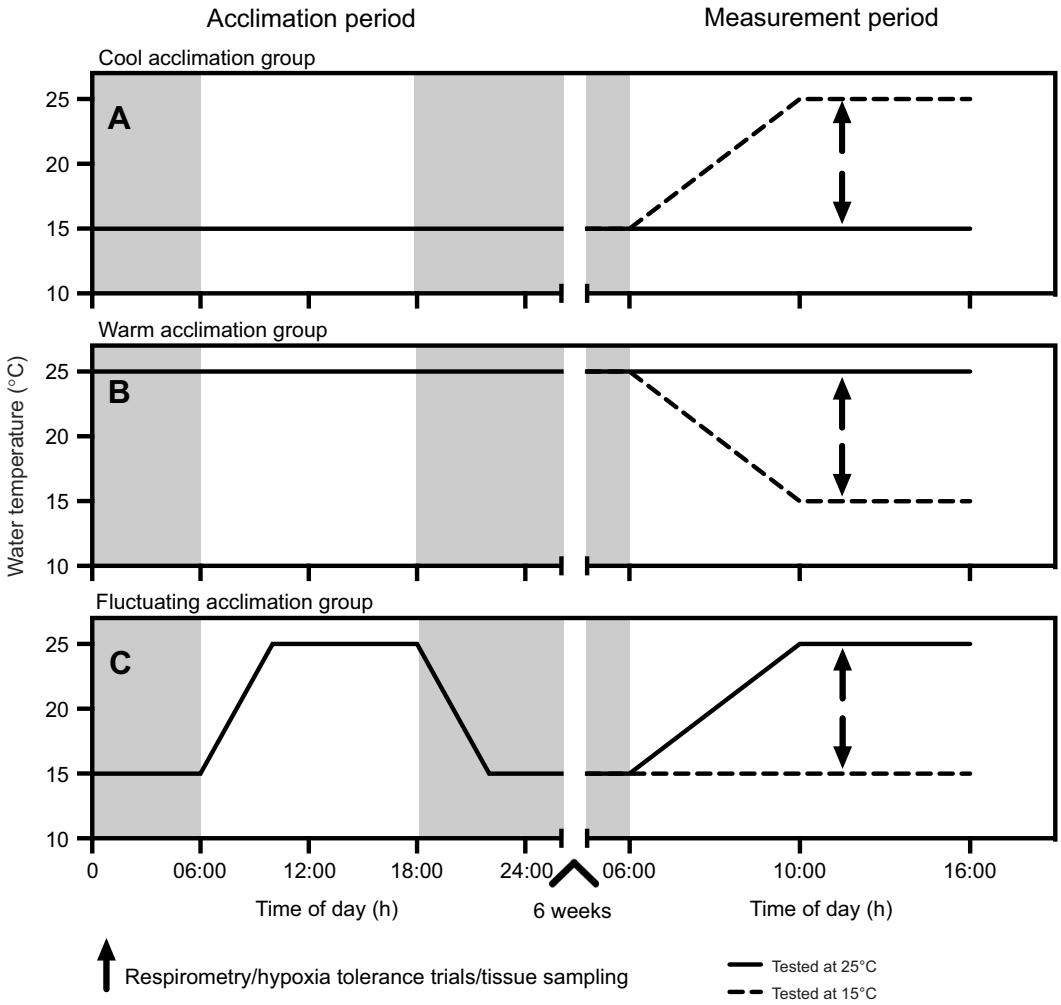

Fig. 1. Experimental design. Killifish were first acclimated for 6 weeks to constant cool (15°C), constant warm (25°C) or diel cycles of temperature (reaching 25°C during the daytime light phase and 15°C during the night-time dark phase; the latter shown by grey shading). These acclimation conditions are shown to the left of the break in the *x*-axis. Following 6 weeks of acclimation, *in vivo* whole-animal measurements and *in vitro* tissue measurements were made in a full-factorial design at both 15°C and 25°C, starting at the times marked by arrows. This was achieved by holding fish overnight at the temperature appropriate for their acclimation group, and then transitioning them over the course of 4 h to the desired test temperature (either the acclimation temperature, solid lines, or the alternative temperature, dashed lines) before experimental measurements or tissue sampling. See Materials and Methods for additional details.

RMR was determined as the lowest $\dot{M}_{O_2}$ measured during measurement periods between 11:00 and 12:00 h in normoxia. We then measured $\dot{M}_{O_2}$ throughout a progressive stepwise hypoxia protocol, where the P_{O_2} of the buffer tank was reduced from ∼20 to 2 kPa in 2 kPa steps (10 min per step) as previously described (Borowiec et al., 2020). P_{crit} was determined by plotting $\dot{M}_{O_2}$ versus P_{O_2}, fitting a linear regression at low P_{O_2} for $\dot{M}_{O_2}$ data points that fell below the RMR value for that individual, and then calculating P_{crit} as the P_{O_2} at which this regression equalled RMR. This method conforms to recent calls for a standardized curve-fitting approach for determining P_{crit} (Reemeyer and Rees, 2019).

Sampling of fish tissues

In a subset of fish from each acclimation group, water temperature was set to the desired test temperature (either 15°C or 25°C) at 06:00 h. At 11:00 h, after ∼1 h of exposure to the test temperature, fish were sampled under resting conditions. Fish were euthanized with a sharp blow to the head followed by spinal transection, and the tail was severed for blood collection. Blood was collected in a heparinized syringe. A portion of blood (10 µl) was added to Drabkin's reagent to determine haemoglobin concentration according to product instructions (Sigma-Aldrich, St Louis, MO, USA). Oxygen dissociation curves were generated at either 15°C or 25°C using a Hemox Analyzer (TCS Scientific, New Hope, PA, USA) using 10 µl of whole blood in 5 ml of buffer (100 mmol l^{-1} Hepes, 50 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, 0.1% bovine serum albumin and 0.2% antifoaming agent). Buffer pH was set to 8.0 when running samples at 15°C and 7.8 when running samples at 25°C to account for the expected variation in blood pH with temperature (Cameron, 1978). The remaining blood was collected in a heparinized capillary tube and centrifuged at 12,700 ***g*** for 5 min to determine haematocrit. Brain, liver and axial white muscles were dissected and immediately freeze-clamped in liquid nitrogen (within 1 min of euthanasia), then stored at −80°C for later analysis of metabolites. Gills were dissected, fixed in 10% formalin for 24 h, then stored in 0.2 mol l^{-1} PBS (274 mmol l^{-1} NaCl, 30.4 mmol l^{-1} Na_2HPO_4, 5.4 mmol l^{-1} KH_2PO_4; pH 7.8) at 4°C for later analysis of gill morphology.

Gill histology

After fixation, the first gill arch was immersed in embedding medium (Shandon Cryomatrix, Fisher Scientific, Runcorn, Cheshire, UK), rapidly frozen in liquid N_2-cooled isopentane, and stored at −80°C for at least 24 h before sectioning. Frozen blocks were sectioned at 10 µm thickness using a cryostat maintained at −20°C (Leica CM 1860). Sections were mounted on glass microscope slides, dried overnight at room temperature, and then stored at −80°C until staining. Sections were stained by incubating in Gills II haematoxylin for 10 min and then in eosin for 2 min, rinsing in distilled water between each step. After staining, sections were dehydrated through progressively increasing concentrations of ethanol (50%, 70%, 95%) for 30 s each before being placed in 100% ethanol for 1 min. Sections were then placed in xylene for at least 1 min before being mounted in Permount Mounting Medium (Thermo Fisher Scientific). For each individual, brightfield images were taken of sections throughout the whole gill arch tissue using a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY, USA). ImageJ (v.1.53q) software (Rasband, 2008) was used to measure heights of lamellae and the interlamellar cell mass (ILCM).

Tissue metabolite assays

Frozen samples of brain, liver and muscle were ground into a fine powder using an insulated mortar and pestle that was pre-cooled with liquid nitrogen, and the powder was stored at −80°C until homogenization. Powdered tissue (20–30 mg) was homogenized for 20 s in 10 µl mg^{-1} tissue of ice-cold 6% $HClO_4$, using the highest setting of a PowerGen 125 electric homogenizer (Thermo Fisher Scientific, Whitby, ON, Canada). The homogenate was vortexed, and a 100 µl aliquot was frozen in liquid nitrogen and stored at −80°C for future analysis of glucose and glycogen. The remaining, unfrozen aliquot was centrifuged at 4°C for 10 min at 10,000 ***g***, and 200 µl of the supernatant was transferred to a new microcentrifuge tube, neutralized using 3 mol l^{-1} K_2CO_3 to 6.8≤pH≤7.2 and centrifuged again under the same conditions. The resulting supernatant was used for quantification of lactate under the following initial assay conditions: 2.5 mmol l^{-1} NAD^+ in glycine buffer (0.6 mol l^{-1} glycine, 0.5 mol l^{-1} hydrazine sulphate, pH 9.4). An initial absorbance measurement was made at 340 nm before adding lactate dehydrogenase (LDH) in excess (5 U ml^{-1}). After a 30-min incubation period, a second absorbance measurement was made. The increase in NADH concentration in the well was calculated using the molar extinction coefficient for NADH (6.22 l $mmol^{-1}$ cm^{-1}). Given that the assay couples lactate to NADH via the LDH reaction, the increase in NADH concentration equals the original molar concentration of lactate in the sample. The assay was run in triplicate at 37°C on a Synergy H1 hybrid multimode microplate reader (Biotek Instruments, Winooski, VT, USA).

To measure glucose and glycogen, the frozen aliquot of acidified homogenate was thawed on ice, and then 100 µl of 400 mmol l^{-1} Na acetate buffer (pH 4.8) and 50 µl of 1 mol l^{-1} K_2CO_3 were added. For liver, samples were also centrifuged (1000 ***g***, 4°C, 5 min) and any debris present on top of the supernatant was removed before the following step. For digestion, 100 µl of each sample was digested with 7 µl of amyloglycosidase (suspended at 4 U l^{-1} in 300 mmol l^{-1} Tris-HCl, 4.05 mmol l^{-1} $MgSO_4$; pH 7.5) for 2 h in a 40°C water bath (vortexing every 15 min), while another 100 µl aliquot from each sample was kept undigested on ice for 2 h. Both digested and undigested fractions were neutralized to 6.8≤pH≤7.2 with 1 mol l^{-1} K_2CO_3, centrifuged at 4°C for 10 min at 10,000 ***g***, and then assayed. Initial assay conditions were as follows: 1 mmol l^{-1} ATP, 0.5 mmol l^{-1} $NADP^+$, 5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} imidazole (pH 7.4). All samples were first depleted of glucose 6-phosphate by adding excess glucose 6-phosphate dehydrogenase (5 U ml^{-1}) and incubating for 30 min, after which an initial NADPH absorbance reading was taken at 340 nm. Glucose content was then determined in both digested samples (containing endogenous free glucose and glucose produced via the breakdown of glycogen) and undigested samples (containing only endogenous free glucose) by measuring the increase in NADPH absorbance elicited 50 min after the addition of the coupling enzyme hexokinase in excess (5 U ml^{-1}). The difference between the glucose contents measured in the digested and undigested samples was used to calculate total glycogen content of the tissue.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 9.4.1, San Diego, CA, USA). We tested for main and interactive effects of acclimation group and test temperature using two-way AVOVA. Data were log or square root transformed, if necessary, to meet the assumptions of the statistical tests. Using the Bonferroni correction to adjust for multiple comparisons, we looked for significant differences between acclimation groups within each test temperature as well as between test temperatures within each acclimation group. A significance level of $P<0.05$ was used for all statistical analyses. All data are reported as means±s.e.m.

RESULTS

Our experimental design allowed us to distinguish effects of test temperature from the effects of past thermal history (Fig. 1). Three acclimation groups were used to assess the effects of thermal history: the cool acclimation group held at 15°C; the warm acclimation group held at 25°C; and the fluctuating acclimation group that was cycled between 15°C at night-time and 25°C during the daytime. Variables of interest were then measured shortly after transfer to each of 15°C and 25°C in a full factorial design. Therefore, transfer of cool-acclimated fish to 25°C was used to assess the effects of acute warming, which were compared with warm-acclimated fish at 25°C to assess plasticity during warm acclimation. Similarly, transfer of warm-acclimated fish to 15°C revealed the effects of acute cooling, which were compared with cool-acclimated fish at 15°C to assess plasticity during cool acclimation. Furthermore, these observations were compared with the fluctuating group to assess whether chronic exposure to daily temperature fluctuations modified the sensitivity to acute temperature changes. This experimental design also allowed us to evaluate whether the effects of acclimation to a constant temperature were advantageous only at that acclimation temperature, or extended broadly across test temperatures.

Hypoxia tolerance

There were strong effects of thermal acclimation and test temperature on t_{LOE}, a key metric of hypoxia tolerance (Fig. 2). This was reflected by significant main effects of acclimation group ($P=0.0207$) and test temperature ($P<0.0001$) as well as a significant interaction ($P<0.0001$) (Table 1). Hypoxia tolerance in the cool acclimation group collapsed when tested at warm temperature, demonstrated by an 11.6-fold decrease in t_{LOE} at 25°C relative to 15°C. The warm acclimation group had greater hypoxia tolerance at 25°C but much worse hypoxia tolerance at 15°C than the cool acclimation group. The fluctuating acclimation group displayed hypoxia tolerance that was similar to that of the cool acclimation group when tested at 15°C, and intermediate between constant

Journal of Experimental Biology (2023) 226, jeb245425. doi:10.1242/jeb.245425

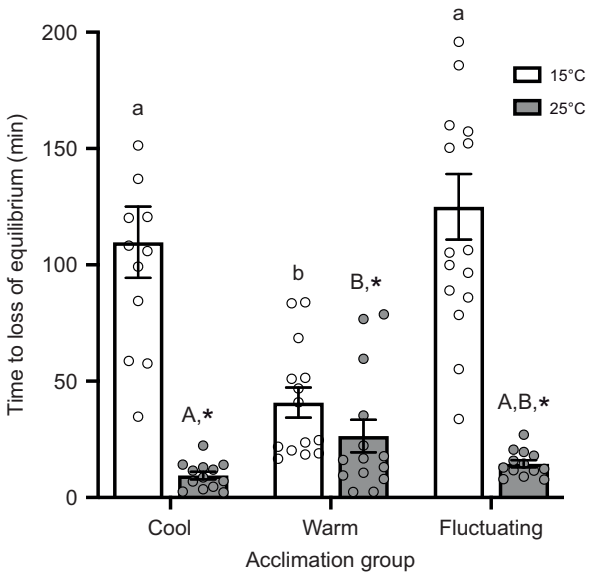

Fig. 2. Acclimation group and test temperature affected time to loss of equilibrium (t_{LOE}) in severe hypoxia. Different test temperatures are distinguished by white (15°C) and grey (25°C) bars. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature, and asterisks indicate significant pairwise differences between test temperatures within acclimation groups. Bars represent means±s.e.m. and circles represent individual values (one individual data point at 238.17 for the cool group tested at 15°C and at 247.22 for the fluctuating group tested at 15°C were beyond the limit of the *y*-axis scale). Sample sizes were as follows: cool group, *N*=12 at 15°C and *N*=13 at 25°C; warm group *N*=14 and 14; fluctuating group, *N*=16 and 13.

acclimation groups when tested at 25°C. Therefore, acclimation to a constant temperature improved hypoxia tolerance at that temperature, whereas acclimation to diel cycles of temperature may have helped maintain hypoxia tolerance across a broader range of test temperatures.

Metabolic rate and critical oxygen tension

Thermal acclimation also had significant effects on RMR, measured as the resting rate of O_2 consumption (Fig. 3A, Table 1). RMR was 2.1- to 2.9-fold higher when fish were tested at 25°C relative to when they were tested at 15°C, which drove a significant effect of test temperature (P<0.0001). However, there was also a significant interaction between acclimation group and test temperature (P=0.007) and a significant main effect of acclimation group (P<0.0001), owing to significantly greater RMR in the cool group than in both warm and fluctuating groups at 25°C but not at 15°C. This suggested that the increase in RMR following acute exposure to elevated temperature, as illustrated in the cool acclimation group, was attenuated over time with acclimation to constant warm or fluctuating temperature regimes.

We also exposed fish to progressive stepwise hypoxia to examine how $\dot{M}_{O_2}$ varied across a range of P_{O_2} (Fig. S1). Consistent with the effects of test temperature on RMR (Fig. 1), $\dot{M}_{O_2}$ was higher on average across a range of high P_{O_2} in fish tested at 25°C relative to 15°C (Fig. S1). $\dot{M}_{O_2}$ declined in more severe levels of hypoxia (Fig. S1), as expected, and the data for $\dot{M}_{O_2}$ as a function of P_{O_2} for each individual were used to calculate P_{crit}. There was a significant interaction (P<0.0001) between acclimation group and test temperature for P_{crit} (Fig. 3B, Table 1). When tested at 15°C, cool-acclimated fish demonstrated significantly lower P_{crit} compared with the warm acclimation group. At 25°C, warm-acclimated fish had the lowest P_{crit}, which was significantly lower than the cool acclimation group, and the fluctuating group were intermediate between the constant acclimation groups. Therefore, the pattern of variation in P_{crit} in the constant acclimation groups was akin to the pattern of variation for t_{LOE}, with both warm and cool groups demonstrating significantly lower P_{crit} when tested at their acclimation temperature, whereas the fluctuating group was intermediate and exhibited less variation in P_{crit} across test temperatures.

Gill morphology

Thermal acclimation had a significant impact on gill morphology (Fig. 4, Table 1), reflected by a main effect of acclimation group

Table 1. Statistical results of two-way ANOVA

	Main effect of acclimation		Main effect of test temperature		Interaction	
Trait	*F*	*P*	*F*	*P*	*F*	*P*
t_{LOE}	$F_{2,76}$=4.083	0.0207	$F_{1,76}$=146.0	<0.0001	$F_{2,76}$=12.85	<0.0001
RMR	$F_{2,60}$=14.88	<0.0001	$F_{1,60}$=167.3	<0.0001	$F_{2,60}$=5.356	0.0072
P_{crit}	$F_{2,57}$=0.8244	0.4436	$F_{1,57}$=0.0591	0.8088	$F_{2,57}$=15.15	<0.0001
ILCM	$F_{2,48}$=7.418	0.0016	$F_{1,48}$=0.4439	0.5084	$F_{2,48}$=0.5441	0.5839
P_{50}	$F_{2,71}$=3.803	0.0270	$F_{1,71}$=21.75	<0.0001	$F_{2,71}$=0.1416	0.8682
Hct	$F_{2,67}$=9.848	0.0002	$F_{1,67}$=12.01	0.0009	$F_{2,67}$=1.955	0.1496
[Hb]	$F_{2,71}$=3.148	0.0490	$F_{1,71}$=21.36	<0.0001	$F_{2,71}$=0.9527	0.3906
MCHC	$F_{2,66}$=1.455	0.2407	$F_{1,66}$=5.770	0.0191	$F_{2,66}$=0.2663	0.7670
Glycogen						
Brain	$F_{2,63}$=11.44	<0.0001	$F_{1,63}$=0.0731	0.7877	$F_{2,63}$=0.1172	0.8896
Liver	$F_{2,44}$=0.1504	0.8608	$F_{1,44}$=0.6075	0.4399	$F_{2,44}$=0.09126	0.9130
Muscle	$F_{2,62}$=1.746	0.1829	$F_{1,62}$=1.416	0.2386	$F_{2,62}$=0.4724	0.6257
Glucose						
Brain	$F_{2,62}$=1.198	0.3087	$F_{1,62}$=3.211	0.0780	$F_{2,62}$=3.949	0.0243
Liver	$F_{2,44}$=2.339	0.1083	$F_{1,44}$=2.217	0.1436	$F_{2,44}$=1.066	0.3530
Muscle	$F_{2,62}$=5.118	0.0088	$F_{1,62}$=2.317	0.1330	$F_{2,62}$=4.345	0.0171
Lactate						
Brain	$F_{2,64}$=15.34	<0.0001	$F_{1,64}$=19.39	<0.0001	$F_{2,64}$=0.6402	0.5305
Liver	$F_{2,64}$=0.6296	0.5361	$F_{1,64}$=0.4290	0.5148	$F_{2,64}$=0.2917	0.7480
Muscle	$F_{2,60}$=7.258	0.0015	$F_{1,60}$=2.103	0.1522	$F_{2,60}$=1.913	0.1565

t_{LOE}, time to loss of equilibrium; RMR, resting metabolic rate; P_{crit}, critical O_2 tension; ILCM, interlamellar cell mass; P_{50}, O_2 tension at 50% haemoglobin saturation; Hct, haematocrit; [Hb], haemoglobin concentration; MCHC, mean corpuscular haemoglobin concentration.

Journal of Experimental Biology (2023) 226, jeb245425. doi:10.1242/jeb.245425

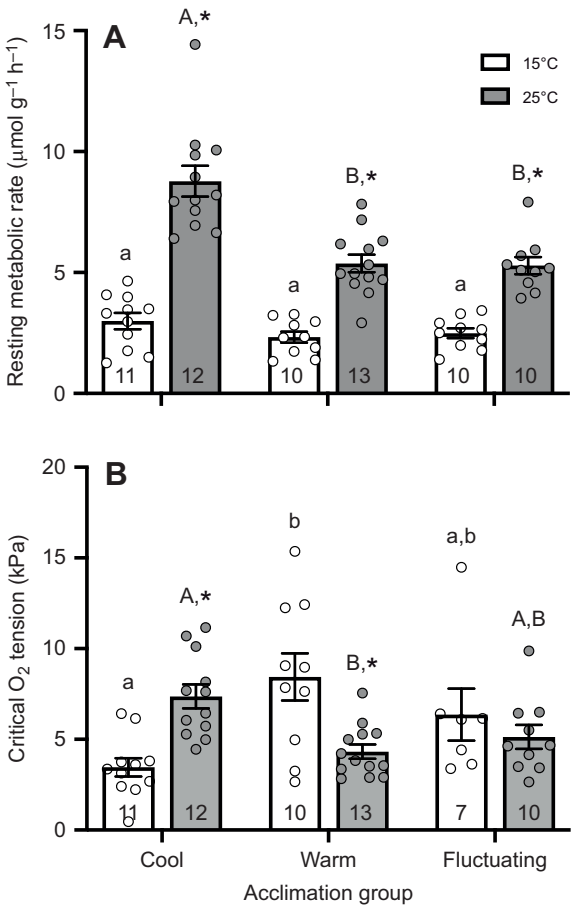

Fig. 3. Resting metabolic rate (RMR) and critical O_2 tension (P_{crit}) varied across acclimation groups and test temperatures. (A) RMR was measured as O_2 consumption rate (μmol O_2 g^{-1} body mass h^{-1}) in normoxia. (B) P_{crit} was calculated from measurements of O_2 consumption rate during exposure to stepwise progressive hypoxia (full set of measurements are shown in Fig. S1). Different test temperatures are distinguished by white (15°C) and grey (25°C) bars. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature, and asterisks indicate significant pairwise differences between test temperatures within acclimation groups. Bars represent means±s.e.m. and circles represent individual values, with samples sizes indicated within each bar.

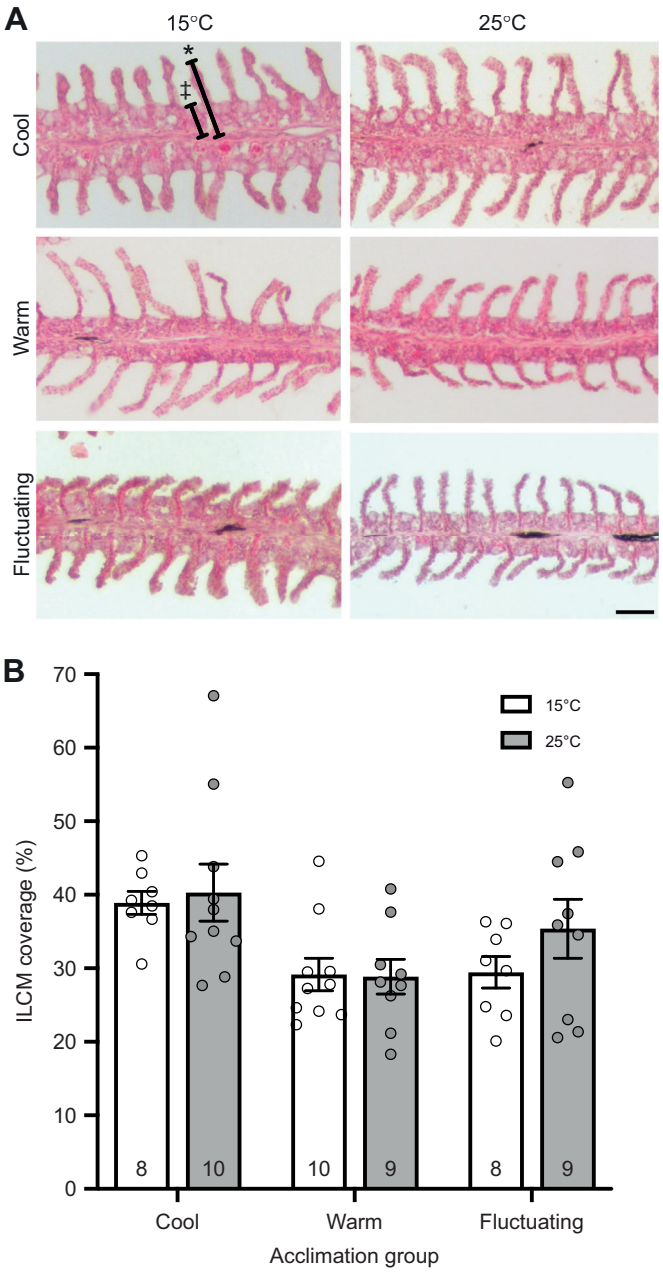

Fig. 4. Gill morphology varied between acclimation groups. (A) Representative images of gills from killifish from each acclimation group (cool, warm or fluctuating) sampled at 15°C or 25°C. Slides are stained with haematoxylin and eosin. *Total lamellar height; ‡ILCM height. Scale bar represents 50 μm and all images are shown at the same scale. (B) Interlamellar cell mass (ILCM) coverage (%) was the quotient of ILCM height and total lamellar height. Different test temperatures are distinguished by white (15°C) and grey (25°C) bars. There was a significant main effect of acclimation temperature on ILCM coverage (P=0.0016), but the pairwise comparisons between acclimation groups were not significant. Bars represent means±s.e.m. and circles represent individual values, with samples sizes indicated within each bar.

(P=0.002) but not test temperature (P=0.508) on the proportion of the lamellae that was covered by ILCM. This effect appeared to be driven by a reduction in ILCM coverage in the warm group compared with the cool group when fish were sampled at either 15°C or 25°C, but the pairwise differences between groups did not reach significance. The fluctuating group displayed ILCM coverage that was not significantly different from either the cool or warm acclimation groups, and was intermediate on average between constant acclimation groups when sampled at 25°C.

Haematology

Thermal acclimation affected haemoglobin–O_2 affinity, measured in intact erythrocytes as the P_{O_2} at 50% saturation (P_{50}) (Fig. 5A, Table 1). This was reflected by a significant effect of acclimation group on P_{50} (P=0.027), driven by lower overall values of P_{50} in the fluctuating group compared with the constant groups. There was also a significant main effect of test temperature (P<0.0001) on P_{50}, such that P_{50} was generally lower at 15°C than at 25°C, and the pairwise difference between temperatures was significant in the warm acclimation group. This expected decrease in haemoglobin–O_2 affinity with test temperature is a direct effect of the overall exothermic nature of haemoglobin oxygenation (Weber and Jensen, 1988).

Journal of Experimental Biology (2023) 226, jeb245425. doi:10.1242/jeb.245425

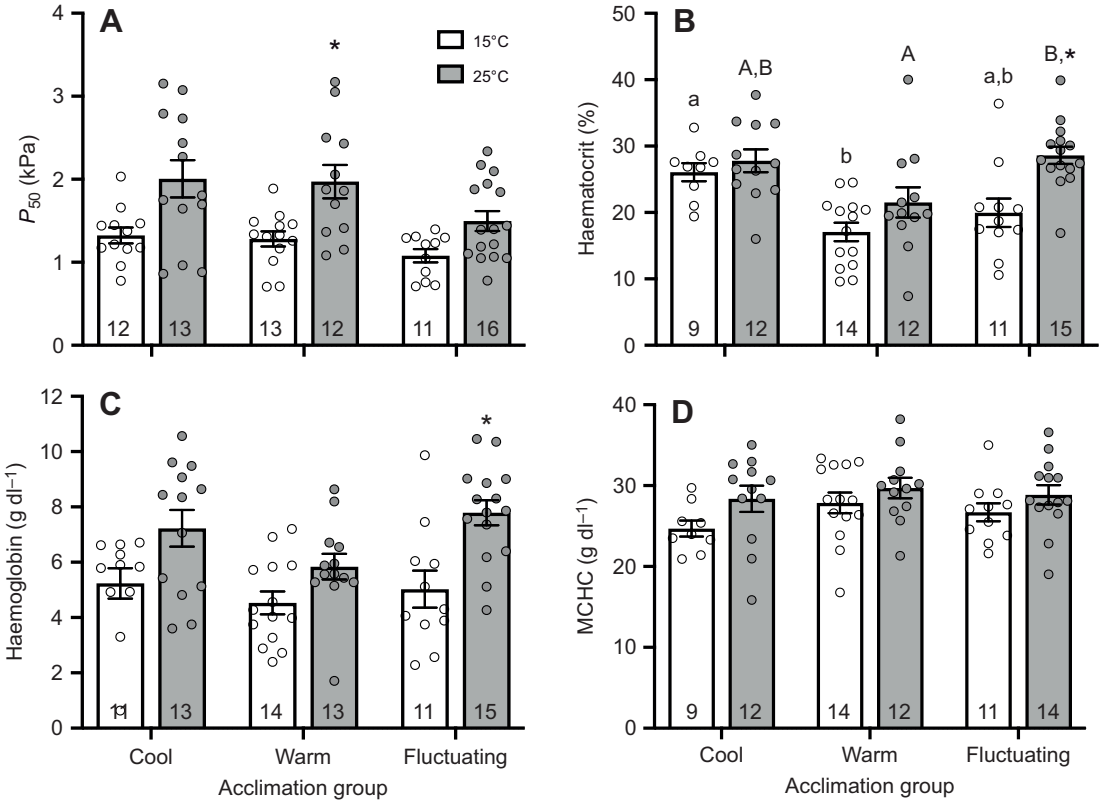

Fig. 5. Haemoglobin–O_2 affinity, haematocrit and blood haemoglobin content varied across acclimation groups and test temperatures. (A) O_2 tension at 50% haemoglobin saturation (P_{50}), measured in intact erythrocytes, (B) haematocrit, (C) whole-blood haemoglobin content (g dl^{-1} blood) and (D) mean corpuscular haemoglobin concentration (MCHC; g dl^{-1} erythrocytes). Different test temperatures are distinguished by white (15°C) and grey (25°C) bars. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature, and asterisks indicate significant pairwise differences between test temperatures within acclimation groups. Bars represent means±s.e.m. and circles represent individual values, with samples sizes indicated within each bar.

Thermal acclimation had some other haematological effects (Fig. 5, Table 1). There were significant main effects of acclimation group (P=0.0002 and P=0.049) and test temperature (P=0.0009 and P<0.0001) on both haematocrit (Fig. 5B) and blood haemoglobin content (Fig. 5C), respectively. Cool-acclimated fish displayed greater haematocrit than warm-acclimated fish, but this difference was not associated with significant corresponding differences in blood haemoglobin content. Haematocrit and blood haemoglobin content were higher at 25°C compared with 15°C in the fluctuating group, reflecting some capacity to rapidly modulate these haematological traits in response to acute changes in temperature. Although the overall pattern of variation appeared to differ slightly between these traits, there was no significant variation in mean corpuscular haemoglobin concentration (MCHC; calculated as the quotient of blood haemoglobin content and haematocrit) across acclimation groups and test temperatures (Fig. 5D).

Tissue metabolites

Thermal acclimation affected glycogen stores in the brain (acclimation effect, P<0.0001), with the highest values in the cool acclimation group (Fig. 6A). However, thermal acclimation had no effect on glycogen stores in the white muscle (Fig. 6B) or liver (Fig. 6C), and glycogen stores were largely unaffected by test temperature (Table 1). There was also significant variation in some other tissue metabolite measurements (Table 2). Brain lactate was elevated in the cool acclimation group (main effect of acclimation group, P<0.0001) but also tended to be higher overall at test temperatures of 25°C compared with 15°C (main effect of test temperature, P<0.0001). There was also a significant acclimation×test temperature interaction on free glucose in the brain (P=0.024), largely driven by elevated values in the cool acclimation group acutely transferred to 25°C. In the muscle, lactate was highest in fish acclimated to fluctuating temperatures (main effect of acclimation group, P=0.002), and there was variation in free glucose across acclimation groups that differed between test temperatures (acclimation×test temperature, P=0.017). In the liver, acclimation group had a significant effect on lactate levels (P=0.046), which appeared to be driven by higher values in the warm acclimation group, but the pairwise differences between groups were not significant.

DISCUSSION

Climate change is leading to rapid change in aquatic environments, increasing the mean and variability of aquatic temperatures, and increasing the incidence of hypoxia. This study examines how thermal history affects the ability of fish to cope with hypoxia through plastic changes in key physiological traits associated with balancing O_2 supply and demand. Thermal history had a strong influence on how hypoxia tolerance changed with acute changes in temperature. Fish acclimated to constant temperatures had the greatest t_{LOE} and lowest P_{crit} at their acclimation temperature, but the lowest t_{LOE} and highest P_{crit} following acute temperature change (Figs 2, 3), suggesting thermal specialization to the acclimation temperature. Effects of acute temperature change on t_{LOE} and P_{crit}

Journal of Experimental Biology (2023) 226, jeb245425. doi:10.1242/jeb.245425

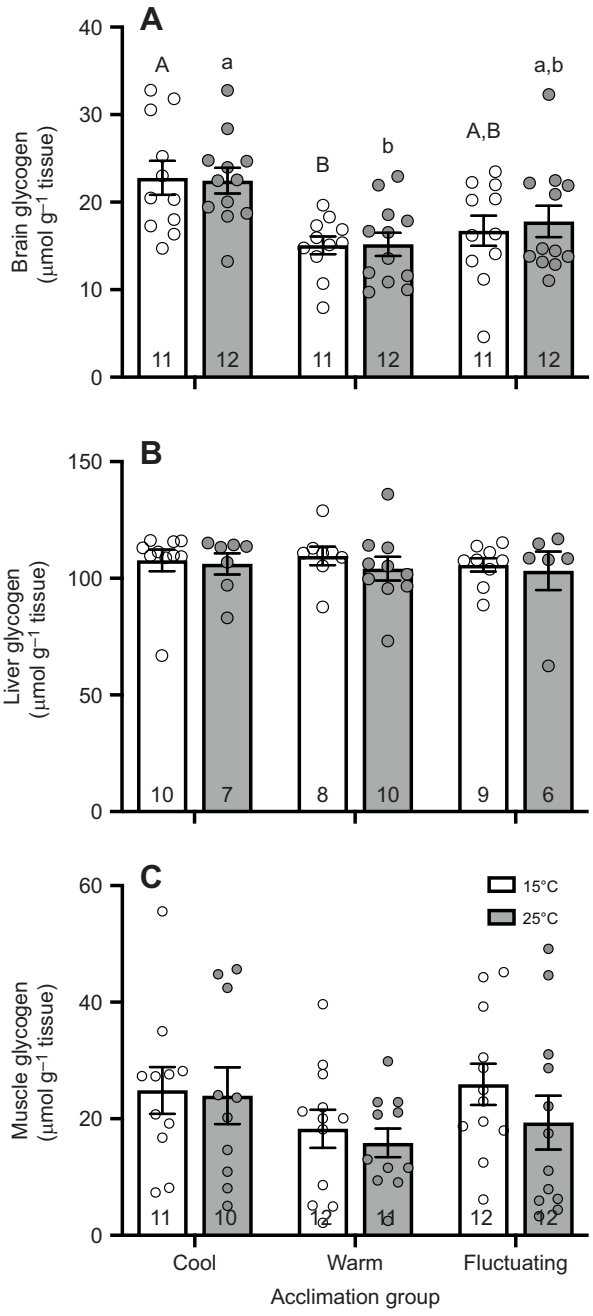

Fig. 6. Brain glycogen content was elevated in the cool acclimation group. Glycogen content (μmol g^{-1} tissue) in the (A) brain, (B) liver and (C) white axial muscle. Different test temperatures are distinguished by white (15°C) and grey (25°C) bars. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature. Bars represent means±s.e.m. and circles represent individual values, with samples sizes indicated within each bar.

were somewhat reduced in fish acclimated to fluctuating conditions, suggesting that they may acquire a more generalist thermal strategy. Variation in these metrics of hypoxia tolerance were associated with variation in physiological and morphological traits associated with O_2 uptake, circulatory O_2 transport and anaerobic metabolism (Figs 4–6). Taken together, these data contribute to the growing appreciation that thermal history and thermal environment can have strong effects on hypoxia tolerance in fish.

Acclimation to constant temperatures leads to thermal specialization and changes in O_2 uptake

Killifish acclimated to constant temperatures appeared to be thermal specialists with respect to hypoxia tolerance, based on our finding that the highest values of t_{LOE} and lowest values of P_{crit} were observed in fish tested at their acclimation temperature (Figs 2, 3). Acute temperature change affected hypoxia tolerance, with acute warming reducing t_{LOE} (i.e. t_{LOE} was lower at 25°C than at 15°C in the cool acclimation group) and acute cooling increased t_{LOE} (i.e. higher t_{LOE} at 15°C than at 25°C in the warm acclimation group). However, among the constant acclimation groups, hypoxia tolerance at a given temperature was greatest (as reflected by higher t_{LOE} and lower P_{crit}) in fish acclimated to that temperature than in fish that were acutely exposed to that temperature. Therefore, in contrast to some previous suggestions (Anttila et al., 2015; Hilton et al., 2008; McBryan et al., 2016; Sollid et al., 2005a), warm acclimation does not lead to general improvements in hypoxia tolerance across temperatures, at least in killifish. If this were the case, warm-acclimated fish would have exhibited highest t_{LOE} and lowest P_{crit} at both test temperatures. However, many previous studies did not use a full factorial design to test cool- and warm-acclimated fish at cool temperatures (Anttila et al., 2015; Hilton et al., 2008; McBryan et al., 2016), precluding the ability to assess whether effects of warm acclimation on hypoxia tolerance are temperature specific.

Improvements in hypoxia tolerance at 25°C were associated with physiological adjustments in warm-acclimated fish that may reduce discrepancies between O_2 supply and O_2 demand in hypoxia. Firstly, warm acclimation led to an apparent regression of the ILCM compared with cool-acclimated killifish (Fig. 4), as previously observed (McBryan et al., 2016). Regression of the ILCM has often been observed with acclimation to warm temperatures (Mitrovic and Perry, 2009; Sollid and Nilsson, 2006; Sollid et al., 2005b), which should increase the functional surface area of the gills and thus increase the capacity for branchial O_2 uptake (Evans et al., 2005; Nilsson and Östlund-Nilsson, 2008). Warm acclimation also reduced resting metabolic rate at 25°C (Fig. 3) (McBryan et al., 2016), which may help reduce O_2 demands in hypoxia. Some other important determinants of O_2 transport were similar between constant acclimation groups, such as the O_2 affinity and content of haemoglobin in the blood (Fig. 5). The lack of any effect of thermal acclimation on haemoglobin–O_2 affinity contrasts previous findings, in which killifish acclimated to 33°C had lower P_{50} than killifish acclimated to 15°C across each test temperature (Chung et al., 2017). This distinction could suggest that haemoglobin–O_2 affinity exhibits relatively little plasticity with acclimation between 15 and 25°C but increases above this range. Nevertheless, improvements in O_2 supply from an increased gill surface area coupled with reduced O_2 demands from a lower resting metabolic rate are likely important for reducing P_{crit} and prolonging t_{LOE} at 25°C in warm-acclimated killifish.

Improvements in hypoxia tolerance in cool-acclimated fish at 15°C cannot be explained by similar mechanisms to those improving hypoxia tolerance in warm-acclimated fish at 25°C. Indeed, cool acclimated fish had greater ILCM coverage, similar O_2 affinity and content of haemoglobin in the blood, and similar metabolic rate at 15°C as compared with warm-acclimated fish. It is possible that other determinants of O_2 transport are enhanced in cool-acclimated fish at 15°C. For example, acute exposure to reduced temperatures can reduce maximal heart rates, but this effect can be partially overcome by acclimation to reduced temperatures (Driedzic and Gesser, 1994; Gilbert and Farrell, 2021; Graham and

Journal of Experimental Biology (2023) 226, jeb245425. doi:10.1242/jeb.245425

Table 2. Effect of acclimation temperature on metabolites in killifish

Metabolite	Test temperature	Metabolite content (μmol g^{-1} tissue)		
		Constant cool	Constant warm	Fluctuating
Brain				
Lactate	15°C	16.04±0.92 (12)[a]	10.53±0.98 (11)[b]	11.47±0.72 (11)[b]
	25°C	18.09±1.23 (12)[a]	14.37±0.55 (12)[b,*]	15.18±0.78 (12)[a,b,*]
Glucose	15°C	1.82±0.14 (11)	2.14±0.27 (11)	2.85±0.54 (11)
	25°C	3.19±0.34 (12)*	2.09±0.43 (12)	2.45±0.26 (12)
Liver				
Lactate	15°C	3.26±0.37 (10)	4.39±0.33 (8)	3.93±0.48 (9)
	25°C	3.30±0.57 (7)	4.01±0.40 (10)	2.43±0.19 (6)
Glucose	15°C	3.70±0.37 (10)	3.09±0.62 (8)	4.57±1.05 (9)
	25°C	6.56±1.25 (7)	3.90±0.66 (10)	3.61±0.43 (6)
Muscle				
Lactate	15°C	17.27±1.31 (11)[a,b]	12.23±1.07 (11)[a]	22.88±2.85 (12)[b]
	25°C	19.03±3.82 (9)	18.07±1.50 (11)	23.98±2.85 (12)
Glucose	15°C	3.26±0.34 (11)[a]	3.62±0.28 (12)[a,b]	5.45±0.85 (12)[b]
	25°C	5.55±0.68 (10)*	3.44±0.29 (11)	5.00±0.58 (12)

Metabolite contents are reported as means±s.e.m., with the sample size in brackets. Dissimilar letters indicate a significant difference in pairwise comparisons between acclimation groups when compared within each test temperature, and asterisks indicate significant pairwise differences between test temperatures within acclimation groups.

Farrell, 1989). If acute exposure to cool temperature limits heart rate and cardiac output in hypoxia, prolonged acclimation to cool temperature could offset this limitation and lead to more effective perfusion of the gills. It is also possible that thermal history alters the strategy killifish use to cope with hypoxia, akin to the shifts in such strategies that occur with acclimation to different patterns of hypoxia exposure (Borowiec et al., 2018). For example, cool acclimation could increase reliance on anaerobic metabolism for coping with periods of O_2 deprivation. In this regard, our observation that cool-acclimated fish had higher levels of glycogen in the brain could suggest that they possess greater fuel reserves to sustain anaerobic glycolysis in this tissue (Fig. 6A). The brain is an energetically demanding organ and is highly susceptible to decreases in O_2 (Soengas and Aldegunde, 2002), and many studies have linked glycogen stores, enzyme activities and the maintenance of ATP levels in the brain to hypoxia sensitivity (Dunn and Hochachka, 1986; Mandic et al., 2013; Saez et al., 2014; Speers-Roesch et al., 2013). Future experiments investigating changes in tissue glycogen and ATP levels following exposure to hypoxia will be useful for strengthening the association between brain glycogen stores and LOE. In contrast, we found that acclimation temperature had no effects on glycogen levels in the liver or muscle (Fig. 6B,C), consistent with previous studies showing no correlation between these traits and hypoxia tolerance across species (Mandic et al., 2013).

Acclimation to diel cycles of temperature reduces thermal sensitivity of hypoxia tolerance

Acclimation to diel cycles of temperature appeared to shift killifish towards becoming thermal generalists with respect to hypoxia tolerance. This was supported by our findings that t_{LOE} in the fluctuating group was intermediate or nearly as high as constant groups tested at their acclimation temperature (Fig. 2), and that P_{crit} was not affected by test temperature in this group (Fig. 3). In many respects, fish in the fluctuating group had similar physiological characteristics as warm-acclimated fish, with similar resting metabolic rates, ILCM coverage and glycogen contents. However, in contrast to the warm acclimation group, fish acclimated to fluctuating temperatures demonstrated rapid and pronounced changes in blood haemoglobin content and haematocrit in response to diel temperature change (Fig. 5B,C). Rapid haematological responses to warm temperature have been observed previously (Kapila et al., 2002; Lowe and Davison, 2005; Muñoz et al., 2018), and may help improve O_2 carrying capacity and blood O_2 transport in hypoxia as long as cardiac output and capillary blood flow is not overly constrained by increases in blood viscosity (Weber and Jensen, 1988). Dynamic increases in capillary haematocrit and erythrocyte flow through capillaries are particularly important for augmenting O_2 transport to muscles during exercise in mammals (Poole, 2019), though the regulation of erythrocyte flow through capillaries during hypoxia and in fish in general is poorly understood. These increases in blood haemoglobin content in the fluctuating group at warm temperature are too rapid to be caused by erythropoiesis, and are likely attributable to splenic contraction, which is known to enable rapid modulation of blood haemoglobin content in killifish and many other species (Borowiec and Scott, 2021; Fänge and Nilsson, 1985; Lai et al., 2006; Yamamoto, 1987; Yamamoto et al., 1985). Increases in haemoglobin–O_2 affinity may also help improve hypoxia tolerance across temperatures, based on the significant effect of acclimation group on P_{50} that was driven by lower P_{50} values in the fluctuating group (Fig. 5A), which could have arisen from adjustments in intracellular concentrations of nucleotide triphosphates or intracellular pH (Dalessio et al., 1991; Nikinmaa, 1990). The latter finding suggests that acclimation to diel cycles of temperature fluctuation leads to some mechanisms of thermal plasticity that are distinct from fish acclimated to constant temperatures, and may contribute to improving hypoxia tolerance across a broad range of temperatures.

Conclusions

Our findings suggest that the interaction between thermal history and thermal environment has significant effects on the ability of fish to cope with hypoxia. Fish experiencing different thermal histories may utilize different physiological mechanisms to cope with hypoxia and exhibit different temperature sensitivities of hypoxia tolerance. Acclimation to constant temperatures appears to result in thermal specialization, as demonstrated by enhanced hypoxia tolerance at the acclimation temperature but lower tolerance following acute temperature change. Contrastingly, fish acclimated

to fluctuating temperatures demonstrate a strategy more consistent with a thermal generalist. Our results thus demonstrate that thermal fluctuations can elicit physiological adjustments that are challenging to predict from those exhibited by fish in constant thermal environments. Determining the impacts of thermal fluctuations and how they interact with other stressors associated with climate change will be vital for predicting how aquatic organisms will cope with environmental change in the future.

Acknowledgements
The authors thank Andy Turko and Brittney Borowiec for technical assistance, as well as Heather Gahs, Emily Gomes-Szoke and Tanisha Warrier for assistance with animal care.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.R.S.; Methodology: M.R.R., G.R.S.; Validation: M.R.R.; Formal analysis: M.R.R.; Investigation: M.R.R.; Resources: G.R.S.; Writing - original draft: M.R.R., G.R.S.; Writing - review & editing: M.R.R., G.R.S.; Supervision: G.R.S.; Funding acquisition: G.R.S.

Funding
This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to G.R.S. (RGPIN-2018-05707). M.R.R. was supported by a NSERC Canada Graduate Scholarship and an Ontario Graduate Fellowship, and G.R.S. is supported by the Canada Research Chairs Program.

Data availability
All data from this study are openly available through figshare: https://doi.org/10.6084/m9.figshare.22203787.v1.

References
Anttila, K., Lewis, M., Prokkola, J. M., Kanerva, M., Seppanen, E., Kolari, I. and Nikinmaa, M. (2015). Warm acclimation and oxygen depletion induce species-specific responses in salmonids. *J. Exp. Biol.* **218**, 1471-1477.

Borowiec, B. G. and Scott, G. R. (2021). Rapid and reversible modulation of blood haemoglobin content during diel cycles of hypoxia in killifish (*Fundulus heteroclitus*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol* **261**, 111054. doi:10.1016/j.cbpa.2021.111054

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

Borowiec, B. G., Crans, K. D., Khajali, F., Pranckevicius, N. A., Young, A. and Scott, G. R. (2016). Interspecific and environment-induced variation in hypoxia tolerance in sunfish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol* **198**, 59-71. doi:10.1016/j.cbpa.2016.04.010

Borowiec, B. G., Hoffman, R. D., Hess, C. D., Galvez, F. and Scott, G. R. (2020). Interspecific variation in hypoxia tolerance and hypoxia acclimation responses in killifish from the family Fundulidae. *J. Exp. Biol.* **223**, jeb209692. doi:10.1242/jeb.209692

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

Burnett, K. G., Bain, L. J., Baldwin, W. S., Callard, G. V., Cohen, S., Di Giulio, R. T., Evans, D. H., Gómez-Chiarri, M., Hahn, M. E., Hoover, C. A. et al. (2007). *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. *Comp. Biochem. Physiol. D Genomics Proteomics* **2**, 257-286. doi:10.1016/j.cbd.2007.09.001

Cameron, J. N. (1978). Regulation of blood pH in teleost fish. *Resp. Physiol.* **33**, 129-144. doi:10.1016/0034-5687(78)90092-0

Chung, D. J., Morrison, P. R., Bryant, H. J., Jung, E., Brauner, C. J., Schulte, P. M. (2017). Intraspecific variation and plasticity in mitochondrial oxygen binding affinity as a response to environmental temperature. *Sci. Rep* **7**, 16238. doi:10.1038/s41598-017-16598-6

Collins, M., Truebano, M., Verberk, W. C. E. P. and Spicer, J. I. (2021). Do aquatic ectotherms perform better under hypoxia after warm acclimation? *J. Exp. Biol* **224**, jeb232512. doi:10.1242/jeb.232512

Dalessio, P. M., DiMichele, L. and Powers, D. A. (1991). Adrenergic regulation of erythrocyte oxygen affinity, pH, and nucleotide triphosphate/hemoglobin ratio in the mummichog, *Fundulus heteroclitus*. *Physiol. Zool.* **64**, 1391-1406. doi:10.1086/physzool.64.6.30158221

Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.-O. and Huey, R. B. (2015). Climate change tightens a metabolic constraint on marine habitats. *Science* **348**, 1132-1135. doi:10.1126/science.aaa1605

Driedzic, W. R. and Gesser, H. (1994). Energy metabolism and contractility in ectothermic vertebrate hearts: hypoxia, acidosis, and low temperature. *Physiol. Rev* **74**, 221-258. doi:10.1152/physrev.1994.74.1.221

Dunn, J. F. and Hochachka, P. W. (1986). Metabolic responses of trout (*Salmo gairdneri*) to acute environmental hypoxia. *J. Exp. Biol* **123**, 229-242. doi:10.1242/jeb.123.1.229

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177. doi:10.1152/physrev.00050.2003

Fänge, R. and Nilsson, S. (1985). The fish spleen: structure and function. *Experientia* **41**, 152-158. doi:10.1007/BF02002607

Ficke, A. D., Myrick, C. A. and Hansen, L. J. (2007). Potential impacts of global climate change on freshwater fisheries. *Rev. Fish Biol. Fisheries* **17**, 581-613. doi:10.1007/s11160-007-9059-5

Gilbert, M. J. H. and Farrell, A. P. (2021). The thermal acclimation potential of maximum heart rate and cardiac heat tolerance in Arctic char (*Salvelinus alpinus*), a northern cold-water specialist. *J. Therm. Biol.* **95**, 102816. doi:10.1016/j.jtherbio.2020.102816

Graham, M. S. and Farrell, A. P. (1989). The effect of temperature acclimation and adrenaline on the performance of a perfused trout heart. *Physiol. Zool.* **62**, 38-61. doi:10.1086/physzool.62.1.30159997

Hilton, Z., Wellenreuther, M. and Clements, K. D. (2008). Physiology underpins habitat partitioning in a sympatric sister-species pair of intertidal fishes. *Funct. Ecol.* **22**, 1108-1117. doi:10.1111/j.1365-2435.2008.01465.x

Hughes, G. M. (1973). Respiratory responses to hypoxia in fish. *Am. Zool.* **13**, 475-489. doi:10.1093/icb/13.2.475

Jung, E. H., Brix, K. V., Richards, J. G., Val, A. L. and Brauner, C. J. (2020). Reduced hypoxia tolerance and survival at elevated temperatures may limit the ability of Amazonian fishes to survive in a warming world. *Sci. Total Environ.* **748**, 141349. doi:10.1016/j.scitotenv.2020.141349

Kapila, R., Kapila, S. and Basade, Y. (2002). Impact of temperature variation on haematology and serum enzymes of *Schizothorax richardsonii* (Gray). *Indian J. Fish.* **49**, 187-192.

Lagerspetz, K. Y. H. (2006). What is thermal acclimation? *J. Therm. Biol.* **31**, 332-336. doi:10.1016/j.jtherbio.2006.01.003

Lai, J. C. C., Kakuta, I., Mok, H. O. L., Rummer, J. L. and Randall, D. (2006). Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. *J. Exp. Biol.* **209**, 2734-2738. doi:10.1242/jeb.02279

Lowe, C. J. and Davison, W. (2005). Plasma osmolarity, glucose concentration and erythrocyte responses of two Antarctic notothenid fishes to acute and chronic thermal change. *J. Fish Biol.* **67**, 752-766. doi:10.1111/j.0022-1112.2005.00775.x

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

Mandic, M., Speers-Roesch, B. and 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. doi:10.1086/667938

McBryan, T. L., Healy, T. M., Haakons, K. L. and Schulte, P. M. (2016). Warm acclimation improves hypoxia tolerance in *Fundulus heteroclitus*. *J. Exp. Biol.* **219**, 474-484. doi:10.1242/jeb.133413

Mitrovic, D. and Perry, S. F. (2009). The effects of thermally induced gill remodeling on ionocyte distribution and branchial chloride fluxes in goldfish (*Carassius auratus*). *J. Exp. Biol.* **212**, 843-852. doi:10.1242/jeb.025999

Muñoz, N. J., Farrell, A. P., Heath, J. W. and Neff, B. D. (2018). Hematocrit is associated with thermal tolerance and modulated by developmental temperature in juvenile chinook salmon. *Physiol. Biochem. Zool.* **91**, 757-762. doi:10.1086/695556

Nikinmaa, M. (1990). *Vertebrate Red Blood Cells – Adaptations of Function to Respiratory Requirements*. New York: Springer-Verlag.

Nilsson, G. E. and Östlund-Nilsson, S. (2008). Does size matter for hypoxia tolerance in fish? *Biol. Rev.* **83**, 173-189. doi:10.1111/j.1469-185X.2008.00038.x

Parmesan, C. (2006). Ecological and evolutionary responses to recent climate change. *Annu. Rev. Ecol. Evol. Syst.* **37**, 637-669. doi:10.1146/annurev.ecolsys.37.091305.110100

Parmesan, C. and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature* **421**, 37-42. doi:10.1038/nature01286

Poole, D. C. (2019). Edward F. Adolph Distinguished Lecture. Contemporary model of muscle microcirculation: gateway to function and dysfunction. *J. Appl. Physiol.* **127**, 1012-1033. doi:10.1152/japplphysiol.00013.2019

Rasband, W. S. (2008). Image J.

Reemeyer, J. E. and Rees, B. B. (2019). Standardizing the determination and interpretation of P_{crit} in fishes. *J. Exp. Biol.* **222**, jeb.210633. doi:10.1242/jeb.210633

Saez, I., Duran, J., Sinadinos, C., Beltran, A., Yanes, O., Tevy, M. F., Martínez-Pons, C., Milán, M. and Guinovart, J. J. (2014). Neurons have an active

glycogen metabolism that contributes to tolerance to hypoxia. *J. Cereb. Blood Flow Metab.* **34**, 945-955. doi:10.1038/jcbfm.2014.33

Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J. Exp. Biol.* **218**, 1856-1866. doi:10.1242/jeb.118851

Schulte, P. M. (2007). Responses to environmental stressors in an estuarine fish: Interacting stressors and the impacts of local adaptation. *J. Therm. Biol.* **32**, 152-161. doi:10.1016/j.jtherbio.2007.01.012

Schulte, P. M., Healy, T. M. and Fangue, N. A. (2011). Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integr. Comp. Biol.* **51**, 691-702. doi:10.1093/icb/icr097

Seebacher, F., Ducret, V., Little, A. G. and Adriaenssens, B. (2015). Generalist–specialist trade-off during thermal acclimation. *R. Soc. Open Sci.* **2**, 140251. doi:10.1098/rsos.140251

Soengas, J. L. and Aldegunde, M. (2002). Energy metabolism of fish brain. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **131**, 271-296. doi:10.1016/S1096-4959(02)00022-2

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

Sollid, J., Weber, R. E. and Nilsson, G. E. (2005a). Temperature alters the respiratory surface area of crucian carp *Carassius carassius* and goldfish *Carassius auratus*. *J. Exp. Biol.* **208**, 1109-1116. doi:10.1242/jeb.01505

Sollid, J., Kjernsli, A., De Angelis, P. M., Røhr, Å. K. and Nilsson, G. E. (2005b). Cell proliferation and gill morphology in anoxic crucian carp. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **289**, R1196-R1201. doi:10.1152/ajpregu.00267.2005

Speers-Roesch, B., Mandic, M., Groom, D. J. E. and 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. doi:10.1016/j.jembe.2013.10.006

Sunday, J. M., Bates, A. E. and Dulvy, N. K. (2012). Thermal tolerance and the global redistribution of animals. *Nature Clim. Change* **2**, 686-690. doi:10.1038/nclimate1539

Tyler, R. M., Brady, D. C. and Targett, T. E. (2009). Temporal and spatial dynamics of diel-cycling hypoxia in estuarine tributaries. *Estuaries Coasts* **32**, 123-145. doi:10.1007/s12237-008-9108-x

Weber, R. E. and Jensen, F. B. (1988). Functional adaptations in hemoglobins from ectothermic vertebrates. *Ann. Rev. Physiol.* **50**, 161-179. doi:10.1146/annurev.ph.50.030188.001113

Yamamoto, K.-I. (1987). Contraction of spleen in exercised cyprinid. *Comp. Biochem. Physiol. A* **87**, 1083-1087. doi:10.1016/0300-9629(87)90043-0

Yamamoto, K., Itazawa, Y. and Kobayashi, H. (1985). Direct observation of fish spleen by an abdominal window method and its application to exercised and hypoxic yellowtail. *J. Ichthyol.* **31**, 427-433.