© 2016. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

RESEARCH ARTICLE

Warm acclimation improves hypoxia tolerance in *Fundulus heteroclitus*

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ABSTRACT

Human activities are increasing both the frequency of hypoxic episodes and the mean temperature of aquatic ecosystems, but few studies have considered the possibility that acclimation to one of these stressors could improve the ability to cope with the other stressor. Here, we used Atlantic killifish, *Fundulus heteroclitus*, to test this hypothesis. Hypoxia tolerance was measured as time to loss of equilibrium in hypoxia (LOE_{hyp}) at 0.4 kPa oxygen. Time to LOE_{hyp} declined from 73.3±6.9 min at 15°C to 2.6±3.8 min at 23°C, and at 30°C no fish could withstand this level of hypoxia. Prior acclimation to warm temperatures significantly increased time to LOE_{hyp}. Hypoxia tolerance of the southern subspecies of killifish, *F. heteroclitus heteroclitus*, was greater than that of the northern subspecies, *F. heteroclitus macrolepidotus*, measured both as critical oxygen tension (P_{crit}) and as time to LOE_{hyp}. Warm acclimation offset the negative effects of temperature on time to LOE_{hyp} to a similar extent in the two subspecies. Warm acclimation increased total lamellar surface area of the gill in both subspecies as a result of regression of an interlamellar cell mass (ILCM). However, differences in total lamellar surface area could not explain differences in time to LOE_{hyp} between the subspecies, suggesting that the lower time to LOE_{hyp} of northern fish is related to their higher routine metabolic rate. These data suggest that thermal plasticity in gill morphology can improve the capacity of this species to tolerate hypoxia, and shows how existing plasticity may help organisms to cope with the complex interacting stressors that they will encounter with increasing frequency as our climate changes.

KEY WORDS: Gill, Critical oxygen tension, Interlamellar cell mass, ILCM, Teleost, Temperature

INTRODUCTION

Understanding the impacts of multiple interacting stressors is critical for predicting how animals will respond to human-induced climate change (Rudd, 2014; Todgham and Stillman, 2013; McBryan et al., 2013). However, most studies of stressors relevant to climate change focus on the effects of single stressors in isolation, even though it is known that stressors may act quite differently when experienced in combination (Wernberg et al., 2012; Crain et al., 2008). In addition, few studies have considered the possibility that the physiological changes that organisms undergo to cope with chronic exposure to one stressor (i.e. changes as a result of acclimation) could alter sensitivity to another stressor, despite the fact that such effects might be expected if there is a mechanistic link between the processes altered by acclimation to one stressor and the processes affected by the other stressor.

In coastal marine ecosystems, high temperature and low oxygen (hypoxia) are critical co-occurring stressors that can have negative effects on organisms (Levin and Breitburg, 2015). Increases in temperature are occurring because of global climate change (Harley et al., 2006), and episodes of hypoxia are increasing in extent and severity as a result of eutrophication due to local factors such as agricultural runoff (Diaz and Rosenberg, 1995, 2008, 2011; Kemp et al., 2009). These stressors have the capacity to reinforce each other because increasing temperature decreases oxygen solubility (García and Gordon, 1992) and simultaneously increases the respiration rates of the microorganisms that are the cause of aquatic hypoxia, worsening the severity of hypoxic events (Iriberri et al., 1985).

For water-breathing ectotherms such as fish, this combination of low oxygen and high temperature is exceptionally challenging, and a level of hypoxia that has modest or no effects at low temperature may be lethal at higher temperatures (Schurmann and Steffensen, 1992). The interactive effects of increased temperature and reduced environmental oxygen are hypothesized to occur because both of these stressors affect aerobic metabolism. As temperature increases, metabolic rate increases exponentially as a result of thermodynamic effects on biochemical reactions, with an associated increase in oxygen demand (Schulte, 2015). Hypoxia, in contrast, limits the availability of environmental oxygen, making it more challenging to meet the increased metabolic demand for oxygen as a result of increased temperature.

Thermal acclimation is known to alter a variety of physiological processes associated with oxygen supply and demand in fish, and can alter the capacity for oxygen uptake by changing gill surface area through regression of an interlamellar cell mass (ILCM) with acclimation to warmer temperatures (Sollid et al., 2005b; Mitrovic and Perry, 2009). These effects of thermal acclimation on processes related to oxygen supply suggest that thermal acclimation could result in changes in hypoxia tolerance. Here, we tested this possibility using the Atlantic killifish (*Fundulus heteroclitus*) as an experimental system.

Atlantic killifish are common in estuaries and salt marshes along the Atlantic coast of North America from Quebec, Canada, to northeastern Florida, USA. These salt marshes are highly variable habitats that undergo daily and seasonal variation in temperature and oxygen levels (Schulte, 2007). Killifish are salt marsh residents that spend their entire lifecycle within the marsh, and thus this species has adapted to cope with these stressors (Schulte, 2013). Killifish are among the most extreme eurytherms known among teleost fishes (Fangue et al., 2006), and they are also exceptionally tolerant of hypoxia (Cochran and Burnett, 1996; Richards et al., 2008). Killifish demonstrate a substantial ability to acclimate to a wide range of temperatures (Fangue et al., 2006), and previous studies have demonstrated that thermal acclimation changes ILCM

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Received 19 October 2015; Accepted 1 December 2015

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

List of symbols and abbreviations

ILCM	interlamellar cell mass
LOE_{hyp}	loss of equilibrium in hypoxia
$\dot{M}_{O_2}$	routine oxygen consumption (mass specific)
P_{crit}	critical oxygen tension
P_{50}	oxygen tension at which haemoglobin is 50% saturated
SEM	scanning electron microscopy

thickness in this species (Barnes et al., 2014). Furthermore, seasonal acclimatization has been shown to alter hypoxia tolerance in the closely related *Fundulus grandis* (Love and Rees, 2002). Therefore, we predicted that acclimation to warm temperatures would result in increased tolerance of hypoxia as a result of increases in gill surface area in killifish.

There is also geographical variation in genetics, morphology and physiology among killifish populations such that northern and southern populations have been designated as separate subspecies (Morin and Able, 1983), with *F. heteroclitus heteroclitus* (Linnaeus 1766) in the south and *F. heteroclitus macrolepidotus* (Walbaum 1792) in the north. The southern subspecies is more tolerant of high temperatures (Fangue et al., 2006) and has a lower routine metabolic rate than does the northern subspecies (Fangue et al., 2009; Healy and Schulte, 2012). Therefore, we predicted that killifish of the southern subspecies would have a greater ability to tolerate hypoxia than would northern killifish as a result of their lower oxygen demand. In contrast, we predicted that northern and southern populations of killifish would show similar plasticity in these traits because both subspecies experience similar extents of seasonal and daily variation in temperature and oxygen in their estuarine habitats (Schulte, 2007), which would be expected to result in strong selection for plasticity in both subspecies (Scheiner, 1993).

MATERIALS AND METHODS

Fish care

Fundulus heteroclitus macrolepidotus (northern subspecies) were collected near Hampton, NH, USA (42°54′46″N) by Aquatic Research Organisms (Inc.). The northern fish used for critical oxygen tension (P_{crit}) determination were collected in March 2008, and those used for all other experiments were collected in March 2012. *Fundulus heteroclitus heteroclitus* (southern subspecies) used for P_{crit} determination were collected near Brunswick, GA, USA (31°7′31″N) by L. Glass (North Carolina State University) in March 2008. The southern fish used for all other experiments were collected near Sapelo Island, GA, USA (31°27′13.6″N) in September 2012. In addition, a group of fish from a geographically intermediate population was collected in May 2008 to determine the body mass scaling relationships for $\dot{M}_{O_2}$ and P_{crit} (Beaverdam Creek, Point Pleasant, NJ, USA; 40°03′0″N). In all cases, fish were collected using un-baited Gee's G40-type minnow traps from publicly accessible land. No collection permits are required for this species. All fish were air shipped to the University of British Columbia and initially held in a single recirculating system with a photoperiod of 12 h light:12 h dark, at a temperature of 15°C in 200 l fibreglass tanks containing artificial seawater at a salinity of 20‰, achieved using Instant Ocean Aquarium Salt (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA). Fish were fed daily to satiation with commercial fish flakes.

Fish were held under these conditions for at least 3 weeks to allow them to recover from transport to the laboratory. They were then individually tagged subcutaneously with Visible Implant Elastomer Tags (Northwest Marine Technology, Inc., Shaw Island, WA, USA), and transferred to 110 l glass aquaria under the same salinity, temperature and photoperiod conditions as in the holding tanks. After 2 weeks, the temperature in the tanks was adjusted to the appropriate experimental acclimation temperature (see below), and the fish were held for at least 6 weeks prior to experimentation. All holding and experimental procedures were approved by the UBC Animal Care Committee on behalf of the Canadian Council on Animal Care and were conducted under approved protocols A07-0288 and A11-0372.

Determination of time to loss of equilibrium in hypoxia

Time to loss of equilibrium in hypoxia (LOE_{hyp}) was determined simultaneously for eight fish. The apparatus consisted of a rectangular Plexiglas tank containing eight plastic beakers (1 l volume) with their sides replaced by 1×1 cm plastic mesh. An individual fish was placed in each beaker 15 min prior to the beginning of the trial. To prevent fish from acquiring oxygen by aquatic surface respiration, plastic mesh was also fitted 3 cm below the water surface in each beaker. The water surface in the tank and beakers was covered with plastic bubble wrap to ensure that oxygen from the air did not diffuse into the water during the trials. Water oxygen levels were reduced by bubbling N_2 gas. Dissolved oxygen was monitored throughout the experiment using a fibre optic oxygen probe (NEO-Fox, Ocean Optics Ltd, Dunedin, FL, USA). A submersible pump was used to ensure adequate water circulation in the experimental tank.

In each trial, oxygen was reduced from 21.2 kPa (100% air saturation) to 0.4 kPa (2% air saturation) over ∼1 h and held at this level by manually adjusting N_2 flow. We selected 0.4 kPa for the hypoxic trials because it is close to the oxygen tension at which haemoglobin is 50% saturated (P_{50}) for *F. heteroclitus* (DiMichele and Powers, 1982). During pilot studies, most fish could maintain equilibrium indefinitely at 0.8 kPa, whereas at 0.3 kPa fish lost equilibrium rapidly and there was little variation among individuals. We defined time to LOE_{hyp} as the time (in minutes) after the apparatus reached 0.4 kPa until the fish lost equilibrium. LOE_{hyp} was determined as the point when a fish was no longer able to respond to gentle movement of the beaker. At the highest temperatures tested, a small number of fish lost equilibrium before the ambient oxygen reached 0.4 kPa. For these individuals, time to LOE_{hyp} was recorded as a negative value (time before the apparatus reached 0.4 kPa). After reaching LOE_{hyp} the fish were weighed and measured, and sex was determined. Fish were then returned to their acclimation tanks.

Two experimental series were performed. In the first series, only fish of the northern subspecies were utilized. To determine the effects of acute temperature increases on hypoxia tolerance, we used fish acclimated to 15°C. Fish were introduced into the LOE_{hyp} apparatus at their acclimation temperature, and after 15 min the temperature in the apparatus was gradually increased by 0.2°C min^{-1} to the appropriate test temperature (20, 23 or 30°C, or held at 15°C as a control). At this point, the hypoxia tolerance trial was conducted as described above.

To assess the effect of thermal acclimation on hypoxia tolerance, the fish used to test the effects of acute temperature increase were allowed to recover from the LOE_{hyp} trial for 24 h at 15°C and then acclimated to the temperature they had been exposed to in the LOE_{hyp} apparatus (15, 20, 23 or 30°C) for at least 6 weeks. Time to LOE_{hyp} was then assessed at the relevant acclimation temperature. Preliminary experiments indicated that time to LOE_{hyp} was not influenced by repeated measurement (P>0.05 for eight fish assayed

Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

on four consecutive days, suggesting that repeated exposure to hypoxia during a trial is not sufficient to cause changes in hypoxia tolerance; data not shown).

In the second experimental series, both the northern and southern subspecies were examined, with four individuals of each subspecies tested simultaneously in the LOE_{hyp} apparatus (with a total of 8 fish per subspecies tested across two trials). To determine the effects of acute temperature increase on hypoxia tolerance, fish were acclimated to 15°C, and time to LOE_{hyp} was determined at 15°C and after acute exposure to 23°C, as described above. The fish were then acclimated to the temperature at which time to LOE_{hyp} was tested (15 or 23°C) for at least 6 weeks, and time to LOE_{hyp} was determined at the relevant acclimation temperature. There was no change in time to LOE_{hyp} at 15°C over this 6 week period (data not shown).

Measurement of routine metabolic rate and P_{crit}

Routine oxygen consumption ($\dot{M}_{O_2}$) and P_{crit} were determined at 20°C and a salinity of 20‰ using closed respirometry, for fish of both subspecies acclimated to these conditions. The apparatus consisted of four glass jars of approximately 220 ml capacity and a multichannel fibre optic oxygen probe (FOXY-R, Ocean Optics Ltd). Each chamber contained a magnetic stir bar and was placed over an individual stir plate to ensure proper mixing. Temperature in the chambers was regulated by partially submerging each chamber in a 20°C water bath. Prior to measurement, one fish was placed in each respirometer overnight (16–20 h) under flow-through conditions. At the start of a trial, a probe was placed in each chamber and the respirometer was sealed to allow measurement of the decrease in oxygen concentration over time, using a sampling rate of once every 15 s. The respirometers were rinsed daily with a 75% ethanol solution, which resulted in negligible bacterial respiration (<1% of total oxygen consumption) in all trials.

Each fish remained in the closed respirometer until the partial pressure of oxygen (P_{O_2}) reached ∼1.5 kPa or until the fish began to show signs of distress. Trials took between 45 and 120 min and fish were generally quiescent for this duration. Any fish that struggled during the trial was removed from the data set. $\dot{M}_{O_2}$ was calculated following Henriksson et al. (2008). The differential solubility of oxygen in water of varying salinities was corrected for using the solubility coefficients, αO_2, at a constant temperature of 20°C (Boutilier et al., 1984).

The change in water [O_2] over time was calculated over sequential 5 min intervals and corrected for fish wet mass and respirometer volume to give $\dot{M}_{O_2}$ (μmol g^{-1} h^{-1}) for each individual. Calculated $\dot{M}_{O_2}$ values for each 5 min interval were then plotted against the mean water P_{O_2} for that interval. P_{crit} is defined as the inflection point where $\dot{M}_{O_2}$ transitions from being independent of environmental P_{O_2} to being dependent on environmental P_{O_2} (Pörtner and Grieshaber, 1993), and was calculated independently for each individual. P_{crit} is often determined using a two-segment linear regression model (Yeager and Ultsch, 1989), but we found this approach to be inaccurate in *F. heteroclitus* because many individuals showed a slight increase in metabolic rate just above the probable P_{crit}. This resulted in a regression with significant negative slope from ∼7 kPa to normoxia, which seemed likely to result in mis-estimation of P_{crit}. We therefore first established the routine $\dot{M}_{O_2}$ for each individual using the slope of the shallowest portion of the dissolved oxygen trace at P_{O_2} greater than 8 kPa where that slope was maintained for at least a 5 min interval. We then defined a horizontal line with *y*-intercept at this routine $\dot{M}_{O_2}$ and determined its intersection with the equation for the linear regression through the calculated $\dot{M}_{O_2}$ values that were more than 12% below the calculated routine $\dot{M}_{O_2}$. We chose this threshold because pilot studies on $\dot{M}_{O_2}$ repeatability indicated that we could accurately and repeatably determine the routine $\dot{M}_{O_2}$ of an individual to within ±12%.

$\dot{M}_{O_2}$ scales with body mass in fish (Clarke and Johnston, 1999), and there is the potential for body mass scaling of P_{crit} as well. Thus, it is important to determine whether differences between populations in either of these parameters are independent of body mass differences between the groups. However, the sample size within each population in this experiment was not sufficient to accurately determine the body mass scaling relationship of these parameters for *F. heteroclitus*. We therefore determined $\dot{M}_{O_2}$ and P_{crit} for a geographically intermediate population of *F. heteroclitus* (*N*=21) and used these data to determine scaling relationships that could be used to calculate residuals of $\dot{M}_{O_2}$ and P_{crit} for both the northern and southern populations to account for the potential effects of body mass on these parameters.

Gill morphometrics

To examine the effects of a wide range of acclimation temperatures on gill morphometrics, we utilized gill samples from northern and southern *F. heteroclitus* from a previous experiment (Healy and Schulte, 2012) in which fish were acclimated to 5, 10, 15, 20, 25, 30 or 33°C (20‰ salinity, 12 h:12 h photoperiod). Gill baskets were preserved in Karnovsky's fixative (25‰ glutaraldehyde, 16% formaldehyde, 0.15 mol l^{-1} PBS buffer, pH 7.4) and transferred to 0.15 mol l^{-1} sodium cacodylate after 24 h of fixation and stored at 4°C until use. All samples were given a random code, and all microscopy and image analysis were performed blind. We cut out the second gill arch from the gill basket and used light microscopy [Olympus Stereomicroscope SZX10; 6.3× magnification, 10× zoom; image capture using cellSens Software (standard)] to determine the number of filaments, filament length, lamellar frequency, lamellar height and the basal length of the lamella (Wegner, 2011). The length of the gill arch was divided into five groups of ∼10 filaments, and we removed one filament from each of these groups for photomicrography. We obtained images in two orientations such that the lamellae were viewed from both the side and the top. Care was taken to ensure that the images were taken at an orientation that provided undistorted measurements. Images were digitized using ImageJ (v1.48). We estimated lamellar frequency based on the distance between five lamellae at a randomly chosen point along the filament. We measured lamellar height and basal length for three lamellae from each filament: one from the base of the filament, one from the middle, and one from the tip. Lamellar surface area was estimated as two times the product of lamellar height and lamellar basal length. Because this approximation does not take into account the potential for gas exchange along the 'top' of the lamella, it may underestimate lamellar surface area; however, analysis of previously published data (e.g. Matey et al., 2008; Brauner et al., 2011) suggests that it results in no more than a 10% reduction in estimated area compared with an approach modelling the lamellae as a half-ellipsoid (e.g. Matey et al., 2008), and it does not change the relationship among groups.

To examine gill structure in more detail, gills sampled in the current experiment from northern and southern *F. heteroclitus* acclimated to 15 and 23°C were used for scanning electron microscopy (SEM) and histological staining. Gills were preserved as described above. For SEM, sections of the second gill arch were dehydrated progressively in ethanol, critically point dried, and then coated in 9 nm of gold palladium by the UBC Bioimaging Facility. These samples were imaged on a Hitachi S-4700 Field Emission Scanning Electron Microscope at 500× magnification. In

Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

preparation for histology, 4 mm pieces of the second gill arch were paraffin wax embedded and serologically sectioned at 5 µm, and mounted and stained with Hematoxylin and Eosin by Wax-it Histology Services (UBC). These sections were imaged using a Motic AE31 inverted microscope at 400× magnification.

Statistical analysis

Statistical analyses were performed using R (v3.0.2). All data are presented as means ± s.e.m. In the first experimental series (effects of acclimation on time to LOE_{hyp} in northern fish), data were analysed using a linear mixed model with test temperature and treatment (acute versus acclimated) as fixed factors, with individual as a random factor. For the second experimental series (effects of subspecies and acclimation on time to LOE_{hyp}), data were analysed using a linear mixed model with test temperature, treatment (acute versus acclimated) and subspecies as fixed factors, with individual as a random factor. *Post hoc* tests were carried out using Tukey's HSD. Differences in $\dot{M}_{O_2}$ and P_{crit} between subspecies were analysed using *t*-tests. Gill morphometric data were analysed using two-way ANOVA with acclimation temperature and subspecies as factors. Because of the large number of treatment groups, and the fact that only a small subset of all possible comparisons is biologically meaningful, we used planned comparisons to assess the effects of acclimation temperature within a subspecies, and the effects of subspecies at each acclimation temperature. These planned comparisons were performed using *t*-tests, and the resulting *P*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method (Thissen et al., 2002). All data were normally distributed and met the assumptions of all tests; α was set at 0.05.

RESULTS

The effect of temperature and thermal acclimation on hypoxia tolerance

Acute exposure to increased temperature had a significant effect on time to LOE_{hyp} of northern killifish (P=0.02; Fig. 1). At 15°C, northern killifish could maintain equilibrium for ∼75 min (73.3±6.9 min) when exposed to hypoxia, whereas at 23°C these fish were only able to maintain equilibrium for ∼3 min (2.6±3.8 min). In fact, at the highest temperature tested (30°C), fish lost equilibrium before the apparatus reached 0.4 kPa (2% air saturation), which we recorded as a negative time to loss of equilibrium. Thermal acclimation increased time to LOE_{hyp} (P=0.01) and there was no significant interaction between test temperature and acclimation temperature (P=0.47), suggesting that acclimation had similar effects across all of the temperatures tested.

Differences between subspecies in metabolic rate and hypoxia tolerance

Fig. 2A shows the effects of ambient P_{O_2} on $\dot{M}_{O_2}$ in both subspecies of killifish. Consistent with previous observations (Healy and Schulte, 2012), fish from the northern subspecies had higher $\dot{M}_{O_2}$ than did fish of the southern subspecies (P=0.03; Fig. 2B). Fish of the northern subspecies also had a significantly higher P_{crit} (P=0.003; Fig. 2C).

Consistent with the differences between subspecies in P_{crit}, there was also a significant difference in time to LOE_{hyp} between subspecies (P=1×10^{-10}; Fig. 3). Acute exposure to increased temperature (23°C) caused a decrease in time to LOE_{hyp} in both subspecies (P=5×10^{-10}) and there was no significant interaction between these factors (P=0.68). Acute thermal exposure caused a similar absolute decrease in time to LOE_{hyp} in the northern and southern subspecies (∼70–80 min). Acclimation increased time to LOE_{hyp} in both subspecies (P=4×10^{-5}) and again there was no significant interaction between these factors (P=0.85).

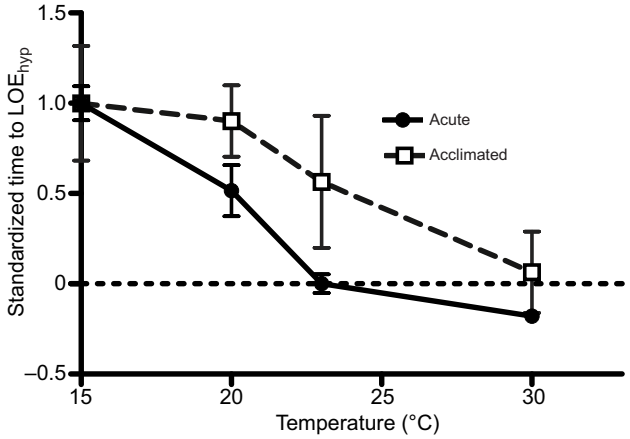

Fig. 1. Standardized time to loss of equilibrium in hypoxia (LOE_{hyp}) in northern killifish (*Fundulus heteroclitus macrolepidotus*) acutely exposed or acclimated to different temperatures. Fish were acclimated to 15°C and tested at the temperature indicated (filled symbols), or acclimated to and tested at their acclimation temperature (open symbols). Time to LOE_{hyp} was determined at 0.4 kPa. Data were normalized to the time to LOE_{hyp} of 15°C-acclimated fish tested at 15°C. Sample sizes: N=6 for all groups except for fish acclimated to 20°C where N=7. All data are reported as means±s.e.m. Effect of acute temperature change P=0.02; effect of acclimation P=0.01; interaction P=0.47.

Because $\dot{M}_{O_2}$ scales with body mass in fish (Clarke and Johnston, 1999), it is important to ensure that the observed differences in $\dot{M}_{O_2}$ and P_{crit} cannot be accounted for by differences in body mass between the populations. We determined the scaling relationships for these parameters using a geographically intermediate population of *F. heteroclitus*. The mass-specific $\dot{M}_{O_2}$ of this population was 8.0±0.64 µg g^{-1} h^{-1}. Log $\dot{M}_{O_2}$ scaled significantly with log body mass (P=0.0001) according to the following equation: y=0.69x+1.04 (R^2=0.53, N=21). Note that these calculations were performed using whole-organism $\dot{M}_{O_2}$ rather than mass-specific $\dot{M}_{O_2}$. Comparison of the residuals from this equation revealed that the $\dot{M}_{O_2}$ of northern and southern populations of *F. heteroclitus* still differed after body mass was taken into account (P=0.004; northern fish residual=0.17±0.03; southern fish residual=0.05±0.02). The P_{crit} of the geographically intermediate population was 4.9±0.02 kPa. Unlike for $\dot{M}_{O_2}$, there was no significant relationship between P_{crit} and body mass (P=0.61, N=21), and therefore no body mass correction was applied to this parameter.

The effect of thermal acclimation and subspecies on gill morphology

Tables 1 and 2 and Fig. 4 summarize the effects of temperature on quantitative measurements of gill morphology in the two subspecies of *F. heteroclitus*. When considered without accounting for body mass (Fig. 4A), total lamellar surface area of both northern and southern fish increased significantly with exposure to increasing temperature (P=4×10^{-11}), and there was no significant difference between subspecies (P=0.10), but there was a significant interaction between subspecies and temperature (P=0.04). Thus, when planned comparisons were used to test the effect of subspecies at each acclimation temperature, we detected a significant difference in lamellar surface area between northern and southern killifish at 15°C (Fig. 4), with northern fish having a higher lamellar surface area than did southern fish. When total lamellar surface area was

Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

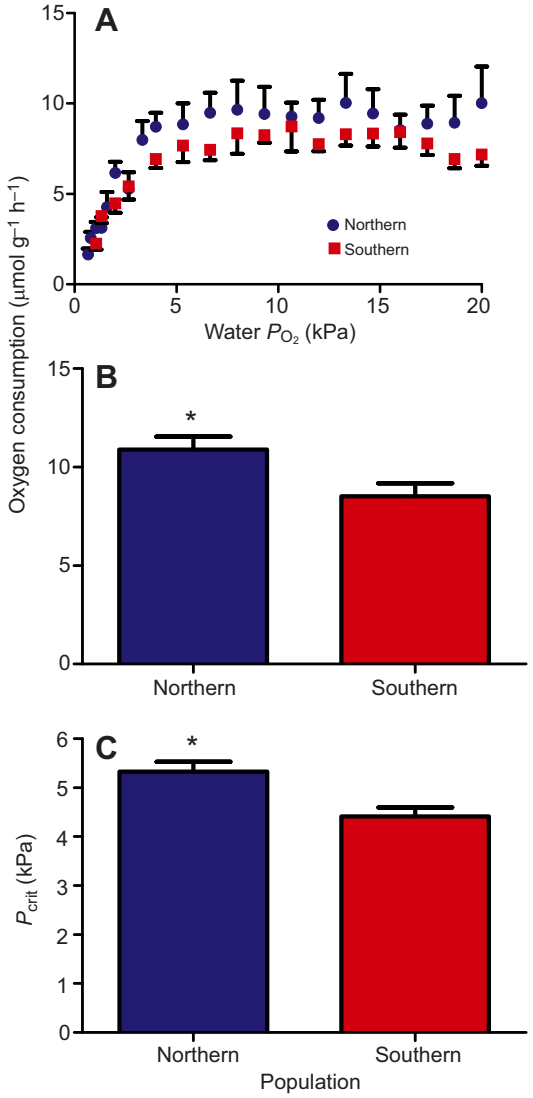

Fig. 2. The critical oxygen tension (P_{crit}) of mass-specific routine oxygen consumption ($\dot{M}_{O_2}$) in the northern and southern subspecies of killifish. (A) The effect of ambient oxygen partial pressure on $\dot{M}_{O_2}$. (B) Comparison of $\dot{M}_{O_2}$ in normoxia between northern and southern killifish. (C) P_{crit} in northern and southern killifish. Asterisks indicate significant differences between subspecies (P<0.05). All data are reported as means±s.e.m. Sample sizes: A: N≥6 for all groups except 20 kPa and <1 kPa, where N=2 for each group; B: N=8 for northern fish and N=16 for southern fish; C: N=20 for northern fish and N=14 for southern fish.

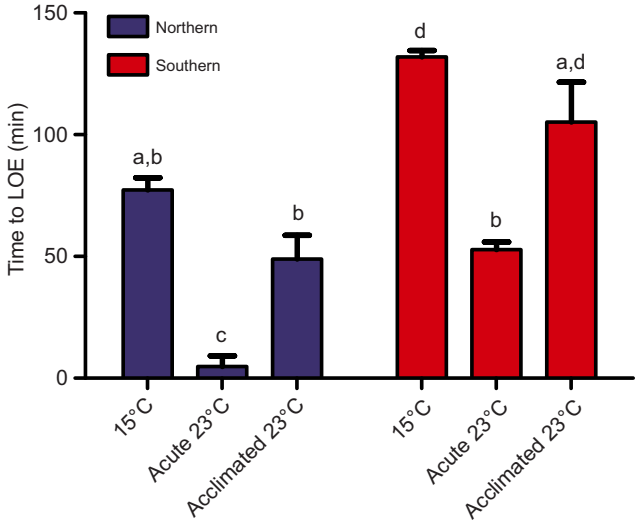

Fig. 3. Time to LOE_{hyp} in northern and southern killifish following exposure to increased temperature. Fish were acclimated to 15°C and tested at 15°C, acclimated to 15°C and tested after acute exposure to 23°C, or acclimated to 23°C and tested at 23°C. Bars sharing the same letter do not differ significantly (P>0.05). All data are reported as means±s.e.m. Sample size: N=8 fish for each group.

expressed relative to body mass (Fig. 4B), there was a significant effect of acclimation temperature (P=6×10^{-9}), a significant difference between subspecies (P=2×10^{-7}) and no significant interaction (P=0.08), with northern fish having consistently higher total lamellar surface area than southern fish at all acclimation temperatures. This difference was detected as significant in planned comparisons at 15 and 33°C (Table 2).

When considered without accounting for body mass (Table 1), lamellar height increased significantly with acclimation temperature (P=2×10^{-16}), and differed between the subspecies (P=6×10^{-5}), with no significant interaction between these factors (P=0.26). Northern fish generally had greater lamellar height, and this difference was detected as significant in planned comparisons at 15°C. Similar patterns were observed when lamellar height was expressed per gram body mass (Table 2); lamellar height increased significantly with acclimation temperature (P=3×10^{-8}), and there was a significant difference between the subspecies (P=2×10^{-7}), with northern fish generally having greater lamellar height; however, in this case there was a significant interaction between the factors (P=0.002). Planned comparisons detected a significant difference between the subspecies at all temperatures except 15°C (Table 2). Taken together, these data suggest that changes in lamellar height explain a substantial proportion of the variation in total lamellar surface area both with acclimation and between the subspecies.

There were also significant effects of acclimation temperature on filament length (P=0.045), but no significant difference between the subspecies (P=0.96). However, there was a significant interaction between subspecies and acclimation temperature (P=0.02) such that the effects of acclimation temperature on filament length were consistent with the patterns for total lamellar surface area for southern fish, but not for northern fish. A somewhat different pattern was observed when filament length was expressed relative to body mass (Table 2). There was no significant effect of acclimation temperature (P=0.96), but there was a significant difference in filament length per body mass between the subspecies (P=0.0003), and a marginally significant interaction (P=0.049), with northern fish generally having greater filament length per body mass, although this difference was not detected using planned comparisons at any acclimation temperature. Taken together, these data suggest that changes with acclimation and differences between subspecies in total lamellar surface area (Fig. 4B) cannot be accounted for by changes in filament length.

Basal lamellar width was also affected by acclimation (P=0.01), and differed between the subspecies (P=2×10^{-7}), with no significant interaction (P=0.11). Northern fish generally had greater basal lamellar width than did southern fish, but these differences were not detected in planned comparisons (Table 1). When basal lamellar width was expressed relative to body mass (Table 2), there was no significant effect of acclimation (P=0.98), a significant difference between the subspecies (P=1×10^{-5}) and a significant interaction between these factors (P=0.008). In general, basal lamellar width

Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

Table 1. Effects of acclimation temperature on gill morphology and body size in killifish

	5°C	15°C	25°C	30°C	33°C
Filament length (mm)					
Northern	1.82±0.11^{a}	1.83±0.06^{a}	1.84±0.17^{a}	1.77±0.03^{a}	1.67±0.08^{a}
Southern	1.52±0.10^{p}	1.47±0.08^{p}	2.06±0.25^{p}	2.06±0.21^{p}	1.90±0.07^{p}
Lamellar frequency (mm^{-1})					
Northern	68.5±0.7^{a,*}	68.3±0.9^{a,*}	71.7±3.5^{a}	67.5±0.1^{a,*}	71.1±0.9^{a}
Southern	79.2±2.3^{p}	77.5±1.0^{p}	74.5±2.8^{p}	77.0±1.2^{p}	77.8±1.1^{p}
Lamellar height (μm)					
Northern	22.9±3.0^{a}	33.8±1.9^{a,*}	52.2±2.4^{b}	59.7±4.0^{b}	61.5±2.7^{b}
Southern	14.7±0.6^{p}	24.3±1.8^{q}	46.3±4.3^{r}	60.2±1.1^{r}	55.8±1.5^{r}
Basal lamellar width (μm)					
Northern	177.6±1.1^{a}	173.8±4.3^{a,*}	178.3±8.8^{a}	178.2±2.7^{a}	177.9±7.5^{a}
Southern	138.3±6.9^{p}	136.6±5.5^{p}	164.6±9.4^{p}	168.5±6.3^{p}	147.6±3.4^{p}
Fish mass (g)					
Northern	4.82±0.55^{a}	4.99±0.28^{a}	4.10±0.12^{a}	3.61±0.19^{a}	3.76±0.12^{a}
Southern	5.48±1.25^{p}	3.87±0.53^{p}	7.85±2.16^{p}	7.54±1.55^{p}	7.28±0.96^{p}
Fish length (cm)					
Northern	7.20±0.20^{a}	7.28±0.14^{a}	7.17±0.07^{a}	7.13±0.23^{a}	7.23±0.08^{a}
Southern	7.36±0.13^{p}	6.43±0.31^{p}	8.10±0.90^{p}	8.10±0.52^{p}	7.98±0.32^{p}
Sample size (*N*)					
Northern	2	6	3	3	4
Southern	5	4	3	4	5

Asterisks indicate a significant difference between subspecies at the acclimation temperature (P<0.05). Values sharing the same letter within a subspecies are not significantly different (P>0.05).

expressed per gram body mass increased with thermal acclimation in northern fish, and decreased in southern fish, which is probably a result of the observed changes in body mass with acclimation.

Lamellar frequency also differed between the subspecies (P=2×10^{-8}), and there were no significant effects of acclimation temperature (P=0.63) or interaction (P=0.25). In this case, southern fish had higher lamellar frequency than did northern fish, which was detected as significant using planned comparisons at 5, 15 and 30°C (Table 1). These differences between subspecies partially offset the effects of differences in lamellar height and basal lamellar width on total lamellar surface area. When lamellar frequency was expressed relative to body mass, there was no significant difference between the subspecies (P=0.35) or an effect of acclimation (0.80), although there was a significant interaction between these factors (P=0.01). Planned comparisons (Table 2) detected few significant differences with acclimation temperature, and no differences between the subspecies at any temperature. Taken together, these data suggest that changes in lamellar frequency are unlikely to account for the observed changes in total lamellar surface area with acclimation, and further emphasize the importance of changes in lamellar height.

To determine whether the observed changes in lamellar height (Tables 1 and 2) could be due to changes in the ILCM, we used SEM and histological staining for a subset of individuals from the second experimental series (acclimated to 15 and 23°C). SEM images (Fig. 5) suggest that the filament may be somewhat thinner at the higher temperature. Histological sections revealed the presence of an ILCM that regressed at 23°C (Fig. 6). This is consistent with the gill morphometry, which indicated an increase in lamellar height and area with increasing temperature (Fig. 4, Tables 1 and 2).

DISCUSSION

The data presented here clearly demonstrate that prior acclimation of killifish to warm temperatures results in improved tolerance of subsequent hypoxia (as measured using time to LOE_{hyp}). This improved tolerance is correlated with a significant increase in gill respiratory surface area as a result of regression of an ILCM in response to warm acclimation. Further, we show that northern and southern subspecies of killifish differ in hypoxia tolerance, but that this difference cannot be accounted for by differences in gill surface area or thermal plasticity of the gill between subspecies. Instead, differences in hypoxia tolerance between subspecies are consistent

Table 2. Gill morphometric parameters per gram body mass in killifish

	5°C	15°C	25°C	30°C	33°C
Filament length (μm g^{-1})					
Northern	0.39±0.07^{a}	0.37±0.02^{a}	0.45±0.03^{a}	0.49±0.03^{a}	0.44±0.03^{a}
Southern	0.32±0.05^{p}	0.40±0.04^{p}	0.30±0.08^{p}	0.29±0.03^{p}	0.28±0.03^{p}
Lamellar frequency (mm^{-1} g^{-1})					
Northern	14.39±1.49^{a,b}	13.90±0.76^{a}	17.58±1.35^{a,b}	18.80±0.98^{a,b}	18.93±0.39^{b}
Southern	17.64±3.68^{p}	21.55±3.74^{p}	12.36±5.20^{p}	11.41±2.05^{p}	11.44±1.43^{p}
Lamellar height (μm g^{-1})					
Northern	4.88±1.18^{a,b,*}	6.83±0.39^{b}	12.72±0.25^{a,*}	16.56±0.83^{c,*}	16.40±1.01^{c,*}
Southern	3.18±0.55^{p}	6.52±0.61^{q}	7.62±3.16^{p,q,r}	8.85±1.43^{r}	8.08±0.82^{r}
Basal lamellar width (μm g^{-1})					
Northern	37.30±4.02^{a}	35.41±2.08^{a}	43.45±1.43^{a}	49.66±3.10^{a}	47.50±3.03^{a,*}
Southern	29.81±5.56^{p}	37.19±4.59^{p}	25.80±8.77^{p}	24.98±4.60^{p}	21.44±2.27^{p}

Asterisks indicate a significant difference between subspecies at the acclimation temperature (P<0.05). Values sharing the same letter within a subspecies are not significantly different (P<0.05).

Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

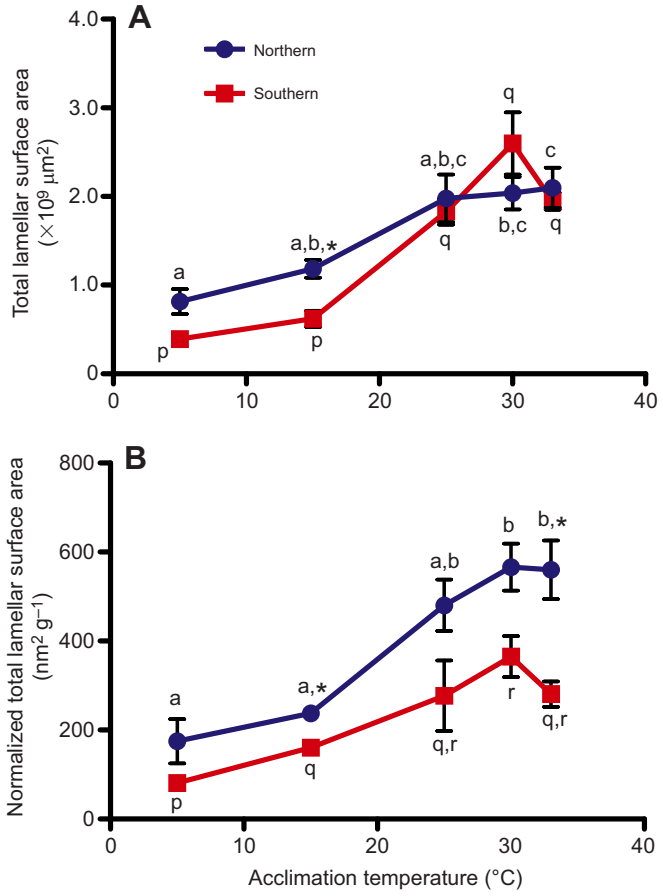

Fig. 4. Total lamellar surface area of the gills of northern and southern killifish. (A) Data for fish acclimated to different temperatures. (B) The same data, expressed relative to body mass. Symbols sharing a letter do not differ within a subspecies. Differences between subspecies at an acclimation temperature are indicated with an asterisk. All data are reported as means±s.e.m. Sample sizes are given in Table 1.

with observed differences in routine metabolic rate. Taken together, these data indicate that processes associated with oxygen supply and demand are underlying mechanistic factors that influence the interacting effects of elevated temperature and hypoxia on fish.

The effect of acute high temperature on hypoxia tolerance

Consistent with the results of many previous studies (Fernandes and Rantin, 1989; Schurmann and Steffensen, 1992; Corkum and Gamperl, 2009; Vaquer-Sunyer and Duarte, 2011), acute exposure to elevated temperatures reduced hypoxia tolerance in both subspecies of killifish (Figs 1 and 3). This decline in hypoxia tolerance could, at least in part, be due to the increase in metabolic rate associated with increased temperature. In killifish, the Q_{10} for aerobic metabolic rate is approximately 1.5–2.5 across this temperature range (Targett, 1978; Healy and Schulte, 2012), and the Q_{10} for the maximal activity of lactate dehydrogenase-B (an important enzyme of anaerobic glycolysis) is ~1.9 (Place and Powers, 1984), and thermal sensitivity does not differ between the subspecies. Therefore, if time to LOE_{hyp} is related solely to the temperature-dependent increase in metabolic rate, one would expect time to LOE_{hyp} to be about half as long for every 10°C increase in temperature. However, our data indicate that time to LOE_{hyp} declined from ~75 min to ~3 min between 15 and 23°C in northern killifish. This suggests that effects on metabolic rate cannot be the sole driver of the interactive effects of temperature and hypoxia on northern killifish at acute time scales.

Both 15 and 23°C are well within the thermal optimum for killifish, and aerobic scope is maintained across this range under normoxic conditions (Healy and Schulte, 2012), suggesting that the large observed change in time to LOE_{hyp} is not simply the result of a stress response. Instead, we suggest that the large decline in time to LOE_{hyp} is more likely to be due to the effects of temperature on the oxygen affinity of haemoglobin. The effects of temperature on haemoglobin oxygen affinity are complex in fish, because they involve both the intrinsic thermal sensitivity of the haemoglobin molecule itself and the effects of small molecule allosteric modifiers and intracellular pH. But in general, increased temperature tends to reduce haemoglobin oxygen affinity in fish (Wood, 1980; Powers, 1980; Weber and Jensen, 1988). This is also likely to be the case in killifish, as the oxygen affinity of stripped haemoglobin has a high thermal sensitivity (Powers et al., 1979).

The effect of thermal acclimation on hypoxia tolerance

Acclimation to warm temperatures markedly improved hypoxia tolerance in killifish, and similar effects have been observed in salmon (Anttila et al., 2015). In killifish, this change was correlated with changes in gill morphology. We observed an increase in gill lamellar surface area with increasing acclimation temperature, which was primarily a result of increases in lamellar height. SEM and histological analysis suggests that these changes are due to regression of an ILCM. Remodelling of gill morphology has primarily been investigated in the context of acclimation to hypoxia (e.g. Crispo and Chapman, 2010), and this has been shown to occur via changes in the ILCM in a variety of fish species (Sollid et al., 2003, 2005a; Nilsson, 2007; Ong et al., 2007; Matey et al., 2008; Turko et al., 2012; Dhillon et al., 2013; Johannsson et al., 2014; Anttila et al., 2015) including killifish (Borowiec et al., 2015). Less is known about the effects of thermal acclimation on gill morphology, but decreases in the ILCM at warmer temperatures have been observed in crucian carp, goldfish (Sollid et al., 2005b; Mitrovic and Perry, 2009) and killifish (Barnes et al., 2014). Decreases in the extent of the ILCM at higher acclimation temperatures and the resulting increase in lamellar surface area are thought to increase oxygen uptake and thus may improve tolerance of hypoxia. Note that the changes in gill morphology in killifish are not as extensive as those that have been observed in the cyprinid fishes. In the cyprinids, almost the entire lamella is covered in the cold (e.g. Sollid et al., 2005b), whereas in killifish, a substantial fraction of the lamella remains exposed even at the lowest temperatures measured (Barnes et al., 2014). However, even these relatively subtle changes are associated with large changes in hypoxia tolerance.

It is unclear whether the ability to remodel the ILCM is broadly distributed across fish taxa, or whether it represents a specialization of fishes occupying habitats that undergo periodic hypoxia (Nilsson, 2007; Dhillon et al., 2013). Thermal acclimation does not improve hypoxia tolerance in two species of coral reef fishes (Nilsson et al., 2010), which suggests that either these species lack the capacity to modify the ILCM or the ILCM is fully regressed at all of the relatively high temperatures tested in these tropical fishes. This second possibility is supported by our observations for killifish, as total lamellar surface area was relatively constant across temperatures from 25 to 33°C (Fig. 4), and similar patterns have been observed in carp and goldfish (Sollid et al., 2005b).

Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

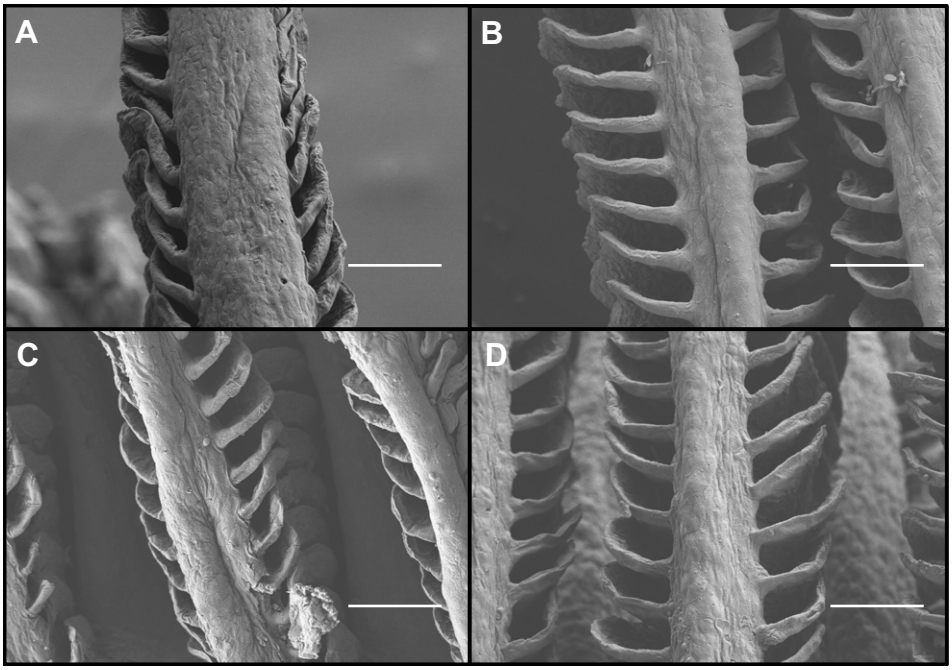

Fig. 5. Scanning electron micrographs of gills from killifish acclimated to 15 and 23°C. (A) Gill from a northern killifish acclimated to 15°C. (B) Gill from a northern killifish acclimated to 23°C. (C) Gill from a southern killifish acclimated to 15°C. (D) Gill from a southern killifish acclimated to 23°C. Scale bars, 50 μm.

Although the evidence presented here suggests that remodelling of the ILCM is likely to contribute to increased hypoxia tolerance with warm acclimation, other mechanisms may also play a role. Changes at any step along the oxygen cascade have the potential to contribute to changes in hypoxia tolerance. For example, in Atlantic salmon (*Salmo salar*), increases in hypoxia tolerance with warm acclimation are not associated with changes in gill structure (Anttila et al., 2015), but instead increases in capillary density in the compact myocardium have been observed in various salmonids (Egginton and Cordiner, 1997; Anttila et al., 2015). Changes in haemoglobin oxygen affinity could also play a role. In *F. heteroclitus*, warm acclimation decreases red blood cell ATP: haemoglobin ratio, causing an increase in haemoglobin oxygen affinity (Greaney and Powers, 1977, 1978; Powers et al., 1986). This could offset the decrease in affinity due to the direct effects of increased temperature, which could improve hypoxia tolerance.

Differences between subspecies

The data presented here indicate that the northern subspecies of killifish is not as tolerant of hypoxia as is the southern subspecies. This difference in hypoxia tolerance was evident when measured either as time to LOE_{hyp} or as P_{crit}. These two measures probably reflect different aspects of tolerance to hypoxia (Richards, 2011, 2009; Farrell and Richards, 2009), and these traits are not correlated

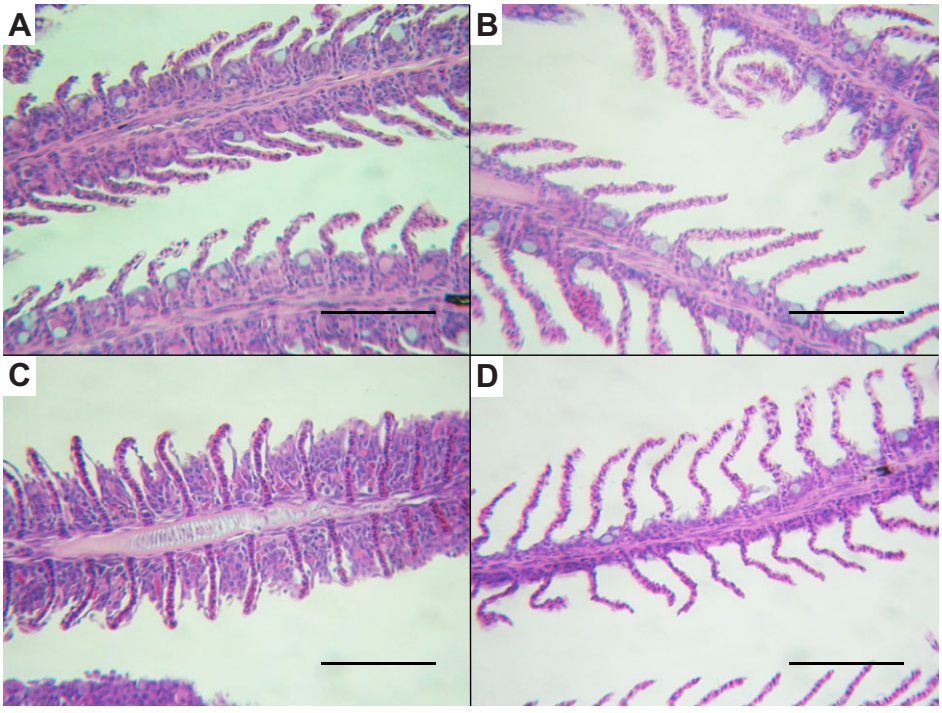

Fig. 6. Histological sections of gills from killifish acclimated to 15 and 23°C, demonstrating regression of the interlamellar cell mass with increased temperature. (A) Gill from a northern killifish acclimated to 15°C. (B) Gill from a northern killifish acclimated to 23°C. (C) Gill from a southern killifish acclimated to 15°C. (D) Gill from a southern killifish acclimated to 23°C. Scale bars, 50 μm.

Journal of Experimental Biology (2016) 219, 474-484 doi:10.1242/jeb.133413

in phylogenetically corrected multispecies comparisons in fish (Dhillon et al., 2013). Instead, P_{crit} is thought to be more closely correlated with haemoglobin P_{50} for oxygen (Mandic et al., 2009; Dhillon et al., 2013). However, the whole-blood haemoglobin P_{50} of killifish is approximately 0.5–0.7 kPa depending on acclimation temperature (DiMichele and Powers, 1982), whereas here we show that P_{crit} is approximately 4.4–5.3 kPa depending on the subspecies. Our current estimates of P_{crit} are somewhat lower than those we have previously obtained for this species (Richards et al., 2008), but are similar to other published values for *F. heteroclitus* (Cochran and Burnett, 1996) and for *F. grandis* (Virani and Rees, 2000). In any case, it is clear that there is a substantial difference between the oxygen tensions at which killifish begin to allow metabolic rate to oxyconform and the oxygen tensions at which there is significant haemoglobin desaturation. We suggest that, at least in killifish, measured P_{crit} may involve a behavioural component. In this study, we monitored routine, rather than standard, oxygen consumption and thus P_{crit} may represent the oxygen tension at which killifish begin to suppress routine activity and transition to standard metabolism and then to oxyconformation.

The differences in time to LOE_{hyp} between killifish subspecies cannot be explained by differences in gill surface area or the plasticity of the ILCM. The northern subspecies tended to have greater lamellar surface area than did the southern subspecies, and this difference was significant when lamellar surface area was expressed relative to body size. This is in direct contrast to the poorer hypoxia tolerance of the northern subspecies. This observation is consistent with the pattern observed in a multi-species comparison of cyprinid fishes in which hypoxia tolerance was inversely correlated with gill surface area in phylogenetically independent comparisons (Dhillon et al., 2013). These authors hypothesized that gill surface area in normoxia is, instead, related to life history and activity patterns. Consistent with this suggestion, northern killifish have a higher routine metabolic rate than do southern killifish. This difference in metabolic demand could be the root of the difference in gill morphology and in hypoxia tolerance between the subspecies.

The two killifish subspecies demonstrated similar plasticity in hypoxia tolerance and in gill remodelling. The plasticity in gill remodelling occurred primarily at temperatures below 25°C, and by 30°C there was no increase in lamellar surface area with increasing acclimation temperature (either in absolute terms or when expressed relative to body mass). Histological analysis suggests that at these temperatures the ILCM is essentially fully regressed. Similarly, hypoxia tolerance showed only modest evidence of improvement with acclimation to 30°C (Fig. 1). The inability to further increase capacity for oxygen extraction via gill remodelling at higher temperatures may play a role in setting limitations on the thermal window of aerobic scope in killifish (Healy and Schulte, 2012). As is the case for gill remodelling and thermal effects on hypoxia tolerance, there is little evidence for divergence in the extent of plasticity in other traits between the northern and southern subspecies of killifish. For example, both northern and southern subspecies alter thermal tolerance (CT_{max}) to similar extents with thermal acclimation (Fangue et al., 2006), and patterns of thermal plasticity in gene expression are generally similar between the subspecies (Dayan et al., 2015). The relatively high plasticity in a variety of traits in killifish is consistent with their highly variable estuarine habitat. In these habitats, both temperature and oxygen can vary across daily and seasonal scales, and thus strong selection for plasticity in processes related to thermal tolerance and oxygen supply would be expected in this species (Scheiner, 1993).

Conclusions

Collectively, the data presented here demonstrate how exposure to high temperature affects hypoxia tolerance in *F. heteroclitus*. Across a range of temperatures not regarded as stressful to this eurythermal fish species, there was a dramatic reduction in hypoxia tolerance with acute temperature increase. We demonstrate that warm acclimation can reverse this reduction in hypoxia tolerance and almost fully compensate for the interactive effects of these stressors at intermediate temperatures. Our data provide evidence that thermal acclimation may improve hypoxia tolerance through increased gill surface area as a result of regression of an interlamellar cell mass as temperature increases. In contrast, differences between the subspecies in hypoxia tolerance cannot be accounted for by differences in gill morphology, and instead are more clearly related to differences in metabolic rate. This observation emphasizes the fact that plasticity and adaptation are acting on different steps in the oxygen cascade in this species.

As our environment continues to be affected by anthropogenic climate change, it will be critical to assess how multiple interacting stressors will affect organisms. Understanding the underlying mechanistic linkages is a key first step towards predicting the responses of organisms to the complex combination of abiotic stressors that are characteristic of both natural and human-impacted environments.

Acknowledgements

We thank the UBC Bioimaging Facility, and particularly Garnet Martens, Kevin Hodgson and Derrick Horne, for assistance with light and electron microscopy.

Competing interests

The authors declare no competing or financial interests.

Author contributions

The experiment was conceived and designed by P.M.S., T.L.M. and T.M.H. T.L.M. performed all time to loss of equilibrium experiments, assisted by T.M.H. K.L.H. performed metabolic rate measurements and measurements of P_{crit}, assisted by T.M.H. All microscopy data were collected by T.L.M. Data were analysed by T.L.M. assisted by T.M.H. and P.M.S. The paper was drafted, reviewed and revised by P.M.S., T.M.H. and T.L.M. All authors commented on the manuscript and approved the submitted version.

Funding

This research was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Undergraduate Student Research Award to T.L.M. and an NSERC Discovery Grant to P.M.S. T.M.H. and K.L.H. were funded through the Discovery Grant to P.M.S.

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