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RESEARCH ARTICLE

Intraspecific variation in the thermal plasticity of mitochondria in killifish

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SUMMARY

Populations of the Atlantic killifish (*Fundulus heteroclitus*) inhabit salt marshes and estuaries along the eastern coast of North America from Newfoundland to northern Florida, and are thus exposed to a large range of temperatures. Previous studies have shown higher whole-organism metabolic rates in the northern subspecies (*F. h. macrolepidotus*) compared with the southern subspecies (*F. h. heteroclitus*) of these fish. Here, we examine phenotypic plasticity in the response to cold temperatures between the two subspecies by acclimating fish to 5, 15 and 25°C and comparing several mitochondrial and muscle properties. The relative area of oxidative muscle *versus* glycolytic muscle fibers was greater in the northern subspecies at the 5 and 15°C acclimation temperatures. However, there were no differences in capillary density between the two subspecies or at different temperatures. Mitochondrial volume and surface densities increased in response to cold temperature acclimation in red and white muscle, but only in the northern killifish. Citrate synthase activities also increased in the northern killifish at 5 and 15°C. The ratio of calculated [free ADP] to [ATP] increased in the 5°C acclimated southern killifish but not in the northern killifish at 5°C when compared with the 15°C acclimation group, suggesting that there are differences in adenylate signaling for mitochondrial respiration between subspecies at low temperature. Taken together, our data indicate that the northern subspecies have a greater ability to increase mitochondrial capacity at colder temperatures compared with the southern subspecies, providing one of the few examples of intraspecific variation in phenotypic plasticity in mitochondrial amount in response to cold temperatures.

Key words: killifish, thermal tolerance, acclimation, mitochondria, phenotypic plasticity.

INTRODUCTION

Because cold decreases the rates of chemical reactions, the rate of diffusion and membrane fluidity, low temperatures pose substantial challenges for ectotherms. Thus, ectotherms that must function at low temperatures often exhibit acclimatory changes that mitigate these effects (for a review, see Guderley, 2004). Mitochondria are of particular significance in the study of cold temperature acclimation, as lower temperatures reduce the mitochondria's ability to produce ATP *via* oxidative phosphorylation, both directly at the level of the mitochondrion and indirectly through limitations on oxygen transport. Thus, acclimation or adaptation to cold temperature requires organisms to counter a potential ATP deficit by enhancing processes involved in oxygen delivery and oxidative capacity (O'Brien, 2011).

Acclimatory increases in oxygen delivery can involve a suite of changes including enhancements to the oxygen supply network to the mitochondria and structural changes within the cell. For example, cold-induced angiogenesis, particularly in the mitochondria-rich red muscle of fishes, can offset reduced diffusion, in addition to providing other benefits such as improved waste removal (Hoppeler et al., 1981; Johnston, 1982; Egginton and Cordiner, 1997; Egginton et al., 2000; Mathieu-Costello et al., 2005; O'Brien, 2011). Another common response during cold temperature acclimation is the proliferation and catabolization of lipids (Jones and Sidell, 1982; Egginton and Sidell, 1989; Sidell and Moerland, 1989; O'Brien, 2011). Lipid droplets in the muscle fibers increase the solubility coefficient of oxygen at low temperatures (Egginton and Sidell, 1989; Sidell, 1998) and provide a preferred fuel substrate. Finally, oxygen delivery can be improved by locating mitochondria in a sub-sarcolemmal position within the cell, closer to the intracellular lipid droplets and the external capillaries (Scott et al., 2009).

Oxidative capacity can be elevated in cold-acclimated organisms *via* changes in a number of physiological processes. Increases in mitochondrial content with cold acclimation have been described in numerous studies (Johnston and Maitland, 1980; Egginton and Sidell, 1989; Johnston et al., 1998; Orczewska et al., 2010), and may be a means of dealing with reduced ATP production by offsetting reduced catalytic capacity of enzymes and decreasing diffusion distances. Increasing the cristae surface density increases oxidative capacity independent of mitochondrial proliferation by increasing respiratory activity per mitochondrion (Guderley, 2004), which also reduces the space taken from myofibrils by the mitochondria within the muscle cell relative to responses involving mitochondrial proliferation alone. Hyperplastic and hypertrophic growth of muscle have also been observed with cold acclimation in fishes, which may be a means of maintaining muscular force generation (Johnston and Maitland, 1980; Sidell, 1980; Egginton and Sidell, 1989; Guderley, 2004). This is an interesting contrast to cold-adapted hibernating mammals, which typically experience muscle atrophy (Egginton, 2002). Increases in the number and/or size of muscle fibers also serve to increase the absolute number and surface area of mitochondria.

Although there are a myriad of physiological mechanisms to deal with cold temperature in fishes, there is substantial variability in how different species respond to low-temperature environments. For example, although increases in mitochondrial volume density were observed in the absence of changes in cristae surface densities in cold-acclimated striped bass (Egginton and Sidell, 1989), the

opposite was observed in cold-acclimated rainbow trout (St Pierre et al., 1998). Similarly, higher citrate synthase and cytochrome oxidase enzyme activities, which are often used as a predictor for higher mitochondrial volume and surface area densities, are not always observed during cold acclimation (Johnston et al., 1998; Lucassen et al., 2006; Grim et al., 2010). However, few studies have examined the traits involved in both oxygen delivery and mitochondrial amount and even fewer have examined variation in these traits at the intraspecific level (but see Lucassen et al., 2006; Morley et al., 2009).

Atlantic or common killifish, *Fundulus heteroclitus* (Linnaeus 1766), have been studied extensively as a model for thermal adaptation (Schulte, 2001; Schulte, 2007). These killifish occupy a steep latitudinal cline along the eastern seaboard of the United States and Canada from northern Florida, USA, to Newfoundland, Canada. Across this range, killifish are exposed to temperatures nearing 40°C at the southern end of the species range during the summer, and temperatures slightly below 0°C during the winter months near the northern limit of the species distribution. The species has been divided into two subspecies: a northern subspecies (*F. h. macrolepidotus*), which occupies a range from northern New Jersey to Newfoundland, and a southern subspecies (*F. h. heteroclitus*), which is found from southern New Jersey to northern Florida (Morin and Able, 1983), with a hybrid zone between them. Importantly, in the context of the present study, the northern subspecies is found in habitats that are routinely at or below 4°C in the winter, whereas temperatures seldom get this low across most of the habitat of the southern subspecies (calculated from NOAA National Estuarine Research Reserve data; http://cdmo.baruch.sc.edu/). Recently, we showed that there is variation between subspecies of killifish in the response of isolated mitochondria to acute thermal challenge (Fangue et al., 2009), but only when fish are acclimated to low temperature and not when fish are acclimated to warm temperatures. In addition, we found that cold acclimation increased citrate synthase activity in the white muscle of northern but not southern killifish, suggesting the possibility of differences in the degree of mitochondrial proliferation in the cold between the subspecies (Fangue et al., 2009). In light of this study, and that of Grim et al. (Grim et al., 2010), who found that white muscle cytochrome oxidase increases with cold acclimation in the northern subspecies of killifish, the objective of the present study was to determine whether there is intraspecific variation in the plasticity of processes involved in oxygen delivery to the mitochondria and mitochondrial amount in killifish in response to acclimation to low temperatures.

MATERIALS AND METHODS

Experimental animals

Adult killifish of the northern subspecies (*F. h. macrolepidotus* Walbaum) were collected from Hampton, NH, USA, and fish of the southern subspecies (*F. h. heteroclitus* Linnaeus) were collected from Morehead City, NC, USA. These collections were made in the summer of 2008. Mean habitat temperatures at the time of collection were 22.0±1.1°C in New Hampshire, and 28.5±1.5°C in North Carolina. Fish were held in a 6000 l recirculating system at 15°C and 10 ppt salinity for a minimum of 4 weeks prior to experimental acclimations. For acclimation (to 5, 15, and 25°C), fish were transferred into six replicate 110 l tanks that were split in half with a flow-through barrier. Ten northern and 10 southern killifish were placed on opposite sides of the barrier in each tank, and the temperature was reduced by 0.5°C day^{-1}, as described by Fangue et al. (Fangue et al., 2006). Once acclimation temperature was reached, fish were held at their prescribed temperatures for 3 weeks. Mean mass was not significantly different between subspecies or acclimation groups (mean ± s.e.m.; northern killifish, 3.60±0.15 g; southern killifish, 3.77±0.28 g). Fish were fed daily with Tetramin® flakes to satiation. At the time of sampling, the water level was slowly lowered in the tank, and an individual fish was carefully scooped into a modified 500 ml plastic beaker half filled with tank water. Tank water containing a lethal dose of the anaesthetic MS-222 buffered with $NaHCO_3$ was slowly added to the beaker and, once anaesthesia had been induced, the animal was immediately killed by decapitation. Tissue samples were then harvested for the various assays described below. The first tissue harvested, for adenylate assays, was a small, thin (less than 2 mm thick) section of white muscle that was immediately frozen in liquid nitrogen. Because of the small size of the tissue, freeze clamping was not required to preserve phosphagen and adenylate levels. All animal procedures complied with the guidelines of the Canadian Council on Animal Care and were approved under the University of British Columbia animal care protocol no. A07-0288.

Muscle histology

Histological analyses were performed on a cross-section of tissue taken across the entire fish behind the anal pore (~2 mm thick). The tissue samples were placed on a cork disc and coated with mounting medium (Fisher Histoprep SH75, Pittsburgh, PA, USA), and then rapidly frozen in 2-methylbutane, which was cooled in liquid N_2. Muscle was sectioned (10 μm) transverse to fiber length in a –20°C cryostat. Cryostat sections were stained for myosin-ATPase (pre-incubated at pH 4.3), succinate dehydrogenase and alkaline phosphatase activities (Deveci et al., 2001). All images were obtained at a magnification of 20× using an Olympus FSX100 Bioimaging Navigator (Tokyo, Japan). Myosin ATPase-stained images were digitized to determine red muscle cross-sectional area. Succinate dehydrogenase-stained images were used to calculate relative oxidative:glycolytic area over the cross-section of fish [(red muscle fiber + pink muscle fiber area):white muscle fiber area]. Alkaline phosphatase-stained images were used to measure capillary density within the red muscle fiber domain and to determine capillary to red muscle fiber area. In all cases, means from six images were calculated per individual, and six individuals were examined per population and temperature group.

Tissue for transmission electron microscopy (TEM) was sampled along the lateral line of the killifish between the pectoral fins and the cut section aligned near the anal pore. Tissue was pinned on a cork disc at a fixed length and fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol l^{-1}), followed by post-fixing in OsO_4 (1%) for 1 h, and was then dehydrated in a graded series with ethanol (30–100%) and embedded in epoxy resin. Ultra-thin sections (0.5 μm) were stained with 4% uranyl acetate and 2% lead citrate. Sections were viewed using a Hitachi H7600 Transmission Electron Microscope (Tokyo, Japan). All TEM analyses were conducted on samples of muscle in cross-section. Four to six individuals per acclimation temperature/subspecies groups and six to eight images per individual were analyzed. Measurements were taken at a final magnification of 60,000× for cristae surface area and 2500× and 1000× for mitochondrial volume density in red muscle and white muscle, respectively. All images were overlaid with a square lattice test pattern consisting of a 96 point grid with a grid size that varied depending on the measurement. Volume density was calculated using the summed point count method (Schmiedl et al., 1990; Weibel, 1979), with a grid spacing of 17.5 μm for white muscle and 4.75 μm for red muscle. Briefly, this method uses the number of intersections of

a grid landing on mitochondria relative to those intersections landing on the reference space as an index of volume density. Cristae density was calculated as a point count of grid intersections (horizontal and vertical) per mitochondria (Egginton and Sidell, 1989) using a 265 nm grid. All histological and TEM samples were analyzed blindly. Samples were prepared in random order and assigned a number, which was cross-referenced to actual sample identifications only after all measurements were recorded to remove any experimenter bias during the measurement process.

Enzyme activity

To measure enzyme activity, frozen white muscle tissue was homogenized in approximately 10 volumes of cold buffer (50 mmol l^{-1} Hepes, 5 mmol l^{-1} EDTA, and 0.1% Triton X-100, pH 7.4 at 20°C) using two passes of 10 s each with a Polytron homogenizer (Fisher Scientific, Nepean, ON, Canada). Following homogenization, each sample was sonicated with one 10 s pass (Kontes Micro Ultrasonic Cell Disrupter KT50, Vineland, NJ, USA). Homogenates were centrifuged at 4°C at 10,000 ***g*** for 2 min. This method effectively releases matrix enzymes such as citrate synthase, but removes enzymes that are tightly bound to the mitochondrial membranes, including the mitochondrial isozyme of creatine kinase, leaving a preparation containing primarily the cytosolic isoform (Iyengar and Iyengar, 1980). The supernatant was aliquoted and frozen in liquid N_2.

Enzyme assays were performed essentially as previously published [citrate synthase (Moyes et al., 1997); lactate dehydrogenase (Moon and Mommsen, 1987); creatine kinase (Bishop et al., 1971)]. All assays, regardless of acclimation group, were performed at a common temperature of 21°C. Briefly, citrate synthase activity was assayed in 50 mmol l^{-1} Tris pH 8.0, 0.3 mmol l^{-1} acetyl CoA, 0.15 mmol l^{-1} DTNB and 0.5 mmol l^{-1} oxaloacetate (oxaloacetate was not added to control wells). All substrate levels were saturating. Lactate dehydrogenase activity was assayed in 50 mmol l^{-1} Tris, pH 7.4, 0.09 mmol l^{-1} NADH and 2.5 mmol l^{-1} pyruvate (omitted from the control). Creatine kinase activity was assayed in 50 mmol l^{-1} Tris pH 7.4, 20 mmol l^{-1} creatine phosphate (omitted from the control), 1.5 mmol l^{-1} ADP, 12 mmol l^{-1} AMP, 1.5 mmol l^{-1} NAD, 20 mmol l^{-1} D-glucose, 6.5 mmol l^{-1} dithiothreitol, 25 mmol l^{-1} $MgCl_2$•H_2O, 2 U ml^{-1} hexokinase (in excess) and 1.5 U ml^{-1} glucose-6-phosphate dehydrogenase.

Adenylates

To measure [ATP], [creatine phosphate] (CrP) and [creatine] (Cr), approximately 50 mg of frozen white muscle was homogenized in 8% perchloric acid with a Polytron homogenizer. Homogenates were centrifuged at 20,000 ***g*** for 10 min (4°C) and the supernatant was neutralized to a pH of 7.6 using potassium carbonate. The neutralized extract was centrifuged again at 20,000 ***g*** for 5 min (4°C) prior to use. [ATP] and [CrP] were analyzed using the coupled creatine kinase, hexokinase and glucose 6-phosphate dehydrogenase reactions, as described in Bergmeyer (Bergmeyer, 1983). Free creatine concentrations were measured with the coupling enzymes creatine kinase, pyruvate kinase, and lactate dehydrogenase according to Bergmeyer (Bergmeyer, 1983). To measure intracellular pH (pH_i), ~100 mg of frozen tissue was ground into a fine powder under liquid N_2 and a metabolic inhibitor (150 mmol l^{-1} KF and 6 mmol l^{-1} nitrilotriacetic acid) was added to prevent H^+ formation (Pörtner, 1990). Measurements were made with a Radiometer G297/G2 capillary microelectrode with a PHM71 acid–base analyzer (Copenhagen, Denmark).

Calculations and statistical analysis

Free cytosolic [ADP] was calculated from measured [ATP], [CrP], [Cr] and pH_i, assuming equilibrium of the creatine kinase reaction:

$$[\mathrm{ADP_{free}}] = \frac{[\mathrm{ATP}][\mathrm{Cr}]}{[\mathrm{CrP}]K'_{\mathrm{CK}}} \, . \quad (1)$$

The equilibrium constants for creatine kinase (K'_{CK}) were adjusted to the acclimation temperature and intracellular pH (Golding et al., 1995; Teague et al., 1996). Free Mg^{2+} was assumed to be 1 mmol l^{-1} (Van Waarde et al., 1990; Jibb and Richards, 2008).

All data are expressed as means ± s.e.m., and were analyzed using a two-way ANOVA with subspecies and temperature as factors using Graphpad Prism (http://graphpad.com). All data met the assumptions of normality and homogeneity of variance. A Tukey's *post hoc* test was used to compare between groups where the interaction term was not significant. In the case of a significant interaction term, the data groups were separated and analyzed independently using a one-way ANOVA and a Tukey's *post hoc* test. A significance level of P<0.05 was used unless otherwise noted.

RESULTS

Muscle histology

Red oxidative muscle fibers were present only along the lateral line, with pink intermediate muscle fiber surrounding the red muscle, and white, glycolytic muscle fibers in the remainder of the tissue (Fig. 1). The different fiber types were easily identifiable using stains indicating succinate dehydrogenase (SDH) and myosin ATPase activities (Fig. 1A,B). Capillary densities were consistent with fiber composition, increasing markedly in density within the red oxidative muscle fibers (Fig. 1C).

The relative amount of oxidative fibers to glycolytic fibers (calculated as the cross-sectional area of red and pink muscle fiber relative to the white muscle fiber area across the whole section through the fish) was significantly greater in the northern subspecies of killifish than in the southern subspecies at 5 and 15°C (two-way ANOVA, subspecies P<0.001), but did not vary with acclimation temperature (two-way ANOVA, P=0.1797), nor was there an interaction between the factors (two-way ANOVA, P=0.7973; Fig. 2A). However, the relative cross-sectional area of oxidative fibers was significantly lower in the intermediate acclimation group. This trend resulted in detection of significant differences between subspecies at 5 and 15°C acclimation temperatures, but not at 25°C. The mean cross-sectional area of a red muscle fiber was lower at the 15°C acclimation temperature than at 5 or 25°C for the northern subspecies of killifish (one-way ANOVA, P=0.03; Fig. 2B). In the southern subspecies, the cross-sectional area of the red muscle fibers was significantly greater at 5°C acclimation temperature compared with both higher acclimation temperatures (one-way ANOVA, P<0.001). Capillary densities (mm^{-2} red muscle) were not significantly different between acclimation temperatures or between subspecies (two-way ANOVA, interaction P=0.799, subspecies P=0.073, temperature P=0.086; Fig. 3A). The number of capillaries per fiber area (mm^2) tended to be highest at the 15°C acclimation temperature (Fig. 3B) as a result of the lower fiber cross-sectional area in both subspecies at this temperature. However, these differences were significant only when comparing the fish acclimated to 15°C with those acclimated to 25°C in the northern subspecies and 5°C in the southern subspecies (Fig. 2B).

Fig. 4 shows representative electron micrographs for red muscle from northern fish acclimated to the three different temperatures. Note the primarily subsarcolemmal location of the mitochondria and the lack of large lipid droplets. Mitochondrial volume densities and

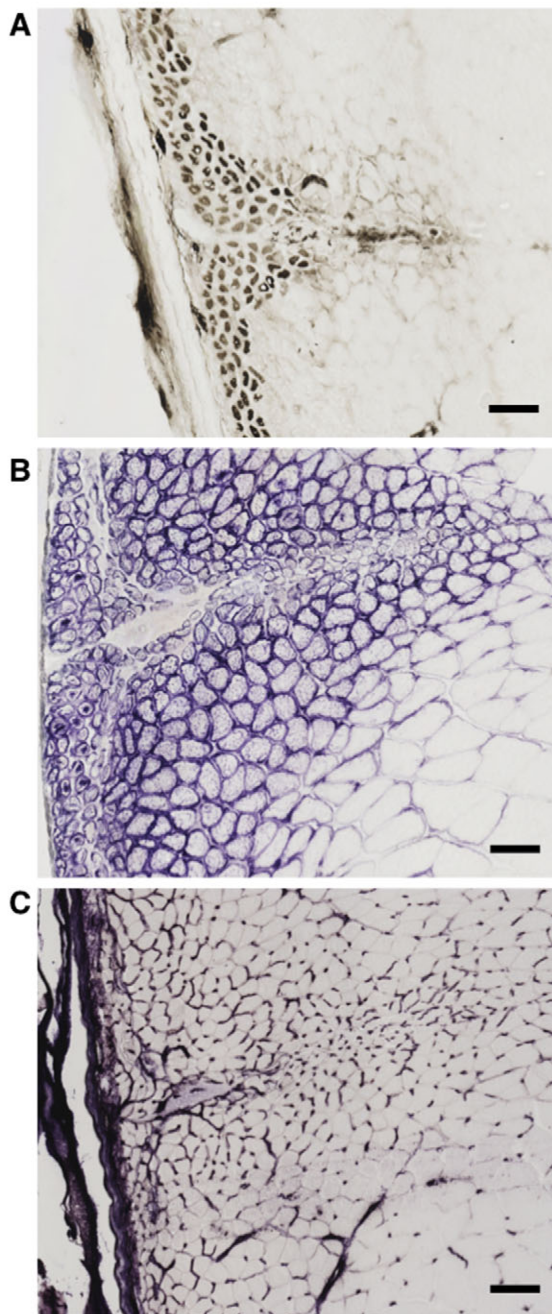

Fig. 1. Histochemical staining of killifish muscle in the area of the lateral line. (A) Myosin ATPase (pH 4.3) differentially stained the oxidative muscle fibers (dark stain) and glycolytic fibers (no stain). (B) Succinate dehydrogenase staining identified regions of high oxidative capacity (i.e. oxidative muscle fibers). (C) Alkaline phosphatase staining identified capillaries. Scale bars, 100 μm.

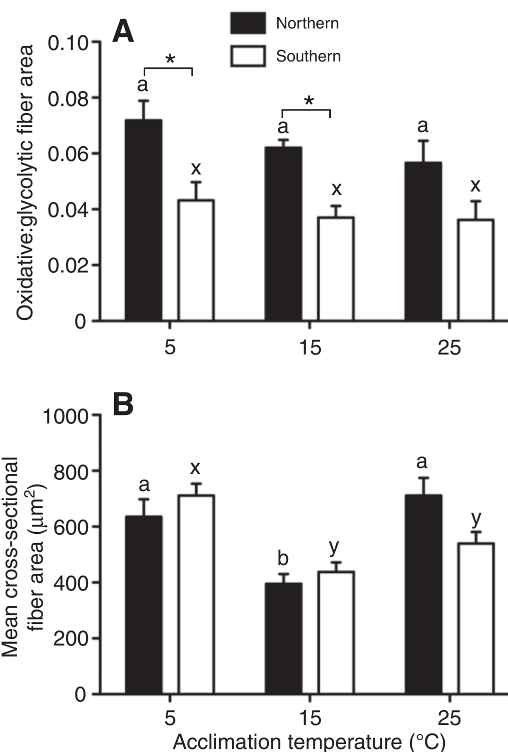

Fig. 2. (A) Muscle fiber composition (expressed as the overall ratio of the area occupied by oxidative muscle to the area occupied by glycolytic muscle) and (B) cross-sectional area of oxidative muscle fibers (μm^2) for northern (black) and southern (white) killifish acclimated to 5, 15 and 25°C. Fiber composition and cross-sectional area are expressed as means ± s.e.m. (N=6). Different letters denote significant differences (P<0.05) between acclimation groups within a subspecies, and an asterisk (*) denotes a significant difference (P<0.05) between populations within an acclimation temperature.

cristae surface densities in the red muscle were significantly higher in 5°C (cold)-acclimated northern fish compared with cold-acclimated southern fish and warm-acclimated northern fish (two-way ANOVA, volume density: interaction P=0.001, subspecies P=0.022, temperature P<0.001; cristae surface density: interaction P=0.003, subspecies P=0.021, temperature P=0.013; Fig. 5A,B). The results in the white muscle were very similar in pattern, although the absolute mitochondrial volume densities and cristae surface densities were much lower. Mitochondrial volume densities and cristae surface densities were significantly higher in the northern subspecies acclimated to 5°C, whereas these densities were the same for the 15°C- and 25°C-acclimated northern subspecies and the southern subspecies at all acclimation temperatures (two-way ANOVA, volume density: interaction P=0.001, subspecies P=0.003, temperature P<0.001; cristae surface density: interaction P=0.024, subspecies P=0.008, temperature P<0.001; Fig. 5C,D).

Enzyme activities

We measured the activity of two cytosolic enzymes (lactate dehydrogenase and creatine kinase) and one mitochondrial enzyme (citrate synthase) in white muscle. Lactate dehydrogenase activities did not differ significantly between the two subspecies within an acclimation temperature, or between acclimation temperatures within a subspecies, although there was a trend towards higher lactate dehydrogenase activities in the northern subspecies (two-way ANOVA, interaction P=0.772, subspecies P=0.056, temperature P=0.254; Table 1). The activities of the cytosolic isoform of creatine kinase were also similar between subspecies and among acclimation temperatures (two-way ANOVA, interaction P=0.028, subspecies P=0.774, temperature P=0.062). However, when the data were analyzed separately we detected significantly lower activities in the northern subspecies acclimated at 25°C (one-way ANOVA, P=0.008; significance assessed using a Bonferroni adjusted α-value of 0.01) (Table 1). White muscle creatine synthase activity differed significantly between the two subspecies at 5 and 15°C (two-way ANOVA, interaction P<0.001, subspecies P<0.001, temperature

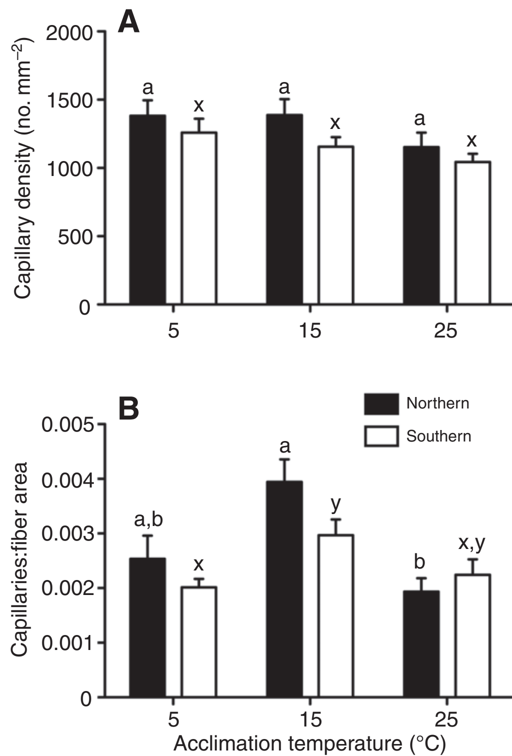

Fig. 3. Capillarity of the oxidative muscle. (A) Capillary density (mm^{-2}) within the cross-section of red muscle and (B) ratio of capillaries to muscle fiber area for northern (black) and southern (white) killifish acclimated to 5, 15 and 25°C. Capillary density and capillaries:muscle fiber area are expressed as means ± s.e.m. (N=6). Different letters denote significant differences (P<0.05) between acclimation groups within a subspecies, and an asterisk (*) denotes a significant difference (P<0.05) between populations within an acclimation temperature.

P<0.001; Table 1). Northern fish had significantly lower creatine synthase activity at 25°C acclimation than at the two colder temperatures (one-way ANOVA, P<0.0001), whereas the southern fish did not (one-way ANOVA, P=0.323).

Adenylates

White muscle [ATP] was affected by temperature acclimation. In the northern subspecies, [ATP] was significantly higher at acclimation temperatures of 15 and 25°C than at 5°C. [ATP] was significantly lower below 25°C for the southern subspecies (two-way ANOVA, interaction P=0.058, subspecies P=0.789, temperature P=0.002; Table 2). White muscle [CrP] of killifish did not differ significantly between acclimation temperatures or subspecies at a given acclimation temperature (two-way ANOVA, interaction P=0.366, subspecies P=0.021, temperature P=0.051). However, *post hoc* tests only detected a decrease at 25°C for the southern subspecies (Table 2). [Cr] did not differ significantly for either subspecies with acclimation temperature, nor did it differ between subspecies at a given acclimation temperature (two-way ANOVA, interaction P=0.256, subspecies P=0.641, temperature P=0.976; Table 2). Intracellular pH decreased with increasing acclimation temperature, but there were no significant differences between subspecies (two-way ANOVA, interaction P=0.010, subspecies P=0.125, temperature P<0.001; Table 2).

The calculated [ADP_{free}] changed significantly with temperature in both subspecies, but the patterns differ between subspecies (two-way ANOVA, interaction P=0.028, subspecies P=0.020, temperature P=0.020; Table 2). In the case of the northern killifish, calculated [ADP_{free}] was significantly lower in fish at 5°C than that observed in northern killifish acclimated at 15 or 25°C, and compared with the southern killifish at the same temperature. Conversely, *post hoc* tests revealed that calculated [ADP_{free}] in the southern subspecies was significantly higher at 5°C than at 15 or 25°C acclimation temperatures, and higher than [ADP_{free}] in the northern subspecies at 5°C. Two-way ANOVA indicated that the ratio of [ADP_{free}] to [ATP] was greater in southern killifish than in northern killifish and increased as temperature decreased in both subspecies (two-way ANOVA, interaction P=0.122, subspecies P=0.020, temperature P<0.0001). By far the largest increase in this ratio occurred in southern killifish between 15 and 5°C, which was the only difference between adjacent temperatures detected as statistically significant in *post hoc* tests (Table 2). As a result, *post hoc* tests only detected significant differences between populations at the lowest acclimation temperature.

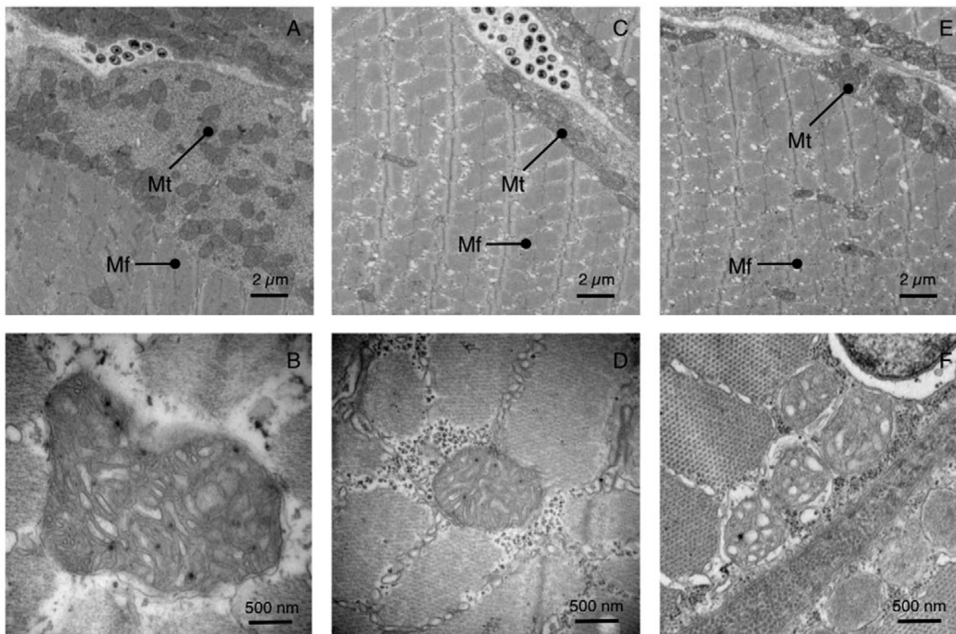

Fig. 4. Transmission electron micrographs of muscle fibers in the area of the lateral line in northern killifish. Mitochondria (A,C,E) and myofibrils (B,D,F) of northern killifish acclimated at 5°C (A,B), 15°C (C,D) and 25°C (E,F). Mt, mitochondria; Mf, myofibril.

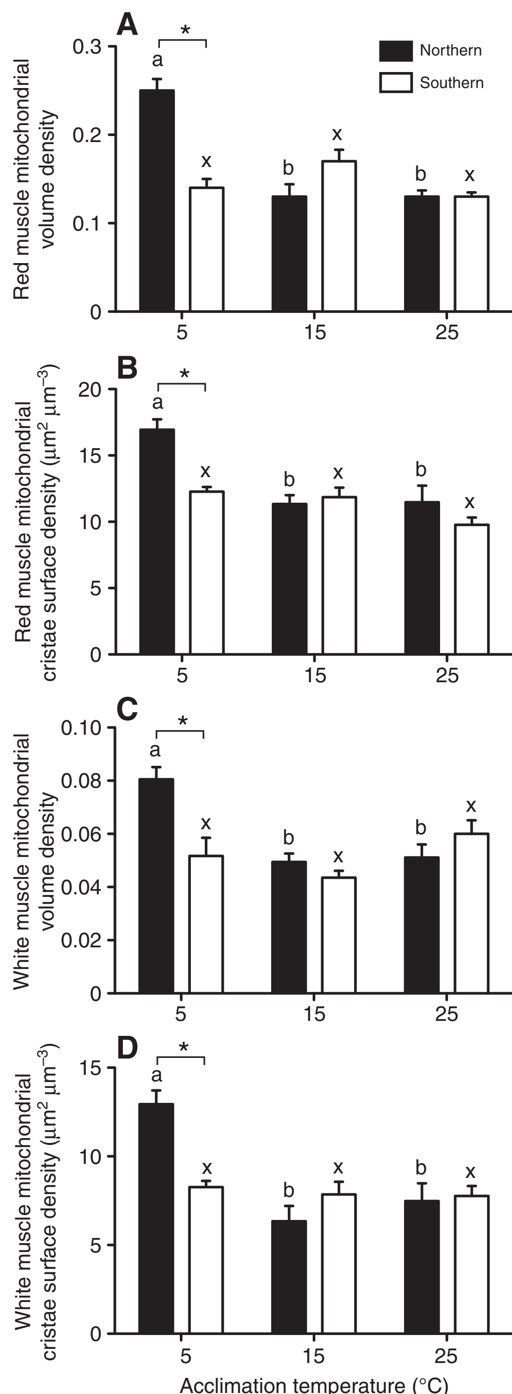

Fig. 5. Mitochondrial density in the muscle of northern (black) and southern (white) killifish. (A) Red muscle mitochondrial volume density, (B) red muscle mitochondrial cristae surface density ($\mu m^2 \mu m^{-3}$), (C) white muscle mitochondrial volume density and (D) white muscle mitochondrial cristae surface density ($\mu m^2 \mu m^{-3}$). Mitochondrial volume densities and cristae surface densities are expressed as means ± s.e.m. (N=4 to 6). Different letters denote significant differences (P<0.05) between acclimation groups within a subspecies, and an asterisk (*) denotes a significant difference (P<0.05) between populations within an acclimation temperature.

DISCUSSION

Interspecific variation in the capacity to modulate muscle and mitochondrial function with cold acclimation has been described in many species of fish (e.g. Egginton and Sidell, 1989; Johnston et al., 1998; St Pierre et al., 1998; Guderley, 2004), but much less is known about the extent of variation in acclimation capacity within a species (but see Lucassen et al., 2006; Morley et al., 2009). In the present study, we show that cold acclimation results in increases in mitochondrial volume density and cristae surface density in both oxidative and glycolytic fibers in the northern subspecies of the Atlantic killifish, but not in the southern subspecies. Furthermore, the area of oxidative fibers relative to glycolytic fibers was greater in the northern subspecies, resulting in a larger total volume of mitochondria per individual and a greater potential for aerobic muscle output. In contrast, the ratio of [ADP_{free}] to [ATP], an important signal that should stimulate mitochondrial respiration *in vivo*, increased with cold acclimation in the white muscle of southern killifish, but to a lesser extent in northern killifish. Taken together, these data demonstrate that killifish from the northern subspecies exhibit substantially greater plasticity in muscle and mitochondrial traits with cold acclimation than do fish from the southern subspecies.

Differences in muscle fiber type

Hyperplastic and hypertrophic growth of oxidative muscle fibers in cold-acclimated fish can aid in prolonged cold temperature tolerance. For example, goldfish (Johnston and Lucking, 1978), striped bass (*Morone saxatilis*) (Egginton and Sidell, 1989) and carp (*Cyprinus carpio*) (Rome et al., 1984) acclimated at lower temperatures have a significantly greater proportion of red muscle fibers compared with fish at warmer acclimation temperatures. Hyperplastic and hypertrophic growth may serve to maintain swimming performance by compensating for reduced muscle forces generated at colder temperatures (Sisson and Sidell, 1987; Rome et al., 1984). In the present study, the proportional area of oxidative muscle fibers to glycolytic muscle fibers was higher in the northern subspecies of killifish at all acclimation temperatures (though not significantly so at 25°C), as determined by succinate dehydrogenase and myosin ATPase staining. This difference is most likely attributable to hyperplastic growth, as the equal cross-sectional area of the red muscle fibers between the two subspecies makes hypertrophy a less likely explanation for the expanded oxidative area. Swim performance (measured as U_{crit}) has been shown to be higher in the northern killifish at all acclimation temperatures ranging from 5.2 to 32.4°C (Fangue et al., 2008). The larger oxidative muscle fiber proportions observed here at all acclimation temperatures is also consistent with the generally higher whole-organism routine oxygen consumption that has been observed in the northern subspecies compared with the southern subspecies (Fangue et al., 2009). The observed differences in the amount of oxidative fibers between killifish subspecies could be the result of genetic differences, or could result from irreversible developmental plasticity. Previous studies have demonstrated the existence of substantial developmental plasticity in these traits in a variety of species of fish (e.g. Johnston et al., 2000; Johnston et al., 2001). Similarly, European sea bass (*Dicentrarchus labrax*) reared at lower temperatures (15°C) have been shown to have a greater total red muscle area than fish reared at higher temperatures (20°C) (Koumoundouros et al., 2009), suggesting that the patterns we observe in killifish could have a similar developmental basis.

Parameters associated with increased oxygen delivery

We found only modest adjustments in parameters likely to increase oxygen delivery at low temperature in killifish. For example, there were no increases in muscle capillarity in either the northern or southern subspecies at low acclimation temperatures (Fig. 3A), and

Table 1. Lactate dehydrogenase, creatine kinase and citrate synthase enzyme activities (μmol min^{-1} g^{-1} wet tissue) in the white muscle of northern and southern killifish subspecies acclimated to 5, 15 and 25°C

Enzyme	Population	5°C	15°C	25°C
LDH	Northern	93.31±5.83	101.56±7.86	89.03±5.13
	Southern	76.46±3.57	83.02±6.30	78.24±3.16
CK	Northern	41.45±3.61^{a}	37.41±2.58^{a}	26.93±2.58^{b}
	Southern	33.56±1.25	35.20±3.94	35.01±2.47
CS	Northern	1.44±0.12^{a,*}	1.79±0.11^{a,*}	0.86±0.08^{b}
	Southern	1.05±0.07^{x}	0.98±0.09^{x}	0.87±0.08^{x}

Significant differences within a population between acclimation temperatures are indicated with different letters. Asterisks indicate significant differences (P<0.05) between populations at a given acclimation temperature (indicated on the northern killifish values). Data are expressed as means ± s.e.m. (N=8). CK, creatine kinase; CS, creatine synthase; LDH, lactate dehydrogenase.

no increases in capillary to fiber ratio (Fig. 3B). This observation was surprising because although very few studies have examined the relationship between cold acclimation and changes in the capillary network, those that have done so (Egginton and Sidell, 1989; Egginton and Cordiner, 1997; Egginton et al., 2000; Egginton, 2002) have observed increases with low temperature. In addition, increases in mitochondrial content (such as those observed here) are often accompanied by an enhanced capillary network (Hoppeler et al., 1981; Johnston, 1982; Mathieu-Costello et al., 1992; Mathieu-Costello et al., 2005). In striped bass acclimated at 5°C *versus* 25°C, the aerobic muscles had a higher capillary to muscle fiber ratio, and capillary density (mm^{-2}) was maintained despite muscle fiber hypertrophy at lower temperatures (Egginton and Sidell, 1989; Egginton, 2002). A similar response was observed in rainbow trout (Egginton and Cordiner, 1997; Egginton et al., 2000). One possible explanation for this difference is that we utilized a relatively short acclimation period (3 weeks) in our study, whereas previous studies have used seasonally acclimatized fish or longer acclimation periods (8–12 weeks). In support of this hypothesis, the capillary to fiber ratio was highest in both northern and southern killifish acclimated to 15°C, the temperature of our long-term holding facility. Alternatively, the primarily subsarcolemmal location of killifish mitochondria compared with the primarily intramyofibrillar location observed in many other fishes [e.g. striped bass (Egginton and Sidell, 1989) and rainbow trout (St Pierre et al., 1998)] might make increasing the number of capillaries per fiber area less important in *F. heteroclitus*.

Similarly, we found no evidence of increases in the number or size of lipid droplets within the muscle, which has been suggested to promote oxygen delivery at colder temperatures when O_2 is readily available but diffusion is reduced (Ellsworth and Pitman, 1990; Desaulniers et al., 1996; St Pierre et al., 1998). In fact, we never detected any lipid droplets in *F. heteroclitus* muscle, even at low acclimation temperatures (despite the fact that we observed lipid droplets in test samples taken from rainbow trout using the same protocol; data not shown). Similarly, in a previous study (Fangue et al., 2008) we did not observe any increases in muscle lipid content with cold acclimation, suggesting that this mechanism of enhancing oxygen delivery is not utilized in *F. heteroclitus*. It is possible that the primarily subsarcolemmal location of the mitochondria in *F. heteroclitus* muscle reduces the need to facilitate intracellular diffusion of oxygen. It has been suggested that the relatively even distribution of mitochondria across red muscle fibers in Antarctic fish may represent an adaptation to facilitate metabolite diffusion in the cold (Archer and Johnston, 1991), but we see no evidence of such a phenomenon in *F. heteroclitus*. In fact, the subsarcolemmal position of mitochondria in *F. heteroclitus* muscle would be expected to result in challenges for the intracellular distribution of metabolites (particularly at low temperatures), as the rates of diffusion for ATP and other metabolites are thought to be approximately two orders of magnitude lower than that of oxygen (Hill, 1965). Interestingly, we observed significant increases in white muscle cytoplasmic creatine kinase activity with cold

Table 2. White muscle [ATP], [Cr_{free}], [CrP], pH_i, [ADP_{free}] and [ADP_{free}]:[ATP] ratio in the northern and southern killifish subspecies acclimated to 5, 15 and 25°C

Measure	Population	5°C	15°C	25°C
[ATP]	Northern	0.48±0.09^{a}	1.70±0.49^{b}	1.52±0.35^{b}
	Southern	0.87±0.17^{x}	0.88±0.25^{x}	2.16±0.37^{y}
[Cr_{free}]	Northern	14.39±0.75^{a}	15.72±1.39^{a}	14.59±0.88^{a}
	Southern	15.30±0.97^{x}	13.61±0.76^{x}	14.72±0.58^{x}
[CrP]	Northern	9.98±0.88^{a}	11.13±0.94^{a}	9.23±1.39^{a}
	Southern	9.68±0.67^{x}	8.74±0.89^{x}	6.55±0.60^{y}
pH_i	Northern	7.33±0.02^{a}	7.26±0.02^{a}	7.13±0.02^{b}
	Southern	7.38±0.03^{x}	7.17±0.03^{y}	7.08±0.02^{z}
[ADP_{free}]	Northern	1.98±1.03^{a,*}	4.58±0.85^{b}	4.11±0.97^{b}
	Southern	7.37±1.75^{x}	3.47±0.84^{y}	3.81±0.47^{y}
[ADP_{free}]:[ATP] ($\times 10^3$)	Northern	3.66±0.32^{a,*}	2.28±0.20^{a,b}	1.58±0.39^{b}
	Southern	5.16±0.49^{x}	2.41±0.31^{yz}	2.01±0.20^{z}

Significant differences within a population between acclimation temperatures are indicated with different letters. Asterisks indicate significant differences (P<0.05) between populations at a given acclimation temperature (indicated on the northern killifish values). Data are expressed as means ± s.e.m. (N=6 to 8). Cr, creatine; CrP, creatine phosphate; pH_i, intracellular pH. [ATP], [Cr_{free}] and CrP are expressed in μmol g^{-1} wet tissue; [ADP_{free}] is expressed in nmol g^{-1} wet tissue.

acclimation in the northern subspecies but not the southern subspecies (Table 1). Creatine kinase may play an important role in shuttling ATP from the mitochondria to the myofibrils in many muscle types (Saks et al., 2007), and thus the increase in creatine kinase activity in northern killifish might indicate an increased reliance on ATP shuttling.

Parameters associated with increased oxidative capacity

In contrast to the modest changes in processes involved in oxygen delivery with cold acclimation, we observed substantial changes in parameters associated with increased oxidative capacity in *F. heteroclitus*. Increases in mitochondrial volume densities and cristae surface areas are often associated with acclimation to colder environments (Johnston and Maitland, 1980; Johnston and Dunn, 1987; Egginton and Sidell, 1989; Sanger, 1993). In conjunction with the increase in oxidative fiber mass, mitochondrial volume density increased in striped bass acclimated to 5°C (Egginton and Sidell, 1989) and in carp at 2°C (Johnston and Maitland, 1980). Similarly, Antarctic fish have a significantly greater mitochondrial volume density than do Mediterranean fish of similar activity patterns (Johnston et al., 1998). Although observations of increasing cristae surface densities during cold acclimation are not as numerous as those for volume densities, higher surface densities have been reported in cold-acclimated rainbow trout (St Pierre et al., 1998). In the present study, both volume density and mitochondrial cristae surface area were higher in the cold-acclimated northern killifish. In contrast, southern killifish demonstrated no response for either variable at all acclimation temperatures. These differences may in part explain the previously observed differences in the oxidative capacity of isolated mitochondria per milligram mitochondrial protein between the subspecies (Fangue et al., 2009). It has been hypothesized that increases in mitochondrial volume density can compensate for reduced catalytic capacity and the diffusion of metabolites at colder temperatures (Tyler and Sidell, 1984) to support higher mitochondrial respiration rates (Fangue et al., 2009).

Increases in mitochondrial volume and/or cristae surface densities during cold acclimation are not observed in all species of fish. Cristae density is not modified by thermal acclimation in goldfish (Tyler and Sidell, 1984) or striped bass (Egginton and Sidell, 1989), despite higher mitochondrial volume densities. This may suggest that increases in volume density are sufficient in overcoming reduced oxidative capacity (Guderley, 2004). In contrast, cold-acclimated rainbow trout increase cristae density without changing volume density (St Pierre et al., 1998). One advantage of increasing cristae surface density and potentially the oxidative capacity per milligram of mitochondrial protein is that the myofibrillar volume density and contractile capacity is not compromised by mitochondrial proliferation (Guderley, 2004). In the case of killifish in the present study, both cristae surface densities and volume densities were elevated in the northern subspecies, suggesting that enhancements to oxidative capacity while minimizing the loss of contractile space are implemented as strategies for cold temperature tolerance.

Mitochondrial enzyme activities

Many studies have shown increased citrate synthase and cytochrome oxidase activities after cold acclimation, or when comparing Antarctic fish to temperate species (Crockett and Sidell, 1990; Battersby and Moyes, 1998; St Pierre et al., 1998; Lucassen et al., 2006). However, several studies have reported a more complex relationship between cold acclimation and enzyme activity, such that activity does not increase in one or both of white muscle or liver tissue (Lucassen et al., 2003; Fangue et al., 2009). Although mitochondrial enzyme activities are often used as proxies for mitochondrial density and activity (e.g. Grim et al., 2010), the relationships between these parameters were far from clear in our data. For example, in northern killifish, mitochondrial volume density did not differ between the two higher acclimation temperatures, but was approximately 60% greater at 5°C (Fig. 5), whereas citrate synthase activity almost doubled from 25 to 15°C, and did not increase further at lower acclimation temperatures. These data clearly indicate that mitochondrial amounts and mitochondrial enzyme activities do not necessarily change in concert. A different pattern was observed in an independent study of the effects of cold acclimation on northern killifish (Grim et al., 2010), in which no changes in citrate synthase activity were observed following 9 days of acclimation to 5°C. To further complicate this picture, in a previous study of thermal acclimation in killifish from our laboratory (Fangue et al., 2009) we observed changes in citrate synthase activities that exactly paralleled the changes in volume density we report here. One possible explanation for these varying findings could relate to differences in the time course and role of changes in enzyme activities relative to structural parameters, which are regulated by independent mechanisms within the cell. However, these discrepancies highlight the question of whether the changes we observed in mitochondrial volume density and cristae surface area have physiological relevance. Our previously published data (Fangue et al., 2009) suggest that differences in the whole-organism metabolic rate between the subspecies of killifish tend to be largest at 5°C, and that maximum mitochondrial oxygen consumption (state III respiration) differs between subspecies only when mitochondria are isolated from fish acclimated to 5°C. These physiological observations are consistent with the changes in mitochondrial ultrastructure that we observe here, and suggest that the changes in mitochondrial amount and ultrastructure could have a functional impact.

The regulation of mitochondrial activity

Although mitochondrial density, cristae surface area, mitochondrial enzyme activities and mitochondrial respiration measured *in vitro* are all potential indices of mitochondrial activity *in vivo*, actual *in vivo* mitochondrial respiration is controlled by a variety of parameters including substrate availability. For example, the availability of ADP is considered to be a potentially important control factor for mitochondrial oxidation *in vivo* (Brand and Murphy, 1987). Here, we observed a large change in the free concentration of ADP in response to cold acclimation in both northern and southern killifish, but these changes were in opposite directions, with an increase in southern killifish and a decrease in northern killifish (Table 2). However, [ATP] also changed with thermal acclimation in northern (but not southern) killifish between the 5 and 15°C groups, and the ADP:ATP ratio is a more appropriate indicator of the stimulatory signal in this case. Similar to the situation with the free concentration of ADP, the ADP:ATP ratio increased substantially with cold acclimation in southern killifish, and changed to a much lesser extent in northern killifish (Table 2). One possible explanation for this observation is that the differences in mitochondrial volume density between subspecies at low temperatures result in differences in the balance between ATP supply and demand, such that southern fish are unable to maintain adequate control over ADP:ATP ratios in the cold. Alternatively, it has been suggested that increases in mitochondrial abundance, such as those that may occur during cold acclimation, enhance the sensitivity of mitochondria to ADP signaling (Dudley et al., 1987; Egginton and

Sidell, 1989). Therefore, it is possible that cold-acclimated northern killifish, with high mitochondrial density, do not require as high an ADP:ATP ratio to stimulate mitochondrial oxygen consumption as do cold-acclimated southern killifish. However, there are contradictions in the literature to the idea that free ADP sensitivity is enhanced during cold acclimation (Blier and Guderley, 1993; Guderley and St-Pierre, 2002). In their study, Blier and Guderley (Blier and Guderley, 1993) reported a decrease in mitochondrial sensitivity to free ADP availability with cold acclimation in rainbow trout. The authors suggest that changes in mitochondrial membrane phospholipids can affect sensitivity to ADP content (van den Thillart and de Bruin, 1981; Blier and Guderley, 1993; Guderley et al., 1997). Northern killifish have been shown to undergo changes in mitochondrial membrane lipids in response to acclimation at 5°C (Grim et al., 2010), suggesting the possibility of a similar mechanism in this subspecies. Lower free ADP concentrations (and ADP:ATP ratios), such as those seen in cold-acclimated northern killifish, have also been hypothesized to reduce the use of glycolytic fuels in favor of substrates such as lipids (Holloszy and Coyle, 1984). High citrate synthase activities, such as those observed in northern killifish acclimated to low and moderate temperatures in the present study, have also been speculated to play a role in lipogenesis (Pörtner, 2002).

Conclusions and perspectives

The results of this study together with those of Fangue et al. (Fangue et al., 2009) provide one of the very few demonstrations of intraspecific variation in the plasticity of mitochondrial function, content and structure in response to low temperature acclimation. To our knowledge, only one other study in fish has examined the variation in mitochondrial properties during cold acclimation within a species (Lucassen et al., 2006). In their study, cod exhibited sequence differences in citrate synthase that were manifested at the functional level, providing a cold-adapted population a greater aerobic capacity at colder temperatures. Differences in plasticity between killifish subspecies could, in principle, be the result of genetic differences or could result from developmental plasticity due to differing thermal experience in the early life history of these wild-caught fish. *Fundulus heteroclitus* provides an ideal model system to distinguish between these possibilities. The ability to cross fish from the northern and southern subspecies, and the availability of natural hybrids on the Atlantic coast of the USA and Canada, offers the potential of identifying whether the differential plasticity observed here is the result of developmental plasticity or has a genetic basis.

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