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Do mitochondrial properties explain intraspecific variation in thermal tolerance?

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SUMMARY

As global temperatures rise, there is a growing need to understand the physiological mechanisms that determine an organism's thermal niche. Here, we test the hypothesis that increases in mitochondrial capacity with cold acclimation and adaptation are associated with decreases in thermal tolerance using two subspecies of killifish (*Fundulus heteroclitus*) that differ in thermal niche. We assessed whole-organism metabolic rate, mitochondrial amount and mitochondrial function in killifish acclimated to several temperatures. Mitochondrial enzyme activities and mRNA levels were greater in fish from the northern subspecies, particularly in cold-acclimated fish, suggesting that the putatively cold-adapted northern subspecies has a greater capacity for increases in mitochondrial amount in response to cold acclimation. When tested at the fish's acclimation temperature, maximum ADP-stimulated (State III) rates of mitochondrial oxygen consumption *in vitro* were greater in cold-acclimated northern fish than in southern fish but did not differ between subspecies at higher acclimation temperatures. Whole-organism metabolic rate was greater in fish of the northern subspecies at all acclimation temperatures. Cold acclimation also changed the response of mitochondrial respiration to acute temperature challenge. Mitochondrial oxygen consumption was greater in cold-acclimated northern fish than in southern fish at low test temperatures, but the opposite was true at high test temperatures. These differences were reflected in whole-organism oxygen consumption. Our data indicate that the plasticity of mitochondrial function and amount differs between killifish subspecies, with the less high-temperature tolerant, and putatively cold adapted, northern subspecies having greater ability to increase mitochondrial capacity in the cold. However, there were few differences in mitochondrial properties between subspecies at warm acclimation temperatures, despite differences in both whole-organism oxygen consumption and thermal tolerance at these temperatures.

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Key words: killifish, acclimation, adaptation, temperature, oxygen limited thermal tolerance, mitochondria.

INTRODUCTION

Understanding the factors that determine the distribution and abundance of species has historically been a central theme in both ecology and environmental physiology (Gaston, 2003; Somero, 2005). Although the pervasive effects of temperature on biochemical and physiological processes are thought to play a fundamental role in establishing biogeographic patterns in aquatic ectotherms (Hochachka and Somero, 2002), the precise mechanisms involved in establishing both the upper and lower thermal limits of organisms are still poorly understood (Pörtner et al., 2006). In light of evidence from polar, temperate and tropical ecosystems demonstrating that climate change is affecting the distribution and local abundance of many marine organisms (Parmesan, 2006; Pörtner and Knust, 2007; Root et al., 2003), it is critical to identify the precise physiological and biochemical mechanisms that define the limits of an organism's thermal niche.

The concept of oxygen and capacity limited thermal tolerance (OLTT) has recently been proposed as a unifying principle for understanding the mechanistic basis of whole-organism thermal tolerance in ectotherms (Pörtner, 2002; Pörtner et al., 2007). The OLTT hypothesis states that an ectotherm's thermal niche is linked to its ability to make physiological adjustments in response to temperature in order to maintain aerobic scope (the difference between maximum and resting metabolic rate). At both low and high temperature extremes, the OLTT hypothesis suggests that organismal performance is limited by the inability to supply oxygen to the respiring mitochondria, i.e. that thermal limitations result from a mismatch between oxygen supply and demand (Pörtner, 2001; Pörtner, 2002; Pörtner et al., 2004). Increasing temperatures result in an increase in the resting oxygen demand, and since the ability of the circulatory and ventilatory systems to supply oxygen has a maximum limit, aerobic scope declines as temperature increases, causing a reduction in performance (reviewed by Pörtner et al., 2004). Decreasing temperatures, in contrast, are thought to cause a decline in the ability of the mitochondria to produce ATP, compromising an organism's ability to perform normal physiological functions including the function of the ventilatory muscles and the circulatory pumps that are needed to supply oxygen to the working tissues at low temperatures. Thus, acclimation or adaptation to the cold is likely to involve increases in mitochondrial density and/or changes in their functional properties to improve ATP production and maintain aerobic scope in the cold. This increase in mitochondrial capacity in the cold, however, may result in a tradeoff during warming because higher mitochondrial capacity increases resting oxygen demand at warmer temperatures. According to the OLTT hypothesis, this increased metabolic rate at warm temperatures would be expected to decrease metabolic scope, thus decreasing the upper thermal limits of the organism. Thus, Pörtner has suggested that '*adjustments of mitochondrial densities and their functional properties appear as*

a critical process in defining and shifting thermal tolerance windows' (Pörtner, 2001).

One particularly interesting aspect of the OLTT framework is that it generates several specific predictions with respect to the relationship between mitochondrial properties and whole-organism responses to temperature: (1) cold-adapted or -acclimated organisms should have greater mitochondrial capacity (either increased mitochondrial amount or increased functional activity), (2) cold-adapted or -acclimated organisms with increased mitochondrial content or function should have higher standard ('resting') metabolic rate relative to warm-adapted organisms and (3) the increased metabolic rate in cold-adapted or -acclimated organisms should be associated with a decrease in thermal tolerance. The purpose of the current study was to test these predictions in a single species that can be acclimated to a wide range of temperatures and that has regional subspecies that are thought to be adapted to different thermal environments. By working within a single eurythermic species, we minimize the effects of phylogenetic differentiation among the organisms being compared and can simultaneously assess the effects of thermal acclimation and adaptation.

Populations of the common killifish (*Fundulus heteroclitus*) have been studied extensively as a model to investigate mechanisms of local thermal adaptation (Schulte, 2001). These fish, which inhabit estuaries and salt marshes along the East Coast of North America from Newfoundland to Northeastern Florida, have a broad capacity to tolerate and acclimate to a wide range of environmental temperatures (Fangue et al., 2006; Powers and Schulte, 1998; Schulte, 2001). The species has been divided into two regional subspecies – a northern form, *F. h. macrolepidotus* Walbaum, and a southern form, *F. h. heteroclitus* Linnaeus (Morin and Able, 1983) – that differ both genetically and physiologically. We have previously shown that these subspecies differ in thermal tolerance such that the putatively cold-adapted northern form has a lower thermal tolerance than the southern form at all acclimation temperatures and that in both subspecies thermal tolerance increases with increasing acclimation temperature (Fangue et al., 2006). In the present study, we assess whole-organism metabolic rate and mitochondrial properties of killifish from the northern and southern subspecies acclimated to a range of temperatures in order to test some of the predictions of the OLTT hypothesis.

MATERIALS AND METHODS

Experimental animals

Adult killifish of the northern subspecies (*Fundulus heteroclitus macrolepidotus*) were collected from Hampton, NH, USA (42°54′46″N), and fish of the southern subspecies (*Fundulus heteroclitus heteroclitus*) were collected from Brunswick, GA, USA (31°7′31″N). All collections were made in late spring of 2004. Fish were acclimated to laboratory conditions as described previously (Fangue et al., 2006). Six replicate 75 liter tanks were used for each acclimation temperature (5°C, 15°C and 25°C), and fish were housed in a split-tank design with northern fish on one side and southern fish on the other to minimize tank effects. Treatment of all experimental animals was in accordance with the University of British Columbia animal care protocol #A01-0180.

Estimation of mitochondrial amount

Four male and four female killifish from each subspecies and temperature acclimation group were sampled for the estimation of mitochondrial amount using mitochondrial enzyme activity and gene expression as proxies. Mean mass was not significantly different between subspecies, acclimation groups or sex (means ± s.e.m.; northern killifish, 7.39±0.72 g; southern killifish, 7.94±0.56 g).

Total RNA was extracted from muscle and liver tissue as described in a previous study (Fangue et al., 2006). Gene-specific primers for citrate synthase (*CS*) and cytochrome *c* oxidase subunit two (*COXII*) were designed using Primer Express software (version 2.0.0; Applied Biosystems, Foster City, CA, USA) from published sequences for *F. heteroclitus*: Accession No. CN983139, *CS* forward 5′ CGG CAT GAC GGA GAT GAA CT 3′, *CS* reverse 5′ GAG GGC CCG GGA CAC A 3′; Accession No. AY735182, *COXII* forward 5′ AGT TTA GGA ATC AAA ATA GAC GCA GTT 3′, *COXII* reverse 5′ CGG GAG GTA ATG AAG GCT GTT 3′; qRT-PCR reactions and melt curve analyses were performed as described previously (Fangue et al., 2006). Results are expressed relative to total RNA used in the reverse transcription reaction.

To assess mitochondrial enzyme activity, frozen liver or muscle tissue was homogenized in 9 volumes of ice-cold buffer (100 mmol l^{-1} Hepes, 5 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT and 0.05% Triton T-100, pH=7.4 at 20°C) using two low-speed passes of 10 s each with a Polytron homogenizer (Fisher Scientific, Nepean, ON, Canada). Cellular debris was removed by a 5 min centrifugation at 2500 ***g*** and 4°C, and preliminary tests ensured the complete release of enzymes from the tissues using this procedure. The remaining supernatant was diluted with buffer containing only 100 mmol l^{-1} Hepes and 5 mmol l^{-1} EDTA, pH=7.4 at 20°C as appropriate for each assay. COX and CS activities were determined as described previously (Moyes et al., 1997). Protein concentrations were determined using the bicinchoninic acid (BCA) method.

Mitochondrial respiration

Four to six fresh killifish livers were pooled for each mitochondrial preparation (approximately 1 g total tissue) to obtain sufficient material for 5–7 respiration assays. Liver tissue was finely diced on cooled glass plates and introduced to 9 volumes of ice-cold isolation medium (250 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 25 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EGTA, 1.5% bovine serum albumin, fraction V 'fatty acid free', pH=7.4 at 20°C) (Bagarinao and Vetter, 1990). Liver tissue was then homogenized on ice by three passes with a motorized Teflon® tissue grinder. The resulting homogenate was centrifuged at 600 ***g*** for 10 min at 4°C to pellet cellular debris. The supernatant was transferred to a new, pre-cooled tube by pouring through glass wool to remove the majority of the fat. The defatted supernatant was then centrifuged at 6000 ***g*** for 10 min at 4°C to pellet the mitochondria, and any remaining fat was carefully removed from the preparation. The pellet was washed twice with isolation media, gently resuspended and kept on ice until all mitochondrial respiration measurements were completed.

Oxygen consumption of isolated mitochondria was measured with an Oroboros Oxygraph 2-k high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria). The oxygen electrodes were calibrated daily with air-saturated assay medium (150 mmol l^{-1} KCl, 25 mmol l^{-1} KH_2PO_4 and 20 mmol l^{-1} Hepes, pH=7.4 at 20°C) (Bagarinao and Vetter, 1990) at each experimental temperature, and zero oxygen measures were made by the addition of sodium dithionite. Oxygen solubility in the assay medium at each temperature was calculated as described previously (Gnaiger and Forstner, 1983). Oxygen consumption of approximately 0.3 mg mitochondrial protein in 1.8 ml air-saturated assay medium was measured following the addition of 0.25 mmol l^{-1} malate to spark the Krebs cycle, and 5 mmol l^{-1} pyruvate was added as the carbon substrate. Pyruvate was selected in order to obtain maximum rates of State III respiration in fish mitochondria

(Johnston et al., 1998). State III rates of oxygen consumption were obtained by adding saturating ADP to a concentration of 0.625 mmol l^{-1}. When all ADP had been phosphorylated, the rate of State IV respiration was measured for 5 min before oligomycin, an inhibitor of mitochondrial F_0F_1-ATPase, was added at 0.625 mmol l^{-1} (State IV_{ol} respiration reflecting proton leak). Respiratory control ratios (as indices of mitochondrial coupling) were determined by dividing State III by State IV (RCR) or State III by State IV_{ol} (RCR_{ol}) (Estabrook, 1967).

Mitochondrial preparations were first assayed at the fish's acclimation temperature (5, 15 or 25°C), and the RCR was calculated to determine the coupling of the preparation. Preparations with RCR values substantially less than 4 were not used for further analysis. Samples of the mitochondrial preparation were assayed at temperatures between 2 and 37°C. The order of assay temperature was randomized, and the final assay of each mitochondrial preparation was repeated at the fish's acclimation temperature to ensure that the preparation had not become progressively uncoupled over the duration of the experiment. All assays for each mitochondrial preparation were completed within 8 h from the start of the mitochondrial isolation, but preparations were often stable for more than 12 h. Within each acclimation temperature group and killifish subspecies, mitochondrial respiration measurements were conducted at each assay temperature for 4–6 independent mitochondrial preparations. Protein concentrations were determined using the BCA method.

Whole-animal respirometry

Mass-specific oxygen consumption (μmol g^{-1} h^{-1}) was determined for northern and southern killifish acclimated to and tested at one of three temperatures (5, 15 or 25°C) or acclimated to 5°C and acutely challenged with increasing measurement temperatures. Mean mass did not differ between subspecies (northern killifish, 5.98±0.25 g, N=22; southern killifish, 6.71±0.34 g, N=21). Fish were placed in 250 ml flow-through respirometers at their acclimation temperature overnight (12–18 h) to recover from handling stress and were not fed during this time. Following the recovery period, an oxygen probe (FOXY-R, Ocean Optics Ltd, Dunedin, FL, USA) was introduced and the respirometer sealed. The decline of oxygen was recorded, and the mass-specific oxygen consumption was calculated as previously described (Sloman et al., 2008). In the acute thermal challenge experiment, six northern 5.49±0.27 g (mean ± s.e.m.) and six southern 6.64±0.82 g killifish were held in respirometers overnight at 5°C, and metabolic rate was measured as previously described. The respirometer was then opened and flushed with well-oxygenated water while the temperature was increased by 5°C at a rate of 0.3°C min^{-1}. The respirometer was then closed and metabolic rate was measured over the course of 30 min. This procedure was repeated such that metabolic rate was measured at temperatures of 5, 10, 15, 20 and 25°C. Oxygen consumption measurements at water temperatures of 30°C could not be determined because, at these temperatures, killifish began to lose equilibrium, leading to heat death.

Statistical analyses

Killifish metabolic rates, mitochondrial respiration measurements, enzyme activities and mRNA data sets were analyzed by multiple analysis of variance (ANOVA) with subspecies, acclimation group and/or assay temperature as factors. Metabolic rate data from the acute thermal challenge experiment were analyzed with a two-factor, repeated-measures ANOVA. All data met the assumptions of normality, and data were log transformed where necessary to meet assumptions of homogeneity of variance. When interaction terms were not significant, *post-hoc* comparisons were performed among the groups with the Student-Newman-Keuls multiple range test (SNK MRT). If the interaction terms were significant, the data were separated and analyzed independently using one-way ANOVA followed by SNK MRT. For all analyses, α was set at 0.05. For the analysis of proton leak (IV_{ol}), slope discontinuities were determined using the Regress algorithm developed by Yeager and Ultsch (Yeager and Ultsch, 1989) for statistical determination of critical points in continuous data sets by determining the intersection of two best fit lines.

RESULTS

Estimation of mitochondrial amount

We measured the activity and mRNA levels of two mitochondrial enzymes: citrate synthase (CS) and cytochrome oxidase (COX) as an indicator of mitochondrial amount (Lucassen et al., 2003; Lucassen et al., 2006; Moyes et al., 1997). Liver CS activity differed significantly between subspecies and with acclimation temperature (two-way ANOVA P=0.013, P<0.001 respectively), and there was no interaction (Table 1). Cold acclimation increased CS activity in both subspecies, and northern fish had significantly higher CS activity compared with southern fish (significant in *post-hoc* tests at 5°C; Table 1). Similar results were observed in muscle (two-way ANOVA, P=0.033 for subspecies and P=0.022 for acclimation temperature), with increased CS activity in the cold and in northern

Table 1. Citrate synthase (CS) and cytochrome *c* oxidase (COX) enzyme activity (units mg^{-1} protein) and mRNA levels (relative to total RNA) in northern (N) and southern (S) killifish subspecies acclimated to 5, 15 or 25°C

	Population	5°C	15°C	25°C
Liver				
CS enzyme activity	N	0.06±0.004^{a,*}	0.04±0.002^{b}	0.04±0.003^{b}
	S	0.05±0.003^{x}	0.04±0.004^{x,y}	0.04±0.002^{y}
CS mRNA	N	1.89±0.352^{a,*}	1.09±0.148^{b,*}	1.25±0.086^{b,*}
	S	0.85±0.136^{x}	0.43±0.112^{x}	0.35±0.060^{x}
Muscle				
CS enzyme activity	N	0.06±0.005^{a,*}	0.04±0.003^{b}	0.04±0.005^{b}
	S	0.04±0.002^{x}	0.03±0.001^{x}	0.04±0.005^{x}
CS mRNA	N	0.84±0.071^{a,*}	0.39±0.064^{b}	0.52±0.093^{b,*}
	S	0.26±0.053^{x}	0.21±0.061^{x}	0.16±0.023^{x}
Liver				
COX enzyme activity	N	0.25±0.012^{a,*}	0.22±0.020^{a}	0.21±0.010^{a}
	S	0.17±0.017^{x}	0.17±0.025^{x}	0.18±0.026^{x}
COX mRNA	N	0.80±0.063^{a}	0.61±0.048^{b}	0.57±0.054^{b}
	S	0.9±0.091^{x}	0.56±0.053^{y}	0.66±0.062^{y}
Muscle				
COX enzyme activity	N	0.04±0.006^{a}	0.05±0.005^{a}	0.05±0.004^{a}
	S	0.05±0.008^{x}	0.05±0.006^{x}	0.05±0.009^{x}
COX mRNA	N	1.19±0.222^{a,*}	0.70±0.108^{b}	1.00±0.151^{a,b}
	S	0.65±0.090^{x}	0.51±0.072^{x}	0.71±0.078^{x}

Significant differences within a population between acclimation temperatures are indicated with different letters. An asterisk (*) indicates a significant difference between populations at a given acclimation temperature (indicated on northern values). Data are expressed as means ± s.e.m. (N=8), and P≤0.05 for all significant comparisons.

fish (Table 1). In both liver and muscle, *CS* mRNA levels showed a similar pattern, as did protein activity, with highest expression observed in cold-acclimated northern fish (two-way ANOVA, liver $P \leq 0.003$ for acclimation temperature and subspecies, muscle $P < 0.001$ for acclimation temperature and subspecies) (Table 1).

In the liver, COX activity was greater in the northern subspecies (two-way ANOVA, $P=0.003$), and there was no significant effect of acclimation temperature or an interaction (Table 1). This pattern was not observed in muscle, however, where there was no significant difference in COX activity between subspecies or acclimation groups (Table 1). Levels of *COXII* mRNA did not correspond with protein activity in either muscle or liver. In liver, *COXII* mRNA levels increased significantly with decreasing acclimation temperature (two-way ANOVA, $P=0.001$), and there was no significant difference between subspecies and no significant interaction (Table 1). In muscle, *COXII* mRNA levels differed significantly between subspecies, with northern fish having higher mRNA levels (two-way ANOVA, $P=0.003$; Table 1), and there was no significant effect of acclimation temperature or an interaction.

These data provide mixed evidence for an increase in mitochondrial amount in northern fish, or with cold acclimation. However, there is a consistent pattern that cold-acclimated northern fish tend to have greater mitochondrial enzyme activity and mRNA levels in most tissues than do southern fish. There is less support for differences in mitochondrial enzyme activity between subspecies when the fish are acclimated to warm temperatures.

Mitochondrial respiration

We measured maximum ADP stimulated (State III) oxygen consumption rates of mitochondria isolated from liver as an index of mitochondrial functional capacity. Respiratory control ratios, RCR (III:IV) as well as RCR_{ol} (III:IV_{ol}; the respiratory control ratio calculated using the respiratory rate of mitochondria inhibited by oligomycin), were measured as an indicator of mitochondrial coupling (Table S1 in supplementary material). Estimates were always greater than 4.0 (RCR) and 10.4 (RCR_{ol}) when assayed at the fish's acclimation temperature. Thus, liver mitochondria from both northern and southern fish were generally highly coupled, with only modest (and non-statistically significant) declines in coupling at the highest assay temperatures at which accurate State III rates could be determined.

Thermal acclimation affected the upper temperature sensitivity of mitochondrial oxidative phosphorylation. State III rates of mitochondria from both northern and southern fish acclimated to 5°C and assayed at 37°C could not be measured, as these mitochondria were insensitive to ADP at this assay temperature whereas mitochondria from 15°C- and 25°C-acclimated fish remained coupled and responsive to ADP up to 37°C, uncoupling only at higher temperatures.

Thermal acclimation had different effects on the response of mitochondria to acute temperature challenge in each killifish subspecies (Fig. 1). Mitochondria from both northern and southern fish acclimated to 25°C showed the expected exponential increase in State III respiratory rates with increasing test temperature. Surprisingly, this pattern was not maintained in mitochondria isolated from fish acclimated at 15°C or 5°C. The acute thermal response curves deviated from the standard exponential form as acclimation temperature decreased, and the extent and nature of this deviation differed between northern and southern fish. As a result, the relationship between the respiratory rates of northern and southern mitochondria at any given test temperature differed, depending on the acclimation temperature of the fish from which

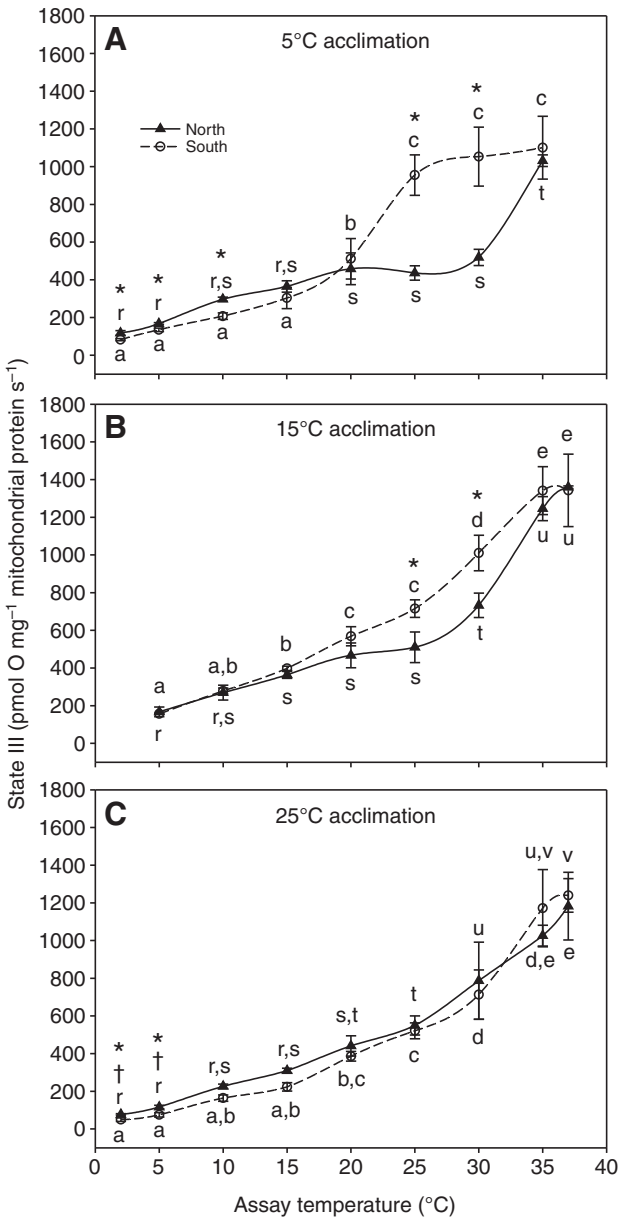

Fig. 1. Temperature dependence of State III (pmol O mg^{-1} mitochondrial protein min^{-1}) respiration rates, determined in mitochondria from 5°C (A), 15°C (B) and 25°C (C) acclimated northern (filled triangles) and southern (open circles) killifish. Significant differences within a population and acclimation temperature are indicated with different letters. An asterisk (*) indicates a significant difference between populations within an acclimation temperature for a given assay temperature. A † indicates a significant difference between the 25°C acclimation group and the 5 and 15°C acclimation groups when assayed at the same temperature (indicated on 25°C acclimation data). Data are expressed as means ± s.e.m. (N=4–6), and $P \leq 0.05$ for all significant comparisons.

the mitochondria were derived. At low test temperatures (2 and 5°C), mitochondria from northern fish had greater State III respiratory rates than those from southern fish when the mitochondria were isolated from fish acclimated to 5 and 25°C but not when mitochondria were isolated from fish acclimated to 15°C. In contrast, at higher test temperatures (25 and 30°C), mitochondria isolated from southern fish had higher State III respiratory rates

than those isolated from northern fish when the fish were acclimated to either 5 or 15°C but not when the fish were acclimated to higher temperatures.

Warm acclimation came at a cost in terms of mitochondrial function in the cold. Warm-acclimated mitochondria (25°C) from both northern and southern fish exhibited State III respiratory rates one-third lower than rates for 5°C- or 15°C-acclimated mitochondria in both subspecies when assayed at low temperatures (compare Fig. 1C with Fig. 1A,B). Interestingly, mitochondria isolated from warm-acclimated southern fish tended to have reduced performance compared with mitochondria from cold-acclimated southern fish, even when assayed at the warm acclimation temperature (25°C). At 25°C, warm-acclimated mitochondria from southern fish respired at approximately one-half the rate of mitochondria isolated from cold-acclimated southern fish (compare Fig. 1C with Fig. 1A). In contrast, mitochondria from northern killifish, regardless of acclimation temperature, had equivalent State III rates between acclimation groups when assayed at 15 and 25°C.

We determined the rates of oxygen consumption for mitochondria inhibited by oligomycin (State IV_{ol}) as an indicator of proton leak. Assay temperature had a significant effect on State IV_{ol}, with rates increasing exponentially with assay temperature in all acclimation groups and for both subspecies (Fig. 2). However the State IV_{ol} rates were always low compared with the ADP-stimulated (State III) rates and demonstrate no evidence of the extreme non-linearities observed in the State III rates, and thus these data cannot explain the unusual shape of the State III oxygen consumption curves, particularly those observed for mitochondria from cold-acclimated fish (Fig. 1A).

Although there were few differences in State IV_{ol} rates between subspecies at any assay temperature, the assay temperature at which these curves reached their inflection point (the largest change in slope between two assay temperatures) differed among acclimation temperature groups such that mitochondria from fish acclimated to 5°C showed an inflection at assay temperatures of 21.8°C for northern and 21.4°C for southern mitochondria. In the 15°C-acclimated groups, the temperature of inflection shifted to 27.5°C for northern mitochondria and 27.1°C for southern mitochondria. The inflection point was 29.8°C for northern mitochondria and 29.7°C for southern mitochondria isolated from fish acclimated to 25°C. When these data were plotted on Arrhenius axes, however, there were no obvious discontinuities (break points) in the slopes (data not shown).

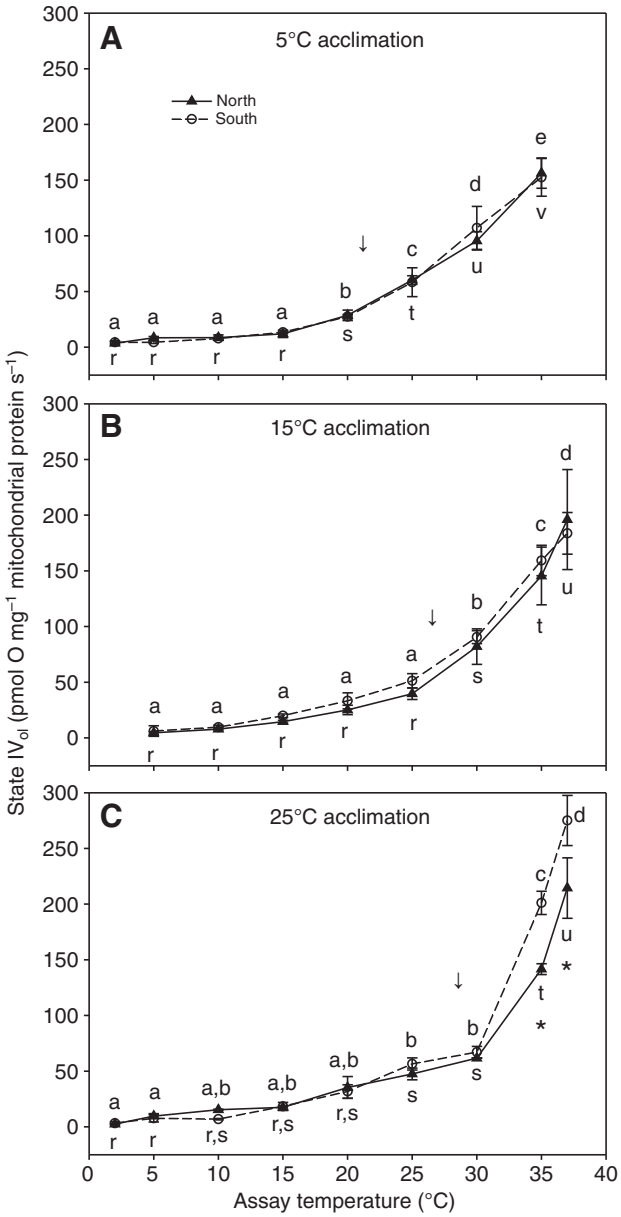

Fig. 2. Temperature dependence of State IV_{ol} (pmol O mg^{-1} mitochondrial protein min^{-1}) respiration rates, determined in mitochondria from 5°C (A), 15°C (B) and 25°C (C) acclimated northern (filled triangles) and southern (open circles) killifish. Significant differences within a population and acclimation temperature are indicated with different letters. An asterisk (*) indicates a significant difference between the 25°C acclimation group and the 5 and 15°C acclimation groups when assayed at the same temperature (indicated on 25°C data). An arrow (↓) indicates the temperature of inflection (the assay temperature where the largest change in slope was observed) within each acclimation temperature group. Data are expressed as means ± s.e.m. (N=4–6), and $P \leq 0.05$ for all significant comparisons.

Whole-organism metabolic rate

To determine whether the differences in mitochondrial properties between subspecies and acclimation groups had any effect on properties at higher levels of organization, we measured whole-organism oxygen consumption of fish from both subspecies acclimated to 5, 15 and 25°C. There was a significant effect of subspecies and acclimation temperature on metabolic rate, with no significant interaction (two-way ANOVA, $P<0.001$ for subspecies and acclimation temperature) (Fig. 3A). As expected, metabolic rate increased with increasing acclimation temperature. Across the whole temperature range, Q_{10} for metabolic rate was approximately 2.3 and was similar between the two subspecies. Q_{10} was slightly greater between 5 and 15°C (2.8) and slightly lower (1.9) at higher temperatures in both killifish subspecies. At all acclimation temperatures, northern fish had a higher metabolic rate than did southern fish.

Due to the striking differences between subspecies in the performance of mitochondria isolated from cold-acclimated killifish in response to acute thermal challenge, we next tested the acute effects of temperature on whole-organism metabolic rate in fish acclimated to 5°C (Fig. 3B). Metabolic rate increased significantly with increasing temperature (two-way repeated-measures ANOVA, $P<0.001$), and there was no significant effect of subspecies. However, there was a significant interaction (P=0.043). As before, the metabolic rate of northern fish was greater than that of southern fish when tested at their acclimation temperature (5°C), but

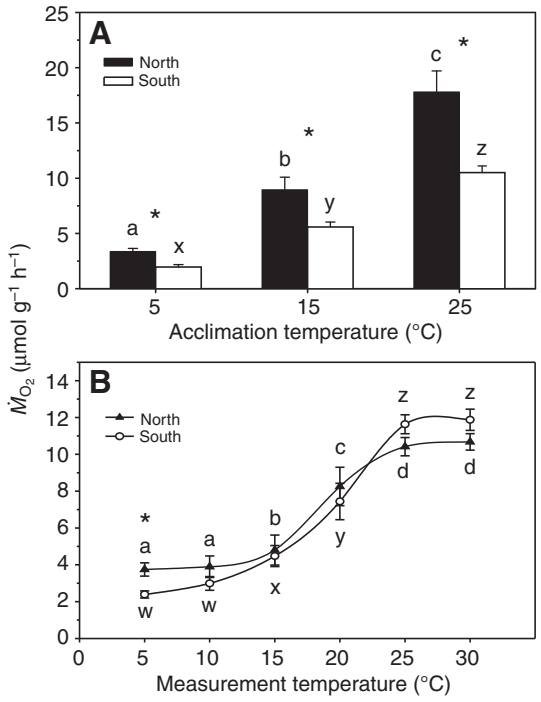

Fig. 3. Metabolic rates (μmol g^{-1} h^{-1}) for northern (black) and southern (white) killifish acclimated to 5, 15 or 25°C (A) or acclimated to 5°C and acutely challenged with increasing temperatures (B). Oxygen consumption rate ($\dot{M}_{O_2}$) values are expressed as means ± s.e.m. (N=6–8 per population and temperature treatment). Different letters indicate significant differences (P≤0.05) between acclimation groups within a population, and an asterisk (*) indicates a significant difference (P≤0.05) between populations within an acclimation temperature.

metabolic rate did not differ between subspecies at any other test temperature. Similar to the results for mitochondrial State III respiratory rates, whole-organism metabolic rates of the two subspecies converged as temperature increased, with the two curves crossing at temperatures above 20°C.

DISCUSSION

The most striking observation presented here is that acclimation temperature had a different effect on the acute response to temperature of mitochondria from each subspecies of killifish (Fig. 1). There were few functional differences between subspecies in mitochondria isolated from fish acclimated to 25°C, except at low assay temperatures, and these mitochondria showed the expected roughly exponential increase in respiratory rates with acute exposure to increasing temperature. In contrast, mitochondria from fish acclimated to 5°C had a much more complex response to acute temperature change, and the shape of the response differed between subspecies. These data demonstrate that there is plasticity in the function of mitochondria in killifish and that this plasticity involves changes in the way in which the mitochondria respond to acute temperature change. Interestingly, the nature and degree of this response differs between the northern and southern subspecies. This complex interaction between subspecies, acclimation temperature and the acute effects of temperature on mitochondrial function has important implications for the OLTT hypothesis because this interaction suggests an additional mechanism whereby organisms may alter their mitochondrial properties and thus adds another layer of complexity to the OLTT hypothesis.

The OLTT hypothesis predicts that both cold acclimation and adaptation should cause an increase in mitochondrial amount. Although there is ample evidence for an increase in mitochondrial amount with cold acclimation in a variety of species (Guderley, 2004; Johnston et al., 1998; Pörtner et al., 2007), we observed only modest, if any, increases in mitochondrial enzyme activity in response to cold acclimation, and the responses differed between enzymes and tissues. It is possible that mitochondrial enzyme activity is simply a poor proxy for mitochondrial amount, as other studies have also observed disconnections between the effects of acclimation on the activities of various mitochondrial enzymes (Lannig et al., 2003; Lucassen et al., 2003; Lucassen et al., 2006). To account for this possibility, we also examined mRNA levels for these genes as an alternative index of mitochondrial amount. *COXII* mRNA has been suggested as a useful measure of the transcription rate of the mitochondrial genome as the entire mitochondrial genome is transcribed simultaneously (Fernandez-Silva et al., 2007). Our results show that cold significantly increases *COXII* mRNA expression in the liver in both northern and southern fish but that this was not the case in muscle. Taken together, these data clearly indicate that there is no simple and uniform response of all tissues to increase mitochondrial amount in response to cold acclimation in *F. heteroclitus*.

Similarly, our data only provide equivocal support for the prediction of the OLTT hypothesis that cold adaptation is associated with increases in mitochondrial amount, as there are few consistent differences in mitochondrial amount between the subspecies across all acclimation temperatures. However, our data do suggest that there may be an interaction between cold adaptation and cold acclimation in this eurythermal species, in that differences between the subspecies in both mitochondrial enzyme activity and mRNA levels were much more pronounced in fish acclimated to low temperatures than in fish acclimated to warm temperatures. Six of the eight variables tested differed significantly between subspecies in cold-acclimated fish while only two of these variables differed between subspecies in warm-acclimated fish. Thus, *Fundulus heteroclitus macrolepidotus* (the northern subspecies) may have somewhat greater capacity to acclimate to the cold by increasing mitochondrial amount than does *Fundulus heteroclitus heteroclitus* (the southern subspecies). A similar pattern has been seen in cod populations distributed across a latitudinal cline. Lucassen et al. showed that, in both muscle and liver, differences between cod populations in mRNA expression and enzyme activities of CS and COX were more significant when fish were acclimated to colder temperatures (Lucassen et al., 2006). Together, these similar observations in two phylogenetically distant fish species suggest that there may be a common pattern of increased plasticity in mitochondrial amount in cold-adapted eurythermal fishes.

The OLTT hypothesis also predicts that mitochondrial function (as measured by State III respiratory rates) should increase with cold acclimation, and substantial previous data in other species support this idea (Bouchard and Guderley, 2003; Guderley and Johnston, 1996; Kraffe et al., 2007; Sommer and Pörtner, 2004). In contrast, our data do not show a strong signal of increased mitochondrial respiratory rates in mitochondria isolated from fish acclimated at lower temperatures, when compared at equivalent assay temperatures. Warm acclimation did, however, result in a shift in the upper thermal sensitivity of mitochondria to acute temperature challenge such that mitochondria from northern and southern fish acclimated to 15 and 25°C remained responsive to ADP at assay temperatures up to 37°C (Fig. 1B,C) whereas the respiratory rates of 5°C-acclimated mitochondria could not be measured at

temperatures greater than 35°C (Fig. 1A). Similarly, Dahlhoff and Somero found that the Arrhenius break temperature for mitochondrial function increased with acclimation temperature in multiple eurythermal species of abalone (Dahlhoff and Somero, 1993). This idea is consistent with the OLTT hypothesis, in that the more thermally sensitive mitochondria from cold-acclimated fish cease to function at a lower temperature upon acute warming, potentially limiting the aerobic scope of cold-acclimated fish at warm temperatures. In addition, we observed that the inflection point of the response of State IV respiration to acute temperature challenge was somewhat different between different acclimation groups (Fig. 2), with mitochondria from warm-acclimated fish having a higher inflection temperature than mitochondria from cold-acclimated fish. Although at present no data are available that bear on the possible mechanistic basis for these differences, a possible explanation could be changes in mitochondrial membrane lipid composition with acclimation, resulting in different fluidity and phase change behaviour. Such changes have been observed in other fish species (e.g. Kraffe et al., 2007) and have been associated with changes in mitochondrial function.

One additional critical issue that must also be considered is that, at present, complete data on the thermal windows of *F. heteroclitus* are lacking. CT_{Max} and CT_{Min} provide an index of the maximum and minimum temperatures at which a fish can survive and thus provide an index of one aspect of the thermal window. The thermal windows for growth and reproduction are, however, likely to be much narrower, and it is this narrower index of the thermal window that is perhaps most relevant to considerations of the OLTT hypothesis. Measurements of the pejus temperature (i.e. the temperature at which aerobic scope begins to decline) may be particularly revealing with respect to the relationship between mitochondrial properties and the thermal window at the organismal level and thus provide a fruitful avenue for further study in this system.

The OLTT hypothesis also predicts that mitochondrial respiratory rate should increase with cold adaptation. Our data provide some support for this idea in that mitochondria from both cold-acclimated and warm-acclimated northern killifish have higher State III respiratory rates than do mitochondria from southern fish when assayed at low temperatures. However, this was not the case for mitochondria from fish acclimated to intermediate temperatures nor for mitochondria assayed at higher temperatures. In fact, at high assay temperatures, mitochondria isolated from intermediate and cold-acclimated southern killifish had higher respiratory rates than did northern mitochondria. This peculiar observation is the result of the complex shape of the acute response to temperature of mitochondria from cold-acclimated fish and the fact that these patterns differ between killifish subspecies.

One interesting aspect of the unusual shape of the mitochondrial temperature curves for cold-acclimated fish is that mitochondria from northern fish have a large zone across which the mitochondria demonstrate limited thermal sensitivity (Q_{10}=1.1, 20–30°C assay temperatures) whereas the State III respiratory rates of southern mitochondria increased with increasing assay temperature across this range (Q_{10}=2.1) and instead had a zone of limited temperature sensitivity (Q_{10}=1.2) over a higher temperature range. Studies comparing acute effects of temperature on mitochondrial respiration with thermal acclimation and adaptation are rare, and most recent studies have found simple linear or exponential increases in State III rates with increasing assay temperatures, with Q_{10}s of 2–3 across a broad range of temperatures (e.g. Johnston et al., 1998; Sommer and Pörtner, 2004). However, early studies of eurythermal marine invertebrates (Newell and Pye, 1970; Newell and Pye, 1971) have provided evidence for a large zone of thermal independence of mitochondrial respiration across environmentally relevant temperature ranges, similar to that observed here for cold-acclimated *F. heteroclitus*. The observation that this region of relative thermal independence is at a higher temperature in mitochondria from cold-acclimated southern fish compared with those for mitochondria from cold-acclimated northern fish is consistent with the observed difference between these subspecies in the position of their thermal windows, which are shifted upwards by approximately 1.5°C in southern fish relative to northern fish (Fangue et al., 2006). However, the observed shift in the thermal window between subspecies is present regardless of acclimation temperature, whereas differences in the shape of the acute response of the mitochondria to temperature change are not present in warm-acclimated fish. This observation suggests that additional mechanisms must be involved in specifying thermal windows in *F. heteroclitus*.

A critical aspect of the OLTT hypothesis is that increases in mitochondrial properties and function as a result of cold adaptation cause increases in whole-organism metabolic rate, which in turn result in decreases in thermal tolerance. To test this hypothesis, we assessed the metabolic rate of northern and southern *F. heteroclitus* acclimated to a range of temperatures. Consistent with the OLTT hypothesis, metabolic rate was greater in northern fish than in southern fish at all acclimation temperatures. As expected under the OLTT framework, this increased metabolic rate is associated with the decreased thermal tolerance that we have observed in the northern subspecies (Fangue et al., 2006). Differences in the metabolic rate of killifish embryos (DiMichele and Westerman, 1997) as well as isolated heart tissue at warm acclimation temperatures (Podrabsky et al., 2000) have also been recorded, with northern fish having higher metabolic rates than southern fish. However, these differences in whole-organism metabolic rate do not relate in any simple way to the differences in mitochondrial properties that we have observed between these subspecies; differences in mitochondrial properties were more evident at low acclimation temperatures whereas the differences in whole-organism metabolic rate between subspecies were present at all acclimation temperatures.

In addition, measurements of metabolic rate at a single acclimation temperature did not capture the effects of acclimation on the acute response of the mitochondria to temperature that we have observed in the mitochondria of this species. To determine whether these mitochondrial properties were manifested at higher levels of organization, we assessed the effects of acute thermal challenge on whole-organism metabolic rate in 5°C-acclimated fish, which demonstrate a non-linear response of mitochondrial respiration to acute thermal challenge. As was the case for mitochondrial respiratory rates, whole-organism metabolic rates differed between subspecies at low temperatures and then converged at intermediate temperatures. At high temperatures (>20°C), however, the differences between subspecies in mitochondrial respiratory rate were much greater than those at the whole-organism level. Thus, the effects observed at the mitochondrial level are partially, but not fully, reflected at the whole-organism level. Taken together, these data support the idea that the relationship between maximum oxygen consumption rates of mitochondria measured *in vitro* and mitochondrial metabolic rates *in vivo* is not a simple one. *In vivo* rates of mitochondrial respiration are influenced by a host of factors including intracellular pH (Moyes et al., 1988), availability and affinity for ADP and NADH (Brand and Murphy, 1987; Guderley and St Pierre, 1999), membrane properties (Kraffe et al.,

2007) and delivery of oxygen and fuels by the circulation (Mathieu-Costello, 1992). It is possible that the oxidative performance of killifish mitochondria *in vivo* is regulated at one or several of these levels.

Implications for whole-organism performance

Unifying principles relating the mechanisms of aerobic capacity modulation to seasonal acclimatization and latitudinal thermal adaptation remain elusive. The data presented here suggest that these two processes may be linked and that thermal adaptation can proceed *via* changes in the ability to acclimate to local temperatures. The potential for complex interactions between acclimation (plasticity) and adaptation may be a critical component of conceptual frameworks such as the OLTT (particularly for temperate zone eurytherms), and consideration of these issues requires careful examination of thermal niche size and shape. The size of the thermal niche as estimated using CT_{Max} and CT_{Min} is similar between killifish subspecies but shifted to slightly higher temperatures in southern fish; killifish populations differ in their thermal tolerance such that southern fish are ~1.5°C more tolerant of high temperatures than northern fish at all acclimation temperatures (Fangue et al., 2006). Swimming performance studies in killifish, regardless of population of origin, have shown that these fish maintain consistent swimming performance across a wide thermal acclimation temperature range of 7–34°C (Q_{10}~1), with performance declining at temperatures outside of this range. These data suggest that killifish have a wide temperature zone over which aerobic scope is maintained and that the boundaries of the thermal niche across which activity can be maintained may be quite similar between the subspecies if sufficient time is allowed for acclimation (Fangue et al., 2008). However, we have also shown that there may be differences between the subspecies in swimming performance in response to acute thermal challenge (Fangue et al., 2008). In the current study, we observed that northern fish have a higher metabolic rate than do southern fish at all acclimation temperatures and that the acute response of metabolic rate to temperature differs between the subspecies in cold-acclimated fish. At the level of the mitochondria, we observed no differences in mitochondrial amount or function between subspecies at warm acclimation temperatures, despite the substantial differences in whole-organism standard metabolic rate in these fish. We also observed differences between populations in mitochondrial amount and function with cold acclimation. Collectively, these data suggest that while northern fish are slightly less thermally tolerant than southern fish, they may be able to maintain high activity across a wider range of temperatures. It has been suggested that high-latitude, cold-adapted eurythermal species exposed to more thermally variable environments may be under strong selection for the maintenance of plasticity, high metabolic rate, large metabolic scope and broad tolerance (Angilletta et al., 2006). Our data support this suggestion and clearly point to the use of contrasting strategies between killifish subspecies and between acclimation temperatures. Thus, we conclude that mitochondrial function and content modulation may differ between subspecies of eurythermal killifish, leading us to suggest the possibility that variation in plasticity may be an important component of local adaptation to a seasonally variable environment in this species.

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Table S1. State IV, RCR (III/IV) and RCR_{ol} (III/IV_{ol}) rates in northern (N) and southern (S) killifish subspecies acclimated to 5, 15 or 25°C and tested at acute temperatures of 2–37°C

Acclimation group	Pop	2°C	5°C	10°C	15°C	20°C	25°C	30°C	35°C	37°C
5°C										
State IV	N	9.79±2.05	27.70±2.66	45.22±3.56	60.71±5.28	79.77±17.60	92.93±11.97	106.77±12.33	189.17±22.24	n/a
	S	8.05±1.94	23.19±3.57	40.25±5.41	60.47±2.51	84.80±14.64	173.36±15.52	170.07±29.64	283.35±18.26	n/a
RCR (III/IV)	N	12.92±1.72	6.99±0.72	6.76±0.79	6.07±0.43	6.03±0.62	4.81±0.48	5.24±1.10	5.71±0.59	n/a
	S	14.88±6.48	7.16±1.36	5.37±0.61	4.93±0.72	5.94±0.47	5.51±0.41	6.67±1.40	3.81±0.38	n/a
RCR_{ol} (III/IV_{ol})	N	45.32±12.04	24.95±3.03	48.13±15.48	47.56±21.99	16.82±2.50	7.33±0.84	5.69±1.02	6.79±0.62	n/a
	S	29.39±10.65	60.41±19.46	28.26±3.77	22.48±3.45	18.28±3.14	18.03±3.48	10.13±0.89	7.10±0.36	n/a
15°C										
State IV	N	n/a	20.94±3.46	31.57±7.34	57.66±4.71	63.37±13.04	90.61±27.24	124.22±27.02	201.68±13.93	324.97±1.90
	S	n/a	21.90±3.25	41.93±5.11	56.23±2.48	90.04±15.36	125.82±6.93	149.59±14.74	257.21±19.08	304.69±41.34
RCR (III/IV)	N	n/a	8.62±1.64	9.24±1.37	6.71±0.48	7.86±1.36	6.43±1.34	6.68±1.22	6.29±0.61	4.18±0.05
	S	n/a	7.60±1.05	6.81±0.53	7.25±0.35	6.75±0.86	5.68±0.07	6.78±0.34	5.25±0.46	4.42±0.24
RCR_{ol} (III/IV_{ol})	N	n/a	47.31±14.89	35.39±1.62	26.45±2.12	19.54±3.21	12.78±0.91	9.94±1.92	9.27±1.32	7.31±1.64
	S	n/a	58.97±44.99	30.89±3.88	21.67±1.68	20.24±4.85	14.65±2.87	11.45±1.62	8.53±0.90	7.26±0.41
25°C										
State IV	N	7.54±1.53	13.93±1.84	27.08±4.88	44.33±5.10	87.90±23.25	90.85±9.96	127.27±35.08	177.34±25.17	298.58±14.98
	S	6.75±3.22	10.87±3.39	27.68±7.72	42.15±2.82	95.15±14.92	130.03±9.32	144.28±52.09	314.57±64.27	341.86±30.94
RCR (III/IV)	N	12.26±3.66	8.49±0.59	9.04±2.03	7.27±0.92	5.45±1.03	6.46±0.71	6.21±0.10	6.05±0.95	4.55±0.00
	S	7.79±3.42	8.65±3.29	6.91±2.06	5.38±0.70	4.08±0.34	4.01±0.28	5.31±1.01	3.75±0.12	3.71±0.38
RCR_{ol} (III/IV_{ol})	N	57.80±30.56	12.98±2.20	14.80±0.92	18.06±1.34	14.57±2.43	12.68±1.71	12.75±3.32	7.27±0.51	5.51±0.50
	S	18.58±3.52	14.04±5.60	24.35±4.27	13.45±2.46	13.02±1.69	10.41±1.86	10.55±1.12	5.79±0.72	4.58±0.42

Data are expressed as means ± s.e.m. (N=4–6).