J Comp Physiol B (2017) 187:463–475
DOI 10.1007/s00360-016-1042-9

ORIGINAL PAPER

Metabolic and regulatory responses involved in cold acclimation in Atlantic killifish, *Fundulus heteroclitus*

Timothy M. Healy[1] · Dillon J. Chung[1] · Kyle G. Crowther[1] · Patricia M. Schulte[1]

Received: 7 June 2016 / Revised: 30 August 2016 / Accepted: 13 October 2016 / Published online: 27 October 2016

Abstract Ectotherms often respond to prolonged cold exposure by increasing mitochondrial capacity via elevated mitochondrial volume density [V_V(mit,f)]. In fish, higher V_V(mit,f) is typically associated with increased expression of nuclear respiratory factor 1 (Nrf1), a transcription factor that induces expression of nuclear-encoded respiratory genes. To examine if *nrf1* expression or the expression of other genes that regulate mitochondrial biogenesis contribute to changes in whole-organism metabolic rate during cold acclimation, we examined the time course of changes in the expression of these genes and in metabolic rate in Atlantic killifish, *Fundulus heteroclitus*. Cold acclimation rapidly decreased metabolic rate, but increased the expression of *nrf1* more gradually, with a time course that depended on how rapidly the fish were transitioned to low temperature. Cold-induced *nrf1* expression was not associated with increases in biochemical indicators of mitochondrial respiratory capacity, suggesting that cold-induced mitochondrial biogenesis may occur without increases in oxidative capacity in this species. These observations imply that changes in *nrf1* expression and metabolic rate due to cold acclimation occur through different physiological mechanisms, and that increases in V_V(mit,f) are likely not directly related to changes in metabolic rate with cold acclimation in this species. However, *nrf1* expression differed between northern and southern killifish subspecies regardless of acclimation temperature, consistent with observed differences in metabolic rate and V_V(mit,f) at 5 °C between these subspecies. Taken together, these results reveal substantial complexity in the regulation of V_V(mit,f) and mitochondrial capacity with temperature in fish and the relationship of these parameters to metabolic rate.

Keywords Gene expression · Temperature · Fish · Mitochondria · Oxygen consumption · Mitochondrial biogenesis

Introduction

Mitochondrial processes are thought to be key mechanisms influencing aerobic scope and organismal performance at low temperatures (e.g., Guderley 2004; Pörtner et al. 2005; Pörtner 2010). One way to increase mitochondrial capacity in the cold is to increase mitochondrial volume density [V_V(mit,f)], as has been observed in several species of fish either directly using electron microscopy (Campbell and Davies 1978; Johnston and Maitland 1980; Egginton and Johnston 1984; Tyler and Sidell 1984; Egginton and Sidell 1989; Guderley 1990; Orczewska et al. 2010; Dhillon and Schulte 2011; O'Brien 2011) or indirectly using assays of citrate synthase (Cs) and cytochrome *c* oxidase (Cox) enzyme amounts and activities (Freed 1965; Shaklee et al. 1977; Jones and Sidell 1982; Kleckner and Sidell 1985; Johnston et al. 1985; Battersby and Moyes 1998; Lucassen et al. 2003, 2006; McClelland et al. 2006; LeMoine et al. 2008; Orczewska et al. 2010). Regulating changes in V_V(mit,f) is challenging as mitochondrial components are encoded in both the nuclear and mitochondrial genomes

Communicated by G. Heldmaier.

Electronic supplementary material The online version of this article (doi:10.1007/s00360-016-1042-9) contains supplementary material, which is available to authorized users.

✉ Timothy M. Healy
healy@zoology.ubc.ca

[1] Department of Zoology, The University of British Columbia, 6270 University Blvd., Vancouver, BC V6T 1Z4, Canada

(e.g., O'Brien 2011), and although the transcriptional mechanisms that coordinate mitochondrial biogenesis are well understood in mammals (e.g., Hock and Kralli 2009), these mechanisms are less well known in other taxa.

In mammals, peroxisome proliferator-activated receptor gamma coactivators are key central regulators of mitochondrial biogenesis. These coactivators interact with downstream transcription factors such as nuclear respiratory factors, estrogen-related receptors and peroxisome proliferator-activated receptors, and these interactions then lead to increased expression levels of nuclear-encoded mitochondrial genes and mitochondrially encoded genes (Hock and Kralli 2009; Scarpulla 2011). In particular, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*ppargc1a*) induces mitochondrial biogenesis in response to cold in mammals (Puigserver et al. 1998). However, in fish, the roles of peroxisome proliferator-activated receptor gamma coactivators are less clear. For example, in several species, the expression of *ppargc1a* decreases with cold acclimation despite increased V_V(mit,f) (McClelland et al. 2006; LeMoine et al. 2008; Bremer and Moyes 2011), and the expression of peroxisome proliferator-activated receptor gamma coactivator 1 beta (*ppargc1b*) increases with cold acclimation but only in some tissues (LeMoine et al. 2008). In contrast, nuclear respiratory factor 1 (*nrf1*) is often upregulated at low temperatures in fish (McClelland et al. 2006; LeMoine et al. 2008; Orczewska et al. 2010; Bremer and Moyes 2011; Bremer et al. 2012), and binding sites for Nrf1 are present in the promoters of many genes involved in mitochondrial respiration and biogenesis, including genes encoding subunits of Cox (Evans and Scarpulla 1990; Chau et al. 1992; Virbasius et al. 1993; Ramachandran et al. 2008; Scarpulla 2008). For example, binding of Nrf1 to the proximal promoter of cytochrome c oxidase subunit IV isoform 1 (*cox4i1*) regulates expression of this subunit in mammals (Dhar et al. 2008). Substantial cold-induction of *cox4i1* expression is common in fish (e.g., Duggan et al. 2011; Bremer et al. 2012), but interestingly, although the proximal promoter of this gene in goldfish (*Carassius auratus*) contains a putative Nrf1 binding site, a recent study demonstrated that the cold-responsiveness of *cox4i1* expression in goldfish is not controlled by the proximal promoter (Gao and Moyes 2016). Despite the growing evidence that Nrf1 is an important protein influencing changes in V_V(mit,f) in many fish species, to our knowledge, no studies have tested intraspecific differences in *nrf1* gene expression between populations that differ in their ability to alter V_V(mit,f) in the cold, which would allow specific tests of the association between *nrf1* expression and metabolic compensation.

Atlantic killifish are small intertidal topminnows found in salt marshes along the eastern seaboard of North America. These habitats experience large fluctuations in temperature both daily and seasonally, and at northern latitudes, killifish survive winter periods when water temperatures can drop to −1.4 °C (Fangue et al. 2006). There are two recognized subspecies of *F. heteroclitus* (Morin and Able 1983): *F. h. macrolepidotus* (northern) ranging from the Gulf of the St. Lawrence River (Canada) to northern New Jersey (USA), and *F. h. heteroclitus* (southern) ranging from New Jersey to northern Florida (USA). Both subspecies have wide thermal tolerance ranges and substantial capacities to acclimate in response to temperature change (Fangue et al. 2006), but the subspecies differ in their responses to low temperature. For example, northern fish increase V_V(mit,f) in white muscle at 5 °C, whereas southern fish do not (Dhillon and Schulte 2011). However, in contrast to the increase in V_V(mit,f) in northern fish, both subspecies decrease routine ($\dot{M}O_{2routine}$) and do not change maximum oxygen consumption ($\dot{M}O_{2max}$) at 5 °C (Healy and Schulte 2012a). Similarly, there is little evidence of cold compensation of mitochondrial function in this species, and either small or no increases in mitochondrial activity are observed with low temperature acclimation (e.g., Fangue et al. 2009; Chung and Schulte 2015; Baris et al. 2016).

In general, when ectotherms respond to cold acclimation through changes in metabolism, there are two strategies of response: (1) increase metabolism to compensate for temperature-mediated decreases in metabolic rate, or (2) suppress metabolic rate to reduce energy consumption in periods of anoxia or low food availability (e.g., Precht 1958; Guderley 1990, 2004). Thus, the increased V_V(mit,f) and decreased $\dot{M}O_{2routine}$ in cold-acclimated killifish are somewhat inconsistent as they would seem to be compatible with strategies one and two, respectively. Consequently, to further examine the metabolic responses of northern killifish to low temperature, we measured the time course of changes in (1) whole-organism oxygen consumption, (2) expression of genes involved in mitochondrial biogenesis, and (3) biochemical markers of mitochondrial capacity over a 6-week acclimation to 5 °C. We then compared the expression of *nrf1* in the two killifish subspecies to test if intraspecific differences in expression of this gene could account for the previously observed differences in the V_V(mit,f) response with cold acclimation between the subspecies.

Methods

Animal collection and laboratory acclimation

Adult killifish were collected from the Taylor River, NH (42°55′24″N, 70°51′5″W) by Aquatic Research Organisms, Inc. (Hampton, NH, USA) in the late summers of 2011 and 2012, and shipped overnight to the University of British Columbia. Following a quarantine period, fish were held in a 6000-L recirculating water system containing City of

Vancouver tap water at 15 °C adjusted to a salinity of 20 ppt with Instant Ocean® Sea Salt (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA), and photoperiod was held constant at 12L:12D. All fish were fed with Nutrafin® Max Tropical Fish Flakes (Hagen, Mansfield, MA, USA) once daily on all days except those immediately prior to experimental measurements or sampling. Fish used for all experiments (below) were of similar sizes with an average mass of 3.70 ± 0.15 g. Animal holding and experimental procedures followed an approved University of British Columbia animal care protocol: A11-0372.

Experimental design

The results presented in this study were obtained from four experiments. Fish collected in 2011 were used in experiments one and two, and fish collected in 2012 were used in experiment three. White muscle tissues from a previously published study (Dhillon and Schulte 2011) were used for experiment four. The details of each experimental design are listed below.

Experiment 1: repeated measures of $\dot{M}O_{2routine}$ during cold acclimation

Twelve fish were uniquely tagged by subepidermal fluorescent elastomer injection (Northwest Marine Technology, Shaw Island, WA, USA), and transferred into two 110-L glass aquaria (6 fish per aquarium) containing City of Vancouver tap water (15 °C, 20 ppt, 12L:12D). After 2 weeks, the fish were divided into two groups of six (cold-acclimation and control). The $\dot{M}O_{2routine}$ of the six fish in the cold-acclimation group was first measured at 15 °C (day 0) according to methods described in the section Measurement of $\dot{M}O_{2routine}$, $\dot{M}O_{2max}$ and aerobic scope, below. After this measurement, the temperature of the oxygen consumption apparatus was acutely decreased to 5 °C by pumping 5 °C water from a separate aquaria into the apparatus over 15 min. Once the apparatus reached 5 °C, $\dot{M}O_{2routine}$ was immediately measured to determine the acute effects of temperature on $\dot{M}O_{2routine}$. These fish were then acclimated to 5 °C for 6 weeks, and $\dot{M}O_{2routine}$ was again measured at 5 °C on days 1, 4, 7, 10, 14, 21, 28 and 42 of acclimation. Control fish were held at 15 °C over the 6 weeks, and $\dot{M}O_{2routine}$ was measured at 15 °C on days 0, 1, 4, 7, 10, 14, 21, 28 and 42 to provide time-matched control values.

Experiment 2: aerobic scope before and after cold acclimation

Twenty-four fish were transferred into four 110-L glass aquaria (6 fish per aquarium; water conditions: 15 °C, 20 ppt, 12L:12D). Two weeks later (day 0), the fish were divided into four groups of six. $\dot{M}O_{2routine}$ and $\dot{M}O_{2max}$ were measured in group one at 15 °C, and in group two immediately after an acute 15-min decrease in temperature to 5 °C, as in experiment 1. Methods for determination of $\dot{M}O_{2routine}$ and $\dot{M}O_{2max}$ are described below. Group three was held at 15 °C for 6 weeks, and group four experienced the same 15-min decrease in temperature to 5 °C as group two and was then held at 5 °C for 6 weeks. After acclimation (day 42), the $\dot{M}O_{2routine}$ and $\dot{M}O_{2max}$ of groups three and four were measured at 15 and 5 °C, respectively (i.e., at their acclimation temperatures).

Experiment 3: gene expression and Cs activity during cold acclimation

One hundred and four fish were transferred to thirteen 110-L glass aquaria held at 15 °C, 20 ppt, 12L:12D (8 fish per aquarium). After 2 weeks, 8 fish (day 0; control) were removed from their aquaria, and euthanized by MS-222 overdose (0.5 g/L) followed by rapid decapitation. Lateral white muscle (immediately posterior to the dorsal fin) was dissected, divided into two sections, snap-frozen, and stored at −80 °C. The remaining 96 fish were divided into three groups (32 fish per group). Group one was held at 15 °C for 40 days, group two experienced a temperature decrease to 5 °C over 20 days (−1 °C every two days) and was then held at 5 °C for 20 additional days, and group three experienced a temperature decrease to 5 °C overnight, and was then held at 5 °C for 40 days. Eight fish per group were sampled on days 5, 20 and 40 using the same protocol as described above for the control fish. Frozen muscle sections (one per fish per assay) were used for analysis of gene expression and citrate synthase enzyme activity (see methods in sections Measurement of gene expression and Citrate synthase activity measurements, below).

Experiment 4: nrf1 gene expression in northern and southern killifish

Killifish white muscle tissue samples from a previously published experiment (Dhillon and Schulte 2011) were used to test for differences in *nrf1* gene expression between northern and southern killifish acclimated to 15 and 5 °C (4 fish per subspecies and acclimation temperature). These tissue samples were originally collected from killifish from New Hampshire, USA (northern) and North Carolina, USA (southern). Gene expression was measured by quantitative real-time polymerase chain reaction (qRT-PCR) as described below.

Measurement of $\dot{M}O_{2routine}$, $\dot{M}O_{2max}$ and aerobic scope

Oxygen consumption measurements were made using methods previously described for killifish (Fangue et al.

2009; Healy and Schulte 2012a, b; McBryan et al. 2016). In brief, to measure $\dot{M}O_{2routine}$, fish were placed in 250-mL respirometers and allowed to acclimate overnight. The next morning, respirometers were sealed, and water oxygen concentrations were measured using NeoFox oxygen probes (Ocean Optics, Dunedin, FL, USA) with a sampling rate of once every 10 s. After 1 h, flow of water was restored to the respirometers. Fish remained quiescent during trials, and the 5-min period that resulted in the lowest constant rate of oxygen consumption was used to calculate $\dot{M}O_{2routine}$. Bacterial respiration was measured on days 0, 10, 21 and 42, and, in all cases, was less than 1% of total respiration, and respirometers were rinsed with 75% ethanol between trials to maintain these low levels of bacterial respiration. When $\dot{M}O_{2max}$ was also measured, fish were removed from their respirometers following measurement of $\dot{M}O_{2routine}$, and chased to exhaustion by hand (minimum 5-min chase) as in Healy and Schulte (2012a). After the chase protocol, fish were immediately returned to the respirometers and the respirometers were sealed (less than 1 min between the end of chase and the start of measurement). The initial rate of post-chase oxygen consumption (first 2–4 min) was used to determine $\dot{M}O_{2max}$. Aerobic scope was calculated (as $\dot{M}O_{2max} - \dot{M}O_{2routine}$) following the trials.

Measurement of gene expression

Total RNA was isolated from muscle tissues using the guanidine isothiocyanate method (Chomczynski and Sacchi 1987) with Ambion™ TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The isolated RNA was then DNase-treated to remove genomic DNA contamination using Ambion™ DNA-*free*™ DNA Removal Kits (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA quantity and integrity were then determined by spectrophotometry and gel electrophoresis, respectively. An Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to reverse-transcribe 2 μg of total RNA of each sample to cDNA following the manufacturer's instructions plus the addition of 5 units of Ambion™ Superase In RNase Inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) per reaction. The gene expression levels of *nrf1*, GA binding protein transcription factor subunit alpha (*gabpa*) (i.e., nuclear respiratory factor 2), *ppargc1a*, *ppargc1b*, cytochrome c oxidase subunit II, mitochondrial (*mt-co2*), *cox4i1* and *18s* ribosomal RNA were then measured by qRT-PCR using methods similar to Fangue et al. (2006) and Healy et al. (2010), using optimized primer concentrations and assay conditions. In brief, qRT-PCR reactions contained 1 μL of cDNA, 4 pmol forward primer, 4 pmol of reverse primer, and 2X Applied Biosystems™ SYBR® Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) for a total volume of 21 μL. For *18s*, cDNA samples were diluted 300× prior to qRT-PCR reactions. Primer pairs and the sequences used for design are found in supplementary online resource one (Table S1). The thermal cycling protocol was as follows: 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 60 s at 60 °C, using an Applied Biosystems™ Prism 7000 sequence analysis system (Thermo Fisher Scientific, Waltham, MA, USA). A pooled sample containing equal parts of each cDNA sample was used to generate a standard curve that was included in every reaction plate. Gene expression for each sample was compared to the standard curve, and then normalized to the gene expression of *18s*. *18s* expression did not differ among acclimation treatments or time points (data not shown). No template and no reverse-transcribed controls were included in the qRT-PCR reactions and indicated insignificant (<1:1024) levels of genomic or other sources of contamination. A melt curve protocol was included at the end of each thermal cycling protocol to confirm the presence of a single amplicon in each reaction.

Citrate synthase activity measurements

Cs enzyme activities were measured as in Mandic et al. (2013). In brief, ~50 mg of white muscle tissue was thawed on ice and then homogenized for 10 s (twice per sample) in 350 μL of homogenization buffer (5 mM EDTA, 50 mM HEPES, 0.1% (v/v) Triton X-100, pH 7.4 at 20 °C) using a PowerGen 125 tissue homogenizer (Fisher Scientific, Ottawa, Canada). Tissue homogenates were centrifuged at 10,000*g* and 4 °C for 2 min. Following centrifugation, supernatants were collected and used to determine CS activity spectrophotometrically with a Spectramax-190 spectrophotometer (Molecular Devices, Sunnyvale, CA). 10 μL of homogenate was added to 200 μL of assay buffer (0.30 mM acetyl-CoA, 0.15 mM DTNB, 50 mM Tris–HCl, pH 8.0 at 25 °C) for analysis. The background rate of absorbance change was measured at 412 nm for 10 min at 25 °C. After this, oxaloacetate was added to a final concentration of 0.5 mM, and absorbance was measured at 412 nm over an additional 10 min. The increase in absorbance over time was converted to Cs activity normalized to the mass of tissue used in each assay.

Statistical analysis

Statistical analyses and figure preparation were performed using R v3.1.2 (R Development Core Team 2014), and alpha was set at 0.05 for all analyses. All data sets satisfied the assumptions of normality and homogeneity of variances, and were analyzed by either repeated-measures

two-way ANOVA (experiment one) or two-way ANOVA (experiments two, three and four) followed by Tukey's post hoc tests.

Results

Experiments 1 and 2: cold acclimation of oxygen consumption rates

The data presented in Fig. 1 demonstrate that decreases in $\dot{M}O_{2routine}$ occur within 24 h of exposure to cold in killifish. In experiment one, there was a significant effect of acclimation treatment ($p = 1.2 \times 10^{-5}$) and time ($p = 4.3 \times 10^{-9}$) on $\dot{M}O_{2routine}$ with a significant interaction ($p = 2.8 \times 10^{-3}$) between these factors. Post hoc tests demonstrated a significant decrease in $\dot{M}O_{2routine}$ following an acute decrease in temperature from 15 to 5 °C ($p < 0.01$), with $Q_{10} = 1.64 \pm 0.12$. Another significant decrease in $\dot{M}O_{2routine}$ was detected after fish had been held at 5 °C for one day ($p < 0.01$; $Q_{10} = 2.99 \pm 0.54$ relative to 15 °C), and continued acclimation to 5 °C did not further affect $\dot{M}O_{2routine}$ ($p = 1.00$ for all), with a Q_{10} of 3.08 ± 0.21 for $\dot{M}O_{2routine}$ at the end of the 42-day acclimation period to 5 °C.

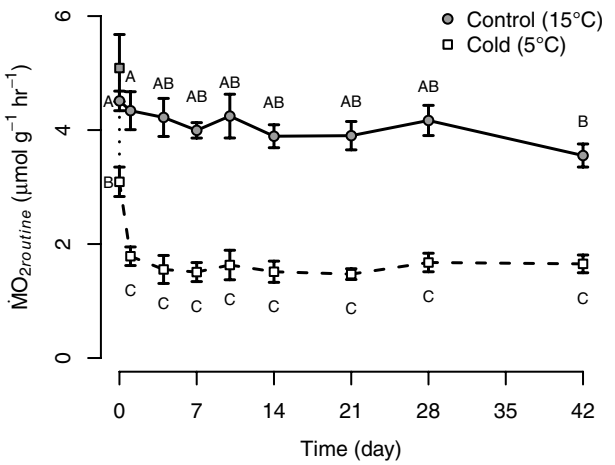

Fig. 1 $\dot{M}O_{2routine}$ over a 6-week 5 °C-acclimation in northern killifish (experiment one). *Grey symbols* indicate $\dot{M}O_{2routine}$ measurements made at 15 °C; *white symbols* indicate measurements made at 5 °C. *Circles connected by solid lines* indicate fish acclimated to the control temperature (15 °C), and *squares* indicate fish in the 5 °C treatment group, with *dashed lines* connecting acclimated groups and a *dotted line* connecting the pre-treatment measurements at 15 °C to those made immediately following acute transfer to 5 °C. The pre-treatment (day 0) measurement at 15 °C in the fish in the 5 °C treatment group is shown, but was not included in statistical comparisons. Data are presented as mean ± SEM ($n = 6$). *Shared letters* indicate groups that do not differ significantly

Although $\dot{M}O_{2routine}$ was relatively consistent in the fish that were held at 15 °C, there was a significant and unexpected decrease in $\dot{M}O_{2routine}$ on day 42 compared to days 0 and 1 ($p < 0.01$ and =0.020, respectively) (Fig. 1). However, we did not observe a similar decrease at 42 days in 15 °C-acclimated fish in experiment two (Tukey: $p = 0.87$) (Fig. 2a). The changes in $\dot{M}O_{2routine}$ at 5 °C in experiment two were consistent with the patterns detected in experiment one (ANOVA—acclimation treatment: $p = 3.8 \times 10^{-7}$, time: $p = 0.13$, interaction: $p = 0.014$) (Fig. 2a).

In experiment two, there were significant effects of acclimation treatment and time on both $\dot{M}O_{2max}$ ($p = 9.9 \times 10^{-10}$ and 0.035, respectively) (Fig. 2b) and aerobic scope ($p = 1.4 \times 10^{-6}$ and 3.2×10^{-3}, respectively) (Fig. 2c) with a significant interaction between factors for $\dot{M}O_{2max}$ ($p = 0.029$). $\dot{M}O_{2max}$ was not different between fish acutely exposed to 5 °C (day 0) and fish acclimated to 5 °C (day 42) ($p = 1.00$). At 5 °C, aerobic scope was higher after acclimation as a result of the reduced $\dot{M}O_{2routine}$ at 42 days, but this increase was not significant in post hoc tests ($p = 0.41$). There was a slight increase in $\dot{M}O_{2max}$ between day 0 and day 42 in fish held at 15 °C ($p = 0.018$), which resulted in an increase in aerobic scope in this group ($p = 0.024$).

Experiment 3: *cox* expression, Cs activity and expression of potential regulators of mitochondrial biogenesis in response to cold acclimation

Cold acclimation had no effect on *mt-co2* gene expression (ANOVA—acclimation treatment: $p = 0.15$, time: $p = 0.16$, interaction: $p = 0.71$), *cox4i1* gene expression (ANOVA—acclimation treatment: $p = 0.09$, time: $p = 0.07$, interaction: $p = 0.29$), and Cs enzyme activity (ANOVA—acclimation treatment: $p = 0.51$, time: $p = 0.61$, interaction: $p = 0.25$) (Table 1). Therefore, we detected no evidence for elevated markers of mitochondrial respiratory capacity in response to cold acclimation in killifish.

Expression levels of *nrf1* increased in 5 °C-acclimated fish after both abrupt and gradual transfer to low temperature (Fig. 3a) (ANOVA—acclimation treatment: $p = 1.5 \times 10^{-4}$, time: $p = 2.7 \times 10^{-5}$, interaction: $p = 0.21$). Post hoc tests detected significantly higher expression of *nrf1* in abruptly transferred 5 °C fish compared to 15 °C-acclimated fish on day 20 ($p = 0.027$), and in gradually transferred 5 °C fish on day 40 compared to day 5 ($p = 3.9 \times 10^{-4}$). In contrast, there were no significant effects of 5 °C-acclimation on the gene expression of *gabpa* (Fig. 3b) (ANOVA—acclimation treatment: $p = 0.53$, time: $p = 0.46$, interaction: $p = 0.11$), and although there were modest changes in the expression of

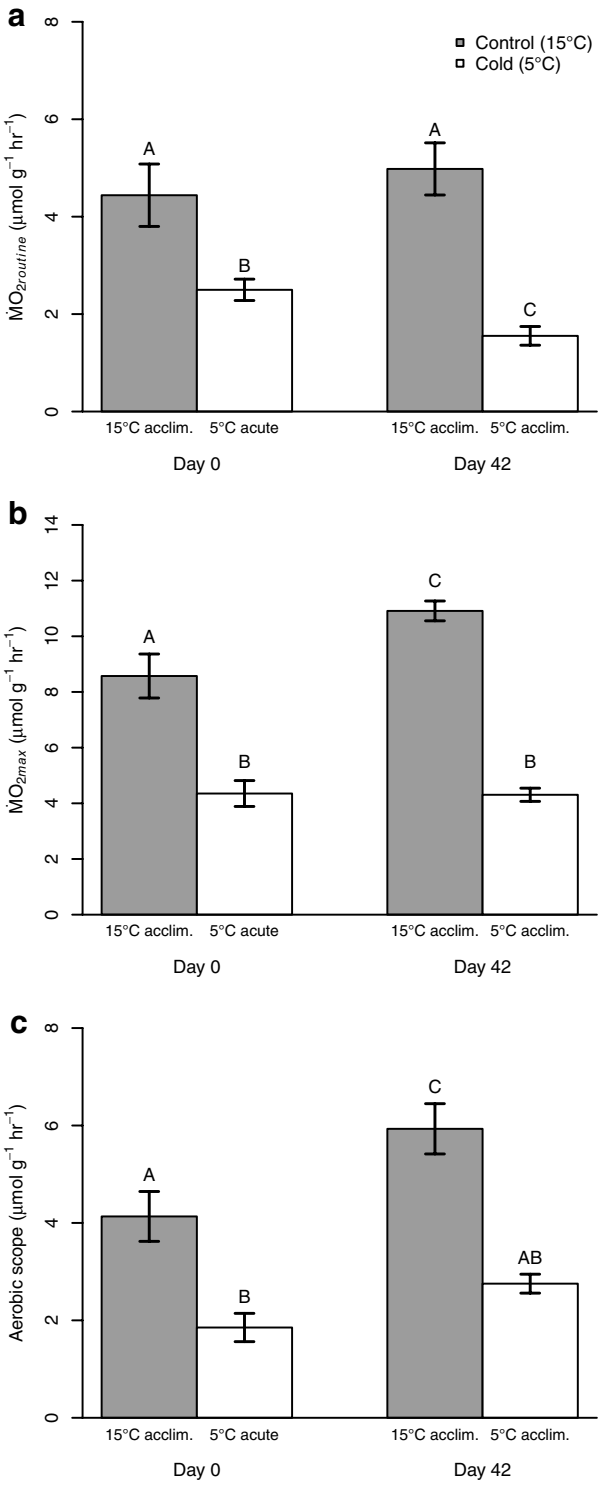

Fig. 2 $\dot{M}O_{2routine}$, $\dot{M}O_{2max}$ and aerobic scope (**a**, **b** and **c**, respectively) before and after acclimation to 5 °C in northern killifish (experiment two). *Grey bars* and *white bars* indicate oxygen consumption measurements made at 15 and 5 °C, respectively. 5 °C acute measurements were made immediately following an acute transfer from 15 to 5 °C, whereas 5 °C acclim. measurements were made after fish were held at 5 °C for 6 weeks. Data are presented as mean ± SEM ($n = 6$). *Shared letters* indicate groups that do not differ significantly

ppargc1a and *ppargc1b*, these were not consistent with increased expression at 5 °C (Fig. 3c, d). For *ppargc1a*, there was a significant effect of acclimation treatment ($p = 3.5 \times 10^{-4}$), but not of time ($p = 0.64$) on gene expression, and there was a significant interaction between factors ($p = 5.3 \times 10^{-3}$). Elevated *ppargc1a* expression was detected for abruptly transferred compared to gradually transferred 5 °C-acclimated fish on day 5 ($p = 0.018$), and for 15 °C-acclimated fish compared to abruptly transferred 5 °C-acclimated fish on day 20 ($p = 6.6 \times 10^{-3}$). For *ppargc1b*, there was a significant effect of time on gene expression ($p = 6.6 \times 10^{-8}$), but neither a significant effect of acclimation treatment ($p = 0.23$), nor an interaction between factors ($p = 0.21$). Post hoc tests detected higher expression on days 20 and 40 than on day 5 in both the 15 °C-acclimated ($p \leq 0.031$) and gradually transferred 5 °C fish ($p \leq 0.034$). In summary, of the transcription factors and coactivators involved in mitochondrial biogenesis that were examined in our study, only *nrf1* demonstrated cold-induced gene expression in northern killifish.

Experiment 4: gene expression of *nrf1* in northern and southern killifish

Because a previous study demonstrated increased V_V(mit,f) in white muscle in response to cold acclimation in northern but not in southern killifish (Dhillon and Schulte 2011), we predicted that *nrf1* gene expression would increase as a result of cold acclimation in only the northern subspecies. Contrary to this prediction, there was a significant effect of acclimation temperature on *nrf1* expression with no interaction between acclimation temperature and subspecies ($p = 9.4 \times 10^{-4}$ and 0.87, respectively), and there was a significant difference in *nrf1* expression between the subspecies ($p = 1.3 \times 10^{-3}$) (Fig. 4). In general, northern fish had higher *nrf1* expression than southern fish at both acclimation temperatures, and both subspecies had higher expression at 5 °C than 15 °C. However, post hoc tests only detected a significant difference between the subspecies at 15 °C ($p = 0.042$ and 0.063 at 15 and 5 °C, respectively), and between the acclimation temperatures in southern fish ($p = 0.034$ and 0.051 for southern and northern fish, respectively).

Discussion

The results presented here clearly demonstrate that decreased $\dot{M}O_{2routine}$ and increased *nrf1* expression in white muscle occur during exposure to low temperature in killifish. Cold exposure decreased $\dot{M}O_{2routine}$ rapidly, whereas *nrf1* expression increased more gradually, indicating that these changes are likely part of separate physiological

Table 1 Biochemical indicators of mitochondrial respiratory capacity (*mt-co2* expression, *cox4i1* expression and Cs activity) during cold acclimation

Acclimation treatment	Day of acclimation	Relative *mt-co2* expression[a] (mean ± SEM) ($n = 8$)	Relative *cox4i1* expression[a] (mean ± SEM) ($n = 8$)	Cs activity (U g-1)[b] (mean ± SEM) ($n = 8$)
Control (15 °C)	Day 0	1.00 ± 0.14	1.00 ± 0.17	1.67 ± 0.21
Control (15 °C)	Day 5	1.16 ± 0.12	1.02 ± 0.19	1.70 ± 0.14
Control (15 °C)	Day 20	1.51 ± 0.25	1.20 ± 0.15	1.44 ± 0.10
Control (15 °C)	Day 40	1.20 ± 0.15	1.64 ± 0.23	1.82 ± 0.23
5 °C (gradual transfer)	Day 5	0.87 ± 0.12	0.82 ± 0.11	1.54 ± 0.12
5 °C (gradual transfer)	Day 20	1.43 ± 0.26	1.55 ± 0.53	1.67 ± 0.13
5 °C (gradual transfer)	Day 40	1.11 ± 0.23	1.19 ± 0.29	1.94 ± 0.22
5 °C (abrupt transfer)	Day 5	1.01 ± 0.17	0.95 ± 0.12	1.55 ± 0.19
5 °C (abrupt transfer)	Day 20	1.06 ± 0.17	0.78 ± 0.10	1.61 ± 0.10
5 °C (abrupt transfer)	Day 40	1.07 ± 0.22	1.08 ± 0.16	1.37 ± 0.20

[a] qRT-PCR expression data were obtained relative to a standard curve generated from a pooled sample, then normalized to the mean value at 15 °C on day 0 to facilitate fold change comparisons among genes

[b] Enzyme units per gram wet mass of white muscle

responses to the cold. However, differences in *nrf1* expression may contribute to differences in $\dot{M}O_{2routine}$ between the killifish subspecies, and may be a component of regulatory differences that cause increased V_V(mit,f) at low temperatures in the white muscle of northern, but not of southern killifish.

Lack of cold compensation of metabolism in killifish

Over a 6-week acclimation to 5 °C, northern killifish did not increase either $\dot{M}O_{2routine}$ or $\dot{M}O_{2max}$, indicating that thermal acclimation does not result in compensation of the thermodynamic effects of low temperature on metabolic rate in these fish. We have previously shown that killifish acutely exposed to 5 °C have higher $\dot{M}O_{2routine}$ and the same $\dot{M}O_{2max}$ as killifish acclimated to 5 °C (Healy and Schulte 2012a, b), which is consistent with the results presented here. One day after fish were transferred to 5 °C, $\dot{M}O_{2routine}$ was lower than immediately after transfer to 5 °C, and additional time at 5 °C did not result in further changes in $\dot{M}O_{2routine}$. Therefore, changes in $\dot{M}O_{2routine}$ in response to cold in killifish occur rapidly. This pattern of a decrease in $\dot{M}O_{2routine}$ between acute transfer and 24 h after cold exposure is consistent with two potential interpretations: (1) killifish undergo inverse compensation by actively suppressing metabolism during the first day of exposure to 5 °C, or (2) killifish do not undergo inverse compensation, and instead, the reduced $\dot{M}O_{2routine}$ at 24 h simply reflects the thermodynamic effects of low temperature, and the somewhat higher $\dot{M}O_{2routine}$ immediately following acute exposure to cold reflects an elevated metabolic demand to increased activity or stress levels when killifish first encounter cold water.

At temperatures between 0 and 40 °C, a 10 °C reduction in temperature typically causes a thermodynamic reduction of metabolic rate by approximately 2- to 3-fold (i.e., $Q_{10} \approx 2$–3, Hochachka and Somero 2002). In our study, the acute Q_{10} for $\dot{M}O_{2routine}$ was ~1.6, the acclimated Q_{10} for $\dot{M}O_{2routine}$ was ~3.0 and the Q_{10} for $\dot{M}O_{2max}$ in either acutely exposed or acclimated fish was ~2.0. Together, these values suggest that most changes in metabolic rate in killifish between 15 and 5 °C are consequences of direct thermodynamic effects. Therefore, the decrease in $\dot{M}O_{2routine}$ over the first day that killifish are held at 5 °C may be due to reduced $\dot{M}O_{2routine}$ after fish recover from stress experienced during acute cold exposure. Alternatively, our data could indicate a modest suppression of $\dot{M}O_{2routine}$ in these fish. Regardless, there is clearly no compensation of metabolic rate at 5 °C in killifish, and therefore, these fish maintain a reduced metabolic state at 5 °C compared to 15 °C.

Cold-inactive organisms usually maintain reduced metabolic rates when exposed to prolonged cold temperatures. For example, organisms that overwinter in frozen anoxic ponds often suppress metabolic rate to extend the time over which metabolism can be fueled through anaerobic processes (e.g., Richards 2009). Although killifish are not necessarily trapped in anoxic pools throughout winter, food supply in their habitats decreases over the winter months (Chidester 1920), and reduced metabolic activity may contribute to overwinter survival. Indeed, this species has been reported to burrow into the mud at the bottom of salt marsh pools during the winter (Chidester 1920). Another fish species from the east coast of North America, the cunner (*Tautogolabrus adspersus*), depresses metabolic rate during seasonal exposure to winter temperatures. However, the $\dot{M}O_{2routine}$ of

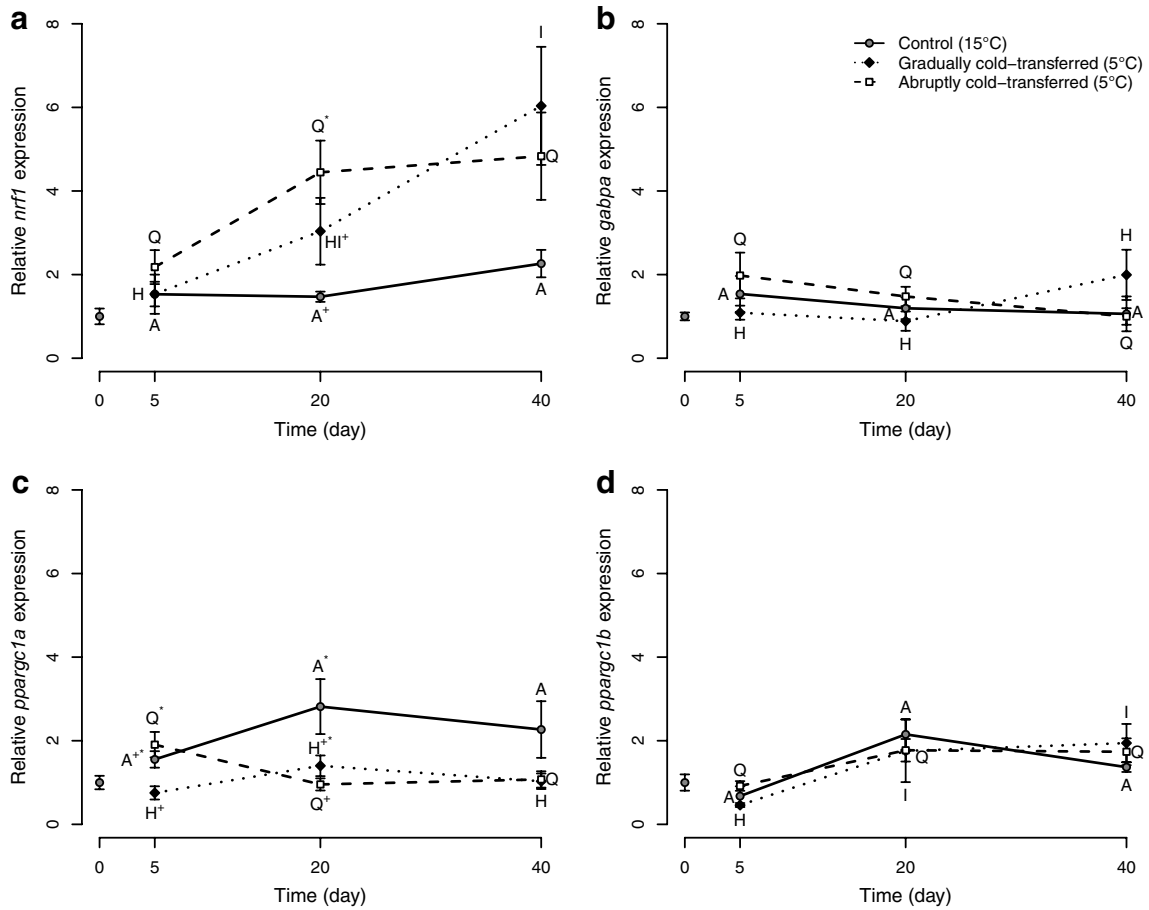

Fig. 3 Gene expression of transcription factors and coactivators involved in mitochondrial biogenesis over a 40-day 5 °C-acclimation in northern killifish (experiment three). **a** *nrf1*, **b** *gabpa*, **c** *ppargc1a*, and **d** *ppargc1b*. Fish held at 15 °C: *grey circles*, fish gradually transferred to 5 °C over days 1–20: *black diamonds*, and fish abruptly transferred to 5 °C on day 0, *white squares*. Control expression values in fish acclimated to 15 °C are shown on day 0, but were not included in statistical comparisons. Data are presented as mean ± SEM ($n = 8$) normalized such that mean day 0 values equal one for all genes facilitating fold change comparisons among genes. *Shared letters* across days indicate groups that do not differ significantly within an acclimation treatment, and shared *symbols* (*, $^{+}$) across acclimation treatments indicate groups that do not differ significantly within a day. If *no symbols* are shown on a day, then there were no significant differences among acclimation treatments on that day

cunner decreases with a Q_{10} of 10.4 when acclimated to cold temperature (Costa et al. 2013), which is substantially more than the decrease in $\dot{M}O_{2routine}$ observed in killifish.

Reduced $\dot{M}O_{2routine}$ in cold-acclimated killifish is likely accompanied by decreased metabolic demand that results in a parallel reduction of mitochondrial oxygen use. However, the maximum respiration rates of liver or heart mitochondria from cold-acclimated killifish are not lower than those of mitochondria from warm-acclimated fish when assayed at a common temperature (Fangue et al. 2009; Chung and Schulte 2015; Baris et al. 2016), and the ratio of [ADP_{free}] to [ATP] is not decreased in the white muscle of cold-acclimated compared to warm-acclimated killifish (Dhillon and Schulte 2011). These studies suggest that the maximum capacity for cellular respiration and the level of at least one signal that regulates cellular respiration are not substantially lower in cold- than warm-acclimated killifish. Therefore, lower maximum rates of mitochondrial respiration at low temperatures in killifish may also primarily reflect thermodynamic effects on cellular reaction rates.

Interestingly, despite the lack of cold compensation of $\dot{M}O_{2routine}$ or $\dot{M}O_{2max}$ in northern killifish, these fish increase V_V(mit,f) in white muscle in response to cold acclimation (Dhillon and Schulte 2011), which would generally be considered an indicator of thermal compensation of metabolism. Divergent patterns for V_V(mit,f) and $\dot{M}O_{2routine}$ in response to cold acclimation have also been demonstrated in eels (*Anguilla* spp.) (Walsh et al. 1983; Egginton and Johnston 1984) and crucian carp (*Carassius carassius*) (Roberts 1966; Johnston and Maitland 1980). The opposite directions of changes in V_V(mit,f) and $\dot{M}O_{2routine}$ in killifish

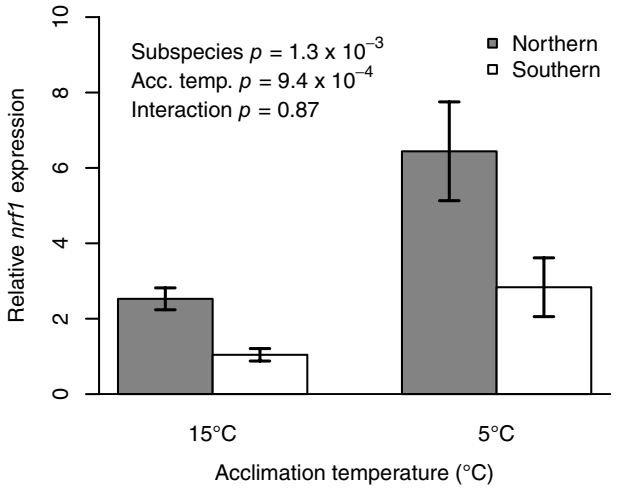

Fig. 4 Intraspecific variation in *nrf1* gene expression in killifish acclimated to 15 and 5 °C (experiment four). Northern subspecies: *grey bars*, and southern subspecies: *white bars*. Data are presented as mean ± SEM ($n = 4$). Significant effects of subspecies ($p = 1.3 \times 10^{-3}$) and acclimation temperature ($p = 9.4 \times 10^{-4}$) with no interaction ($p = 0.87$) were detected by two-way ANOVA

in response to cold suggest that these effects are unlikely to share a mechanistic or a functional link. Consistent with this suggestion, reduction of $\dot{M}O_{2routine}$ at 5 °C occurred rapidly, whereas *nrf1* expression increased more gradually when fish were acclimated to 5 °C, reaching levels approximately fourfold higher in 5 °C-acclimated compared to 15 °C-acclimated fish after 20 days of acclimation.

Time courses of thermal acclimation

Relatively few studies have examined the time courses of phenotypic changes that occur as a result of thermal acclimation. In killifish and other fish species, changes in thermal tolerance limits are generally complete within 1 week of acclimation to warm temperatures compared to between 3 and 6 weeks of acclimation to cold temperatures (e.g., Cossins et al. 1977; Healy and Schulte 2012b). In goldfish, the time courses of changes in thermal tolerance are correlated with changes in membrane fluidity (Cossins et al. 1977), and although mechanistic changes in membrane phospholipid compositions in rainbow trout (*Oncorhynchus mykiss*) can occur over a range of time courses, increases in long-chain polyunsaturated phospholipids occur after approximately 2–3 weeks of exposure to cold (Hazel and Landrey 1988a, b), which is consistent with the idea that most responses to low temperature exposure occur over 3–6 weeks.

Matching the general time frame for cold acclimation outlined above, changes in *nrf1* expression in killifish white muscle as a result of exposure to low temperatures occur over the first 3–6 weeks of acclimation. Similarly, in three-spined stickleback (*Gasterosteus aculeatus*), *nrf1* expression is elevated in liver tissue during the first 4 weeks of acclimation to cold temperatures (Orczewska et al. 2010). However, in stickleback, oxidative muscle *nrf1* expression only increases after 9 weeks of acclimation (Orczewska et al. 2010).

The time courses for changes in mitochondrial processes during cold acclimation have been investigated either directly through measurements of mitochondrial respiration rates (Bouchard and Guderley 2003), or indirectly through changes in the expression levels or activities of metabolic enzymes (Lehmann 1970; Sidell et al. 1973; Bouchard and Guderley 2003; Lucassen et al. 2003; Orczewska et al. 2010). Typically, there is an early and transient increase in *cs* mRNA followed by a gradual increase in Cs activity over 2–9 weeks in liver tissue, whereas there are no significant effects of cold acclimation associated with Cox (Lucassen et al. 2003; Orczewska et al. 2010). In oxidative muscle, both *cs* mRNA and Cs activity increase gradually over 4–10 weeks of cold acclimation (Bouchard and Guderley 2003; Orczewska et al. 2010), but changes associated with Cox vary among species. In stickleback oxidative muscle, effects of cold acclimation on Cox are similar to those on Cs (Orczewska et al. 2010), whereas Cox is unaffected by cold acclimation in rainbow trout oxidative muscle (Bouchard and Guderley 2003). Together, the results discussed above clearly demonstrate that the time courses for changes in metabolic enzymes as a result of cold acclimation can vary among species and tissues, and our results in killifish emphasize the potential for differences among species, as we observed no changes in either Cs or Cox during acclimation to 5 °C. Alternatively, it is possible that the differences between our results and those observed previously are a consequence of ending our experiment after 6 weeks of acclimation, because previous studies detect significant effects after up to 10 weeks of exposure to cold.

As discussed above, the majority of decreases in $\dot{M}O_{2routine}$ as a result of cold acclimation in killifish are likely a consequence of thermodynamic effects, but there is also the possibility of a modest suppression of $\dot{M}O_{2routine}$ during the first day of acclimation to 5 °C. This decrease in $\dot{M}O_{2routine}$ would be indicative of a rapid effect of cold acclimation. Previous studies have demonstrated a range of time courses for changes in $\dot{M}O_{2routine}$ among different species after transfer to low temperatures. For example, data from American eels (*A. rostrata*) suggest rapid effects of acclimation to 5 °C on $\dot{M}O_{2routine}$ similar to the changes we observed in killifish (Walsh et al. 1983), whereas acclimation to 10 °C in European eels (*A. anguilla*) results in a more gradual decrease in $\dot{M}O_{2routine}$ (Egginton and Johnston 1984).

In the current study, we examined the time courses of metabolic responses to prolonged exposure to cold

temperatures without variation in photoperiod. These experiments are useful to identify mechanistic responses that occur specifically as a result of reductions in temperature. However, in nature, seasonal decreases in temperature are associated with fewer hours of light per day, and this variation in photoperiod can result in differences in thermal tolerance that are consistent with seasonal changes in temperature (e.g., Healy and Schulte 2012b) and in variation in metabolic capacities (e.g., Guderley et al. 2001; Martin et al. 2009). It is possible that differences in photoperiod could also impact the time courses of changes in these traits, and this possibility remains to be investigated more thoroughly.

Role of *nrf1* gene expression in cold acclimation in killifish

Our data suggest that increased *nrf1* gene expression is an important acclimatory response to cold temperatures in killifish, as in other fish species (McClelland et al. 2006; LeMoine et al. 2008; Orczewska et al. 2010; Bremer and Moyes 2011; Bremer et al. 2012). Killifish that were gradually transferred to low temperature increased *nrf1* expression more slowly than fish that experienced an abrupt change in temperature implying that cold-induced expression of *nrf1* is regulated in a manner dependent on the timing of cold exposure. In contrast, none of the other regulatory genes examined in this study (*gabpa*, *ppargc1a* and *ppargc1b*) demonstrated evidence of cold-induced expression. Furthermore, northern fish have higher *nrf1* expression levels than southern fish in white muscle, consistent with higher V_V(mit,f) in this tissue in northern than in southern fish, at least, at 5 °C (Dhillon and Schulte 2011), suggesting a possible role for Nrf1 in regulating V_V(mit,f). However, several lines of evidence indicate that increased *nrf1* gene expression alone does not necessarily lead to higher V_V(mit,f) in killifish: (1) V_V(mit,f) does not change between 15 and 5 °C in white muscle in southern killifish (Dhillon and Schulte 2011), whereas, here, we show that *nrf1* expression increases at 5 °C in this subspecies, and (2) northern and southern fish have the same V_V(mit,f) in white muscle at 15 °C (Dhillon and Schulte 2011), whereas, here, we show that northern fish have higher expression of *nrf1* than southern fish at 15 °C. Most likely, these mismatches indicate that although *nrf1* gene expression is a key part of the response to cold acclimation, downstream regulatory differences also impact changes in V_V(mit,f). Overexpression of *nrf1* in transgenic mice does not increase mitochondrial respiratory capacity (Baar et al. 2003), similarly indicating the role of additional regulatory processes in mitochondrial biogenesis. As a result, cold-induced *nrf1* expression in northern killifish is potentially one of several signals that combine to increase V_V(mit,f) in response to prolonged cold, and differences in other regulatory signals between the subspecies may explain why southern fish increase *nrf1* expression in response to cold acclimation, but do not increase V_V(mit,f).

Interestingly, despite the observed changes in *nrf1* expression and the known changes in V_V(mit,f) in killifish, *mt-co2* and *cox4i1* gene expression and Cs enzyme activity do not increase with cold acclimation. Increased *nrf1* expression after cold acclimation is typically associated with an increase in Cox or Cs in fish (McClelland et al. 2006; LeMoine et al. 2008; Orczewska et al. 2010; Bremer and Moyes 2011). However, previous studies in killifish white muscle have also detected relatively little evidence for increases in these indicators with cold acclimation. Dhillon and Schulte (2011) found no change in Cs activity between 15 and 5 °C-acclimation treatments, and Fangue et al. (2009) detected a significant increase in this indicator, but the change was modest with similar magnitude to the trend for higher expression in gradually transferred 5 °C fish compared to 15 °C-acclimated fish in our study. Fangue et al. (2009) did find a significant increase in *cs* mRNA at 5 °C in northern fish, but there was no evidence for increases in *mt-co2* with cold acclimation. One possible explanation for the lack of change in Cs activity and Cox subunit gene expression in the current study is that changes in these parameters require longer than a 6-week acclimation period. In three-spined stickleback oxidative muscle, the expression levels of *mt-co2* and *cox4i1* increase between 4 and 9 weeks of acclimation (Orczewska et al. 2010). However, V_V(mit,f) is elevated in northern killifish white muscle after 6 weeks (Dhillon and Schulte 2011), and increases in V_V(mit,f) were only assessed in stickleback oxidative muscle after 9 weeks. Another possible explanation for the lack of change in Cs and Cox is that oxidative capacity is unchanged in response to cold acclimation even though V_V(mit,f) increases in killifish.

Differences in V_V(mit,f) without parallel changes in oxidative capacity have been observed in notothenioid fish, as the hearts of Antarctic icefish have increased V_V(mit,f) compared to other species, but do not have elevated oxidative capacities (Feller et al. 1985; Johnston and Harrison 1987; O'Brien and Sidell 2000; Urschel and O'Brien 2008; O'Brien and Mueller 2010; O'Brien 2011). The higher V_V(mit,f) observed in icefish is thought to improve movement of oxygen to the mitochondria as oxygen molecules diffuse more easily through the larger network of hydrophobic intracellular membranes (Sidell 1998). Consequently, V_V(mit,f) in killifish white muscle may increase to facilitate oxygen diffusion at low temperatures. However, killifish increase cristae surface density at 5 °C (Dhillon and Schulte 2011), whereas this parameter is not higher in icefishes than in other notothenioid species (O'Brien and Sidell 2000), and icefish with elevated V_V(mit,f) do not have increased *nrf1* gene expression relative to other

notothenioid species (Urschel and O'Brien 2008). Therefore, even if differences among notothenioids occur for similar reasons to changes with acclimation temperature in killifish, the cellular mechanisms controlling these responses are likely not shared.

The above discussion identifies that the relationship between *nrf1* expression and changes in V_V(mit,f) and function during cold acclimation in killifish is somewhat unclear, because *nrf1* expression and V_V(mit,f) increase with cold acclimation, whereas typical markers of cellular respiratory capacity do not. Target genes of Nrf1 are not known to include genes involved in the production of the mitochondrial membrane (O'Brien and Mueller 2010; O'Brien 2011; Scarpulla et al. 2012), which is opposite to the expectations of a change in V_V(mit,f) but not in oxidative capacity. However, relatively little is known about the mechanisms controlling the induction of mitochondrial membrane components (e.g., O'Brien 2011). For example, translocator and maintenance protein 41 and prohibitin have only relatively recently been identified as candidates involved in inner mitochondrial membrane assembly in yeast (Kutik et al. 2008, Osman et al. 2009). It seems unlikely that Nrf1 induces expression of genes encoding membrane components differently in killifish compared to other species, and therefore, we hypothesize two potential roles of cold-induced *nrf1* expression in these fish: (1) elevated *nrf1* expression leads to increased production of structural proteins in the mitochondria consistent with increased volume density but not respiration, or (2) increased *nrf1* expression induces genes that contribute to an overall shift in oxidative ATP production towards utilizing lipid-based substrates which is a common response to low temperature in many fish. In northern killifish, cold acclimation causes reductions in [ADP_{free}] in white muscle (Dhillon and Schulte 2011), and lower [ADP_{free}] may favor the use of lipid substrates over glycolytic ones (Holloszy and Coyle 1984). If a shift towards lipid oxidation occurs in both northern and southern killifish, this second hypothesis could account for the increased expression of *nrf1* in the southern subspecies even though this subspecies does not increase V_V(mit,f).

Nrf1 could affect lipid oxidation through feedback regulation of peroxisome proliferator-activated receptor gamma coactivators, which interact with peroxisome proliferator-activated receptors that regulate the expression of genes controlling lipid oxidation (e.g., Hock and Kralli 2009). In mammals, Nrf1 can increase Ppargc1a activity through induction of myocyte enhancer factor 2A (*mef2a*) (Ramachandran et al. 2008). However, Mef2a acts as an activator by increasing transcription of *ppargc1a*, and *ppargc1a* expression was not induced at low temperature in our study. Therefore, if Nrf1 affects lipid oxidation via peroxisome proliferator-activated receptors and Ppargc1a in killifish, it likely occurs through an unknown downstream pathway that changes Ppargc1a activity through post-translational modifications or interactions.

Taken together, the above discussion highlights that changes in metabolic rate and V_V(mit,f) are separate aspects of cold acclimation in killifish, which likely have unrelated controlling mechanisms. Consequently, increases in V_V(mit,f) may not directly contribute to differences in $\dot{M}O_{2routine}$ or aerobic scope with cold acclimation. Therefore, if aerobic scope limits performance at low temperatures (e.g., Pörtner 2010), changes in V_V(mit,f) are likely a parallel mechanism in killifish to improve the delivery of oxygen to its site of use in the mitochondrion.

Conclusions

Here, we show that nuclear respiratory factor 1 gene expression increases in a time-course-dependent manner during cold acclimation in killifish, and that intraspecific variation in the expression of *nrf1* likely contributes to intraspecific differences in mitochondrial volume density at low temperature. However, indicators of mitochondrial respiratory capacity do not increase in parallel with *nrf1* expression, suggesting that higher mitochondrial volume densities may not be associated with increases in oxidative capacity in this species. Similarly, there is no evidence that killifish compensate for the thermodynamic effects of low temperature on $\dot{M}O_{2routine}$ or $\dot{M}O_{2max}$. These results highlight the diversity of metabolic strategies that fishes can use to respond to changes in environmental temperature and the complexities of the cellular mechanisms that control these responses.

Acknowledgments This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to PMS, NSERC Canada Graduate Scholarships to DJC and TMH, and an NSERC Undergraduate Student Research Award to KGC.

Compliance with ethical standards

Ethics approval All procedures performed in this study were in accordance with an approved University of British Columbia animal care Protocol: A11-0372.

References

Baar K, Song Z, Semenkovich CF, Jones TE, Han DH, Nolte LA, Ojuka EO, Chen M, Holloszy JO (2003) Skeletal muscle overexpression of nuclear respiratory factor 1 increases glucose transport capacity. FASEB J 17:1666–1673. doi:10.1096/fj.03-0049com

Baris TZ, Crawford DL, Oleksiak MF (2016) Acclimation and acute temperature effects on population differences in oxidative

phosphorylation. Am J Physiol Regul Integr Comp Physiol 310:185–196. doi:10.1152/ajpregu.00421.2015
Battersby BJ, Moyes CD (1998) Influence of acclimation temperature on mitochondrial DNA, RNA, and enzymes in skeletal muscle. Am J Physiol Regul Integr Comp Physiol 275:905–912
Bouchard P, Guderley H (2003) Time course of the response of mitochondria from oxidative muscle during thermal acclimation of rainbow trout, *Oncorhynchus mykiss*. J Exp Biol 206:3455–3465. doi:10.1242/jeb.00578
Bremer K, Moyes CD (2011) Origins of variation in muscle cytochrome *c* oxidase activity within and between fish species. J Exp Biol 214:1888–1895. doi:10.1242/jeb.053330
Bremer K, Monk CT, Gurd BJ, Moyes CD (2012) Transcriptional regulation of temperature-induced remodeling of muscle bioenergetics in goldfish. Am J Physiol Regul Integr Comp Physiol 303:150–158. doi:10.1152/ajpregu.00603.2011
Campbell CM, Davies PS (1978) Temperature acclimation in the teleost, *Blennius pholis*, changes in enzyme activity and cell structure. Comp Biochem Physiol B 61:165–167. doi:10.1016/0305-0491(78)90235-3
Chau CA, Evans MJ, Scarpulla RC (1992) Nuclear respiratory factor 1 activation sites in genes encoding the γ-subunit of ATP synthase, eukaryotic initiation factor 2α, and tyrosine aminotransferase. J Biol Chem 267:6999–7006
Chidester FE (1920) The Behavior of *Fundulus heteroclitus* on the salt marshes of New Jersey. Amer Nat 54:551–557
Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159. doi:10.1016/0003-2697(87)90021-2
Chung DJ, Schulte PM (2015) Mechanisms and costs of mitochondrial thermal acclimation in a eurythermal killifish (*Fundulus heteroclitus*). J Exp Biol 218:1621–1631. doi:10.1242/jeb.120444
Cossins AG, Friedlander MJ, Prosser CL (1977) Correlations between behavioural temperature adaptations by goldfish and the viscosity and fatty acid composition of their synaptic membranes. J Comp Physiol 120:109–121. doi:10.1007/BF00619309
Costa IASF, Driedzic WR, Gamperl AK (2013) Metabolic and cardiac responses of cunner *Tautogolabrus adspersus* to seasonal and acute changes in temperature. Physiol Biochem Zool 86:233–244. doi:10.1086/669538
Dhar SS, Ongwijitwat S, Wong-Riley MT (2008) Nuclear respiratory factor 1 regulates all ten nuclear-encoded subunits of cytochrome c oxidase in neurons. J Biol Chem 283:3120–3129. doi:10.1074/jbc.M707587200
Dhillon RS, Schulte PM (2011) Intraspecific variation in the thermal plasticity of mitochondria in killifish. J Exp Biol 214:3639–3648. doi:10.1242/jeb.057737
Duggan AT, Kocha KM, Monk CT, Bremer K, Moyes CD (2011) Coordination of cytochrome c oxidase gene expression in the remodelling of skeletal muscle. J Exp Biol 214:1880–1887. doi:10.1242/jeb.053322
Egginton S, Johnston IA (1984) Effects of acclimation temperature on routine metabolism, muscle mitochondrial volume density and capillary supply in the elver (*Anguilla anguilla* L.). J Therm Biol 9:165–170. doi:10.1016/0306-4565(84)90016-0
Egginton S, Sidell BD (1989) Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. Am J Physiol Regul Integr Comp Physiol 256:1–9
Evans MJ, Scarpulla RC (1990) NRF-1: a *trans*-activator of nuclear-encoded respiratory genes in animal cells. Genes Dev 4:1023–1034. doi:10.1101/gad.4.6.1023
Fangue NA, Hofmeister M, Schulte PM (2006) Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. J Exp Biol 209:2859–2872. doi:10.1242/jeb.02260
Fangue NA, Richards JG, Schulte PM (2009) Do mitochondrial properties explain intraspecific variation in thermal tolerance? J Exp Biol 212:514–522. doi:10.1242/jeb.024034
Feller G, Goessens G, Gerday C, Bassleer R (1985) Heart structure and ventricular ultrastructure of hemoglobin- and myoglobin-free icefish *Channichthys rhinoceratus*. Cell Tissue Res 242:669–676. doi:10.1007/BF00225436
Freed J (1965) Changes in activity of cytochrome oxidase during adaptation of goldfish to different temperatures. Comp Biochem Physiol 14:651–659. doi:10.1016/0010-406X(65)90252-5
Gao G, Moyes CD (2016) Evaluating the role of NRF-1 in regulation of the goldfish COX4-1 gene in response to temperature. J Exp Biol. doi:10.1242/jeb.141184
Guderley H (1990) Functional significance of metabolic responses to thermal acclimation in fish muscle. Am J Physiol Regul Integr Comp Physiol 259:245–252
Guderley H (2004) Metabolic responses to low temperature in fish muscle. Biol Rev 79:409–427. doi:10.1017/S1464793103006328
Guderley H, Leroy PH, Gagné A (2001) Thermal acclimation, growth, and burst swimming of threespine stickleback: enzymatic correlates and influence of photoperiod. Physiol Biochem Zool 74:66–74. doi:10.1086/319313
Hazel JR, Landrey SR (1988a) Time course of thermal adaptation in plasma membranes of trout kidney. I. Headgroup composition. Am J Physiol Regul Integr Comp Physiol 255:622–627
Hazel JR, Landrey SR (1988b) Time course of thermal adaptation in plasma membranes of trout kidney. II. Molecular species composition. Am J Physiol Regul Integr Comp Physiol 255:628–634
Healy TM, Schulte PM (2012a) Thermal acclimation is not necessary to maintain a wide thermal breadth of aerobic scope in the common killifish (*Fundulus heteroclitus*). Physiol Biochem Zool 85:107–119. doi:10.1086/664584
Healy TM, Schulte PM (2012b) Factors affecting plasticity in whole-organism thermal tolerance in common killifish (*Fundulus heteroclitus*). J Comp Physiol B 182:49–62. doi:10.1007/s00360-011-0595-x
Healy TM, Tymchuk WE, Osborne EJ, Schulte PM (2010) Heat shock response of killifish (*Fundulus heteroclitus*): candidate gene and heterologous microarray approaches. Physiol Genomics 41:171–184. doi:10.1152/physiolgenomics.00209.2009
Hochachka PW, Somero GN (2002) Biochemical adaptation: mechanism and process in physiological evolution. Oxford University Press, New York
Hock MB, Kralli A (2009) Transcriptional control of mitochondrial biogenesis and function. Annu Rev Physiol 71:177–203. doi:10.1146/annurev.physiol.010908.163119
Holloszy JO, Coyle EF (1984) Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J Appl Physiol 56:831–838
Johnston IA, Harrison P (1987) Morphometrics and ultrastructure of myocardial tissue in Notothenioid fishes. Fish Physiol Biochem 3:1–6. doi:10.1007/BF02183988
Johnston IA, Maitland B (1980) Temperature acclimation in crucian carp, *Carassius carassius* L., morphometric analyses of muscle fibre ultrastructure. J Fish Biol 17:113–125. doi:10.1111/j.1095-8649.1980.tb02746.x
Johnston IA, Sidell BD, Driedzic WR (1985) Force-velocity characteristics and metabolism of carp muscle fibres following temperature acclimation. J Exp Biol 119:239–249
Jones PD, Sidell BD (1982) Metabolic responses of striped bass (*Morone saxatilis*) to temperature acclimation. II. Alterations in metabolic carbon sources and distributions of fiber types in locomotory muscle. J Exp Zool 219:163–171. doi:10.1002/jez.1402190205
Kleckner NW, Sidell BD (1985) Comparison of maximal activities of enzymes from tissues of thermally acclimated and naturally acclimatized chain pickerel (*Esox niger*). Physiol Zool 58:18–28

Kutik S, Rissler M, Guan XL, Guiard B, Shui G, Gebert N, Heacock PN, Rehling P, Dowhan W, Wenk MR, Pfanner N, Wiedemann N (2008) The translocator maintenance protein Tam41 is required for mitochondrial cardiolipin biosynthesis. J Cell Biol 183:1213–1221. doi:10.1083/jcb.200806048

Lehmann J (1970) Veränderungen der Enzymaktivitäten nach einem Wechsel der Adaptationstemperatur, untersucht am Seitenrumpfmuskel des Goldfisches (*Carassius auratus* L.). Int Rev Hydrobiol 55:763–781. doi:10.1002/iroh.19700550505

LeMoine CMR, Genge CE, Moyes CD (2008) Role of the PGC-1 family in the metabolic adaptation of goldfish to diet and temperature. J Exp Biol 211:1448–1455. doi:10.1242/jeb.014951

Lucassen M, Schmidt A, Eckerle LG, Pörtner H-O (2003) Mitochondrial proliferation in the permanent vs. temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. Am J Physiol Regul Integr Comp Physiol 285:1410–1420. doi:10.1152/ajpregu.00111.2003

Lucassen M, Koschnick N, Eckerle LG, Pörtner H-O (2006) Mitochondrial mechanisms of cold adaptation in cod (*Gadus morhua* L.) population from different climatic zones. J Exp Biol 209:2462–2471. doi:10.1242/jeb.02268

Mandic M, Speers-Roesch B, Richards JG (2013) Hypoxia tolerance in sculpins is associated with high anaerobic enzyme activity in brain but not in liver or muscle. Physiol Biochem Zool 86:92–105. doi:10.1086/667938

Martin N, Kraffe E, Guderley H (2009) Effect of day length on oxidative capacities of mitochondria from red muscle of rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol Part A 152:599–603. doi:10.1016/j.cbpa.2009.01.010

McBryan TL, Healy TM, Haakons KL, Schulte PM (2016) Warm acclimation improves hypoxia tolerance in *Fundulus heteroclitus*. J Exp Biol 219:474–484. doi:10.1242/jeb.133413

McClelland GB, Craig PM, Dhekney K, Dipardo S (2006) Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (*Danio rerio*). J Physiol 577:739–751. doi:10.1113/jphysiol.2006.119032

Morin RP, Able KW (1983) Patterns of geographic variation in the egg morphology of the fundulid fish, *Fundulus heteroclitus*. Copeia 1983:726–740. doi:10.2307/1444339

O'Brien KM (2011) Mitochondrial biogenesis in cold-bodied fishes. J Exp Biol 214:275–285. doi:10.1242/jeb.046854

O'Brien KM, Mueller IA (2010) The unique mitochondrial form and function of Antarctic Channichthyid icefishes. Integr Comp Biol 50:993–1008. doi:10.1093/icb/icq038

O'Brien KM, Sidell BD (2000) The interplay among cardiac ultrastructure, metabolism and the expression of oxygen-binding proteins in Antarctic fishes. J Exp Biol 203:1287–1297

Orczewska JI, Hartleben G, O'Brien KM (2010) The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation. Am J Physiol Regul Integr Comp Physiol 299:352–364. doi:10.1152/ajpregu.00189.2010

Osman C, Haag M, Potting C, Rodenfels J, Dip PV, Wieland FT, Brugger B, Westermann B, Langer T (2009) The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. J Cell Biol 184:583–596. doi:10.1083/jcb.200810189

Pörtner H-O (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213:881–893. doi:10.1242/jeb.037523

Pörtner H-O, Lucassen M, Storch D (2005) Metabolic biochemistry: its role in thermal tolerance and in the capacities of physiological and ecological function. In: Farrell AP, Steffensen JF (eds) Fish physiology, volume 22: physiology of polar fishes. Elsevier, San Diego, pp 79–154. doi:10.1016/S1546-5098(04)22003-9

Precht H (1958) Concepts of the temperature adaptation of unchanging reaction systems of cold-blooded animals. In: Prosser CL (ed) Physiological adaptations. American Physiology Society, Washington, DC, pp 50–78

Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 92:829–839. doi:10.1016/S0092-8674(00)81410-5

Ramachandran B, Yu G, Gulick T (2008) Nuclear respiratory factor 1 controls myocyte enhancer factor 2A transcription to provide a mechanism for coordinate expression of respiratory chain subunits. J Biol Chem 283:11935–11946. doi:10.1074/jbc.M707389200

Richards JG (2009) Metabolic and molecular responses of fish to hypoxia. In: Richards JG, Farrell AP, Brauner CJ (eds) Fish physiology, volume 27: Hypoxia. Elsevier, San Diego, pp 443–485. doi:10.1016/S1546-5098(08)00010-1

Roberts JL (1966) Systemic versus cellular acclimation to temperature by poikilotherms. Helgol Wiss Meeresunters 14:451–465. doi:10.1007/BF01611638

Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev 88:611–638. doi:10.1152/physrev.00025.2007

Scarpulla RC (2011) Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. Biochim Biophys Acta 1813:1269–1278. doi:10.1016/j.bbamcr.2010.09.019

Scarpulla RC, Vega RB, Kelly DP (2012) Transcriptional integration of mitochondrial biogenesis. Trends Endocrin Met 23:459–466. doi:10.1016/j.tem.2012.06.006

Shaklee JB, Christiansen JA, Sidell BD, Prosser CL, Whitt GS (1977) Molecular aspects of temperature acclimation in fish: contributions of changes in enzyme activities and isozyme patterns to metabolic reorganization in the green sunfish. J Exp Zool 201:1–20. doi:10.1002/jez.1402010102

Sidell BD (1998) Intracellular oxygen diffusion: the roles of myoglobin and lipid at cold body temperature. J Exp Biol 201:1118–1127

Sidell BD, Wilson FR, Hazel J, Prosser CL (1973) Time course of thermal acclimation in goldfish. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 84:119–127. doi:10.1007/BF00697602

Tyler S, Sidell BD (1984) Changes in mitochondrial distribution and diffusion distances in muscle of goldfish upon acclimation to warm and cold temperatures. J Exp Zool 232:1–9. doi:10.1002/jez.1402320102

Urschel MR, O'Brien KM (2008) High mitochondrial densities in the hearts of Antarctic icefishes are maintained by an increase in mitochondrial size rather than mitochondrial biogenesis. J Exp Biol 211:2636–2638. doi:10.1242/jeb.018598

Virbasius CA, Virbasius JV, Scarpulla RC (1993) NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. Genes Dev 7:2431–2445. doi:10.1101/gad.7.12a.2431

Walsh PF, Foster GD, Moon TW (1983) The effects of temperature on metabolism of the American eel *Anguilla rostrata* (LeSueur): compensation in the summer and torpor in the winter. Physiol Zool 56:532–540