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RESEARCH ARTICLE

Thermal acclimation and subspecies-specific effects on heart and brain mitochondrial performance in a eurythermal teleost (*Fundulus heteroclitus*)

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ABSTRACT

Mitochondrial performance may play a role in setting whole-animal thermal tolerance limits and their plasticity, but the relative roles of adjustments in mitochondrial performance across different highly aerobic tissues remain poorly understood. We compared heart and brain mitochondrial responses to acute thermal challenges and to thermal acclimation using high-resolution respirometry in two locally adapted subspecies of Atlantic killifish (*Fundulus heteroclitus*). We predicted that 5°C acclimation would result in compensatory increases in mitochondrial performance, while 33°C acclimation would cause suppression of mitochondrial function to minimize the effects of high temperature on mitochondrial metabolism. In contrast, acclimation to both 33 and 5°C decreased mitochondrial performance compared with fish acclimated to 15°C. These adjustments could represent an energetic cost-saving mechanism at temperature extremes. Acclimation responses were similar in both heart and brain; however, this effect was smaller in the heart, which might indicate its importance in maintaining whole-animal thermal performance. Alternatively, larger acclimation effects in the brain might indicate greater thermal sensitivity compared with the heart. We detected only modest differences between subspecies that were dependent on the tissue assayed. These data demonstrate extensive plasticity in mitochondrial performance following thermal acclimation in killifish, and indicate that the extent of these responses differs between tissues, highlighting the importance and complexity of mitochondrial regulation in thermal acclimation in eurytherms.

KEY WORDS: Killifish, Thermal performance, Oxidative phosphorylation, Local adaptation, Temperature, Fish

INTRODUCTION

Ambient temperature (T_a) constrains whole-organism performance, and this is especially true for ectotherms. These constraints are due, at least in part, to temperature effects on biochemical reaction rates and, by extrapolation, aerobic metabolism (Hochachka and Somero, 2002; Schulte, 2015; Guderley and St-Pierre, 2002; Pörtner, 2001; Pörtner and Farrell, 2008). Declines in aerobic performance at thermal extremes are thought to occur because of an inability to deliver O_2 to systemic tissues, possibly owing to effects on cardiac function (Pörtner, 2001; Somero, 2010; Iftikar and Hickey, 2013). Alternatively, temperature-induced declines in neural function are suggested to constrain thermal performance limits (Somero and DeVries, 1967; Ern et al., 2015; Cossins, 1977; Jastroch et al., 2007; Miller and Stillman, 2012). However, the relative importance of these organ systems in setting an organism's thermal performance limits is still subject to debate (Clark et al., 2013; Ern et al., 2015).

One mechanism that could underlie cardiac and neural failure is mitochondrial dysfunction. For example, the capacity for heart mitochondrial ATP synthesis declines at temperatures preceding heart failure in *Notolabrus celidotus* (Iftikar and Hickey, 2013). However, no studies have examined the possibility of mitochondrial failure being associated with temperature-induced declines in neural performance. Comparisons of mitochondrial function among tissues and within the same organism are thus imperative as differences in thermal sensitivity and plasticity of mitochondrial function may reveal the relative importance of particular tissues in setting thermal tolerance limits.

There is substantial evidence for thermally induced plasticity in mitochondrial structure and function in ectotherms (Guderley and St-Pierre, 2002; Seebacher et al., 2010). Low-temperature acclimation is associated with increased mitochondrial oxidative phosphorylation (OXPHOS) capacity, mitochondrial volume density and alterations in mitochondrial membrane composition (Chung and Schulte, 2015; Egginton and Johnston, 1984; Grim et al., 2010; Fangue et al., 2009; Kraffe et al., 2007; Dhillon and Schulte, 2011; Schnell and Seebacher, 2008). In contrast, high-temperature acclimation has been associated with changes in mitochondrial membrane fatty acid saturation and lowered mitochondrial respiratory capacity (Chung and Schulte, 2015; Guderley and Johnston, 1996; Khan et al., 2014; Strobel et al., 2013; Baris et al., 2016a; Fangue et al., 2009). These changes may induce trade-offs causing mitochondrial function to decline at temperatures that were not previously harmful, which may account for shifts in whole-animal thermal tolerance following acclimation (Fangue et al., 2009; Chung and Schulte, 2015).

Natural selection acting on mitochondrial function has the potential to improve performance in subspecies that experience different thermal regimes (Castellana et al., 2011; Guderley, 2011; Pörtner, 2001). Indeed, differences in whole-animal and mitochondrial thermal performance among *Drosophila* species occurs because of divergences in mitochondrial DNA sequence and incompatibilities between mitochondrial and nuclear genomes (Pichaud et al., 2013; Hoekstra et al., 2013). Although these studies provide support for selection enhancing thermal performance, much remains to be discovered about whether these mechanisms constrain or enhance mitochondrial acclimation capacity and the consequences for whole-animal performance.

Here we utilize Atlantic killifish [*Fundulus heteroclitus* (Linnaeus 1766)], a profoundly eurythermal teleost, to examine plasticity and putatively adaptive variation in mitochondrial

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List of symbols and abbreviations

CCO	cytochrome *c* oxidase
CS	citrate synthase
CT	critical thermal
CT_{max}	critical thermal maximum
ETS	electron transport system
FCCP	carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone
OXPHOS	oxidative phosphorylation
RCR	respiratory control ratio
SUIT	substrate uncoupler inhibitor titration
T_a	ambient temperature
T_{assay}	assay temperature
T_b	core body temperature
TMPD	*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine

performance with temperature. These animals reside in intertidal salt marshes along the Atlantic coast of North America. In these estuarine habitats they experience large diel and seasonal fluctuations in T_a. Their ability to survive [critical thermal (CT) limits=−1 to 41°C] and acclimate (acclimation limits=2 to 35°C) over a large thermal range may be due to a thermally robust mitochondrial physiology (Fangue et al., 2009; Chung and Schulte, 2015) and acclimation capacity (Chung and Schulte, 2015; Fangue et al., 2009; Baris et al., 2016a). In addition, northern and southern subspecies of *F. heteroclitus* have undergone local adaptation to their thermal environments and have diverged for a number of traits such as whole-animal metabolic rates and thermal tolerance (Schulte, 2001; Fangue et al., 2006, 2009). Despite the existence of genetic polymorphisms in genes encoding mitochondrial proteins that differentiate northern and southern killifish (Whitehead, 2009; Mckenzie et al., 2016), few clear differences in mitochondrial function exist between these subspecies (Baris et al., 2016a; Fangue et al., 2009), although differentiation in mitochondrial function has been examined in only heart (Baris et al., 2016a) and liver (Fangue et al., 2009), and nothing is known about the function of brain mitochondria in this species.

In this study, we assessed the relative responses of brain and heart mitochondria to thermal acclimation (5, 15 and 33°C) in northern and southern *F. heteroclitus*. This design allows us to improve understanding of the mechanisms that underlie the potential failure of these tissues during thermal stress and to identify the relative contribution of each tissue to setting organismal thermal tolerance limits. We measured mitochondrial performance using high-resolution respirometry to quantify changes in function through each component of the mitochondrial electron transport system (ETS). We addressed the following questions. (1) Does acclimation to 5 and 33°C alter mitochondrial function through compensatory increases and suppression of activity respectively? (2) Do heart and brain mitochondrial performance differ in their acute and acclimation thermal responses, which might reveal a greater contribution of one tissue to whole-animal thermal tolerance? (3) Do locally adapted killifish subspecies exhibit differences in mitochondrial function that could underlie the previously observed differences in whole-organism metabolic rates and thermal tolerance? In this way, we provide a greater mechanistic understanding of the mitochondrion's role in the setting of whole-animal thermal tolerance limits.

MATERIALS AND METHODS

Animals

Experiments were conducted following University of British Columbia approved animal care protocol A11-0372. Wild-caught adult southern killifish (*Fundulus heteroclitus heteroclitus*) were collected from Jekyll Island, Georgia (31°02′N, 81°25′W), in June 2014. Northern killifish (*Fundulus heteroclitus macrolepidotus*) were collected in Ogden's Pond estuary, Nova Scotia (45°71′N, 61° 90′W), in September 2014. Fish were estimated to be 1 to 2 years old based on length at catch (>5 cm; Valiela et al., 1977). Fish were housed at the University of British Columbia Aquatics Facility in 190 liter recirculating tanks with biological filtration at T_a=15±2°C, 20 ppt salinity and 12 h:12 h light:dark photoperiod prior to experimentation. Fish were fed once daily to satiation (Tetrafin Max, Rolf C. Hagen Inc., Montreal, QC, Canada). After at least 10 months of laboratory holding (July 2015), fish were distributed into 114 liter tanks with T_a=5, 15 or 33°C at 20 ppt salinity and 12 h:12 h light:dark. We chose 33°C for our high-temperature acclimation as this is the point at which effects on whole-organism aerobic metabolism are first observed, while also avoiding substantial induction of breeding physiology which occurs at lower temperatures (i.e. 30°C; Healy and Schulte, 2012; Matthews, 1939). We chose 5°C as our low acclimation temperature because this is the temperature at which effects on whole-organism aerobic metabolism are first observed (Healy and Schulte, 2012). Fish were acclimated for a minimum of 4 weeks prior to sampling.

Heart permeabilization

Following thermal acclimation, fish were removed from their holding tanks and euthanized by cervical dislocation and weighed at 09:00 h PST prior to the daily feeding. Each day, five killifish were sampled. Hearts were excised, weighed and transferred to a single Petri dish containing ice-cold BIOPS solution (2.77 mmol l^{-1} CaK_2EGTA, 7.23 mmol l^{-1} K_2EGTA, 5.77 mmol l^{-1} Na_2·ATP, 6.56 mmol l^{-1} $MgCl_2·6H_2O$, 20 mmol l^{-1} taurine, 15 mmol l^{-1} Na-phosphocreatine, 20 mmol l^{-1} imidazole, 0.5 mmol l^{-1} dithiothreitol, 50 mmol l^{-1} MES hydrate, 1 g l^{-1} fatty-acid-free BSA, pH 7.1 at 0°C). From this point forward, hearts were not individually tracked, making it impossible to associate a specific preparation with a whole-organism mass. For each heart, the bulbus was removed and the whole ventricle was teased into one fiber bundle (duration 3 min) using sharp forceps. Fiber bundles from different fish were not mixed. Individual fiber bundles were placed into 3 ml of ice-cold BIOPS solution in a 12-well tissue culture plate (one bundle per well). 30 µl of saponin (final concentration 0.05 mg ml^{-1}) was added to each well and ventricle fibers were shaken (80 rpm) on ice for 30 min. Following the saponin treatment, fibers were washed three times for 5 min in ice-cold MiRO5 (0.5 mmol l^{-1} EGTA, 3 mmol l^{-1} $MgCl_2·6H_2O$, 60 mmol l^{-1} lactobionic acid, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} HEPES, 110 mmol l^{-1} sucrose, 1 g l^{-1} fatty-acid-free BSA, pH 7.1 at 25°C). Individual fiber bundles were blotted on filter paper and weighed (5–10 mg) prior to respiration assays.

Brain permeabilization

Brains were also dissected from the killifish, and brain permeabilization was achieved using methods similar to those for heart tissues, with the exception that brains from the five different individuals sampled on a given day were pooled. Brains were weighed, pooled in ice-cold BIOPS solution and cut into approximately 2 mm^3 pieces using a scalpel and sharp forceps. All brain pieces were transferred into one 3 ml aliquot of ice-cold BIOPS solution to which 30 µl of saponin (0.05 mg ml^{-1}) was added. Following saponin treatment and three washes in ice-cold MiRO5, randomly selected brain pieces were blotted on filter paper and weighed out into 20–35 mg tissue pools immediately prior to

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respiration assays. Randomization of brain pieces following pooling and mincing of brain pieces prevented the inclusion of fish mass in analyses of respiration data.

Substrate uncoupler inhibitor titration protocol

Flux through the mitochondrial ETS and OXPHOS apparatus was assessed using a substrate uncoupler inhibitor titration (SUIT) protocol modified from Iftikar and Hickey (2013). Permeabilized tissue respiration rates were measured using a high-resolution respirometry system (O2k MiPNet Analyzer, Oroboros Instruments, Innsbruck, Austria). Pre-weighed tissue samples were added to chambers containing 2 ml of air-equilibrated MiRO5. Oxygen electrodes were calibrated across a range of O_2 tensions (350 nmol ml^{-1} to O_2 depleted) at each assay temperature (T_{assay}=5, 15, 33, 37°C) to account for temperature effects and background O_2 consumption by the probes. Zero calibration of the probes at each assay temperature was achieved using a yeast suspension. During the assay, O_2 tension was maintained between 350 and 200 nmol ml^{-1} by injecting O_2 into the gas phase above the medium to maintain the partial pressure gradient to the mitochondria. Respiration rates were normalized to tissue wet mass.

State II respiration fueled through ETS complex I (LEAK-I) was achieved through the addition of pyruvate (10 mmol l^{-1}) and malate (2 mmol l^{-1}). A saturating quantity of ADP (2.5 mmol l^{-1}) was introduced to the chamber followed by glutamate (10 mmol l^{-1}) to assess complex-I-linked state III respiration (oxidative phosphorylation, OXP-I). State III respiration fueled through complexes I and II (OXP-I,II) was measured through the addition of succinate (10 mmol l^{-1}). Complex I- and II-linked state IV respiration (LEAK-I,II) was estimated through the introduction of carboxyatractyloside (5 µmol l^{-1}). We used carboxyatractyloside as an inhibitor because of oligomycin's suppressive effects on respiration in similar preparations (Baris et al., 2016b). Substrate oxidation capacity (ETS-I,II, fueled through ETS complexes I and II) was achieved by fully uncoupling mitochondria with repeated additions of carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP, 0.5 µmol l^{-1}). This was followed by inhibition of ETS complexes I, II and III through the sequential addition of rotenone (0.5 µmol l^{-1}, dissolved in ethanol; yielding ETS-II), malonate (5 mmol l^{-1}) and antimycin A (2.5 µmol l^{-1}), respectively. Apparent cytochrome *c* oxidase (ETS complex IV; CCO) capacity was measured through the addition of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mmol l^{-1}) and ascorbate (2 mmol l^{-1}). We accounted for auto-oxidation of TMPD and ascorbate through chemical background corrections at each assay temperature. Mitochondrial coupling was estimated using the ratio of OXP-I and LEAK-I (respiratory control ratio; RCR-I) and OXP-I,II and LEAK-I,II (RCR-I,II). Limitations on OXPHOS capacity by ETS capacity were assessed using the ratio of OXP-I,II to ETS-I,II.

Citrate synthase assay

We approximated changes in heart and brain mitochondrial quantity following thermal acclimation by measuring changes in citrate synthase (CS) activity (Srere, 1969; Larsen et al., 2012) in a different subset of fish than those used for mitochondrial assays. Tissues were frozen in liquid N_2 and stored at −80°C until they were ready to be assayed. Frozen tissues were thawed on ice and homogenized individually in 250 or 350 µl (heart and brain, respectively) of homogenization buffer [5 mmol l^{-1} EDTA, 50 mmol l^{-1} HEPES, 0.1% (v/v) Triton X-100, pH 7.4 at 20°C] using two 10 s passes of a tissue homogenizer (PowerGen 125, Fisher Scientific, Ottawa, Canada). Homogenized samples were centrifuged (10,000 ***g*** for 2 min at 4°C) and the resulting supernatant was used for the assay. Tissue homogenate was diluted (21-fold) in assay buffer (0.30 mmol l^{-1} acetyl-CoA, 0.15 mmol l^{-1} DTNB, 50 mmol l^{-1} Tris-HCl, pH 8.0 at 25°C) and a background rate of change of absorbance was measured at 412 nm for 10 min using a Molecular Devices Spectramax-190 at 25°C. This was followed by addition of oxaloacetate (21.5 mmol l^{-1} final concentration) and reaction rate was monitored for 10 min at 412 nm. CS activity was corrected to protein concentration as determined by Bradford assay with BSA as a standard (Bradford, 1976).

Statistical analyses

All data are presented as means±s.e.m.; sample size (*n*) is indicated in the relevant figure and table captions. All statistical tests were completed using R software (version 3.0.2) with α=0.05.

Thermal acclimation effects on whole-animal, heart and brain mass, and cardiosomatic and craniosomatic indices were assessed using separate two-way ANOVAs with acclimation temperature and subspecies as factors. The effects of subspecies, assay temperature and acclimation temperature on LEAK-I; OXP-I; OXP-I,II; LEAK-I,II; ETS-I,II; ETS-II; CCO; RCR-I; RCR-I,II; and OXP-I,II/ETS-I, II were assessed using separate three-way ANOVAs for hearts and brains. Heart and brain CS activity were analyzed using two-way ANOVAs with acclimation temperature and subspecies as factors. We ran Shapiro–Wilk and Bartlett's tests to confirm normal distributions and homogeneity of variance in our data. Our data conformed to the assumption of homogeneity of variance but were not always normally distributed (data were typically slightly right-skewed). Given that ANOVAs are robust to moderate deviations from normality, we proceeded with parametric analysis (Harwell et al., 1992).

RESULTS

Whole-animal and tissue-specific mass

We measured whole-animal and tissue wet masses following thermal acclimation to estimate the overall energetic status of the animals. Northern and southern killifish acclimated to 5°C had the highest whole-animal mass, whereas 33°C acclimated fish had the lowest ($P_{acclimation}$<0.001; Fig. 1A). Northern killifish had greater whole-animal mass compared with southern killifish ($P_{subspecies}$<0.05). This subspecies effect may be influenced by differences in initial mass that we are unable to account for. No interaction effect was detected ($P_{subspecies×acclimation}$=0.195).

Acclimation to 5°C was associated with greater heart mass in both subspecies of killifish compared with 15°C control fish. In contrast, acclimation to 33°C was associated with lower heart mass ($P_{acclimation}$<0.001; Fig. 1B). Northern killifish exhibited greater heart mass at all acclimation temperatures, particularly following acclimation to 5°C ($P_{acclimation×subspecies}$<0.05, $P_{subspecies}$<0.001). Acclimation to 5°C was associated with a greater cardiosomatic index compared with 15°C control fish and a lower index following acclimation to 33°C ($P_{acclimation}$<0.05; Fig. 1D). Northern killifish had a greater cardiosomatic index than southern killifish and there was no significant interaction of subspecies and acclimation effects ($P_{subspecies}$<0.001, $P_{subspecies×acclimation}$=0.469).

Unlike acclimation effects on the heart, 5°C acclimation did not result in greater brain mass when compared with 15°C control fish (Fig. 1C). In contrast, acclimation to 33°C resulted in significantly lower brain mass compared with control fish ($P_{acclimation}$<0.001). Northern killifish had greater brain mass than southern killifish

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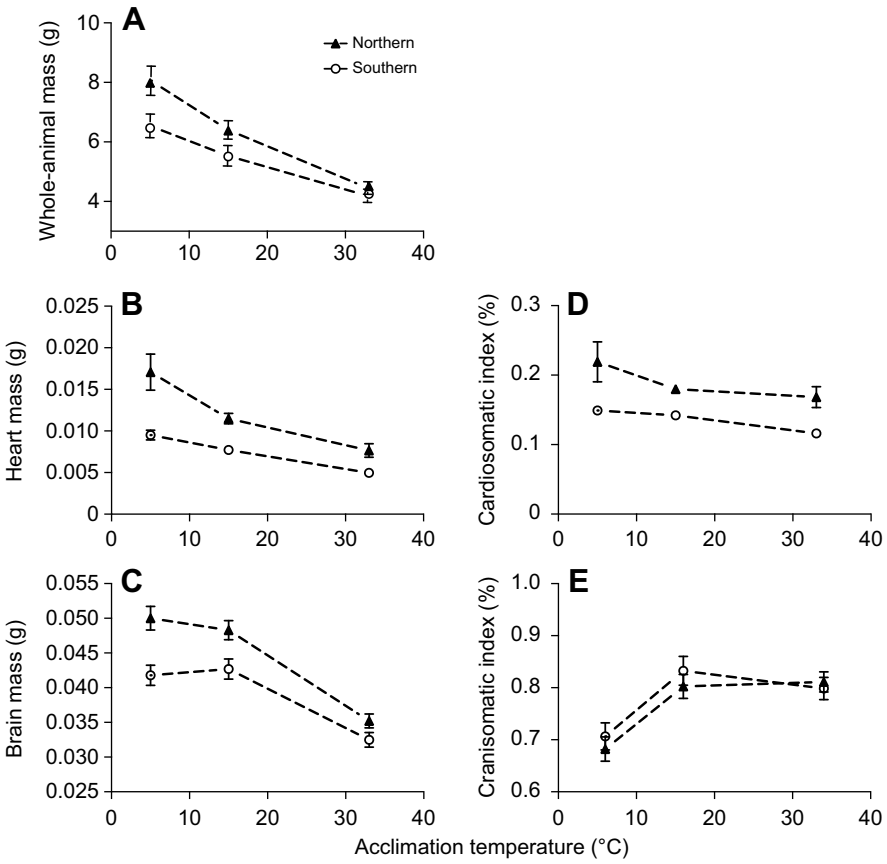

Fig. 1. Thermal acclimation (5, 15 or 33°C) effects on whole-animal, heart, and brain wet mass and somatic indices from northern (black triangle) and southern (open circle) subspecies of *Fundulus heteroclitus*. Killifish were thermally acclimated for 4 weeks prior to sampling. Cardiosomatic (D) and craniosomatic (E) indices were calculated as the ratio of tissue mass (B and C, respectively) to whole-animal mass (A). Data are means±s.e.m.; see Results for associated statistics (n=49–56).

($P_{subspecies}$<0.001). No significant interaction effect was detected ($P_{subspecies×acclimation}$=0.146). Craniosomatic index was lower in 5°C acclimated killifish compared with 15°C control fish and there was no difference between 15 and 33°C acclimated killifish (Fig. 1E, $P_{acclimation}$<0.001). We did not detect any significant subspecies or interaction effects on craniosomatic index ($P_{subspecies}$=0.544, $P_{subspecies×acclimation}$=0.623).

Heart OXPHOS and LEAK

We measured OXPHOS (state III) and LEAK (state II or IV) respiration fueled through ETS complex I, and I and II in tandem to estimate changes in mitochondrial respiration resulting from our treatments. Our respiration rates were marginally lower than those measured in hearts from *Notolabrus celidotus* (OXPHOS fueled through complex I=25 pmol O_2 mg tissue^{-1} s^{-1}, T_{assay}=15°C; Iftikar et al., 2015). There was no significant subspecies effect on heart mitochondrial OXPHOS (OXP-I, OXP-I,II) or LEAK (LEAK-I, LEAK-I,II) respiration when fueled through ETS complex I, or I and II (Fig. 2, see Table 1 for *P*-values). Increases in assay temperature caused an increase of these mitochondrial parameters in both subspecies. In addition, there was a significant interaction between subspecies and assay temperature on OXP-I,II; which was driven by marginally lower acute thermal sensitivity in southern killifish when compared to northern killifish at higher assay temperatures ($P_{subspecies×assay}$<0.05; Fig. 2E,F).

Unlike the relatively modest differences in respiration rates between the subspecies, there was a large effect of thermal acclimation on OXPHOS and LEAK respiration. Acclimation to 5 and 33°C resulted in lower OXPHOS and LEAK respiration compared with 15°C acclimated fish, at most assay temperatures. However, at the highest assay temperatures (i.e. 33 to 37°C), 5°C acclimated killifish from both subspecies exhibited an increase in thermal sensitivity that was not present in 15 or 33°C acclimated fish, such that the respiration in this group became similar to that of the 15°C acclimated group at the highest assay temperature (Fig. 2, see Table 1 for *P*-values).

Heart maximum mitochondrial capacity

Killifish heart ETS capacity (i.e. maximum substrate oxidation) through ETS complexes I and II in tandem (ETS-I,II), and ETS complex II (ETS-II), and apparent CCO capacity responded similarly to previously described OXPHOS and LEAK parameters (Fig. 3). Increases in assay temperature increased respiration rates for both subspecies, acclimation to 5 and 33°C resulted in lower rates of respiration compared with 15°C controls, and there were relatively few differences between subspecies (see Table 1 for *P*-values).

However, we observed significant interaction effects between subspecies and assay temperature on ETS-I, ETS-I,II and CCO that are most likely a result of marginally lower respiration rates in southern killifish compared with northern killifish at high assay temperatures (Fig. 3, see Table 1 for *P*-values). We also detected an interaction effect between acclimation and assay temperature on ETS-I,II, which occurred because of acclimation temperature-specific thermal sensitivity changes between T_{assay}=33 and 37°C ($P_{acclimation×assay}$<0.05; Fig. 3A,B). CCO was subject to an interaction effect between subspecies and thermal acclimation which is most likely due to 5°C acclimated northern fish exhibiting lower respiration compared with 33°C acclimated individuals and a reversed acclimation effect in southern killifish; nevertheless, these effects were modest ($P_{subspecies×acclimation}$<0.05; Fig. 3E,F).

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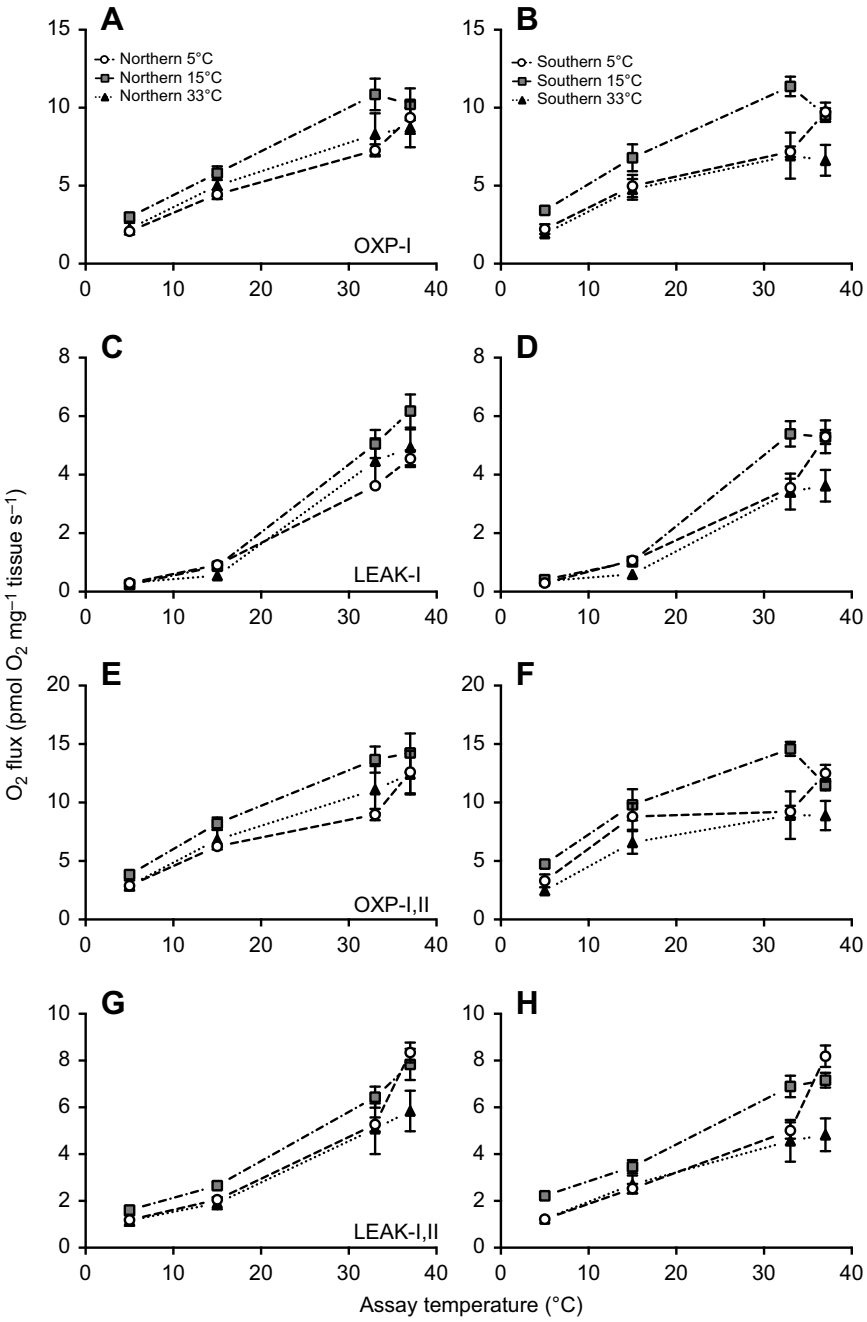

Fig. 2. Coupled heart mitochondrial respiration from northern and southern killifish acclimated to 5 (open circle), 15 (grey square) or 33°C (black triangle) for 4 weeks. Permeabilized heart preparations from northern (A,C,E,G) and southern (B,D,F,H) subspecies were subjected to a substrate uncoupler inhibitor titration protocol. Oxidative phosphorylation (OXP; A,B,E,F; state III) and LEAK (C,D,G,F; state II or IV) respiration were measured through electron transport system (ETS) complex I (A–D; pyruvate, malate and glutamate as substrates) and complexes I and II in tandem (E–H; complex I substrates and succinate). Data are means±s.e.m.; see Table 1 for associated statistics (n=7–8).

Heart mitochondrial control ratios

RCR was calculated to provide insight into the contribution of LEAK to OXPHOS capacity, and as a means of estimating changes in mitochondrial coupling among our treatments (Fig. 4).

We did not detect any significant subspecies effects on heart RCR-I (Fig. 4A,B, see Table 1 for P-values). As assay temperature increased, RCR-I decreased and became indistinguishable among acclimation groups ($P_{\text{acclimation}\times\text{assay}}$<0.001; Table 1). As assay temperature decreased between 15 and 5°C we observed a decrease of RCR-I in 33°C acclimated heart mitochondria. We observed the opposite effect in both 5 and 15°C acclimated fish.

In both subspecies, heart RCR-I,II was highest at T_{assay}=15°C and declined at all other assay temperatures (P_{assay}<0.001; Fig. 4C,D). Thermal acclimation effects were different between subspecies, with 33 and 5°C acclimation resulting in the greatest RCR-I,II values in northern and southern killifish, respectively ($P_{\text{acclimation}\times\text{subspecies}}$<0.005).

We calculated OXP-I,II/ETS-I,II to identify potential limitations on OXPHOS capacity by substrate oxidation capacity (Fig. S1, see Table 1 for P-values). We detected a significant subspecies effect that was driven by a marginally greater ratio in southern killifish compared with their northern counterparts (Fig. S1A,B). As assay temperature increased between 33 and 37°C, the ratio of OXP-I,II/ETS-I,II also increased. No significant acclimation effects or interaction effects were detected.

Brain OXPHOS and LEAK

We assessed the effects of thermal acclimation and local adaptation on brain mitochondrial function, as declines in performance may underlie temperature-induced failure of the nervous system. We did

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Table 1. *P*-values for three-way ANOVAs of *Fundulus heteroclitus* heart mitochondrial parameters

	P-value						
Parameter	Subspecies	Acclimation temperature	Assay temperature	Subspecies×Acclimation	Subspecies×Assay	Acclimation×Assay	Subspecies×Acclimation×Assay
OXP-I	0.522	**<0.001**	**<0.001**	0.159	0.516	**<0.05**	0.969
LEAK-I	0.362	**<0.001**	**<0.001**	0.103	0.329	**<0.01**	0.234
OXP-I,II	0.532	**<0.001**	**<0.001**	0.078	**<0.05**	0.076	0.924
LEAK-I,II	0.884	**<0.001**	**<0.001**	0.607	0.100	**<0.001**	0.949
ETS-I,II	0.058	**<0.001**	**<0.001**	0.086	**<0.05**	**<0.05**	0.832
ETS-II	0.182	**<0.001**	**<0.001**	0.051	**<0.01**	0.079	0.742
CCO	0.716	**<0.001**	**<0.001**	**<0.05**	**<0.05**	0.239	0.259
RCR-I	0.776	**<0.01**	**<0.001**	0.540	0.129	**<0.001**	0.704
RCR-I,II	**<0.01**	0.464	**<0.001**	**<0.005**	0.341	0.241	0.561
OXP-I,II/ETS-I,II	**<0.01**	0.065	**<0.001**	0.912	0.155	0.213	0.103

Significant *P*-values are in bold. OXP, oxidative phosphorylation; ETS, maximum mitochondrial substrate oxidation capacity; CCO, apparent cytochrome *c* oxidase capacity; RCR, respiratory control ratio (OXP/LEAK); I, flux fueled through ETS complex I; II, flux fueled through ETS complex II; n=7–8.

not detect differences between subspecies when measuring brain OXPHOS (state III) and LEAK (state II or IV) respiration fueled through ETS complex I and ETS complexes I and II in tandem (Fig. 5, see Table 2 for *P*-values). As T_{assay} increased, we observed that respiration increased in all groups and that acclimation to 5 and 33°C resulted in lower OXPHOS and LEAK respiration compared with 15°C control fish ($P_{acclimation×assay}$<0.001; Fig. 5).

Brain maximum mitochondrial capacity

Brain ETS (ETS-I,II; ETS-II) and apparent CCO capacity exhibited responses that were similar to those observed for OXPHOS and LEAK parameters. There were large interaction effects between thermal acclimation and assay temperature that were driven by lower ETS capacity and CCO respiration in 5 and 33°C acclimated killifish compared with 15°C acclimated fish, particularly at high assay temperatures (Fig. 6, see Table 2 for *P*-values).

We detected significant subspecies effects on ETS-II and CCO in the brain. In both cases, southern killifish had marginally greater capacity compared with their northern counterparts (Fig. 6C–F; see Table 2 for *P*-values). There was a trend toward a similar significant subspecies effect on ETS-I,II ($P_{subspecies}$=0.087; Fig. 6A,B).

Brain mitochondrial control ratios

RCR fueled through ETS complex I (RCR-I) in brain mitochondria decreased as T_{assay} increased ($P_{subspecies×acclimation×assay}$<0.01; Fig. 7A,B, see Table 2 for *P*-values). At T_{assay}=5°C, brain RCR-I was greatest in 15°C acclimated northern killifish whereas 5°C acclimated southern killifish maintained the highest RCR-I. As T_{assay} increased to 15°C and higher, differences among acclimation treatments were removed.

Brain RCR-I,II did not differ between subspecies ($P_{subspecies}$=0.116; Fig. 7C,D, see Table 2 for *P*-values). At low T_{assay}, differences among thermal acclimation treatments were apparent, with 5°C acclimated killifish maintaining the highest RCR-I,II followed by 15 and 33°C acclimation groups. As T_{assay} increased, RCR-I,II decreased and differences among thermal acclimation treatments were removed ($P_{acclimation×assay}$<0.05).

The ratio of brain OXP-I,II/ETS-I,II increased at T_{assay} extremes of 5 and 37°C ($P_{subspecies×acclimation×assay}$<0.05; Fig. S1C,D, see Table 2 for *P*-values). In general, northern killifish exhibited greater differences among acclimation treatments, particularly at T_{assay}=5 and 15°C. Overall, 33°C acclimated killifish maintained the greatest OXP-I,II/ETS-I,II, except when southern killifish were assayed at T_{assay}=5°C and this ratio became indistinguishable among acclimation treatments. In contrast, 5°C acclimated fish maintained the lowest OXP-I,II/ETS-I,II ratio except at T_{assay}=33 and 37°C in northern killifish, when this ratio increased substantially.

Heart and brain citrate synthase activity

We measured whole heart and brain CS activity to estimate changes in mitochondrial content, a mechanism that could account for observed acclimation and subspecies effects on mitochondrial performance.

Heart CS activity was not significantly altered by acclimation ($P_{acclimation}$=0.190; Fig. 8A). Southern killifish exhibited greater heart CS activity compared with northern killifish at most acclimation temperatures ($P_{subspecies}$<0.001). No significant interaction effects were detected ($P_{subspecies×acclimation}$=0.190).

We detected a significant interaction effect of subspecies and thermal acclimation on brain CS activity ($P_{acclimation×subspecies}$<0.05; Fig. 8B). In general, northern killifish exhibited greater activity compared with southern killifish ($P_{subspecies}$<0.05, n=7). Northern killifish acclimated to 5 and 33°C exhibited a decrease in brain CS activity compared with 15°C controls ($P_{acclimation}$<0.05, n=7). In contrast, southern killifish acclimated to 5°C exhibited a small increase in brain CS activity but 33°C acclimation resulted in a decline. These data indicate that mitochondrial content as estimated by CS activity does not account for our observed acclimation and subspecies effects on mitochondrial performance.

DISCUSSION

In this study, we compared the effects of thermal acclimation and local adaptation on heart and brain mitochondrial function to assess the relative contributions of these tissues to setting whole-organism thermal tolerance limits. Tissue comparisons revealed that heart and brain mitochondria responded similarly to thermal acclimation. Acclimation to both 5 and 33°C decreased heart and brain mitochondrial performance when compared with 15°C acclimated fish (Figs 2–7). These effects were greatest in the brain, suggesting that cardiac mitochondrial performance may be protected or that brain mitochondria are more susceptible to negative effects of acclimation. In contrast, subspecies differences were modest and tissue-specific. Northern killifish exhibited marginally greater maximum ETS capacity (substrate oxidation capacity) and apparent CCO capacity in the heart (Fig. 3), whereas southern killifish exhibited slightly greater maximum capacity in the brain (Fig. 6). Subspecies effects were not apparent for OXPHOS or LEAK respiration, indicating the potential for subspecies differentiation in parameters associated with maximum enzyme

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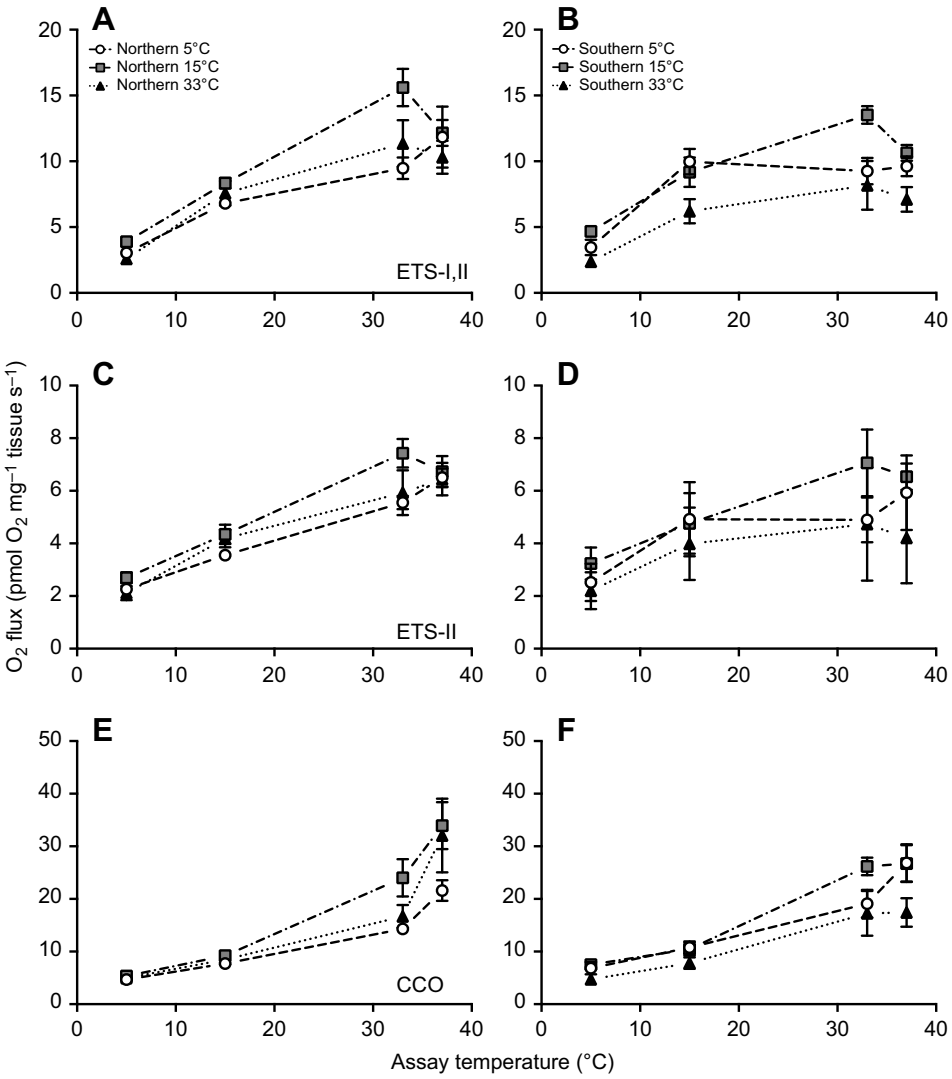

Fig. 3. Maximum heart mitochondrial substrate oxidation capacity (ETS) and apparent cytochrome *c* oxidase (CCO) capacity from northern and southern *Fundulus heteroclitus* acclimated to 5 (open circle), 15 (grey square) or 33°C (black triangle) for 4 weeks. Substrate oxidation capacity was measured in northern (A,C,E) and southern (B,D,F) subspecies through ETS complexes I and II in tandem (A,B; pyruvate, malate, glutamate and succinate as substrates) or complex II alone (C,D; succinate as a substrate, rotenone as a complex I inhibitor). Data are means±s.e.m.; see Table 1 for associated statistics (n=7–8).

capacity and not coupled respiration (Figs 2, 5). These data strongly suggest a role for mitochondrial function in the process of whole-animal thermal acclimation and a modest role for heart or brain mitochondrial capacity in setting subspecies-specific whole-organism thermal tolerance limits.

Does acclimation to 33°C result in a suppression of mitochondrial activity?

We predicted that acclimation to 33°C would cause a decline in mitochondrial performance to counteract the unsustainable mitochondrial O_2 consumption and ROS production suggested to occur following acute high-temperature shifts (Abele et al., 2002; Hochachka and Somero, 2002). Our observed decline in heart and brain mitochondrial capacity supports the prediction of decreased mitochondrial capacity at high temperatures (Figs 2–7). Similar declines in mitochondrial function have been observed previously in heart and liver mitochondria in *F. heteroclitus* (Baris et al., 2016a; Chung and Schulte, 2015) and in other ectotherms (Khan et al., 2014; Guderley and Johnston, 1996; Strobel et al., 2013). Acclimation to 33°C results in a decline in routine oxygen consumption in *F. heteroclitus*, suggesting that this temperature causes a collapse of aerobic metabolism or that active metabolic suppression is taking place (Healy and Schulte, 2012). Interestingly, these effects may occur at lower temperatures for heart mitochondria than the whole organism, as acclimation to 28°C causes declines in mitochondrial respiration in killifish (Baris et al., 2016a), whereas whole-organism metabolic rates are maintained (Healy and Schulte, 2012). Declines in whole-animal and tissue mass (Fig. 1) following 33°C acclimation may be indicative of an energetic mismatch induced by insufficient food supply and high energetic demands (Chung and Schulte, 2015). These data provide support for a mismatch of organism-level energetic supply and demand with increasing temperature and potential sub-lethal costs associated with high-temperature acclimation; this may account for our observed mitochondrial suppression with potential consequences for the fitness of these animals (Salin et al., 2016; Lemoine and Burkepile, 2012; Iles, 2014).

Does acclimation to 5°C result in compensation of mitochondrial activity?

We predicted that acclimation to 5°C would cause an increase in mitochondrial capacity to compensate for decreases in enzyme function and mitochondrial membrane fluidity associated with acute low-temperature shifts (Guderley, 2004; Guderley and Johnston, 1996; Oellermann et al., 2012; Chung and Schulte, 2015; Fangue et al., 2009; Dos Santos et al., 2013). In contrast, we observed

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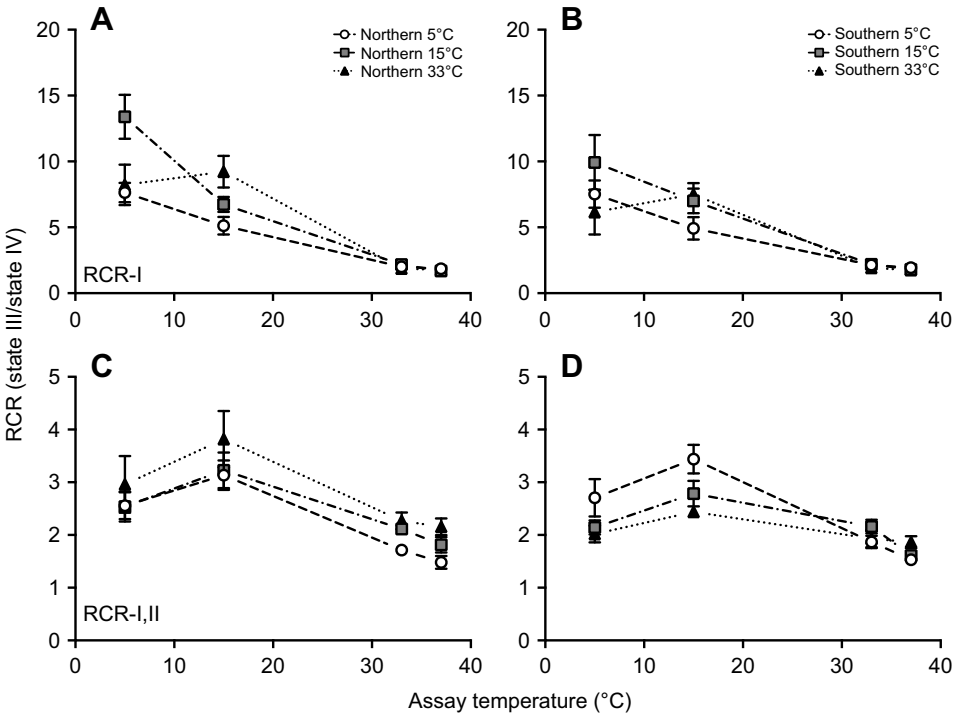

Fig. 4. Heart respiratory control ratios (RCR; the ratio of state III to state IV respiration) from northern and southern *Fundulus heteroclitus* acclimated to 5 (open circle), 15 (grey square) or 33°C (black triangle) for 4 weeks. Northern (A,C) and southern (B,D) *F. heteroclitus* RCR were calculated from respiratory states with flux through ETS complex I alone (A,B; pyruvate, malate and glutamate as substrates) or complexes I and II in tandem (C,D; complex I substrates and succinate). Data are means±s.e.m.; see Table 1 for associated statistics (n=7–8).

decreased heart and brain mitochondrial performance following acclimation to low temperatures (Figs 2–7). This decrease might be a result of an active suppression of metabolism (Richards, 2010; Precht, 1958). There are few clear demonstrations of low-temperature-associated metabolic suppression in fishes (Campbell et al., 2008; Costa et al., 2013; Crawshaw, 1984). At the whole-animal level, routine metabolism measured at 5°C is lower in 5°C acclimated *F. heteroclitus* than in 15°C acclimated fish, which could point to a role for metabolic suppression (Healy and Schulte, 2012). Alternatively, decreases in metabolic rate following low-temperature acclimation may indicate entrance into quiescent states with reductions in spontaneous activity. Disentangling these metabolic and behavioral effects is difficult because of the challenge of assessing these traits at low temperatures. Nevertheless, our observed decrease in heart and brain mitochondrial performance following acclimation to 5°C indicates a role for metabolic suppression in the killifish acclimation response.

Mechanisms of mitochondrial suppression

One mechanism that may account for decreased mitochondrial capacity following thermal acclimation is lower mitochondrial content. However, acclimation to 5°C is associated with an increase in mitochondrial volume density in *F. heteroclitus* white muscle (Dhillon and Schulte, 2011). We estimated mitochondrial quantity by measuring heart and brain CS activity (Fig. 8) (Larsen et al., 2012). Thermal acclimation exerted limited effects on CS activity when compared with our mitochondrial respiration data, and subspecies effects were the opposite in the heart (Figs 2–7). Although the use of a single mitochondrial marker may be insufficient to assess changes in mitochondrial content (reviewed by Moyes et al., 1998), our data do suggest that limited changes in mitochondrial amount occur in these tissues following thermal acclimation and indicate a larger role for intrinsic changes in mitochondrial properties (e.g. mitochondrial lipid remodeling).

Our observation that declines in mitochondrial performance following acclimation to 5 and 33°C were not specific to ETS complex I is intriguing given that we have previously demonstrated highly specific effects of thermal acclimation on ETS complex I in *F. heteroclitus* liver mitochondria (Chung and Schulte, 2015). A lack of ETS complex specificity in heart mitochondria has also been demonstrated in *F. heteroclitus* acclimated to 28°C (Baris et al., 2016a). But this phenomenon is not universal, as ETS complex-specific responses to thermal acclimation occur in other tissues in *F. heteroclitus* and other species (Dos Santos et al., 2013; Chung and Schulte, 2015). This lack of ETS complex specificity may be a methodological artifact, as the SUIT protocol used here provides a mixture of substrates simultaneously, whereas traditional mitochondrial respirometry experiments often supply ETS complex I and II substrates in separate assays. Supplying substrates together may decrease the ability to assess the contribution of each ETS complex to total mitochondrial flux. Under conditions where mitochondrial respiration is assessed through complex II alone (i.e. ETS-II), we observed identical acclimation effects to those observed when both substrates are presented together (Figs 3C,D, 6C,D). We thus conclude that heart and brain mitochondria respond to thermal acclimation with a general decrease in ETS function that is not specific to complex I.

A potential mechanism for constraining mitochondrial performance is a limitation on OXPHOS capacity by ETS capacity (Fig. S1, OXP-I,II/ETS-I,II). In general, heart OXPHOS capacity was not constrained by ETS capacity (i.e. the ratio was approximately 1) and was insensitive to acute temperature shifts (Fig. S1A,B; except at T_{assay}=37°C). This contrasts with Baris et al.'s (2016b) observation of acute temperature effects on this parameter [direct comparisons between these studies should be made carefully as Baris et al. (2016b) assessed effects on complexes I and II separately whereas we make this comparison through complexes I and II in tandem]. In the brain, OXP-I,II/ETS-I,II was acclimation-temperature dependent, indicating that these effects may play a role in altering mitochondrial performance (Fig. S1C,D). As T_{assay} increased in both tissues, OXP-I,II/ETS-I,II increased, which may be a consequence of OXPHOS limitations by substrate

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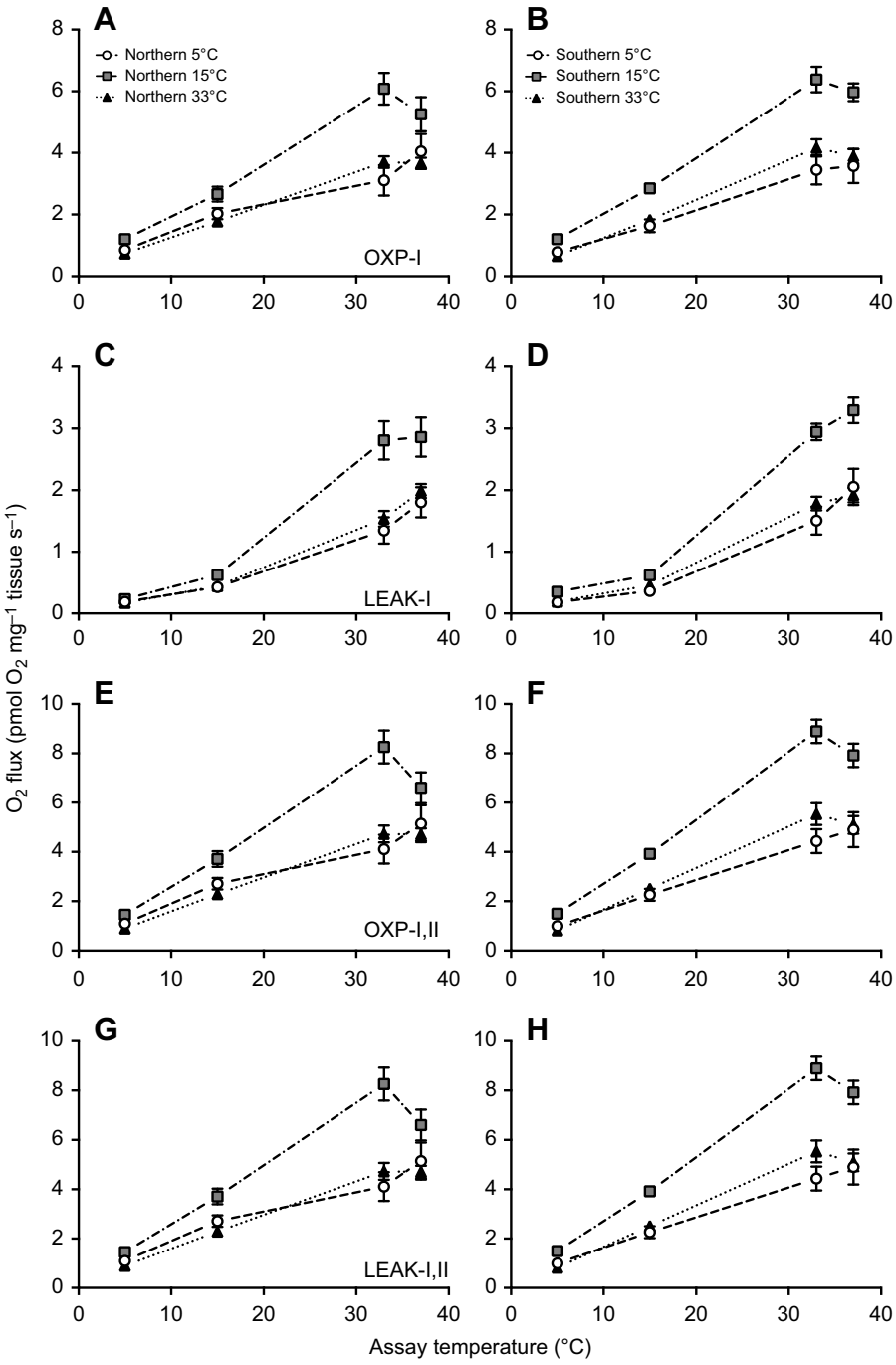

Fig. 5. Coupled brain mitochondrial respiration from northern and southern killifish acclimated to 5 (open circle), 15 (grey square) or 33°C (black triangle) for 4 weeks. Permeabilized brain preparations from northern (A,C,E,G) and southern (B,D,F,H) subspecies were subjected to a substrate uncoupler inhibitor titration protocol. Oxidative phosphorylation (OXP; A,B,E,F; state III) and LEAK (C,D,G,H; state II or IV) respiration were measured through ETS complexes I (A–D; pyruvate, malate and glutamate as substrates) and complexes I and II in tandem (E–H; complex I substrates and succinate). Data are means±s.e.m.; see Table 2 for associated statistics (n=7–8).

oxidation capacity or increased LEAK (Figs 2, 5). Subspecies effects were modest and tissue dependent. Although these data point to a potential role of substrate oxidation limiting OXPHOS capacity, the inconsistent direction and tissue specificity mainly serve to highlight the complexity of these processes.

Do heart and brain mitochondrial performance differ?

A novel aspect of this study is our comparison of heart and brain mitochondrial thermal responses, as any differences we observe might reveal a greater contribution of one tissue to setting organismal thermal tolerance limits. Acclimation to 5 and 33°C clearly lowers both heart and brain mitochondrial capacity (Figs 2–7). However, decreases in mitochondrial capacity with acclimation are larger in the brain (Figs 2–7). This may reflect a preferential decrease in brain mitochondrial function and at least partially sustained heart mitochondrial performance. This lends support to the idea that cardiac performance is important for maintaining whole-animal thermal performance. Alternatively, these larger decreases in brain mitochondrial function may be a consequence of mitochondrial failure occurring at lower temperatures in the brain when compared with the heart. These scenarios are not mutually exclusive, but they at least indicate differences in the way that these tissues respond to thermal acclimation. However, these effects are not universal, as Yan and Xie (2015) detected no difference in the responses of heart and brain mitochondrial function to winter acclimation in *Silurus meridionalis*.

One potential complication of interpreting our brain mitochondrial respiration data is that we are unable to account

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Table 2. *P*-values for three-way ANOVAs of *Fundulus heteroclitus* brain mitochondrial parameters

	P-value						
Parameter	Subspecies	Acclimation temperature	Assay temperature	Subspecies×Acclimation	Subspecies×Assay	Acclimation×Assay	Subspecies×Acclimation×Assay
OXP-I	0.605	**<0.001**	**<0.001**	0.351	0.736	**<0.001**	0.876
LEAK-I	0.200	**<0.001**	**<0.001**	0.741	0.685	**<0.001**	0.889
OXP-I,II	0.221	**<0.001**	**<0.001**	0.245	0.498	**<0.001**	0.894
LEAK-I,II	0.221	**<0.001**	**<0.001**	0.831	0.822	**<0.001**	0.767
ETS-I,II	0.087	**<0.001**	**<0.001**	0.660	0.326	**<0.001**	0.577
ETS-II	**<0.05**	**<0.001**	**<0.001**	0.542	0.694	**<0.001**	0.592
CCO	**<0.005**	**<0.001**	**<0.001**	0.218	0.132	**<0.001**	0.955
RCR-I	**<0.001**	0.058	**<0.001**	0.475	**<0.05**	0.178	**<0.01**
RCR-I,II	0.116	**<0.05**	**<0.001**	0.827	0.345	**<0.05**	0.981
OXP-I,II/ETS-I,II	**<0.005**	**<0.001**	**<0.001**	0.079	0.411	**<0.005**	**<0.05**

Significant *P*-values are in bold. OXP, oxidative phosphorylation; ETS, maximum mitochondrial substrate oxidation capacity; CCO, apparent cytochrome *c* oxidase capacity; RCR, respiratory control ratio (OXP/LEAK); I, flux fueled through ETS complex I; II, flux fueled through ETS complex II; *n*=7–8.

for differences in brain-region-specific mitochondrial performance (Nicholls and Ferguson, 2013). Indeed, Jastroch et al. (2007) demonstrated a brain-region-specific induction of UCP1 mRNA expression in *Cyprinus carpio* following acclimation to 8°C. Although we are unable to account for these differences, the randomization of brain pieces in our assays would suggest a decrease in overall brain mitochondrial function.

Our data demonstrating similar thermal acclimation responses in heart and brain are intriguing when compared with thermal acclimation responses in liver mitochondria. Northern *F. heteroclitus* exhibit increased liver mitochondrial capacity in the cold and decreased capacity following high-temperature acclimation (Chung and Schulte, 2015). These tissue-specific mitochondrial responses might be a consequence of each tissue's *in vivo* function. Tissues involved with maintaining aerobic metabolism may be suppressed during cold acclimation to decrease energetic requirements. In contrast, cold-compensated liver function might allow *F. heteroclitus* to continue processing food, and lay down

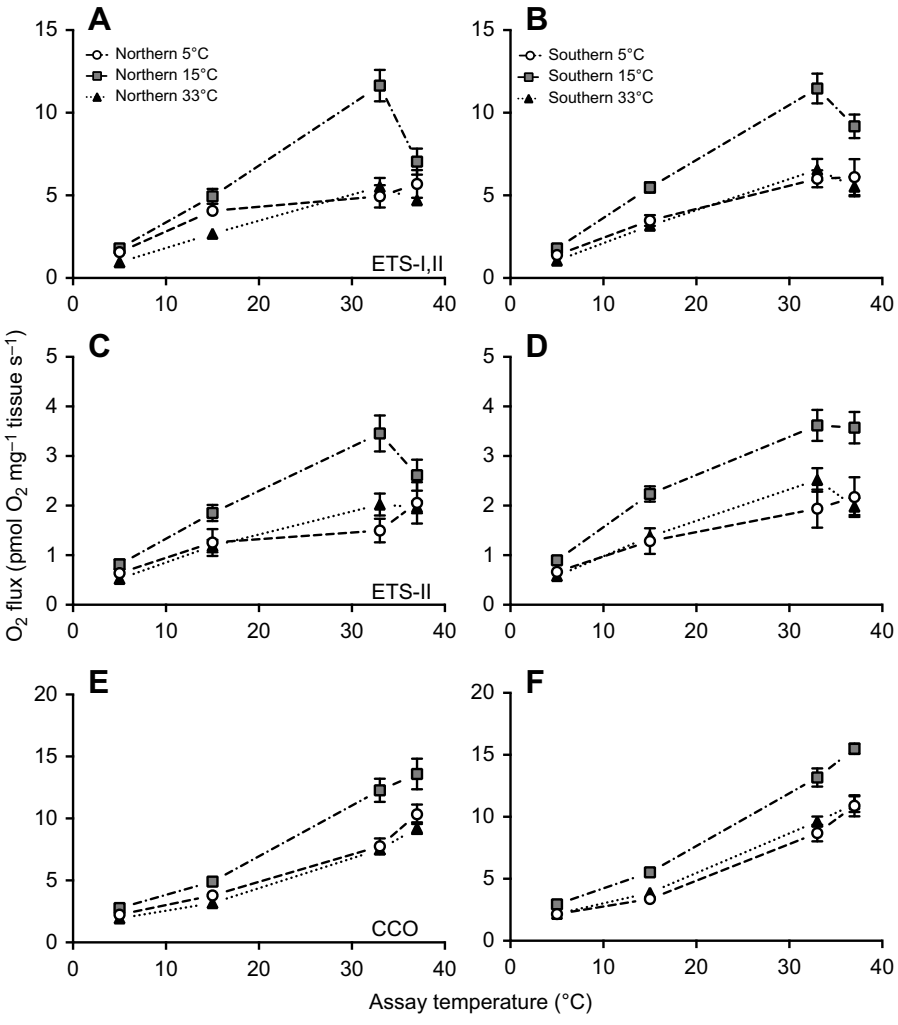

Fig. 6. Maximum brain mitochondrial substrate oxidation capacity (ETS) and apparent CCO capacity from northern and southern *Fundulus heteroclitus* acclimated to 5 (open circle), 15 (grey square) or 33°C (black triangle) for 4 weeks. Substrate oxidation capacity was measured in northern (A,C,E) and southern (B,D,F) subspecies through ETS complexes I and II in tandem (A,B; pyruvate, malate, glutamate and succinate as substrates) or complex II alone (C,D; succinate as a substrate, rotenone as a complex I inhibitor). Data are means±s.e.m.; see Table 2 for associated statistics (*n*=7–8).

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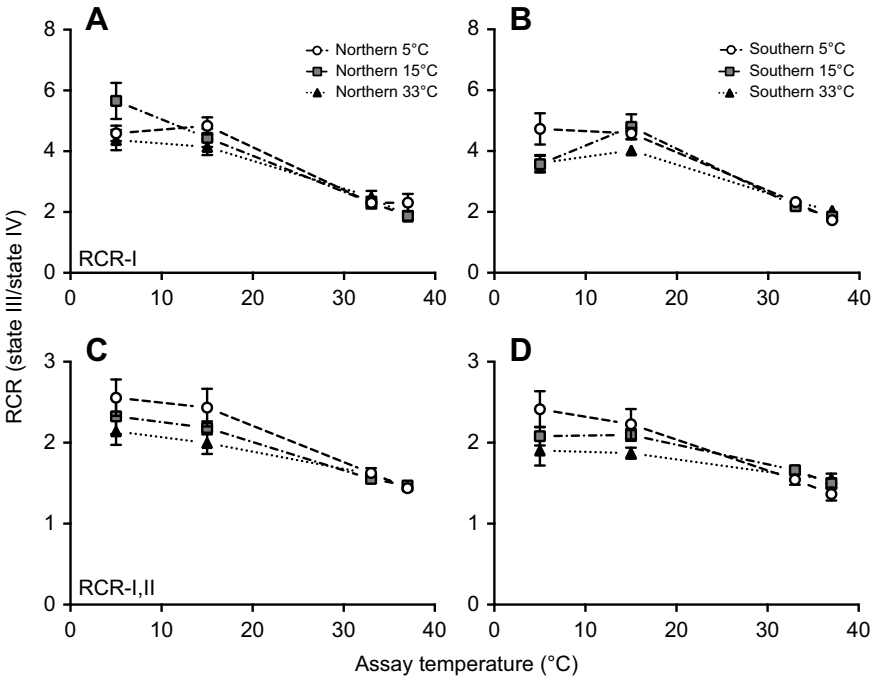

Fig. 7. Brain RCR (the ratio of state III to state IV respiration) from northern and southern *Fundulus heteroclitus* acclimated to 5 (open circle), 15 (grey square) or 33°C (black triangle) for 4 weeks. Northern (A,C) and southern (B,D) *F. heteroclitus* RCR were calculated from respiratory states with flux through ETS complex I alone (A,B; pyruvate, malate and glutamate as substrates) or complex I and II in tandem (C,D; complex I substrates and succinate). Data are means±s.e.m.; see Table 2 for associated statistics (n=7–8).

energy stores for use once low temperatures are removed (Crawshaw, 1984). Indeed, cold acclimation is associated with greater whole-animal and tissue mass (Fig. 1). But an increase in energy stores seems counterintuitive, as winter conditions are associated with a reduced abundance of prey items (Chidester, 1920). We thus may be detecting a confounding effect of our photoperiod (12 h:12 h light: dark), feeding (once daily *ad libitum*) and low-temperature treatment. By combining this pseudo-winter condition with excess food, we could be artificially sustaining liver function.

As a general objective, we wanted to determine whether mitochondrial modifications following thermal acclimation were associated with the setting of organism-level thermal tolerance limits. Fangue et al. (2006) previously demonstrated shifts in *F. heteroclitus* whole-animal upper thermal tolerance limits following thermal acclimation [i.e. critical thermal maximum (CT_{max}) increases from 31 to 41°C following acclimation to 5 and 33°C, respectively]. If mitochondrial function sets these thermal tolerance limits, we would expect mitochondrial dysfunction to occur at temperatures approaching CT_{max} (i.e. between T_{assay}=33 and 37°C). In 5°C acclimated fish (CT_{max}=~31°C), we observed few obvious signs of mitochondrial dysfunction at T_{assay}=33°C. However, we observed a steep increase in oxygen consumption between T_{assay}=33 and 37°C, indicating mitochondrial failure at temperatures exceeding whole-organism thermal tolerance limits (Figs 2, 3, 5, 6). In contrast, in 33°C acclimated fish (CT_{max}=~41°C) most respiratory parameters did not increase between T_{assay}=33 and 37°C. This violates the expectation of increases in oxygen consumption with temperature, again suggesting that mitochondrial dysfunction occurs between 33 and 37°C, which approaches the temperatures of whole-organism thermal failure. Similarly, for 15°C acclimated fish (CT_{max}=~37°C), we observed level or declining oxygen consumption between T_{assay}=33 and 37°C. Although it is difficult to make causal connections between acute temperature effects on mitochondrial performance and declines in tissue or organism function (though see Iftikar and Hickey, 2013), our results are nonetheless suggestive of a role of declining mitochondrial function in the setting of upper thermal tolerance limits in warm-acclimated fish.

Do locally adapted subspecies exhibit differences in mitochondrial respiratory function?

One important objective of this study was to determine the role that mitochondria play in differentiating northern and southern subspecies of killifish. We predicted that northern killifish would exhibit greater mitochondrial respiration compared with their southern counterparts, consistent with the greater whole-animal metabolic rate of northern killifish (Fangue et al., 2009), but that the maximal temperatures at which mitochondrial performance can be sustained would be lower, consistent with the lower whole-organism thermal tolerance of the northern subspecies (Fangue et al., 2006). We demonstrate that differences between subspecies in substrate oxidation and apparent CCO capacity are modest (Figs 3, 6) and are absent in coupled respiratory states (Figs 2, 5), and that the effects of acute high temperature exposure are similar between the subspecies. We observed subspecies differences in CS activity, although the direction of this difference depended on the tissue being assayed (Fig. 8). These results indicate that there are subspecies differences in mitochondrial content, which is intriguing given the lack of subspecies differences in mitochondrial respiration. For the heart, where the southern subspecies has greater heart CS activity, this suggests that respiration per unit of mitochondrion may be lower in southern fish. In contrast, in the brain, where the northern subspecies has greater CS activity at the 15°C acclimation temperature only, this suggests the possibility that respiration per unit mitochondrion may be lower in northern fish. Alternatively, if CS activity is not a strong indicator of mitochondrial amount, these data hint at a potential mismatch between tricarboxylic acid cycle capacity and ETS flux. When considered at the level of the tissue, however, these data indicate that tissue-specific differences in mitochondrial function likely play only modest roles in setting subspecies-specific whole-animal metabolic rate and thermal performance limits.

Intra-specific comparisons of mitochondrial function often reveal modest differences. For example, thermally acclimated (11 and 18°C) subtropical individuals of *Sepia officinalis* exhibit marginally greater OXPHOS capacity compared with their temperate counterparts (Oellermann et al., 2012). In addition, small subspecies effects on mitochondrial performance are also

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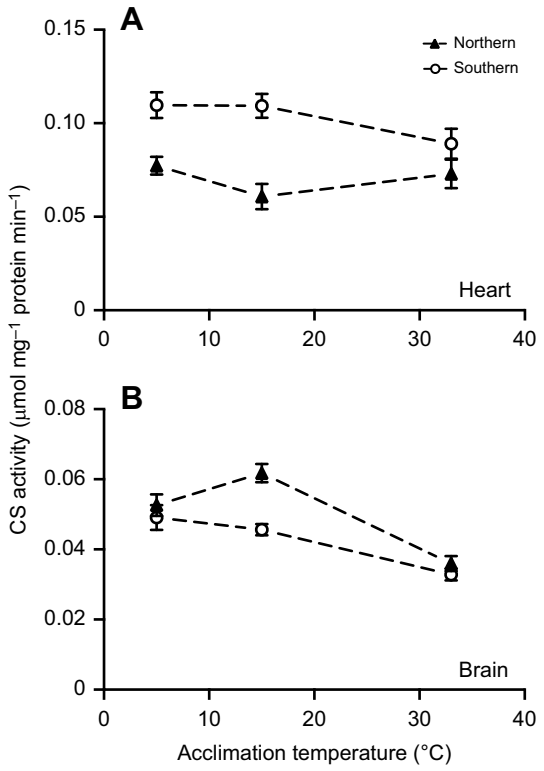

Fig. 8. Whole heart and brain citrate synthase (CS) activity. Northern (black triangle) and southern (open circle) *Fundulus heteroclitus* were acclimated to 5, 15 and 33°C for 4 weeks. Data are means±s.e.m.; see Results for associated statistics (*n*=7).

observed in *F. heteroclitus* hearts and livers (Baris et al., 2016a; Fangue et al., 2009). Our data are consistent with these observations of limited subspecies effects (Figs 2–7). Modest subspecies differences are perhaps unsurprising as mitochondrial genes are often subject to neutral and purifying selection and functional differences may only be detected under specific conditions (Melo-Ferreira et al., 2014; Silva et al., 2014), and in general this is true among species of *Fundulus* as well (Whitehead, 2009). However, mitochondrial respirometry assays often assess function under saturating substrate conditions to ensure a maximum enzyme velocity. This is a distinctly non-physiological condition and may mask nuanced intra-specific differences in performance that could manifest as differences in whole-organism metabolic rate.

Although the sizes of our subspecies effects are not large, it is important to consider their ramifications in the context of the whole organism. Indeed, northern and southern killifish whole-animal CT limits differ by approximately 1.5°C, while whole-animal acute thermal tolerance extends over a 30°C range (CT limits of −1 to 34°C in 12.5°C acclimated fish; Fangue et al., 2006). Our observation of sustained heart and brain mitochondrial function over a similarly large range of acute temperatures (5 to 37°C) and small differences between subspecies pairs well with these whole-organism data, and suggests that if mitochondrial function does set thermal tolerance limits, the size of those differences might be quite small. It is important to note, however, that making clear connections between our mitochondrial data and subspecies differences in whole-animal thermal tolerance is difficult as our subspecies effects were variable between tissues and among our different respiratory states (Figs 2–7). It is nevertheless possible that our observed variation in mitochondrial properties contributes to the mechanistic bases for these subspecies differences in whole-organism thermal tolerance and warrants further investigation.

Conclusions

In this study, we provide an assessment of thermal acclimation effects on brain and heart mitochondrial function in genetically distinct northern and southern subspecies of the Atlantic killifish. Our most striking observation was that acclimation to both 5 and 33°C caused a large decline in mitochondrial performance. These acclimation effects may occur as an energetic cost-saving mechanism at low temperatures and as a result of energetic mismatches following high-temperature acclimation. Heart and brain mitochondrial function declined following acclimation to 5 and 33°C. The size of these effects was larger in the brain, perhaps indicating some level of preserved cardiac mitochondrial function or larger pathological changes in brain mitochondria. We detected small, tissue-specific subspecies effects. These results indicate that the contribution of mitochondrial capacity to differentiating subspecies-specific whole-animal thermal tolerance limits is likely small. Although not measured here, OXPHOS efficiency (P/O ratio) and reactive oxygen species dynamics likely play important roles in thermal acclimation and local adaptation. Investigating these mechanisms will provide a more complete understanding of the role of mitochondria in these processes. Overall, we demonstrate a consistent decrease in mitochondrial performance following acclimation to thermal extremes, with the extent of modification depending on the tissue being assayed. These data highlight the importance and complexity of mitochondrial function in *F. heteroclitus* and improve our understanding of the responses of eurythermal organisms to thermal acclimation in general.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.J.C. and P.M.S. contributed to the conception, design, data interpretation and revision of this article. D.J.C and H.J.B. contributed to data collection. D.J.C. completed data analysis and drafted the article.

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Supplementary information

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