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RESEARCH ARTICLE

High temperature impairs mitochondrial function in rainbow trout cardiac mitochondria

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ABSTRACT

Mitochondria provide cellular energy through oxidative phosphorylation, and thus temperature-induced constraints on mitochondrial function may be crucial to animal aerobic scope and thermal tolerance. Here, we report the effect of temperature in the range 5–30°C on respiration rates of isolated cardiac mitochondria from rainbow trout (*Oncorhynchus mykiss*) studied by high-resolution respirometry and spectrophotometric enzyme activity assays. Arrhenius breakpoint temperature analysis indicated that mitochondrial respiration rates under phosphorylating and fully uncoupled conditions increased exponentially up to 20°C, but stopped increasing at higher temperatures. In contrast, respiration rates measured under non-phosphorylating leak conditions continued to increase up to 30°C. The decrease in the ratio between phosphorylating and uncoupled respiration at high temperature indicated that phosphorylation was gradually impaired with increasing temperature, possibly because of the steadily increasing proton leak across the membrane. In addition, we found that complex I (NADH dehydrogenase) activity decreased above 20°C, similarly to mitochondrial respiration, and that complex I was unstable in the presence of detergents, suggesting that it may be particularly sensitive to changes in its interaction with membrane phospholipids. In contrast, complex II (succinate dehydrogenase) maintained activity at temperatures above 20°C, although succinate oxidation was insufficient to compensate for the loss of complex I activity in intact mitochondria. Together, these results indicate that the temperature-induced decrease in cardiac mitochondrial function coincides with the temperature at which trout aerobic scope peaks, and is largely due to impaired phosphorylation and complex I activity.

KEY WORDS: Complex I, Fish, Heart, Mitochondria, Respiration, Temperature

INTRODUCTION

Temperature is an important abiotic factor constraining physiology and biogeography of animals (Addo-Bediako et al., 2000; Root et al., 2003; Sunday et al., 2011; Sunday et al., 2012), particularly aquatic ectotherms, whose body temperature strongly follows that of the environment. However, the physiological mechanisms that set the upper thermal limits of animals and determine their aerobic scope have remained controversial (Helmuth et al., 2005; Jutfelt et al., 2018; MacMillan, 2019; Wang et al., 2014).

Mitochondrial function is central in maintaining cellular homeostasis as mitochondrial respiration is responsible for most of the animal's oxygen consumption and provides most of the cellular energy production. While acute warming in ectotherms leads to increased mitochondrial respiration and capacity of the mitochondrial enzyme activity, temperatures above the thermal limit of the mitochondria lead to destabilization of mitochondrial enzyme complexes, decreased coupling of the electron transport chain (ETC) and the phosphorylation system, destabilization of the mitochondrial membranes, Ca^{2+} overload and oxidative stress (Chung and Schulte, 2020; Qian et al., 2004). Available data on liver mitochondria reviewed recently by Chung and Schulte (2020) suggest that decreases in mitochondrial respiration are unlikely to determine organismal upper thermal limits in fish, whereas studies on the effect of temperature on mitochondria from other tissues, such as the heart, are less conclusive (Birkedal and Gesser, 2003; Christen et al., 2018; Chung et al., 2017; Gerber et al., 2020; Iftikar and Hickey, 2013; Iftikar et al., 2014).

As the metabolic rate and thereby oxygen requirement increase exponentially with increasing temperature, it has been hypothesized that oxygen supply to the heart limits cardiac performance and thereby whole-animal acute thermal tolerance (Clark et al., 2008; Eliason et al., 2011; Pörtner and Farrell, 2008), although this model does not seem to be generally valid in fish species (Gräns et al., 2014; Norin et al., 2014; Wang et al., 2014).

The rainbow trout (*Oncorhynchus mykiss*) is a cold-water stenotherm with an upper thermal limit between 27 and 32°C, depending on acclimation temperature (Chen et al., 2015; Currie et al., 1998), and a peak in aerobic scope at around 20°C (Chen et al., 2015). Trout is an example of a species that is not limited by oxygen delivery to the heart at high temperatures (Motyka et al., 2017), at least in part due to the presence of coronary arteries supplying the heart with well-oxygenated blood (Ekström et al., 2019; Morgenroth et al., 2021). Thus, this species allows disentangling contributions from limitations in oxygen supply to the heart and oxygen consumption by the cardiac mitochondria in determining the thermal tolerance of this species. Furthermore, mitochondrial complex I (NADH dehydrogenase) from trout heart is highly unstable in the presence of detergents when analysed by Blue Native PAGE (BN-PAGE), in contrast to other vertebrates' enzymes (Bundgaard et al., 2020), suggesting that in the trout heart this enzyme may be particularly sensitive to changes in membrane properties with increasing temperature.

Here, we investigated the effect of temperature on mitochondrial respiration rates in isolated trout heart mitochondria using breakpoint analysis of Arrhenius plots to determine temperature limits (Nickerson et al., 1989). Furthermore, we investigated the effect of temperature on the activity of mitochondrial complex I and II, where electrons enter the ETC, to address their contribution to the observed temperature effects. We hypothesized that thermally impaired mitochondrial function may contribute to determine the aerobic scope of this species, and specifically that mitochondrial respiration rates would decrease at a temperature below the upper thermal limit of the rainbow trout, and that complex I activity may be particularly affected.

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MATERIALS AND METHODS

Animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (N=15, mass 435.27±13.9 g, mean±s.e.m.) were purchased from a commercial supplier and kept at 16°C in aquaria with a recirculation system on a 12 h:12 h light:dark cycle and fed to satiation with trout pellets every other day.

Preparation of isolated mitochondria

Animals were killed with a blow to the head and the ventricle was dissected out and immediately placed in ice-cold STE buffer [250 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris, 0.5 mmol l^{-1} EGTA; pH 7.4 with 0.2% (w/v) fatty acid-free bovine serum albumin (BSA)]. Heart mitochondria were prepared on ice as previously described (Bundgaard et al., 2018). Briefly, the heart was rinsed with STE to remove blood and finely homogenized with a razor blade and a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700 ***g*** at 4°C for 5 min; the supernatant was filtered through a cheesecloth to remove cellular debris and centrifuged twice at 10,000 ***g*** at 4°C for 10 min. The pellet was gently rinsed to remove damaged mitochondria before final resuspension in STE without BSA. Protein content was determined with a bicinchoninic acid (BCA) assay using BSA as a standard. A portion of mitochondria were kept at −80°C for later determination of citrate synthase, complex I and II activities and analysis with BN-PAGE.

High-resolution respirometry

The remaining portion of freshly isolated mitochondria was immediately used to determine mitochondrial respiration rate at temperatures ranging from 5 to 30°C using two Oroboros O2K-oxygraphs (Oroboros Instruments, Innsbruck, Austria) run in parallel. Respiration rate at each temperature was measured in duplicate and systematically randomized to prevent bias of the order of measurements, with maximum 60 min between measurements at different temperatures. Mitochondria were stored on ice in the meantime. This delay in measuring did not affect the measured respiration rate (data not shown).

The instrument was calibrated daily at each temperature with air-saturated respiration buffer [220 mmol l^{-1} mannitol, 70 mmol l^{-1} sucrose, 10 mmol l^{-1} Hepes, 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} KPO_4^-; pH 7.4 with 0.3% (w/v) fatty acid-free BSA]. Representative traces illustrating the respiration protocol used is shown in Fig. 1. Isolated mitochondria (0.025–0.065 mg ml^{-1}) were added to each chamber and 2.5 mmol l^{-1} malate and 5 mmol l^{-1} pyruvate were added to initiate state II complex I-dependent non-phosphorylating respiration (LEAK MP), before 1 mmol l^{-1} ADP was added to stimulate state III complex I-dependent phosphorylating respiration (P MP). Addition of the complex II substrate succinate (10 mmol l^{-1}) then initiated state III complex I+II-dependent phosphorylating respiration (P MPS). Addition of 10 µmol l^{-1} cytochrome *c* was added to test the integrity of the outer mitochondrial membrane, which resulted in an increase in respiration rate >10% in ∼5% of the experiments, randomly distributed between samples and test temperatures. These experiments were excluded from the study. The complex V inhibitor oligomycin (Omy, 2.5 µmol l^{-1}) was then added, resulting in non-phosphorylating state IV respiration. To determine maximal uncoupled respiration rate of the ETC, 5–15 µl carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was titrated until the respiration rate reached a plateau. Finally, 2.5 µmol l^{-1} antimycin A (AMA) was added to inhibit complex III to correct for non-mitochondrial oxygen consumption rate.

Enzyme activity assays

V_{max} determinations were measured on a spectrophotometer (Cary 60 UV-Vis; Agilent Technologies, Santa Clara, CA, USA) (complex I and II) or microplate reader (SpectraMax iD3; Molecular Devices, San Jose, CA, USA) (citrate synthase) on the first thaw of frozen mitochondria.

Complex I activity

Complex I activity was measured as the rotenone-sensitive NADH: ubiquinone dehydrogenase activity based on Trounce et al. (1996). Isolated mitochondria (50 µg protein) were added to a cuvette with 120 mmol l^{-1} KCl, 10 mmol l^{-1} EDTA, 10 mmol l^{-1} Hepes and

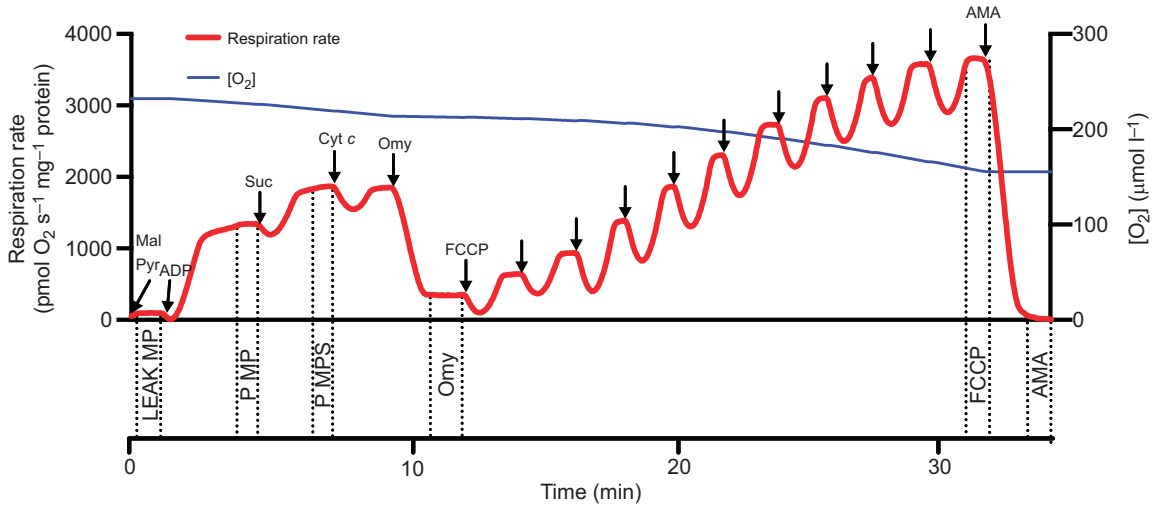

Fig. 1. Representative trace of the respiration protocol for isolated trout heart mitochondria. Malate (Mal, 2.5 mmol l^{-1}) and pyruvate (Pyr, 5 mmol l^{-1}) were added to stimulate complex I (CI)-dependent leak respiration (LEAK MP), followed by addition of adenosine diphosphate (ADP, 1 mmol l^{-1}) to stimulate CI-dependent phosphorylating respiration (P MP) and then addition of succinate (Suc, 10 mmol l^{-1}) stimulated CI+CII-dependent phosphorylating respiration (P MPS). Cytochrome *c* (Cyt *c*, 10 µmol l^{-1}) was added as a quality control, before oligomycin (Omy, 2.5 µmol l^{-1}) was added to inhibit complex V and initiate leak respiration followed by titration with the uncoupler FCCP to induce maximal uncoupled respiration. Finally, non-mitochondrial oxygen consumption was measured in the presence of the complex III inhibitor antimycin A (AMA, 2.5 µmol l^{-1}).

0.05% dodecylmaltoside (DDM) (w/v), pH 7.4 with 2 mmol l^{-1} KCN, 300 nmol l^{-1} AMA and 100 µmol l^{-1} NADH at temperatures in the range 5–30°C and incubated for ∼1 min to ensure activation of complex I. To start the assay, 0.1 mmol l^{-1} decylubiquinone (an electron acceptor) was added and the oxidation of NADH was followed at 340–380 nm using extinction coefficient ε_{NADH} of 6.22 mmol l^{-1} cm^{-1} (Estornell et al., 1993).

Complex II activity

Complex II activity was determined as described (Spinazzi et al., 2012) as the reduction of 2,6-dichlorophenolindophenol (DCPIP) over time in a cuvette containing 25 mmol l^{-1} KPO_4^-, pH 7.5 with 20 mmol l^{-1} succinate, 80 µmol l^{-1} DCPIP and 300 µmol l^{-1} KCN at temperatures in the range 5–30°C. Isolated mitochondria (∼50 µg protein) were incubated in the cuvette for 5 min to ensure the full activation of complex II, before 50 µmol l^{-1} decylubiquinone was added to initiate the assay. The reduction of DCPIP was followed at 600 nm using extinction coefficient ε_{DCPIP} 20.7 mmol l^{-1} cm^{-1}.

Citrate synthase activity

Complex I and complex II activities were both normalized to citrate synthase (CS) activity, a proxy for mitochondrial density (Larsen et al., 2014). CS activity was measured at 28°C in 100 mmol l^{-1} Tris, pH 8 with 0.1% (w/v) Triton X-100 with 5 µmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 36 µmol l^{-1} acetyl-CoA. The assay was started by addition of 38 µmol l^{-1} oxaloacetate, and activity was measured as the formation of 1,3,5-trinitrobenzene (TNB) at 412 nm using extinction coefficient ε_{TNB}=13.6×10^3 mol l^{-1} cm^{-1} (Srere, 1969).

BN-PAGE

Mitochondrial complexes of trout (*N*=3) and mouse (*N*=3; *Mus musculus*, Linneaus 1758) heart mitochondria were separated on BN-PAGE as previously described (Bundgaard et al., 2018). Mouse heart mitochondrial complexes were used to standardize the size of trout complexes and to compare with well-described and stable mitochondrial complexes (Letts et al., 2016; Schägger and Pfeiffer, 2001). Mice were euthanized by cervical dislocation and mouse heart mitochondria were isolated as described for trout in the 'Preparation of isolated mitochondria' section. Briefly, mitochondrial complexes from both species were extracted on ice with 3 g g^{-1} protein of the harshest detergent dodecylmaltoside (DDM) for 15 min, with 1.5 g g^{-1} protein of the gentler detergent lauryl maltose neopentyl glycol (LMNG) for 1 h, and with 3 g g^{-1} protein of the most gentle detergent digitonin for 5 min. The extracted complexes were diluted 1:10 with 5% Coomassie Brilliant Blue G-250 and 100 µg protein was loaded per lane. In-gel complex I activity was determined by incubation for 15 min at room temperature with 150 µmol l^{-1} NADH and 1 mg ml^{-1} nitroblue tetrazolium, which precipitates as a purple stain at bands with NADH dehydrogenase activity. The sizes of the complexes were determined from an exponential fit (r^2=0.99) of the migration distance of the mouse complexes extracted with DDM using the molecular masses: CI (complex I, NADH dehydrogenase), 880 kDa; CV (complex V, F_1F_0-ATP synthase), 600 kDa; $CIII_2$ (complex III, ubiquinone-cytochrome *c* oxidoreductase), 460 kDa; CIV (complex IV, cytochrome *c* oxidase), 200 kDa; CII (complex II, succinate dehydrogenase), 140 kDa (Letts et al., 2016).

Calculations and statistics

The activation energy of mitochondrial respiration and enzymatic reactions can be calculated from the Arrhenius equation $\ln(r) = -(E_a/R)\times(1/T)$, where r is the rate of the process investigated, E_a is the activation energy (kJ mol^{-1}), R is the gas constant (kJ^{-1} mol^{-1}) and T is the absolute temperature (K). The E_a for a given process was derived from the slope of the Arrhenius plot, with rates expressed as µmol O_2 min^{-1} mg^{-1}, µmol NADH min^{-1} CS^{-1} or µmol succinate min^{-1} CS^{-1}. A linear Arrhenius plot within a given temperature range indicates a constant activation energy of the system, while a change in the slope of the linear plot at a given temperature (breakpoint) indicates a change in functional and by inference structural properties of the system (Nickerson et al., 1989).

The temperature coefficient (Q_{10}) of the mitochondrial respiration rates under various conditions (LEAK MP, P MP, P MPS, Omy and FCCP) and enzyme activities of complex I and II were calculated within the linear part of the Arrhenius plot as the ratio between rates for a 10°C increase in temperature.

Statistical differences (P<0.05) in the effect of test temperature on complex I and II activity and the ratio between state III respiration rate and maximal uncoupled respiration (P MPS:FCCP) was determined using a one-way ANOVA with Tukey's multiple comparisons test. Temperature effects on respiration rate were determined with a two-way ANOVA with Tukey's multiple comparisons test. Data were analysed using GraphPad Prism 9 (version 9.0.0), and are presented as means±s.e.m.

RESULTS

The mitochondrial respiration rate in isolated trout heart mitochondria increased with increasing temperature for all measured respiration states up to 20°C (Fig. 2A). At temperatures above 20°C, respiration rates of the ETC under phosphorylating (P MP and P MPS) and non-phosphorylating, uncoupled (FCCP) conditions no longer increased (Fig. 2A), as reflected by the breakpoint in the slopes of Arrhenius plots at around 20°C (Fig. 2B) for P MP, P MPS and FCCP. Addition of succinate (P MPS) did not prevent the decrease in respiration rate above 20°C. These findings indicate that the ETC and/or phosphorylation system of trout heart mitochondria have a critical upper thermal limit between 20 and 25°C.

In contrast, LEAK respiration (LEAK MP and Omy) continued to increase with increasing temperatures (Fig. 2A), indicated by linear Arrhenius plots within the whole range of temperatures investigated (Fig. 2B). This finding indicates that in trout heart mitochondria, the proton leakiness of the inner mitochondrial membrane increases with temperature, as expected due to increase in fluidity for example, and that the integrity of the inner mitochondrial membrane is not affected at temperatures up to at least 30°C, as no breakpoints are detected.

The linearity of the Arrhenius plots reveals that the increase in respiration rate with temperature followed the same temperature coefficient (Q_{10}) up to 20°C for P MP (1.76±0.16), P MPS (1.75±0.15) and FCCP (2.38±0.09) and up to 30°C for LEAK MP (1.82±0.21) and Omy (2.21±0.13) (means±s.e.m.; Fig. 2C; Table 1). The calculated activation energies (E_a) for the investigated processes are listed in Table 1, and were in the range 35.53–58.63 kJ mol^{-1}. These values are very close to those reported for other species of fish (Blier and Lemieux, 2001; Gerber et al., 2020; Hardewig et al., 1999; Hilton et al., 2010; Iftikar and Hickey, 2013; Iftikar et al., 2014).

The ratio between maximal phosphorylating respiration (P MPS) and maximal uncoupled respiration (FCCP) at each temperature indicates whether electron transport or the phosphorylation system are limiting for oxygen consumption (Gnaiger, 2009). A ratio close to 1 indicates that electron transport is limiting, as there is limited scope for increasing oxygen consumption with uncoupling, whereas a ratio close to 0 indicates that the phosphorylation system is limiting, as increase in uncoupling increases oxygen

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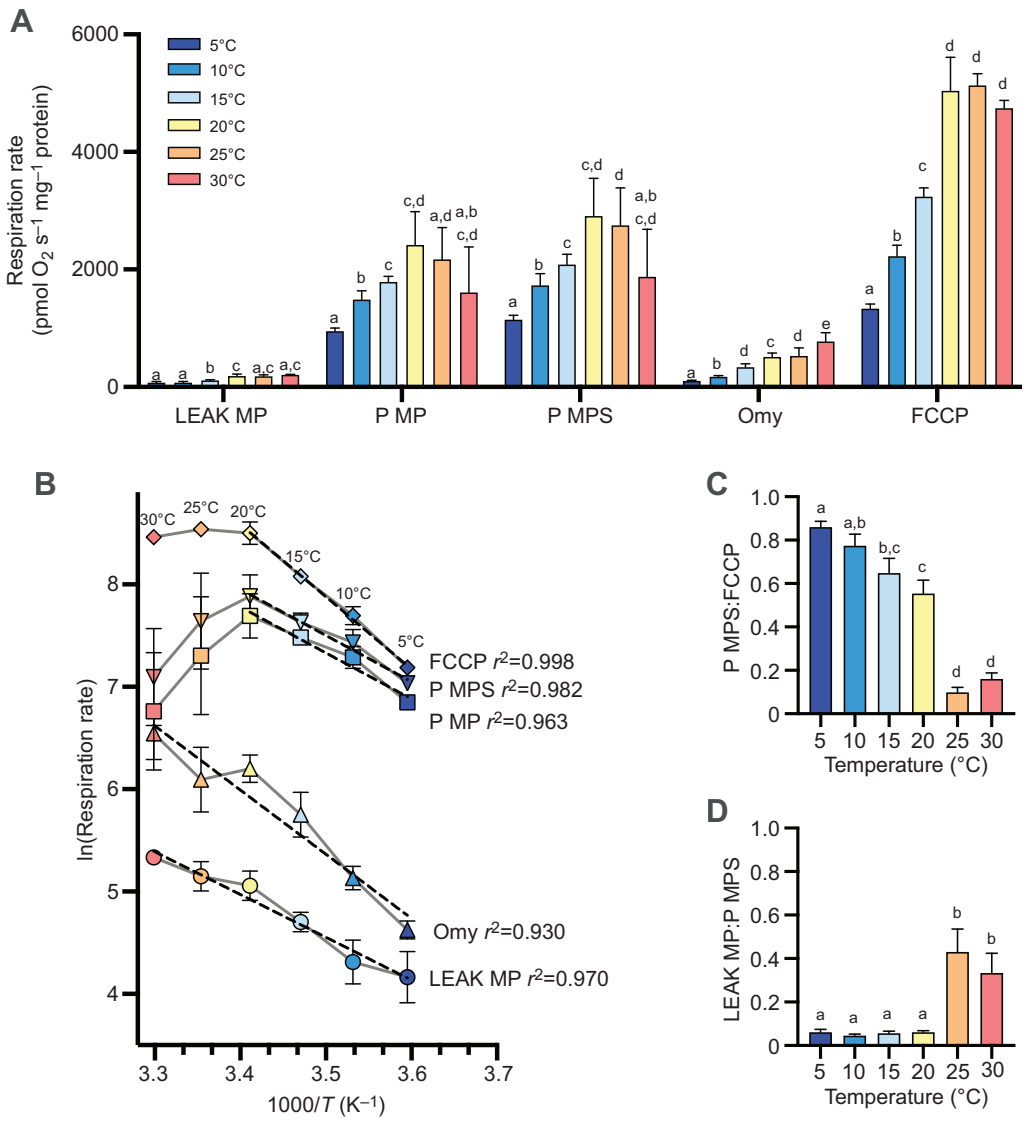

Fig. 2. Mitochondrial respiration rate and temperature effects in isolated trout heart mitochondria. (A) Respiration rate of isolated trout heart mitochondria at 5, 10, 15, 20, 25 and 30°C under leak conditions (LEAK MP and Omy), phosphorylating conditions (P MP and P MPS) and maximal uncoupled respiration (FCCP). (B) Arrhenius plots for the respiration rate as a function of temperature for each respiratory condition, with linear regressions of the linear parts shown as dashed lines with respective r^2 values. (C) Ratio between phosphorylating and maximal uncoupled respiration (P MPS:FCCP) at each test temperature. (D) Ratio between leak respiration and maximal phosphorylating respiration (LEAK MP:P MPS) at each test temperature. Two-way ANOVA showed a significant effect of temperature on respiration rate (P=0.029) and dissimilar lowercase letters denote significant differences between test temperatures for A, C and D (Tukey's multiple comparisons test, α<0.05). Values are expressed as means±s.e.m.; N=4–5. In some cases, the error bar is not visible because it is too small.

consumption (Gnaiger, 2009). As the P MPS:FCCP ratio decreased significantly with increasing temperature, particularly at 25 and 30°C (Fig. 2D), our data show that the phosphorylation system becomes the main limiting factor to oxygen consumption of trout heart mitochondria above 20°C.

The ratio between leak respiration (LEAK MP) and maximal phosphorylating respiration (P MPS) reflects the degree of coupling of the mitochondria. The significant increase in LEAK MP:P MPS from ∼0.05 at 5–20°C to ∼0.4 at 25 and 30°C (Fig. 2D) suggests that increased proton permeability inhibits phosphorylating respiration above 20°C.

To investigate the effect of temperature on the ETC further, we tested the effect of temperature specifically on complex I and II enzyme activity (Fig. 3). We found that complex I activity decreased

Table 1. Temperature coefficient and activation energy for leak, phosphorylating with complex I and complex I+II substrates, leak and maximal respiration rates and complex I and II activity

	Q_{10}	E_a (kJ mol^{-1})	Temperature range (°C)
Respiration state		(kJ mol O_2^{-1})	
LEAK MP	1.82±0.21[a]	35.53±5.10[a]	5–30
P MP	1.76±0.16[a]	36.87±6.14[a]	5–20
P MPS	1.75±0.15[a]	37.09±5.67[a]	5–20
Omy	2.21±0.13[a]	54.94±4.52[a,b]	5–30
FCCP	2.38±0.09[a]	58.63±2.51[b]	5–20
Enzyme activity		[kJ mol (NADH or DCPIP)$^{-1}$]	
Complex I	2.04±0.49	37.74±14.54	5–20
Complex II	1.42±0.06	24.58±3.19*	10–30

Temperature coefficient (Q_{10}) and activation energy (E_a) were determined within the linear range determined from the Arrhenius plots (means±s.e.m. are shown). Different lower case letters denote significant differences within respiration states determined by Tukey's multiple comparisons test (α<0.05). *Significant differences between Q_{10} and E_a for complex I and complex II activity, determined by unpaired t-tests (P<0.05).

Journal of Experimental Biology (2021) 224, jeb242382. doi:10.1242/jeb.242382

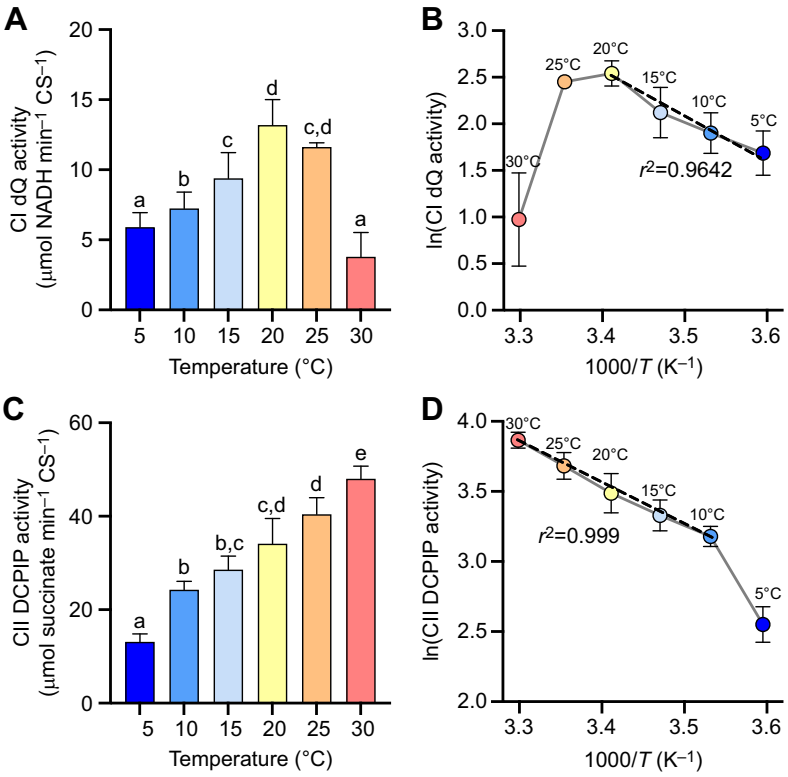

Fig. 3. Effect of temperature on the activity of complex I and complex II. (A) Complex I (CI) activity in isolated trout heart mitochondria measured as the oxidation of NADH at 340–380 nm using decylubiquinone (dQ) as electron acceptor relative to citrate synthase (CS) activity at 5, 10, 15, 20, 25 and 30°C. (B) Corresponding Arrhenius plot with linear regression in the linear range shown as a dashed line. (C) Complex II (CII) activity in isolated trout heart mitochondria in the same temperature range as for CI, measured as the reduction of DCPIP at 600 nm with succinate as electron donor. (D) Corresponding Arrhenius plot with linear regression in the linear range shown as a dashed line. Values are shown as means±s.e.m.; N=4–5. One-way ANOVA showed a significant effect of temperature (P<0.05) and dissimilar lowercase letters denote significant differences (Tukey's multiple comparisons test, α<0.05).

at temperatures above 20°C (Fig. 3A,B), as illustrated by the breakpoint in the Arrhenius plot (Fig. 3B), suggesting that electron transfer through this complex contributes to limit oxygen consumption above 20°C. Complex II activity continued to increase exponentially with temperature from 10 to 30°C (Fig. 3C) with a constant Q_{10}, as illustrated by the linearity of the Arrhenius plot (Fig. 3D). However, there was a breakpoint in the Arrhenius plot at around 10°C, indicating that complex II activity is negatively impacted at lower temperatures (Fig. 3D).

As complex I was found to be sensitive to high temperatures (Fig. 3A,B), and trout complex I was previously shown to be sensitive to detergents (Bundgaard et al., 2020), we investigated whether the stability of complex I enzyme might be affected by the interaction with the membrane lipids by extracting mitochondrial membrane complexes using three detergents with different strengths (representative gel shown in Fig. 4). Qualitative BN-PAGE experiments with in-gel measurements of complex I activity revealed that trout complex I was stable with the gentle detergent digitonin (Fig. 4A), but fell apart with the harsher detergents LMNG (Fig. 4B) and DDM (Fig. 4C). In contrast, mouse complex I was stable in the presence of all detergents used (Fig. 4D–F). These results indicate that the decreased activity of complex I at temperatures above 20°C (Fig. 3A,B) may derive from an altered interaction with the lipid membrane. Both protein staining intensities and complex I activity staining were visibly more prominent in mouse than in trout heart mitochondria (Fig. 4), showing a higher density of complexes in heart mitochondria from mouse compared with trout, in accordance with previous comparisons between endo- and ectothermic mitochondria (Else and Hulbert, 1985; Hulbert and Else, 1989).

DISCUSSION

Decreases in mitochondrial function at high temperatures have been suggested to be a crucial factor in determining the physiological basis for upper thermal limits in animals (Blier et al., 2014; Iftikar and Hickey, 2013; Wang et al., 2014), but possibly not in fish (Chung and Schulte, 2020). To provide evidence based on mitochondria isolated from heart, which may play a more crucial role in temperature tolerance in fish than liver (Chung and Schulte, 2020), we performed a rigorous analysis of how temperature affects respiration rate in isolated rainbow trout heart mitochondria. A major result of this investigation is that the mitochondrial respiration rate increases exponentially up to 20°C, but decreases at temperatures above 20°C (Fig. 2). As this coincides with the peak in aerobic scope at 20°C and is well below the upper thermal limit for this species of 27–32°C (Chen et al., 2015; Currie et al., 1998), this finding suggests that mitochondrial function may be one of the first factors limiting rainbow trout heart function when temperature suddenly increases.

The larger decrease in phosphorylating respiration relative to the decrease in uncoupled respiration rate above 20°C (Fig. 2A,B) is reflected in the decrease in the P MPS:FCCP ratio (Fig. 2C). As phosphorylating respiration depends on both the ETC and the phosphorylation system, while uncoupled respiration depends only on the ETC, the decrease in the P MPS:FCCP ratio (Fig. 2C) indicates that the phosphorylation system is particularly impaired at temperatures above 20°C. The significant increase in the LEAK MP:P MPS ratio above 20°C (Fig. 2D) suggests a higher degree of mitochondrial uncoupling (i.e. proton leak) at high temperatures, which would contribute to limit proton supply to the phosphorylation system. However, the absence of a breakpoint in leak respiration (Fig. 2B) suggests that the integrity of the inner mitochondrial membrane in the rainbow trout heart is not compromised at high temperatures, and that the impairment of the phosphorylation system is probably due to other factors as well. The mismatch between oxygen consumption rate of the ETC and the phosphorylation system above 20°C would lead to a decrease in mitochondrial ATP production, and thereby impair the ability to support the higher energy requirements at higher temperatures. In

Journal of Experimental Biology (2021) 224, jeb242382. doi:10.1242/jeb.242382

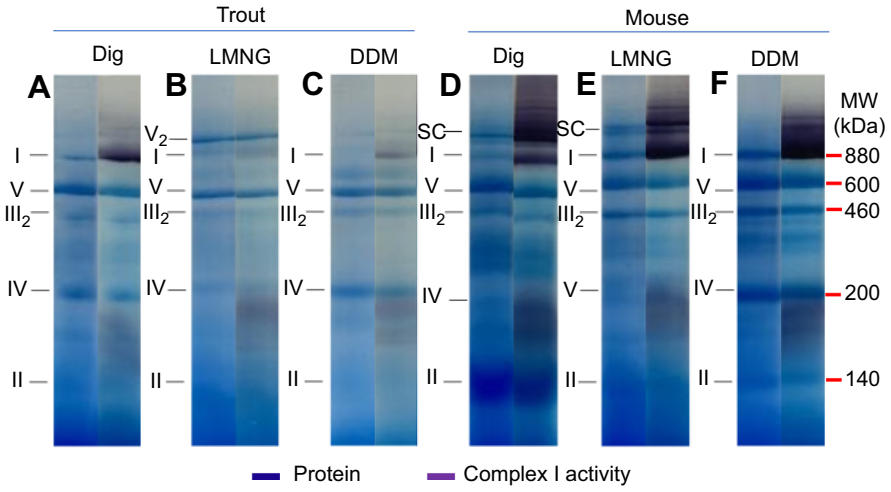

Fig. 4. Mitochondrial membrane complexes from trout and mouse heart mitochondria separated by BN-PAGE. (A–C) Trout mitochondria; (D–F) mouse mitochondria. The same gel was stained to visualize native protein complexes (blue) and to detect in-gel complex I NADH-reductase activity (purple). Complexes were extracted using the detergents digitonin (Dig; A and D), lauryl maltose neopentyl glycol (LMNG, B and E) and dodecylmaltoside (DDM, C and F). The size of of protein complexes (shown on the right, in kDa) was estimated from the size of mouse complexes separated by DDM (Letts et al., 2016). The gel is representative of results from three individuals from each species. SC, supercomplex; V_2, complex V dimer; I, complex I; V, complex V; III_2, complex III dimer; IV, complex IV; II, complex II.

support of our finding, a similar decrease in the function of the phosphorylation system at high temperatures with heat stress has also been suggested previously in rainbow trout heart fibres (Birkedal and Gesser, 2003), in heart mitochondria from another salmonid, the arctic char (*Salvelinus alpinus*) (Christen et al., 2018) and in heart mitochondria from the New Zealand wrasse (*Notolabrus celidotus*) (Iftikar and Hickey, 2013).

While a decrease in the activity of the phosphorylation system explains the observed decrease in mitochondrial phosphorylating oxygen consumption at temperatures above 20°C (Fig. 2A,B; P MP, P MPS), we also observed a breakpoint in the uncoupled respiration, which is independent of the phosphorylation system (Fig. 2A,B; FCCP). This indicates that the activity of the ETC was also negatively affected above 20°C. Specifically, we found that complex I activity decreases above 20°C (Fig. 3A), indicated by the breakpoint in the Arrhenius plot (Fig. 3B), closely reflecting the pattern seen with intact mitochondria (Fig. 2A,B).

In contrast to complex I, complex II activity continued to increase exponentially with increasing temperature above 20°C (Fig. 3C,D), indicating that it does not play a major role in impairing coupled mitochondrial respiration at high temperatures. Interestingly, the presence of a breakpoint in the Arrhenius plot for complex II activity at around 10°C (Fig. 3D) suggests that complex II activity is instead compromised at low temperatures. The different effect of temperature on complex I and II suggests that complex I and II may complement each other at temperatures outside the range 10–20°C and that complex II may at least in part compensate for the decrease in complex I activity above 20°C. However, addition of the complex II substrate succinate did not prevent the decrease in respiration rate of the ETC above 20°C. This suggests that as long as NADH is available, electron transfer through complex II in the ETC is insufficient to compensate for the limitations on the phosphorylation system at high temperatures (Fig. 2).

Rainbow trout complex I was structurally highly unstable as judged by the presence of a weak complex I band compared with mouse complex I on BN-PAGE, even when extracted with the gentle detergent digitonin (Fig. 4). This is consistent with our previous findings (Bundgaard et al., 2020) and suggests that trout complex I stability is highly dependent on the interaction with the membrane phospholipids. Phospholipid composition has been shown to change with warm acclimation in the oxidative muscle mitochondria of rainbow trout (Kraffe et al., 2007) and in mitochondria from other species (Chung et al., 2018; Ekström et al., 2017; Gerber et al., 2020; Grim et al., 2010). Changes in phospholipid composition, for example in the structurally important phospholipid cardiolipin (Baker et al., 2016; Hoch, 1992), with acclimation could perhaps prevent structural changes to complex I with increasing temperature, and thereby preserve enzyme function.

Our results contribute to previous work on the influence of mitochondrial function on thermal tolerance, and suggest that proper function of heart mitochondria is more critical in determining the thermal tolerance of fish compared with liver mitochondria, which most of the previous work is based on (Chung and Schulte, 2020), consistent with the general view that the heart may be one of the first organs to be limited by heat stress (Clark et al., 2008; Eliason et al., 2011; Pörtner and Farrell, 2008). Alternatively, the rainbow trout might be an exception to the general rule, as oxygen delivery to the heart is not a limiting factor with heat stress in this species (Motyka et al., 2017), due at least in part to the coronary circulation (Ekström et al., 2019; Morgenroth et al., 2021). Finally, our study, along with others (Birkedal and Gesser, 2003; Christen et al., 2018; Iftikar et al., 2014) indicates that some specific properties of mitochondrial function, in particular the phosphorylation system, appear more sensitive to heat stress than oxygen consumption by the ETC *per se*.

In conclusion, this study shows that the acute tolerance of the rainbow trout heart to high temperatures may be limited by a decrease in mitochondrial function, particularly due to impaired phosphorylation caused by increased proton leak and decreased complex I activity.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.F., A.B.; Methodology: A.B.; Validation: A.F., A.B.; Formal analysis: J.M.; Investigation: J.M.; Resources: A.F.; Data curation: A.F., A.B.; Writing - original draft: A.B.; Writing - review & editing: J.M., A.F., A.B.; Visualization: J.M., A.B.; Supervision: A.F., A.B.; Project administration: A.F., A.B.; Funding acquisition: A.B.

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