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RESEARCH ARTICLE

Thermal sensitivity and phenotypic plasticity of cardiac mitochondrial metabolism in European perch, *Perca fluviatilis*

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ABSTRACT

Cellular and mitochondrial metabolic capacity of the heart has been suggested to limit performance of fish at warm temperatures. We investigated this hypothesis by studying the effects of acute temperature increases (16, 23, 30, 32.5 and 36°C) on the thermal sensitivity of 10 key enzymes governing cardiac oxidative and glycolytic metabolism in two populations of European perch (*Perca fluviatilis*) field-acclimated to 15.5 and 22.5°C, as well as the effects of acclimation on cardiac lipid composition. In both populations of perch, the activity of glycolytic (pyruvate kinase and lactate dehydrogenase) and tricarboxylic acid cycle (pyruvate dehydrogenase and citrate synthase) enzymes increased with acute warming. However, at temperatures exceeding 30°C, a drastic thermally induced decline in citrate synthase activity was observed in the cold- and warm-acclimated populations, respectively, indicating a bottleneck for producing the reducing equivalents required for oxidative phosphorylation. Yet, the increase in aspartate aminotransferase and malate dehydrogenase activities occurring in both populations at temperatures exceeding 30°C suggests that the malate–aspartate shuttle may help to maintain cardiac oxidative capacities at high temperatures. Warm acclimation resulted in a reorganization of the lipid profile, a general depression of enzymatic activity and an increased fatty acid metabolism and oxidative capacity. Although these compensatory mechanisms may help to maintain cardiac energy production at high temperatures, the activity of the electron transport system enzymes, such as complexes I and IV, declined at 36°C in both populations, indicating a thermal limit of oxidative phosphorylation capacity in the heart of European perch.

KEY WORDS: Citrate synthase, Fatty acid oxidation, Lipid composition, Temperature acclimation, Tricarboxylic acid cycle

INTRODUCTION

Temperature is a key factor influencing the biology of all ectothermic animals including fish. With predicted increases in average global temperatures, as well as an increased frequency of transient extreme heat waves (Diffenbaugh and Field, 2013), fish populations will likely be more exposed to temperatures outside of their thermal tolerance range in the future. Temperature tolerance has been suggested to be an important determinant of species geographical distribution (Sunday et al., 2012), yet the physiological mechanisms determining the upper critical thermal maximum (CT_{max}) are still not well understood (Beitinger and Lutterschmidt, 2011; Clark et al., 2013).

Acute warming in fish typically elicits an exponential increase in metabolic rate (i.e. oxygen consumption rate) with a temperature coefficient (i.e. Q_{10}) of ~2–3 (Clark et al., 2008; Ekström et al., 2016a; Gollock et al., 2006; Sandblom et al., 2016). The increased tissue oxygen demand is met by an increased cardiac output, mainly via an increased heart rate. However, at temperatures close to the upper thermal maximum, cardiac function (i.e. heart rate and cardiac output) often declines, suggesting that cardiac failure may be related to thermal tolerance limits (Clark et al., 2008; Ekström et al., 2016a, 2014; Farrell, 2009; Gollock et al., 2006; Mendonca and Gamperl, 2010).

Recent evidence suggests that the mitochondrion, which provides the majority of the ATP required for myocardial contractility and cellular ion homeostasis, plays a central role in the impairment of cardiac function at high temperature (Blier et al., 2014; Hilton et al., 2010; Iftikar and Hickey, 2013; Iftikar et al., 2014). Indeed, mitochondrial efficiency and respiration rate have been shown to be impaired at high temperatures, possibly owing to the failure of several metabolic enzymes and mitochondrial complexes that govern substrate oxidation, electron transport and oxidative phosphorylation (Hilton et al., 2010; Iftikar and Hickey, 2013; Iftikar et al., 2014; Lemieux et al., 2010). For example, Iftikar and colleagues (Iftikar and Hickey, 2013; Iftikar et al., 2014) recently demonstrated that cardiac failure occurred at a temperature similar to that at which the activity of mitochondrial complex II (CII), cytochrome *c* oxidase [i.e. complex IV (CIV)] and the electron transport system (ETS; comprising the activity of complexes I and III, from now on abbreviated CI+CIII) started to decrease in three New Zealand wrasse species (*Notolabrus celidotus*, *Notolabrus fucicola* and *Thalassoma lunare*). Similarly, failure of mitochondrial CI and CIII during acute warming in ventricular tissues of Atlantic wolffish (*Anarhichas lupus*) resulted in declining mitochondrial efficiency and respiration rates (Lemieux et al., 2010).

Thus, while mitochondrial function and ATP production may be limited by an acute temperature increase, the synergistic interactions between the metabolic pathways driving these processes make it difficult to pinpoint the limiting step(s). It has been suggested that the loss of synchronicity at extreme temperatures, among processes involved in the transformation of the reducing equivalents [i.e. nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$)], may constitute a crucial limiting factor for cellular ATP production, as these processes expressed different thermal sensitivities (Blier et al., 2014; Lemieux et al., 2010; Pichaud et al., 2011). However, the links between these processes, their thermal sensitivities and cardiac function remain underexplored in fish.

Thermal acclimation (i.e. phenotypic plasticity) is likely to mitigate some of the negative effects of temperature on myocardial

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List of symbols and abbreviations

AAT	aspartate aminotransferase
ATP	adenosine triphosphate
CS	citrate synthase
CT_{max}	critical thermal maximum
CI	mitochondrial complex I
CI+CIII	joint activity of mitochondrial complexes I and III
CII	mitochondrial complex II
CIII	mitochondrial complex III
CIV	mitochondrial complex IV
ETF	electron transport flavoprotein
ETS	electron transport system
$FADH_2$	flavin adenine dinucleotide
FAME	fatty acid methyl ester
HOAD	hydroxy acyl CoA dehydrogenase
INT	p-iodonitrotetrazolium violet
LDH	lactate dehydrogenase
MDH	malate dehydrogenase
MUFA	monounsaturated fatty acid
NADH	nicotinamide adenine dinucleotide
PDH	pyruvate dehydrogenase
PK	pyruvate kinase
PUFA	polyunsaturated fatty acid
Q_{10}	temperature coefficient
SFA	saturated fatty acid
UQ_1	ubiquinone 1

ATP production. For example, alterations in membrane fatty acid composition are known to play an important role in the adjustments of enzymatic function and mitochondrial transmembrane proton leak, which may improve mitochondrial ATP production at high temperatures (Brookes et al., 1998; Hulbert and Else, 1999; Kraffe et al., 2007). In addition, the transcription of enzymes governing cardiac glycolytic, tricarboxylic acid and oxidative metabolic pathways increased following warm acclimation from 13 to 19°C, but declined at higher acclimation temperatures in the eurythermal goby species *Gillichthys mirabilis*. Furthermore, warm acclimation resulted in downregulated expression of hydroxyacyl-coenzyme A dehydrogenase (HOAD), indicating a reduced fatty acid oxidative capacity, whilst lactate dehydrogenase (LDH) transcription was elevated, indicating a potential increased importance of anaerobic metabolism at high temperatures in these fish (Jayasundara et al., 2015).

Although these findings indicate that the enzymatic ATP generating machinery of the fish heart can exhibit considerable thermal plasticity with increasing temperatures, to our knowledge, there is limited information on the effects of long-term thermal field acclimation on the acute temperature sensitivity and resilience of key enzymatic components in fish. This information is crucial for understanding the potential impacts of global warming on cellular mechanisms, and hence whole-animal metabolic and physiological processes in fish populations exposed to a warming climate.

In the present study, we examined the thermal sensitivity and plasticity of the enzymatic machinery in cardiac tissues from two populations of European perch (*Perca fluviatilis* Linnaeus 1758) sourced from the Baltic Sea or from a unique, artificially heated ecosystem in the Baltic Sea, the 'Biotest' enclosure. The Biotest enclosure is a man-made basin in the Baltic archipelago that receives heated cooling water from a nearby nuclear power plant, which increases the average water temperature by 5–10°C compared with the adjacent archipelago (see Fig S1 and Hillebrand et al., 2010; Sandblom et al., 2016; Sandström et al., 1995). Hence, these populations of perch stem from two different thermal environments (reference: 15.5°C; Biotest: 22.5°C at the time of the present study), which have resulted in differences between populations in regards to their tolerance to high temperatures (Ekström et al., 2016a; Sandblom et al., 2016). For example, previous studies have revealed that the Biotest fish have a significantly elevated CT_{max} compared with reference fish (29.8–30.8°C and 32.0–33.0°C in reference and Biotest fish, respectively). Moreover, cardiovascular performance (i.e. heart rate and cardiac output) declined dramatically between 28 and 29°C in reference fish during acute warming, while these variables were maintained or even increased continuously until CT_{max} was reached in Biotest fish (Ekström et al., 2016a; Sandblom et al., 2016). To determine whether these differences in cardiovascular and whole-animal thermal tolerance between populations can be explained by differences in cardiac metabolic capacities, we evaluated the activities of key enzymes involved in different metabolic pathways. We first evaluated the glycolytic enzymes pyruvate kinase (PK), which produces pyruvate, and LDH, which may both produce (from lactate) and consume pyruvate, and hence resides at the aerobic and anaerobic node of glycolysis. LDH activity level could also be important to maintain proper redox status (NADH/NAD) in the cytoplasm. We then evaluated the oxidative metabolism pathway by measuring the activity of enzymes governing pyruvate oxidation [pyruvate dehydrogenase (PDH)], the tricarboxylic acid cycle [citrate synthase (CS) and malate dehydrogenase (MDH)], enzymes involved in fatty acid and protein catabolism [HOAD and aspartate aminotransferase (AAT), respectively], as well as crucial components of the mitochondrial respiration chain participating in electron transfer and creation of the proton gradient (CI, CI+CIII and CIV), which ultimately produces the vast majority of cellular ATP. The enzymatic activities were measured at the respective acclimation temperatures and at temperatures known to constitute the thermal maximum for these populations. We hypothesized that a temperature-dependent failure of any of these enzymes would constrain subsequent steps of aerobic and/or anaerobic ATP production, and may therefore constitute a limiting factor for cardiac and possibly whole-animal thermal tolerance. Furthermore, we hypothesized that the increased CT_{max} of Biotest fish (Ekström et al., 2016a; Sandblom et al., 2016) may be linked to a reduced thermal sensitivity of the metabolic machinery, a change in substrate exploitation and/or a modulation of the proportion of catalytic capacities of dehydrogenase and oxidative processes that may improve the metabolic capacity and ATP production at critically high temperatures. Finally, we aimed to elucidate whether enzymatic catalytic capacities could be linked to a reorganization of the mitochondrial membrane lipid composition owing to changes in the proportions and saturation level of fatty acids, which is known to occur during acclimation.

MATERIALS AND METHODS

Experimental animals

Adult perch of mixed sex were caught by hook and line from the intake channel leading into the power plant cooling system (reference, 14.3±0.2°C), and from the cooling water outlet into the Biotest enclosure (Biotest, 22.8±0.3°C), i.e. downstream of the power plant (for detailed information, see Sandblom et al. 2016). Fish were held in 1200 liter holding tanks supplied with a continuous flow of aerated brackish water (~5 ppt) either from the Baltic Sea (15.4±0.4°C during the sampling period) or from the Biotest enclosure (22.3±0.7°C during the sampling period). Fish were held in these conditions for at least 5 days prior to tissue

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collection. The fish were kept outdoors, thus maintaining the natural photoperiod, and were not fed. All experiments were performed in agreement with ethical permit 65-2012 from the animal ethics committee in Gothenburg, Sweden.

Tissue/sample preparation

Fish were killed by a sharp cranial blow. After determining mass, fork length and condition of the fish, the heart was removed and the ventricle was dissected free and blotted, followed by determination of the ventricular wet mass. The tissues were transferred to liquid nitrogen and were thereafter kept at −80°C until homogenization in a sucrose buffer (1:10 mass-specific dilution in buffer) containing 250 mmol l^{-1} sucrose, 20 mmol l^{-1} Tris, 40 mmol l^{-1} KCl, 2 mmol l^{-1} EGTA, pH 7.4 (Spinazzi et al., 2012). Homogenates were then centrifuged at 750 ***g*** for 5 min and kept at −80°C until processing. The post-freeze stability of homogenates was evaluated and validated by: (1) comparing activities of CS, CI, CI+CIII and CIV (as described below) between freshly prepared homogenates and frozen homogenates, which revealed <5% difference in activity at 23°C (n=2 and n=3 for Biotest and reference fish, respectively); and (2) comparing the current values for enzymatic activities (in U g^{-1} of tissue) with previously reported data from cardiac tissue of teleost fishes (see Table S2).

Determination of enzymatic activity

Enzymatic activities were determined using an EnVision® Multilabel Plate Reader with temperature control (PerkinElmer, Waltham, MA, USA). The plate reader was placed in a thermostatically controlled room at 15°C. Enzymatic activities for both populations (n=20 from each population for each assay) were determined at 16°C (Reference perch acclimation temperature), 23°C (Biotest perch acclimation temperature), 30°C (reference perch CT_{max}), 32.5°C (Biotest perch CT_{max}) and 36°C (temperature constituting the thermal extreme for this species) (Ekström et al., 2016a; Sandblom et al., 2016).

The path length was determined by calculating the height (h) of the volume in the well (including the bottom thickness) according to the microplate specifications with the following formula:

$$h = (4V)/(\pi d^2), \quad (1)$$

where V is the final volume of the reaction and d is the diameter of the well. Enzymatic activities were normalized to protein concentration, which was determined using the bicinchoninic acid method (Smith et al., 1985), with BSA as standard and expressed as U mg^{-1} protein, where U represents 1 µmol of substrate transformed to product in 1 min. Most data available on cardiac tissue of teleost fish are presented as U g^{-1} tissue in the literature and for Table S2, our data on enzymatic activities were therefore normalized to wet tissue mass (similar patterns were found for both normalization types). Protocols for measuring enzyme activities were modified and adapted from Thibeault et al. (1997) (for LDH, PDH, CS, HOAD and CIV), Bergmeyer (1983) (for CI+CIII and MDH), Pelletier et al. (1994) (for AAT and PK), and from Janssen et al. (2007) (for CI). In all cases, the enzymatic reactions were initiated with the addition of substrates and the blank was measured without substrates, except for PK, MDH, AAT and CI+CIII, where the reaction medium without sample was used. All measurements were performed at least in duplicate.

PK (EC 2.7.1.40) activity was determined by following the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} µmol^{-1}) for 4 min in a reaction medium containing 50 mmol l^{-1} imidazole-Hcl, 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl, 5 mmol l^{-1} ADP, 0.15 mmol l^{-1} NADH, 5 mmol l^{-1} phosphoenolpyruvate and 0.6 U ml^{-1} lactate dehydrogenase, pH 7.4.

LDH (EC1.1.1.27) catalytic capacity was measured in a 100 mmol l^{-1} potassium phosphate buffer complemented with 0.16 mmol l^{-1} NADH and 0.4 mmol l^{-1} pyruvate (omitted from the blank), pH 7.0, by recording the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} µmol^{-1}) for 4 min.

PDH (EC 1.2.4.1) activity was evaluated using the reduction of p-iodonitrotetrazolium violet (INT) at 490 nm (ε=15.9 ml cm^{-1} µmol^{-1}) for 4 min when homogenates were incubated in 50 mmol l^{-1} tris-HCl, 0.1%(v/v) triton-X100, 1 mmol l^{-1} $MgCl_2$ and 1 mg ml^{-1} BSA complemented with 2.5 mmol l^{-1} NAD, 0.5 mmol l^{-1} EDTA, 0.1 mmol l^{-1} coenzyme A, 0.1 mmol l^{-1} oxalate, 0.6 mmol l^{-1} INT, 6 U ml^{-1} lipoamide dehydrogenase, 0.2 mmol l^{-1} thiamine pyrophosphate and 5 mmol l^{-1} pyruvate (omitted from the blank), pH 7.8.

CS (EC 4.1.3.7) activity was measured at 412 nm for 4 min by following the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, ε=13.6 ml cm^{-1} µmol^{-1}) using a 100 mmol l^{-1} imidazole-HCl buffer containing 0.1 mmol l^{-1} DTNB, 0.1 mmol l^{-1} acetyl-CoA and 0.15 mmol l^{-1} oxaloacetic acid (omitted from the blank), pH 8.0.

For MDH (EC 1.1.1.37) activity, the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} µmol^{-1}) was recorded for 4 min using a 100 mmol l^{-1} potassium phosphate buffer with 0.2 mmol l^{-1} NADH and 0.5 mmol l^{-1} oxaloacetic acid, pH 7.5.

AAT (EC 2.6.1.1) activity was determined by following the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} µmol^{-1}) for 4 min in a reaction medium containing 100 mmol l^{-1} potassium phosphate, 0.32 mmol l^{-1} NADH, 10 mmol l^{-1} alpha ketoglutarate, 22 mmol l^{-1} aspartate, 0.025 mmol l^{-1} pyridoxal phosphate and 0.6 U ml^{-1} malate dehydrogenase, pH 7.0.

HOAD (EC 1.1.1.35) catalytic capacity was evaluated in a 100 mmol l^{-1} triethanolamine-HCl buffer complemented with 5 mmol l^{-1} EDTA, 1 mmol l^{-1} KCN, 0.115 mmol l^{-1} NADH and 0.05 mmol l^{-1} acetoacyl CoA (omitted from the blank), pH 7.0, by recording the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} µmol^{-1}) for 4 min.

CI (EC 1.6.5.3) activity was measured by following the reduction of 2,6-dichloroindophenol (DCPIP) at 600 nm (ε=19.1 ml cm^{-1} µmol^{-1}). Briefly, CI oxidizes NADH and the electrons produced reduce the ubiquinone 1 (UQ_1) that subsequently delivers the electrons to DCPIP. After incubation of homogenates in a 100 mmol l^{-1} potassium phosphate buffer containing 0.5 mmol l^{-1} EDTA, 3 mg ml^{-1} BSA, 1 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} KCN, 4.2 µmol l^{-1} antimycin A, 75 µmol l^{-1} DCPIP and 65 µmol l^{-1} UQ_1, pH 7.5, for 5 min in the plate reader set at the desired assay temperature, 0.14 mmol l^{-1} NADH was added to start the reaction, which was recorded for 10 min. The same reaction with 1 µmol l^{-1} rotenone was followed in parallel and the specific CI activity represented by the rotenone-sensitive activity was calculated.

CI+CIII activity was measured by following the reduction of INT at 490 nm (ε=15.9 ml cm^{-1} µmol^{-1}) for 4 min using a 100 mmol l^{-1} potassium phosphate, 0.85 mmol l^{-1} NADH, 2 mmol l^{-1} INT, 0.03% (v/v) triton X-100, pH 8.5 reaction medium.

CIV (EC 1.9.3.1) activity was determined by following oxidation of reduced cytochrome c at 550 nm (ε=19.1 ml cm^{-1} µmol^{-1}) for 4 min in a reaction medium containing 100 mmol l^{-1} potassium phosphate and 0.05 mmol l^{-1} equine heart cytochrome c, pH 8.0. Cytochrome c was reduced by the addition of sodium dithionite (4 mmol l^{-1}) and the solution was bubbled with air to eliminate the

excess of reducing agent. Reactions were run against a reference of 0.05 mmol l^{-1} cytochrome *c* oxidized with 0.33% (w/v) potassium ferricyanide.

Determination of perch heart lipid profile

The lipid profile of the perch heart was determined by quantifying the presence of saturated [hexadecanoic (16:0) and octadecanoic (18:0)] and monounsaturated [palmitoleic (16:1), vaccenic (18:1) and nervonic (24:1n9)] fatty acids (SFA and MUFA, respectively), as well as polyunsaturated fatty acids (PUFA) including the omega-3 [docosapentaenoic (20:5n3), eicosapentaenoic (22:5n3) and docosahexaenoic (22:6n3)] and omega-6 [linoleic (18:2n6) and arachidonic (20:4n6)] fatty acids.

Fatty acid transmethylation

The direct transesterification method used for fatty acid transesterification was adapted from Lepage and Roy (1984). Briefly, heart tissue was homogenized (1:10 dilution in buffer) in 100 mmol l^{-1} potassium phosphate, 1 mmol l^{-1} EDTA buffer, pH 7.5, and 30 mg of tissue was spiked with 0.1 mg of internally added tridecanoic acid and tricosanoic acid (Nu-Check Prep, Elysian, MN, USA). Direct trans-methylation was performed with 3 ml of a freshly prepared 3% sulfuric acid (H_2SO_4) methanol solution. The reaction was performed in test vials, with Teflon screw caps, at 90°C in a dry bath for 1 h. Vials were vortexed before the dry bath, after 30 min and at the end of the procedure. Vials were subsequently cooled at 4°C and 5 ml of water was added. Fatty acid methyl esters (FAMEs) were extracted by adding 1 ml of hexane followed by centrifugation (3000 ***g***) for 10 min at room temperature. The hexane was collected in a test tube and was evaporated using nitrogen. The hexane extraction was repeated three times. FAMEs were suspended in 100 µl of toluene prior to the analysis in the gas chromatograph.

Gas chromatographic conditions

FAMEs were separated and quantified by gas chromatography (Trace Ultra 100, Thermo Fisher Scientific, Waltham, MA, USA) using a 60 m×0.32 mm i.d. capillary column (DB-23, Agilent Technologies Canada, Mississauga, ON, Canada). Helium was used as carrier gas (230 kPa constant pressure) and temperature vaporization was set at 230°C with a split injection of 100 ml min^{-1}. The thermal protocol was programmed to increase the temperature from 50 to 140°C at a rate of 25°C min^{-1}, followed by an increase (heating rate: 3.0°C min^{-1}) to 195°C, at which the temperature was maintained for 5 min, followed by a final increase to 225°C (heating rate: 4.0°C min^{-1}) which was maintained for 5 min. Individual methyl esters were identified by comparison with known standards (Supelco 37 Component FAME mix and GLC-569).

Chemicals

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, United States), except GLC-569, which was obtained from NuCheck Prep.

Calculations and statistics

Relative ventricular mass was calculated as: Relative ventricular mass=Ventricle mass/Body mass. Fish condition factor was calculated as: Condition factor=(100×Body mass)/Body length^3. The temperature dependence of the enzymatic catalytic activities, i.e. Q_{10}, was calculated as:

$$Q_{10} = (R_{T2}/R_{T1})^{10/(T2-T1)}, \quad (2)$$

where R_{T1} represents the enzymatic rate at a given initial temperature (T1) and R_{T2} represents the rate following an increase in enzymatic assay temperature (T2). The peroxidation index (PI) quantifies the susceptibility of the phospholipid fatty acids to peroxidative damage and is calculated according to Hulbert et al. (2007) as:

$$\begin{aligned} PI = \Sigma[&(0.025 \times \text{monoenoics})(1 \times \text{dienoics})(2 \times \text{trienoics}) \\ &\times (4 \times \text{tetraenoics})(6 \times \text{pentaenoics}) \\ &(8 \times \text{hexaenoics})]. \end{aligned} \quad (3)$$

The unsaturation index (UI) is a quantitative measurement of the amount of double bonds per 100 acyl chains and is calculated according to Hulbert et al. (2007) as:

$$\begin{aligned} UI = \Sigma[&(1 \times \text{monoenoics})(2 \times \text{dienoics})(3 \times \text{trienoics}) \\ &(4 \times \text{tetraenoics})(5 \times \text{pentaenoics})(6 \times \text{hexaenoics})]. \end{aligned} \quad (4)$$

Statistical analyses were performed using the R platform (http://www.r-project.org). Enzymatic catalytic capacities were modeled with a linear mixed effects model, using the 'lme' function of the 'nlme' package (Pinheiro et al., 2014), and treating population (reference and Biotest) and temperature (16, 23, 30, 32.5 and 36°C) as fixed effects. Activity of each enzyme was determined on 20 different fish sampled in each population, and was measured at the five temperatures for each fish. We therefore treated fish (a specific ID was given for each fish of both populations) and assay temperature within fish as random effects. Residual maximum likelihood was used to estimate the variance of the model parameters. Normality of residuals was checked and data were ln transformed when required. The significance of the fixed effects was estimated using a Type II ANOVA to assess the change in deviance using Wald chi-square (χ^2) tests. Multiple comparisons were then tested with pairwise comparisons of the least-squares means using adjusted *P*-values with significance set at $P<0.05$. Statistical differences between populations regarding morphological variables and lipid profiles were determined using *t*-tests with significance set at $P<0.05$.

RESULTS

Thermal effects on glycolysis

While there was no change in cardiac PK activity between 16 and 23°C in either population (Fig. 1A, Table S1), the catalytic activity increased significantly at 30°C ($P<0.001$). PK activity was further augmented at 32.5°C in both populations ($P<0.001$), at which the activity plateaued and was maintained following the subsequent increase to 36°C in both reference and Biotest fish.

An initial increase in LDH activity was observed between 16 and 23°C in both populations ($P<0.001$ and $P=0.002$ for reference and Biotest fish, respectively; Fig. 1B, Table S1). The activity plateaued at 23°C in reference fish but increased further at 30°C in Biotest fish ($P<0.001$), at which the activity stabilized and did not change further at higher temperatures (Fig. 1B, Table 1). Both populations displayed relatively modest thermal sensitivities, with Q_{10} values ranging between 1.0 and 1.6 among all assay temperatures (Table 1). The LDH activity of the Biotest fish was significantly lower compared with reference fish at all temperatures (Table S1).

Temperature effects on pyruvate oxidation and tricarboxylic acid cycle

The activity of PDH increased with temperature in both populations (Fig. 2A, Table S1). In reference fish, the activity increased

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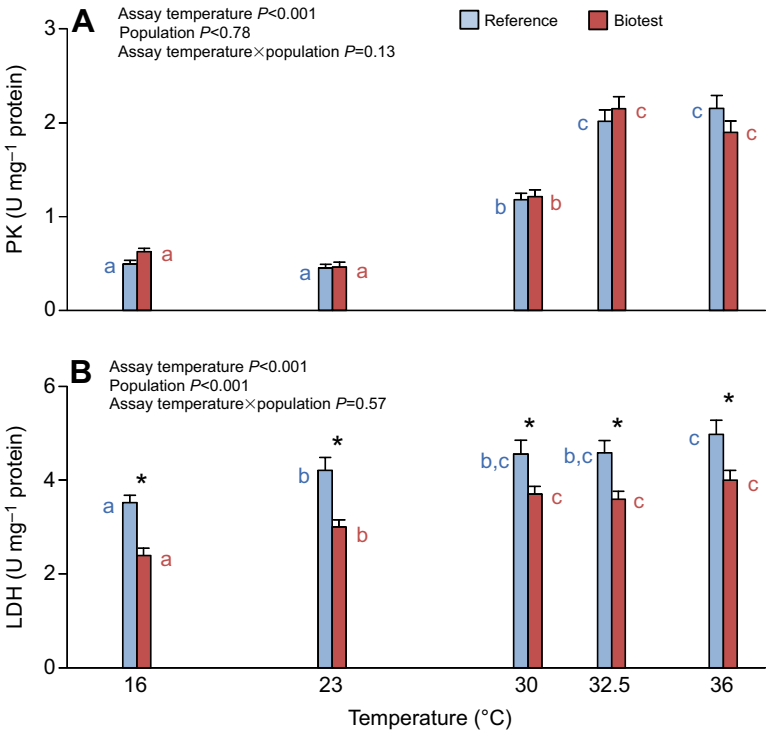

Fig. 1. Maximum catalytic capacity of glycolytic enzymes in the perch heart during a thermal challenge. Determination of maximum catalytic capacity of (A) pyruvate kinase (PK) and (B) lactate dehydrogenase (LDH) from ventricular tissues of two differentially field-acclimated populations (reference: 15.5°C, *n*=20; Biotest: 22.5°C, *n*=20) of European perch, *Perca fluviatilis*. The enzymes were measured at assay temperatures representing the acclimation temperatures (16 and 23°C) and critically high temperatures (30, 32.5 and 36°C) for these populations. Values are means±s.e.m. *Statistically significant differences between populations. Dissimilar letters denote statistical differences within treatment groups between assay temperatures.

significantly until 30°C (P=0.004 between 16 and 23°C; P<0.001 between 23 and 30°C), followed by a significant decline at 32.5°C (P<0.001; Table S1) and a modest but significant increase at 36°C (P=0.025; Table S1). In Biotest fish, the activity only increased significantly between 23 and 30°C (P<0.001) and remained unaltered at higher temperatures. The catalytic activity of PDH was found to be significantly lower in Biotest compared with reference fish at 23°C (P=0.018), 30°C (P<0.001) and 36°C (P=0.038), even though a non-significant trend for a decreased activity in the Biotest fish was also observed at 16°C (P=0.079) and 32.5°C (P=0.062).

There were no differences between populations in cardiac CS activity at 16°C (Fig. 2B), but the activity was found to be significantly lower in Biotest compared with reference fish at all other temperatures tested (P=0.007 at 23°C; P<0.001 at 30 and 32.5°C; P=0.031 at 36°C). Increasing assay temperatures resulted in a steady elevation of CS activity from 16 to 30°C in reference and Biotest fish (P<0.001), at which the activity peaked in both populations. At temperatures above 30°C, there was a drastic and progressive decrease of activity (P≤0.001 for both populations) as well as thermal sensitivity in both populations (Q_{10}<1; Table 1).

Cardiac MDH activity increased significantly from 16 to 30°C in both populations (P<0.001; Fig. 2C), where it reached maximum values followed by a leveling off in both populations. The activity was found to be lower in Biotest compared with reference fish at all assay temperatures (P<0.001). Overall, the thermal dependence of cardiac MDH activity was low and maintained, with Q_{10} ranging between 0.9 and 1.3 at all assay temperatures in both populations of perch (Table 1).

Thermal effects on amino acid and fatty acid catabolism

The activity of cardiac AAT was significantly lower in Biotest fish at most assay temperatures (P<0.001) except at 23°C (Fig. 3A).

Table 1. Temperature coefficients (Q_{10}) of enzyme activity for key cardiac metabolic enzymes from two differentially field-acclimated populations of European perch, *Perca fluviatilis*, captured at a reference site in the Baltic Sea (15.5°C) and from the chronically heated Biotest enclosure (22.5°C)

	16–23°C		23–30°C		30–32.5°C		32.5–36°C	
	Reference	Biotest	Reference	Biotest	Reference	Biotest	Reference	Biotest
PK	0.91±0.06^{a}	0.82±0.16^{a}	3.87±0.49^{b}	3.88±0.44^{b}	2.46±0.32^{b}	4.33±1.08^{b}	1.46±0.31^{a}	1.12±0.17^{a}
LDH	1.33±0.11	1.56±0.15^{a}	1.15±0.06	1.44±0.10^{a}	1.04±0.05	1.19±0.0.25^{b}	1.40±0.17	1.60±0.25^{a}
PDH	1.26±0.06^{a}	1.20±0.09^{a}	1.49±0.05^{a}	1.38±0.07^{a}	0.73±0.04^{b}	0.85±0.13^{b}	1.58±0.22^{a}	1.66±0.25^{a}
CS	2.60±0.06^{a,*}	2.34±0.09^{a}	1.91±0.05^{b}	1.82±0.10^{b}	0.74±0.03^{c}	0.54±0.04^{c}	0.34±0.01^{d}	0.36±0.01^{d}
MDH	1.30±0.04^{a}	1.34±0.05^{a}	1.13±0.03^{b}	1.27±0.06^{a}	0.97±0.02^{c}	0.92±0.06^{b}	0.96±0.02^{c}	1.02±0.05^{b}
HOAD	1.41±0.13^{a,b}	1.35±0.10^{a}	1.02±0.02^{b,c}	1.19±0.24^{a}	0.91±0.05^{c}	0.82±0.10^{b}	1.87±0.16^{a,*}	5.22±0.64^{c}
CI	1.16±0.07^{a}	1.02±0.06^{a}	1.18±0.12^{a}	0.95±0.03^{a}	1.36±0.11^{a}	2.41±0.58^{b}	0.70±0.04^{b,*}	0.94±0.06^{a}
CI+CIII	1.51±0.05^{a}	1.54±0.05^{a}	0.97±0.03^{b,*}	1.17±0.03^{b}	1.11±0.87^{b,*}	0.87±0.05^{c}	0.61±0.04^{c}	0.62±0.08^{d}
CIV	1.31±0.04^{a}	1.61±0.11^{a}	0.85±0.03^{b,*}	1.57±0.37^{a}	1.74±0.13^{a,*}	3.65±0.56^{b}	0.72±0.24^{b}	0.73±0.16^{c}

	16–23°C		23–32.5°C		32.5–36°C	
	Reference	Biotest	Reference	Biotest	Reference	Biotest
AAT	0.86±0.07^{a,*}	1.35±0.07^{a}	2.01±0.09^{b,*}	1.68±0.07^{b}	1.46±0.12^{c,*}	1.53±0.14^{a,b}

*Statistically significant differences between populations. Dissimilar letters denote statistical differences between temperature ranges. Note the different temperature range for AAT. See List of symbols and abbreviations for definitions.

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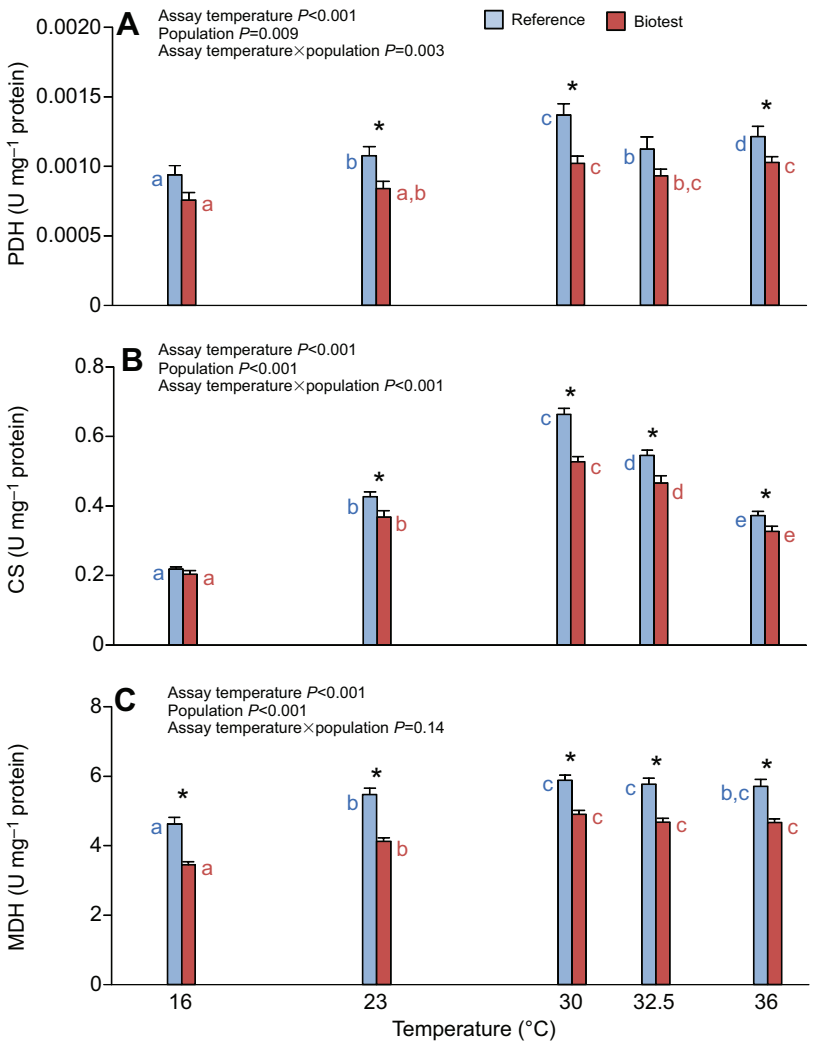

Fig. 2. Maximum catalytic capacity of tricarboxylic acid cycle enzymes in the perch heart during a thermal challenge. Determination of maximum catalytic capacity of (A) pyruvate dehydrogenase (PDH), (B) citrate synthase (CS) and (C) malate dehydrogenase (MDH) from ventricular tissues of two differentially field-acclimated populations (reference: 15.5°C, *n*=20; Biotest: 22.5°C, *n*=20) of European perch, *Perca fluviatilis*. The tricarboxylic enzymes were measured at assay temperatures representing the acclimation temperatures (16 and 23°C) and critically high temperatures (30, 32.5 and 36°C) for these populations. Values are means ±s.e.m. *Statistically significant differences between populations. Dissimilar letters denote statistical differences within treatment groups between assay temperatures.

Unfortunately, the enzymatic assay for cardiac AAT at 32.5°C failed, resulting in a loss of data for this temperature in both populations. In reference fish, the activity initially decreased between 16 and 23°C (P=0.001), followed by a significant substantial increase at 32.5°C (P<0.001 between 23 and 32.5°C; Q_{10}=2; Table 1), and a modest but significant increase at 36°C (P<0.001 between 32.5 and 36°C). In Biotest fish, the activity gradually and significantly increased from 16 to 36°C (P=0.02 between 16 and 23°C; P<0.001 between 23 and 32.5°C as well as between 32.5 and 36°C).

The activity of cardiac HOAD remained unaltered between 16 and 32.5°C in both populations, followed by an increase in activity between 32.5 and 36°C (P=0.001 for reference and P<0.001 for Biotest; Fig. 3B, Table S1). The increase in activity corresponded to a Q_{10} of 1.9 in the reference fish, while the activity increased more drastically in the Biotest fish (Q_{10}=5.2, Table 1). Interestingly, while the activity of HOAD was significantly lower in Biotest compared with reference fish at 32.5°C (P=0.001), it was significantly higher in the Biotest fish at 36°C (P<0.001).

Effects of temperature on mitochondrial complexes

There were significant differences between populations in CI activity, with a substantially lower activity in Biotest compared with reference fish at 23°C (P=0.006), 30°C (P<0.001), 32.5°C (P<0.001) and 36°C (P=0.001) (Fig. 4A, Table S1). In reference fish, the activity increased progressively with temperature from 16 to 32.5°C (with significance reached between 30 and 32.5°C, P<0.001), after which the activity declined (P=0.006 between 32.5 and 36°C, Q_{10}<1; Table 1). There were, however, almost no changes in the CI activity in Biotest fish (Q_{10}~1 from 16 to 30°C), with the exception of a small but significant increase between 30 and 32.5°C (P=0.049, Q_{10}=2.4; Table 1), after which the activity leveled off and remained unchanged at 36°C.

CI+CIII activity was similarly influenced by the assay temperatures (Fig. 4B, Table S1), and the activity initially increased between 16 and 23°C in both reference and Biotest fish (P<0.001), but then remained unchanged between 23 and 32.5°C in both populations (Q_{10} values ~1; Table 1). The activity subsequently decreased drastically at 36°C in both populations (P<0.001, Q_{10} values <1; Table 1).

Cardiac CIV activity did not differ between populations between 16 and 32.5°C, but was higher in Biotest fish at 36°C (P=0.016; Fig. 4C; Table S1). In reference fish, the activity of CIV increased modestly with temperature and was significantly elevated at 32.5°C compared with previous temperatures (P<0.001, P=0.005 and P<0.001 for 16, 23 and 30°C, respectively). In Biotest fish, the activity increased between 16 and 23°C (P=0.003), did not change between 23 and 30°C, and further increased between 30 and 32.5°C (P<0.001). At 36°C, the activity declined in both populations (P<0.001 and P=0.030 for reference and Biotest fish, respectively; Q_{10} values ≤1; Table 1).

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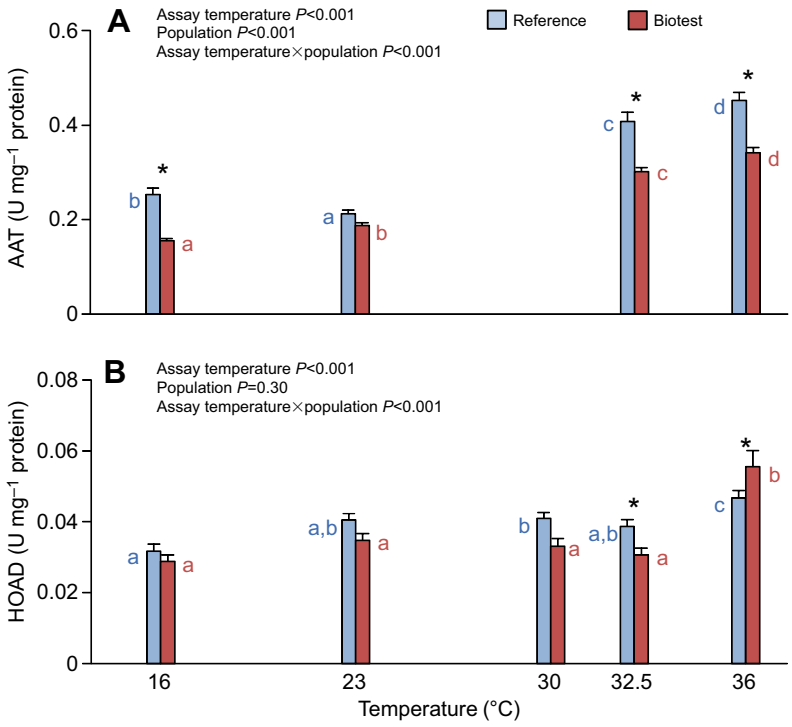

Fig. 3. Maximum catalytic capacity of enzymes governing amino acid and fatty acid catabolism in the perch heart during a thermal challenge. Determination of maximum catalytic capacity of (A) aspartate aminotransferase (AAT) and (B) hydroxy-acyl Coenzyme A dehydrogenase (HOAD) from ventricular tissues of two differentially field-acclimated populations (reference: 15.5°C, *n*=20; Biotest: 22.5°C, *n*=20) of European perch, *Perca fluviatilis*. The enzymes were measured at assay temperatures representing the acclimation temperatures (16 and 23°C) and critically high temperatures (30, 32.5 and 36°C) for these populations. Values are means±s.e.m. *Statistically significant differences between populations. Dissimilar letters denote statistical differences within treatment groups between assay temperatures.

The concomitant increase in CIV and decrease in CI activity in the Biotest population resulted in depression of CI/CIV ratio when measured at higher temperatures (0.12±0.03, 0.13±0.05, 0.07±0.01, 0.06±0.01 and 0.06±0.01 at 16, 23, 30, 32.5 and 36°C, respectively) whereas this ratio was constant in reference fish (0.10±0.02, 0.09±0.01, 0.11±0.02, 0.10±0.02 and 0.10±0.01 at 16, 23, 30, 32.5 and 36°C, respectively).

Morphological variables and lipid composition of the perch heart

Reference fish had a higher relative ventricular mass than the Biotest fish, but there were no significant differences in body mass, body length or condition factor between populations (Table 2).

The lipid profiles were significantly different between the two populations of perch (Table 3). Hearts of Biotest fish had higher proportions of 16:0, 18:0 and total SFA (all $P<0.001$). Total MUFA was lower in Biotest fish ($P=0.002$), which was mainly due to a lower proportion of 18:1 ($P<0.001$). While the amount of omega-6 was not different between populations, the Biotest fish displayed lower total omega-3 ($P<0.001$), including 20:5n-3, 22:5n-3 and 22:6n-3 (all $P<0.001$), as well as lower total PUFA ($P<0.001$). This resulted in a lower omega-3/omega-6 ratio in Biotest compared with reference fish ($P<0.001$, Table 3). The peroxidation and unsaturation indices were significantly lower in Biotest compared with reference fish (Table 3).

Table 2. Morphological characters of the two differentially field-acclimated populations of European perch, *Perca fluviatilis*, captured at a reference site in the Baltic Sea (15.5°C) and from the chronically heated Biotest enclosure (22.5°C)

	Reference	Biotest
Body mass (g)	415±50	396±44
Body length (mm)	302±10	293±8
Condition factor (%)	1.4±0.04	1.5±0.03
Relative ventricle mass (×10^{-3})	68.5±1.51*	61.6±1.54

*Statistically significant differences ($P\leq0.05$) between populations.

DISCUSSION

Acute effects of temperature on metabolism in the perch heart

The enzyme activity values reported here are all well within the range previously reported in cardiac tissue from various fishes (see Table S2).

Despite the increased capacity for pyruvate synthesis (PK) and anaerobic capacity (LDH) with increasing temperatures, the concomitant decrease of CS and PDH activities occurring

Table 3. Lipid composition of hearts from European perch, *Perca fluviatilis*, captured at a reference site in the Baltic Sea (15.5°C) and from the chronically heated Biotest enclosure (22.5°C)

Fatty acid	Reference	Biotest	*t*-test *P*-value
Hexadecanoic acid (16:0)	15.47±0.38	19.86±0.57	<0.001
Octadecanoic acid (18:0)	8.23±0.23	12.13±0.35	<0.001
Saturated fatty acids	25.46±0.64	34.47±0.9	<0.001
Palmitoleic acid (16:1)	1.89±0.13	2.12±0.12	0.190
Vaccenic acid (18:1)	18.81±0.40	16.28±0.38	<0.001
Nervonic acid (24:1n9)	1.971±0.12	1.87±0.13	0.586
Monounsaturated fatty acids	23.20±0.53	20.84±0.50	0.002
Linoleic acid (18:2n6)	1.62±0.05	1.78±0.09	0.126
Arachidonic acid (20:4n6)	9.45±0.21	9.45±0.28	0.987
Omega-6	12.81±0.25	13.39±0.38	0.213
α-Linolenic acid (18:3n3)	0.33±0.02	0.58±0.05	<0.001
Docosapentaenoic acid (20:5n3)	6.37±0.20	5.06±0.21	<0.001
Eicosapentaenoic acid (22:5n3)	1.80±0.05	1.46±0.06	<0.001
Docosahexaenoic acid (22:6n3)	29.60±0.80	23.82±1.00	<0.001
Omega-3	38.69±0.95	31.50±1.19	<0.001
Ratio omega-3/omega-6	3.04±0.09	2.38±0.11	<0.001
Peroxidation index	332.81±7.50	278.08±9.46	<0.001
Unsaturated index	290.96±5.34	247.92±6.92	<0.001

The values (mean±s.e.m.) represent the sum of the average lipid content for each lipid class, i.e. saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids (omega-3 and omega-6 fatty acids).
Fatty acyl chains with values lower than 1 g/100 g are not shown except for omega-3 precursor 18:3n3.

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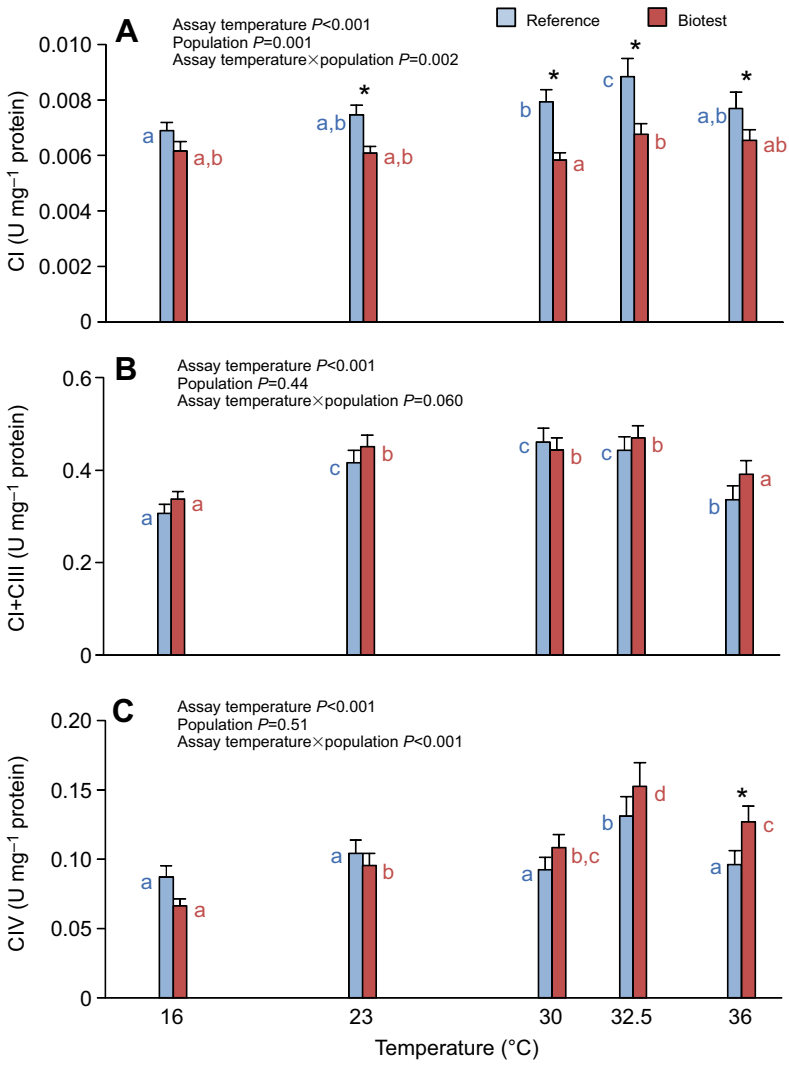

Fig. 4. Maximum catalytic capacity of mitochondrial complexes in the perch heart during a thermal challenge. Determination of maximum catalytic capacity of (A) complex I (CI), (B) complexes I and III (CI+CIII) and complex IV (CIV) of the electron transport system from ventricular tissues of two differentially field-acclimated populations (reference: 15.5°C, n=20; Biotest: 22.5°C, n=20) of European perch, *Perca fluviatilis*. The enzymes were measured at assay temperatures representing the acclimation temperatures (16 and 23°C) and critically high temperatures (30, 32.5 and 36°C) for these populations. Values are means±s.e.m. *Statistically significant differences between populations. Dissimilar letters denote statistical differences within treatment groups between assay temperatures.

between 30 and 32.5°C, particularly in reference fish acclimated to lower environmental temperature, might suggest an impaired capacity to oxidize pyruvate in the perch heart at high temperatures. Furthermore, the drastic decline in CS activity at temperatures exceeding 30°C could indicate a potential bottleneck for further steps of the substrate oxidation processes of the tricarboxylic acid cycle at higher temperatures. While these findings contrast with observations in other species displaying a progressive increase in CS and PDH activity with temperature (Lemieux et al., 2010; Swimmer et al., 2004), the current findings suggest an impaired capacity to reduce NAD and FAD, and hence to provide the electrons necessary to drive the ETS, ultimately impairing ATP production in the perch heart at high temperatures. It is, however, important to note that the *in vitro* enzymatic activities observed in the present study might not completely reflect actual metabolic flux of living tissue *in vivo*, but rather the overall catalytic capacity at key steps (Johnston, 1977; Suarez et al., 1997; West et al., 1993). Therefore, the relative changes and differences between populations regarding the activity of these enzymes is what is really significant, as this provides important information concerning the modulations of mitochondrial capacity in response to warming.

The elevation of AAT activity between 30 and 36°C, concomitant with the high MDH activity, suggests that the malate–aspartate shuttle could potentiate the production and transport of cytosolic NADH across the inner mitochondrial membrane at these high temperatures (Hochachka et al., 1979; Safer, 1975). This mechanism may compensate for the decline in CS activity by maintaining the provision of NADH to CI for the transport of electrons in the mitochondria, as is also implied by strong positive correlations between MDH, AAT and CI (Pearson's product moment r=0.70 between MDH and AAT, r=0.61 between AAT and CI and r=0.71 between MDH and CI at 32.5°C, all P<0.001).

The increased PK activity would also elevate pyruvate concentrations at 32.5°C, which, in combination with increased LDH and decreased PDH activities in both populations of perch, could contribute to channeling pyruvate to lactate and hence potentiate anaerobic glycolysis, a response that has been reported in other fish species (Iftikar et al., 2014).

In spite of these potential compensatory measures to maintain NADH and ATP production in mitochondria, the decline of mitochondrial CI (in reference fish only), CI+CIII and CIV at 36°C should ultimately constrain and limit oxidative phosphorylation and ATP production in the perch heart of both populations. These findings are in agreement with previous findings demonstrating an impairment of the ETS, and in turn, mitochondrial respiration capacity, at high temperature in heart mitochondria of several New Zealand wrasse species (Iftikar and Hickey, 2013; Iftikar et al., 2014, 2015) and Atlantic wolffish (Lemieux et al., 2010). In fact, we suggest that the concomitant decline in substrate oxidation of

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tricarboxylic acid cycle intermediates would eventually decrease NADH and $FADH_2$ production, which may also represent an underlying mechanism for the decline of mitochondrial respiration capacity at high temperature (Blier et al., 2014; Pichaud et al., 2011). However, this hypothesis needs further support, for example, by conducting a thorough *in vivo* evaluation of the effects of acute warming on NADH and $FADH_2$ levels. Furthermore, increased LDH activity and an increased reliance on anaerobic glycolysis to supply ATP would result in lowering of internal cardiac pH, which could ultimately impair enzymatic activity, and also *in vivo* heart function (e.g. cardiac contractility) (Clark et al., 2008; Driedzic and Gesser, 1994; Iftikar and Hickey, 2013; Iftikar et al., 2014; Steinhausen et al., 2008).

Collectively, these findings suggest that the enzymatic machinery of the cardiac cells might constitute a limiting factor for cellular energy production, which would in turn constrain heart function at high temperatures despite the relative plasticity of mitochondrial metabolism.

Effects of thermal field acclimation on enzymatic function in the perch heart

As expected, the reference fish had a greater relative ventricular mass compared with Biotest fish (Ekström et al., 2016c; Sandblom et al., 2016). Chronic exposure to warmer temperatures typically results in a reduced thermal sensitivity (i.e. thermal compensation) of numerous physiological traits relating to metabolic, enzymatic and cardiovascular functions at a given temperature (Ekström et al., 2016a,b; Sandblom et al., 2016; Seebacher et al., 2015). Indeed, the warm-acclimated Biotest fish displayed a lower activity of LDH, PDH, CS, MDH, AAT and CI at almost all assay temperatures. These findings are indicative of a downregulation of aerobic metabolism in the heart and are consistent with previous studies demonstrating a depression of enzymatic thermal sensitivity and a downregulated expression of enzymes governing glycolysis, tricarboxylic acid cycle and mitochondrial respiration following acclimation and adaptation to warmer habitats in fish (Jayasundara et al., 2015; West et al., 1999). This is also in agreement with observed reductions in whole-animal routine oxygen consumption rate, as well as reduced cardiac power output, heart rate and cardiac output, which are indicative of a reduced cardiac oxygen demand in Biotest perch at given test temperatures (Ekström et al., 2016a; Sandblom et al., 2016).

Additionally, as CS activity is often regarded as a reliable marker for mitochondrial content (Larsen et al., 2012), the decreased activity in CS, but also PDH, MDH (being enzymes located in the mitochondrial matrix) as well as CI, may reflect a reduction in mitochondrial content following warm acclimation. Indeed, this scenario would comply with previous observations of an inverse relationship between cardiac mitochondrial density and habitat temperature in fish (Shiels et al., 2011). It is therefore likely that the reduction and thermal compensation of routine metabolic rate and cardiac functions in the Biotest perch (Ekström et al., 2016a; Sandblom et al., 2016), could be partly explained by lowered mitochondrial content and hence metabolic activity in the myocardium.

While there were no obvious beneficial effects of warm acclimation on the thermal sensitivity of most of the enzymes investigated, there was an observed increase in HOAD and CIV activity at temperatures exceeding 32.5°C. This suggests that the warm-acclimated Biotest perch have an increased capacity to oxidize fatty acids for aerobic energy production, which may potentiate cardiac functions at high temperatures. Furthermore, the lower activity of LDH in the Biotest population could help maintain intra-cardiac lactate levels and hence pH within homeostatic levels, possibly facilitating cardiac contractility at high temperatures.

Interestingly, the depression of the CI/CIV ratio in Biotest fish at higher temperature indicates a significant change in the proportion of the complex that feeds ETS with electrons (CI) and the proportion of the complex that oxidizes ETS through reduction of molecular oxygen (CIV), suggesting a lower reduction capacity of ETS for a given electron flux in mitochondria from warm-acclimated fish at higher temperature. This could prevent accelerated ROS production at high temperature and supports the hypothesis that oxidative stress management could be a metabolic parameter that delineates high temperature limits (Blier et al., 2014).

Collectively, these mechanisms may contribute to explain the increased cardiac and whole-animal thermal tolerance observed in the warm-acclimated Biotest fish (Ekström et al., 2016a; Sandblom et al., 2016).

Lipid composition and enzymatic function in the perch heart

The fatty acid levels of reference fish are similar to values previously reported in perch from the Baltic Sea (Polak-Juszczak and Komar-Szymczak, 2009). The present data demonstrate that warm acclimation induced an increase in saturated fatty acid content, a decrease in omega-3 and a decrease in the unsaturation index in the Biotest fish heart, which is consistent with previous findings regarding the influence of chronic temperature exposure on membrane composition in fish (Calabretti et al., 2003; Hazel, 1995; Kraffe et al., 2007; Skalli et al., 2006). While the lower unsaturation index may reflect intra-population differences in dietary preferences and/or feeding rates in Biotest compared with reference fish, the decreased levels of unsaturated fatty acids may reflect the increased usage of fatty acids for oxidative energy production, as is implied by the increased HOAD activity of Biotest perch.

The degree of unsaturation of membrane lipids has been proposed to be positively correlated to activity of membrane-bound enzymes and transmembrane proton leak rates, resulting in an increased enzyme activity and whole-animal metabolic rate (Brookes et al., 1998; Hulbert and Else, 1999). Conversely, the lower PUFA observed in the Biotest perch may partly explain the lower activity of some of the membrane-associated enzymes observed here, as well as the reduction in mitochondrial proton leak previously observed in the warm-acclimated Biotest fish (N.P., A.E., P. Rowinski, F. Sundström, P.U.B. and E.S., unpublished observation), consequently depressing cardiac metabolic activity (as indicated by reduced cardiac power output) as previously observed in Biotest fish (Ekström et al., 2016a). The higher level of SFA and lower content of PUFA also significantly contributed in the reduction of the peroxidation index (PI) of the membranes, which is known to be associated with a higher resistance to oxidative stress, as shown in bivalves (see Munro and Blier, 2012). This result is also in line with modified proportions of CI/CIV observed in the chronically warm Biotest population, which could also prevent high-temperature-induced ROS production in the warm-acclimated Biotest fish (Banh et al., 2016; Blier et al., 2014).

Physiological implications of reduced oxidative capacity in the fish heart

The current findings suggest that aerobic ATP production in the perch heart may become impaired at critically high temperatures, which could prove detrimental for maintaining cardiac functions. Indeed, Iftikar and colleagues have demonstrated a linkage between decreasing mitochondrial ATP production and the maintenance of heart rate at high temperatures in New Zealand wrasse (Iftikar and Hickey, 2013; Iftikar et al., 2014).

Impaired aerobic ATP production could impact myocardial function in several ways. For example, while the decline of heart rate and cardiac contractility has previously been attributed to altered ionic trans-membrane ion conductance across the sarcolemma [owing to failing Na^+ channel function or a denaturation of Na^+/K^+-ATPase (Lennard and Huddart, 1991; Vornanen et al., 2014)], this may instead reflect an ATP limitation of Na^+/K^+-ATPase at high temperatures. Furthermore, impaired Na^+/K^+-ATPase activity may also lead to a reduced influx of K^+, causing extracellular hyperkalemia, which further impairs myocardial conductivity, rhythmicity and contractility of hearts from fish and other ectotherms (Chapman and Rodrigo, 1987; Hove-Madsen and Gesser, 1989; Kalinin and Gesser, 2002; Nielsen and Gesser, 2001).

Previous studies conducted on these populations of perch revealed that heart rate and cardiac output declined between 28 and 29°C in reference fish, while these variables were maintained or increased continuously in Biotest fish until CT_{max} was reached at 32–33°C (Ekström et al., 2016a; Sandblom et al., 2016). Therefore, the failure of the substrate oxidation system observed between 30 and 32.5°C in both populations does not fully coincide with the temperature at which the heart failed in these previous studies. An explanation for this mismatch might lie in the thermal history of the perch used in the present and previous studies. Prior to the experiments, the fish of the present study experienced a natural extreme thermal event, which resulted in peak summer temperatures that were 3.5–5.9°C higher than in previous years, when the other experiments were performed (see Fig. S1). Thus, the Biotest fish used here may represent a thermally selected sub-sample of perch, likely with a higher thermal tolerance limit than the Biotest perch used in the previous studies.

Furthermore, it is possible that heart failure in perch may coincide with the loss of regulation and catalytic capacities of the mitochondrial system as a whole, which cannot be summarized entirely by the functions of the individual parts. Instead, it may reflect a collapse of the interactions between the integrated components. We can further infer from the current data obtained on *in vitro* enzymatic activities, that the mitochondria in the perch heart might not be able to maintain its metabolic capacity at critically high temperatures, when the capacity of its enzymatic machinery is compromised. However, how this relates to the *in vivo* situation is currently unknown and warrants further investigation.

Conclusions and perspectives

The present study provides evidence for an impairment of the substrate oxidation system in the perch heart at high temperatures, which most likely plays a crucial role in determining the upper critical thermal maximum for mitochondrial function, and, consequently, cardiac and whole-animal performance at high temperatures. Furthermore, while thermal field acclimation resulted in modifications to alleviate detrimental thermal effects on certain aspects of myocardial ATP production, highlighting the plasticity of the oxidative pathway, the failure of mitochondrial complexes in the ETS at high temperatures may represent a mechanism setting the maximum thermal limit for cardiac ATP production and function in perch. Collectively, this multi-level failure of the functional components involved with myocardial energy production at high temperatures may contribute to setting the limit for cardiac and whole-animal thermal tolerance in species of fish such as the European perch. Such limitation of mitochondrial metabolism in the fish heart could constitute a crucial factor for the resilience of fish to acute thermal events, such as heat waves, and be an important determinant of species' geographical distribution patterns in the future (Blier et al., 2014; Iftikar et al., 2014; Seebacher et al., 2015; Sunday et al., 2012).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

N.P., E.S. and A.E. conceived and designed the experiments. A.E., N.P. and B.-A.D.C. performed the experiments and analyzed the data. All authors contributed to the data interpretation and in drafting the manuscript. All authors gave final approval for publication.

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Supplementary information

Supplementary information available online at
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References

Banh, S., Wiens, L., Sotiri, E. and Treberg, J. R. (2016). Mitochondrial reactive oxygen species production by fish muscle mitochondria: Potential role in acute heat-induced oxidative stress. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **191**, 99-107.

Beitinger, T. L. and Lutterschmidt, W. I. (2011). Measures of thermal tolerance. In *Encyclopedia of Fish Physiology: From Genome to Environment* (ed. A. P. Farrell), pp. 1695-1702. New York: Elsevier Inc.

Bergmeyer, H. U. (1983). *Methods of enzymatic analysis. Vol II: Samples, Reagents, Assessment of Results*. Weinheim: Verlag-chemie.

Blier, P. U., Lemieux, H. and Pichaud, N. (2014). Holding our breath in our modern world: will mitochondria keep the pace with climate changes? *Can. J. Zool.* 591-601.

Brookes, P. S., Buckingham, J. A., Tenreiro, A. M., Hulbert, A. J. and Brand, M. D. (1998). The proton permeability of the inner membrane of liver mitochondria from ectothermic and endothermic vertebrates and from obese rats: correlations with standard metabolic rate and phospholipid fatty acid composition. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **119**, 325-334.

Calabretti, A., Cateni, F., Procida, G. and Favretto, L. G. (2003). Influence of environmental temperature on composition of lipids in edible flesh of rainbow trout (*Oncorhynchus mykiss*). *J. Sci. Food Agric.* **83**, 1493-1498.

Chapman, R. A. and Rodrigo, G. C. (1987). The negative inotropic effect of raised extracellular potassium and caesium ions on isolated frog atrial trabeculae. *Q. J. Exp. Physiol.* **72**, 561-570.

Clark, T. D., Sandblom, E., Cox, G. K., Hinch, S. G. and Farrell, A. P. (2008). Circulatory limits to oxygen supply during an acute temperature increase in the Chinook salmon (*Oncorhynchus tshawytscha*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R1631-R1639.

Clark, T. D., Sandblom, E. and Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771-2782.

Diffenbaugh, N. S. and Field, C. B. (2013). Changes in ecologically critical terrestrial climate conditions. *Science* **341**, 486-492.

Driedzic, W. R. and Gesser, H. (1994). Energy metabolism and contractility in ectothermic vertebrate hearts: hypoxia, acidosis, and low temperature. *Physiol. Rev.* **74**, 221-258.

Ekström, A., Jutfelt, F. and Sandblom, E. (2014). Effects of autonomic blockade on acute thermal tolerance and cardioventilatory performance in rainbow trout, *Oncorhynchus mykiss*. *J. Therm. Biol.* **44**, 47-54.

Ekström, A., Brijs, J., Clark, T. D., Gräns, A., Jutfelt, F. and Sandblom, E. (2016a). Cardiac oxygen limitation during an acute thermal challenge in the European perch: Effects of chronic environmental warming and experimental hyperoxia. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **311**, R440-R449.

Ekström, A., Hellgren, K., Gräns, A., Pichaud, N. and Sandblom, E. (2016b). Dynamic changes in scope for heart rate and cardiac autonomic control during warm acclimation in rainbow trout. *J. Exp. Biol.* **219**, 1106-1109.

Ekström, A., Jutfelt, F., Fredrik Sundstrom, L., Adill, A., Aho, T. and Sandblom, E. (2016c). Chronic environmental warming alters cardiovascular and haematological stress responses in European perch (Perca fluviatilis). *J. Comp. Physiol. B* **186**, 1023-1031.

Farrell, A. P. (2009). Environment, antecedents and climate change: lessons from the study of temperature physiology and river migration of salmonids. *J. Exp. Biol.* **212**, 3771-3780.

Gollock, M. J., Currie, S., Petersen, L. H. and Gamperl, A. K. (2006). Cardiovascular and haematological responses of Atlantic cod (*Gadus morhua*) to acute temperature increase. *J. Exp. Biol.* **209**, 2961-2970.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42.

Hillebrand, H., Soininen, J. and Snoeijs, P. (2010). Warming leads to higher species turnover in a coastal ecosystem. *Glob. Change Biol.* **16**, 1181-1193.

Hilton, Z., Clements, K. D. and Hickey, A. J. R. (2010). Temperature sensitivity of cardiac mitochondria in intertidal and subtidal triplefin fishes. *J. Comp. Physiol. B* **180**, 979-990.

Hochachka, P. W., Storey, K. B., French, C. J. and Schneider, D. E. (1979). Hydrogen shuttles in air versus water breathing fishes. *Comp. Biochem. Physiol. B* **63**, 45-56.

Hove-Madsen, L. and Gesser, H. (1989). Force frequency relation in the myocardium of rainbow trout. Effects of K+ and adrenaline. *J. Comp. Physiol. B* **159**, 61-69.

Hulbert, A. J. and Else, P. L. (1999). Membranes as possible pacemakers of metabolism. *J. Theor. Biol.* **199**, 257-274.

Hulbert, A. J., Pamplona, R., Buffenstein, R. and Buttemer, W. A. (2007). Life and death: metabolic rate, membrane composition, and life span of animals. *Physiol. Rev.* **87**, 1175-1213.

Iftikar, F. I. and Hickey, A. J. (2013). Do mitochondria limit hot fish hearts? Understanding the role of mitochondrial function with heat stress in *Notolabrus celidotus*. *PLoS ONE* **8**, e64120.

Iftikar, F. I., MacDonald, J. R., Baker, D. W., Renshaw, G. M. and Hickey, A. J. (2014). Could thermal sensitivity of mitochondria determine species distribution in a changing climate? *J. Exp. Biol.* **217**, 2348-2357.

Iftikar, F. I., Morash, A. J., Cook, D. G., Herbert, N. A. and Hickey, A. J. (2015). Temperature acclimation of mitochondria function from the hearts of a temperate wrasse (*Notolabrus celidotus*) *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **184**, 46-55.

Janssen, A. J. M., Trijbels, F. J. M., Sengers, R. C. A., Smeitink, J. A. M., van den Heuvel, L. P., Wintjes, L. T. M., Stoltenborg-Hogenkamp, B. J. M. and Rodenburg, R. J. T. (2007). Spectrophotometric assay for complex i of the respiratory chain in tissue samples and cultured fibroblasts. *Clin. Chem.* **53**, 729-734.

Jayasundara, N., Tomanek, L., Dowd, W. W. and Somero, G. N. (2015). Proteomic analysis of cardiac response to thermal acclimation in the eurythermal goby fish *Gillichthys mirabilis*. *J. Exp. Biol.* **218**, 1359-1372.

Johnston, I. A. (1977). A comparative study of glycolysis in red and white muscles of the trout (*Salmo gairdneri*) and mirror carp (Cyprinus carpio). *J. Fish Biol.* **11**, 575-588.

Kalinin, A. and Gesser, H. (2002). Oxygen consumption and force development in turtle and trout cardiac muscle during acidosis and high extracellular potassium. *J. Comp. Physiol. B* **172**, 145-151.

Kraffe, E., Marty, Y. and Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions. *J. Exp. Biol.* **210**, 149-165.

Larsen, S., Nielsen, J., Hansen, C. N., Nielsen, L. B., Wibrand, F., Stride, N., Schroder, H. D., Boushel, R., Helge, J. W., Dela, F. et al. (2012). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J. Physiol.* **590**, 3349-3360.

Lemieux, H., Tardif, J.-C., Dutil, J.-D. and Blier, P. U. (2010). Thermal sensitivity of cardiac mitochondrial metabolism in an ectothermic species from a cold environment, Atlantic wolffish (*Anarhichas lupus*). *J. Exp. Mar. Biol. Ecol.* **384**, 113-118.

Lennard, R. and Huddart, H. (1991). The effect of thermal stress on electrical and mechanical responses and associated calcium movements of flounder heart and gut. *Comp. Biochem. Physiol. A Physiol.* **98**, 221-228.

Lepage, G. and Roy, C. C. (1984). Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J. Lipid Res.* **25**, 1391-1396.

Mendonca, P. C. and Gamperl, A. K. (2010). The effects of acute changes in temperature and oxygen availability on cardiac performance in winter flounder (*Pseudopleuronectes americanus*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **155**, 245-252.

Munro, D. and Blier, P. U. (2012). The extreme longevity of *Arctica islandica* is associated with increased peroxidation resistance in mitochondrial membranes. *Aging Cell* **11**, 845-855.

Nielsen, J. S. and Gesser, H. (2001). Effects of high extracellular [K^+] and adrenaline on force development, relaxation and membrane potential in cardiac muscle from freshwater turtle and rainbow trout. *J. Exp. Biol.* **204**, 261-268.

Pelletier, D., Dutil, J.-D., Blier, P. U. and Guderley, H. (1994). Relation between growth rate and metabolic organization of white muscle, liver and digestive tract in cod, *Gadus morhua*. *J. Comp. Physiol. B* **164**, 179-190.

Pichaud, N., Ballard, J. W., Tanguay, R. M. and Blier, P. U. (2011). Thermal sensitivity of mitochondrial functions in permeabilized muscle fibers from two populations of *Drosophila simulans* with divergent mitotypes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R48-R59.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. and R Core Team (2014). nlme: linear and nonlinear mixed effects models. R package version 3.1-117. Available at http://CRAN.R-project.org/package=nlme.

Polak-Juszczak, L. and Komar-Szymczak, K. (2009). Fatty acid profiles and fat contents of commercially important fish from Vistula Lagoon. *Pol. J. Food Nutr. Sci.* **59**, 225-229.

Safer, B. (1975). The metabolic significance of the malate-aspartate cycle in heart. *Circ. Res.* **37**, 527-533.

Sandblom, E., Clark, T. D., Grans, A., Ekstrom, A., Brijs, J., Sundstrom, L. F., Odelstrom, A., Adill, A., Aho, T. and Jutfelt, F. (2016). Physiological constraints to climate warming in fish follow principles of plastic floors and concrete ceilings. *Nat. Commun.* **7**, 11447.

Sandström, O., Neuman, E. and Thoresson, G. (1995). Effects of temperature on life history variables in perch. *J. Fish Biol.* **47**, 652-670.

Seebacher, F., White, C. R. and Franklin, C. E. (2015). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nature Clim. Change* **5**, 61-66.

Shiels, H. A., Di Maio, A., Thompson, S. and Block, B. A. (2011). Warm fish with cold hearts: thermal plasticity of excitation-contraction coupling in bluefin tuna. *Proc. Biol. Sci.* **278**, 18-27.

Skalli, A., Robin, J. H., Le Bayon, N., Le Delliou, H. and Person-Le Ruyet, J. (2006). Impact of essential fatty acid deficiency and temperature on tissues' fatty acid composition of European sea bass (Dicentrarchus labrax). *Aquaculture* **255**, 223-232.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

Spinazzi, M., Casarin, A., Pertegato, V., Salviati, L. and Angelini, C. (2012). Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nat. Protoc.* **7**, 1235-1246.

Steinhausen, M. F., Sandblom, E., Eliason, E. J., Verhille, C. and Farrell, A. P. (2008). The effect of acute temperature increases on the cardiorespiratory performance of resting and swimming sockeye salmon (*Oncorhynchus nerka*). *J. Exp. Biol.* **211**, 3915-3926.

Suarez, R. K., Staples, J. F., Lighton, J. R. B. and West, T. G. (1997). Relationships between enzymatic flux capacities and metabolic flux rates: nonequilibrium reactions inmuscleglycolysis. *Proc. Natl. Acad. Sci. USA* **94**, 7065-7069.

Sunday, J. M., Bates, A. E. and Dulvy, N. K. (2012). Thermal tolerance and the global redistribution of animals. *Nature Clim. Change* **2**, 686-690.

Swimmer, Y., McNaughton, L., Moyes, C. and Brill, R. (2004). Metabolic biochemistry of cardiac muscle in three tuna species (bigeye, *Thunnus obesus*; yellowfin, *T. albacares*; and skipjack, *Katsuwonus pelamis*) with divergent ambient temperature and oxygen tolerances. *Fish Physiol. Biochem.* **30**, 27-35.

Thibeault, M., Blier, P. U. and Guderley, H. (1997). Seasonal variation of muscle metabolic organization in rainbow trout (*Oncorhynchus mykiss*). *Fish physiol. Biochem.* **16**, 139-155.

West, T. G., Arthur, P. G., Suarez, R. K., Doll, C. J. and Hochachka, P. W. (1993). *In vivo* utilization of glucose by heart and locomotory of exercising rainbow trout (*Onchorhynchys mykiss*). *J. Exp. Biol.* **177**, 63-79.

West, J. L., Bailey, J. R., Almeida-Val, V. M. F., Val, A. L., Sidell, B. D. and Driedzic, W. R. (1999). Activity levels of enzymes of energy metabolism in heart and red muscle are higher in north-temperate-zone than in Amazonian teleosts. *Can. J. Zool.* **77**, 690-696.

Vornanen, M., Haverinen, J. and Egginton, S. (2014). Acute heat tolerance of cardiac excitation in the brown trout (*Salmo trutta fario*). *J. Exp. Biol.* **217**, 299-309.