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Dynamic changes in cardiac mitochondrial metabolism during warm acclimation in rainbow trout.

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Summary statement

Mitochondrial oxygen consumption of cardiac permeabilized fibers decreases rapidly after acute warming and then remains stable during the subsequent warm acclimation process in rainbow trout.

Abstract

Although the mitochondrial metabolism responses to warm acclimation have been widely studied in fish, the time course of this process is less understood. Here, we characterise changes of rainbow trout (*Oncorhyncus mykiss*) cardiac mitochondrial metabolism during acute warming from 10 to 16˚C, and during the subsequent warm acclimation for 39 days (D). We repeatedly measured mitochondrial O_2 consumption in cardiac permeabilized fibers and functional integrity of mitochondria (*i.e.* mitochondrial coupling and cytochrome c effect) at two assay temperatures (10 and 16°C), as well as citrate synthase (CS) and lactate dehydrogenase (LDH) activities at room temperature. LDH and CS activities significantly increased between D0 (10ºC acclimated fish) and D1 (acute warming to 16˚C), while mitochondrial O_2 consumption measured at respective *in vivo* temperatures did not change. Enzymatic activities and mitochondrial O_2 consumption rates significantly decreased by D2, and remained stable during warm acclimation (D2-39). The decrease in rates of O_2 between D0 and D1 coincided with an increased cytochrome c effect and a decreased mitochondrial coupling, suggesting a structural/functional impairment of mitochondria during acute warming. We suggest that after two days of warm acclimation, a new homeostasis is reached, which may involve removal of dysfunctional mitochondria. Interestingly, from D2 onward, there was a lack of differences in mitochondrial O_2 consumption rates between the assay temperatures, suggesting that warm acclimation reduces the acute thermal sensitivity of mitochondria. This study provides significant knowledge on the thermal sensitivity of cardiac mitochondria that is essential to delineate the contribution of cellular processes to warm acclimation.

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Introduction

Temperature is a fundamental environmental factor affecting all metabolic and physiological processes in ectothermic animals such as fish. Thus, global warming and the anticipated increased frequency of extreme thermal events will impose constraints on ectothermic organisms, which may ultimately result in geographical redistribution of species and altered population structures (Sunday et al., 2010). However, the capacity of fish to maintain homeostasis and thermally adjust metabolic processes may provide resilience in a warming environment (Seebacher et al., 2015). This may be particularly true for mitochondrial metabolism, which uses oxygen to produce ATP that is central to all physiological processes. Although responses to both acute and chronic temperature increases have been widely studied in fish, the question of whether and how fish can dynamically modulate mitochondrial functions in response to both acute (*i.e.* rapid) and chronic (*i.e.* long-term) warming exposure is largely unresolved, although it has vast ecological implications.

During acute warming in fish, whole animal oxygen consumption rate generally increases with a temperature coefficient (Q_{10}) of 2-3 (Clark et al., 2008; Sandblom et al., 2016). When the warm exposure is prolonged, acclimation (*i.e.* reversible phenotypic plasticity) may allow some species to fully or partially mitigate the direct thermodynamic effects on oxygen consumption rate and key cardiovascular variables such as heart rate (Ekström et al., 2016a; Sandblom et al., 2016). Such thermal responses at the whole animal level can be linked to the need to redirect fuel reserves to power the metabolic pathways leading to the aerobic production of ATP by mitochondria (Iftikar and Hickey, 2013). Indeed, mitochondrial oxygen consumption and citrate synthase (CS) activity (a key enzyme of the tricarboxylic acid cycle and a proxy for aerobic metabolism) have been shown to follow a similar pattern as the whole animal metabolic rate during both acute and chronic temperature changes, although on a different scale (White et al., 2011). Specifically, acute warming of either whole animals (*in vivo*) or isolated tissues (*in vitro*)

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from ectotherms generally leads to an increase in mitochondrial oxygen consumption rates and in the overall catalytic capacities of metabolic enzymes (Hochachka and Somero, 1973). This is principally due to decreased activation energy of enzymatic reactions, and an increased probability of interaction between enzymes and substrates (Aledo et al., 2010). Moreover, the fluidity of mitochondrial membranes increases during the initial stage of warming due to modifications of phospholipid composition (Hazel and Landrey 1988a; Hazel and Landrey, 1988b; Hazel, 1992; Kraffe et al., 2007). This can influence the capacity of membrane-bound enzymes, such as the complexes of the electron transport system (ETS) that depend on the physical state of the lipid environment in which they function (Kraffe et al., 2007; Martin et al., 2013). Acute warming can also result in adverse effects subsequent to increased mitochondrial functions, such as unsustainable oxygen consumption and increased production of reactive oxygen species (Abele et al., 2002; Chung and Schulte, 2015). However, during warm acclimation for several weeks, decreases in rates of mitochondrial O_2 consumption and enzyme catalytic capacities, as well as adjustments of membrane architecture via changes in phospholipid proportions and fatty acid composition are typically observed, probably to limit these adverse effects (Hochachka and Somero, 1973; Hazel and Landrey, 1988a; Hazel and Landrey, 1988b, Guderley and Johnston, 1996; Guderley and St-Pierre, 2002; Kraffe et al., 2007; Fangue et al., 2009; Chung and Schulte, 2015). Nonetheless, a shortcoming of most laboratory acclimation experiments is that only "snapshots" of information are obtained (*e.g.* direct comparisons between fixed thermal acclimation groups), which preclude a more detailed determination of the dynamics of the acclimation process (Somero, 2015). Thus, the time required for thermal acclimation to be completed is often assumed rather than properly assessed, which may lead to biased conclusions regarding the acclimation status of the various parameters of interest (but see Ekström et al., 2016).

Moreover, due to the complexity of the thermal acclimation process, the different components of the mitochondrial metabolism affected by temperature are not clear, as they may depend on the time course and the intensity of the thermal exposure (Hazel, 1972; Sidell, 1983; Blier and Guderley, 1993; Hazel, 1995; St-Pierre et al., 1998; Guderley, 2004; Itoi et al., 2003; Kraffe et al., 2007; Iftikar et al., 2014; Jayasundara et al., 2015). For example, in isolated mitochondria from the red muscle of rainbow trout, *Oncorhynchus mykiss*, a temperature increase from 5 to 9°C over 2 days (Bouchard and Guderley, 2003) or from 5 to 15°C over 3 days (Kraffe et al.,

2007), did not significantly change rates of pyruvate oxidation (state 3 and state 4: oxygen consumption in presence and absence of ADP, respectively, when pyruvate is provided to the mitochondria). However, additional warming from 9 to 13°C over another two days resulted in an increase of state 3, followed by a decrease after further warming to 15°C, with the lowest values occurring after 8 weeks at 15°C (Bouchard and Guderley, 2003). Moreover, activities of key enzymes of mitochondrial metabolism such as cytochrome c oxidase (COX), *i.e.* the mitochondrial complex IV reducing O_2, as well as CS, were not affected during the first four weeks of warm acclimation in red muscle of trout (Bouchard and Guderley, 2003). In contrast, in white muscle, warming from 9°C to either 23 or 28°C over 1 or 2 days, respectively, significantly decreased CS activity in white sucker (*Castotomus commersoni)* and remained low for another 8 days (Hardewig et al., 2000).

The discrepencies between the abovementioned studies could be attibuted to the thermal regime tested, as a greater temperature increase will most likely impose significant metabolic and physiological constraints due to thermal stress. Another explanation could reside in the distinct metabolic needs between the tissues tested, as red muscle is predominantly oxidative while white muscle is mainly glycolytic. Surprisingly, the mitochondrial dynamic changes occurring in the heart, a highly oxidative tissue that is central to the overall oxygen transporting capacity of all vertebrates, have never been investigated during warm acclimation. Even so, it was recently shown that cardiac tissue of *Silurus meridionalis* displayed metabolic over-compensation with seasonal acclimatization, contrasting with other tissues (Yan and Xie, 2015). In fact, it has recently been suggested that thermal sensitivity of cardiac mitochondrial metabolism might be the very parameter determining species distribution during warming (Iftikar et al., 2014).

Here, we characterised the thermal sensitivity of mitochondrial metabolism in the heart of rainbow trout, *Oncorhynchus mykiss*, during acute warming *in vivo* from 10 to 16ºC. We then followed the subsequent dynamic modifications with chronic warm acclimation at 16ºC. For this purpose, we measured mitochondrial oxygen consumption at several steps of the electron transport system (ETS) in cardiac permeabilized fibers at two assay temperatures (10 and 16°C), as well as activities of CS and lactate dehydrogenase (LDH, marker of anaerobic metabolism) at room temperature, throughout the *in vivo* thermal exposure protocol at different intervals for 39 days (D). We hypothesized that the heart, as an aerobic, high ATP demanding organ, might

display a significant and rapid increase of mitochondrial capacities in response to an *in vivo* temperature increase to cover the metabolic costs that are essential for the maintenance of heart function. However, after an initial adjustment phase, warm acclimation would decrease these capacities below initial values to mitigate any deleterious effects.

Materials and methods

Experimental animals, temperature protocol and tissue sampling

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were purchased from a local fish farm (Antens laxodling AB, Alingsås, Sweden) and acclimated to 9.5 ±0.5°C for eight weeks in a 500L holding tank supplied with aerated freshwater from a recirculating system with a 12:12 h diurnal light:dark cycle. The temperature was controlled using a 9kW heater (K060, Värmebaronen, Kristianstad, Sweden). During the last week of the acclimation, the temperature was ~ 10ºC and therefore constitutes the initial acclimation temperature and was subsequently used as one of the assay temperatures.

A group of six fish (n = 6) was netted from the holding tank at 10ºC (*i.e.* D0) and killed with a sharp blow to the head. The water was then heated at a rate of 0.5ºC h^{-1} to reach the final temperature of 16ºC after 12 hours. A second similar sampling of fish was then performed approximately 1 hour after the water temperature had stabilized at 16°C (*i.e.* D1, n = 6). Thereafter, five subsequent samplings were performed, including at D2, D4, D11, D25 and D39 of warm acclimation to 16°C (n = 8 for each sampling day). At each sampling, individual body mass (BM) and the fork length (FK) were determined. The temperature range for the study was chosen as it is well within the range of temperatures experienced by these fish in the hatchery environment, and because we rationalized that a 6ºC temperature increase over 12 hours would be large enough to cause clear metabolic and physiological changes without causing an excessive stress.

The heart was quickly excised and placed in an ice-cold BIOPS relaxing solution containing 10 mM Ca-EGTA buffer, 0.1 µM free Ca^{2+}, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1 (Veksler et al., 1987; Letellier et al., 1992) before further dissection. The heart was then emptied of blood, the bulbus and atrium removed, and the ventricle was weighed and cut in three parts. Specifically, the ventricle apex was cut in two halves, which were permeabilized for mitochondrial respiration experiments (see below). The remaining part of the ventricle was stored

at -80°C for later enzymatic analyses. All experimental procedures were covered by ethical permit 65-2012 from the regional animal ethics committee in Gothenburg, Sweden.

Mitochondrial oxygen consumption experiments

Preparation of permeabilized cardiac fibers and mitochondrial respirometry setup

All preparation steps were conducted at 4°C. The parts of the apex were mechanically permeabilized in BIOPS using fine tweezers and were further chemically permeabilized by incubating the cardiac fibers in BIOPS complemented with 50 µg mL^{-1} saponin under continuous mixing. After this, the fibers were washed for 10 min in respiration medium containing 140 mM KCl, 5 mM KH_2PO_4, 20 mM HEPES, 3 mM $MgCl_2$, 0.5% (w/v) BSA, pH 7.2. The fibers were then blotted, weighed (3.3 -9.9 mg) using a Sartorius BP110S 0.1 mg readability (Sartorius, Göttingen, Germany), and immediately transferred into glass mini chambers (Loligo® Systems ApS, Tjele, Denmark) filled with air-saturated respiration medium. Mitochondrial respiration for each half of the apex was determined at assay temperatures of 10 and 16°C to match the temperatures used in the *in vivo* acclimation experiment. It took around one and a half hour to process and perform the experiments on each part. Thus, one of the halves of the apex was immediately processed for permeabilization after dissection, whereas the other part was stored in BIOPS solution until the first experiment had been performed and the system had been equilibrated at the next experimental temperature. To ensure that differences in the timing of the experiments did not bias the results, the order of assay temperatures was randomized between experimental runs. Additionally, the testing order was selected as a random factor in the statistical model (see below).

The chambers were immersed in a recirculating water bath for temperature control and equipped with oxygen sensor spots OXSP5 (Pyro Science GmbH, Aachen, Germany) fixed on the inner surface wall. Oxygen concentration was measured using FireStingO_2 probes connected to a FireStingO_2 fiber-optic oxygen meter (Pyro Science GmbH, Aachen, Germany) recording the signal emitted from the sensor spots. The exact volume of each chamber was determined by weighing the empty chambers, filling them with distilled water and closing them with their respective lids (so the excess water was removed), and then weighing them again. Constant

stirring of the medium inside the chamber was accomplished using mini stirrers connected to a control unit (Loligo® Systems ApS, Tjele, Denmark) set at 800 rpm. Background O_2 consumption (*e.g.* any oxygen consumed by the sensor spots) was evaluated and found to be negligible for all chambers throughout the experiments. The system was calibrated with zero oxygen after oxygen removal by sodium dithionite addition and with air-saturated respiration medium equilibrated at either 10 or 16°C. To test if the oxygen diffusion in permeabilized fibers was limiting the O_2 consumption by cardiac mitochondria (Gnaiger, 2009), we performed several pilot experiments at two oxygen concentration ranges; *i.e.* between 15 and 100% and between 80 and 150% of air-saturated oxygen conditions. We did not detect any significant differences between these two conditions in any of the parameters measured (less than 5% difference in the rates obtained between both conditions, n = 4 for each temperature) and therefore conducted all experiments between 15 and 100% of air-saturated oxygen concentration.

Substrate-uncoupler-inhibitor titration (SUIT) protocol for mitochondrial oxygen consumption

All measurements were expressed as means of mass-specific mitochondrial O_2 consumption rates expressed as pmol O_2 s^{-1} mg^{-1} (pmol of O_2 consumed per second per mg of permeabilized fibers) ± s.e.m. and are presented with the abbreviation(s) of the complex(es) contributing to the electron flux, followed by the state of respiration (complex-STATE, see Pichaud et al., 2013). All substrate concentrations were optimized to stimulate maximum rates of mitochondrial O_2 consumption. Pyruvate and malate (5mM and 0.5mM respectively) were first injected in the chambers to monitor the LEAK state (or state 2') for complex I (CI-LEAK), which is a resting state of non-phosphorylating respiration when mitochondrial oxygen consumption is maintained mainly to compensate for the proton leak (Gnaiger, 2009). After stabilization of the signal, excess ADP (5mM) was added to measure the OXPHOS state (or state 3) for complex I (CI-OXPHOS), which evaluated the mitochondrial oxygen consumption when the transport of electrons from complex I is coupled to the production of ATP. Subsequent injection of cytochrome c (10 µM) allowed evaluation of the functional integrity of the outer mitochondrial membrane (CIc-OXPHOS): a 5-15% increase of O_2 indicates damages of the outer mitochondrial membrane, leaking of endogenous cytochrome c and therefore dysfunctional mitochondria (Kuznetsov et al., 2008). Sequential injections of different compounds were then performed to measure additional mitochondrial O_2 consumption rates: succinate (10 mM) to determine maximum OXPHOS with

convergent electron flux from complex I and complex II (CI+CII-OXPHOS); FCCP (titration of 0.25 µM steps) to trigger uncoupled respiration for complex I and complex II as a measure of maximum ETS capacity (CI+CII-ETS); rotenone (0.5 µM) to inhibit complex I and measure the mitochondrial O_2 consumption due to the electron input through complex II in the uncoupled state (CII-ETS); and antimycin A (2.5 µM) to inhibit complex III and measure residual O_2 consumption (*i.e.* the oxygen consumed by oxidative side reactions occurring in permeabilized fibers) which was used to correct all the mitochondrial O_2 consumption rates.

After inhibition of complexes I and III, COX activity was measured to determine the maximal efficiency of this complex to consume oxygen. For this measurement, new respiration medium equilibrated at the respective assay temperatures was injected in the chamber to raise the oxygen concentration in the chamber and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, 0.5mM) and ascorbate (2mM) were added. Due to autoxidation of TMPD and ascorbate, chemical backgrounds were evaluated at the end of each experiment after inhibition of COX by sodium azide (100 mM) and were subtracted from the activity (Gnaiger et al., 1998).

Calculation of mitochondrial respiration ratios, temperature coefficients (Q_{10}), and morphological variables.

Two different ratios were calculated to evaluate the functionality and the quality of the mitochondrial preparations used in the experiment : (i) the P/L ratio (CI-OXPHOS/CI-LEAK) which is usually taken as a good indicator of mitochondrial quality and of mitochondrial coupling (Gnaiger, 2009; Iftikar et al., 2014); and (ii) the cytochrome c effect (CIc-OXPHOS/CI-OXPHOS) to evaluate the structural integrity of the outer mitochondrial membrane (Kuznetsov et al., 2008).

Temperature coefficients (Q_{10}) of mitochondrial respiration were calculated using the following formula:

$$Q_{10} = (R_2/R_1)^{10/(T2-T1)}$$

Two different Q_{10} values were calculated:

(i) the acclimation temperature Q_{10} was calculated from mean mitochondrial O_2 consumption rates obtained at the initial water temperature of 10˚C and at subsequent acclimation days after

warming to 16˚C, with R_1 representing the rates of mitochondrial O_2 consumption measured during D0 at T1=10°C and R_2 representing the rates of mitochondrial O_2 consumption measured during the other acclimation days (D1-39) at T2=16°C.

(ii) the assay temperature Q_{10} was calculated for individual fish (expressed as means ± s.e.m.) at each sampling day using the mitochondrial O_2 consumption rates obtained with the two *in vitro* assay temperatures (10 and 16°C) where R_1 represents the rates of mitochondrial O_2 consumption measured at T1=10°C and R_2 represents the rates of mitochondrial O_2 consumption measured at T2=16°C.

The relative ventricular mass (RVM) was calculated as:

RVM = Ventricle mass/BM

The fish condition factor was calculated as:

Condition factor = $(100*BM)/FL^3$

with BM in g and FL in cm.

Enzymatic analyses

The activities of LDH and CS were assayed on the frozen ventricular tissue samples. Homogenization of tissue was performed in 50 mM potassium phosphate buffer, pH 7.2. After centrifugation for 5 min at 500 *g*, the freshly prepared supernatant was used directly for enzymatic activity measurements. Both enzymes were measured in triplicates using a microplate reader (SpectraMax 290 microplate reader, Molecular Devices, Sunnyvale, CA, United States) at room temperature (24°C), according to Ekström et al. (2016b). While this approach did not allow us to infer the direct effects of temperature on enzymatic function, changes in their activities still provide a good general proxy for changes in aerobic (CS) and anaerobic (LDH) enzymatic capacities during warm acclimation. For LDH activity, homogenates were incubated with a 100 mM potassium phosphate buffer complemented with 0.16 mM, 0.4 mM NADH, and 0.4 mM pyruvate (omitted for the control), pH 7.2. Activity was measured at 340 nm for 4 min (ε_{340} = 6.22 mL cm^{-1} μmol^{-1}). CS activity was measured by incubating homogenates with a 100 mM

imidazole-HCl buffer complemented with 0.1 mM 5,5'-Dithiobis(2-nitrobenzoic acid), 0.1 mM acetylCoA and 0.15 mM oxaloacetate (omitted for the control), pH 8.0. Activity was measured at 412 nm for 4 min (ε_{412} = 13.6 mL cm^{-1} μmol^{-1}). All enzymatic activities are expressed as U g^{-1} of tissue, where U is 1µmol of substrate transformed per minute.

Statistical analyses

All statistical analyses were performed with the R software (version 3.1.0, Free Software Foundation; Boston, MA, USA). For the rates of mitochondrial O_2 consumption, and ratios calculated with these rates, the data were fitted to a linear mixed effects model, using the 'lme' function of the 'nlme' package. The acclimation days (*i.e.* D0, D1, D2, D4, D11, D25 and D39) and the *in vitro* assay temperatures (10 and 16°C) were included as fixed effects (acclimation day and assay temperature factors), whereas the order of testing was included as a random effect (see *Preparation of permeabilized cardiac fibers and mitochondrial respirometry setup*). For enzymatic activities, *in vitro* Q_{10} calculations, and morphological variables, one-way ANOVAs were performed, considering the acclimation day as fixed effect. In all cases, Tukey's HSD tests were performed when an effect was detected in the models. Normality and homogeneity of variances were verified beforehand using plots of residuals and Levene's tests respectively, and data were ln transformed when required. Statistical significance was set at $P<0.05$.

Results

F-values and significance from the mixed model on the different mitochondrial O_2 consumption rates studied are presented in Table 1. Results from one-way ANOVAs on enzymatic activites and assay temperature Q_{10} values, as well as specific comparisons obtained for all the parameters with posthoc Tukey´s HSD tests are presented below.

Mass specific mitochondrial O_2 consumption rates

In vivo temperature effects: differences between acclimation days

The mass specific mitochondrial O_2 consumption rates at the temperatures the fish experienced *in vivo* throughout the temperature exposure protocol (*i.e.* 10ºC for D0 and 16ºC for D1-39) are illustrated by the red line in Figure 1. Almost all mitochondrial O_2 consumption rates increased slightly (but not significantly) between D0 and D1, decreased significantly between D1 and D2, and then remained unchanged for the rest of the warm acclimation period. The relatively minor increase between D0 and D1 resulted in acclimation temperature Q_{10} values slightly higher than 1.0 for CI-LEAK, CI-OXPHOS and CI+CII-OXPHOS (1.32, 1.18 and 1.13 respectively). The Q_{10} values calculated between D0 and D1 for CI+CII-ETS, CII-ETS and COX were close to 1.0 (1.06, 0.94 and 0.99 respectively), suggesting that these parameters were virtually unaffected by the acute temperature increase. However, between D0 and D2-39, the acclimation temperature Q_{10}s were always <1.0 (data not shown) suggesting metabolic over-compensation.

In vitro temperature effects: differences between assay temperatures across acclimation days

For CI-LEAK, no specific differences were detected with posthoc tests between the two assay temperatures across acclimation days (Fig. 1), although a general effect of assay temperature was detected with the linear mixed model (Table 1). Fish acclimated to 10ºC yielded the highest CI-LEAK measured at the assay temperature of 16ºC, which then decreased during warm acclimation with corresponding values at D2-39 being significantly different from D0 (Fig. 1).

For CI-OXPHOS, CI+CII-OXPHOS, and for CI+CII-ETS, a significant decrease was observed between D0 and D2-39 when measured at 16°C, but the only significant difference when assayed at 10ºC was observed between D0 and D39 (Fig. 1). Moreover, significant differences were detected between the assay temperatures for CI-OXPHOS, CI+CII-OXPHOS and CI+CII-ETS at D0 and D1, with mitochondrial O_2 consumption rates being significantly lower when measured at 10°C (Fig. 1). These differences seem mainly driven by the capacity of complex I because when this complex was inhibited (*i.e.* CII-ETS), there were no differences across acclimation days or between assay temperatures (Fig. 1). The COX activity exhibited the same general pattern as CI-OXPHOS, CI+CII-OXPHOS and CI+CII-ETS. At the assay temperature of 16°C, a decrease occurred with significantly higher mitochondrial O_2 consumption rates at D0 than when fish were warm acclimated from D2 to 39 (Fig. 1). Similarly, when assayed at 10°C, COX activity was significantly higher in 10ºC acclimated fish compared to most subsequent warm acclimation days (*i.e.* D1, D2, D4 and D39). Collectively, these results indicate a rapid resetting of mitochondrial metabolism, which appeared to be completed from the second day of the warm acclimation protocol as there were no further changes throughout the remainder of the acclimation period.

The assay temperature Q_{10} are presented in Table 2. These Q_{10}s calculated for CI-LEAK, CI-OXPHOS, CI+CII-OXPHOS, CI+CII-ETS and COX were all influenced by the acclimation days ($F_{6,45}$=2.64, P=0.028; $F_{6,45}$=4.72, P<0.001; $F_{6,45}$=5.48, P<0.001; $F_{6,45}$=3.73, P=0.004; $F_{6,45}$=2.84, P=0.020 respectively). However, only the assay temperature Q_{10}s for CI-OXPHOS, CI+CII-OXPHOS and COX were significantly different among acclimation days, as detected with posthoc tests (Table 2). For these three parameters, a slight increase was observed immediately upon warming between D0 and D1, followed by a significant decrease between D1 and D2 (Table 2). These values then remained relatively stable throughout the warm acclimation protocol and were close to those obtained in the 10ºC acclimated fish at D0.

Ratio for mitochondrial functionality and quality

All preparations showed well-coupled mitochondrial respiration (P/L > 5, Fig. 2A). P/L was not influenced by *in vitro* assay temperature (Table 1), suggesting that CI-LEAK and CI-OXPHOS were similarly affected by the increase in assay temperature. However, P/L was significantly

influenced by acclimation days (Table 1), with values significantly decreasing between D0 and D1, and then remaining unchanged throughout the warm acclimation period, although there were no significant differences in P/L measured between D0 and D4-25 (Fig. 2A). The calculated cytochrome c effect (Fig. 2B) showed a strong acclimation day effect, as it was significantly increased upon warming at D1, followed by a significant decrease on D2 (Table 1). At D2 this cytochrome c effect was still significantly higher than D0 and than D4-39, but lower than D1 (Fig. 2B). At warm acclimation D4 and onwards, the cytochrome c effect returned to the values measured at D0 in 10ºC acclimated fish (Fig. 2B).

When considering these parameters measured at the respective acclimation temperatures (*i.e.* measured at 10ºC for D0 and at 16ºC for D1-39), P/L were not significantly different. An increase of cytochrome c effect was observed between D0 and D1-2, but afterwards, it returned to initial values and was also significantly lower than at D1-2 (Table 2).

Enzymatic analysis

Both LDH and CS activities were significantly influenced by the acclimation days ($F_{6,48}$ = 4.04, P=0.002 for CS; $F_{6,48}$=2.41, P=0.04 for LDH), with similar patterns across warm acclimation for both enzymes (Fig. 3). The activity of both enzymes increased significantly with *in vivo* warming and peaked on D1, but then rapidly decreased and were not significantly different from the initial values at D0 for the duration of the warm acclimation period (with significance detected for CS between D1 and D25 and between D1 and D39).

Morphological variables

All morphological variables are reported in Table 3. Although no significant differences were detected for body mass, fork lenght and condition factor between fish acclimated to 10ºC and 16ºC, the relative ventricular mass decreased significantly after 25 days of warm acclimation (P=0.006 between D0 and D25; P=0.012 between D0 and D39).

Discussion

In the fish heart, mitochondrial metabolism is essential for the provision of ATP and might therefore be a prime target for adjustments should thermal changes occur. This study examined the effects of both acute and chronic warming on the cardiac mitochondrial metabolism of rainbow trout transitioning from 10 to 16 ºC, a temperature increase that can be considered benign for this species. We worked on farmed rainbow trout and from an ecological perspective, our inferences about these fish are limited. However, they still represent a good model for studying the effects of temperature during warm acclimation. The current study revealed a general elevation of aerobic and anaerobic metabolism after acutely increasing the temperature from 10 to 16ºC (*i.e.* between D0 and D1), as observed by the changes in CS and LDH activities, respectively. Contrary to predictions, however, mitochondrial capacities as measured by O_2 consumption rates did not change significantly across the same measurement days (D0 and D1), and at D2 and onwards, a significant decrease of most mitochondrial O_2 consumption rates and enzymatic activities was observed with warm acclimation. During the initial two days of acute temperature shift, an impairment of the outer mitochondrial membrane likely occurred as indicated by the increased cytochrome c effect, which likely caused a small reduction in mitochondrial coupling (*i.e.* P/L). Interestingly, this impairment of the outer mitochondrial membrane was not observed from D4 onwards suggesting a restoration of cell homeostasis. Moreover, acute thermal insensitivity of mitochondrial capacities was observed from D4, as indicated by lack of differences in mitochondrial O_2 consumption rates between assay temperatures. This might serve to widening the zone of thermal independence and therefore the tolerance to unpredicted temperature variations.

No differences were detected in COX activity during the warm acclimation process. This complex is thought to act as an electron sink and to limit the ETS capacity (Arnold, 2012), and it has been suggested that thermally induced changes in COX affect respiratory regulation mainly through an impact on the redox state of the ETS (Blier and Lemieux, 2001). However, this complex is usually found to have an excess capacity that is required for adequate functioning of mitochondria at the different temperatures encountered by the species, thus allowing the ETS to be mainly in an oxidized state and to ensure a sharp thermodynamic gradient in the ETS under

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most thermal conditions (Blier and Lemieux, 2001; Hilton et al., 2010; Blier et al., 2013). Surprisingly, we did not find such an excess as the values for COX were only slightly higher than those for CI+CII-OXPHOS. Usually, this excess is more pronounced when assayed *in vitro* at high temperature (Hilton et al., 2010). It is therefore possible that the assay temperature employed here was not high enough to display such excess, and that COX functional capacity was not affected by raising the water temperature between D0 and D1. The substantial *in vitro* thermal sensitivity of COX as indicated by the assay temperature Q_{10}s probably reflects an adjustment of the redox state of the ETS due to simultaneous *in vitro* and *in vivo* temperature changes, which is also seen at the level of complex I and complex II during the OXPHOS state (*i.e.* CI-OXPHOS and CI+CII-OXPHOS). From D2 onwards, however, mitochondria demonstrated limited thermal sensitivity. While this result might seem surprising, it has already been demonstrated that in some marine invertebrates (Newell and Pye, 1970; Newell and Pye, 1971) and in fish (Fangue et al., 2009), mitochondrial respiration can exhibit a large zone of thermal independence across environmentally relevant temperatures, which seems to be the case here after an initial critical period. This might result in an upward shift of the zone of thermal independence.

The modifications observed for CI-LEAK, CI-OXPHOS, CI+CII-OXPHOS and CI+CII-ETS with warm acclimation suggest that the capacity of complex I was primarily affected, as the rate of mitochondrial oxygen consumption was unchanged when this complex was inhibited during CII-ETS. The complex I of the ETS is one of the main contributors to the proton motive force allowing the phosphorylation of ADP into ATP. Indeed, our results are in accordance with the hypothesis that this complex is a target for modification during thermal acclimation (Efremov et al., 2010), which was recently elegantly demonstrated in *Fundulus heteroclitus* liver (Chung and Schulte, 2015).

Between D0 and D1, significant increases of LDH and CS activities were detected, which were not related to changes in morphological variables such as condition factor or relative ventricular mass, suggesting an increase in aerobic and anaerobic cardiac capacity. However, from D2 onwards, the activities of both LDH and CS returned to values observed at D0 before warming. CS activity displayed the lowest values at D25 and D39 with significant differences detected when compared to D1. Interestingly, we also observed a decrease of relative ventricular mass for

these days suggesting a structural remodeling of the cardiac tissue. This remodeling was not surprising and likely constitutes a compensatory mechanism as a reduced ventricular mass could still sufficiently maintain cardiac output as the force and rate of ventricular contraction typically increase at high temperature (Gamperl and Farrell, 2004; Ekström et al., 2016a).

A reduced mitochondrial coupling (decreased P/L) was also observed between D0 and D1. This P/L reduction coincided with an increased mitochondrial O_2 consumption after addition of exogenous cytochrome c. Cytochrome c is a peripheral protein of the mitochondrial inner membrane that is only loosely bound and is essential for the transport of electrons between complex III and complex IV. If the outer membrane of mitochondria is damaged, the endogenous cytochrome c can be released and the addition of exogenous cytochrome c will increase the mitochondrial oxygen consumption (Hand and Menze, 2008; Kuznetsov et al., 2008; Iftikar and Hickey, 2013). The increased cytochrome c effect observed between D0 and D1 with acute warming therefore suggests an impaired mitochondrial functional capacity. When measuring mitochondrial functions in isolated mitochondria from red muscle, Guderley and Bouchard (2003), as well as Kraffe et al. (2007), found that warming neither changed the rates of pyruvate oxidation (CI-OXPHOS) nor COX activity during the first week of warm acclimation (from either 5 to 9ºC over two days (Bouchard and Guderley, 2003) or from 5 to 15ºC over three days (Kraffe et al., 2007)). However, these studies used isolated mitochondria from red muscle and a much slower rate of thermal change (4°C over 2 days for the first and second weeks of the warm acclimation and 2°C over 1 day for the third week in Guderley and Bouchard, 2003; 10°C over 3 days in Kraffe et al., 2007), which might have prevented the mitochondrial loss of function observed in our study. Therefore, the use of different tissues and thermal regimes might explain the discrepancies between these earlier studies and the present study when comparing the initial phase of the warm acclimation. On the long-term, however, the results are quite similar to those found for isolated mitochondria from red muscle in the previous studies, both at the level of enzymatic activities and mitochondrial oxygen fluxes, when the temperature was increased from 9ºC to 15ºC (Bouchard and Guderley, 2003).

The impaired mitochondrial functions suggested by the cytochrome c effect and the decreased P/L between D0 and D1 might also be explained by a change in mitochondrial membrane composition resulting in membrane destabilization. Indeed, it has been shown in both trout

muscle (Kraffe et al., 2007) and plasma membranes from trout kidney (Hazel and Landrey, 1988a; Hazel and Landrey, 1988b) that the proportions of phospholipid classes and phospholipid headgroup composition represent early detectable responses following an increase in water temperature. For example, when the temperature was increased from 4.5 to 9°C within 8 hours, a decline of monounsaturated species of phosphatidylcholine was observed in plasma membranes from trout kidney (Hazel and Landrey, 1988a; Hazel and Landrey, 1988b), constituting a rapid and central mechanism for regulation of physical and functional properties of cell membranes (Hazel, 1995). Considering that these changes can rapidly occur with a mild temperature increase of 4.5ºC over 8 hours (Hazel and Landrey, 1988a; Hazel and Landrey, 1988b), it is therefore likely that such modifications are involved in the increased cytochrome c effect observed in our study after an increase of 6ºC over 12 hours. Moreover, it has also been suggested that changes in oxidative capacities and COX activity occurring during warm acclimation could be related to modifications of the composition of cardiolipin (Kraffe et al., 2007), a minor lipid class found in mitochondrial membranes that plays an important role in the micro-environment of the oxidative phosphorylation enzymes (Paradies et al., 2002; Schlame et al., 2005). Interestingly, it has been shown that cardiolipin anchors cytochrome c to the inner mitochondrial membrane and that externalization of cardiolipin (for example due to peroxidation) to the outer mitochondrial membrane releases cytochrome c in the cytoplasmic space (Chu et al., 2013; Bradley et al., 2016). Thus, it is possible that the rapid modifications in mitochondrial capacities and the increased cytochrome c effect observed in our study at D1 might be related to modifications of mitochondrial cardiolipin. This could subsequently lead to membrane destabilization during the initial phase of the warm acclimation, but further studies are required to verify that.

The increase in mitochondrial O_2 consumption following addition of cytochrome c was not seen on sampling days after D2, which exhibited values similar to before the temperature increase. This could indicate that dysfunctional mitochondria had been eliminated from the cell after D2. In fish, mitochondrial volume density varies with water temperature and thermal acclimation can induce changes in the cristae surface density of mitochondria as well as mitochondrial volume density (Tyler and Sidell, 1984; Guderley and Johnston, 1996; Guderley et al., 1997; St Pierre et al., 1998; Guderley, 2004; White et al., 2011; Dhillon and Schulte, 2011), but how and when such changes occur during the acclimation process is unknown. Two different mechanisms are believed to be used by the cell to ensure mitochondrial homeostasis and survival; apoptosis and

mitophagy, but to the best of our knowledge none of these have been considered in the context of thermal acclimation. Of interest, both processes are potently regulated by cardiolipin and its function to anchor cytochrome c to the inner mitochondrial membrane, and have been shown to be triggered by cardiolipin modifications (Sorice et al., 2004; Chu et al., 2013; Bradley et al., 2016). Release of cytochrome c triggers apoptosis and cell death (Goldstein et al., 2000), and it is therefore possible that the increased cytochrome c effect we observed upon warming induced this mechanism. Another mechanism that may also be triggered when mitochondria are impaired is mitophagy, *i.e.* the destruction of dysfunctional mitochondria by autophagy (Kim et al., 2007). Interestingly, mitophagy is believed to be part of a more complex response, the mitochondrial unfolded-protein response, which is a stress response triggered by accumulation of unfolded or misfolded proteins in the mitochondrial matrix and subsequent activation of molecular chaperones such as heat shock protein 60 (Hsp60) and mitochondrial Hsp70 (Haynes et al., 2013; Jovaisaite et al., 2014). Even though little information is available about Hsp60 in fish (but see Buckley et al., 2006), it has been demonstrated that after acute heat stress (Currie et al., 2000; Deane and Woo, 2005), or during mild warming (Logan and Somero, 2011), the transcript abundance of Hsp70 is initially increased, but then decreases with further acclimation (Currie et al., 2000; Deane and Woo, 2005). It is therefore possible that after the temperature was raised from 10 to 16ºC on D1 in the present study, alteration of cardiolipin leading to mitochondrial impairment resulted in the triggering of the mitochondrial unfolded-protein response and stimulation of mitophagy to re-establish the mitochondrial homeostasis after D2. This hypothesis might be worth exploring by measuring transcript levels and/or protein levels of Hsp60 and mitochondrial Hsp70. Additionally, protein levels of autophagic marker such as the microtubule-associated protein 1-light chain 3B protein (MAP1-LC3B) in parallel with measurement of mitochondrial protein levels could indicate the occurrence of mitophagy during the time course of acclimation to warmer temperatures.

In summary, we show that the cardiac mitochondrial metabolism in rainbow trout is rapidly altered following an acute increase in environmental temperature. The small increase of mitochondrial oxygen consumption, as well as a significant increase of LDH and CS activities probably reflect an adjustment of both anaerobic and aerobic metabolism. On D2 the significant decrease of mitochondrial capacities indicates that the temperature increase induces a structural modification of mitochondrial membrane phospholipids such as cardiolipin, leading to functional

impairment, which was supported by the increase of the cytochrome c effect at D1 and D2. We suggest that these impaired mitochondria are likely removed by mitophagy, which allowed the re-establishment of cell homeostasis after the second day of acclimation to the higher temperature. Moreover, from D2 onwards, we observed that rates of mitochondrial O_2 consumption were similar when measured at either of the the two assay temperatures suggesting thermal independence, which might help the fish to maintain cardiac mitochondrial capacities accross environmentally relevant temperature variations during warm acclimation.

The current findings of reestablishment of cellular and mitochondrial homeostasis within 2 days following acute warming contrast with the much more prolonged and complex acclimation responses of integrated cardiovascular functions following an almost identical acclimation protocol in trout (Ekström et al., 2016a). Indeed, the scope for heart rate, *i.e.* the difference between resting and maximum heart rates, represents a key determinant of overall cardiovascular oxygen transport capacity (Farrell et al., 2009), and was found to exhibit a more complex warm acclimation pattern. This pattern was characterised by an initial increase due to increases in both resting and maximum heart rate, followed by a rapid decline on D2 due to reduced maximum heart rate, and then a subsequent increase due to a gradually increasing cholinergic tone reducing the resting heart rate (Ekström et al., 2016a). Consequently, it took at least 39 days for heart rate scope to reach a new steady state following this temperature increase. Thus, there are striking differences in warm acclimation rates between integrated cardiovascular functions, determining oxygen transport capacity at the whole animal level, and mitochondrial functions underlying oxidative capacities at the cellular level as observed in the present study. These findings not only highlight the need for detailed time course investigations at multiple levels of organisation to delineate the different mechanisms involved in warm acclimation, but also to reveal potential time-dependent constraints on aerobic metabolic rate with environmental warming. Such information would further our understanding of the ecological impacts of global warming, which is of paramount importance considering the anticipation of more frequent heat waves and increasing water temperatures challenging fish populations throughout the world.

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Competing interests

The authors declare no conflict of interest.

Author contributions

N.P. and E.S. conceived and designed the experiments. N.P. performed the experiments, analysed data and drafted the manuscript. N.P., A.E., E.S. and K.H. participated in the experimental work and maintenance of fish. All authors contributed feedback to the writing process and approved the manuscript.

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Tables

Table 1. F-values from the linear mixed model on mass-specific mitochondrial oxygen consumption rates.

	Denominator *df*	Acclimation Day Num *df* = 6	Assay Temperature Num *df* = 1	Interaction Num *df* = 6
P/L (CI-OXPHOS/CI-LEAK)	89	3.38**	0.46	0.95
Cytochrome c effect	89	24.8***	1.6	1.5
O_2 consumption rates				
CI-LEAK	89	4.67***	8.43**	2.28*
CI-OXPHOS	89	10.67***	12.14***	4.05**
CI+CII-OXPHOS	89	11.35***	13.05***	3.78**
CI+CII-ETS	89	10.90***	12.10***	2.85*
CII-ETS	89	2.08	0.52	1.07
COX	89	11.43***	24.76***	2.36*

*P<0.05, **P<0.01, ***P<0.001

Table 2. Thermal sensitivity (Q_{10}) calculated between assay temperatures (10 and 16°C).

Acclimation Day (***in vivo*** **temperature)**	Day 0 (10°C)	Day 1 (16°C)	Day 2 (16°C)	Day 4 (16°C)	Day 11 (16°C)	Day 25 (16°C)	Day 39 (16°C)
Q_{10} for mitochondrial O_2 consumption rates:							
CI-LEAK	1.57 ± 0.16	1.71 ± 0.17	1.15± 0.28	1.16 ± 0.14	1.12 ± 0.17	1.01 ± 0.10	1.28 ± 0.08
CI-OXPHOS	1.67 ± 0.15^{a,b}	2.21 ± 0.32^{a}	1.06 ± 0.14^{b}	1.10 ± 0.08^{b}	1.27 ± 0.20^{b}	1.01 ± 0.11^{b}	1.17 ± 0.14^{b}
CI+CII-OXPHOS	1.66± 0.15^{a,b}	1.91 ± 0.13^{a}	1.04 ± 0.10^{c}	1.12 ± 0.08^{b,c}	1.26 ± 0.21^{b,c}	1.01 ± 0.10^{c}	1.19 ± 0.14^{b,c}
CI+CII-ETS	1.55 ± 0.12	1.74 ± 0.09	1.03 ± 0.13	1.16 ± 0.10	1.28 ± 0.21	1.02 ± 0.09	1.22 ± 0.11
CII-ETS	1.10 ± 0.33	1.35 ± 0.19	1.34 ± 0.23	0.86 ± 0.14	1.09 ± 0.10	1.22 ± 0.22	1.33 ± 0.16
COX	1.46 ± 0.11^{a,b}	2.12 ± 0.23^{a}	1.17 ± 0.16^{b}	1.20 ± 0.09^{b}	1.46 ± 0.28^{a,b}	1.20 ± 0.15^{b}	1.34 ± 0.18^{a,b}

Values are presented as means±s.e.m. Dissimilar letters denote significant differences between the different days of the experiment.

Table 3. Morphological variables of rainbow trout (*Oncorhynchus mykiss*) sampled during the acclimation protocol.

Acclimation Day (*in vivo* Temperature)	BM (g)	FL (mm)	CF	RVM (%)
0 (10ºC)	38.8±1.9	155.2±6.1	1.08±0.06	0.102±0.005[a]
1 (16ºC)	37.9±1.8	151.8±6.4	1.07±0.06	0.102±0.005[a]
2 (16ºC)	37.5±1.8	149.5±6.7	1.07±0.06	0.103±0.005[a]
4 (16ºC)	37.7±2.0	152.2±6.6	1.05±0.07	0.103±0.006[a]
7 (16ºC)	40.1±2.4	157.8±7.3	1.06±0.06	0.104±0.006[a]
11 (16ºC)	37.3±1.8	149.6±6.4	1.04±0.05	0.097±0.005[a,b]
25 (16ºC)	38.5±1.9	154.2±6.7	1.04±0.06	0.087±0.004[b]
39 (16ºC)	39.8±2.0	157.4±6.9	1.03±0.07	0.091±0.004[b]

Mean values ±s.e.m. for body mass (BM), fork length (FL), condition factor (CF) and relative ventricle mass (RVM) measured on fish acclimated to 10°C and during the warm acclimation to 16°C. Letters denote statistical differences between acclimation days.

Figures

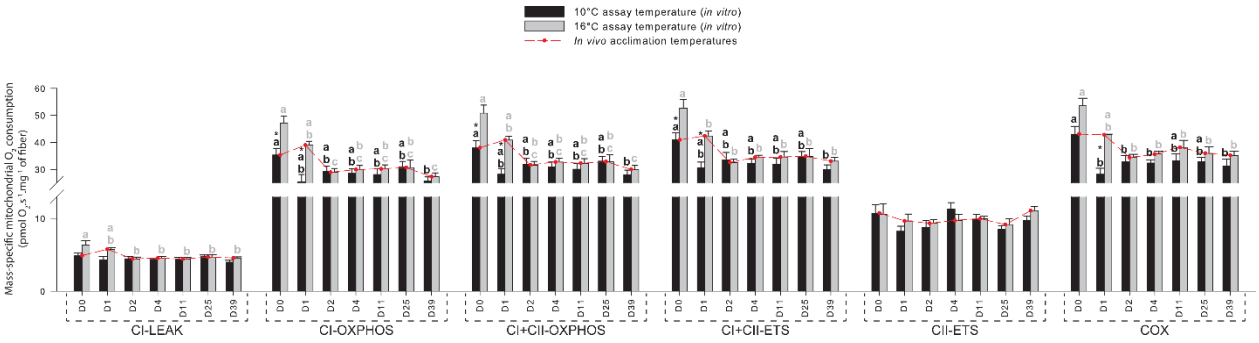

Figure 1. Effect of warm acclimation on mass-specific mitochondrial O_2 consumption rates measured in permeabilized cardiac fibers from rainbow trout (*Oncorhynchus mykiss*). Mitochondrial functions were measured in presence of pyruvate+malate (CI-LEAK), +ADP (CI-OXPHOS), +succinate (CI+CII-OXPHOS), +FCCP (CI+CII-ETS), +rotenone (CII-ETS), +TMPD+ascorbate (COX). Bars represent the results obtained when measurements were performed at the two different assay temperatures, 10 (black bars) and 16˚C (grey bars), and the red line represents the results obtained when measurements were performed at the temperatures experienced *in vivo* by the fish before (*i.e.* on D0 at 10˚C, n=6) and during warm acclimation (on D1-39 at 16°C, n=6 for D1 and n=8 for D2-39). Results are means ± s.e.m. Statistical differences were obtained with posthoc Tukey's test when an effect of the interaction acclimation day×assay temperature was observed with the linear mixed model. * depicts differences between the two *in vitro* assay temperatures assayed the same acclimation day; small letters depict differences between acclimation days at each assay temperature (black letters for 10°C and grey letters for 16°C).

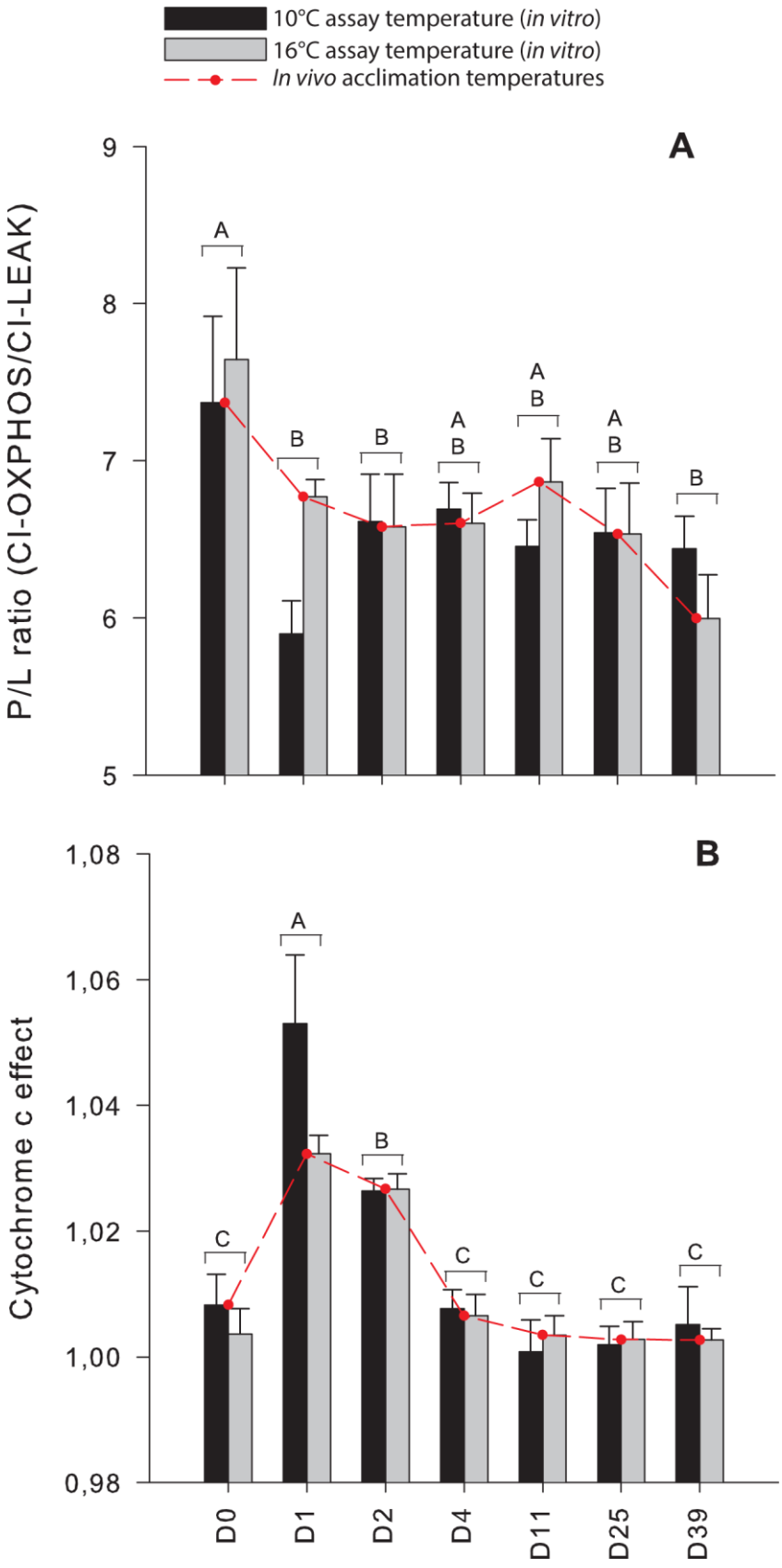

Figure 2. Effect of warm acclimation on the mitochondrial coupling and outer mitochondrial membrane integrity. Data are presented as means ± s.e.m. and illustrates (A) Mitochondrial coupling (P/L = CI-OXPHOS/CI-LEAK) and (B) Cytochrome c effect calculated after and before injection of cytochrome c (CIc-OXPHOS/CI-OXPHOS). Bars represent the results obtained when measurements were performed at 10 (black bars) and 16˚C (grey bars), and the red line represents the results obtained when measurements were performed at the temperatures experienced *in vivo* by the fish before (*i.e.* on D0 at 10˚C, n=6) and during warm acclimation (on D1-39 at 16°C, n=6 for D1 and n=8 for D2-39). Dissimilar capital letters denote statistical differences obtained with posthoc Tukey's test between acclimation days when an effect of acclimation day was observed with the linear mixed model.

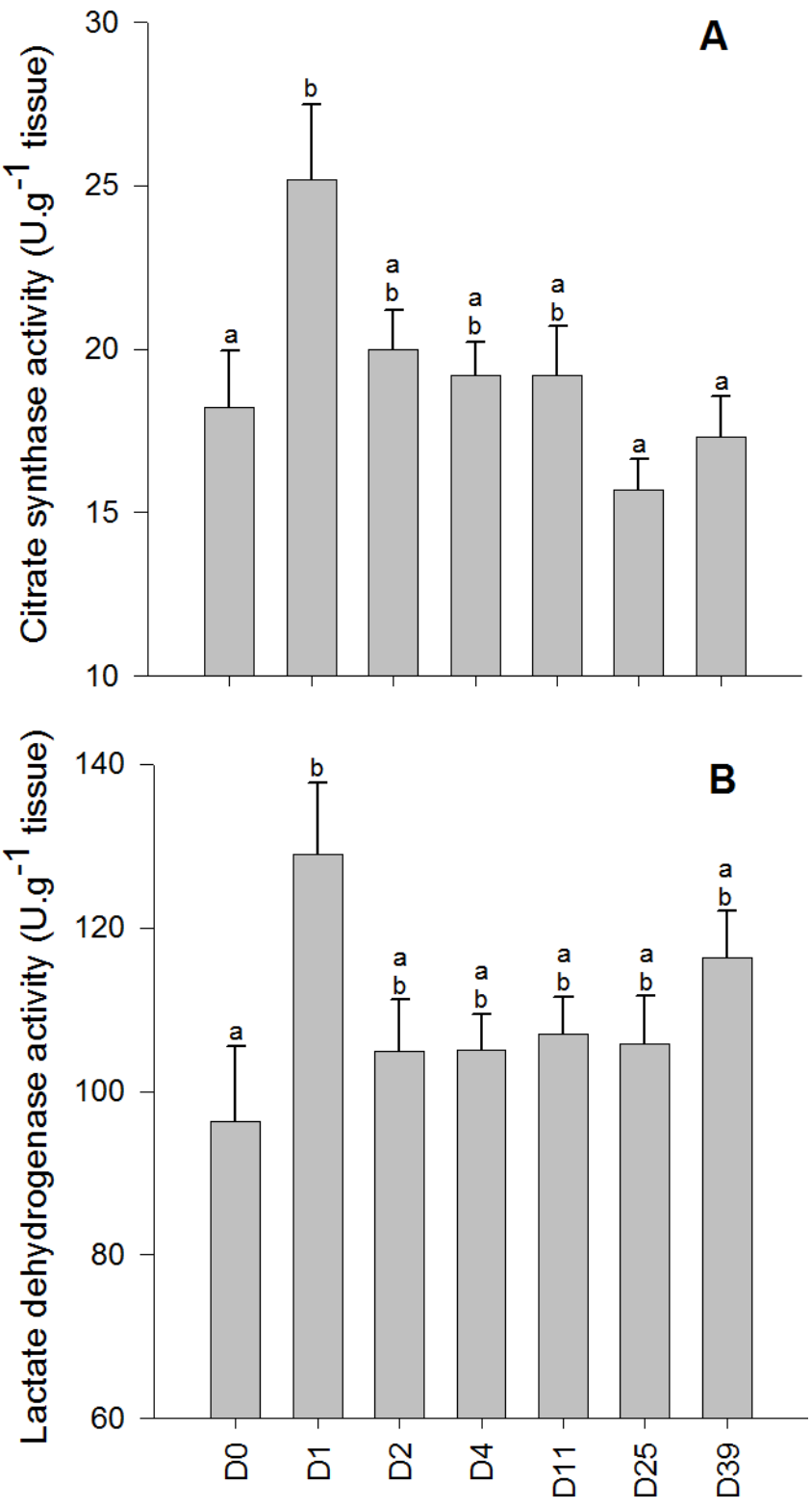

Figure 3. Enzymatic activities of citrate synthase and lactate dehydrogenase measured in the heart of rainbow trout (*Oncorhynchus mykiss*). Results are presented as means ± s.e.m. and illustrate (A) Citrate synthase and (B) Lactate dehydrogenase activites expressed as $U.g^{-1}$ of tissue, where U is 1µmol of substrate transformed per minute. n=6 for D0 and D1; n=8 for D2-39. Dissimilar letters denote statistical differences between acclimation days obtained with posthoc Tukey's test when an effect was detected with one-way ANOVA.