RESEARCH ARTICLE

Dynamic changes in cardiac mitochondrial metabolism during warm acclimation in rainbow trout
Nicolas Pichaud1,2,*, Andreas Ekström2, Kim Hellgren2 and Erik Sandblom2

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 characterized the changes of rainbow trout (Oncorhynchus mykiss) cardiac mitochondrial metabolism during acute warming from 10 to 16°C, and during the subsequent warm acclimation for 39 days. We repeatedly measured mitochondrial oxygen consumption in cardiac permeabilized fibers and the functional integrity of mitochondria (i.e., mitochondrial coupling and cytochrome c effect) at two assay temperatures (10 and 16°C), as well as the activities of citrate synthase (CS) and lactate dehydrogenase (LDH) at room temperature. LDH and CS activities significantly increased between day 0 (10°C acclimated fish) and day 1 (acute warming to 16°C) while mitochondrial oxygen consumption measured at respective in vivo temperatures did not change. Enzymatic activities and mitochondrial oxygen consumption rates significantly decreased by day 2, and remained stable during warm acclimation (days 2–39). The decrease in rates of oxygen between day 0 and day 1 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 2 days of warm acclimation, a new homeostasis is reached, which may involve the removal of dysfunctional mitochondria. Interestingly, from day 2 onwards, there was a lack of differences in mitochondrial oxygen 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.

KEY WORDS: Warm acclimation, Electron transport system, Fish, Heart, Mitochondria, Temperature

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 a geographical redistribution of species and altered population structures (Sunday et al., 2012). 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 (Q10) 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 (Ifikar 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., 2012). Specifically, acute warming of either whole animals (in vivo) or isolated tissues (in vitro) 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,b; Hazel, 1995; 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 the rates of mitochondrial oxygen 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,b; 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., 2016a).

Moreover, due to the complexity of the thermal acclimation process, the different components of 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, 1995; Sidell, 1983; Blier and Guderley, 1993; 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 2 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 oxygen, as well as CS, were not affected during the first four weeks of warm acclimation in the 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 discrepancies between the above-mentioned studies could be attributed to the thermal regime tested, as a greater temperature increase will most likely impose significant metabolic and physiological constraints because of thermal stress. Another explanation could reside in the distinct metabolic needs between the tissues tested, as red muscle is predominantly oxidative whereas 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 the cardiac tissue of Silurus meridionalis displayed metabolic over-compensation with seasonal acclimatization, compared 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).

In this study, we characterized the thermal sensitivity of mitochondrial metabolism in the heart of rainbow trout, Oncorhynchus mykiss, during acute warming in vivo from 10 to 16°C. We then followed the subsequent dynamic modifications with chronic warm acclimation at 16°C. For this purpose, we measured mitochondrial oxygen consumption at several steps of the ETS in cardiac permeabilized fibers at two assay temperatures (10 and 16°C), as well as the activities of CS and lactate dehydrogenase (LDH, a marker of anaerobic metabolism) at room temperature, throughout the in vivo thermal exposure protocol at different intervals for 39 days. 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 8 weeks in a 500 l 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 9 kW heater (K060, Värmebaronen, Kristianstad, Sweden). During the last week of the acclimation, the temperature was approximately 10°C 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 10°C (i.e. day 0) and killed with a sharp cranial blow. The water was then heated at a rate of 0.5°C h−1 to reach the final temperature of 16°C after 12 h. A second similar sampling of fish was then performed approximately 1 h after the water temperature had stabilized at 16°C (i.e. day 1, N=6). Thereafter, five subsequent samplings were performed, including at days 2, 4, 11, 25 and 39 of warm acclimation to 16°C (N=8 for each sampling day). At each sampling, individual body mass (Mb) and the fork length (FL) were determined. The temperature range for this 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 6°C temperature increase over 12 h 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 mmol l−1 Ca-EGTA buffer, 0.1 µM free Ca2+, 20 mmol l−1 imidazole, 20 mmol l−1 taurocholic acid, 50 mmol l−1 K-MES, 0.5 mmol l−1 dithiothreitol, 6.56 mmol l−1 MgCl2, 5.77 mmol l−1 ATP, 15 mmol l−1 phosphocreatine, pH 7.1 (Vekslser 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 into three parts. Specifically, the ventricle apex was cut into two halves, which were permeabilized for mitochondrial respiration experiments (see ‘Preparation of permeabilized cardiac fibers and mitochondrial respirometry setup’, 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 mmol l−1 KCl, 5 mmol l−1
mitochondrial oxygen consumption rates expressed as mass-specific. All measurements were expressed as means of mass-specific mitochondrial oxygen consumption rates. For each half of the apex, the measurement was determined at assay temperatures of 10 and 16°C to match the temperatures used in the in vivo acclimation experiment. It took approximately 1.5 h 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 half 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 ‘Statistical analyses’, 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 FireStingO2 probes connected to a FireStingO2 fiber-optic oxygen meter (Pyro Science) 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) set at 800 rpm. Background oxygen 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 adding sodium dithionite 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 oxygen consumption by cardiac mitochondria (Gnaiger, 2009), several pilot experiments at two oxygen concentration ranges were performed; 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 (≤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 oxygen consumption rates expressed as pmol O2 s⁻¹ mg⁻¹ 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 oxygen consumption. Pyruvate and malate (5 and 0.5 mmol l⁻¹, respectively) were first injected into 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 (5 mmol l⁻¹) 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 μmol l⁻¹) allowed for evaluation of the functional integrity of the outer mitochondrial membrane (C1c-OXPHOS); a 5–15% increase of oxygen 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 oxygen consumption rates: succinate (10 mmol l⁻¹) to determine maximum OXPHOS with convergent electron flux from complex I and complex II (CI+CII-OXPHOS); FCCP (titration of 0.25 μmol l⁻¹ steps) to trigger uncoupled respiration for complex I and complex II as a measure of maximum ETS capacity (CI+CII-ETS); rotenone (0.5 μmol l⁻¹) to inhibit complex I and measure the mitochondrial oxygen consumption due to the electron input through complex II in the uncoupled state (CII-ETS); and antimycin A (2.5 μmol l⁻¹) to inhibit complex III and measure residual oxygen consumption (i.e. the oxygen consumed by oxidative side reactions occurring in permeabilized fibers) which was used to correct all the mitochondrial oxygen 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 into the chamber to raise the oxygen concentration in the chamber and N, N',N',N'-tetramethyl-p-phenylenediamine ([TMPD] 0.5 mmol l⁻¹) and ascorbate (2 mmol l⁻¹) were added. Because of autoxidation of TMPD and ascorbate, chemical backgrounds were evaluated at the end of each experiment after inhibition of COX by sodium azide (100 mmol l⁻¹) and were subtracted from the activity (Gnaiger et al., 1998).

**Calculation of mitochondrial respiration ratios, temperature coefficients (Q10) 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 (C1c-OXPHOS/C1c-LEAK) to evaluate the structural integrity of the outer mitochondrial membrane (Kuznetsov et al., 2008).

Temperature coefficients (Q10) of mitochondrial respiration were calculated using the following formula:

\[ Q_{10} = \left( \frac{R_2}{R_1} \right)^{10/(T_2-T_1)}. \]  

Two different Q10 values were calculated: (i) the acclimation temperature Q10 was calculated from mean mitochondrial oxygen consumption rates obtained at the initial water temperature of 10°C and at subsequent acclimation days after warming to 16°C, with R1 representing the rates of mitochondrial oxygen consumption measured during day 0 at T1=10°C and R2 representing the rates of mitochondrial oxygen consumption measured during the other acclimation days (days 1–39) at T2=16°C; and (ii) the assay temperature Q10 was calculated for individual fish (expressed as mean±s.s.e.m.) at each sampling day using the mitochondrial oxygen consumption rates obtained with the two in vitro assay temperatures (10 and 16°C) where R1 represents the rates of mitochondrial oxygen consumption measured at T1=10°C and R2...
represents the rates of mitochondrial oxygen consumption measured at $T_2=16{\text{°C}}$.

The relative ventricular mass (RVM) was calculated as:

$$RVM = \text{ventricle mass}/M_b.$$  \hfill (2)

The fish condition factor was calculated as:

$$\text{Condition factor} = (100M_b)/FL^3,$$  \hfill (3)

with $M_b$ 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 mmol l$^{-1}$ 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, USA) 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 mmol l$^{-1}$ potassium phosphate buffer complemented with 0.16 mmol l$^{-1}$, 0.4 mmol l$^{-1}$ NADH, and 0.4 mmol l$^{-1}$ pyruvate (omitted for the control), pH 7.2. Activity was measured at 340 nm for 4 min ($e_{340} = 6.22$ ml cm$^{-1}$ μmol$^{-1}$). CS activity was measured by incubating homogenates with a 100 mmol l$^{-1}$ imidazole-HCl buffer complemented with 0.1 mmol l$^{-1}$ 5,5′-dithiobis(2-nitrobenzoic acid), 0.1 mmol l$^{-1}$ acetylCoA and 0.15 mmol l$^{-1}$ oxaloacetate (omitted for the control), pH 8.0. Activity was measured at 412 nm for 4 min ($e_{412} = 13.6$ ml cm$^{-1}$ μmol$^{-1}$). All enzymatic activities are expressed as U g$^{-1}$ of tissue, where U is 1 μmol 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 oxygen 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. 0, 1, 2, 4, 11, 25 and 39 days) 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 the ’Preparation of permeabilized cardiac fibers and mitochondrial respirometry setup’ section). For enzymatic activities, in vitro $Q_{10}$ values, as well as specific comparisons with $post hoc$ Tukey’s HSD tests were presented below.

**RESULTS**

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

**Mass-specific mitochondrial oxygen consumption rates**

**In vivo temperature effects: differences between acclimation days**

The mass-specific mitochondrial oxygen consumption rates at the temperatures the fish experienced in vivo throughout the temperature exposure protocol (i.e. 10°C for day 0 and 16°C for days 1–39) are illustrated by the red line in Fig. 1. Almost all mitochondrial oxygen consumption rates increased slightly (but not significantly) between day 0 and day 1, decreased significantly between day 1 and day 2, and then remained unchanged for the rest of the warm acclimation period. The relatively minor increase between day 0 and day 1 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 day 0 and day 1 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 day 0 and days 2–39, the acclimation temperature $Q_{10}$ values 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 $post hoc$ 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 10°C yielded the highest CI-LEAK measured at the assay temperature of 16°C, which then decreased during warm acclimation with corresponding values at days 2–39 being significantly different from day 0 (Fig. 1). For CI-OXPHOS, CI+CII-OXPHOS and CI+CII-ETS, a significant decrease was observed between day 0 and 10°C.
and days 2–39 when measured at 16°C, but the only significant difference when assayed at 10°C was observed between day 0 and day 39 (Fig. 1). Moreover, significant differences were detected between the assay temperatures for CI-OXPHOS, CI+CII-OXPHOS and CI+CII-ETS at day 0 and day 1, with mitochondrial oxygen 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 oxygen consumption rates at day 0 than when fish were warm acclimated from days 2 to 39 (Fig. 1). Similarly, when assayed at 10°C, COX activity was significantly higher in 10°C acclimated fish compared with most subsequent warm acclimation days (i.e. days 1, 2, 4 and 39). Collectively, these results indicate a rapid resetting of mitochondrial metabolism, which appeared to be completed from day 2, 4 and 39. Collectively, these results indicate a rapid resetting of mitochondrial metabolism, which appeared to be completed from day 2, 4 and 39. Moreover, significant differences were detected between assay temperatures measured at the two different assay temperatures, 10°C (black bars) and 16°C (gray 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 day 0 at 10°C, n=6) and during warm acclimation (on days 1–39 at 16°C, N=6 for day 1 and N=8 for days 2–39). Results are means±s.e.m. Statistical differences were obtained with post hoc Tukey’s test when an effect of the interaction acclimation day×assay temperature was observed with the linear mixed model. An asterisk depicts the differences between the two in vitro assay temperatures assayed on the same acclimation day; dissimilar lowercase letters depict differences between acclimation days at each assay temperature (black letters for 10°C and gray letters for 16°C).

### 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 day 0 and day 1, and then remaining unchanged throughout the warm acclimation period, although there were no significant differences in P/L measured between day 0 and days 4–25 (Fig. 2A). The calculated cytochrome c effect (Fig. 2B) showed a strong acclimation day effect, as it was significantly increased upon warming at day 1, followed by a significant decrease on day 2 (Table 1). At day 2 this cytochrome c effect was still significantly higher than at day 0 and at days 4–39 but lower than at day 1 (Fig. 2B). At warm acclimation day 4 and onwards, the cytochrome c effect returned to the values measured at day 0 in 10°C acclimated fish (Fig. 2B).

When considering these parameters measured at the respective acclimation temperatures (i.e. measured at 10°C for day 0 and at 16°C for days 1–39), P/L were not significantly different. An increase of cytochrome c effect was observed between day 0 and day 2, 4 and 39 when measured at 16°C, but the only significant difference when assayed at 10°C was observed between day 0 and day 39 (Fig. 1). Moreover, significant differences were detected between the assay temperatures for CI-OXPHOS, CI+CII-OXPHOS and CI+CII-ETS at day 0 and day 1, with mitochondrial oxygen 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 oxygen consumption rates at day 0 than when fish were warm acclimated from days 2 to 39 (Fig. 1). Similarly, when assayed at 10°C, COX activity was significantly higher in 10°C acclimated fish compared with most subsequent warm acclimation days (i.e. days 1, 2, 4 and 39). 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 $Q_{10}$ values are presented in Table 2. These $Q_{10}$ values calculated for CI-LEAK, CI-OXPHOS, CI+CII-OXPHOS, CI+CII-ETS and COX were all influenced by the acclimation days, as detected with post hoc tests (Table 2). For these three parameters, a slight increase was observed immediately upon warming between day 0 and day 1, followed by a significant decrease between day 1 and day 2 (Table 2). These values then remained relatively stable throughout the warm acclimation protocol and were close to those obtained in the 10°C acclimated fish at day 0.

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

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

Values are presented as means±s.e.m. Dissimilar letters denote significant differences between the different days of the experiment.
days 1–2, but afterwards, it returned to initial values and was also significantly lower than at days 1–2 (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 day 1, but then rapidly decreased and was not significantly different from the initial values at day 0 for the duration of the warm acclimation period (with significance detected for CS between day 1 and day 25 and between day 1 and day 39).

**Morphological variables**

All morphological variables are reported in Table 3. Although no significant differences were detected for $M_b$, FL and condition factor between fish acclimated to 10 and 16°C, the RVM decreased significantly after 25 days of warm acclimation ($P=0.006$ between day 0 and day 25; $P=0.012$ between day 0 and day 39).

**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°C, 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 present study revealed a general elevation of aerobic and anaerobic metabolism after acutely increasing the temperature from 10 to 16°C (i.e. between day 0 and day 1), as observed by the changes in the activities of CS and LDH, respectively. Contrary to predictions, however, mitochondrial capacities as measured by oxygen consumption rates did not change significantly across the same measurement days (day 0 and day 1), and at day 2 and onwards, a significant decrease of most mitochondrial oxygen consumption rates and enzymatic activities was observed with warm acclimation. During the initial 2 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 day 4 onwards, suggesting a restoration of cell homeostasis. Moreover, acute thermal insensitivity of mitochondrial capacities was observed from day 4, as indicated by lack of differences in mitochondrial oxygen consumption rates between assay temperatures. This might serve to widen 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 most thermal conditions (Blier and Lemieux, 2001; Blier et al., 2013; Hilton et al., 2010). Surprisingly, we did not find such an excess as the values for COX were only slightly higher than those for CI+CII-ETS. 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 in this study was not high enough to display such excess, and that COX functional capacity was not affected by raising the water temperature between day 0 and day 1. The substantial in vitro thermal sensitivity of COX as indicated by the assay temperature Q10 values 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 day 2 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, 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 in this study after an initial crucial 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 the liver of Fundulus heteroclitus (Chung and Schulte, 2015).

Between day 0 and day 1, significant increases of LDH and CS activities were detected, which were not related to changes in morphological variables such as condition factor or RVM, suggesting an increase in aerobic and anaerobic cardiac capacity. However, from day 2 onwards, the activities of both LDH and CS returned to values observed at day 0 before warming. CS activity displayed the lowest values at day 25 and day 39 with significant differences detected when compared with day 1. Interestingly, we also observed a decrease of RMV 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 day 0 and day 1. This P/L reduction coincided with an increased mitochondrial oxygen consumption after the 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 day 0 and day 1 with acute warming therefore suggests an impaired mitochondrial functional capacity. When measuring mitochondrial functions in isolated mitochondria from red muscle, Bouchard and Guderley (2003) as well as Krafje 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 9°C over 2 days (Bouchard and Guderley, 2003) or from 5 to 15°C over 3 days (Krafje 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. In the long term, however, the results are quite similar to those found for isolated mitochondria from red muscle in previous studies, both at the level of enzymatic activities and mitochondrial oxygen fluxes, when the temperature was increased from 9 to 15°C (Bouchard and Guderley, 2003).

The impaired mitochondrial functions suggested by the cytochrome c effect and the decreased P/L between day 0 and day 1 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,b) 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 h, a decline of monounsaturated species of phosphatidylcholine was observed in plasma membranes from trout kidney (Hazel and Landrey, 1988a,b), 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 4.5°C over 8 h (Hazel and Landrey, 1988a,b), it is therefore likely that such modifications are involved in the increased cytochrome c effect observed in our study after an increase of 6°C over 12 h. 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 (e.g. due to peroxidation) to the outer mitochondrial membrane releases cytochrome c in the cytoplasmic space (Bradley et al., 2016; Chu et al., 2013). Thus, it is possible that the rapid modifications in mitochondrial capacities and the increased cytochrome c effect observed in our study at day 1 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 this.

The increase in mitochondrial oxygen consumption following the addition of cytochrome c was not seen on sampling days after day 2, which exhibited values similar to those seen before the temperature increase. This could indicate that dysfunctional mitochondria had been eliminated from the cell after day 2. 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 (Dhillon and Schulte, 2011; Guderley, 2004; Guderley and Johnston, 1996; Guderley et al., 1997; St-Pierre et al., 1998; Tyler and Sidell, 1984; White et al., 2012) 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 has 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 (Bradley et al., 2016; Chu et al., 2013; Sorice et al., 2004). The 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 the 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; Jovaisa 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 16°C on day 1 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 day 2. This hypothesis might be worth exploring by measuring transcript levels and/or protein levels of Hsp60 and mitochondrial Hsp70. Additionally, protein levels of an 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 conclusion, 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 in the activities of LDH and CS, probably reflect an adjustment of both anaerobic and aerobic metabolism. On day 2 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 day 1 and day 2. 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 day 2 onwards, we observed that rates of mitochondrial oxygen consumption were similar when measured at either of the two assay temperatures, suggesting thermal independence, which might help the fish to maintain cardiac mitochondrial capacities across environmentally relevant temperature variations during warm acclimation.

The current findings of re-establishment 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 characterized by an initial increase due to increases in both resting and maximum heart rate, followed by a rapid decline on day 2 owing 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 organization 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 competing or financial interests.

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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