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RESEARCH ARTICLE

Acclimation to warm temperatures has important implications for mitochondrial function in Atlantic salmon (*Salmo salar*)

Lucie Gerber*, Kathy A. Clow and Anthony K. Gamperl

ABSTRACT

In fish, the capacity of thermal acclimation to preserve cardiac mitochondrial function under future warming scenarios is important to understand given the central roles that cardiac energy metabolism and performance play in this taxa's thermal tolerance. We acclimated Atlantic salmon to 12 and 20°C (for >2 months), and investigated the effects of acute and chronic warming on cardiac mitochondrial respiration and reactive oxygen species (ROS) production (release rate) using high-resolution fluorespirometry. Further, we compared the sensitivity of mitochondrial respiration to nitric oxide (i.e. the NO IC_{50}), and assessed the mitochondrial response to anoxia–reoxygenation (AR). Acute exposure to 20°C increased maximal mitochondrial respiration by ∼55%; however, the mitochondria's complex I respiratory control ratio was 17% lower and ROS production was increased by ≥60%. Acclimation to 20°C: (1) preserved mitochondrial coupling and aerobic capacity; (2) decreased the mitochondria's ROS production by ∼30%; (3) increased the mitochondria's NO IC_{50} by ∼23%; and (4) improved mitochondrial membrane integrity at 20°C. AR did not affect mitochondrial function at 12°C, but acute exposure to 20°C and AR depressed maximal mitochondrial respiration (by ∼9%) and coupling (by ∼16%) without impacting ROS production. Finally, warm acclimation did not improve the capacity of mitochondria to recover from AR, indicating that there was no 'cross-tolerance' between these challenges. Our findings provide compelling evidence that thermal plasticity of cardiac mitochondrial function contributes to the Atlantic salmon's capability to survive at ≥20°C for prolonged periods, but call into question whether this plasticity may allow them to withstand high temperatures when combined with other stressors.

KEY WORDS: Anoxia–reoxygenation, Citrate synthase, Nitric oxide, Reactive oxygen species, Mitochondrial respiration, Superoxide dismutase

INTRODUCTION

With climate warming, temperatures in many aquatic environments may reach levels that have direct negative effects on fish physiological performance (Sandblom et al., 2016 and references therein). As a fish's critical thermal maximum is approached, one of the first organs to fail is the heart (Farrell, 2002; Iftikar and Hickey, 2013; Iftikar et al., 2014; Haverinen and Vornanen, 2020), making cardiac function a primary determinant of fish thermal tolerance (Eliason and Anttila, 2017). Cardiac functionality is highly dependent on ATP production and, therefore, on the capacity of mitochondria to produce ATP (Rodnick and Gesser, 2017). Impairment of cardiac mitochondrial function at elevated temperatures is a threat to myocardial oxidative function, and the importance of mitochondrial plasticity in limiting cardiac dysfunction (i.e. heart failure) and shaping thermal tolerance has been suggested by several studies (Iftikar and Hickey, 2013; Iftikar et al., 2014; Chung et al., 2017; Christen et al., 2018; O'Brien et al., 2018). Acute warming first results in an increase in oxidative phosphorylation (OXPHOS) that enables tissues to meet their higher metabolic demands and to sustain cellular energy (ATP) production. However, mitochondrial capacity becomes compromised (i.e. plateaus or declines) at temperatures close to the fish's upper thermal limit. This is due to alterations in crucial mitochondrial processes during acute warming such as the rate of proton leak, coupling efficiency (respiratory control ratio; RCR), the integrity of mitochondrial membranes and the functionality of protein complexes (notably complex I; Hilton et al., 2010; Lemieux et al., 2010; Iftikar and Hickey, 2013; Iftikar et al., 2014; Rodnick et al., 2014; Christen et al., 2018; O'Brien et al., 2018). The effect of chronic warming on mitochondrial physiology is less understood, but long-term acclimation under laboratory conditions may help to improve our understanding of the plasticity of cardiac mitochondrial function and its role in fish thermal tolerance (Iftikar et al., 2015; Baris et al., 2016; Chung et al., 2017; Leo et al., 2017; Pichaud et al., 2017; Howald et al., 2019; Pichaud et al., 2019). Moreover, the effect of thermal acclimation on the oxidative status (e.g. the level of reactive oxygen species production) experienced by cardiac mitochondria is largely unknown, despite the important role of oxidative processes in defining a fish's responses to environmental change (Birnie-Gauvin et al., 2017). An increase in ROS production during acute warming was reported for permeabilized cardiac fibers from a New Zealand wrasse (*Notolabrus celidotus*; Iftikar and Hickey, 2013) and isolated cardiac mitochondria of Arctic char (*Salvelinus alpinus*; Christen et al., 2018). Nevertheless, the effect of chronic exposure to increased temperatures on reactive oxygen species (ROS) production rates by cardiac mitochondria has only been investigated in permeabilized cardiac fibers of *N. celidotus*, and was comparable between 15°C- and 21°C-accclimated animals (Iftikar et al., 2015). In addition, although the combined effects of warming (acute or chronic) and O_2 limitation on cardiac mitochondrial function have been explored, most data on how these combined factors affect fish mitochondrial function have been conducted using the liver as the model tissue (e.g. Onukwufor et al., 2016; Sappal et al., 2015a,b).

Nitric oxide (NO) plays an important role in adjustments of cardiac function and aerobic metabolism (Fago and Jensen, 2015; Imbrogno et al., 2018) by inducing vasodilation (Fago et al., 2012), reversibly inhibiting mitochondrial respiration (Gerber et al., 2019) and controlling breathing (Perry et al., 2016) in fish. However, the role of NO in regulating mitochondrial respiration when ectotherms

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are warmed, and tissues likely experience hypoxic stress, has not been examined. It is possible that the widespread actions of NO interfere with mitochondrial bioenergetic processes during thermal acclimation. For instance, we recently showed that mitochondrial sensitivity to inhibition by NO was altered by hypoxic acclimation (Gerber et al., 2019), a link between thermosensitivity and responsiveness to NO has been suggested in homeotherms (Simon, 1998), and the involvement of NO in heat defense (i.e. the control of breathing and the related hypoxic ventilatory response) has been demonstrated in rats (Nakano et al., 2001).

The present study assessed the thermal dependence and plasticity of cardiac mitochondrial function when Atlantic salmon were warmed to 20°C and held at this temperature for approximately 2 months. We hypothesized that: (1) although several mitochondrial processes and/or functions would be compromised in this species by acute warming, they would be largely compensated for by warm acclimation; and (2) that the role of NO in regulating mitochondrial respiration would be altered by acclimation to high temperatures. To test our hypotheses, we measured isolated cardiac mitochondrial function following *in vivo* acclimation to 20°C (for 62–72 days), and *in vitro* acute warming (from 12°C to 20°C) alone or in combination with an *in vitro* anoxia–reoxygenation (AR) event (i.e. the absence of oxygen for 10 min). We measured mitochondrial O_2 consumption, the thermal sensitivity of respiration (Q_{10}), the coupling and efficiency of oxidative phosphorylation (RCR and P:O ratio), outer membrane integrity using cytochrome *c* and ROS production/release rate (estimated as H_2O_2 flux) when complex I alone (owing to its known thermal sensitivity) and both complexes I+II were stimulated. In addition, we investigated whether/how warm acclimation altered oxidative capacity [by measuring citrate synthase (CS) activity], antioxidant defense capacity [by measuring superoxide dismutase (SOD) activity] and mitochondrial sensitivity to inhibition by NO (by measuring NO IC_{50}).

The Atlantic salmon was chosen as a model to study the thermal sensitivity of cardiac mitochondria because many facets of its cardiac response to environmental challenges are well characterized (Anttila et al., 2013, 2014, 2015; Penney et al., 2014; Hvas et al., 2017; Leeuwis et al., 2019). Considerable cardiac and aerobic plasticity has been observed in Atlantic salmon during thermal stress (Anttila et al., 2014; Hvas et al., 2017). Finally, the 12°C-acclimated salmon used in this study have a critical thermal maximum (CT_{max}) of ∼25.8°C, the temperature where cardiac arrhythmias (f_{Hmax}) appear is 22.9°C (A.K.G., K.A.C., R. M. Sandrelli, L.G. and E. F. C. Peroni, unpublished data) and salmon exposed to an incremental temperature increase of 1°C per week begin dying at 22°C (Gamperl et al., 2020). Thus, the temperature used for warm acclimation (i.e. 20°C) is close to, but just below, this species' CT_{max}, f_{Hmax} and incremental temperature tolerance (IT_{max}).

MATERIALS AND METHODS

Experimental animals and temperature acclimation

Atlantic salmon were obtained from Cape d'Or Sustainable Seafood Inc. (Advocate Harbour, Nova Scotia, Canada) and initially maintained at the Dr Joe Brown Aquatic Research Building [JBARB, Memorial University of Newfoundland (MUN)] in a 4000 liter tank supplied with aerated seawater (32 ppt) at 10±1°C and a 12 h:12 h light:dark photoperiod for 1 month. Thereafter, the fish were randomly distributed into four 2200 liter circular tanks (∼30 fish per tank) receiving 12±1°C aerated seawater and a 14 h:10 h light:dark photoperiod at the Laboratory for Atlantic Salmon and Climate Change Research (LASCCR, Ocean Sciences Centre, MUN). After 2 weeks of acclimation to the tanks, two control tanks were maintained at 12°C, while the temperature was gradually increased in the two other tanks (by ∼0.25°C every day) until the temperature reached 20°C. The tanks were then maintained at 12 or 20°C for 2 months before sampling (the experiments began). Fish were fed a commercial marine fish diet (Europa; Skretting Inc.) at ∼1.5% body mass day^{-1} during this period.

The experiments below were completed within 10 days; hence the salmon were acclimated between 62 and 72 days to 12 or 20°C. The experiments were performed on pairs of animals daily, i.e. one 12°C- and one 20°C-acclimated fish.

All experimental procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee of MUN (protocol 16-92-KG).

Mitochondrial isolation

We used the protocol outlined in Gerber et al. (2019) with the exception that the composition of the respiration medium was modified by replacing 2 mmol l^{-1} EDTA with 1 mmol l^{-1} EGTA. Briefly, fish were killed by a blow to the head before measuring their fork length and body mass. Then, the ventricle was excised, cut in half, rinsed briefly with ice-cold isolation medium (in mmol l^{-1}: 230 mannitol; 75 sucrose; 20 HEPES; 1 EGTA, pH 7.4), blotted dry and weighed. A small piece of ventricle was removed, weighed and snap-frozen in liquid nitrogen prior to being kept at −80°C for later use in enzymatic assays. The rest of the ventricle was thoroughly minced and split into four aliquots to facilitate homogenization. Each aliquot was re-suspended in three volumes of isolation medium in an ice-cooled glass-homogenizer, and gently homogenized using six passes of a loose-fitting motor-driven Teflon pestle. The crude homogenates were then combined and centrifuged at 800 ***g*** for 10 min at 4°C to remove cell debris, and the resulting supernatant was centrifuged at 8000 ***g*** for 10 min at 4°C to pellet the mitochondria. The lipid layer and the supernatant were carefully removed, and the resulting mitochondrial pellet was washed twice by gentle re-suspension in ice-cold isolation medium containing 10 mg ml^{-1} of BSA (fatty acid free; Sigma Chemical Co., CAS 9048-46-8), and then centrifuged at 8000 ***g*** for 10 min at 4°C. This final pellet was weighed, and then gently re-suspended in four volumes of ice-cold respiration medium [in mmol l^{-1}: 160 KCl; 30 HEPES; 10 KH_2PO_4; 1 EGTA; 10 mg ml^{-1} BSA (fatty acid free), pH 7.4]. The protein content of the mitochondrial suspensions was assayed using the Bradford assay with BSA as a standard (Thermo Fisher Scientific, Waltham, MA, USA), and the contribution of BSA to the measured protein levels was accounted for using respiration medium with BSA as a blank. Mitochondrial suspensions were placed on ice for 30 min to rest before being used for high-resolution respirometry.

Experimental protocols

Mitochondrial physiology

The physiology of isolated cardiac mitochondria from 12°C- and 20°C-acclimated salmon was assessed using high-resolution respirometry (O2k-polarographic O_2 sensor, Oroboros Instruments, Innsbruck, Austria), fluorometry (O2k-Fluo LED2-module, Oroboros Instruments), amperometry (O2k-MultiSensor system, Oroboros Instruments) and an ISO-NOP NO sensor (World Precision Instruments, Sarasota, FL, USA). We conducted two separate experiments at two assay temperatures (12°C and 20°C) on the mitochondria from 12°C- and 20°C-acclimated fish, which are described in detail below and shown in Figs S1 and S2. The experimental duration varied between 60 and 120 min. O2k-sensor

calibration and correction for background oxygen flux were performed at each assay temperature (12 and 20°C) following standard operating procedures (O2k-SOP; Fasching and Gnaiger, 2018; Gnaiger, 2018), and the oxygen solubility factor (FM) of the KCl experimental medium at 12°C and 20°C was used to ensure accurate oxygen concentration measurements (O2k-SOP; Gnaiger, 2010). All parameters were recorded in real-time using DatLab v 7.3 software (Oroboros Instruments). In each experiment, mitochondrial suspensions were injected into calibrated 2 ml O2k-chambers containing 12°C or 20°C respiration medium that was 100% air-equilibrated.

Measurement of coupled mitochondrial respiration and ROS production/release rates

In experiment 1, we measured cardiac mitochondrial respiration and ROS production/release rates simultaneously. ROS production/release rates were estimated by measuring extra-mitochondrial H_2O_2 using Amplex® UltraRed (AmR, 10 µmol l^{-1}), horseradish peroxidase (HRP, 3 U ml^{-1}), SOD (25 U ml^{-1}) and the Green Fluorescence-Sensor of the O2k-Fluo LED2-Module (with gain and LED intensity set to 1000 and 500 mV, respectively). The ROS signal was calibrated by the addition of H_2O_2 (0.1 µmol l^{-1}) following standard operating procedures (O2k-SOP; Krumschnabel et al., 2019; Makrecka-Kuka et al., 2015). In experiment 1 (see Fig. S1A), mitochondria (0.25 mg protein ml^{-1}) were added to 2 ml of 100% air-equilibrated respiration medium at 12°C or 20°C. Then the complex I (CI) substrates glutamate (15 mmol l^{-1}) and malate (2 mmol l^{-1}), and ADP (200 µmol l^{-1}), were added to fuel the electron transport system (ETS) prior to measuring oxidative phosphorylation (OXPHOS, State 3 respiration) via complex I (P_{GM}) with a second addition of ADP (200 µmol l^{-1}). After the depletion of ADP, leak respiration (State 4) via CI ($L_{T,GM}$) was measured. Thereafter, succinate (5 mmol l^{-1}) was added, followed by ADP (200 µmol l^{-1}) to measure OXPHOS respiration (State 3) via CI+II (P_{GMS}). After the depletion of ADP, leak respiration rate (State 4) via CI+CII ($L_{T,GMS}$) was measured. Then, the effect of a 10 min anoxic bout on mitochondrial respiration and ROS release rates via CI+II was measured. To decrease the O_2 level in the chamber, an excess of ADP (700 µmol l^{-1}) was added, and the mitochondria consumed all the ADP and returned to State 4 before they became anoxic. After 10 min of anoxia, the chamber oxygen level was returned to pre-anoxic levels by lifting the chamber stoppers. Then, ADP (200 µmol l^{-1}) was added to measure OXPHOS respiration and leak respiration post-anoxia. This protocol also allowed for the determination of mitochondrial P_{50} [i.e. partial oxygen pressure of oxygen (in kPa) at which the respiration rate was half of maximal] during leak respiration.

Measurement of the inhibition of mitochondrial respiration by exogenous NO

In experiment 2 (see Fig. S2A), we measured ventricular mitochondrial respiration and NO concentration in the chambers simultaneously. An NO sensor (ISO-NOP, World Precision Instruments) was connected to one of the amperometric channels of the O2k-MultiSensor system following a standard protocol (O2k-SOP; Fasching and Gnaiger, 2016). The NO sensors were maintained in a polarized state using a pre-polarizer (NSA-3, World Precision Instruments) to allow for quick stabilization of the signal once connected to the O2k instrument. The NO sensors were calibrated as described elsewhere (Gerber et al., 2019) and following the manufacturer's recommendations (WPI instruction manual, Calibration of NO Sensor by Decomposition of SNAP – Method 2). Briefly, the O2k chamber windows were covered with custom-made 3D-printed caps and the lights inside the chambers were turned off. The NO sensors were calibrated daily at 12°C or 20°C in O2k chambers filled with 2 ml of 0.1 mol l^{-1} $CuCl_2$ solution by the sequential addition of known SNAP concentrations (stock solution 0.05 mmol l^{-1}) over a range of 0–1000 nmol l^{-1} of NO using the TIP2k syringe. Following calibration, the chamber was thoroughly rinsed and filled with respiration medium, and the NO sensors were allowed 30 min to stabilize under these new conditions (respiration medium at 12°C or 20°C). Then, the mitochondria's sensitivity to NO (NO IC_{50}) was measured using the protocol described in Gerber et al. (2019). Briefly, in this experiment (Fig. S2A), mitochondria (0.25 mg protein ml^{-1}) were added to 100% air-equilibrated 2 ml-calibrated chambers filled with respiration medium at 12°C or 20°C. Substrates of CI and CII (glutamate, 15 mmol l^{-1}; malate, 2 mmol l^{-1}; and succinate, 5 mmol l^{-1}) were added, followed by ADP (200 µmol l^{-1}), to fuel the ETS and assess mitochondrial coupling. Then, an excess amount of ADP (1 mmol l^{-1}) was added, and the NO donor PAPANONOate (20 and 10 µmol l^{-1} at 12°C and 20°C, respectively) was added to the chamber at ∼60% air saturation to initiate NO release at a rate of ∼20 nmol min^{-1}. This slow increase in NO levels in the chambers gradually inhibited respiration, and allowed for the determination of mitochondrial NO IC_{50} (the concentration of NO that inhibited 50% of maximal respiration). Once the maximal respiration rate was inhibited by ∼75%, and the O_2 concentration in the chamber had plateaued, excess oxyhemoglobin [OxyHb, 20 µmol l^{-1}, see Gerber et al. (2019) for preparation of OxyHb] was added to reverse NO's inhibition of respiration and to confirm that the effect observed was due to the release of NO and not to the addition of the NO donor molecule (PAPANONOate) per se. Excess OxyHb scavenged all the NO and stopped the PAPANONOate-mediated NO release, allowing for calculation of the NO sensors' zero drift. Reversal of NO inhibition allowed mitochondrial OXPHOS respiration to be re-established, and once leak respiration was established, the chamber oxygen level was returned to 60% air saturation by lifting the chamber stoppers, and OXPHOS respiration was initiated by the addition of excess ADP (1 mmol l^{-1}). This served as a control for comparison with OXPHOS respiration in the presence and absence of NO (not shown). Cytochrome *c* (10 µmol l^{-1}) was also added during the plateau at maximal respiration to assess outer mitochondrial membrane integrity. PAPANONOate (20 mmol l^{-1}) was prepared daily, and immediately prior use, in ice-cold respiration medium (without BSA) for each experiment. Recordings of NO concentration were corrected for baseline and zero drift before data analysis.

Calculation of mitochondrial respiration coupling, efficiency, integrity and thermal sensitivity

Respiratory control (RCR; i.e. State 3/State 4) and P:O (i.e. [$ADP_{injected}$ in µmol]/[O_2 consumed in µ-atoms]) ratios with CI and CI+II substrates were calculated to evaluate mitochondrial coupling and the efficiency of ATP synthesis, respectively. The effect of cytochrome *c* on mitochondrial respiration [i.e. (CI+CII $OXPHOS_{Cyt\ c}$−CI+CII OXPHOS)/CI+CII $OXPHOS_{Cyt\ c}$] and the temperature coefficient [Q_{10}; i.e. $(R_2/R_1)^{10/(T_2-T_1)}$] were calculated to evaluate the integrity of the outer mitochondrial membrane and the thermal sensitivity of mitochondrial respiration, respectively.

Enzyme assays

Enzymatic activities were measured at room temperature (25°C) using a 96-well microplate reader (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA, USA).

Table 1. *P*-values for repeated measures three-way ANOVAs of Atlantic salmon cardiac mitochondrial parameters

	Acclimation	Complex	Temperature	Acclimation× Complex	Acclimation× Temperature	Complex× Temperature	Acclimation×Complex× Temperature	
O_2 OXPHOS	0.3329	**<0.0001**	**<0.0001**	0.4828	0.118	**0.0008**	**0.027**	Fig. 1
O_2 leak	0.7957	**<0.0001**	**<0.0001**	0.3736	0.19	**<0.0001**	**0.0332**	Fig. 1
RCR	0.4115	**<0.0001**	**0.0035**	0.7296	**0.0196**	0.108	0.0599	Fig. 1
PO ratio	**0.0184**	**<0.0001**	0.3281	0.2816	0.1907	0.4548	0.4548	Fig. 1
ROS OXPHOS	**0.0209**	**0.0396**	**<0.0001**	0.5643	0.1354	**0.0354**	0.3696	Fig. 2
ROS leak	0.178	**<0.0001**	**<0.0001**	0.3665	0.391	**0.0001**	0.204	Fig. 2
%ROS/O_2 OXPHOS	**0.0077**	**<0.0001**	0.2547	0.286	0.0565	0.6843	0.0888	Fig. 2
%ROS/O_2 leak	0.2822	0.432	0.0308	0.6057	0.5493	0.2132	0.9928	Fig. 2
	Acclimation	Anoxia	Temperature	Acclimation× Anoxia	Acclimation× Temperature	Anoxia× Temperature	Acclimation×Anoxia× Temperature	
O_2 OXPHOS AR	0.3864	**<0.0001**	**<0.0001**	0.4457	0.7024	**<0.0001**	**0.0017**	Fig. 3
O_2 leak AR	0.4923	**0.0001**	**<0.0001**	0.0589	0.0664	**<0.0001**	0.6351	Fig. 3
ROS OXPHOS AR	0.0688	0.1825	**<0.0001**	0.156	0.1365	0.4798	0.4953	Fig. 3
ROS leak AR	0.3885	**0.0013**	**<0.0001**	0.0789	0.5242	0.0786	0.1869	Fig. 3
%ROS/O_2 OXPHOS AR	**0.0071**	**0.0002**	**0.0101**	0.2495	0.471	**0.02**	0.0897	Fig. 3
%ROS/O_2 leak AR	0.5879	**0.0035**	**0.0035**	0.0916	0.5817	0.519	0.8101	Fig. 3
RCR AR	0.1143	**<0.0001**	**<0.0001**	0.052	0.4089	**<0.0001**	**<0.0001**	Fig. 3

Significant *P*-values (<0.05) are in bold. OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio; PO, efficiency of ATP synthesis; %ROS/O_2, ROS release rate per unit of respirational O_2 flux; AR, anoxia–reoxygenation.

CS activity was determined on both ventricle homogenates and mitochondrial suspensions from both 12°C- and 20°C-acclimated fish using the method described by Treberg et al. (2007), and used as an indicator of mitochondrial density and aerobic (oxidative) capacity. A snap-frozen piece of ventricle was thoroughly minced, re-suspended in four volumes of ice-cold homogenization buffer (25 mmol l^{-1} HEPES, 2 mmol l^{-1} EDTA and 0.5% Triton X-100, pH 7.0) and homogenized using a Polytron homogenizer (Kinematica Inc., Bohemia, NY, USA). The resulting crude homogenate and thawed mitochondrial suspensions were further diluted in 2 and 19 volumes of homogenization buffer, respectively, and centrifuged at 2000 ***g*** for 5 min at 4°C. CS activities for the ventricle homogenates and mitochondrial suspensions were expressed as U g $tissue^{-1}$ and as U mg $protein^{-1}$, respectively.

SOD activity was assessed using the SOD Assay Kit-WST (Sigma Aldrich) following the manufacturer's protocol. The thawed mitochondrial suspensions were diluted in 9 volumes of homogenization buffer to lyse the mitochondrial membrane. Then, the thawed mitochondrial suspensions were centrifuged at 13,000 ***g***, and the resulting supernatant was further diluted in 13 volumes of dilution buffer (SOD Kit). Using the WST-1 inhibition curve and SOD standards, the concentration of SOD (in U mg^{-1} mitochondrial protein) in the mitochondrial suspensions was determined.

Statistical analyses

Statistical analyses were performed using Prism 8 software (GraphPad Inc., La Jolla, CA, USA). Effects of complex (or anoxia, Fig. 3), acclimation and assay temperature on all mitochondrial respiration data were assessed using repeated measures three-way ANOVAs. Groups that differed by only one factor (Sidak's multiple comparison tests) are shown in the graphs, whereas *P*-values and significant interactions between main effects are shown in Table 1. The NO IC_{50}, mito-P_{50}, Q_{10} and enzymatic activity data were analyzed using repeated measures two-way ANOVAs, followed by Sidak's multiple comparison tests as shown in Table 2 or described in the Results. The morphometric data were analyzed using unpaired two-tailed *t*-tests. The level of statistical significance for all analyses was $P<0.05$, and all data are presented as means±s.e.m.

RESULTS

Morphometric variables

There was no significant difference in body mass (831.3±28.9 versus 898.6±60.2 g; *t*-test, $F=4.311$, $P=0.3411$) or in relative ventricular mass (0.066±0.003 versus 0.071±0.00; *t*-test, $F=1.713$, $P=0.2126$) between 12°C- and 20°C-acclimated fish. There was also no difference in cardiac mitochondrial yield, the ratio of mitochondrial pellet mass to ventricular wet mass (0.049±0.002 versus 0.047±0.002; *t*-test, $F=0.8024$, $P=0.4024$) or the protein concentration of the mitochondrial suspension (50.44±3.6 mg ml^{-1} versus 51.73±2.6 mg ml^{-1}; Mann–Whitney test, $P=0.7308$) between 12°C- and 20°C-acclimated fish.

Effect of acute and chronic warming on mitochondrial respiration, coupling, efficiency, thermal sensitivity and integrity

Respiration rates during OXPHOS (State 3; Fig. 1A) and leak states (State 4; Fig. 1B) were higher when measured at 20°C as compared with 12°C, and when using substrates of CI+CII as compared with CI alone (by approximately 55 and 85%, respectively; Fig. 1, Table 1). In addition, although acclimation to 20 versus 12°C did not significantly alter mitochondrial respiration rates at 20°C, there were subtle differences in the thermal sensitivity of respiration between acclimation groups and between complexes (i.e. I versus I and II) when expressed as Q_{10} values. For example,

Table 2. Thermal sensitivity (Q_{10} values) calculated between assay temperatures (12°C and 20°C) for cardiac mitochondrial OXPHOS and leak respiration

	12°C-acclimated salmon	20°C-acclimated salmon
CI-leak	2.23±0.21	2.07±0.15
CI-OXPHOS	1.77±0.41	2.23±0.45*
CI+CII-leak	2.27±0.13	2.07±0.14*
CI+CII-OXPHOS	1.75±0.15	1.93±0.34#

Asterisks (*) indicate a significant ($P<0.05$) difference between acclimation groups within a respiration state, whereas a number sign (#) indicates a significant difference between complexes within a respiration state. Values are presented as means±s.e.m., $N=7$ per group.

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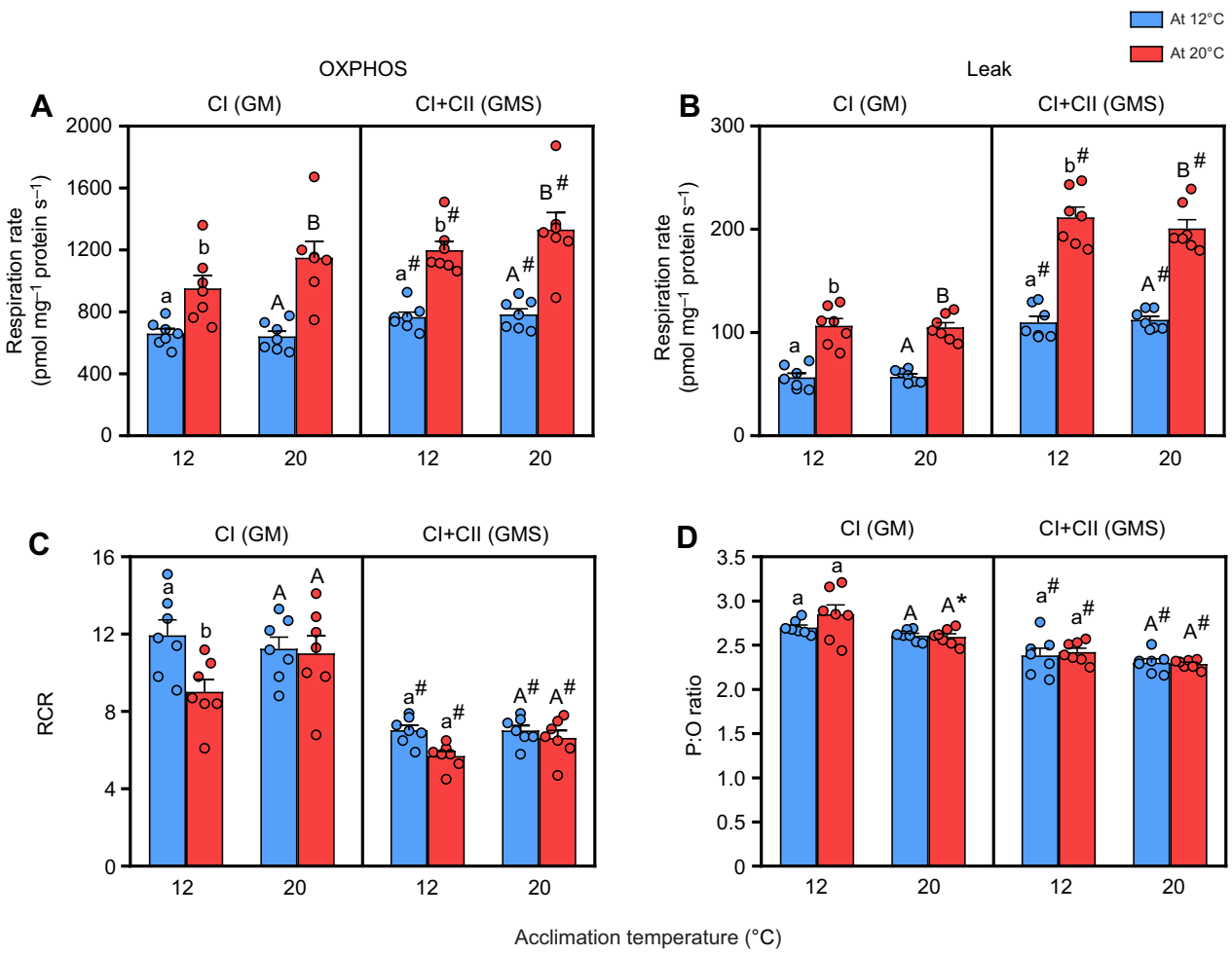

Fig. 1. Mitochondrial respiration, respiratory control ratio and efficiency of cardiac mitochondria from Atlantic salmon acclimated at 12°C and 20°C, and measured at both temperatures. (A) Respiration rate during oxidative phosphorylation (OXPHOS; State 3) with substrates for CI (GM) and CI+CII (GMS). (B) Respiration rate during leak (State 4) measured with substrates of CI (GM) and CI+II (GMS). (C) Respiratory control ratio (RCR; State 3/State 4). (D) The efficiency of OXPHOS respiration as determined using the flux ratios of ADP to atomic oxygen (P:O ratios) in the presence of substrates of CI (GM) and CI+II (GMS). Asterisk (*) indicates a significant (P<0.05) difference between acclimation groups. A number sign (#) indicates a significant (P<0.05) difference between complexes within an acclimation temperature/test temperature combination, whereas dissimilar lowercase and uppercase letters indicate a significant difference between test temperatures within an acclimation temperature/complex combination. Results of the statistical analysis are presented in Table 1. Bars are means±s.e.m., whereas circles represent individual values (N=7 per group).

12°C-acclimated salmon had higher (P<0.05) Q_{10} values for leak respiration than 20°C-acclimated fish (~2.25 versus 2.07), but lower Q_{10} values for OXPHOS respiration (~1.76 versus 1.93–2.23; Table 2). In 20°C-acclimated fish, the Q_{10} value for CI-OXPHOS (2.23±0.45) was significantly greater than for CI+CII-OXPHOS (1.93±0.34; Table 2).

With regards to mitochondrial coupling (i.e. RCR values; Fig. 1C) and efficiency (i.e. P:O ratio; Fig. 1D), the biggest influence was that of the mitochondrial complexes; RCR and P:O values for complex I+II were approximately ~30–40% (~6–7 versus ~9–12, respectively) and ~15% (~2.3–2.4 versus ~2.6–2.8, respectively) lower as compared with complex I. There was also an overall effect of test temperature on mitochondrial coupling, and of acclimation temperature on the P:O ratio (see Table 1). RCR values measured at 20°C were lower than those at 12°C; this effect was most notable in 12°C-acclimated fish for CI-fueled respiration (Fig. 1C). The P:O ratio was higher in 12°C-acclimated fish as compared with 20°C-acclimated fish, an effect that was most notable in 12°C-acclimated fish for CI-fueled respiration at 20°C (Fig. 1D). There was no effect of acclimation or test temperature on the P_{O_2} at which leak respiration rate was half of maximal (i.e. the P_{50}), with mito-P_{50} values of ~0.04 kPa for all groups (data not shown).

Effect of acute and chronic warming on ROS release rates

There was an overall effect of acclimation temperature on ROS production/release rate during OXPHOS (Fig. 2A), but not for leak respiration (Fig. 2B). This acclimation effect was most notable during CI+CII OXPHOS at 20°C, both when measured as ROS production/release rate (Fig. 2A) and when expressed as a percentage of O_2 flux (%ROS/O_2; Fig. 2C). With regards to the latter, the mitochondrial ROS production/release rate from 20°C-acclimated fish was ~30% lower as compared with 12°C-acclimated fish (Fig. 2A,C). In both acclimation groups, ROS release rates during both OXPHOS (Fig. 2A) and leak (Fig. 2B) states were higher (by ~55–78% and ~52–62%) when measured at 20°C than at 12°C. Although adding the CII substrate succinate increased ROS production/release rate during leak respiration (Fig. 2B; by ~90–111%) in both groups and at both test temperatures, it decreased %ROS/O_2

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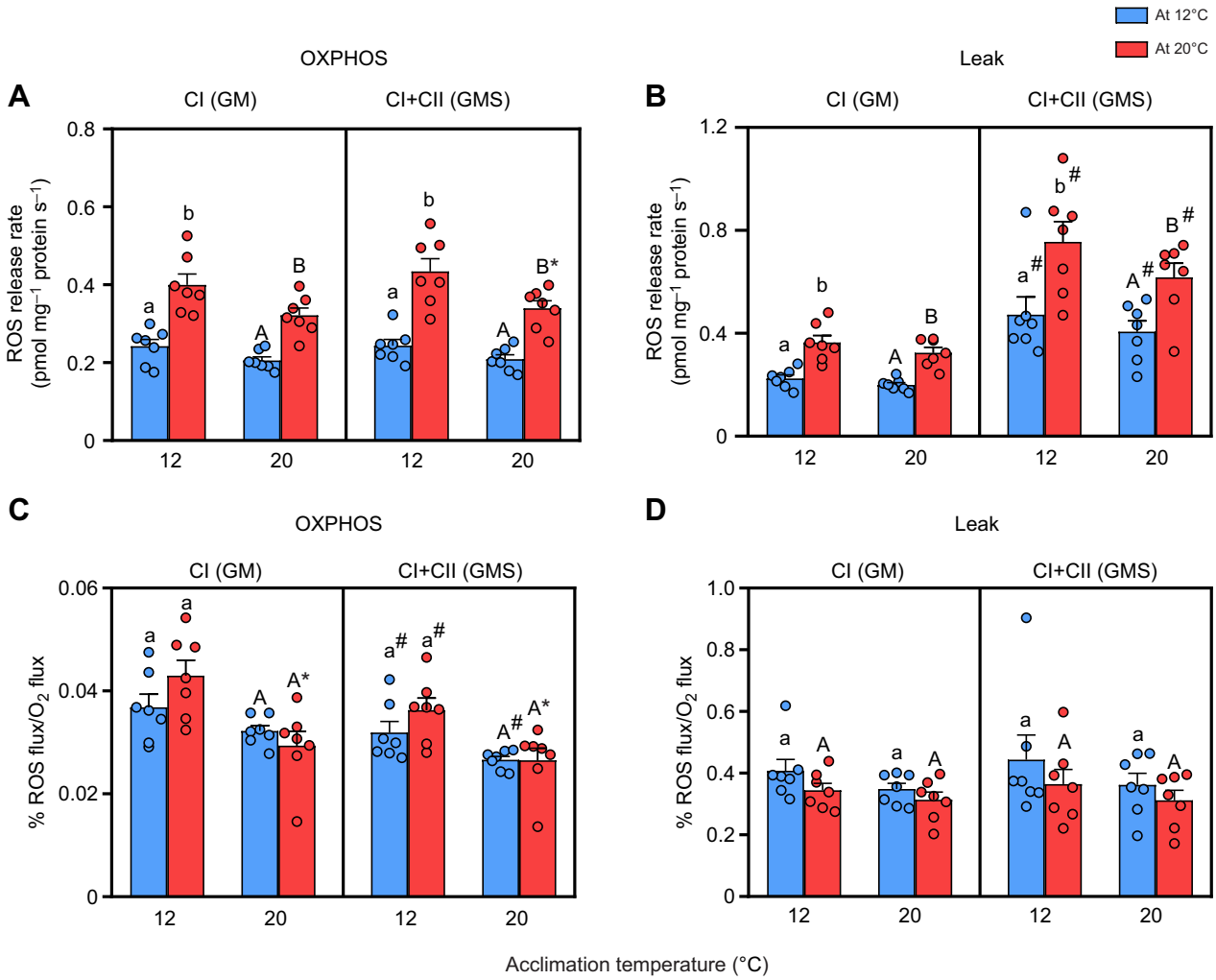

Fig. 2. Mitochondrial reactive oxygen species (ROS) release rate in cardiac mitochondria from Atlantic salmon acclimated at 12°C and 20°C, and measured at both temperatures. (A) ROS release rate during oxidative phosphorylation (OXPHOS) with substrates for CI (GM) and CI+CII (GMS). (B) ROS release rates during leak respiration when measured with substrates of CI (GM) and CI+II (GMS). ROS release rate expressed as a percentage of respirational (O_2) flux during (C) OXPHOS and (D) leak respiration. Other details are as in Fig. 1.

flux during OXPHOS in all groups except 20°C-acclimated fish at 20°C (Fig. 2C).

Effect of AR on mitochondrial respiration, ROS production/release rate and coupling

Acclimation did not alter the mitochondrial response to AR (Fig. 3). However, temperature and anoxic exposure had interactive effects on mitochondrial respiration. For example, although anoxic exposure decreased OXPHOS (Fig. 3A; by ∼9–16%) and increased leak respiration (Fig. 3B; by 12%) at 20°C in both groups, it had no effect when the mitochondria were tested at 12°C. Only a limited effect of AR on mitochondrial ROS production was observed when measured as ROS release rate (Fig. 3C,D). However, when expressed as a percentage of O_2 flux (i.e. %ROS/O_2 flux; Fig. 3E,F), mitochondrial ROS production was increased consistently by anoxic exposure in the OXPHOS state (this effect most notable in 20°C-acclimated fish tested at 20°C). In contrast, ROS production/release rate was generally decreased post-anoxia in the leak state (this effect was most notable for 12°C-acclimated fish at both test temperatures). Finally, RCR values were significantly lower (by ∼10–20%) post-anoxia as compared with pre-anoxia in all groups, with the exception of 20°C-acclimated fish tested at 12°C (Fig. 3G).

Effect of thermal acclimation on mitochondrial sensitivity to nitric oxide

The release of NO (at ∼20 nmol l^{-1} min^{-1}) using the NO donor PAPANONOate progressively inhibited CI+CII stimulated cardiac mitochondrial OXPHOS respiration in both acclimation groups and at both test temperatures (12°C and 20°C; Fig. 4A and Fig. S2). The concentration of NO that inhibited 50% of maximal mitochondrial respiration (i.e. the mitochondrial sensitivity to NO or NO IC_{50}) was not affected by acclimation when measured at 12°C. However, it was higher in 20°C-acclimated fish as compared with 12°C-acclimated fish when tested at 20°C (71.53 versus 54.73 nmol l^{-1}, respectively; *post hoc* t=2.497, P=0.0392).

Enzymatic activity

Thermal acclimation did not alter CS activities in the myocardium (ventricle) or in the mitochondria themselves, and mitochondrial SOD activities in the mitochondria were not different (Table 3).

Mitochondrial integrity

The integrity of the mitochondrial outer membrane was altered by test temperature (two-way ANOVA, F=9.137, P=0.0116), but not

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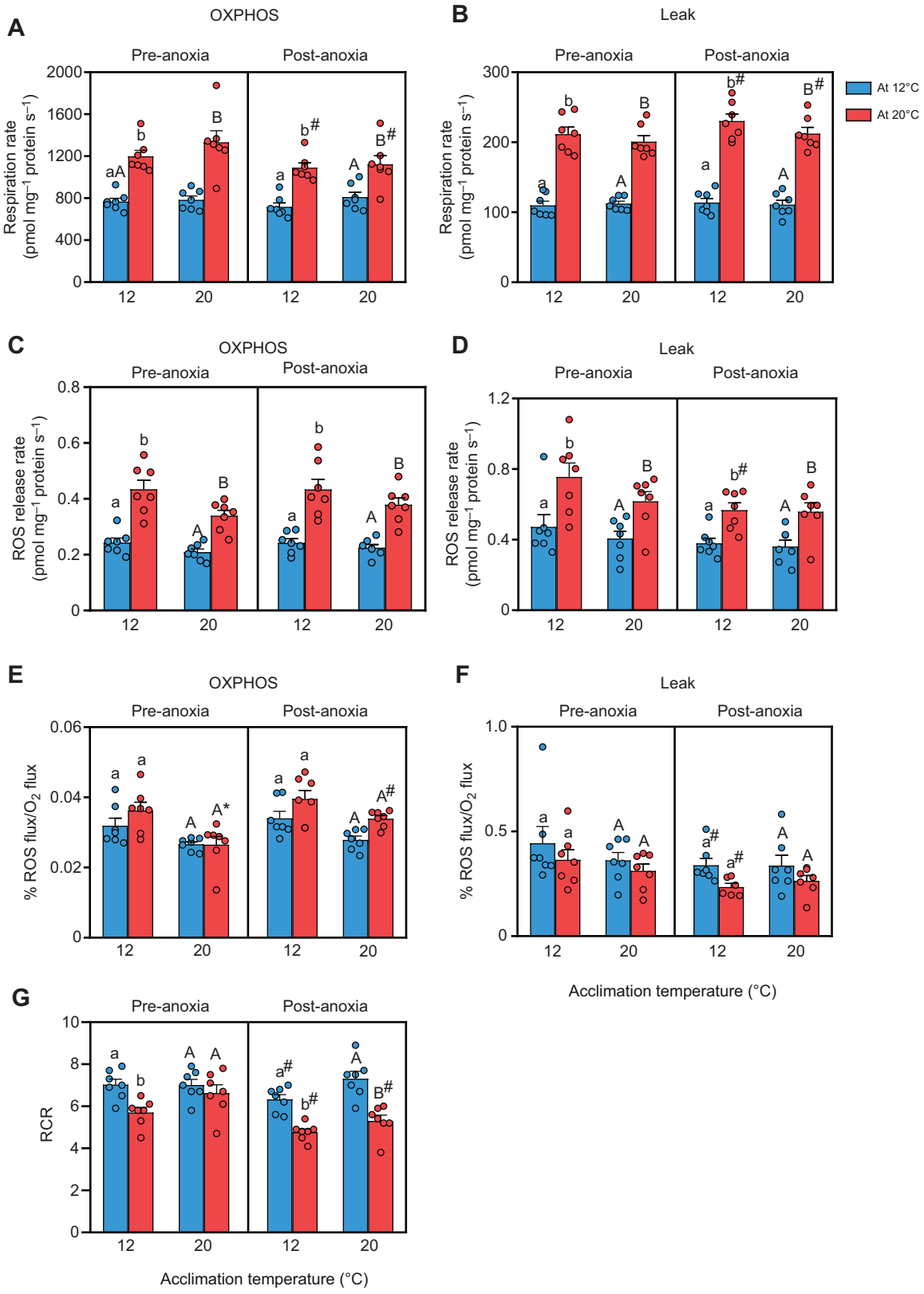

Fig. 3. Pre- and post-anoxia (10 min duration) mitochondrial respiration and ROS release rate for cardiac mitochondria from Atlantic salmon acclimated at 12°C and 20°C, and measured at both temperatures. All parameters were measured in the presence of substrates for CI+II. Respiration during (A) OXPHOS (State 3) and (B) leak (State 4) respiration pre- and post-anoxia. ROS release rate during (C) OXPHOS and (D) leak respiration pre- and post-anoxia. ROS release rate expressed as a percentage of respirational (O_2) flux during (E) OXPHOS and (F) leak respiration pre- and post-anoxia. (G) RCR (State 3/State 4) pre- and post-anoxia. Asterisk (*) indicates a significant (P<0.05) difference between acclimation groups. A number sign (#) indicates a significant (P<0.05) difference between complexes within an acclimation temperature/test temperature combination, whereas dissimilar lowercase and uppercase letters indicate a significant difference between test temperatures within an acclimation temperature/pre- post-anoxia combination. Results of the statistical analysis are presented in Table 1. The pre-anoxia CI+CII data are also shown in Figs 1 and 2. Other details are as in Fig. 1.

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Table 3. Myocardial and cardiac mitochondrial citrate synthase (CS) activity, and cardiac mitochondrial superoxide dismutase (SOD) activity, in Atlantic salmon acclimated at 12°C and 20°C

	12°C-acclimated salmon	20°C-acclimated salmon	Two-tailed unpaired *t*-test
Mitochondrial CS activity (U mg protein^{-1})	0.83±0.043	0.81±0.049	F=1.310, P=0.8174
Tissue CS activity (U g tissue^{-1})	11.20±0.41	10.64±0.61	F=2.103, P=0.6067
Mitochondrial SOD activity (U mg protein^{-1})	1.85±0.14	2.07±0.18	F=1.636, P=0.3657

There were no significant effects of acclimation temperature. Values are presented as means±s.e.m., N=7 per group.

by acclimation temperature (two-way ANOVA, F=0.4310, P=0.5250). For example, although a comparable increase in OXPHOS was reported after the addition of cytochrome *c* in 12°C- and 20°C-acclimated fish at 12°C (~7.2%), at 20°C, mitochondria from 20°C-acclimated salmon displayed the lowest cytochrome *c*-induced increase in oxygen consumption (~3.6%; *post hoc* t=3.158, P=0.0181; Fig. 5).

DISCUSSION

There is growing evidence that mitochondrial capacity may limit cardiac performance and, hence, play a major role in determining fish thermal tolerance (Iftikar and Hickey, 2013; Iftikar et al., 2014; Penney et al., 2014; Christen et al., 2018; O'Brien et al., 2018; Little et al., 2020). Thus, we investigated the response(s) of Atlantic salmon cardiac mitochondria to acute and chronic warming (to 20°C). Our results provide evidence that Atlantic salmon cardiac mitochondria can withstand acute and chronic warming to 20°C, and that this likely contributes to this species' capability for cardiac acclimation to warm temperatures (Anttila et al., 2014) and their high aerobic capacity across a wide range of temperatures (Hvas et al., 2017). Further, it is noteworthy that while acute warming increased ROS production/release rate and decreased RCR, these heat-induced effects were largely mitigated by warm acclimation to 20°C. Overall, our findings support the idea that thermal plasticity of cardiac mitochondrial function contributes to shaping fish cardiac performance and thermal tolerance.

Effects of acute warming to 20°C on cardiac mitochondrial function

Acute warming leads to an increase in physiological and biological rates, and the mitochondrial response to warming plays a key role in meeting the temperature-dependent increase in cardiac energy metabolism and function (Iftikar and Hickey, 2013; Iftikar et al., 2014, 2015; Ekström et al., 2016, 2017; Little et al., 2020). As expected, cardiac mitochondrial respiration was higher at 20°C as compared with 12°C (Fig. 1), and the magnitude of the increase was comparable to that reported in other fish species using permeabilized cardiac fibers (Chung et al., 2017; Hilton et al., 2010; Howald et al., 2019; Iftikar and Hickey, 2013; Iftikar et al., 2014) or isolated mitochondria (Christen et al., 2018; Ekström et al., 2017; Lemieux et al., 2010; O'Brien et al., 2018; Rodnick et al., 2014). Furthermore, the Q_{10} of 1.75 (for CI+CII OXPHOS between 12°C and 20°C; see Table 2 for details) during acute warming is similar to previously reported values for cardiac performance (i.e. heart rate, Q_{10} of 1.87 from 12 to 20°C; Anttila et al., 2014) and whole-organism O_2 consumption ($\dot{M}_{O_2}$, Q_{10} of 1.79 from 12 to 20°C; Leeuwis et al., 2019) in this species. This strongly suggests that cardiac mitochondrial function is finely tuned to meet the increased energy demands of the heart and whole organism when faced with elevated temperatures. Yet, some of the parameters measured suggest that mitochondrial functional integrity, and hence the mitochondria's capacity to efficiently produce ATP, was impaired. Most notable was the decrease in CI-dependent RCR following acute warming, which indicates a loss of CI coupling (Fig. 1C). This lower RCR in acutely warmed fish was driven primarily by an increase in leak respiration that was not compensated for by the increase in OXPHOS respiration (Fig. 1A,B), and this agrees with previous studies on fish cardiac

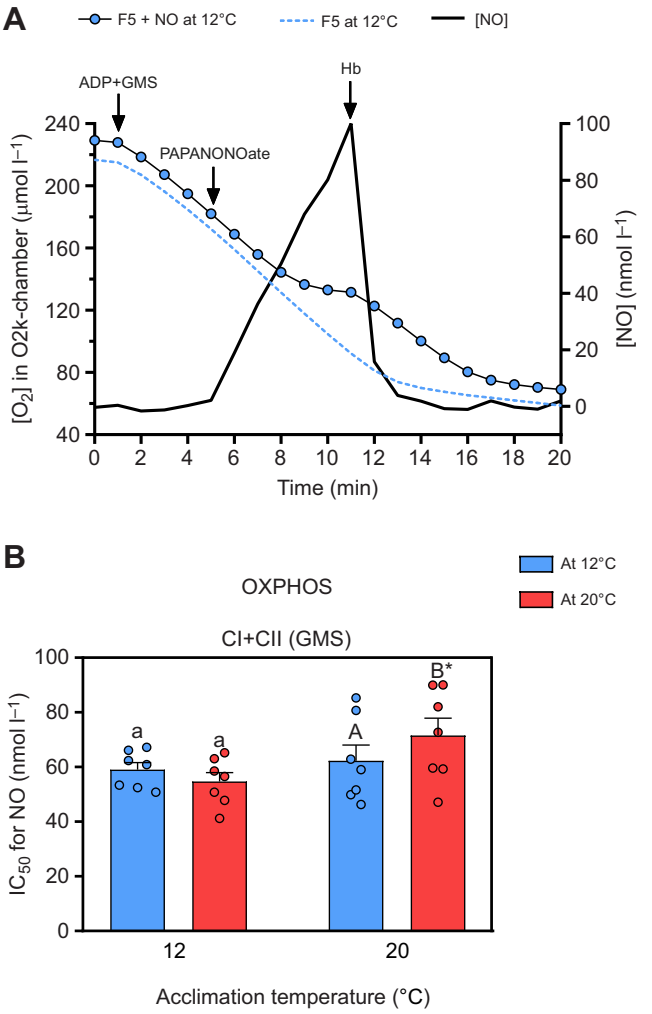

Fig. 4. Inhibition of State 3 (OXPHOS) respiration by nitric oxide (NO) in mitochondria isolated from Atlantic salmon acclimated at 12°C and 20°C, and measured at both temperatures. (A) Representative traces of mitochondrial respiration (O_2 consumption) in the absence (dotted line) or presence (blue circles) of the NO donor PAPANONOate, and of NO production (solid line). Cardiac mitochondria were incubated with CI+II substrates (GMS) and excess ADP. PAPANONOate was then added at ~60% air saturation during OXPHOS respiration to release NO at a rate of ~20 nmol l^{-1} min^{-1}. O_2 consumption by the mitochondria was gradually inhibited by NO release. When O_2 consumption was inhibited (i.e. the chamber O_2 level plateaued), hemoglobin (Hb) was added in excess to scavenge NO, and this reversed the NO inhibition of mitochondrial State 3 respiration. (B) Concentration of NO at which mitochondrial respiration rate was half of maximal (i.e. the IC_{50}). An asterisk (*) indicates a significant (P<0.05) difference between acclimation groups, whereas dissimilar letters indicate a significant difference between test temperatures within an acclimation group. In B, bars are means±s.e.m., whereas circles represent individual values (N=7 per group).

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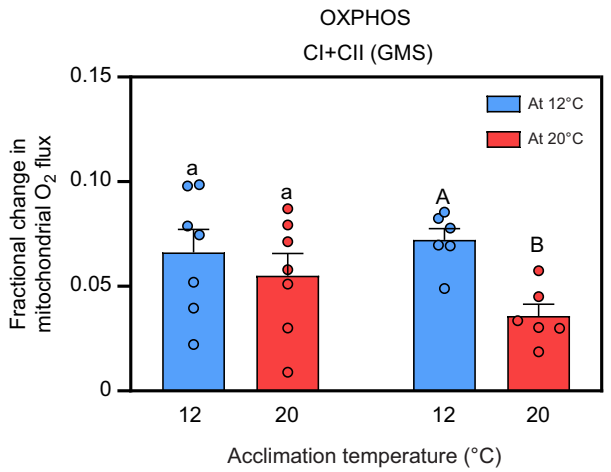

Fig. 5. Fractional increase in State 3 (OXPHOS) respiration with cytochrome *c* addition in cardiac mitochondria isolated from Atlantic salmon acclimated at 12°C and 20°C, and measured at both temperatures. The integrity of the outer mitochondrial membrane was measured in the presence of substrates for CI+II (GMS) and excess ADP. A change in O_2 flux above 0.1 (i.e. >10% increase in O_2 flux) suggests a loss of outer membrane integrity. Dissimilar lowercase and uppercase letters indicate a significant (P<0.05) difference between test temperatures within an acclimation group. There was no significant difference between acclimation groups. Bars are means±s.e.m., whereas circles represent individual values (N=7 in the cold-acclimated group and N=6 in the warm-acclimated group).

mitochondria indicating that CI is a thermally sensitive complex and a putative limiting step of the ETS (Iftikar and Hickey, 2013; Iftikar et al., 2014, 2015; Ekström et al., 2017; Christen et al., 2018). The impairment of CI's capacity for electron flow in response to acute warming could result from a decreased mitochondrial capacity for NADH production that constrains mitochondrial ATP production from NADH-linked substrates. Chung and Schulte (2015) showed that suppression of mitochondrial respiration in the liver of killifish (*Fundulus heteroclitus*) when acclimated to high temperatures (33°C) was primarily caused by lower respiration rates through CI. However, the RCR of CI+II was not lower when 12°C fish were acutely exposed to 20°C (Fig. 1C), and thus, it appears that CII may exert a higher level of control on mitochondrial respiration during acute warming, and result in mitochondrial function and integrity being fairly independent of temperature from 12 to 20°C in this species. This hypothesis is supported by the unaltered P:O ratios when mitochondria from 12°C-acclimated fish were acutely warmed to 20°C. This could underlie a temporary compensatory response in an attempt to conserve metabolic efficiency despite the loss of CI coupling during acute warming.

Another notable mitochondrial response to acute warming was a large (~65–78%) increase in absolute ROS release rate (Fig. 2A). An increase in ROS production during acute warming has also been reported in permeabilized cardiac fibers of wrasse from New Zealand (Iftikar and Hickey, 2013) and isolated cardiac mitochondria of Arctic char (Christen et al., 2018), although the increase in the latter species was not until the temperature reached 25°C. The elevated level of ROS production could be due to an imbalance between ROS production rates and antioxidant defense capacities, and possibly lead to the opening of the mitochondrial permeability transition pore (Murphy, 2009). The latter would increase inner membrane permeability, a hypothesis supported by the higher fractional increase in CI+CII respiration following the addition of NADH when mitochondria from 12°C-acclimated salmon were exposed to 20°C (Gerber et al., 2020).

Influence of acclimation to 20°C on cardiac physiology

Relative ventricular mass

Relative ventricular mass (RVM) does not change (Klaiman et al., 2011; Hevrøy et al., 2013, 2015; Keen et al., 2016), or decreases slightly (Anttila et al., 2015), when fish are acclimated to temperatures between 15 and 19°C. These data agree with the present study, and the results of Gamperl et al. (2020), who showed that the RVM of Atlantic salmon was not significantly affected by acclimation to 20°C for periods of up to a month. However, these authors also showed that RVM does increase when Atlantic salmon were pushed further (i.e. to 23°C). Overall, these data suggest that changes in RVM in this species are only seen when temperatures approach their maximum rearing temperature, where an increase in heart (ventricular mass) may help to compensate for the negative effects of high temperature on cardiac pumping capacity.

Mitochondrial respiration, efficiency and ROS production

Although the response of cardiac mitochondria to acute heat stress has been investigated in a number of studies, only limited and very recent information is available on how cardiac mitochondrial function might respond to chronic warming (Iftikar et al., 2015; Baris et al., 2016; Chung et al., 2017; Ekström et al., 2017; Leo et al., 2017; Pichaud et al., 2017, 2019; Howald et al., 2019). In the present study, we acclimated Atlantic salmon for 62–72 days to 20°C, and show that chronic exposure to 20°C mitigated some of the negative effects of an acute change from 12 to 20°C on mitochondrial function. For example, as compared with mitochondria from 12°C-acclimated fish acutely warmed to 20°C, 20°C-acclimated fish had a higher CI-RCR value, a higher Q_{10} for CI between 12 and 20°C, lower CI and CI+CII ROS production, and the lowest fractional increase in CI+CII respiration after the addition of cytochrome *c* (indicative of better outer membrane integrity) (Figs 1 and 2, Table 2). Furthermore, warm-acclimation did not alter CS activities in the myocardium or the mitochondria themselves (Table 3), suggesting that mitochondrial abundance and oxidative capacities in the heart were not altered (St-Pierre and Boutilier, 2001; Guderley and St-Pierre, 2002), consistent with the maintained mitochondrial respiratory function. Our observation that CS activity was not altered with warm acclimation is consistent with Chung et al. (2017), who showed that the activity of this enzyme was not affected by acclimating killifish to 33°C as compared with 15°C. Further, it is consistent with the results of Pichaud et al. (2017) for rainbow trout. Although these authors reported a temporary increase in heart CS activity when temperature was increased from 10 to 16°C, CS activity returned to control values after a few days of acclimation.

One benefit of warm acclimation (to 20°C) was the apparent preservation of CI functionality (i.e. maintained CI-RCR; Fig. 1 and Table 1), and this was confirmed in our subsequent study, which reports: improved mitochondrial capacity following 20°C acclimation until acute test temperature reached 26°C; and that enhanced CI functionality compensated for a loss of CII capacity induced by exposure to warm temperatures (Gerber et al., 2020). Collectively, these studies (present study; Gerber et al., 2020) support the central role of CI (i.e. CI capacity, thermal sensitivity and plasticity) in defining thermal tolerance, cardiac dysfunction (Iftikar et al., 2015; Christen et al., 2018) and acclimation potential in fish (Strobel et al., 2013). However, this acclimatory response (i.e. the enhancement of CI-dependent respiration) in cardiac mitochondria of warm-acclimated fish as compared with cold-acclimated fish is in contrast with that reported for other fish species. In another salmonid (the rainbow trout, *Oncorhynchus mykiss*), a

loss of cardiac mitochondrial functionality was primarily due to a loss of CI coupling and oxidative capacity when this species was acclimated to 16°C versus 10°C (Pichaud et al., 2017). This disparity is likely explained by the temperature of acclimation used in Pichaud et al. (2017) versus that used here; i.e. 16°C would be considered a preferred/optimum temperature for these species, whereas 20°C is closer to their IT_{max} (upper thermal tolerance) (Hvas et al., 2017; Gamperl et al., 2020). However, Iftikar et al. (2015) reported that wrasse cardiac mitochondrial function at 20°C was not higher in 20°C- versus 15°C-acclimated fish when measured at 20°C. Both Baris et al. (2016) and Chung et al. (2017) found that killifish acclimated to 28–33°C versus 12–15°C had a lower OXPHOS-I respiration rate when measured at 28–38°C. Similarly, in the European perch (*Perca fluvitalis*), the catalytic capacity of CI was decreased by warm acclimation (Ekström et al., 2017; Pichaud et al., 2019). One potential reason that salmon show a compensatory increase in, rather than a downregulation of, mitochondrial respiration is that, unlike many other temperate teleost species, the salmon is cold-active and has a high aerobic/metabolic capacity. It is possible that such species show a different acclimatory response when exposed to temperatures approaching their thermal limits for prolonged periods as compared with species such as the wrasse, killifish and European perch. This hypothesis would be consistent with Hvas et al. (2017), who reported that salmon acclimated to 23°C had a slightly higher maximum metabolic rate at this temperature than at 18 and 15°C. However, this hypothesis needs to be examined experimentally.

It is noteworthy that mitochondrial function was not affected by acute cooling as none of the parameters measured at 12°C in the 20°C-acclimated fish differed from those of the 12°C-acclimated fish. This is in agreement with two recent studies showing that cardiac mitochondria from European perch from a chronically heated habitat and warm-acclimated European seabass did not lose their mitochondrial capacity at lower temperatures (Howald et al., 2019; Pichaud et al., 2019).

The thermal sensitivity of ROS production and its role in the response to environmental changes (Blier et al., 2014; Birnie-Gauvin et al., 2017) are still under investigation, and there is limited information on the effects of chronic warming on mitochondrial ROS production. Chung and Schulte (2015) estimated liver mitochondrial ROS production rates (under State 4; no ADP+succinate+rotenone with or without Antimycin A) and free radical leak in killifish after 4 weeks of acclimation to 5, 15 or 33°C using the same Amplex UltraRed assay that we used here (Treberg et al., 2015), but did not detect any acclimation effect on ROS production or free radical leak. Here, we estimated ROS release rates in response to acute and chronic exposure to 20°C under more experimental/respiratory conditions (i.e. with CI substrates alone and both CI+CII substrates in presence and absence of ADP), and report that a clear benefit of warm acclimation in Atlantic salmon was a significant reduction in cardiac mitochondrial ROS production (Fig. 2 and Table 1). Indeed, relative ROS production/release (per respirational O_2 flux) was decreased by ∼30% at 20°C in mitochondria from warm-acclimated fish as compared with mitochondria acutely exposed to 20°C (i.e. from cold-acclimated fish) (Fig. 2). The mitigation in ROS production observed in chronically warmed fish is likely related to the preservation versus loss of CI functionality (i.e. CI coupling) reported in the present study and in Gerber et al. (2020). Indeed, CI is an important site of ROS production, and its impairment is known to induce a burst in ROS production (Murphy, 2009). A reduction in ROS release following warm acclimation could also be the result of a long-term antioxidant response (Banh et al., 2016), but no change in the mitochondrial activity of the antioxidant enzyme SOD was observed following warm acclimation (Table 3). Nonetheless, we have to acknowledge that other important antioxidant enzyme activities, such as those of catalase and glutathione peroxidase, were not assessed.

It is also well known that temperature influences mitochondrial membrane composition and fluidity (Hazel, 1995; Kraffe et al., 2007), which can affect: (1) proton leak (see Seebacher et al., 2010 for a review); (2) the oxidative capacity of mitochondria (see Guderley and St-Pierre, 2002 for a review); and (3) ROS production (see Blier et al., 2014 for a review). During thermal acclimation, considerable changes in fatty acid composition occur in the membranes of fish liver mitochondria (Strobel et al., 2013; Chung et al., 2018). Although cardiac mitochondrial membrane remodeling during chronic warming has not been investigated per se, a re-organization of the heart's lipid profile was observed in European perch following warm acclimation (Ekström et al., 2017) and chronic hypoxia has been shown to alter the lipid composition of cardiac mitochondria in the sablefish (*Anoplopoma fimbria*; Gerber et al., 2019). Nonetheless, although the fractional increase in O_2 flux after cytochrome *c* (an indication of outer mitochondrial integrity) measured at 20°C was the lowest in warm-acclimated salmon in this study (Fig. 5) and that of Gerber et al. (2020) (∼7% versus 3% at 20°C in 12°C- versus 20°C-acclimated fish), the effect of NADH on O_2 flux (an indication of inner mitochondrial membrane integrity) increased with test temperature and was not different (and possibly even marginally higher) in 20°C- versus 12°C-acclimated fish tested at temperatures from 20 to 28°C (Gerber et al., 2020). This latter result strongly suggests that changes in inner mitochondrial fatty acid composition (i.e. membrane integrity) were not related to the lower ROS release rates observed for 20°C-acclimated salmon.

NO effects on the sensitivity of mitochondrial respiration

Although an acute temperature increase from 12 to 20°C did not affect the sensitivity of mitochondrial respiration to NO, we found that the NO IC_{50} increased by 30% with warm acclimation as compared with both 12°C-acclimated fish measured at 20°C and 20°C-acclimated fish measured at 12°C (Fig. 4). Thus, this research shows that NO's modulation of mitochondrial respiration is sensitive to long-term/prolonged changes in temperature. That NOS/NO signaling in the fish heart is temperature sensitive is supported by two previous studies. In the European eel (*Anguilla anguilla*), NO modulation of the Frank–Starling response was evident at the fish's acclimation temperature, but not when 20°C (summer) hearts were tested at 10°C (winter) and vice versa (Amelio et al., 2013). In addition, Jørgensen et al. (2014) reported an increase in the expression of inducible NO synthase in the myocardium of Atlantic salmon when acclimated to 19°C for 3 and 8 weeks. The mechanism(s) mediating the loss of mitochondrial NO sensitivity in warm acclimated salmon is/are unknown, but could be the result of transitional changes in cytochrome *c* oxidase (CCO) function or in the affinity of CCO for its substrates – O_2, cytochrome *c* and NO (see Lesnefsky et al., 2001; Kraffe et al., 2007; Hilton et al., 2010). A diminished NO sensitivity may delay NO's inhibition of mitochondrial respiration and allow oxidative phosphorylation to better satisfy the increased metabolic demands associated with heat stress. However, there is not enough information or data in the literature for us to predict whether this effect (i.e. reduction in NO sensitivity) is detrimental or beneficial with regards to prolonged exposure to elevated temperatures close to

a fish's IT_{max}. Arguments both in favor and against this hypothesis can be envisioned given the ubiquitous role of NO in the regulation of mitochondrial signaling (Erusalimsky and Moncada, 2007; Palacios-Callender et al., 2004; Chouchani et al., 2013) and of the cardiovascular system (Moncada et al., 1991; Flögel et al., 2010; Imbrogno et al., 2018; Carenvale et al., 2020).

Warm acclimation does not lessen the relatively minor impacts of AR on salmon mitochondrial function

The effects of O_2 limitation at elevated temperatures on fish mitochondrial respiration and ROS production have received very limited attention (Sappal et al., 2015a,b; Onukwufor et al., 2016). Further, no previous study has looked at the effects of both an acute temperature increase and acclimation to the same high temperature on mitochondrial function after AR. In liver mitochondria from 10°C-acclimated trout acutely tested at 5, 13 and 25°C, Onukwufor et al. (2016) reported that AR resulted in a large decrease in State 3 respiration at 13 and 25°C (by ∼30–40% after 15 min of AR), increases in State 4 respiration of ∼1.8- and 3.1-fold, respectively, and that these changes resulted in a much greater decrease in the mitochondria's RCR at 25°C versus 13°C (from ∼7 to 3 versus 2, respectively). In general, our results for cardiac mitochondria exposed to 10 min of AR agree with those of Onukwufor et al. (2016), in that the impact of AR on mitochondrial function was greater at higher temperatures. However, we show that AR at 12°C had no impact on these parameters, and that even at 20°C the effects were relatively minor (changes of ∼10–15% for all parameters) (Fig. 3). Sappal et al. (2015b) exposed liver mitochondria from 11°C- and 20°C-acclimated fish to AR for 10 min at their acclimation temperature. They showed that exposure of liver mitochondria to AR also resulted in large changes in the above three parameters in both 11°C- and 20°C-acclimated fish, and that acclimation to 20°C versus 11°C worsened the deleterious effects of AR on mitochondrial respiration. In contrast, we showed that AR at 12°C had no effects on mitochondrial respiration, and that the overall effects of AR on mitochondrial respiration at 20°C were relatively minor and not affected by acclimation temperature (Fig. 3A,B,G). These results clearly show that the response of salmon cardiac mitochondria to temperature acclimation and AR are much different than those of trout liver mitochondria (these are both salmonid species with similar upper thermal and hypoxia tolerances; Beitinger et al., 2000; Barnes et al., 2011; Remen et al., 2013; Williams et al., 2019). What factor(s) underlie these differences in response are not known, but perhaps the fact that the majority of the salmonid heart is perfused by venous blood of low oxygen content and P_{O_2} (Farrell and Smith, 2017) plays a role. Further, and importantly, there was no indication at all in the present study that acclimation to high temperatures affects the tolerance of mitochondrial respiration to anoxia exposure and/or O_2 limitation. This result agrees with the majority of studies that have examined aspects of fish cardiorespiratory physiology, and show that there is no, or very little, 'cross-tolerance' between hypoxia and high temperature exposure in this taxa (Anttila et al., 2015; Motyka et al., 2017; Levesques et al., 2019; McBryan et al., 2016).

Post-anoxia, ROS production/release was unchanged or slightly lower in both acclimation groups, with the exception that fish acclimated to and tested at 20°C had a slightly higher ROS production/release rate (Fig. 3E,F), Given that the latter effect was relatively small, and largely related to the fact that acclimation to 20°C decreased ROS production/release at 20°C as compared to fish acclimated to 12°C, our results agree with those of Onukwufor et al. (2016), who showed that AR had no effect on State 4 ROS production/release in rainbow trout.

Summary and perspectives

This study provides several novel insights into how acclimation alters the Atlantic salmon's mitochondrial response to temperatures (20°C) near its maximum rearing temperature (∼23°C; Gamperl et al., 2020), and key information with regards to the mechanistic basis for this species' cardiac and aerobic plasticity in the face of warming temperatures (Anttila et al., 2014; Hvas et al., 2017). For example, within the limits of this study, we found that: the salmon's cardiac mitochondria are resilient to warming in the range of 12–20°C; and that although acclimation to 20°C does not constrain or diminish mitochondrial function at 12°C, it protects mitochondrial coupling and decreases ROS production at 20°C. Yet, the two acclimation groups differed very little with respect to their cardiac mitochondrial responses to warming and O_2 limitation (AR). These data strongly suggest that mitochondrial function does not experience 'cross-tolerance' to the major environmental challenges of high temperature and O_2 limitation, and questions the ability of acclimation to one of these challenges to preserve cardiac mitochondrial function and integrity under climate change scenarios where hypoxia and high temperatures often co-occur.

The question of whether this thermal plasticity preserves or improves mitochondrial function (and thus, potentially salmon upper thermal tolerance) has been addressed in a companion manuscript to this work (Gerber et al., 2020). In that paper, we confirm many of the findings of this study, but also provide compelling evidence that warm acclimation to 20°C does not result in a large trade-off in Atlantic salmon mitochondrial function (i.e. increased oxidative phosphorylation does not result in heightened ROS production), and that it protects mitochondrial functional capacity until temperatures reach at least 24°C versus 20°C in 12°C-acclimated fish. However, questions remain about the acclimatory mechanisms responsible for the mitigation of ROS production at high temperatures, and why there are so disparate responses between temperate teleost species with regards to the mitochondrial acclimatory response to warm temperatures (i.e. those approaching the maximum temperatures they can tolerate; e.g. the present study versus Iftikar et al. 2015; Ekström et al. 2017; Baris et al. 2016 and Chung et al. 2017). Further, the importance of the adjustment in mitochondrial NO sensitivity with prolonged warming, and its implications for cardiovascular protection and functionality, remain to be determined. Clearly, these are critical questions with regard to mitochondrial function and cardiac performance at high temperatures that are germane not only to our understanding of fish physiology and thermal tolerance, but also to the management and conservation of fish species as the impacts of climate change on aquatic species intensify (Helmuth, 2009; Cooke et al., 2013; Speers-Roesch and Norin, 2016; Norin and Metcalfe, 2019; Little et al., 2020).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.G., A.K.G.; Methodology: L.G., K.A.C., A.K.G.; Validation: L.G., A.K.G.; Formal analysis: L.G., K.A.C.; Investigation: L.G., K.A.C.; Resources: A.K.G.; Data curation: L.G., K.A.C.; Writing - original draft: L.G., A.K.G.; Writing - review & editing: L.G., K.A.C., A.K.G.; Visualization: L.G., K.A.C., A.K.G.; Supervision: A.K.G.; Project administration: A.K.G.; Funding acquisition: A.K.G.

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Supplementary information

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References

Amelio, D., Garofalo, F., Capria, C., Tota, B. and Imbrogno, S. (2013). Effects of temperature on the nitric oxide-dependent modulation of the Frank-Starling mechanism: the fish heart as a case study. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **164**, 356-362. doi:10.1016/j.cbpa.2012.10.037

Anttila, K., Dhillon, R. S., Boulding, E. G., Farrell, A. P., Glebe, B. D., Elliott, J. A. K., Wolters, W. R. and Schulte, P. M. (2013). Variation in temperature tolerance among families of Atlantic salmon (*Salmo salar*) is associated with hypoxia tolerance, ventricle size and myoglobin level. *J. Exp. Biol.* **216**, 1183-1190. doi:10.1242/jeb.080556

Anttila, K., Couturier, C. S., Øverli, Ø., Johnsen, A., Marthinsen, G., Nilsson, G. E. and Farrell, A. P. (2014). Atlantic salmon show capability for cardiac acclimation to warm temperatures. *Nat. Commun.* **5**, 6-11. doi:10.1038/ncomms5252

Anttila, K., Lewis, M., Prokkola, J. M., Kanerva, M., Seppanen, E., Kolari, I. and Nikinmaa, M. (2015). Warm acclimation and oxygen depletion induce species-specific responses in salmonids. *J. Exp. Biol.* **218**, 1471-1477. doi:10.1242/jeb.119115

Banh, S., Wiens, L., Sotiri, E. and Treberg, J. R. (2016). Mitochondrial reactive oxygen species production by fish muscle mitochondria: potential role in acute heat-induced oxidative stress. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **191**, 99-107. doi:10.1016/j.cbpb.2015.10.001

Baris, T. Z., Crawford, D. L. and Oleksiak, M. F. (2016). Acclimation and acute temperature effects on population differences in oxidative phosphorylation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **310**, R185-R196. doi:10.1152/ajpregu.00421.2015

Barnes, R. K., King, H. and Carter, C. G. (2011). Hypoxia tolerance and oxygen regulation in Atlantic salmon, *Salmo salar* from a Tasmanian population. *Aquaculture* **318**, 397-401. doi:10.1016/j.aquaculture.2011.06.003

Beitinger, T. L., Bennett, W. A. and McCauley, R. W. (2000). Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Env. Biol. Fish.* **58**, 237-275. doi:10.1023/A:1007676325825

Birnie-Gauvin, K., Costantini, D., Cooke, S. J. and Willmore, W. G. (2017). A comparative and evolutionary approach to oxidative stress in fish: a review. *Fish Fisheries* **18**, 928-942. doi:10.1111/faf.12215

Blier, P. U., Lemieux, H. and Pichaud, N. (2014). Holding our breath in our modern world: Will mitochondria keep the pace with climate changes? *Can. J. Zool.* **92**, 591-601. doi:10.1139/cjz-2013-0183

Carenvale, C., Syme, D. A. and Gamperl, A. K. (2020). Effects of hypoxic acclimation, muscle strain and contraction frequency on nitric oxide-mediated myocardial performance in steelhead trout (*Oncorhynchus mykiss*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **318**, R214-R226. doi:10.1152/ajpregu.00107.2019

Chouchani, E. T., Methner, C., Nadtochiy, S. M., Logan, A., Pell, V. R., Ding, S., James, A. M., Cochemé, H. M., Reinhold, J., Lilley, K. S. et al. (2013). Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. *Nat. Med.* **19**, 753-759. doi:10.1038/nm.3212

Christen, F., Desrosiers, V., Dupont-Cyr, B. A., Vandenberg, G. W., Le François, N. R., Tardif, J.-C., Dufresne, F., Lamarre, S. G., Blier, P. U. (2018). Thermal tolerance and thermal sensitivity of heart mitochondria: mitochondrial integrity and ROS production. *Free Radic. Biol. Med.* **116**, 11-18. doi:10.1016/j.freeradbiomed.2017.12.037

Chung, D. J. and Schulte, P. M. (2015). Mechanisms and costs of mitochondrial thermal acclimation in a eurythermal killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **218**, 1621-1631. doi:10.1242/jeb.120444

Chung, D. J., Bryant, H. J. and Schulte, P. M. (2017). Thermal acclimation and subspecies-specific effects on heart and brain mitochondrial performance in a eurythermal teleost (*Fundulus heteroclitus*). *J. Exp. Biol.* **220**, 1459-1471. doi:10.1242/jeb.151217

Chung, D. J., Sparagna, G. C., Chicco, A. J. and Schulte, P. M. (2018). Patterns of mitochondrial membrane remodeling parallel functional adaptations to thermal stress. *J. Exp. Biol.* **221**, jeb174458. doi:10.1242/jeb.174458

Cooke, S. J., Sack, L., Franklin, C. E., Farrell, A. P., Beardall, J., Wikelski, M. and Chown, S. L. (2013). What is conservation physiology? Perspectives on an increasingly integrated and essential science. *Conserv. Physiol.* **1**. doi:10.1093/conphys/cot001

Ekström, A., Brijs, J., Clark, T. D., Gräns, A., Jutfelt, F. and Sandblom, E. (2016). Cardiac oxygen limitation during an acute thermal challenge in the European perch: effects of chronic environmental warming and experimental hyperoxia. *Am. J. Physiol. Integr. Comp. Physiol.* **311**, R440-R449. doi:10.1152/ajpregu.00530.2015

Ekström, A., Sandblom, E., Blier, P. U., Dupont Cyr, B.-A., Brijs, J. and Pichaud, N. (2017). Thermal sensitivity and phenotypic plasticity of cardiac mitochondrial metabolism in European perch, *Perca fluviatilis*. *J. Exp. Biol.* **220**, 386-396. doi:10.1242/jeb.150698

Eliason, E. J. and Anttila, K. (2017). Temperature and the cardiovascular system. In *Fish Physiology – The Cardiovascular System Development, Plasticity and Physiological Responses* (ed. A. K. Gamperl, T. E. Gillis, A. P. Farrell and C. J. Brauner), pp. 235-297. Elsevier.

Erusalimsky, J. D. and Moncada, S. (2007). Nitric oxide and mitochondrial signaling: From physiology to pathophysiology. *Arterioscler. Thromb. Vasc. Biol.* **27**, 2524-2531. doi:10.1161/ATVBAHA.107.151167

Fago, A. and Jensen, F. B. (2015). Hypoxia tolerance, nitric oxide, and nitrite: lessons from extreme animals. *Physiol.* **30**, 116-126. doi:10.1152/physiol.00051.2014

Fago, A., Jensen, F. B., Tota, B., Feelisch, M., Olson, K. R., Helbo, S., Lefevre, S., Mancardi, D., Palumbo, A., Sandvik, G. K. et al. (2012). Integrating nitric oxide, nitrite and hydrogen sulfide signaling in the physiological adaptations to hypoxia: a comparative approach. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **162**, 1-6. doi:10.1016/j.cbpa.2012.01.011

Farrell, A. P. (2002). Cardiorespiratory performance in salmonids during exercise at high temperature: Insights into cardiovascular design limitations in fishes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **132**, 797-810. doi:10.1016/S1095-6433(02)00049-1

Farrell, A. P. and Smith, F. (2017). Cardiac form, function and physiology. In *Fish Physiology* (ed. A. K. Gamperl, T. E. Gillis, A. P. Farrell and C. J. Brauner), Vol. 36, pp. 155-264. Elsevier.

Fasching, M. and Gnaiger, E. (2016). O_2k-MultiSensor system with amperometric sensors: NO, H_2S, H_2O_2. *Mitochondr. Physiol. Netw.* **15.05**, 1-11.

Fasching, M. and Gnaiger, E. (2020). O_2k Quality Control 2: Instrumental oxygen background correction and accuracy of oxygen flux. *Mitochondr. Physiol. Netw.* **14.06**, 1-16.

Flögel, U., Fago, A. and Rassaf, T. (2010). Keeping the heart in balance: the functional interactions of myoglobin with nitrogen oxides. *J. Exp. Biol.* **213**, 2726-2733. doi:10.1242/jeb.041681

Gamperl, A. K., Ajiboye, O. O., Zanuzzo, F. S., Sandrelli, R. M., Peroni, E. F. C. and Beemelmanns, A. (2020). The impacts of increasing temperature and moderate hypoxia on the production characteristics, cardiac morphology and haematology of Atlantic salmon (*Salmo salar*). *Aquaculture* **519**, 734874. doi:10.1016/j.aquaculture.2019.734874

Gerber, L., Clow, K. A., Katan, T., Emam, M., Leeuwis, R. H. J., Parrish, C. C. and Gamperl, A. K. (2019). Cardiac mitochondrial function, nitric oxide sensitivity and lipid composition following hypoxia acclimation in sablefish. *J. Exp. Biol.* **222**, jeb208074. doi:10.1242/jeb.208074

Gerber, L., Clow, K. A., Mark, F. C. and Gamperl, A. K. Improved mitochondrial function in salmon (*Salmo salar*) following high temperature acclimation suggests that there are cracks in the proverbial 'ceiling'. *Sci. Rep.* **10**, 21636. doi:10.1038/s41598-020-78519-4

Gnaiger, E. (2010). Oxygen calibration and solubility in experimental media. *Surgery* **12**, 1-12.

Gnaiger, E. (2018). O2k Quality Control 1: polarographic oxygen sensors and accuracy of calibration. *Mitochondrial Physiol. Netw.* 06.03, 1-20.

Guderley, H. and St-Pierre, J. (2002). Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. *J. Exp. Biol.* **205**, 2237-2249.

Haverinen, J. and Vornanen, M. (2020). Atrioventricular block, due to reduced ventricular excitability, causes the depression of fish heart rate in fish at critically high temperatures. *J. Exp. Biol.* **223**, jeb.225227.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42. doi:10.1146/annurev.ph.57.030195.000315

Helmuth, B. (2009). From cells to coastlines: how can we use physiology to forecast the impacts of climate change? *J. Exp. Biol.* **212**, 753-760. doi:10.1242/jeb.023861

Hevrøy, E. M., Hunskår, C., de Gelder, S., Shimizu, M., Waagbø, R., Breck, O., Takle, H., Sussort, S. and Hansen, T. (2013). GH–IGF system regulation of attenuated muscle growth and lipolysis in Atlantic salmon reared at elevated sea temperatures. *J. Comp. Physiol. B.* **183**, 243-259. doi:10.1007/s00360-012-0704-5

Hevrøy, E. M., Tipsmark, C. K., Remø, S. C., Hansen, T., Fukuda, M., Torgersen, T., Vikeså, V., Olsvik, P. A., Waagbø, R. and Shimizu, M. (2015). Role of the GH-IGF-1 system in Atlantic salmon and rainbow trout post-smolts at elevated water temperature. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **188**, 127-138. doi:10.1016/j.cbpa.2015.06.030

Hilton, Z., Clements, K. D. and Hickey, A. J. R. (2010). Temperature sensitivity of cardiac mitochondria in intertidal and subtidal triplefin fishes. *J. Comp. Physiol.* **180**, 979-990. doi:10.1007/s00360-010-0477-7

Howald, S., Cominassi, L., LeBayon, N., Claireaux, G. and Mark, F. C. (2019). Future ocean warming may prove beneficial for the northern population of European seabass, but ocean acidification will not. *J. Exp. Biol.* **222**, jeb213017. doi:10.1242/jeb.213017

Hvas, M., Folkedal, O., Imsland, A. and Oppedal, F. (2017). The effect of thermal acclimation on aerobic scope and critical swimming speed in Atlantic salmon, *Salmo salar*. *J. Exp. Biol.* **220**, 2757-2764. doi:10.1242/jeb.154021

Iftikar, F. I. and Hickey, A. J. R. (2013). Do mitochondria limit hot fish hearts? Understanding the role of mitochondrial function with heat stress in *Notolabrus celidotus*. *PLoS ONE* **8**, e64120. doi:10.1371/journal.pone.0064120

Iftikar, F. I., Macdonald, J. R., Baker, D. W., Renshaw, G. M. C. and Hickey, A. J. R. (2014). Could thermal sensitivity of mitochondria determine species distribution in a changing climate? *J. Exp. Biol.* **217**, 2348-2357. doi:10.1242/jeb.098798

Iftikar, F. I., Morash, A. J., Cook, D. G., Herbert, N. A. and Hickey, A. J. R. (2015). Temperature acclimation of mitochondria function from the hearts of a temperate wrasse (*Notolabrus celidotus*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **184**, 46-55. doi:10.1016/j.cbpa.2015.01.017

Imbrogno, S., Filice, M., Cerra, M. C. and Gattuso, A. (2018). NO, CO, and H_2S: What about gasotransmitters in fish and amphibian hearts? *Acta Physiol.* **223**, e13035. doi:10.1111/apha.13035

Jørgensen, S. M., Castro, V., Krasnov, A., Torgersen, J., Timmerhaus, G., Hevrøy, E. M., Hansen, T. J., Susort, S., Breck, O. and Takle, H. (2014). Cardiac responses to elevated seawater temperature in Atlantic salmon. *BMC Physiol.* **14**, 2. doi:10.1186/1472-6793-14-2

Keen, A. N., Fenna, A. J., McConnell, J. C., Sherratt, M. J., Gardner, P. and Shiels, H. A. (2016). The dynamic nature of hypertrophic and fibrotic remodeling of the fish ventricle. *Front. Physiol.* **6**, 427. doi:10.3389/fphys.2015.00427

Klaiman, J. M., Fenna, A. J., Shiels, H. A., Macri, J. and Gillis, T. E. (2011). Cardiac remodeling in fish: strategies to maintain heart function during temperature change. *PLoS ONE* **6**, e24464. doi:10.1371/journal.pone.0024464

Kraffe, E., Marty, Y. and Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions. *J. Exp. Biol.* **210**, 149-165. doi:10.1242/jeb.02628

Krumschnabel, G., Fontana-Ayoub, M., Fasching, M. and Gnaiger, E. (2019). O_2k-FluoRespirometry: HRR and simultaneous determination of H_2O_2 production with Amplex UltraRed. *Mitochondr. Physiol. Netw.* 20.14, 1-6.

Leeuwis, R. H. J., Nash, G. W., Sandrelli, R. M., Zanuzzo, F. S. and Gamperl, A. K. (2019). the environmental tolerances and metabolic physiology of sablefish (*Anoplopoma fimbria*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **231**, 140-148. doi:10.1016/j.cbpa.2019.02.004

Lemieux, H., Tardif, J.-C., Dutil, J.-D. and Blier, P. U. (2010). Thermal sensitivity of cardiac mitochondrial metabolism in an ectothermic species from a cold environment, Atlantic wolffish (*Anarhichas lupus*). *J. Exp. Mar. Bio. Ecol.* **384**, 113-118. doi:10.1016/j.jembe.2009.12.007

Leo, E., Kunz, K. L., Schmidt, M., Storch, D., Pörtner, H.-O. and Mark, F. C. (2017). Mitochondrial acclimation potential to ocean acidification and warming of polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*). *Front. Zool.* **14**, 1-12. doi:10.1186/s12983-017-0205-1

Lesnefsky, E. J., Gudz, T. I., Moghaddas, S., Migita, C. T., Ikeda-Saito, M., Turkaly, P. J. and Hoppel, C. L. (2001). Aging decreases electron transport complex III activity in heart interfibrillar mitochondria by alteration of the cytochrome *c* binding site. *J. Mol. Cell. Cardiol.* **33**, 37-47. doi:10.1006/jmcc.2000.1273

Levesques, K. D., Wright, P. A. and Bernier, N. (2019). Cross talk without cross tolerance: effect of rearing temperature on the hypoxia response of embryonic zebrafish. *J. Physiol. Biochem. Zool.* **92**, 349-364. doi:10.1086/703178

Little, A. G., Loughland, I. and Seebacher, F. (2020). What do warming waters mean for fish physiology and fisheries? *J. Fish Biol.* **97**, 328-340. doi:10.1111/jfb.14402

Makrecka-Kuka, M., Krumschnabel, G. and Gnaiger, E. (2015). High-resolution respirometry for simultaneous measurement of oxygen and hydrogen peroxide fluxes in permeabilized cells, tissue homogenate and isolated mitochondria. *Biomol.* **5**, 1319-1338. doi:10.3390/biom5031319

McBryan, T. L., Healy, T. M., Haakons, K. L. and Schulte, P. M. (2016). Warm acclimation improves hypoxia tolerance in *Fundulus heteroclitus*. *J. Exp. Biol.* **219**, 474-484. doi:10.1242/jeb.133413

Moncada, S., Palmer, J. M. and Higgs, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109-142.

Motyka, R., Norin, T., Petersen, L. H., Huggett, D. B. and Gamperl, A. K. (2017). Long-term hypoxia exposure alters the cardiorespiratory physiology of steelhead trout (*Oncorhynchus mykiss*), but does not affect their upper thermal tolerance. *J. Therm. Biol.* **68**, 149-161. doi:10.1016/j.jtherbio.2016.03.007

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem. J.* **417**, 1-13. doi:10.1042/BJ20081386

Nakano, H., Lee, S.-D. A., Ray, A. D., Krasney, J. A. and Farkas, G. A. (2001). Role of nitric oxide in thermoregulation and hypoxic ventilatory response in obese zucker rats. *Am. J. Respir. Crit. Care Med.* **164**, 437-442. doi:10.1164/ajrccm.164.3.2010142

Norin, T. and Metcalfe, N. B. (2019). Ecological and evolutionary consequences of metabolic rate plasticity in response to environmental change. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 1768. doi:10.1098/rstb.2018.0180

O'Brien, K. M., Rix, A. S., Egginton, S., Farrell, A. P., Crockett, E. L., Schlauch, K., Woolsey, R., Hoffman, M. and Merriman, S. (2018). Cardiac mitochondrial metabolism may contribute to differences in thermal tolerance of red- and white-blooded Antarctic notothenioid fishes. *J. Exp. Biol.* **221**, jeb177816. doi:10.1242/jeb.177816

Onukwufor, J. O., Kibenge, F., Stevens, D. and Kamunde, C. (2016). Hypoxia-reoxygenation differentially alters the thermal sensitivity of complex I basal and maximal mitochondrial oxidative capacity. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **201**, 87-94. doi:10.1016/j.cbpa.2016.06.033

Palacios-Callender, M., Quintero, M., Hollis, V. S., Springett, R. J. and Moncada, S. (2004). Endogenous NO regulates superoxide production at low oxygen concentrations by modifying the redox state of cytochrome *c* oxidase. *Proc. Nat. Acad. Sci. USA* **101**, 7630-7635. doi:10.1073/pnas.0401723101

Penney, C. M., Nash, G. W. and Kurt Gamperl, A. (2014). Cardiorespiratory responses of seawater-acclimated adult Arctic char (*Salvelinus alpinus*) and Atlantic salmon (*Salmo salar*) to an acute temperature increase. *Can. J. Fish. Aquat. Sci.* **71**, 1096-1105. doi:10.1139/cjfas-2013-0569

Perry, S., Kumai, Y., Porteus, C. S., Tzaneva, V. and Kwong, R. W. M. (2016). An emerging role for gasotransmitters in the control of breathing and ionic regulation in fish. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **186**, 145-159. doi:10.1007/s00360-015-0949-x

Pichaud, N., Ekström, A., Hellgren, K. and Sandblom, E. (2017). Dynamic changes in cardiac mitochondrial metabolism during warm acclimation in rainbow trout. *J. Exp. Biol.* **220**, 1674-1683. doi:10.1242/jeb.152421

Pichaud, N., Ekström, A., Breton, S., Sundström, F., Rowinski, R., Blier, P. U. and Sandblom, E. (2019). Cardiac mitochondrial plasticity and thermal sensitivity in a fish inhabiting an artificially heated ecosystem. *Sci. Rep.* **9**, 17832. doi:10.1038/s41598-019-54165-3

Remen, M., Oppedal, F., Imsland, A. K., Olsen, R. E. and Torgersen, T. (2013). Hypoxia tolerance thresholds for post-smolt Atlantic salmon: dependency of temperature and hypoxia acclimation. *Aquaculture* **416**, 41-47. doi:10.1016/j.aquaculture.2013.08.024

Rodnick, K. J. and Gesser, H. (2017). Cardiac energy metabolism. In *The Cardiovascular System: Morphology, Control and Function*, Vol. 36A (ed. A. K. Gamperl, T. E. Gillis, A. P. Farrell and C. J. Brauner), pp. 317-367. San Diego: Academic Press.

Rodnick, K. J., Gamperl, A. K., Nash, G. W. and Syme, D. A. (2014). Temperature and sex dependent effects on cardiac mitochondrial metabolism in Atlantic cod (*Gadus morhua L.*). *J. Therm. Biol.* **44**, 10-118. doi:10.1016/j.jtherbio.2014.02.012

Sandblom, E., Clark, T. D., Gräns, A., Ekström, A., Brijs, J., Sundström, L. F., Odelström, A., Adill, A., Aho, T. and Jutfelt, F. (2016). Physiological constraints to climate warming in fish follow principles of plastic floors and concrete ceilings. *Nat. Commun.* **7**, 1-8. doi:10.1038/ncomms11447

Sappal, R., Fast, M., Stevens, D., Kibenge, F., Siah, A. and Kamunde, C. (2015a). Effects of copper, hypoxia and acute temperature shifts on mitochondrial oxidation in rainbow trout (*Oncorhynchus mykiss*) acclimated to warm temperature. *Aquat. Toxicol.* **169**, 46-57. doi:10.1016/j.aquatox.2015.10.006

Sappal, R., MacDougald, M., Fast, M., Stevens, D., Kibenge, F., Siah, A. and Kamunde, C. (2015b). Alterations in mitochondrial electron transport system activity in response to warm acclimation, hypoxia-reoxygenation and copper in rainbow trout, *Oncorhynchus mykiss*. *Aquat. Toxicol.* **165**, 51-63. doi:10.1016/j.aquatox.2015.05.014

Seebacher, F., Brand, M. D., Else, P. L., Guderley, H., Hulbert, A. J. and Moyes, C. D. (2010). Plasticity of oxidative metabolism in variable climates: molecular mechanisms. *Physiol. Biochem. Zool.* **83**, 721-732. doi:10.1086/649964

Simon, E. (1998). Nitric oxide as a peripheral and central mediator in temperature regulation. *Amino Acids* **14**, 87-93. doi:10.1007/BF01345248

Speers-Roesch, B. and Norin, T. (2016). Ecological significance of thermal tolerance and performance in fishes: new insights from integrating field and laboratory approaches. *Funct. Ecol.* **30**, 842-884. doi:10.1111/1365-2435.12652

St-Pierre, J. and Boutilier, R. G. (2001). Aerobic capacity of frog skeletal muscle during hibernation. *Physiol. Biochem. Zool.* **74**, 390-397. doi:10.1086/320428

Strobel, A., Graeve, M., Poertner, H. O. and Mark, F. C. (2013). Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic nototheniid fish, *Notothenia rossii* and *Lepidonotothen squamifrons*. *PLoS ONE* **8**, e68865. doi:10.1371/journal.pone.0068865

Treberg, J. R., MacCormack, T. J., Lewis, J. M., Almeida-Val, V. M. F., Val, A. L. and Driedzic, W. R. (2007). Intracellular glucose and binding of hexokinase and phosphofructokinase to particulate fractions increase under hypoxia in heart of the Amazonian armored catfish (*Liposarcus pardalis*). *Physiol. Biochem. Zool.* **80**, 542-550. doi:10.1086/520129

Treberg, J. R., Munro, D., Banh, S., Zacharias, P. and Sotiri, E. (2015). Differentiating between apparent and actual rates of H_2O_2 metabolism by isolated rat muscle mitochondria to test a simple model of mitochondria as regulators of H_2O_2 concentration. *Redox Biol.* **5**, 216-224. doi:10.1016/j.redox.2015.05.001

Williams, K. J., Cassidy, A. A., Verhille, C. E., Lamarre, S. G. and MacCormack, T. J. (2019). Diel cycling hypoxia enhances hypoxia tolerance in rainbow trout (*Oncorhynchus mykiss*): evidence of physiological and metabolic plasticity. *J. Exp. Biol.* **222**, jeb206045. doi:10.1242/jeb.206045