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RESEARCH ARTICLE

Cardiac mitochondrial function, nitric oxide sensitivity and lipid composition following hypoxia acclimation in sablefish

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ABSTRACT

In fishes, the effect of O_2 limitation on cardiac mitochondrial function remains largely unexplored. The sablefish (*Anoplopoma fimbria*) encounters considerable variations in environmental oxygen availability, and is an interesting model for studying the effects of hypoxia on fish cardiorespiratory function. We investigated how *in vivo* hypoxia acclimation (6 months at 40% then 3 weeks at 20% air saturation) and *in vitro* anoxia-reoxygenation affected sablefish cardiac mitochondrial respiration and reactive oxygen species (ROS) release rates using high-resolution fluorespirometry. Further, we investigated how hypoxia acclimation affected the sensitivity of mitochondrial respiration to nitric oxide (NO), and compared mitochondrial lipid and fatty acid (FA) composition between groups. Hypoxia acclimation did not alter mitochondrial coupled or uncoupled respiration, or respiratory control ratio, ROS release rates, P_{50} or superoxide dismutase activity. However, it increased citrate synthase activity (by ∼20%), increased the sensitivity of mitochondrial respiration to NO inhibition (i.e., the NO IC_{50} was 25% lower), and enhanced the recovery of respiration (by 21%) and reduced ROS release rates (by 25–30%) post-anoxia. In addition, hypoxia acclimation altered mitochondrial FA composition [increasing arachidonic acid (20:4ω6) and eicosapentaenoic acid (20:5ω3) proportions by 11 and 14%, respectively], and SIMPER analysis revealed that the phospholipid:sterol ratio was the largest contributor (24%) to the dissimilarity between treatments. Overall, these results suggest that hypoxia acclimation may protect sablefish cardiac bioenergetic function during or after periods of O_2 limitation, and that this may be related to alterations in mitochondrial sensitivity to NO and to adaptive changes in membrane composition (fluidity).

KEY WORDS: Anoxia-reoxygenation, Citrate synthase, Fatty acid composition, Mitochondrial respiration, Reactive oxygen species, Superoxide dismutase

INTRODUCTION

The frequency and severity of hypoxia in marine environments are expected to increase because of climate change, and this is likely to impact natural habitats where many fishes live (Breitburg et al., 2018; Diaz and Rosenberg, 2008). However, the ability and strategies to cope with hypoxic events vary considerably between species, and so does their tolerance to periods of oxygen limitation (Bickler and Buck, 2007; Galli and Richards, 2014; Hochachka and Lutz, 2001). Mitochondrial integrity and function are key determinants of fish stress tolerance, including hypoxia tolerance (Sokolova, 2018) as O_2 limitation leads to a cascade of events at the mitochondrial level (Pamenter, 2014). Mitochondria are the primary site of O_2 consumption, and thus, oxygen limitation alters mitochondrial respiration and impacts tissue ATP supply and metabolic functions that rely on aerobic respiration. In addition, mitochondria are a major source of reactive oxygen species (ROS), notably superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which are produced during electron transfer through the electron transport system (ETS) and may result in oxidative stress (Munro and Treberg, 2017; Murphy, 2009).

Most studies on the effects of hypoxia and/or anoxia-reoxygenation (AR) on mitochondrial function in fishes have used tissues such as muscle and liver (Borowiec et al., 2015; Du et al., 2016; Onukwufor et al., 2017; Ransberry et al., 2016; Sappal et al., 2015a,b). The only two studies that have examined the effects of chronic hypoxia on cardiac mitochondrial function provide conflicting information. Cook et al. (2013), using permeabilized cardiac fibres and isolated mitochondria, showed that complex I and II respiration were not affected in juvenile snapper (*Pagrus auratus*) when these fish were acclimated to 10.2–12.1 kPa for 6 weeks. In contrast, Hickey et al. (2012) reported that oxidative phosphorylation was halved in permeabilized ventricle fibres from the hypoxia-intolerant shovelnose ray (*Aptychotrema rostrata*) following a 2 h *in vivo* hypoxic insult (i.e. at 40% of each species' critical oxygen tension), but preserved in the hypoxia-tolerant epaulette shark (*Hemiscyllum ocellatum*).

The heart is a particularly important organ for studying the impacts of O_2 limitation on mitochondrial function as cardiac function is recognised as being critical to hypoxia tolerance and survival (Gamperl and Driedzic, 2009; Stecyk, 2017; Tota et al., 2011). The spongy myocardium (which comprises all or the majority of the heart in fishes; Farrell and Smith, 2017) is the last in line to be perfused and receives particularly oxygen-poor blood under hypoxic conditions, but must often elevate its function to ensure the proper oxygenation of other tissues (Farrell and Stecyk, 2007; Stecyk, 2017). Furthermore, cardiac nitrosative signalling is a well-recognized modulator of the physiological response and adaptation of lower vertebrates (including fishes) to hypoxia (Fago and Jensen, 2015; Fago et al., 2012; Gattuso et al., 2018; Imbrogno et al., 2018). For example, NO influences mitochondrial signalling and has numerous beneficial and protective effects on mitochondria during O_2 limitation (Taylor and Moncada, 2010), including the modulation of mitochondrial O_2 consumption and ROS production (Davidson and Duchen, 2006; Erusalimsky and Moncada, 2007; Guzik et al., 2002; Korge et al., 2008; Palacios-Callender et al., 2004). These effects are mainly due to NO's competition for O_2 binding sites on cytochrome oxidase (complex IV) (Brown, 2001; Cleeter et al., 1994; Cooper et al., 2008) and the

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List of symbols and abbreviations

AMPL	acetone mobile polar lipid
AR	anoxia-reoxygenation
ARA	arachidonic acid
CCO	cytochrome c oxidase (complex IV)
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
E_{PM}	uncoupled respiration with pyruvate and malate
E_{PMS}	uncoupled respiration with pyruvate, malate and succinate
$E_{S(Rot)}$	uncoupled respiration with succinate and rotenone
E_{TM}	uncoupled respiration with TMPD and ascorbate
ETS	electron transport system
FA	fatty acid
FCCP	carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazone
FFA	free fatty acid
J_{max}	Maximal respiration
$L_{N\ PM}$	leak respiration in the absence of ATP with pyruvate and malate
$L_{T\ PM}$	leak respiration in the presence of ATP with pyruvate and malate
$L_{T\ PMS}$	leak respiration in the presence of ATP with pyruvate, malate and succinate
MUFA	monounsaturated fatty acid
NO IC_{50}	[NO] at which mitochondrial respiration rate is half of maximal (IC_{50})
OXPHOS	oxidative phosphorylation
PL:ST	phospholipid to sterol ratio
P_{PM}	oxphos respiration with pyruvate and malate
P_{PMS}	oxphos respiration with pyruvate, malate and succinate
P_{50}	partial pressure of oxygen (P_{O_2}) at which mitochondrial respiration rate is half of maximal
P:S	polyunsaturated to saturated fatty acid ratio
PUFA	polyunsaturated fatty acid
RCR	respiratory control ratio
RM	respiration medium
ROS	reactive oxygen species
SFA	saturated fatty acid
SIMPER	similarity percentage
SOD	superoxide dismutase
TAG	triacylglycerol
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine

S-nitrolysation of complex I (Brown and Borutaite, 2004; Chouchani et al., 2013; Clementi et al., 1998). In addition, mitochondrial sensitivity to NO (i.e. the concentration that inhibits mitochondrial respiration by 50%; IC_{50}) and NO binding to the ETS are good indicators of the ability of NO to control mitochondrial respiration (Brookes et al., 2003; Shiva et al., 2001). In mammals, mitochondrial sensitivity to NO is altered by hypoxic events and acclimation (Aguirre et al., 2010; Brookes et al., 2001; Venkatraman et al., 2003; Zelickson et al., 2011). However, the ability of NO to influence mitochondrial respiration and the effect of hypoxia acclimation on mitochondrial sensitivity to NO have not been investigated in fishes.

Membrane remodelling is also known to accompany changes in environmental conditions, and can strongly influence mitochondrial and cardiac performance. For example, acute hypoxic stress increases cell membrane fatty acid (FA) saturation and decreases membrane fluidity in mice brain tissue and rat aortic endothelial cells (Duan et al., 1999; Ledoux et al., 2003). Chung et al. (2018) recently reported that changes in phospholipid FA composition are the primary signature of thermal acclimation in killifish (*Fundulus heteroclitus*) and that these changes are linked to hepatic mitochondrial performance. Furthermore, Vagner et al. (2019) showed that the highly unsaturated fatty acid (HUFA) content of cell membranes, which is known to be tightly regulated by temperature (Pethybridge et al., 2015), influences the cardiorespiratory function of marine fishes. Yet, the effect of hypoxia acclimation on mitochondrial membrane FA composition and fluidity in fishes, and thus on mitochondrial function and performance, have also not been examined.

In the present study, we used sablefish (*Anoplopoma fimbria* Pallas 1814) to: (1) investigate how *in vivo* long-term hypoxia acclimation (i.e. ∼6 months at 40% followed by 3 weeks at 20% air saturation), and *in vitro* AR, affected cardiac mitochondrial respiration and ROS release rates; (2) determine how normoxia versus hypoxia acclimation affects the oxygen kinetics (P_{50}, RCR and P:O ratio) of cardiac mitochondrial function and its sensitivity to NO; (3) measure superoxide dismutase (SOD) and citrate synthase (CS) activities in cardiac mitochondria and the myocardium as indicators of endogenous antioxidant and oxidative capacity/mitochondrial biogenesis in these fish, respectively; and finally, (4) compare cardiac mitochondrial lipid classes and FAs in normoxia- versus hypoxia-acclimated fish. The sablefish was selected for these studies as it is a naturally evolved hypoxia-tolerant species (Leeuwis et al., 2019) that encounters important variations in oxygen availability in its natural environment and through its life history. For example, whereas juveniles live in-shore, the adults inhabit cold and deep waters (up to 1500 m) that are often severely hypoxic (Mason et al., 1983; Moser et al., 1994). Further, although hypoxia-tolerance is usually associated with inactivity, a comatose state, dormancy and hypometabolism in vertebrates (Bickler and Buck, 2007; Boutilier, 2001), sablefish remain active at the P_{O_2} levels chosen for the long-term hypoxia acclimation and are thus an interesting comparative model.

MATERIALS AND METHODS

Experimental animals and hypoxia acclimation

Juvenile sablefish (<1 g) were obtained from the NOAA Manchester Research Station (Port Orchard, Washington, USA) and reared for ∼1 year at the Dr Joe Brown Aquatic Research Building (JBARB, Memorial University of Newfoundland) in 3000 l tanks supplied with aerated seawater (32 p.p.t.) at 10±1°C and a 12 h:12 h light: dark photoperiod. Thereafter, 60 fish were randomly distributed between two 1.2 m^3 tanks receiving 10°C seawater at an oxygen partial pressure (P_{O_2}) of 19–20 kPa (i.e. normoxic water). After a 2 week period of acclimation, one tank was kept normoxic while the other was made hypoxic. The P_{O_2} in this latter tank was gradually lowered over 3 weeks (by 2 kPa every 4 days) to 8±1 kPa (40% air saturation) using a custom-designed solenoid valve system (Electronics Workshop, Memorial University of Newfoundland) described elsewhere (Motyka et al., 2017; Petersen and Gamperl, 2010a), and by reducing seawater flow into the hypoxic tank from 12 to 5 l min^{-1}. This oxygen level was maintained for 6 months. Thereafter, the P_{O_2} in the hypoxic tank was lowered over 2 weeks (by 1 kPa every 4 days) to 4±1 kPa (20% air saturation), and maintained at this P_{O_2} level for 3 additional weeks before sampling began. The experiments took 10 days, hence the sablefish were maintained between 21 and 31 days at this final oxygen level. A P_{O_2} of 4 kPa is only slightly above the hypoxic limit for this species at this temperature (P_{crit} ∼3.3 kPa, 16% air saturation, Leeuwis et al., 2019). This P_{O_2} level was only used for ∼1 month because sablefish stop or reduce their feeding close to their P_{crit} (our personal observations) and we wanted the normoxia- and hypoxia-

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acclimated sablefish to have similar morphological and fitness characteristics when sampled. Fish were hand fed a commercial marine fish diet (Europa; Skretting Inc.) at ∼0.65% body mass day^{-1} during the experiment. However, if the hypoxic fish did not consume this amount, the normoxic fish were fed an equally reduced ration. Water ammonia and nitrite levels were checked weekly, and values did not exceed 0.006 and 0.1 ppm, respectively.

All experimental procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (protocol no. 16-92-KG).

Mitochondrial isolation

Fish were killed by a blow to the head, fish length and body mass were measured, and then the heart (ventricle) was excised and weighed. After allowing the heart to beat for several minutes in cold isolation medium (230 mmol l^{-1} mannitol, 75 mmol l^{-1}sucrose, 20 mmol l^{-1} HEPES, 1 mmol l^{-1} EGTA, pH 7.4) to remove red blood cells from the lumen, a small piece of ventricle was removed, weighed and snap-frozen in liquid nitrogen and kept at −80°C for later use in enzymatic assays. Then the rest of the ventricle was cut in half, rinsed briefly with isolation medium, blotted dry, and placed in a Petri dish filled with ice-cold isolation medium. The ventricle halves were thoroughly minced using fine scissors and a razor blade, and the pieces split into four aliquots to facilitate homogenization. Each aliquot was resuspended 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 (i.e. free fatty acids released during tissue disruption) and the supernatant were carefully removed, and the resulting mitochondrial pellet was washed twice by gentle resuspension in ice-cold isolation medium containing 10 mg ml^{-1} BSA (fatty acid free; Sigma, CAS 9048-46-8) and then centrifuged at 8000 ***g*** for 10 min at 4°C. This final pellet was weighed, and then gently resuspended in four volumes of ice-cold respiration medium (RM) [160 mmol l^{-1} KCl, 30 mmol l^{-1} HEPES, 10 mmol l^{-1} KH_2PO_4, 2 mmol l^{-1} EDTA, 10 mg ml^{-1} BSA (fatty acid free), pH 7.4]. The total time required to isolate the mitochondria was 45 min. The protein content of the mitochondrial suspensions was measured using a 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 RM with BSA as a blank.

Mitochondrial physiology

The physiology of isolated ventricular mitochondria from normoxia- and hypoxia-acclimated sablefish was assessed using high-resolution respirometry (O2k polarographic O_2 sensor, Oroboros Instruments, Innsbruck, Austria), fluorometry (O2k Fluo LED2-module, Oroboros Instruments) and amperometry (O2k MultiSensor system, Oroboros Instruments; and an ISO-NOP NO sensor, World Precision Instruments, Sarasota, FL). We conducted 4 separate experiments on the mitochondria from one normoxia- and one hypoxia-acclimated fish daily, which are described in detail below and shown in Figs S1 and S2. The experimental duration varied between 60 min and 150 min. All parameters were recorded in real time using DatLab 7 software (Oroboros Instruments), and O2K sensor calibration, O_2 background flux, internal zero drift and the response delay were all accounted for following the manufacturer's standard operating procedures (O2k; Fasching and Gnaiger, 2018; Gnaiger, 2018, 2016).

Measurement of coupled mitochondrial respiration and ROS release rates

In experiment 1, ventricular mitochondrial respiration and ROS release rates were simultaneously measured using a protocol modified from Du et al. (2016). ROS release rates were estimated by measuring extramitochondrial 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). This detection system measures extramitochondrial H_2O_2 levels (i.e. release rate), and thus, only estimates ROS production by the mitochondria (Munro et al., 2016; Treberg et al., 2015). The ROS signal was calibrated daily by the addition of H_2O_2 (0.1 µmol l^{-1}) following standard operating procedures (O2k; Krumschnabel et al., 2019).

In experiment 1, (see Fig. S1A) mitochondria (0.375 mg protein ml^{-1}) were added to 2 ml of 100% air-equilibrated RM at 10°C, and leak respiration without adenylates (State 2; L_N) was stimulated by the addition of a saturating concentration of complex I (CI) substrates: pyruvate, 10 mmol l^{-1} and malate, 2 mmol l^{-1}. Then, an optimal concentration of ADP (400 µmol l^{-1}; as determined in preliminary experiments) was added to assess the maximal capacity for oxidative phosphorylation (OXPHOS, state 3) via complex I (P_{PM}). After depletion of ADP, leak respiration (state 4) via CI ($L_{T\ PM}$) was measured. To determine the capacity for OXPHOS respiration (state 3) via CI+II (P_{PMS}), a saturating concentration of substrate for CII (succinate, 5 mmol l^{-1}) was then added followed by ADP (400 µmol l^{-1}). After the depletion of ADP, leak respiration rate (state 4) with ATP via CI+CII ($L_{T\ PMS}$) was measured. These measurements also allowed for the determination of the respiratory control ratio (RCR; i.e. state 3/state 4) and for an estimation of the efficiency of ATP synthesis (P:O ratio, i.e. [$ADP_{injected}$ in µmol]/[O_2 consumed in µ atoms]) with CI and CI+II substrates and 400 µmol l^{-1} ADP. Finally, to assess the effect of anoxia on respiration and ROS release rates via CI+II during OXPHOS respiration (P_{PMS}), an excess of ADP (1.2 mmol l^{-1}) was added to the chamber. The mitochondria consumed all of the O_2 and became anoxic while in OXPHOS respiration. After 10 min of anoxia, the O_2 level was raised to 50–60 µmol l^{-1} by lifting the stoppers of the chambers and OXPHOS respiration was re-established until the mitochondria consumed all ADP and entered into leak respiration. Then, to assess the effect of anoxia on ROS release rates via CI+II during leak respiration ($L_{T\ PMS}$), the mitochondria were allowed to consume all the O_2 again, and entered a second bout of anoxia while in the leak state. After 10 min, the chamber was reoxygenated to 50–60 µmol l^{-1} again by lifting the stopper of the chamber, and leak respiration via CI+II ($L_{T\ PMS}$) was re-established. 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] and catalytic efficiency [i.e. maximum respiration rate (J_{max})/P_{50})] during OXPHOS and leak respiration.

Measurement of uncoupled mitochondrial respiration

In experiment 2 (see Fig. S1B), the capacity for electron transport by the mitochondrial complexes was assessed using the mitochondrial uncoupler FCCP [carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazone]. Ventricular mitochondria (0.375 mg protein ml^{-1}), a saturating concentration of CI substrates (pyruvate 10 mmol l^{-1} and

malate 2 mmol l^{-1}) and excess ADP (1.2 mmol l^{-1}) were sequentially added to the 2 ml 100% air-equilibrated chambers at 10°C to initiate maximal OXPHOS respiration via CI. Prior to uncoupling, inner mitochondrial membrane integrity was assessed by the addition of cytochrome C (10 µmol l^{-1}) and the increase in O_2 consumption was always less than 10%. This indicated that the mitochondria had good inner membrane integrity (Galli et al., 2013). CI's capacity for electron transport was obtained after uncoupling the mitochondria with 1.5 µmol l^{-1} of FCCP (as determined in preliminary experiments by the sequential/incremental addition of 0.5 µmol l^{-1} FCCP). A saturating concentration of succinate (5 mmol l^{-1}) was then added to determine the CI+II capacity for electron transport before the CI inhibitor rotenone (2.5 µmol l^{-1}) was added to obtain CII's capacity for electron transport alone. Finally, TMPD (0.5 mmol l^{-1}) and ascorbate (10 mmol l^{-1}) were added to assess CIV's capacity for electron transport, and sodium azide (100 mmol l^{-1}) was added to account for the auto-oxidation of TMPD and to terminate the experiment.

Measurement of exogenous NO production and inhibition of mitochondrial respiration

In experiment 3 (see Fig. S2A), ventricular mitochondrial respiration and NO concentration in the chamber were simultaneously measured using an NO sensor (ISO-NOP, World Precision Instruments, Sarasota, FL) connected to one of the amperometric channels of the O2k MultiSensor system following standard operating procedures (O2k; Fasching and Gnaiger, 2016). The NO sensors were maintained in a polarized state between experiments using a pre-polarizer [NSA-3, World Precision Instruments (WPI), Sarasota, FL, USA] to allow quick stabilization of the signal once connected to the O2k instrument. The NO sensors were calibrated daily by the convenient and accurate $CuCl_2$ method as described by the manufacturer (WPI). Following calibration, the chamber was thoroughly rinsed, and filled with RM and the NO sensors were allowed 30 min to stabilize at 10ºC. For the calibrations and experiments, the lights were turned off and the chamber windows were covered with custom-made 3D-printed caps. Mitochondrial sensitivity to NO (NO IC_{50}) was determined using a protocol adapted from previous studies (Brookes et al., 2001; Mantena et al., 2009; Shiva et al., 2001; Venkatraman et al., 2003). Briefly, in this experiment (Fig. S2A), mitochondria (0.375 mg protein ml^{-1}) were injected into calibrated 2 ml 100% air-equilibrated chambers filled with RM at 10°C. A saturating concentration of substrates (10 mmol l^{-1} pyruvate, 2 mmol l^{-1} malate and 5 mmol l^{-1} succinate) and an optimal concentration of ADP (400 µmol l^{-1}) were then added 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 µmol l^{-1}) was added to the chamber at ∼60% air saturation to initiate NO release. The rate of NO release by PAPANONOate (15–20 nmol l^{-1} min^{-1}) in our experiments was similar to the low physiological fluxes of NO (i.e. 20 nmol l^{-1} min^{-1}) used by Dikalov et al. (2017). This increase in chamber NO levels gradually inhibited respiration, and allowed for the determination of mitochondrial NO IC_{50}. Once the maximal respiration rate was inhibited by ∼75%, and the O_2 concentration in the chamber had plateaued, excess oxyhaemoglobin (10 µmol l^{-1}, see below) was added to reverse NO's inhibition of respiration. This was done to confirm that the observed effect was due to the release of NO and not to the addition of the NO donor molecule (PAPANONOate) per se. Excess oxyhaemoglobin scavenged all the NO and stopped the PAPANONOate-mediated NO release, allowing for calculation of the NO sensor's zero drift. Reversal of NO inhibition allowed mitochondrial OXPHOS respiration to be re-established until all the ADP was consumed. 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. Cytochrome C (10 µmol l^{-1}) was also added during the plateau at maximal respiration to assess inner mitochondrial membrane integrity. PAPANONOate (20 mmol l^{-1}) was prepared daily, and immediately prior to use, in ice-cold RM (without BSA) for each experiment (3 and 4). Recordings of NO concentration were corrected for baseline and zero drift before data analysis.

Oxyhaemoglobin for these experiments was obtained from anesthetized Atlantic salmon. Blood was withdrawn from the caudal vessels using a heparinized syringe (10 ml) and the red blood cells were isolated from plasma by centrifugation (1 min at 800 ***g***). The red blood cells were then lysed in air-saturated MilliQ water (×10), and the haemolysate was centrifuged to remove cell debris (1 min at 2000 ***g***). Aliquots of haemolysate were kept at −20°C until use. The concentration of oxyhaemoglobin in the haemolysate was determined using a previously described procedure (Jensen, 2007). Briefly, the haemolysate was further diluted (×20) in air-saturated MilliQ water and transferred into a 1 cm^2, 1 ml cuvette. The concentration of oxyhaemoglobin (mmol l^{-1}) in the haemolysate was estimated using the absorbance at the two characteristic peaks of the oxyhaemoglobin spectrum (i.e. at 541 and 577 nm) using a spectrophotometer (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA) and their respective coefficients of extinction (i.e. ε=13.8 and 14.6 l $mmol^{-1}$ cm^{-1}).

Measurement of NO production and mitochondrial consumption of NO

In experiment 4 (Fig. S2B), NO consumption by the mitochondria was assessed by measuring NO production by PAPANONOate in the absence and presence of mitochondria (and substrates) using a protocol modified from Venkatraman et al. (2003). Briefly, PAPANONOate (20 µmol l^{-1}) was added to the chamber containing the RM alone to determine the NO release rate in the absence of mitochondria over the time period required in experiment 3 to inhibit ∼75% of mitochondrial respiration; i.e. ∼8 min. NO production from PAPANONOate was non-linear, and started to plateau after ∼20 min (see Fig. S2B). Once NO concentration in the chamber had plateaued at a concentration of ∼250–300 nmol l^{-1}, mitochondria (0.375 mg protein ml^{-1}) from normoxia- or hypoxia-acclimated fish were added to the O2k chambers and allowed to consume/metabolize the NO. Once a new plateau was reached, substrates of CI+II were added and the mitochondria were allowed to consume/metabolize NO in leak respiration (state 2), then ADP was added to initiate OXPHOS respiration and determine NO consumption by the mitochondria via CI+II during OXPHOS respiration. Oxyhaemoglobin was then added in excess (10 µmol l^{-1}) to scavenge all remaining NO and determine the NO sensor's internal drift during the experimental period, and to terminate the experiment.

Enzymatic assays

Superoxide dismutase (SOD) and citrate synthase (CS) activities were determined in the ventricle and mitochondrial suspensions from both normoxia- and hypoxia-acclimated fish. The snap-frozen piece of ventricle was placed in an ice-cooled Petri dish, thoroughly minced using fine scissors, and resuspended 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). This suspension was then

homogenized with a Polytron homogenizer (Kinematica Inc., Bohemia, NY, USA). The resulting crude homogenate and thawed mitochondrial suspensions were split into two aliquots because two different centrifugation speeds and dilution buffers were used for the CS and SOD enzymatic assays. For CS activity, the ventricular aliquots and thawed mitochondrial suspensions were further diluted in 4 and 19 volumes of homogenization buffer, respectively, and centrifuged at 2000 ***g*** for 5 min at 4°C. For the assay of SOD activity, the thawed mitochondrial suspensions were first diluted in six volumes of homogenization buffer to lyse the mitochondrial membrane. Then, both the ventricular aliquots and thawed mitochondrial suspensions were centrifuged at 13,000 *g*, and the resulting supernatants were further diluted in nineteen and thirteen volumes of dilution buffer (SOD Kit), respectively. The resulting supernatants were used for enzymatic activity assays that were performed at room temperature (25°C) using a 96-well microplate reader (SpectraMax M2^{e}, Molecular Devices, Sunnyvale, CA). SOD activity was assessed using a SOD Assay Kit-WST (Sigma-Aldrich) following the manufacturer's protocol and CS activity was measured using the method described by Treberg et al. (2007). CS and SOD activities for the ventricle homogenates and mitochondrial suspensions were expressed as U g tissue^{-1} and as U mg protein^{-1}, respectively. The protein content of the mitochondrial suspensions was determined using a Bradford assay (with BSA as a standard), and the contribution of BSA to the measured protein levels was accounted for using RM with BSA as a blank. Protein contents were not significantly different between groups.

Chemicals

Most chemicals used in the above procedures were purchased from Sigma-Aldrich (Mississauga, ON, Canada), except for PAPANONOate and SNAP, which were obtained from Cayman Chemical (Ann Arbor, MI, USA), and Amplex UltraRed, which was from Thermo Fisher Scientific (Waltham, MA USA). All reagents for the lipid work were Omnisolv HPLC grade.

Measurement of mitochondrial FA and lipid classes

For lipid analyses, isolated mitochondria (0.2 g ml^{-1}) were placed in 15 ml glass test tubes that had been fired in a muffle furnace (at 450°C for at least 6 h). The glass tubes were then filled with 2 ml of chloroform (HPLC grade), flushed with nitrogen, and covered with Teflon-lined caps that were further sealed with Teflon tape before being stored at −20°C until lipid extractions were performed.

Lipid extraction

Lipid samples were extracted according to Parrish (1999). Samples were homogenized in a mixture of ice-cold chloroform:methanol (2:1), using a metal rod coated with Teflon. Chloroform-extracted water was added to bring the ratio of chloroform:methanol:water to 8:4:3. The sample was then sonicated for 4 min in an ice bath, and centrifuged at 3000 rpm for 2 min at room temperature. A double pipetting technique was used (i.e. by placing a 2 ml lipid clean glass Pasteur pipette inside a 1 ml glass pipette) to remove the bottom organic layer without disturbing the top aqueous layer. Chloroform was then added back to the extraction test tube and the entire procedure was repeated three times. All organic layers were pooled into a separate lipid-cleaned vial. Finally, samples were concentrated under a flow of nitrogen gas.

Lipid class separation

An Iatroscan Mark 6 TLC-FID (thin-layer chromatography-flame ionization detector) (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), silica coated Chromarods, and a three-step development method (Parrish, 1987) were used to determine the lipid class composition. The lipid extracts were applied to the Chromarods and focused to a narrow band using 100% acetone. The first development system was hexane:diethyl ether:formic acid (98.95:1.0:0.05). The rods were developed for 25 min, removed from the system for 5 min to dry and replaced for 20 min. The second development was for 40 min in hexane:diethyl ether:formic acid (79:20:1, v/v/v). The final development system had two steps, the first was in 100% acetone for two 15 min periods, followed by two 10 min periods in chloroform:methanol:chloroform-extracted water (5:4:1, v/v/v). The rods were dried in a constant humidity chamber prior to each solvent system. After each system, the rods were partially scanned in the Iatroscan and the data were collected using Peak Simple software (SRI Instruments, version 3.67, Torrance, CA, USA,). The Chromarods were calibrated using standards from Sigma Chemicals (St Louis, MO, USA).

Fatty acid methyl ester (FAME) derivatization

Fifty microlitres from each lipid extract were transferred into a separate lipid-cleaned 15 ml glass vial, and concentrated under a flow of nitrogen until reaching complete dryness. Then, 1.5 ml of methylene chloride and 3 ml of Hilditch reagent (1.5 sulfuric acid:98.5 anhydrous methanol) were added to each vial, and this was followed by vortexing and 4 min of sonication (Fisher Scientific FS30, Pittsburgh, PA, USA). Then, vials were filled with nitrogen, capped and heated at 100°C for 1 h. Subsequently, 0.5 ml of saturated sodium bicarbonate solution and 1.5 ml of hexane were added to each vial, followed by vortexing, and then the upper organic layer was removed into a separate lipid-clean glass vial. Each sample was dried and refilled with ∼0.5 ml hexane. Finally, vials were filled with nitrogen, capped, sealed with Teflon tape, and sonicated for 4 min to resuspend the fatty acids. All FAMEs were analyzed on a HP 6890 GC-FID (gas chromatography-flame ionization detector system) equipped with a 7683 autosampler (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). Fatty acid peaks were identified with standards (PUFA 1, PUFA 3, FAME and a Supelco 37 component FAME mixture; Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada). Finally, chromatograms were integrated using the Agilent openLab data analysis system, version 2.2 (Santa Clara, CA, USA). FA data are expressed as a percentage of total FAME.

Statistical analyses

Statistical analyses were performed using GraphPad Prism v7.04 software (La Jolla, CA, USA). Data were analysed using repeated-measures two-way ANOVAs followed by Bonferroni's multiple comparison tests, except: (1) the NO IC_{50}, morphometric and enzymatic, and lipid and FA data, which were analysed using unpaired two-tailed Student's *t*-tests; and (2) NO release rate, which was analysed using a one-way ANOVA followed by Tukey's multiple comparison tests. The level of statistical significance was $P \leq 0.05$, although comparisons at $P \leq 0.10$ are also shown. Multivariate analyses of lipid classes and fatty acids were also undertaken using PRIMER software v.6.1.16 and PERMANOVA v.1.0.6 (PRIMER-E, Plymouth, UK), and as part of the lipid/FA analyses, the contributions of various groups were investigated using the SIMPER function. This function provides the percentage of dissimilarity within the studied factors and the relative contribution of each variable to the observed dissimilarity. The relative contribution of lipids to the overall profile was also analysed using a permutational multivariate

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ANOVA based on distances (PERMANOVA). All data are presented as means±s.e.m.

RESULTS

Morphometric variables

There was no difference in body mass (1.44±0.17 vs 1.40±0.09 kg) and length (50.2±2.1 vs 50.5±0.8 cm), or in relative ventricular mass (0.079±0.006 and 0.068±0.002%), between normoxia- and hypoxia-acclimated fish, respectively. There was also no difference in ventricular mitochondrial yield, calculated as the mitochondrial pellet to ventricular wet mass ratio, between normoxia- (0.155±0.031) and hypoxia-acclimated (0.131±0.017) fish.

Coupled mitochondrial respiration and ROS release rates

Mitochondrial respiration (Fig. 1A,B) and % ROS/O_2 flux (Fig. 1C,D) were not altered by hypoxia acclimation. In both groups, oxidative (OXPHOS) respiration (P_{PMS}) and leak respiration (L_{PMS}) via CI+II were ∼1.1-fold and 3.3-fold higher, respectively, as compared with OXPHOS respiration (P_{PM}, Fig. 1A) and leak respiration (L_{PM}, Fig. 1B) via CI alone. In addition, leak respiration via CI in the absence of ATP ($L_{N\ PM}$) was ∼3.7-fold higher than leak respiration in the presence of ATP ($L_{T\ PM}$), and was comparable to leak respiration via CI+II ($L_{T\ PMS}$, Fig. 1B). The % ROS/O_2 flux during OXPHOS respiration increased (by ∼12%) following the addition of the CII substrate succinate in the normoxia-acclimated fish, but remained unchanged in the hypoxia-acclimated fish (Fig. 1C). Both the presence of ATP ($L_{T\ PM}$) and the addition of the CII substrate succinate ($L_{T\ PMS}$) increased the %ROS/O_2 flux during leak respiration in the two acclimation groups (Fig. 1D).

Mitochondrial respiratory control, oxygen affinity, catalytic and OXPHOS efficiency

The respiratory control ratio (RCR) in the presence of CI (PM) and CI+II substrates (PMS) was also not altered by hypoxia acclimation. However, it was reduced greatly by the presence of the CII substrate succinate (from ∼36 to ∼12; Fig. 2A), as would be expected based on the greatly increased leak respiration rate after this substrate was added (Fig. 1B). The P_{50} during both leak ($L_{T\ PMS}$) and OXPHOS (P_{PMS}) respiration via CI+II was also not altered by hypoxia acclimation. However, the P_{50} was >2-fold higher in P_{PMS} compared with $L_{T\ PMS}$ (Fig. 2B; ∼0.06 vs ∼0.026 kPa, respectively). Hence, catalytic efficiency (J_{max}/P_{50}) was ∼5.5-fold higher in P_{PMS} compared with $L_{T\ PMS}$ (Fig. 2C). Finally, while the efficiency of OXPHOS respiration (P:O ratio) was not altered by hypoxia acclimation or substrate in normoxia-acclimated fish, the addition of the CII substrate succinate lowered the P:O ratio (by ∼6%) in the hypoxia-acclimated group (Fig. 2D).

Uncoupled mitochondrial respiration

The uncoupler FCCP was used to achieve maximal mitochondrial respiration. This parameter was not altered by hypoxia acclimation, but was highly dependent on the complexes involved (Fig. 3). CI-dependent uncoupled respiration (E_{PM}) was ∼6-fold greater than that for CII ($E_{S(Rot)}$; i.e. after the addition of rotenone). The addition of TMPD and ascorbate, after rotenone, resulted in an increase

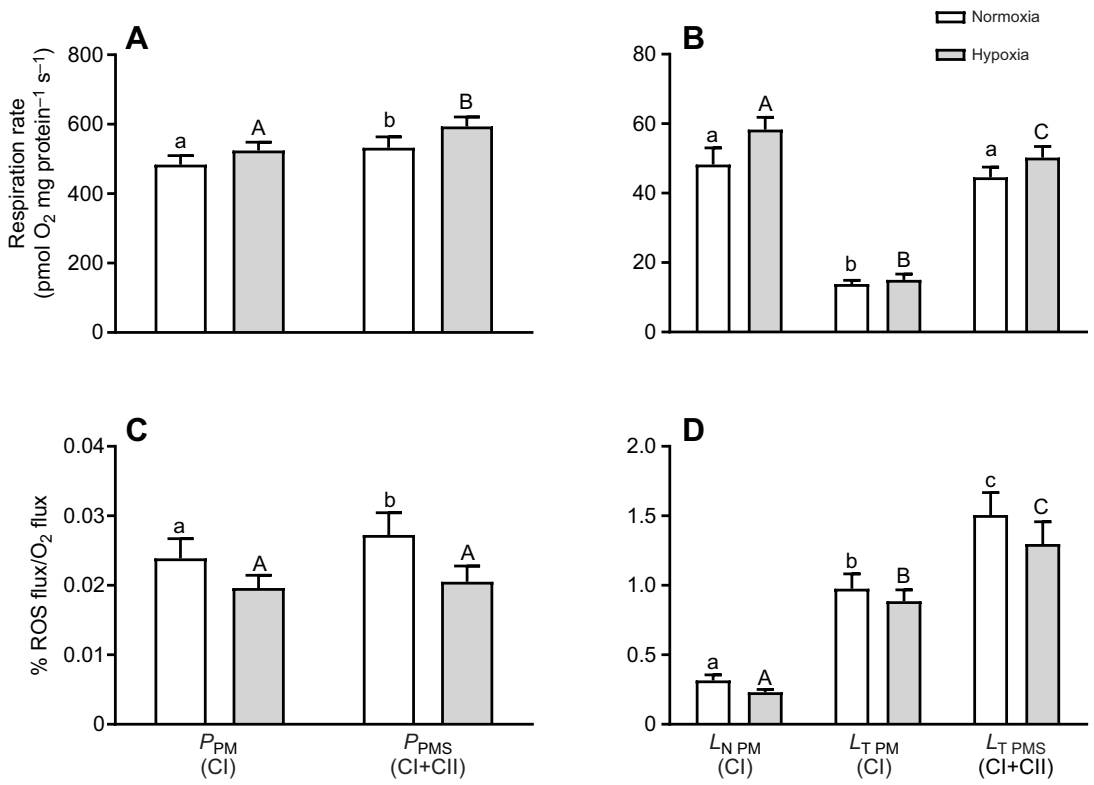

Fig. 1. Coupled respiration and ROS release rates in ventricular mitochondria from sablefish acclimated to normoxia or hypoxia. (A) Respiration rate measured during OXPHOS with ADP and substrates of CI (P_{PM}) and CI+CII (P_{PMS}). (B) Leak respiration rate measured in the absence of ATP (L_N), and in the presence of ATP (L_T) with substrates of CI ($L_{T\ PM}$) and CI+II ($L_{T\ PMS}$). (C) ROS release rates, expressed as percentage of O_2 flux, during OXPHOS respiration with ADP and substrates of CI (P_{PM}) and CI+II (P_{PMS}). (D) ROS release rates, expressed as percentage of O_2 flux, during leak respiration in the absence of ATP (L_N), and in the presence of ATP (L_T) and substrates of CI ($L_{N\ PM;}$ $L_{T\ PM}$) and CI+II ($L_{T\ PMS}$). Asterisks (*) and plus signs (+) in all figures indicate a significant difference between groups at $P \leq 0.05$ and $P \leq 0.10$, respectively, whereas letters (lower case or capital) indicate a significant difference within a group. Values are means±s.e.m., N=8.

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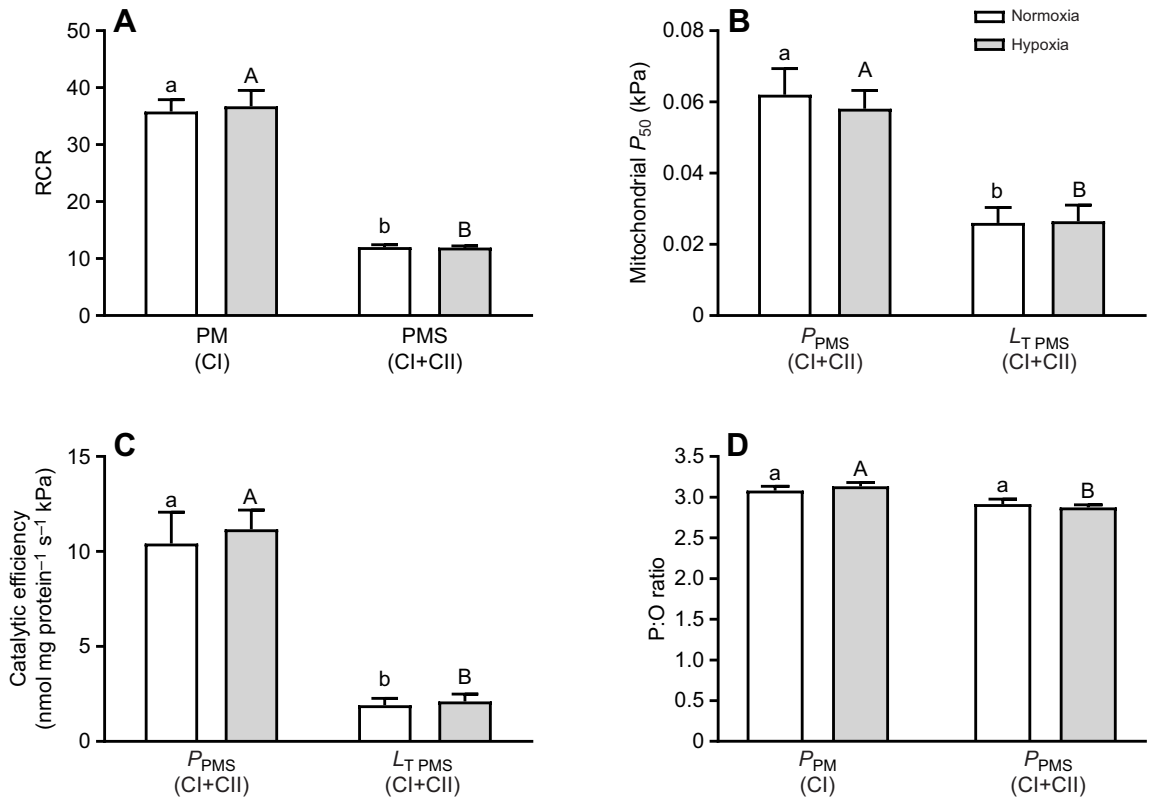

Fig. 2. Mitochondrial respiratory control ratio, oxygen affinity (P_{50}), catalytic efficiency and P:O ratio for ventricular mitochondria from sablefish acclimated to normoxia or hypoxia. (A) Respiratory control ratio (RCR) measured with substrates of CI (PM) and CI+CII (PMS). (B) Mitochondrial P_{50} measured during OXPHOS respiration (P_{PMS}) and during leak respiration ($L_{T\ PMS}$) with substrates of CI+II (PMS). (C) Apparent catalytic efficiency (J_{max}/P_{50}) measured during OXPHOS respiration (P_{PMS}) and during leak respiration ($L_{T\ PMS}$) with substrates of CI+II (PMS). (D) P:O ratios in the presence of ADP and substrates of CI (PM) and CI+II (PMS). Other details are as in Fig. 1.

in respiration that was ∼25% lower than that recorded during CI+II-dependent uncoupled respiration (E_{PMS}).

Effect of anoxia on mitochondrial respiration and ROS release rates

Brief exposure to anoxia reduced OXPHOS respiration (P_{PMS}) capacity via CI+II upon reoxygenation. However, OXPHOS respiration after the anoxia bout (post-anoxia 1) was higher (by ∼21%) in mitochondria from hypoxia-acclimated fish (Fig. 4A). Anoxia had no effect on leak respiration in the normoxia-acclimated group, and there was no difference in post-anoxic L_{PMS} respiration between the two groups. Nonetheless, leak respiration in the hypoxia-acclimated fish was enhanced (by ∼15%) compared with pre-anoxic values (Fig. 4B). Brief exposure to anoxia only increased % ROS/O_2 flux in the normoxia-acclimated group (by ∼37%) during OXPHOS respiration (Fig. 4C), and although the % ROS/O_2 flux post-anoxia during leak respiration increased drastically in both groups, the increase was much less in hypoxia- versus normoxia-acclimated fish (by ∼142% vs ∼194%, respectively Fig. 4D). These differences resulted in % ROS/O_2 flux during P_{PMS} and L_{PMS} being significantly lower in hypoxia-acclimated fish post-anoxia (by ∼27% and ∼31%, respectively).

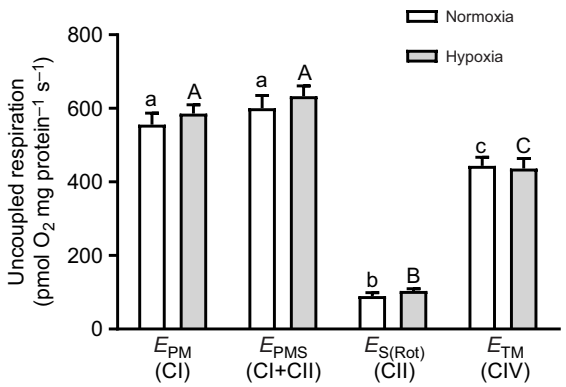

Fig. 3. Uncoupled respiration in ventricular mitochondria from sablefish acclimated to normoxia or hypoxia. Respiration rate in the presence of the uncoupler FCCP and excess ADP, and with saturating concentrations of substrates of CI (E_{PM}), CI+II (E_{PMS}), following the blockade of CI ($E_{S(Rot)}$, with rotenone) and after stimulating complex IV [with TMPD and ascorbate (E_{TM}); the latter corrected for CIV auto-oxidation using sodium azide]. Other details are as in Fig. 1.

Effect of NO on mitochondrial respiration

NO production by PAPANONOate and its inhibition of OXPHOS respiration were evaluated over an O_2 range from ∼160 to 110 µmol l^{-1} (Fig. 5A). The release of NO by the NO donor PAPANONOate gradually inhibited mitochondrial respiration during OXPHOS respiration in both normoxia- and hypoxia-acclimated fish (Fig. 5B). However, hypoxia acclimation enhanced mitochondrial sensitivity to NO. This led to a steeper respiration rate vs [NO] relationship and a NO IC_{50} that was 25% lower in hypoxia-acclimated fish (i.e. 94.1 nmol l^{-1} vs 126.5 nmol l^{-1} in normoxia-acclimated fish; Fig. 5C). The release of NO by

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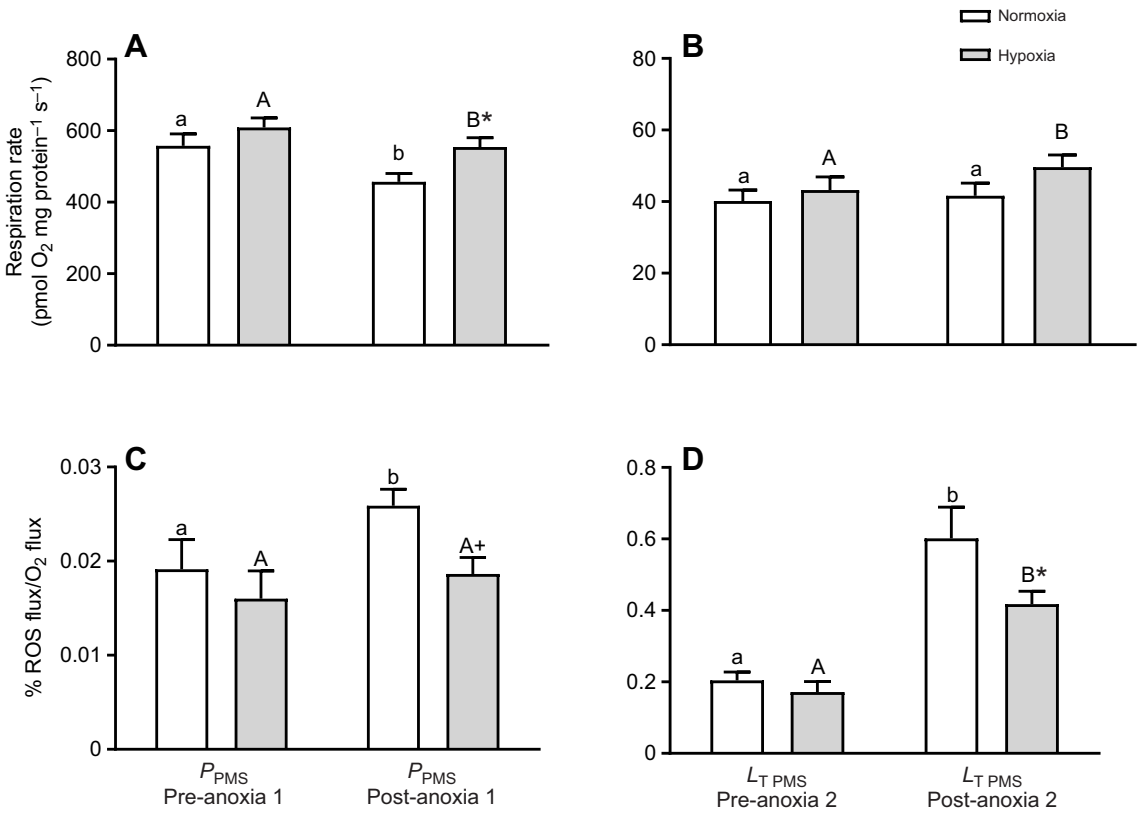

Fig. 4. Effect of *in vitro* anoxia on respiration and ROS release rates in ventricular mitochondria from sablefish acclimated to normoxia or hypoxia. (A) Respiration rate during OXPHOS respiration measured with excess ADP and substrates of CI+II (P_{PMS}) before and after the first 10 min bout of anoxia. (B) Leak respiration rate measured in the presence of ATP and substrates of CI+II ($L_{T\ PMS}$) before and after the second 10 min bout of anoxia. (C) ROS release rates, expressed as percent of O_2 flux, during OXPHOS respiration measured before and after the first 10 min bout of anoxia. (D) ROS release rates, expressed as percentage of O_2 flux, during leak respiration measured before and after the second 10 min bout of anoxia. Other details are as in Fig. 1.

PAPANONOate inhibited >80% of OXPHOS respiration in both normoxia- and hypoxia-acclimated fish, and its effect was completely reversed by the addition of oxyhaemoglobin (Fig. 5D). This confirmed that the effect of PAPANONOate was due to NO release and not the NO donor molecule per se, and that the inhibition was reversible.

NO consumption by mitochondria

The NO concentration reached in the chamber after 8 min (i.e. the time required to inhibit OXPHOS by ∼75%), and hence the calculated rate of NO release following PAPANONOate addition was lower in the presence of mitochondria (Fig. 6A; Fig. S2A) compared with that of buffer alone (Fig. 6A; Fig. S2B). Indeed, NO release rate was 0.46 nmol l^{-1} s^{-1} in buffer alone versus 0.33 and 0.24 nmol l^{-1} s^{-1} in the presence of ventricular mitochondria (in OXPHOS state) from normoxia- and hypoxia-acclimated fish, respectively; the effect of acclimation was not significant (Fig. 6B). This effect was confirmed by adding mitochondria to the chamber after NO levels had stabilized (∼20 min after PAPANONOate addition; Fig. S2B; Fig. 6C). The addition of mitochondria from normoxia- and hypoxia-acclimated fish to the chamber reduced [NO] in the chamber (i.e. from 280 to 145 nmol l^{-1} and from 270 to 112 nmol l^{-1}, respectively) and this was associated with a prolonged period of negative NO flux values (Fig. S2B). The addition of substrates of CI+II (PMS) and ADP, and thus entry of mitochondria into OXPHOS respiration, produced a similar but much smaller, and not significant, effect (Fig. S2B; Fig. 6C).

Enzymatic assays

Hypoxia acclimation increased CS activity in both the ventricular homogenates (by ∼19%, Fig. 7A), and the mitochondrial suspensions (by ∼21%, Fig. 7B). In contrast, activity of the antioxidant enzyme superoxide dismutase (SOD) was not different when measured in the ventricular myocardium (Fig. 7C) or in isolated mitochondria (Fig. 7D).

Mitochondrial lipid analyses

Lipids in the cardiac mitochondria consisted of 84–89% phospholipid, and there were only minor proportions of hydrocarbons, triacylglycerols, free fatty acids, alcohols, sterols and acetone mobile polar lipids. There were no significant differences in the lipid classes, when calculated as a proportion (%), or in total lipids when calculated as a concentration (mg g^{-1} wet weight), between the two acclimation groups (Table 1); although the phospholipid to sterol (PL:ST) ratio was ∼50% higher in mitochondria from hypoxia-acclimated fish.

The isolated mitochondria from the two experimental groups did not show differences for most of the fatty acids detected. However, the proportions (% of total FA, Table 2) of both 20:4ω6 and 20:5ω3 were higher in the mitochondria of the hypoxia-acclimated fish (by 11% and 14%, respectively). The proportions of 18:3ω4 were also increased in the hypoxia-acclimated fish, while the opposite was shown for 18:3ω6. Finally, the sum of 20:4ω6+20:5ω3 was higher (by 13%) in the hypoxia-acclimated fish.

Principal coordinates analysis (PCO) showed that PCO1 explained 44.7%, while PCO2 accounted for 24.9%, of the variability in the data, and that the normoxia- and hypoxia-acclimated fish formed separate

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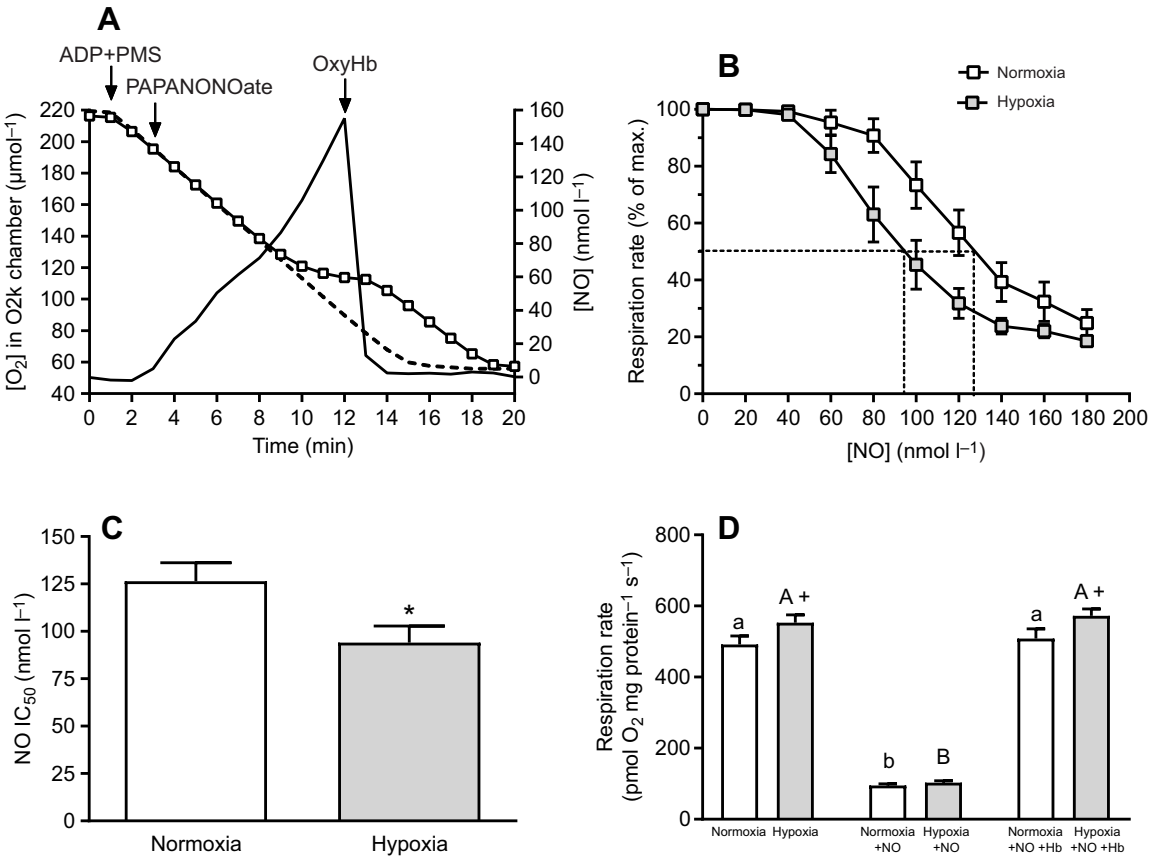

Fig. 5. Effect of nitric oxide (NO) on respiration in ventricular mitochondria from sablefish acclimated to normoxia or hypoxia. (A) Representative traces of mitochondrial O_2 consumption in the absence (dotted line) or presence (white squares) of PAPANONOate, and of NO production (solid line). Mitochondria were incubated with CI+CII substrates (PMS) and excess ADP to initiate OXPHOS respiration. PAPANONOate was added at 60% air saturation during OXPHOS respiration to release NO, which gradually inhibited O_2 consumption by the mitochondria. When O_2 consumption was inhibited (plateaued), oxyhaemoglobin (oxyHb) was added in excess to scavenge NO; this reversed NO's inhibition of mitochondrial respiration and allowed the mitochondria to consume all remaining ADP and re-established leak respiration. (B) The inhibition curve of mitochondrial respiration (% of maximal respiration rate) by NO during OXPHOS respiration with excess ADP and substrates of CI+II. This relationship was used to determine the NO IC_{50} for each group. (C) Concentration of NO at which mitochondrial respiration rate was half of maximal (IC_{50}). (D) Initial OXPHOS respiration rate (P_{PMS}), after the addition of PAPANONOate and after the addition of oxyHb. Other details are as in Fig. 1.

clusters. The hypoxia-acclimated fish were clustered on the top half of the plot, and were associated with ω6 polyunsaturated FAs (PUFAs): 18:2ω6, 20:4ω6, and with the ω3 PUFAs (18:3ω3, 20:5ω3 and 22:5ω3). Furthermore, the mitochondria from hypoxia-acclimated fish were associated with 16:1ω7 and 18:1ω9, the sums of MUFA and 20:4ω6+20:5ω3, and with the ratios of PUFA to SFA (P:S) and phospholipids to sterols (PL:ST). The normoxia-acclimated fish were clustered on the bottom half of the plot, and were associated with 16:0, 18:0, 22:6ω3, 22:4ω6, and the sums of SFA and ω3 fatty acids. Similarity percentage (SIMPER) analysis demonstrated that the main drivers of the differences between acclimation groups were PL:ST (24.3% contribution), 22:1ω7 (7.6%), 16:4ω1 (7.5%), 22:4ω6 (5.4%), 18:2ω6 (4.2%) and 18:1ω9 (4.1%).

PERMANOVA pairwise tests of the data presented in the PCO (Fig. 8) showed that the hypoxia and normoxia treatments were not different from each other. However, when normalization and transformation were performed, a significant difference was observed between these treatments.

DISCUSSION

Morphometric and mitochondria metrics

Based on the morphometric data, there did not appear to be any negative effects of hypoxia acclimation on the sablefish. Body mass and length, ventricular mass and cardiac mitochondrial yield were all unaltered by hypoxia acclimation, and no mortality was reported during the acclimation period. Thus, at the organism level, it appears that sablefish have the ability to cope with low O_2 availability for extended periods, even when the P_{O_2} level is close to their P_{crit}. This is consistent with their life history, as adult sablefish are bathydemersal and are exposed to oxygen levels as low as 2 kPa (Moser et al., 1994).

Effect of hypoxia acclimation on respiratory capacity and control

Cardiac mitochondrial respiration was maintained in sablefish following exposure to chronic hypoxia (Fig. 1A,B). A similar response to hypoxia has been reported in permeabilized ventricular fibres of the hypoxia-tolerant epaulette shark (*Hemiscyllum ocellatum*; Hickey et al., 2012) following acute hypoxic stress (2 h) and in cardiac mitochondria and permeabilized fibres of the snapper (*Pagrus auratus*; Cook et al., 2013) following long-term hypoxia acclimation (6 weeks). This response is likely tissue dependent, as the heart might be more resilient/robust to the effects of hypoxia owing to its crucial function and position in the circulation. Such an effect was observed in the common frog (*Rana temporaria*), where 4 months of hypoxic submergence did not alter

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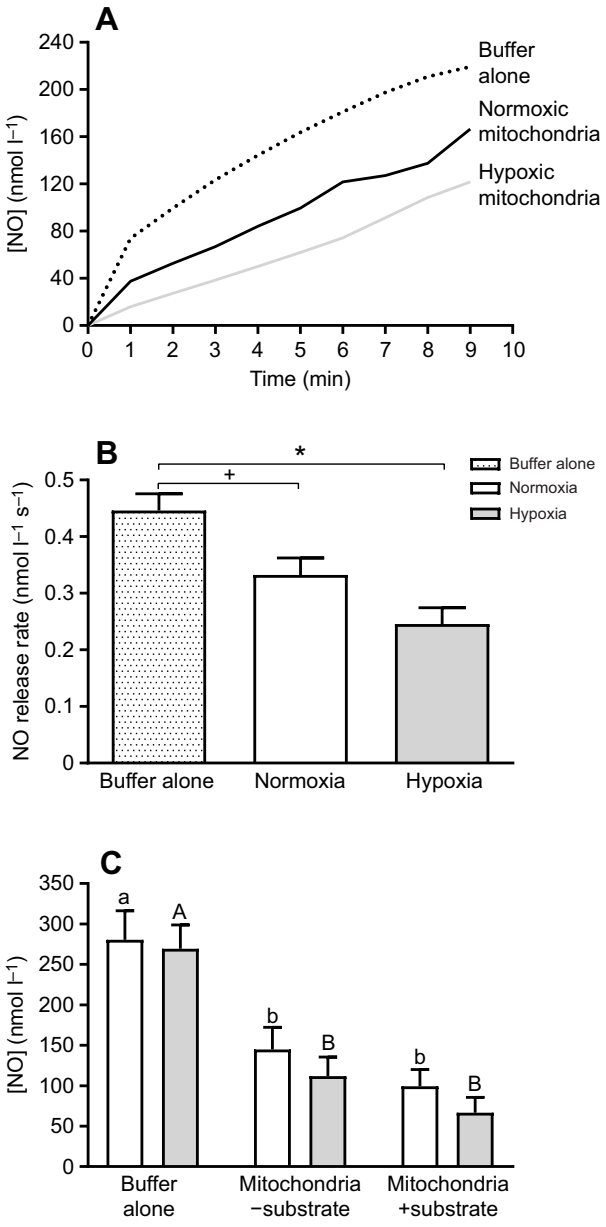

Fig. 6. NO release by PAPANONOate and NO consumption by ventricular mitochondria from sablefish acclimated to normoxia or hypoxia. (A) NO concentration (in nmol l^{-1}) reached in the chamber following addition of PAPANONOate in the RM alone (experiment 4) and in the presence of mitochondria from normoxia- and hypoxia-acclimated fish (with substrates of CI+II and excess ADP, experiment 3) over 8 min (i.e. the time required to inhibit ∼75% of mitochondrial respiration). (B) Calculated NO release rate (nmol l^{-1} s^{-1}) by PAPANONOate in the presence of mitochondria (experiment 3), and substrates compared with the NO release rate in the presence of RM alone (experiment 4) over the same time period. (C) NO concentration (in nmol l^{-1}) in the chamber ∼20 min after PAPANONOate was added to the RM alone, and after addition of mitochondria and substrates of CI+CII and excess ADP; experiment 4. Other details are as in Fig. 1.

the oxygen consumption of cardiomyocytes (Currie and Boutilier, 2001), whereas this condition reduced respiration rate by half in skeletal muscle (St-Pierre et al., 2000). However, the maintenance of mitochondrial function following prolonged O_2 limitation does not appear to be a phenomenon exclusive to cardiac mitochondria as long-term hypoxia acclimation of killifish (∼30 days) did not alter cytochrome c oxidase (CCO) activity in the heart, muscle and brain (Borowiec et al., 2015), or hepatic mitochondrial respiration (Du et al., 2016).

In this study, we also report that several physiological indicators of respiratory control and metabolic state [i.e., RCRs, mitochondrial P_{50}, OXPHOS P:O ratio and catalytic efficiency (J_{max}/P_{50})] were not altered by long-term hypoxia acclimation (Fig. 2). The comparable RCR values (Fig. 2A), and absence of an effect of hypoxia acclimation on FCCP-stimulated mitochondrial respiration (Fig. 3), indicate that there was no loss of ETS coupling and agree with the maintained mitochondrial respiration reported in this study following prolonged O_2 limitation (Fig. 1A,B). Further, the similar P_{50} values between groups (Fig. 2B) suggest that the O_2 dependence of complex IV (CCO), the primary site of O_2 consumption in the mitochondrion, was not altered by hypoxia acclimation. This agrees with previous studies in fish (Cook et al., 2013; Du et al., 2016), rats (Costa et al., 1997), birds (Scott et al., 2009) and amphibians (Currie and Boutilier, 2001). Collectively, these findings suggest that sablefish mitochondrial respiration and control are not negatively affected by chronic hypoxia (21–31 days at 4 kPa). However, whether sablefish mitochondrial function is impacted by more severe hypoxic conditions (e.g. when the P_{O_2} is below the species' P_{crit}) is not known.

The activity of citrate synthase (CS), a marker of oxidative capacity, increased by a similar amount (proportion) in both the ventricular homogenates (Fig. 7A) and the mitochondrial suspensions (Fig. 7B) following hypoxia acclimation (i.e. by ∼20%). This finding suggests that there was a compensatory response to sustain cardiac mitochondrial capacity in hypoxia-acclimated sablefish (e.g. see Figs 1–3), and that this primarily involved changes in the intrinsic properties of the mitochondria and not in the abundance (volume) of these organelles (St-Pierre and Boutilier, 2001). This interpretation would be consistent with: (1) studies on thermal acclimation and evolutionary adaptation to temperature, which show that changes in the number of mitochondria and in oxidative capacity per milligram of mitochondrial protein are complementary strategies that can alter tissue oxidative capacity (e.g. see Guderley and St-Pierre, 2002); and (2) St-Pierre and Boutilier (2001), who reported that changes in CS activity at the level of the mitochondrion played a major role in determining tissue CS activity in the hearts of hibernating (i.e. cold and severely hypoxic) frogs (*Rana temporaria*). In contrast to our study, previous studies on fish report no change in heart homogenate CS activity following hypoxia acclimation (Borowiec et al., 2015; Cook et al., 2013).

Effect of hypoxia acclimation on oxidative stress (ROS release and SOD activity)

One widespread strategy to limit ROS production upon low oxygen stress is to decrease OXPHOS respiration to prevent the ETS from working in reverse and the consequent back flow of electrons to complexes III, II and I (Chouchani et al., 2016; Murphy, 2009). Such a response was observed in the hypoxia-sensitive shovelnose ray, where the respiration and ROS production of permeabilized cardiac fibres were decreased by a similar amount following 2 h of acute hypoxia (Hickey et al., 2012). Hypoxia-acclimated sablefish, however, maintained their cardiac mitochondrial capacity and efficiency without an enhancement of ROS release rates (Fig. 1). The absence of an effect of hypoxia acclimation on mitochondrial respiration and ROS release rates is consistent with what was observed for cardiac fibres from the hypoxia-tolerant epaulette shark when exposed to acute (2 h) hypoxia (Hickey et al., 2012). Further, Du et al. (2016) reported that while mitochondria from the

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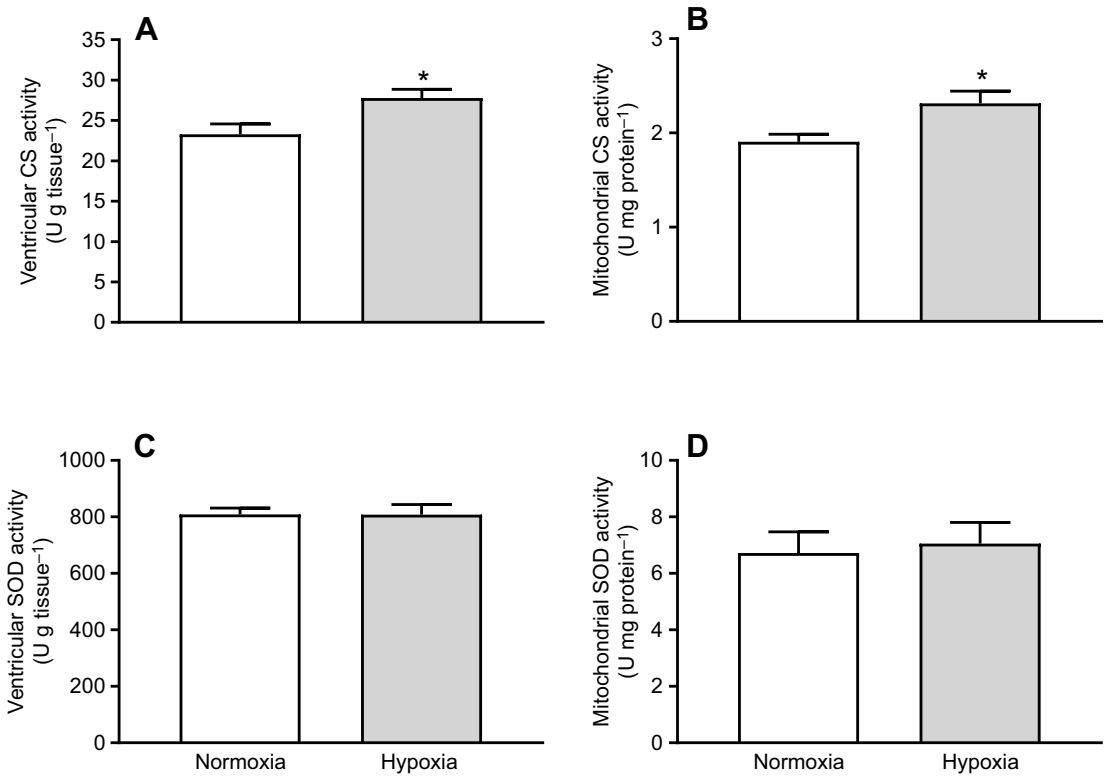

Fig. 7. Citrate synthase (CS) and superoxide dismutase (SOD) activity in ventricular tissue and mitochondria from sablefish acclimated to normoxia or hypoxia. Activity of citrate synthase in (A) ventricular homogenates (in U g tissue^{-1}) and (B) mitochondrial suspensions (U mg protein^{-1}; *N*=8 normoxic fish and *N*=7 hypoxic fish). Activity of the antioxidant enzyme SOD in (C) ventricular homogenates (in U g tissue^{-1}; *N*=6) and (D) mitochondrial suspensions (U mg protein^{-1}; *N*=8 normoxic fish and *N*=6 hypoxic fish). Other details are as in Fig. 1.

hypoxia-tolerant killifish also did not reduce their respiration when exposed to chronic hypoxia, they were able to reduce their ROS release rate by ∼50%. These data suggest that some mechanisms (plasticity) exist(s) in hypoxia-tolerant fish species to protect mitochondrial bioenergetic function during periods of environmental O_2 limitation.

To further investigate whether hypoxia acclimation affected the degree of oxidative stress experienced by the sablefish heart, we assessed the antioxidant defence capacity of their heart by measuring the activity of the enzyme superoxide dismutase (SOD) in both the ventricular myocardium and mitochondrial homogenates. The lack of a change in SOD activity following hypoxia acclimation suggests that antioxidant defences in the sablefish heart were already sufficient to catalyse the amount of ROS produced and to protect the myocardium from ROS-related damage (Fig. 7C,D). This is consistent with the unchanged mitochondrial ROS release rates reported in this study following hypoxia acclimation (Fig. 1C,D). While many animals increase their endogenous anti-oxidant capacity in response to oxygen limitation to prepare for oxidative stress (e.g. see Du et al., 2016), a strategy called POS (see Storey, 1996 and Hermes-Lima et al., 2015), there are also examples of animals for which no increase in antioxidant capacity was reported under hypoxia/anoxia (Leveelahti et al., 2014 and references therein). These latter studies are consistent with what we report here for the sablefish, and suggest that like many hypoxia-tolerant species, sablefish have evolved a naturally high antioxidant capacity that protects tissues from oxidative damage during O_2 limitation (Hermes-Lima and Zenteno-Savín, 2002).

Table 1. Lipid class composition (% of total lipids) of ventricular mitochondria from sablefish acclimated to normoxia or hypoxia

	Hypoxia	Normoxia	*P*-value
Hydrocarbons	2.1±1.3	2.3±1.1	0.93
Triacylglycerols (TAG)	0.004±0.004	1.5±1.2	0.30
Free fatty acids (FFA)	0.8±0.4	2.1±0.8	0.18
Alcohols	2.2±0.7	2.9±0.7	0.54
Sterols	4.6±2.3	4.3±1.5	0.91
Acetone mobile polar lipids (AMPL)	0.8±0.5	2.8±1.0	0.11
Phospholipids	89.1±1.6	83.5±2.5	0.10
Phospholipids/sterols	59.1±16.8	39.6±13.5	0.39
Total lipids (mg g^{-1} wet weight)	30.4±3.8	37.1±6.4	0.40

Values are means±s.e.m, *N*=7 normoxic fish and *N*=8 hypoxic fish.

Effect of anoxia-reoxygenation (AR) on mitochondrial respiration and ROS release rates

In vitro AR impaired cardiac mitochondrial function as reflected by a decrease in OXPHOS capacity in both acclimation groups, and by an increase in leak respiration in the hypoxia-acclimated fish (Fig. 4A,B). This agrees with previous studies on fish, rat and turtle cardiac mitochondria, which report mitochondrial dysfunction after AR events (Galli and Richards, 2014 and references therein). In Du et al. (2016), a transient increase in leak respiration after reoxygenation was reported, which was due to the ETS working in reverse and the accumulation of ADP. However, our data do not suggest reversal of the ETS and ATP consumption by complex V, as no transient increase was observed in leak respiration after anoxia in either acclimation group (Fig. S1). It is possible that cardio-protective mechanisms prevent complex V from operating in

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Table 2. Fatty acid composition (% of total fatty acids) of ventricular mitochondria from sablefish acclimated to normoxia or hypoxia

	Hypoxia	Normoxia	*P*-value
16:0	11.9±0.5	12.2±0.2	0.57
16:1ω7	0.9±0.1	0.8±0.04	0.24
16:4ω1	1.0±0.2	0.7±0.2	0.23
18:0	8.5±0.2	9.0±0.1	0.12
18:1ω9	7.7±0.6	7.4±0.4	0.68
18:1ω7	6.3±0.2	6.2±0.3	0.73
18:2ω6	2.0±0.2	1.7±0.1	0.12
18:3ω6	0.18±0.02	0.22±0.01	**0.04**
18:3ω4	0.23±0.02	0.18±0.01	**0.05**
18:3ω3	0.4±0.03	0.3±0.04	0.19
20:1ω9	0.8±0.1	0.7±0.2	0.61
20:4ω6 arachidonic acids (ARA)	3.6±0.1	3.2±0.1	**0.05**
20:4ω3	0.8±0.1	0.6±0.1	0.09
20:5ω3 eicosapentaenoic acids (EPA)	7.8±0.4	6.7±0.2	**0.04**
22:5ω3 docosapentaenoic acids (DPA)	3.9±0.2	3.4±0.1	0.09
22:6ω3 docosahexaenoic acids (DHA)	37.2±1.4	38.8±1.0	0.37
Σ saturated fatty acids (SFA)	21.6±0.6	21.6±0.6	0.20
Σ monounsaturated fatty acids (MUFA)	17.0±0.9	17.0±0.9	0.90
Σ polyunsaturated fatty acids (PUFA)	60.9±0.7	60.9±0.7	0.33
PUFA/SFA	2.8±0.1	2.7±0.1	0.13
Σ ω3	51.3±0.8	51.3±0.8	0.85
22:6ω3/20:5ω3	4.9±0.4	4.9±0.4	0.08
20:5ω3/20:4ω6	2.3±0.1	2.1±0.1	0.67
Terrestrial (18:3ω3+18:2ω6)	2.4±0.2	2.4±0.2	0.12
20:4ω6+22:5ω6	4.4±0.1	4.0±0.1	0.06
20:4ω6+20:5ω3	11.4±0.5	9.9±0.3	**0.02**

Values are means±s.e.m., *N*=6 normoxic fish and *N*=7 hypoxic fish. Significant values are indicated in bold.

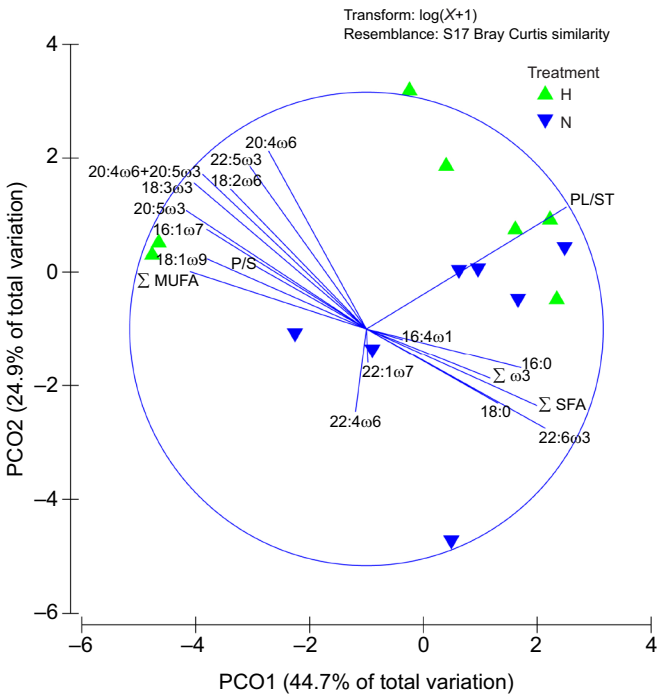

Fig. 8. Principal coordinates analysis (PCO) of lipid and FA composition (%) (r^2>0.35) of ventricular mitochondria from sablefish acclimated to normoxia or hypoxia. PL and ST represent the lipid classes of phospholipid and sterol, respectively. Σ SFA and Σ MUFA represent total saturated fatty acids and total monosaturated fatty acids, respectively. P:S is the ratio between polyunsaturated and saturated fatty acids. *N*=7 normoxic fish and *N*=6 hypoxic fish.

reverse in sablefish, and this should be investigated in more detail in the future.

Interestingly, acclimation to hypoxia lessened the decrease in OXPHOS respiration after anoxia, diminished the post-anoxic increase in ROS release rate during OXPHOS respiration and lessened ROS release rates post-anoxia during the leak state (Fig. 4). While sablefish cardiac mitochondria were not insensitive to anoxia exposure, hypoxia acclimation appears to allow them to be more efficient at oxidative phosphorylation and better defended against ROS damage following anoxia exposure, and this suggests that hypoxia acclimation may prevent reoxygenation damage and protect cardiac mitochondrial function post-anoxia. Our results contrast with those of Hickey et al. (2012) where *in vitro* AR elevated the % ROS/O_2 flux in permeabilized cardiac fibres from hypoxia-exposed epaulette sharks but did not affect the ROS release rate in hypoxia-intolerant shovelnose rays. However, a reduction in ROS release rates following *in vivo* hypoxia acclimation and *in vitro* AR was also reported in liver mitochondria from hypoxia-acclimated killifish (Du et al., 2016). The potential mechanisms underlying reductions in ROS production following hypoxia acclimation or AR bouts are not well understood, but were recently discussed in depth by Du et al. (2016). It is possible that the reduced ROS release rate post-anoxia in hypoxia-acclimated sablefish resulted from a decrease in superoxide produced by mitochondria as a by-product of electron transport, or an increase in membrane proton conductance. Increases in proton conductance are thought to limit ROS generation by allowing the mitochondria to operate at a lower membrane potential (Cunha et al., 2011). This increased proton conductance would be expected to be associated with elevated leak respiration, and this parameter increased following anoxic exposure in hypoxia-acclimated, but not in normoxia-acclimated sablefish (Fig. 4B). However, it is unlikely that the lower ROS release rate reported was related to alterations in ROS elimination/degradation. Although the activity of other mitochondrial enzymes that degrade H_2O_2 were not measured in this study (e.g. glutathione peroxidase and catalase), there was no difference in SOD activity between the acclimation groups.

Effect of NO on mitochondrial respiration and mitochondrial sensitivity to NO

In the present study, we showed that NO inhibited sablefish cardiac mitochondrial respiration using the NO donor PAPANONOate and high-resolution respirometry (Fig. 5; Fig. S2) and that this inhibition was completely reversed by the addition of oxyhaemoglobin (Fig. 5A, D). These responses were both anticipated/expected as NO competes with oxygen for binding to the active site of the terminal enzymatic complex of the ETS (i.e. CCO), and haemoglobin is a known scavenger/inactivator of NO (Borutaite et al., 2000; Brown, 2001; Kelm, 1999). Interestingly, we also observed a lower rate of NO release in the presence of mitochondria compared with that in buffer alone (Fig. 6). This was likely due to substrate-independent consumption of NO by the mitochondria's membranes (Shiva et al., 2001; Venkatraman et al., 2003).

At an O_2 level of ~120 µmol l^{-1}, we report that the IC_{50} value (i.e. the concentration of NO at which mitochondrial respiration was reduced by 50%) was ~126 nmol l^{-1} in normoxia-acclimated fish, compared to only ~94 nmol l^{-1} in hypoxia-acclimated fish at 10°C. This increase in mitochondrial sensitivity to NO would be beneficial under limiting O_2 conditions as it would prevent the development of near-anoxic conditions in the heart, and the ETS from working in reverse. This is the first report of an IC_{50} for NO in fish, although numerous IC_{50} values have been reported for various mammalian tissues and conditions; these values ranging from ~150 to ~400 nmol NO l^{-1} (Brookes et al., 2003; Brown, 2001 and

references therein; King et al., 2016; Venkatraman et al., 2003). In agreement with our study, an increase in the sensitivity of mitochondrial respiration to NO inhibition was observed in mitochondria isolated from the liver of rats and mice following chronic alcohol consumption; a condition known to induce hypoxia in the liver (King et al., 2016; Venkatraman et al., 2003; Zelickson et al., 2011). These data suggest an important role for NO in mitochondrial regulation, and may have implications for hypoxic vasodilation, O_2 sensing and cardioprotection.

In the present study, we measured the IC_{50} over a pre-determined range of O_2 (~110–160 µmol O_2 l^{-1}; ~7–10 kPa P_{O_2}). This O_2 level is slightly higher than *in vivo* blood oxygen levels as the majority of the fish heart receives its O_2 supply from venous blood with a low P_{O_2} (~4.1–6.4 kPa; Ekström et al., 2016 and references therein; Farrell and Smith, 2017; Petersen and Gamperl, 2010b), and the sablefish heart is composed entirely of spongy myocardium (i.e. it has no coronary blood supply; personal observations). Given that NO inhibits mitochondrial respiration by competing with O_2, it is possible that the IC_{50} for NO would be reduced at lower levels of oxygen. Nonetheless, our value for IC_{50} (~126 nmol NO l^{-1} in normoxia-acclimated fish) is very similar to that measured for rat liver mitochondria at physiological/tissue O_2 levels (an IC_{50} of 140 nmol l^{-1} at 5–10 µmol O_2 l^{-1}; Brookes et al., 2003).

Mitochondrial lipids

The observed changes in 20:4ω6, 20:5ω3, 18:3ω6 and 18:3ω4 content (Table 2 and Fig. 8) suggest that these fatty acids play an important role in the maintenance of mitochondrial function (e.g. see Figs 1–4) during *in vivo* chronic hypoxia. The preservation of mitochondrial membrane integrity and fluidity is dependent upon finding a balance between the proportion of unsaturated versus saturated fatty acids and cholesterol content. The mitochondria from hypoxic sablefish had higher proportions of both 20:4ω6 and 20:5ω3 (Table 2), and a ~50% higher phospholipid:sterol (PL:ST) ratio (Table 1). Although this latter difference was not significant, the SIMPER analysis revealed that this ratio was the largest contributor to the dissimilarity between treatments (~24%) (Fig. 8). A relationship between PL:ST and membrane fluidity in fish has been demonstrated in previous studies (Arts and Kohler, 2009; Farkas et al., 2001). However, the concept of homeoviscous adaptation has been primarily investigated in the context of temperature changes (Chung et al., 2018; Grim et al., 2010), and very little is known about the impacts of hypoxia on membrane fluidity in mammals or fish. In the only study that we could find that examined the effects of prolonged hypoxia on cell membrane FA, Ledoux et al. (2003) reported that rat aortic endothelial cells exposed to acute hypoxic stress (i.e. 0% O_2 for 18 h) had reduced membrane fluidity that was related to a decrease in the ratio of polyunsaturated to saturated fatty acids. The discrepancy between Ledoux et al. (2003) and our results [i.e. increase in the ratio of phospholipid: sterol and in polyunsaturated (20:4ω6 and 20:5ω3) FA following long-term hypoxia] could be related to the diversity of adaptations to hypoxia, differences between species and/or differences in how short- and long-term hypoxia/anoxia exposure affect membrane composition and fluidity. For instance, Duan et al. (1999) reported that brain phospholipid levels were elevated by one preconditioning bout of hypoxia (8 min), but that 4 bouts (ranging in duration from 8 to 67 min) resulted in phospholipid levels that were closer to control levels (mice only exposed to normoxia).

The hypoxic sablefish could have potentially responded to low oxygen acclimation by reducing the β-oxidation of fatty acids, and increasing tissue lipid content, as previously shown for the heart of hypoxic rats (Whitmer et al., 1978). This could change the major fatty acid constituents of the whole tissue, including the mitochondria. For example, Huss et al. (2001) showed that hypoxia decreased the oxidative capacity of mitochondrial fatty acids, through inhibition of transcript for the rate-limiting enzyme of fatty acid oxidation, carnitine palmitoyltransferase. Thus, the observed negative association between hypoxia acclimation and 16:0 (Fig. 8) could be related to lower FA oxidation in the hypoxia-acclimated fish as part of a cellular adaptive mechanism. Finally, the hypoxia-acclimated fish showed an increase in the proportions of 20:4ω6 (~11%), 20:5ω3 (~14%) and the sum of 20:4ω6+20:5ω3 (~13%). These alterations in FA composition could play a role in eicosanoid metabolism, since 20:4ω6 and 20:5ω3 compete as substrates for eicosanoid synthesis (Calder, 2007, 2013), and as such, may have influenced the health (i.e. immune response) and inflammatory status of the hypoxia-acclimated fish (Friesen et al., 2013). However, direct measurements of eicosanoids and the sablefish's (fish) immune function following hypoxia acclimation are necessary to test this hypothesis.

Mitochondrial responses to hypoxia in fishes

The maintenance of mitochondrial function is essential for hypoxia tolerance because of its central role in the production of cellular energy and ROS for metabolic and redox homeostasis, respectively (Pamenter, 2014; Solaini et al., 2010). In the present study, we showed that the cardiac mitochondria of sablefish can endure chronic hypoxia as neither steady-state mitochondrial respiration or any indicators of respiratory control [i.e. respiratory control ratio (RCR), mitochondrial O_2 affinity (P_{50}), P:O ratio, uncoupling with FCCP] were altered following hypoxia acclimation. In addition, hypoxia acclimation did not appear to induce oxidative stress, as mitochondrial ROS release rates during steady-state mitochondrial respiration and superoxide dismutase (SOD) activity were unchanged. This maintenance of cardiac mitochondrial function appears to be associated with alterations in the intrinsic properties of the mitochondria. Indeed, we showed that: (1) CS activity increased by ~20% in hypoxia-acclimated fish; (2) cardiac mitochondria from hypoxia-acclimated fish were more resilient to anoxia-reoxygenation events as evidenced by improved OXPHOS respiration and reduced ROS release rates post-anoxia; (3) hypoxia acclimation increased the sensitivity of mitochondrial respiration to inhibition by NO; and (4) hypoxia acclimation resulted in a remodelling of mitochondrial lipid (FA) composition. Overall, these findings suggest that the hypoxia-tolerant sablefish may possess plastic traits related to mitochondrial function that can be adjusted by hypoxia acclimation to defend (maintain) cardiac mitochondrial and bioenergetic function during periods of O_2 limitation.

However, it is not clear to what extent these data can be extrapolated to other fish species. With regards to several of the measured mitochondrial parameters (e.g. CS and SOD activities, and ROS release rates), there were discrepancies in the responses as compared with those reported in other studies (e.g. Borowiec et al., 2015, Cook et al., 2013, Du et al., 2016; Hickey et al., 2012). These contrasting findings with regards to the mitochondria's response to hypoxia may be due to tissue or methodological differences (i.e. in the duration and severity of hypoxia, in study temperature), or related to species-specific behavioural strategies to survive long-term hypoxia. For example, our studies were conducted at a relatively cold temperature (10°C) and the sablefish in our experiment remained active at this temperature and continued to feed at 40% and 20% air saturation. These variable mitochondrial

responses were also probably shaped by the phylogenetic and evolutionary histories of the species and their native hypoxic environments (Borowiec et al., 2015; Currie and Boutilier, 2001; Devaux et al., 2019; Galli and Richards, 2014; Mandic and Regan, 2018). Clearly, more research is needed before we can understand what factors (mechanisms) allow mitochondrial physiology to be plastic in the face of O_2 limiting conditions, and characterize how different metabolic strategies relate to the severity and temporal nature of the hypoxic environments that fishes inhabit.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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References
Aguirre, E., Rodríguez-Juárez, F., Bellelli, A., Gnaiger, E. and Cadenas, S. (2010). Kinetic model of the inhibition of respiration by endogenous nitric oxide in intact cells. *Biochim. Biophys. Acta Bioenerg.* **1797**, 557-565. doi:10.1016/j.bbabio.2010.01.033

Arts, M. T. and Kohler, C. C. (2009). Health and condition in fish: the influence of lipids on membrane competency and immune response. In *Lipids in Aquatic Ecosystems* (ed. M. Kainz, M. Brett and M. Arts), pp. 237-256. New York, NY: Springer.

Bickler, P. E. and Buck, L. T. (2007). Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annu. Rev. Physiol.* **69**, 145-170. doi:10.1146/annurev.physiol.69.031905.162529

Borowiec, B. G., Darcy, K. L., Gillette, D. M. and Scott, G. R. (2015). Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **218**, 1198-1211. doi:10.1242/jeb.114579

Borutaite, V., Budriunaite, A. and Brown, G. C. (2000). Reversal of nitric oxide-, peroxynitrite- and S -nitrosothiol-induced inhibition of mitochondrial respiration or complex I activity by light and thiols. *Biochim. Biophys. Acta* **1459**, 405-412. doi:10.1016/S0005-2728(00)00178-X

Boutilier, R. G. (2001). Mechanisms of cell survival in hypoxia and hypothermia. *J. Exp. Biol.* **204**, 3171-3181.

Breitburg, D., Levin, L. A., Oschlies, A., Grégoire, M., Chavez, F. P., Conley, D. J., Garçon, V., Gilbert, D., Gutiérrez, D., Isensee, K. et al. (2018). Declining oxygen in the global ocean and coastal waters. *Science* **359**, eaam7240. doi:10.1126/science.aam7240

Brookes, P. S., Zhang, J., Dai, L., Zhou, F., Parks, D. A., Darley-Usmar, V. M. and Anderson, P. G. (2001). Increased sensitivity of mitochondrial respiration to inhibition by nitric oxide in cardiac hypertrophy. *J. Mol. Cell. Cardiol.* **33**, 69-82. doi:10.1006/jmcc.2000.1276

Brookes, P. S., Kraus, D. W., Shiva, S., Doeller, J. E., Barone, M.-C., Patel, R. P., Lancaster, J. R. and Darley-Usmar, V. (2003). Control of mitochondrial respiration by NO, effects of low oxygen and respiratory state. *J. Biol. Chem.* **278**, 31603-31609. doi:10.1074/jbc.M211784200

Brown, G. C. (2001). Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim. Biophys. Acta* **1504**, 46-57. doi:10.1016/S0005-2728(00)00238-3

Brown, G. C. and Borutaite, V. (2004). Inhibition of mitochondrial respiratory complex I by nitric oxide, peroxynitrite and S-nitrosothiols. *Biochim. Biophys. Acta* **1658**, 44-49. doi:10.1016/j.bbabio.2004.03.016

Calder, P. C. (2007). Immunomodulation by omega-3 fatty acids. *Prostaglandins, Leukot. Essent. Fat. Acids* **77**, 327-335. doi:10.1016/j.plefa.2007.10.015

Calder, P. C. (2013). n-3 Fatty acids, inflammation and immunity: new mechanisms to explain old actions. *Proc. Nutr. Soc.* **72**, 326-336. doi:10.1017/S0029665113001031

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

Chouchani, E. T., Pell, V. R., James, A. M., Work, L. M., Saeb-Parsy, K., Frezza, C., Krieg, T. and Murphy, M. P. (2016). A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury. *Cell Metab.* **23**, 254-263. doi:10.1016/j.cmet.2015.12.009

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

Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S. and Schapira, A. H. V. (1994). Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide: Implications for neurodegenerative diseases. *FEBS Lett.* **345**, 50-54. doi:10.1016/0014-5793(94)00424-2

Clementi, E., Brown, G. C., Feelisch, M. and Moncada, S. (1998). Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc. Natl. Acad. Sci. USA* **95**, 7631-7636. doi:10.1073/pnas.95.13.7631

Cook, D. G., Iftikar, F. I., Baker, D. W., Hickey, A. J. R. and Herbert, N. A. (2013). Low-O_2 acclimation shifts the hypoxia avoidance behaviour of snapper (*Pagrus auratus*) with only subtle changes in aerobic and anaerobic function. *J. Exp. Biol.* **216**, 369-378. doi:10.1242/jeb.073023

Cooper, C. E., Mason, M. G. and Nicholls, P. (2008). A dynamic model of nitric oxide inhibition of mitochondrial cytochrome c oxidase. *Biochim. Biophys. Acta* **1777**, 867-876. doi:10.1016/j.bbabio.2008.03.021

Costa, L. E., Méndez, G. and Boveris, A. (1997). Oxygen dependence of mitochondrial function measured by high-resolution respirometry in long-term hypoxic rats. *Am. J. Physiol.* **273**, C852-C858. doi:10.1152/ajpcell.1997.273.3.C852

Cunha, F. M., Caldeira da Silva, C. C., Cerqueira, F. M. and Kowaltowski, A. J. (2011). Mild mitochondrial uncoupling as a therapeutic strategy. *Curr. Drug Targets* **12**, 783-789. doi:10.2174/138945011795528778

Currie, S. and Boutilier, R. G. (2001). Strategies of hypoxia and anoxia tolerance in cardiomyocytes from the overwintering common frog, *Rana temporaria*. *Physiol. Biochem. Zool.* **74**, 420-428. doi:10.1086/320424

Davidson, S. M. and Duchen, M. R. (2006). Effects of NO on mitochondrial function in cardiomyocytes: pathophysiological relevance. *Cardiovasc. Res.* **71**, 10-21. doi:10.1016/j.cardiores.2006.01.019

Devaux, J. B. L., Hickey, A. J. R. and Renshaw, G. M. C. (2019). Mitochondrial plasticity in the cerebellum of two anoxia-tolerant sharks: contrasting responses to anoxia/re-oxygenation. *J. Exp. Biol.* **222**, jeb191353. doi:10.1242/jeb.191353

Diaz, R. J. and Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science* **321**, 926-929. doi:10.1126/science.1156401

Dikalov, S. I., Mayorov, V. I. and Panov, A. V. (2017). Physiological levels of nitric oxide diminish mitochondrial superoxide: potential role of mitochondrial dinitrosyl iron complexes and nitrosothiols. *Front. Physiol.* **8**, 907. doi:10.3389/fphys.2017.00907

Du, S. N. N., Mahalingam, S., Borowiec, B. G. and Scott, G. R. (2016). Mitochondrial physiology and reactive oxygen species production are altered by hypoxia acclimation in killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **219**, 1130-1138. doi:10.1242/jeb.132860

Duan, C.-L., Yan, F.-S., Lu, G.-W., Liu, H.-M. and Yin, N. (1999). Changes in phospholipids and free fatty acids in the brains of mice preconditioned by hypoxia. *NeuroSignals* **8**, 261-266. doi:10.1159/000014596

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

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. *Physiology* **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

Farkas, T., Fodor, E., Kitajka, K. and Halver, J. E. (2001). Response of fish membranes to environmental temperature. *Aquac. Res.* **32**, 645-655. doi:10.1046/j.1365-2109.2001.00600.x

Farrell, A. P. and Smith, F. (2017). Cardiac form, function and physiology. In *The Cardiovascular System: Morphology, Control and Function* (ed. A. Kurt Gamperl, Todd E. Gillis, Anthony P. Farrell and C. J. B.), pp. 155-264. Cambridge, MA: Elsevier Inc.

Farrell, A. P. and Stecyk, J. A. W. (2007). The heart as a working model to explore themes and strategies for anoxic survival in ectothermic vertebrates. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **147**, 300-312. doi:10.1016/j.cbpa.2007.01.021

Fasching, M. and Gnaiger, E. (2016). O2k-Multisensor system with amperometric sensors: NO, H_2S, H_2O_2. *Mitochondrial Physiol. Netw.* **15.05**, 1-11.

Fasching, M. and Gnaiger, E. (2018). O2k quality control 2: instrumental oxygen background correction and accuracy of oxygen flux. *Mitochondrial Physiol. Netw.* **14.6**, 1-14.

Friesen, E., Balfry, S. K., Skura, B. J., Ikonomou, M. and Higgs, D. A. (2013). Evaluation of poultry fat and blends of poultry fat with cold-pressed flaxseed oil as supplemental dietary lipid sources for juvenile sablefish (*Anoplopoma fimbria*). *Aquac. Res.* **44**, 300-316. doi:10.1111/j.1365-2109.2012.03239.x

Galli, G. L. J. and Richards, J. G. (2014). Mitochondria from anoxia-tolerant animals reveal common strategies to survive without oxygen. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **184**, 285-302. doi:10.1007/s00360-014-0806-3

Galli, G. L. J., Lau, G. Y. and Richards, J. G. (2013). Beating oxygen: chronic anoxia exposure reduces mitochondrial F1FO-ATPase activity in turtle (*Trachemys scripta*) heart. *J. Exp. Biol.* **216**, 3283-3293. doi:10.1242/jeb.087155

Gamperl, A. K. and Driedzic, W. R. (2009). Cardiovascular function and cardiac metabolism. In *Hypoxia* (ed. J. G. Richards, A. P. Farrell and C. J. Brauner), pp. 301-360. Cambridge, MA: Elsevier Inc.

Gattuso, A., Garofalo, F., Cerra, M. C. and Imbrogno, S. (2018). Hypoxia tolerance in teleosts: Implications of cardiac nitrosative signals. *Front. Physiol.* **9**, 1-13. doi:10.3389/fphys.2018.00366

Gnaiger, E. (2016). O2k-calibration by DatLab. *Mitochondrial Physiol. Netw.* **19.18**, 1-12.

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

Grim, J. M., Miles, D. R. B. and Crockett, E. L. (2010). Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. *J. Exp. Biol.* **213**, 445-452. doi:10.1242/jeb.036939

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.

Guzik, T. J., West, N. E. J., Pillai, R., Taggart, D. P. and Channon, K. M. (2002). Nitric oxide modulates superoxide release and peroxynitrite formation in human blood vessels. *Hypertension* **39**, 1088-1094. doi:10.1161/01.HYP.0000018041.48432.B5

Hermes-Lima, M. and Zenteno-Savín, T. (2002). Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp. Biochem. Physiol. C* **133**, 537-556. doi:10.1016/S1096-4959(02)00146-X

Hermes-Lima, M., Moreira, D. C., Rivera-Ingraham, G. A., Giraud-Billoud, M., Genaro-Mattos, T. C. and Campos, É. G. (2015). Preparation for oxidative stress under hypoxia and metabolic depression: revisiting the proposal two decades later. *Free Radic. Biol. Med.* **89**, 1122-1143. doi:10.1016/j.freeradbiomed.2015.07.156

Hickey, A. J. R., Renshaw, G. M. C., Speers-Roesch, B., Richards, J. G., Wang, Y., Farrell, A. P. and Brauner, C. J. (2012). A radical approach to beating hypoxia: depressed free radical release from heart fibres of the hypoxia-tolerant epaulette shark (*Hemiscyllum ocellatum*). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **182**, 91-100. doi:10.1007/s00360-011-0599-6

Hochachka, P. and Lutz, P. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **130**, 435-459. doi:10.1016/S1096-4959(01)00408-0

Huss, J. M., Levy, F. H. and Kelly, D. P. (2001). Hypoxia inhibits the peroxisome proliferator-activated receptor alpha/retinoid X receptor gene regulatory pathway in cardiac myocytes. *J. Biol. Chem.* **276**, 27605-27612. doi:10.1074/jbc.M100277200

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

Jensen, F. B. (2007). Nitric oxide formation from nitrite in zebrafish. *J. Exp. Biol.* **210**, 3387-3394. doi:10.1242/jeb.008748

Kelm, M. (1999). Nitric oxide metabolism and breakdown. *Biochim. Biophys. Acta* **1411**, 273-289. doi:10.1016/S0005-2728(99)00020-1

King, A. L., Mantena, S. K., Andringa, K. K., Millender-Swain, T., Dunham-Snary, K. J., Oliva, C. R., Griguer, C. E. and Bailey, S. M. (2016). The methyl donor S-adenosylmethionine prevents liver hypoxia and dysregulation of mitochondrial bioenergetic function in a rat model of alcohol-induced fatty liver disease. *Redox Biol.* **9**, 188-197. doi:10.1016/j.redox.2016.08.005

Korge, P., Ping, P. and Weiss, J. N. (2008). Reactive oxygen species production in energized cardiac mitochondria during hypoxia/reoxygenation: modulation by nitric oxide. *Circ. Res.* **103**, 873-880. doi:10.1161/CIRCRESAHA.108.180869

Krumschnabel, G., Fontana-Ayoub, M., Fasching, M. and Gnaiger, E. (2019). O2-FluoRespirometry: HRR and simultaneous determination of H_2O_2 production with Amplex UltraRed. *Mitochondrial Physiol. Netw.* **20.14**, 1-6.

Ledoux, S., Runembert, I., Koumanov, K., Michel, J. B., Trugnan, G. and Friedlander, G. (2003). Hypoxia enhances ecto-5′-nucleotidase activity and cell surface expression in endothelial cells. *Circ. Res.* **92**, 848-855. doi:10.1161/01.RES.0000069022.95401.FE

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

Leveelahti, L., Rytkönen, K. T., Renshaw, G. M. C. and Nikinmaa, M. (2014). Revisiting redox-active antioxidant defenses in response to hypoxic challenge in both hypoxia-tolerant and hypoxia-sensitive fish species. *Fish Physiol. Biochem.* **40**, 183-191. doi:10.1007/s10695-013-9835-1

Mandic, M. and Regan, M. D. (2018). Can variation among hypoxic environments explain why different fish species use different hypoxic survival strategies? *J. Exp. Biol.* **221**, jeb161349. doi:10.1242/jeb.161349

Mantena, S. K., Vaughn, D. P., Andringa, K. K., Eccleston, H. B., King, A. L., Abrams, G. A., Doeller, J. E., Kraus, D. W., Darley-Usmar, V. M. and Bailey, S. M. (2009). High fat diet induces dysregulation of hepatic oxygen gradients and mitochondrial function *in vivo*. *Biochem. J.* **417**, 183-193. doi:10.1042/BJ20080868

Mason, C., Beamish, R. J. and McFarlane, G. A. (1983). Sexual maturity, fecundity, spawning, and early life history of sablefish (*Anoglopoma fimbria*) off the Pacific coast of Canada. *Can. J. Fish. Aquat. Sci.* **40**, 2126-2134. doi:10.1139/f83-247

Moser, H. G., Chakter, R., Smith, P. E., Lo, N. C. H., Ambrose, D. A., Meyer, C. A., Sandknop, E. M. and Watson, W. (1994). Early life history of sablefish, *Anoplopoma fimbria*, off Washington, Oregon, and California, with application to biomass estimation. *Calif. Coop. Ocean. Fish. Investig. Rep.* **35**, 144-159.

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

Munro, D. and Treberg, J. R. (2017). A radical shift in perspective: mitochondria as regulators of reactive oxygen species. *J. Exp. Biol.* **220**, 1170-1180. doi:10.1242/jeb.132142

Munro, D., Banh, S., Sotiri, E., Tamanna, N. and Treberg, J. R. (2016). The thioredoxin and glutathione-dependent H_2O_2 consumption pathways in muscle mitochondria: involvement in H_2O_2 metabolism and consequence to H_2O_2 efflux assays. *Free Radic. Biol. Med.* **96**, 334-346. doi:10.1016/j.freeradbiomed.2016.04.014

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

Onukwufor, J. O., Stevens, D. and Kamunde, C. (2017). Combined effects of cadmium, temperature and hypoxia-reoxygenation on mitochondrial function in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **182**, 129-141. doi:10.1016/j.aquatox.2016.11.015

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. Natl. Acad. Sci. USA* **101**, 7630-7635. doi:10.1073/pnas.0401723101

Pamenter, M. E. (2014). Mitochondria: a multimodal hub of hypoxia tolerance. *Can. J. Zool.* **92**, 569-589. doi:10.1139/cjz-2013-0247

Parrish, C. C. (1987). Separation of aquatic lipid classes by chromarod thin-layer chromatography with measurement by Iatroscan flame ionization detection. *Can. J. Fish. Aquat. Sci.* **44**, 722-731. doi:10.1139/f87-087

Parrish, C. C. (1999). Determination of total lipid, lipid classes, and fatty acids in aquatic samples. In *Lipids in Freshwater Ecosystems* (ed. M. T. Arts and B. Wainmann), pp. 4-10. New York: Springer-Verlag.

Petersen, L. H. and Gamperl, A. K. (2010a). *In situ* cardiac function in Atlantic cod (*Gadus morhua*): effects of acute and chronic hypoxia. *J. Exp. Biol.* 213, 820-830. doi:10.1242/jeb.033753

Petersen, L. H. and Gamperl, A. K. (2010b). Cod (*Gadus morhua*) cardiorespiratory physiology and hypoxia tolerance following acclimation to low oxygen. *Physiol. Biochem. Zool.* **84**, 18-31. doi:10.1086/657286

Pethybridge, H. R., Parrish, C. C., Morrongiello, J., Young, J. W., Farley, J. H., Gunasekera, R. M. and Nichols, P. D. (2015). Spatial patterns and temperature predictions of tuna fatty acids: tracing essential nutrients and changes in primary producers. *PLoS ONE* **10**, e0131598. doi:10.1371/journal.pone.0131598

Ransberry, V. E., Blewett, T. A. and McClelland, G. B. (2016). The oxidative stress response in freshwater-acclimated killifish (*Fundulus heteroclitus*) to acute copper and hypoxia exposure. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **179**, 11-18. doi:10.1016/j.cbpc.2015.08.001

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

Scott, G. R., Egginton, S., Richards, J. G. and Milsom, W. K. (2009). Evolution of muscle phenotype for extreme high altitude flight in the bar-headed goose. *Proc. R. Soc. B Biol. Sci.* **276**, 3645-3653. doi:10.1098/rspb.2009.0947

Shiva, S., Brookes, P. S., Patel, R. P., Anderson, P. G. and Darley-Usmar, V. M. (2001). Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome c oxidase. *Proc. Natl. Acad. Sci. USA* **98**, 7212-7217. doi:10.1073/pnas.131128898

Sokolova, I. (2018). Mitochondrial adaptations to variable environments and their role in animals' stress tolerance. *Integr. Comp. Biol.* **58**, 519-531. doi:10.1093/icb/icy017

Solaini, G., Baracca, A., Lenaz, G. and Sgarbi, G. (2010). Hypoxia and mitochondrial oxidative metabolism. *Biochim. Biophys. Acta Bioenerg.* **1797**, 1171-1177. doi:10.1016/j.bbabio.2010.02.011

Stecyk, J. A. W. (2017). Cardiovascular responses to limiting oxygen levels. In *The Cardiovascular System: Development, Plasticity and Physiological Responses* (ed. A. K. Gamperl, T. E. Gillis, A. P. Farrell and C. J. Brauner), pp. 299-371. Cambridge, MA: Elsevier Inc.

Storey, K. B. (1996). Oxidative stress: animal adaptation in nature. *Brazilian J. Med. Biol. Res.* **29**, 1715-1733.

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

St-Pierre, J., Brand, M. D. and Boutilier, R. G. (2000). The effect of metabolic depression on proton leak rate in mitochondria from hibernating frogs. *J. Exp. Biol.* **203**, 1469-1476.

Taylor, C. T. and Moncada, S. (2010). Nitric oxide, cytochrome c oxidase, and the cellular response to hypoxia. *Arterioscler. Thromb. Vasc. Biol.* **30**, 643-647. doi:10.1161/ATVBAHA.108.181628

Tota, B., Angelone, T., Mancardi, D. and Cerra, M. C. (2011). Hypoxia and anoxia tolerance of vertebrate hearts: an evolutionary perspective. *Antioxid. Redox Signal.* **14**, 851-862. doi:10.1089/ars.2010.3310

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

Vagner, M., Pante, E., Viricel, A., Lacoue-labarthe, T., Zambonino-Infante, J.-L., Quazuguel, P., Dubillot, E., Huet, V., Le Delliou, H. et al. (2019). Ocean warming combined with lower omega-3 nutritional availability impairs the cardio-respiratory function of a marine fish. *J. Exp. Biol.* **222**, jeb187179. doi:10.1242/jeb.187179

Venkatraman, A., Shiva, S., Davis, A. J., Bailey, S. M., Brookes, P. S. and Darley-Usmar, V. M. (2003). Chronic alcohol consumption increases the sensitivity of rat liver mitochondrial respiration to inhibition by nitric oxide. *Hepatology* **38**, 141-147. doi:10.1053/jhep.2003.50293

Whitmer, T., Idell-wenger, J. A., Hershey, S., Rovetto, J. and Neely, R. (1978). Control of fatty acid metabolism in ischemic and hypoxic hearts. *J. Bioclogical Chem.* **253**, 4305-4309.

Zelickson, B. R., Benavides, G. A., Johnson, M. S., Chacko, B. K., Venkatraman, A., Landar, A., Betancourt, A. M., Bailey, S. M. and Darley-Usmar, V. M. (2011). Nitric oxide and hypoxia exacerbate alcohol-induced mitochondrial dysfunction in hepatocytes. *Biochim. Biophys. Acta Bioenerg.* **1807**, 1573-1582. doi:10.1016/j.bbabio.2011.09.011