The Journal of Experimental Biology 214, 3732-3741

doi:10.1242/jeb.062042

## RESEARCH ARTICLE

# Inter-relationship between mitochondrial function and susceptibility to oxidative stress in red- and white-blooded Antarctic notothenioid fishes

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Accepted 30 August 2011

## SUMMARY

**It is unknown whether Antarctic fishes can defend themselves against oxidative stress induced by elevations in temperature. We hypothesized that Antarctic icefishes, lacking the oxygen-binding protein hemoglobin, might be more vulnerable to temperature-induced oxidative stress compared with red-blooded notothenioids because of differences in their mitochondrial properties. Mitochondria from icefishes have higher densities of phospholipids per mg of mitochondrial protein compared with red-blooded species, and these phospholipids are rich in polyunsaturated fatty acids (PUFA), which can promote the formation of reactive oxygen species (ROS). Additionally, previous studies have shown that multiple tissues in icefishes have lower levels of antioxidants compared with red-blooded species. We quantified several properties of mitochondria, including proton leak, rates of ROS production, membrane composition and susceptibility to lipid peroxidation (LPO), the activity of superoxide dismutase (SOD) and total antioxidant power (TAOP) in mitochondria isolated from hearts of icefishes and red-blooded notothenioids. Mitochondria from icefishes were more tightly coupled than those of red-blooded fishes at both 2°C and 10°C, which increased the production of ROS when the electron transport chain was disrupted. The activity of SOD and TAOP per mg of mitochondrial protein was equivalent between icefishes and red-blooded species, but TAOP normalized to mitochondrial phospholipid content was significantly lower in icefishes compared with red-blooded fishes. Additionally, membrane susceptibility to peroxidation was only detectable in icefishes at 1°C and not in red-blooded species. Together, our results suggest that the high density of mitochondrial phospholipids in hearts of icefishes may make them particularly vulnerable to oxidative stress as temperatures rise.**

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/22/3732/DC1

Key words: Antarctic fish, mitochondria, oxidative stress.

## INTRODUCTION

Notothenioid fishes have inhabited the thermally stable and cold environment of the Southern Ocean for ~10–12 million years (Eastman, 1993). Water temperatures south of the Antarctic Polar Front range between –1.9°C and 3°C, and fluctuate minimally on a seasonal basis (Littlepage, 1965; Eastman, 1993; Hunt et al., 2003). Notothenioids possess an array of adaptations that make them extraordinarily well-suited for life in the cold, including antifreeze proteins and cold-stable microtubules (Cheng and Detrich, 2007). The ability of notothenioids to withstand elevations in temperature is less clear. The upper incipient lethal temperature limit of three species of notothenioids was determined to be between only 5°C and 7°C (Somero and DeVries, 1967). However, more recent studies have revealed that notothenioids have a limited capacity to acclimate to warmer temperatures, as evidenced by changes in thermal tolerance, cardiac function and antifreeze levels in response to an increase in temperature (Jin and DeVries, 2006; Podrabsky and Somero, 2006; Franklin et al., 2007; Bilyk and Devries, 2011). The ability of Antarctic fishes to withstand warming of the Southern Ocean, which is occurring very rapidly in the western Antarctic Peninsula (WAP) region (Vaughan et al., 2003), will be dependent, at least in part, on their ability to maintain mitochondrial function over a range of temperatures.

Channichthyid icefishes are among the most notable families of Antarctic notothenioids, distinguished by their lack of expression of the oxygen-binding protein, hemoglobin (Hb) (Ruud, 1954). Six of the 16 species of icefishes also lack the intracellular oxygen-binding protein myoglobin (Mb) in their heart ventricle, which stores and facilitates the diffusion of oxygen within oxidative muscle (Sidell et al., 1997; Moylan and Sidell, 2000). The loss of Hb expression reduces the blood oxygen-carrying capacity of icefishes to one-tenth that of red-blooded species (Ruud, 1954). Icefishes have likely survived without Hb and Mb because of their cold, well-oxygenated environment, together with several modifications in their cardiovascular system, which enhance oxygen delivery (reviewed by Sidell and O'Brien, 2006). However, as global temperatures rise, the loss of Hb may become disadvantageous. The solubility of oxygen in blood plasma is inversely correlated with temperature, so that as temperature increases, blood oxygen-carrying capacity of icefishes is more likely to decline compared with Hb-expressing fish. Current empirical evidence supports this conjecture. The critical thermal maximum, defined as the temperature at which fish lose the ability to right themselves, is positively correlated with hematocrit and is 1.5–3.0°C lower in icefishes compared with red-blooded notothenioids (Beers and Sidell, 2011).

Differences in the properties of mitochondria between red- and white-blooded notothenioids may impact the balance between oxygen supply and demand and could therefore influence thermal tolerance. Mitochondrial function is inextricably linked to structure, which is strikingly different between red- and white-blooded notothenioids. Mitochondria from icefishes are 1.1–1.4-times larger with more loosely packed inner membranes (cristae) compared with mitochondria from red-blooded notothenioids (O'Brien and Sidell, 2000). Alterations in mitochondrial morphology can affect several aspects of mitochondrial function, including proton leak (Porter et al., 1996), which occurs when protons leak across the inner mitochondrial membrane from the intermembrane space into the mitochondrial matrix, bypassing the ATP synthase enzyme (reviewed by Brand, 2005). Proton leak is affected by the composition of mitochondrial membranes (Brookes et al., 1998) and is positively correlated with the density of inner mitochondrial membranes (Porter et al., 1996), the activity of uncoupling proteins (UCPs) (Brand et al., 2004) and the adenosine nucleotide translocase (ANT) enzyme (Brand et al., 2005) and, notably, temperature (Chamberlin, 2004; Jastroch et al., 2007). As temperature and proton leak increase, more oxygen will be required by the respiratory chain to generate a proton gradient and maintain ATP production. This may be particularly problematic for icefishes with a reduced blood oxygen-carrying capacity compared with red-blooded species.

Compared with red-blooded species, icefishes may also be less thermally tolerant because properties of their mitochondria could place them at a greater risk for oxidative damage as temperature increases. Oxidative stress occurs when the rate of production of reactive oxygen species (ROS) exceeds antioxidant defenses, resulting in oxidatively damaged macromolecules (reviewed by Halliwell, 2011). The majority of ROS are produced by complexes I and III of the mitochondrial respiratory chain when electrons leak from redox centers and react with oxygen, forming superoxide (reviewed by Turrens, 2003). Rates of ROS production are influenced by rates of cellular respiration, the degree of mitochondrial coupling and the presence of polyunsaturated fatty acids (PUFAs). Studies of mitochondria isolated from ectotherms have shown that as temperature increases, oxygen consumption and the production of ROS increases (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004). This effect may be magnified *in vivo* in the presence of PUFAs, which propagate the formation of ROS *via* the lipid peroxidation (LPO) cycle (reviewed by Girotti, 1998; Crockett, 2008). Mitochondria from icefishes may be particularly vulnerable to oxidative damage at elevated temperatures because they are more lipid-rich compared with those from red-blooded fishes (O'Brien and Mueller, 2010). Levels of two of the major mitochondrial phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), are 1.3–1.4-times higher per mg of mitochondrial protein in mitochondria from the icefish *Chaenocephalus aceratus* compared with those from the red-blooded species *Notothenia coriiceps*. Potentially compounding the problem, oxidative muscles of icefishes have mitochondrial densities up to 2.3-times higher than red-blooded species (reviewed by O'Brien and Mueller, 2010). The high density of lipid-rich mitochondria in oxidative muscles of icefishes enhances oxygen delivery in the absence of Hb and Mb (reviewed by O'Brien, 2011). Although beneficial at the current cold temperature of the Southern Ocean, mitochondria and tissues rich in polyunsaturated phospholipids may be a liability as temperature increases because they promote the formation of ROS (Cosgrove et al., 1987). Moreover, previous studies suggest that icefishes have a lower capacity to detoxify ROS compared with red-blooded species (Cassini et al., 1993; Witas et al., 1984). The activity of superoxide dismutase (SOD) is 6-times lower and the activity of catalyse enzyme is 4-times lower in hearts of the icefish *Chionodraco hamatus* compared with the red-blooded species *Pagothenia bernacchii* (Cassini et al., 1993). Low levels of antioxidants coupled with high levels of PUFAs may make icefishes especially vulnerable to oxidative stress as the Southern Ocean warms. The WAP, where animals for this study were obtained, is one of the fastest warming regions on Earth (Schofield et al., 2010), with sea surface temperatures increasing more than 1°C since 1951 (Meredith and King, 2005). The top 100 m of continental shelf waters in the WAP vary annually by ~3°C whereas deeper waters, originating as circumpolar deep water (CDW), are more thermally stable and range between 1°C and 1.5°C (Barnes and Peck, 2008; Clarke et al., 2009).

We hypothesized that differences in mitochondrial structure and function between red- and white-blooded notothenioids might contribute to differences in thermal tolerance. To test this hypothesis, we measured proton leak, mitochondrial membrane composition, rates of ROS production, susceptibility of mitochondrial membranes to LPO, the activity of SOD and total antioxidant power (TAOP) in mitochondria isolated from hearts of red- and white-blooded notothenioids. Mitochondria from heart ventricles were used because the heart is highly aerobic and contains a high density of mitochondria. Additionally, we have developed techniques for isolating intact, well-coupled mitochondria from heart tissue (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). Most measurements were made at temperatures close to physiological temperature (1–2°C) and at the elevated temperature of 10°C to determine if mitochondrial susceptibility to oxidative stress induced by warming differs between red- and white-blooded notothenioid fishes.

## MATERIALS AND METHODS

### Tissue collection

*Chaenocephalus aceratus* (–Hb/–Mb) (Lönnberg), *Chionodraco rastrospinosus* (–Hb/+Mb) (Dewitt and Hureau), *Notothenia coriiceps* (+Hb/+Mb) (Richardson) and *Gobionotothen gibberifrons* (+Hb/+Mb) (Lönnberg) were captured in Dallmann Bay (64°S, 62°W) during the austral autumn of 2009 using an otter trawl deployed from the ARSV *Laurence M. Gould*. *Notothenia coriiceps* were also captured using baited traps. Fish were maintained in circulating seawater tanks onboard the *Laurence M. Gould* and then transferred to circulating seawater tanks at the U.S. Antarctic Research Station, Palmer Station, where they were held at 0±1°C. Animals were killed by a sharp blow to the head followed by transecting the spinal cord. Heart ventricles were quickly excised and frozen in liquid nitrogen unless experiments required fresh tissue. Frozen tissues were stored at –80°C. All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care Committee (134774-2).

### Mitochondrial isolation

Heart ventricles were excised as described above, placed in ice-cold Ringer solution (240 mmol $l^{-1}$ NaCl, 2.5 mmol $l^{-1}$ $MgCl_2$, 5.0 mmol $l^{-1}$ KCl, 2.5 mmol $l^{-1}$ $NaHCO_3$, 5.0 mmol $l^{-1}$ $NaH_2PO_4$, pH 8.0), and allowed to contract several times to clear blood from the ventricular lumen. Two ventricles from *C. rastrospinosus* or *N. coriiceps*, and 5–6 ventricles from *G. gibberifrons* were pooled for each mitochondrial preparation. Ventricles were homogenized in 8 volumes of ice-cold isolation buffer [0.1 mol $l^{-1}$ sucrose, 140 mmol $l^{-1}$ KCl, 10 mmol $l^{-1}$ EDTA, 5 mmol $l^{-1}$ $MgCl_2$, 20 mmol $l^{-1}$ Hepes, 0.5% fatty acid-free bovine serum albumin (BSA), pH 7.3 at 4°C] using

a Tekmar Tissuemizer (TeledyneTekmar, Cincinnati, OH, USA) set at low speed for 3 s. Homogenization was completed by hand using a 40 ml Tenbroeck ground glass homogenizer (Wheaton, Millville, NJ, USA). Mitochondria were separated by differential centrifugation as described previously by Urschel and O'Brien (Urschel and O'Brien, 2009). Mitochondrial pellets were resuspended in assay buffer (173 mmol $l^{-1}$ sucrose, 135 mmol $l^{-1}$ KCl, 5 mmol $l^{-1}$ $KH_2PO_4$, 20 mmol $l^{-1}$ Hepes, 0.5% fatty acid-free BSA, pH 7.3 at 4°C). BSA was omitted from all buffers for isolating mitochondria used to quantify rates of ROS production because BSA interferes with detecting resorufin. Despite the lack of BSA, the respiratory control ratio (RCR) was >5 in mitochondria, indicating well-coupled mitochondria. For analyzing mitochondrial membrane composition, measuring the susceptibility of mitochondrial membranes to peroxidation, and the activity of SOD and TAOP, heart ventricles were homogenized in 8 volumes of isolation buffer without sucrose, and BSA was omitted from wash buffer. Mitochondria were resuspended in 10 mmol $l^{-1}$ Tris buffer, pH 7.3 at 4°C, frozen in liquid nitrogen and stored at –80°C for these measurements. Protein concentration of all mitochondrial preparations was determined using the bicinchoninic acid (BCA) assay (Smith et al., 1985).

The RCR was measured at 2°C and 10°C to verify the quality of mitochondria prior to measuring proton leak, and only mitochondria with an RCR >5 were used. Mitochondria were resuspended in oxygenated assay buffer (173 mmol $l^{-1}$ sucrose, 135 mmol $l^{-1}$ KCl, 5 mmol $l^{-1}$ $KH_2PO_4$, 20 mmol $l^{-1}$ Hepes, 0.5% fatty acid-free BSA, pH 7.3 at each assay temperature) as described previously by Urschel and O'Brien (Urschel and O'Brien, 2009). Mitochondrial state III respiration rates were measured using a Strathkelvin oxygen electrode (Strathkelvin Instruments, North Lanarkshire, Scotland, UK) with 5 mmol $l^{-1}$ pyruvate, 1 mmol $l^{-1}$ malate and 0.6 mmol $l^{-1}$ ADP added as substrates. State III respiration rates were measured for 3–8 min following the addition of ADP, and oxygen consumption was measured for an additional 3 min following ADP depletion to determine state IV respiration rates.

### Proton leak

Proton leak was measured in isolated mitochondria at 2°C and 10°C. Rates of state II respiration were quantified using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK), and mitochondrial membrane potential was measured using a $TPMP^+$-sensitive electrode as described by Brand (Brand, 1995). Assay buffer (173 mmol $l^{-1}$ sucrose, 135 mmol $l^{-1}$ KCl, 5 mmol $l^{-1}$ $KH_2PO_4$, 20 mmol $l^{-1}$ Hepes, 0.5% fatty acid-free BSA, pH 7.3 at assay temperature) was oxygenated for 5 min with constant stirring. Mitochondria were then added, along with 5 µmol $l^{-1}$ rotenone, 1 µg µl$^{-1}$ oligomycin and 80 ng µl$^{-1}$ nigericin. A $TPMP^+$ standard curve was generated by adding five aliquots of 0.5 µmol $l^{-1}$ $TPMP^+$. State II respiration rates were measured for 3–12 min following the addition of 4 mmol $l^{-1}$ succinate. State II respiration was then gradually inhibited by adding increasing concentrations of malonate every 3 min, beginning with 0.2 mmol $l^{-1}$ and increasing to a final concentration of 3.2 mmol $l^{-1}$. Mitochondria were then uncoupled by adding 0.29 µmol $l^{-1}$ carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) to correct for drift of the $TPMP^+$-sensitive electrode. All measurements were made in duplicate in 4–5 mitochondrial preparations per species. Rates of state II respiration were plotted against mitochondrial membrane potential to obtain proton leak curves. Mitochondrial membrane potential was calculated from the response of the $TPMP^+$-sensitive electrode according to the Nernst equation.

Non-specific binding of $TPMP^+$ to the membrane was measured at 2°C and 10°C in two mitochondrial preparations from *C. aceratus*, *C. rastrospinosus* and *N. coriiceps* as described by Lotscher et al. (Lotscher et al., 1980). Non-specific binding of $TPMP^+$ to mitochondrial membranes of *G. gibberifrons* was assumed to be equivalent to that of *N. coriiceps*. Mitochondrial matrix volume was measured in *C. aceratus* and *N. coriiceps* at 1°C as described by Brand (Brand, 1995). Previous studies have shown that mitochondrial matrix volume does not change with temperature (Chamberlin, 2004). Measurements were made in duplicate in 4–6 mitochondrial preparations per species. Mitochondrial matrix volume of *C. rastrospinosus* was determined by plotting mitochondrial surface-to-volume ratio against mitochondrial matrix volume for *N. coriiceps* and *C. aceratus*, and using previous stereological measurements of mitochondrial surface-to-volume ratio for *C. rastrospinosus* (O'Brien and Sidell, 2000). Mitochondrial matrix volume of *G. gibberifrons* was assumed to be the same as the matrix volume of *N. coriiceps* because the mitochondrial surface-to-volume ratio is equivalent between these two species (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). Mitochondrial matrix volume and corrections factors for non-specific binding of $TPMP^+$ are shown in Table S1 (supplementary table).

### Rates of ROS production

Rates of ROS production were measured in mitochondria isolated from *C. aceratus* and *N. coriiceps* at 2°C and 10°C by monitoring the rate of formation of resorufin at 572 nm using a Perkin-Elmer Lambda 40 spectrophotometer (Perkin-Elmer Corp., Waltham, MA, USA) as described by Chen et al. (Chen et al., 2003). Mitochondria were incubated in assay buffer (173 mmol $l^{-1}$ sucrose, 135 mmol $l^{-1}$ KCl, 5 mmol $l^{-1}$ $KH_2PO_4$, 20 mmol $l^{-1}$ Hepes, pH 7.3 at assay temperature) containing 50 µmol $l^{-1}$ Amplex Red, 0.2 U $ml^{-1}$ horseradish peroxidase, 30 U $ml^{-1}$ SOD and 5 mmol $l^{-1}$ succinate for 60 min. 5 µmol $l^{-1}$ rotenone or 10 µmol $l^{-1}$ antimycin A were added to inhibit complex I or III, respectively. 1 mmol $l^{-1}$ malate and 2.5 mmol $l^{-1}$ pyruvate were used as substrates in place of succinate when the respiratory chain was inhibited with rotenone. Hydrogen peroxide was serially diluted (0 µmol $l^{-1}$ to 1 µmol $l^{-1}$) to create a standard curve for calculating rates of ROS production. All measurements were done in duplicate in 10 mitochondrial preparations per species.

### Susceptibility to LPO

The susceptibility of mitochondrial membranes to LPO was quantified using the fluorometric probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY 581/591) as described previously (Drummen et al., 2002; Grim et al., 2010). LPO was induced with hydroxyl radicals generated by the Fenton reaction between $Cu^{2+}$ (as copper sulfate) and cumene hydroperoxide (CumOOH). Membranes were diluted to 0.05 mg $ml^{-1}$ protein using 20 mmol $l^{-1}$ Chelex®-Tris (pH 7.4). A working BODIPY stock (1 mmol $l^{-1}$ in 100% ethanol) was diluted to 10 µmol $l^{-1}$ with 20 mmol $l^{-1}$ Chelex®-Tris (pH 7.4). This probe solution was further diluted to a final concentration of 148 nmol $l^{-1}$ with the 0.05 mg $ml^{-1}$ protein solution (final protein concentration of 0.05 mg $ml^{-1}$). The probe was dispersed within the membrane by stirring slowly in the dark at 4°C for 60 min. Subsequently, LPO was induced in duplicate cuvettes containing 2.5 ml of membrane/probe solution by adding 38 µl of 822 µmol $l^{-1}$ $CuSO_4$ and 82 µl of 20 mmol $l^{-1}$ Chelex®-Tris (pH 7.4), followed 5 min later by the addition of 38 µl of 3.3 mmol $l^{-1}$ CumOOH and 82 µl of 20 mmol $l^{-1}$ Chelex®-Tris (pH 7.4) (total volume of 2.74 ml and final

inductant concentrations of 11.5 µmol l$^{-1}$ and 46 µmol l$^{-1}$, respectively). The inductant load was titrated at 10°C until a small (~3.5–6 min), linear slope was observed, and subsequent increases in inductant load did not substantially increase linear rates of LPO. This titration step ensured that the same inducant challenge would be sufficient to produce a measurable rate of LPO when assayed at 1°C. Fluorescence decay was followed at both 10°C and 1°C at excitation/emission wavelengths of 568 nm/590 nm, using an LS-50B spectrofluorometer (Perkin-Elmer Corp.). Linear portions of the decay slope represented rates of LPO, and an extinction coefficient of 139,444 l mol$^{-1}$ cm$^{-1}$ was used in all calculations (Drummen et al., 2004). Rates of LPO were normalized to protein content and phospholipid content measured according to Rouser et al. (Rouser et al., 1970).

### Lipid extraction

Lipids were extracted and analyzed from mitochondria of *C. aceratus* and *N. coriiceps* as described by Yang et al. (Yang et al., 2009). Briefly, mitochondria were homogenized in 3 volumes (vol./wt of mitochondria) of chloroform/methanol (1:2). The extraction mixture was further diluted with 1 volume (vol./wt of mitochondria) of chloroform and 1 volume (vol./wt of mitochondria) of distilled water. The extraction mixture was centrifuged (10 min, 0.5 ***g***) to separate the chloroform and aqueous methanol, and then chloroform was carefully removed. 2 ml of chloroform/methanol (1:1) was added to the remaining aqueous phase and the chloroform separated and removed as described above. The chloroform was dried under nitrogen stream, resuspended in 4 ml of chloroform/methanol (1:1), re-extracted in 1.8 ml of 20 mmol l$^{-1}$ aqueous LiCl and dried as described above. The lipid extracts were resuspended in chloroform/methanol (1:1) at a final volume of 500 µl mg$^{-1}$ protein and further diluted with chloroform/methanol/isopropanol (1:2:4) to a final concentration of less than 50 pmol total phospholipid µl$^{-1}$ prior to mass spectroscopy analysis. Lipids were analyzed using a TSQ Quantum Ultra Plus triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA).

The equation used for calculating unsaturation index (UI) was modified from Hulbert et al. (Hulbert et al., 2007) by Grim et al. (Grim et al., 2010) to account for the presence of four acyl chains in cardiolipin, resulting in a maximum of 24 double bonds per molecule of cardiolipin (6 double bonds within each of its 4 acyl chains):

$$\mathrm{UI} = \sum_{n=24}^{n=0} n \times \text{mol\% of fatty acids containing } n \text{ double bonds.} \quad (1)$$

### SOD activity (EC 1.15.1.1)

The activity of SOD was measured in isolated mitochondria using a modified method (Crapo et al., 1978) of the xanthine oxidase (XO)/cytochrome *c* protocol originally described by McCord and Fridovich (McCord and Fridovich, 1969). This method is based on the ability of SOD to inhibit the reduction of cytochrome *c* by superoxide. The reaction mixture contained 50 mmol l$^{-1}$ potassium phosphate (pH 7.8), 0.1 mmol l$^{-1}$ EDTA, 0.05 mmol l$^{-1}$ xanthine, 0.01 mmol l$^{-1}$ acetylated cytochrome *c* (equine heart), 0.01 mmol l$^{-1}$ KCN and ~0.07 U XO. The final concentration of XO was determined empirically so that the reduction of cytochrome *c*, detected at 550 nm, occurred at a rate of 0.02 O.D. min$^{-1}$. All assays were performed in duplicate at 5.0±0.5°C using a Perkin-Elmer Lambda 40 spectrophotometer (Perkin-Elmer Corp.). Temperature was regulated using a refrigerated, circulating water bath connected to the spectrophotometer. One unit of activity is defined as the amount of SOD needed to inhibit the reduction of cytochrome *c* by 50%.

### TAOP

TAOP was measured in isolated mitochondria using a TAOP kit (Kit #TA02, Oxford Bioresearch, Manchester Hills, MI, USA). This kit uses a colorimetric endpoint assay that determines the reduction of copper coupled to bathocuprione. Samples were diluted 80-fold, which included an initial 2-fold dilution in PBS followed by a 40-fold dilution in the buffer provided by the manufacturer, as per the manufacturer's instructions. Standard curves using uric acid (0–2 mmol l$^{-1}$) were measured with each set of samples. Both standards and samples were run in duplicate. TAOP is expressed in uric acid equivalents per mg of protein or per µmol of phospholipid.

### Statistical analyses

Significant differences in the rates of state II respiration measured at a common membrane potential, rates of ROS production within a species at different temperatures and between species at a common temperature, mean LPO susceptibility (normalized to protein content and phospholipid content), metrics of phospholipid content (abundance of individual phospholipid species and phospholipid classes), membrane unsaturation, activity of SOD and TAOP were compared using a Student's *t*-test and the software JMP (JMP5 or JMP7; SAS, Cary, NC, USA). Data were log transformed as necessary to maintain assumptions of normality. Data not meeting assumptions of normality or homogeneity of variance were compared with Wilcoxon rank sums or Welch ANOVA (JMP5), respectively. Significance was set at $P<0.05$. Unless otherwise noted, data are presented as means ± standard error of the mean (s.e.m.).

## RESULTS

### Proton leak

Proton leak was greater in mitochondria isolated from red-blooded notothenioids compared with icefishes at both 2°C and 10°C (Fig. 1). To compare proton leak among species or treatments, state II respiration rates are compared at the highest common membrane potential (e.g. Brookes et al., 1998; Jastroch et al., 2007). State II respiration rates were 16.1-times higher in mitochondria from *N. coriiceps* and 17.2-times higher in mitochondria from *G. gibberifrons* compared with *C. aceratus* at a common membrane potential of 190 mV at 2°C (Table 1). State II respiration rates were 4.4-times higher in mitochondria from *C. rastrospinosus* compared with *C. aceratus* at a common membrane potential of 190 mV at 2°C (Table 1). State II respiration rates were not significantly different between mitochondria of *N. coriiceps* and *G. gibberifrons* at 190 mV and 2°C ($P>0.05$, Table 1).

Proton leak was higher in mitochondria of all four species at 10°C compared with 2°C, but remained lower in icefishes compared with red-blooded notothenioids (Fig. 1A,B). Proton leak was lowest in mitochondria of *C. aceratus* at a common membrane potential of 147 mV at 10°C compared with all other species (Fig. 1B, Table 1). State II respiration rates were 3.7-times higher in mitochondria of *C. rastrospinosus*, 9.2-times higher in *N. coriiceps* and 12.2-times higher in *G. gibberifrons* compared with *C. aceratus*, but were not significantly different between mitochondria of *N. coriiceps* and *G. gibberifrons* at 147 mV and 10°C ($P>0.05$, Table 1). Together, these findings indicate that proton leak is lowest in mitochondria of *C. aceratus*, intermediate in *C. rastrospinosus* and highest in *N. coriiceps* and *G. gibberifrons* at 10°C (Fig. 1B).

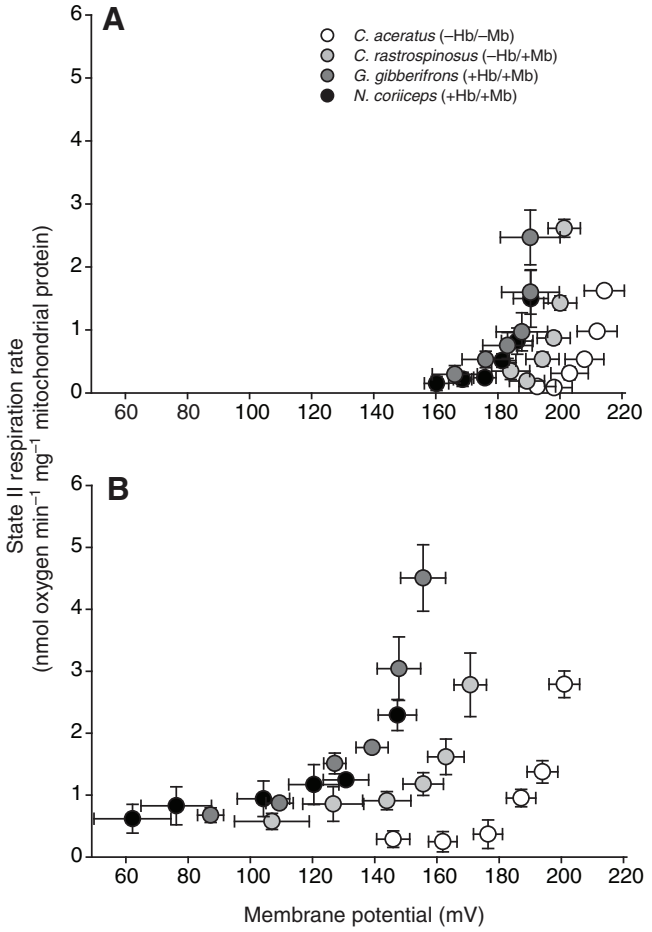


Fig. 1. Proton leak in mitochondria isolated from heart ventricle of *Chaenocephalus aceratus*, *Chionodraco rastrospinosus*, *Notothenia coriiceps* and *Gobionotothen gibberifrons* at 2°C (A) and 10°C (B). State II respiration rates were quantified with succinate as the substrate and plotted against membrane potential obtained by titration with the complex II inhibitor, malonate. *N*=4–5.

### Rates of ROS production

Rates of ROS production were not significantly different between mitochondria from *C. aceratus* and *N. coriiceps* at 2°C or 10°C ($P$>0.05, Fig. 2). In both species, rates of ROS production were significantly higher at 10°C compared with 2°C ($P$<0.05, Fig. 2). However, rates of ROS production were significantly higher in mitochondria of *C. aceratus* at both temperatures compared with *N. coriiceps* when the respiratory chain was interrupted with either antimycin A or rotenone ($P$<0.05, Fig. 2). Similar to rates of ROS production in untreated mitochondria, rates of ROS production were significantly higher in mitochondria of both species treated with antimycin A or rotenone at 10°C compared with 2°C ($P$<0.05, Fig. 2).

### Susceptibility to LPO

When rates of LPO were measured at a temperature that approaches physiological temperature for these animals (1°C), significant LPO was observed in mitochondrial membranes prepared from *C. aceratus* whereas LPO was undetectable in membranes from *N. coriiceps* (Fig. 3A,B). Rates of LPO were significantly higher in mitochondria from *C. aceratus* at 10°C compared with 1°C ($P$<0.05, Fig. 3A,B) and higher in *N. coriiceps* at 10°C compared with 1°C, but our inability to detect LPO at 1°C in *N. coriiceps* precluded statistical analysis. Rates of LPO, measured at 10°C, were similar between *C. aceratus* and *N. coriiceps* when normalized to either protein ($P$>0.05, Fig. 3A) or phospholipid content ($P$>0.05, Fig. 3B).

### Lipid composition

We sought to determine if differences in the composition of mitochondrial membranes might influence susceptibility to LPO. Mitochondrial membranes from *C. aceratus* had a 1.6-times higher content of phospholipid compared with *N. coriiceps* as indicated by increased levels of hydrolysable phosphate ($P$<0.01, Table S2 in supplementary material). Twenty-two phospholipid species were in abundances greater than 1 mol% for at least one of the study species, and the contents of nearly half (45%) of these phospholipids varied between species ($P$<0.05, Table S2 in supplementary material). Four phospholipids were particularly abundant (>5 mol%), and all were highly unsaturated containing at least 5 double bonds (Table S2 in supplementary material). One was identified as major phosphatidylcholine (MPC) D16:0-20:5 or D16:1-20:4, one as lysophosphosphatidylcholine (LPC) 22:6, and two as PEs D18:1-22:6 or D18:2-22:5 and D18:0-22:6 or D18:1-22:5. All but LPC 22:6 were significantly elevated in mitochondrial membranes from *C. aceratus*. Of the four phospholipid classes quantified [cardiolipin (CL), MPC, LPC and PE], two classes were significantly different between the mitochondrial membranes of *C. aceratus* and *N. coriiceps*. Total content of MPC was 1.2-times higher in *C. aceratus* compared with *N. coriiceps* ($P$<0.05, Table 2). In contrast, LPC were 1.6-fold more abundant in *N. coriiceps*, relative to *C. aceratus* ($P$<0.05, Table 2). Additionally, while the UI of all LPC was 1.8-times higher in membranes from *N. coriiceps*, relative to *C. aceratus* ($P$<0.05, Table 3), total membrane UI was similar between the species ($P$>0.05, Table 3).

### Antioxidant levels

Maximal activities of SOD and TAOP per mg of mitochondrial protein were equivalent between mitochondria isolated from heart ventricles of the icefish *C. aceratus* compared with the red-blooded species *N. coriiceps* ($P$>0.05, Fig. 4A,B). In contrast, when TAOP

Table 1. State II oxygen consumption rates of mitochondria isolated from hearts of red- and white-blooded notothenioid fishes

| | At 2°C and 190 mV | At 10°C and 147 mV |
|---|---|---|
| *Chaenocephalus aceratus* | 0.093* | 0.249* |
| *Chionodraco rastrospinosus* | 0.409* | 0.910* |
| *Gobionotothen gibberifrons* | 1.598±0.348 | 3.042±0.513 |
| *Notothenia coriiceps* | 1.499±0.456 | 2.293±0.251 |

Oxygen consumption is given as nmol oxygen min$^{-1}$ mg$^{-1}$ protein.
*Oxygen consumption determined by interpolation.
Values are expressed as means ± s.e.m. unless determined by interpolation.
*N*=4–5. *P*>0.05.

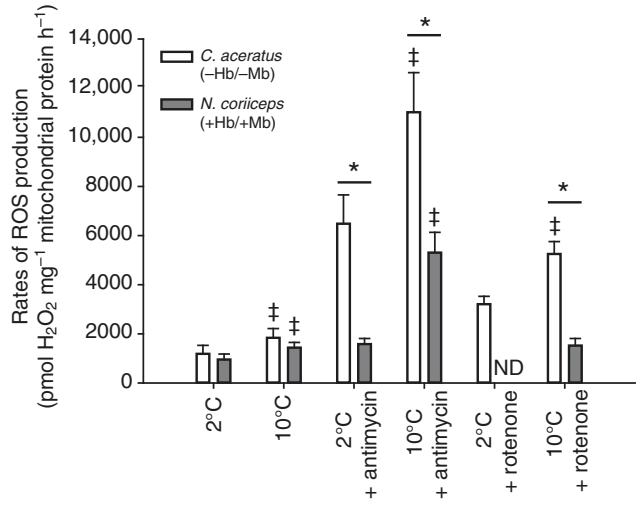


Fig. 2. Rates of ROS production in mitochondria isolated from heart ventricle of *Chaenocephalus aceratus* and *Notothenia coriiceps* at 2°C and 10°C. Rates of reactive oxygen species (ROS) production were quantified by monitoring the formation of resorufin in the presence and absence of the respiratory chain inhibitors antimycin A and rotenone. ND=not detected. *N*=10. Significant differences between species at common temperatures and treatments are indicated by asterisks. Significant differences within a species at different temperatures and treatments are indicated by double daggers. $P<0.05$.

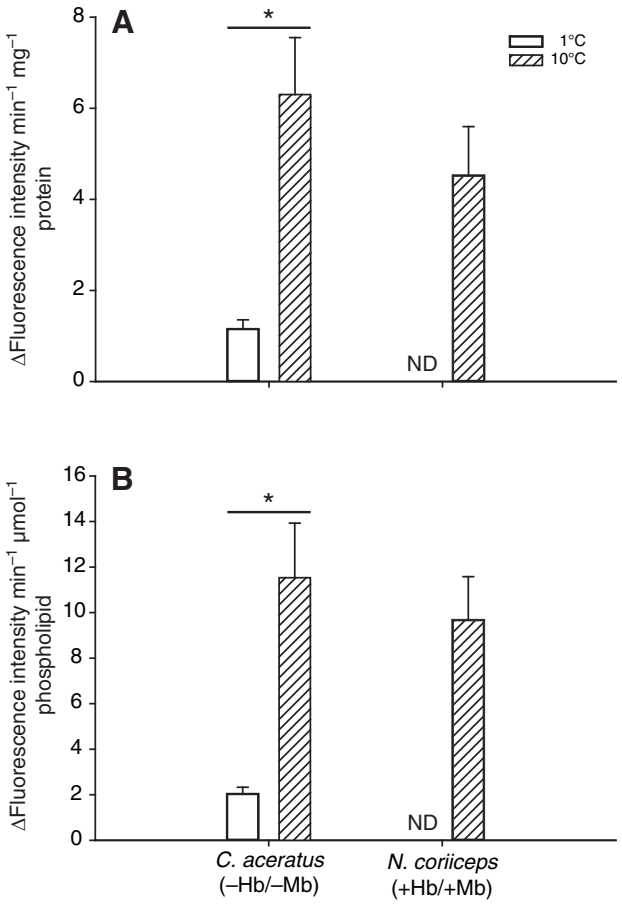


Fig. 3. Rates of lipid peroxidation (LPO) in mitochondrial membranes of *Chaenocephalus aceratus* and *Notothenia coriiceps* at 1°C and 10°C, normalized to protein content (A) and phospholipid content of mitochondrial membranes (B). LPO was induced in membrane preparations using hydroxyl radicals produced by the Fenton reaction between $Cu^{2+}$ and cumene hydroperoxide. ND=not detected. *N*=6. Significant differences within a species at different temperatures are indicated by asterisks. $P<0.05$.

was normalized to phospholipid content, levels were 1.4-times higher in mitochondria from *N. coriiceps* compared with *C. aceratus* ($P<0.05$, Fig. 4B).

## DISCUSSION

This is the first study to examine how differences in mitochondrial architecture between red- and white-blooded notothenioids impact mitochondrial function and susceptibility to oxidative stress. Mitochondria from icefishes are more tightly coupled compared with those from red-blooded species. Although potentially beneficial under normal, physiological conditions, a high degree of coupling can promote the formation of ROS when the electron transport chain is disrupted. Additionally, mitochondria from icefishes are rich in phospholipids, and our results suggest that the capacity to protect against LPO may be lower in icefishes compared with red-blooded species.

### Mitochondria from icefishes are tightly coupled

Previous studies have suggested that high mitochondrial densities in oxidative muscle of polar fishes, rich in PUFAs (Johnston et al., 1998), may be energetically costly (Guderley, 2004) because proton leak positively scales with UI. Our data suggest otherwise. The UI of mitochondrial membrane phospholipids is 1.9–2.0-times higher in Antarctic notothenioid fishes compared with mitochondria isolated from oxidative muscle of rainbow trout (*Oncorhynchus mykiss*) acclimated to 5°C (Kraffe et al., 2007), yet mitochondria from all four Antarctic teleosts measured here were more tightly coupled compared with those from temperate teleosts. State II respiration rates were ~61-times higher in mitochondria isolated from liver of common carp (*Cyprinus carpio*) warm-acclimated to 20°C, and 40-times higher in carp cold-acclimated to 8°C compared with *N. coriiceps* at 2°C when compared at a common membrane potential of 162 mV or 169 mV, respectively (Jastroch et al., 2007). Similarly, state II respiration rates were 50-times higher in mitochondria isolated from liver of cold-acclimated rainbow trout (*O. mykiss*) maintained at 4–8°C compared with *N. coriiceps* at 2°C when compared at the common membrane potential of 166 mV (Brookes et al., 1998). These differences may be overestimated because proton leak was measured at 25°C in mitochondria isolated from carp and at 20°C in rainbow trout, which in both cases was higher than the acclimation temperature of the fish (Brookes et al., 1998; Jastroch et al., 2007). However, in studies where proton leak was measured at the acclimation temperature of the animal, proton leak was still higher in the temperate species compared with Antarctic fishes. Rates of state II respiration were 10-times higher in mitochondria isolated from hearts of rainbow trout, and 11-times higher in mitochondria from oxidative muscle of rainbow trout at 15°C compared with *N. coriiceps* at 2°C when compared at a common membrane potential of 185 mV (Leary et al., 2003).

The greater proton leak in mitochondria from red-blooded notothenioids compared with icefishes may be due to their higher density of inner mitochondrial membranes per mitochondrion (Archer and Johnston, 1991; O'Brien and Sidell, 2000). Porter and colleagues determined that 69% of the difference in proton leak among mammals can be accounted for by differences in the density

Table 2. Relative abundance of selected phospholipid classes in mitochondrial membranes of red- and white-blooded notothenioid fishes

| | *Chaenocephaus aceratus* (–Hb/–Mb) | *Notothenia coriiceps* (+Hb/+Mb) |
|---|---|---|
| CL | 5.31±0.30 | 6.37±0.55 |
| MPC | 34.77±1.00 | 30.14±1.42* |
| LPC | 11.59±1.76 | 18.82±1.93* |
| PE | 48.33±1.74 | 44.67±1.59 |
| Total | 100% | 100% |

Values are expressed as means ± s.e.m. $N$=6. Significant differences in abundance of individual phospholipids between species are indicated with asterisks. $P$<0.05. CL, cardiolipin; MPC, major phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine.

of inner mitochondrial membranes (Porter et al., 1996). The surface density of inner mitochondrial membranes per volume of mitochondria is 1.4–1.5-times higher in heart ventricles of red-blooded notothenioids compared with icefishes (Archer and Johnston, 1991; O'Brien and Sidell, 2000), providing a greater surface area across which protons can leak. Studies have shown that plotting state II respiration rates per mg of cytochrome *c* oxidase (CCO) creates different leak curves compared with those obtained by plotting state II respiration rates per mg of mitochondrial protein because the ratio between the respiratory components and other mitochondrial proteins may vary among species or tissue types (Leary et al., 2003). Although we did not measure the maximal activity of CCO in isolated mitochondria, state III respiration rates and the maximal activity of citrate synthase (CS) per mg of mitochondrial protein are equivalent between red- and white-blooded notothenioids (data not shown), suggesting that CCO activity is likely similar as well. Thus, the differences we observed in proton leak between red- and white-blooded notothenioids would likely also be apparent when plotted as state II respiration rates per mg of CCO.

The contribution of lipid composition to proton leak in Antarctic fishes is unclear. Omega-6 fatty acids 20:3(*n*-6) and 20:4(*n*-6), and omega-3 fatty acid 22:6(*n*-3) promote leak (Brookes et al., 1998) whereas myristic acid (14:0), palmitoleate acid [16:1(*n*-7)], oleic acid [18:1(*n*-9)], the omega-6 fatty acid 18:3(*n*-6) and the omega-3-fatty acid 18:3(*n*-3) inhibit leak (Brookes et al., 1998). Levels of the proton leak-stimulating 22:6 LPC is 2.2-times higher in *N. coriiceps* compared with *C. aceratus*, which might contribute to the greater proton leak in *N. coriiceps*. However, there are no clear differences in other proton leak-stimulating or proton leak-inhibiting phospholipids, or UI between the two species. One limitation of our study is that lipid composition was quantified in a mixture of inner and outer mitochondrial membranes. Differences in the composition of inner mitochondrial membranes among icefishes and red-blooded notothenioids might provide more insight to the extent which membrane composition mediates proton leak.

Proton leak increased in all species in response to an increase in temperature. Temperature coefficient ($Q_{10}$) values for protein- or enzyme-mediated reactions are typically between two and three (Hochachka and Somero, 2002). The high $Q_{10}$ for proton leak (>20) suggests that the increase in leak in response to temperature is not mediated by changes in activity of either UCPs or ANT (reviewed by Echtay, 2007), but rather by lipids. Moreover, proton leak was measured at 2°C and 10°C in mitochondria isolated from animals held at the same temperature (0±1°C), excluding the possibility that the temperature-induced increase in proton leak could be mediated by changes in the expression of UCPs or ANT.

### Mitochondria from icefishes have a greater potential to produce high levels of ROS than those from red-blooded notothenioids

The majority of ROS are produced by the mitochondrial respiratory chain where ~0.15% of oxygen consumed is converted to ROS (St-Pierre et al., 2002). Rates of ROS production were similar between mitochondria isolated from heart ventricles of *C. aceratus* and *N. coriiceps* at both 2°C and 10°C. Similar rates of ROS production are consistent with their similar rates of state III respiration at both 2°C and 10°C (data not shown). Rates of ROS production increased 1.5–1.6-times in response to an increase in temperature from 2°C to 10°C, which is similar to values obtained in mitochondria isolated from other ectotherms. For example, ROS production increased 2.5-times under state III conditions and 2.1-times under state IV+ conditions (state IV in the presence of oligomycin) in mitochondria isolated from the gill of the Antarctic bivalve *Laternula elliptica* in response to an 8°C increase in temperature, from 1°C to 9°C (Heise et al., 2003). Similarly, ROS production increased 1.8-times under state III conditions and 1.9-times under state IV+ conditions in mitochondria isolated from mantle tissue of mud clams (*Mya arenaria*) in response to a 10°C increase in temperature, from 5°C to 15°C (Abele et al., 2002).

Disruption of electron transfer at complex I with rotenone or complex III with antimycin A resulted in a significant increase in rates of ROS production in both species. Similar to mammalian cardiac mitochondria (Turrens and Boveris, 1980), more ROS is produced by complex III than complex I in Antarctic fishes. Rates of ROS production increased up to 6.0-times upon the addition of

Table 3. Unsaturation indices of select phospholipid classes in mitochondrial membranes of red- and white-blooded notothenioid fishes

| | *Chaenocephaus aceratus* (–Hb/–Mb) | *Notothenia coriiceps* (+Hb/+Mb) |
|---|---|---|
| CL | 71.70±4.01 | 78.29±7.17 |
| MPC | 188.95±6.44 | 172.05±8.52 |
| LPC | 55.82±8.39 | 97.87±10.50* |
| PE | 299.19±10.00 | 271.69±9.57 |
| Total | 615.65±3.84 | 619.90±2.88 |

Unsaturation index (UI) calculated following the modifications of Hulbert et al. (2007) by Grim et al. (2010). Values are expressed as means ± s.e.m. $N$=6. Significant differences in abundance of individual phospholipids between species are indicated with asterisks. $P$<0.05. CL, cardiolipin; MPC, major phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine.

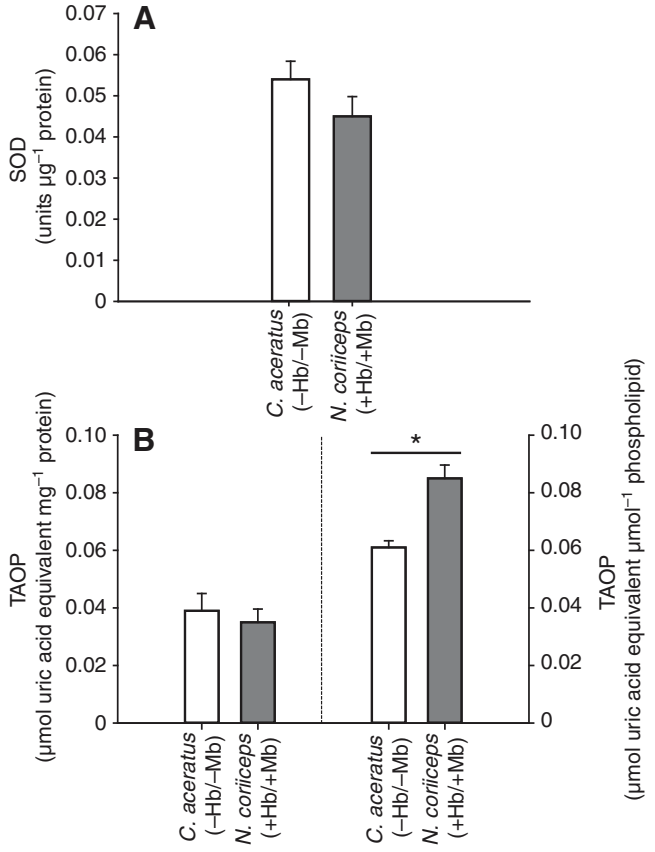


Fig. 4. Antioxidant capacity of isolated mitochondria from red- and white-blooded notothenioid fishes. (A) Activity of superoxide dismutase (SOD) was measured at 5°C in mitochondria isolated from heart ventricles of *Chaenocephalus aceratus* and *Notothenia coriiceps*. *N*=6. (B) Total antioxidant power (TAOP) of mitochondria from *C. aceratus* and *N. coriiceps* normalized per mg of mitochondrial protein or phospholipid content of mitochondrial membranes. *N*=6. Significant differences between species are indicated by asterisks. $P<0.05$.

antimycin A, but only up to 2.9-times following the addition of rotenone. There was a much greater increase in the production of ROS in mitochondria from *C. aceratus* compared with *N. coriiceps* when electron transfer was disrupted with either rotenone or antimycin A. This difference is most likely attributable to the higher mitochondrial membrane potential of *C. aceratus* compared with *N. coriiceps* because the formation of ROS increases exponentially as membrane potential exceeds 140 mV (Kadenbach et al., 2010).

As temperature increases, rates of ROS production increase because cellular respiration increases, but ROS formation will also increase *in vivo* if tissues become hypoxic (Guzy and Schumacker, 2006). Studies in both yeast and mammals have shown that similar to the effect of antimycin A, hypoxia increases the life span of ubisemiquinone (QH), which is formed during electron transfer between ubiquinol and complex III, and which leaks electrons to oxygen (Guzy and Schumacker, 2006; Guzy et al., 2007). There is evidence that the heart ventricle of fishes is likely one of the first tissues to become hypoxic as temperature increases (Farrell, 2002). Like many teleosts, Antarctic fishes have a serial circulation, in which the heart ventricle is oxygenated by venous blood entering the ventricular lumen (Davie and Farrell, 1991; Zummo et al., 1995). Blood oxygen content will likely decline as temperature and metabolic rate increases, resulting in inadequate oxygen delivery to the heart. Icefishes may be especially vulnerable to cardiac hypoxia because of the low oxygen-carrying capacity of their blood. Moreover, if hearts become hypoxic, the production of ROS will increase to a greater extent in icefishes because of their higher mitochondrial membrane potential and higher density of mitochondrial membranes rich in PUFAs, which promote ROS formation *via* the LPO cycle (reviewed in Girotti, 1998). However, the degree to which the formation of ROS is propagated by lipid radicals depends on the susceptibility of the membrane to LPO and levels of antioxidants.

### Susceptibility of mitochondria to LPO and antioxidant capacity differs between icefishes and red-blooded notothenioids

When measured at a physiologically relevant temperature (1°C), mitochondrial membranes from heart ventricles of *C. aceratus* appeared to be more susceptible to LPO than those from *N. coriiceps*. Susceptibility to LPO is related to phospholipid content and composition; unsaturated fatty acids are more likely to become oxidized than saturated ones (Cosgrove et al., 1987), and phospholipids with ethanolamine head groups are more likely to undergo oxidation than other phospholipid classes (Wang et al., 1994). Mitochondrial membrane UI is similar between *C. aceratus* and *N. coriiceps*, and PE/PC values are somewhat lower in *C. aceratus* (1.39) than in *N. coriiceps* (1.48). Based on these lipid characteristics alone, we would predict rates of LPO to be somewhat higher in the mitochondrial membranes of *N. coriiceps* compared with those from *C. aceratus*. However, regardless of the normalization criterion employed, significant LPO was detected in mitochondrial membranes from *C. aceratus* but not in membranes from *N. coriiceps* at 1°C. These data are in agreement with previous studies (Grim et al., 2010) that showed phospholipid composition alone is not an accurate predictor of LPO susceptibility. Other properties of membranes, such as antioxidant defenses, must also be considered.

Among low molecular weight antioxidants, liposoluble vitamin E is thought to be the first line of defense against LPO (Halliwell and Gutteridge, 2007). Dunlap et al. reported that the red-blooded species *G. gibberifrons* had on average 7.4-times higher total vitamin E levels [including α-tocopherol and marine-derived tocopherol (MDT)], than *C. aceratus*, while percent MDT was generally elevated in tissues of icefishes (Dunlap et al., 2002). While MDT has a greater efficiency than α-tocopherol for inhibiting LPO at low temperature (Yamamoto et al., 2001), the relatively low abundance of this vitamin E constituent (maximum 14%) in *C. aceratus* (Dunlap et al., 2002) makes it likely that the vitamin E content of *G. gibberifrons* and perhaps in *N. coriiceps* as well, imparts a greater level of defense against LPO, despite the lower MDT content.

The activity of SOD was similar per mg of mitochondrial protein in mitochondria isolated from heart ventricles of *C. aceratus* and *N. coriiceps*. Similarly, total antioxidant potential per mg of mitochondrial protein was equivalent in *C. aceratus* compared with *N. coriiceps*. Nevertheless, TAOP, which includes lipid-soluble antioxidants such as vitamin E, normalized per unit of phospholipid was significantly lower in mitochondria from *C. aceratus* compared with *N. coriiceps*. We did not quantify phospholipid content in mitochondria used for measuring SOD activity, but using mean values obtained for mitochondria prepared for measuring TAOP reveals that SOD activity per unit of phospholipid was 1.5-times lower in mitochondria from *C. aceratus* compared with *N. coriiceps*. Lower levels of TAOP and SOD per unit of phospholipid may

explain the greater susceptibility of mitochondrial membranes from *C. aceratus* to peroxidation compared with *N. coriiceps*. *In vivo*, the activity of CCO and CS per g of ventricular mass is equivalent between red-blooded notothenioids and icefishes (O'Brien and Sidell, 2000), suggesting rates of production of ROS are similar per g of tissue. However, mitochondrial densities and thus phospholipid densities are 2-fold higher per g of ventricular tissue of *C. aceratus* compared with *N. coriiceps* (Urschel and O'Brien, 2008), and the activity of both SOD and catalase per g of tissue is 4–6-times lower in icefishes compared with red-blooded species (Cassini et al., 1993). Taken together, these data suggest mitochondrial membranes, as well as the mitochondrial-rich heart ventricles of icefishes may be at a greater risk for oxidative damage as temperature and ROS production increases. Current studies are aimed at determining whether oxidative stress is greater in hearts of icefishes compared with red-blooded species exposed to elevated temperatures.

## Conclusions

Current climate trends suggest a warmer future for fishes inhabiting the Southern Ocean and particularly the WAP region (Clarke et al., 2007). Elevations in temperature will be challenging for Antarctic notothenioids, which seem to have a reduced capacity to adjust to fluctuations in temperature compared with temperate and more Northern fish species (Jin and DeVries, 2006; Buckley and Somero, 2009). Oxidative stress is one challenge imposed by elevated temperatures (Parihar and Dubey, 1995; Heise et al., 2006; Lushchak and Bagnyukova, 2006a; Lushchak and Bagnyukova, 2006b). Icefishes may be more susceptible to oxidative damage because of their greater capacity to produce ROS under some conditions, which may be propagated by high densities of polyunsaturated phospholipids within cardiac muscle. We do not know if small, incremental increases in temperature, as would be experienced during global warming might induce antioxidant defenses, or if similar to the heat-shock response (Hofmann et al., 2000), Antarctic fishes have lost the ability to elevate antioxidant defenses and combat oxidative stress. Future studies will address this question.

## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| ANT | adenosine nucleotide translocase |
| BCA | bicinchoninic acid |
| BSA | bovine serum albumin |
| CII-BODIPY 581/591 | 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid |
| CCO | cytochrome *c* oxidase |
| CL | cardiolipin |
| CS | citrate synthase |
| CumOOH | cumene hydroperoxide |
| FCCP | carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone |
| Hb | hemoglobin |
| LPC | lysophosphatidylcholine |
| LPO | lipid peroxidation |
| Mb | myoglobin |
| MDT | marine-derived tocophenol |
| MPC | major phosphatidylcholine |
| PBS | phosphate buffered saline |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PUFAs | polyunsaturated fatty acids |
| QH | ubisemiquinone |
| $Q_{10}$ | temperature coefficient |
| RCR | respiratory control ratio |
| ROS | reactive oxygen species |
| SOD | superoxide dismutase |
| TAOP | total antioxidant power |
| $TPMP^+$ | methyltriphenylphosphonium |
| UCP | uncoupling protein |
| UI | unsaturation index |
| XO | xanthine oxidase |

## ACKNOWLEDGEMENTS

First and foremost, we would like to acknowledge the numerous contributions of Dr Bruce D. Sidell to the field of comparative physiology and biochemistry of Antarctic fishes. His passion for the Antarctic inspired us all. Thanks to the Crew and Masters of the ARSV *Laurence M. Gould* and the Raytheon support staff at Palmer Station, Antarctica. Without their skillful assistance, this work would not have been possible. Thanks to Julie Buckingham and Dr Martin Brand for teaching I.A.M. how to quantify proton leak and for kindly providing the $TPMP^+$-sensitive electrodes. We also thank Dr Xianlin Han at Washington University School of Medicine for analyzing the phospholipid composition of mitochondrial membranes.

## FUNDING

This work was supported by grants from the National Science Foundation [ANT 0741301 to K.M.O. and E.L.C.; work by J.M.B was supported by ANT-0739637 to Bruce D. Sidell].

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Table S1. Mitochondrial matrix volume and correction factors for non-specific $TPMP^+$ binding

| | *Chaenocephaus aceratus* | *Chionodraco rastrospinosus* | *Gobionotothen gibberifrons* | *Notothenia coriiceps* |
|---|---|---|---|---|
| Matrix volume (μl $mg^{-1}$ protein) | 1.52 | 0.91* | 0.51** | 0.51 |
| $TPMP^+$ binding (per mg protein) 2°C | 0.896±0.046 | 0.920±0.044 | 0.991** | 0.991±0.005 |
| $TPMP^+$ binding (per mg protein) 10°C | 0.974±0.021 | 0.986±0.001 | 0.964** | 0.964±0.014 |

*Based on stereological measurements (O'Brien and Sidell, 2000).

**Assumed to be similar to *N. coriiceps* based on sterological measurements (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008).

Non-specific $TPMP^+$ binding is expressed as means ± s.e.m. $N$=2–6.

Table S2. Abundance of individual phospholipid species in mitochondrial membranes of *Chaenocephaus aceratus* and *Notothenia coriiceps*

| | Possible species | *C. aceratus* | *N. coriiceps* |
|---|---|---|---|
| CL | | | |
| | 18:2-18:0-22:6-22:6<br>22:6-22:6-18:1-18:1 22:6-20:4-20:3-18:1 | 1.12±0.08 | 0.67±0.10* |
| | 20:4-20:4-22:6-22:5<br>18:2-22:6-22:6-22:5<br>22:6-22:6-22:6-18:1<br>22:6-22:6-20:4-20:3 | 0.85±0.07 | 0.99±0.07 |
| MPC | | | |
| | D16:0-18:1<br>D16:1-18:0 | 3.66±0.09 | 1.79±0.10* |
| | D16:1-20:5 | 2.37±0.08 | 1.75±0.26 |
| | D16:0-20:5<br>D16:1-20:4 | 6.17±0.42 | 3.83±0.35* |
| | D16:1-22:6<br>D18:2-20:5 | 2.24±0.22 | 1.85±0.22 |
| | D16:0-22:6<br>D18:1-20:5<br>D18:2-20:4 | 16.76±1.12 | 17.81±0.90 |
| | D18:1-22:6 | 2.53±0.26 | 2.18±0.20 |
| | D18:0-22:6<br>D18:1-22:5 | 1.04±0.06 | 0.93±0.05 |
| LPC | | | |
| | 20:5 | 2.63±0.44 | 2.11±0.27 |
| | 22:6 | 6.11±0.91 | 13.33±1.56* |
| PE | | | |
| | D16:0-20:5<br>D16:1-20:4 | 1.48±0.15 | 1.79±0.20 |
| | P16:0-22:6<br>P18:1-20:5<br>A18:2-20:5<br>P18:2-20:4<br>P18:1-20:4<br>P18:0-20:5<br>A18:1-20:5<br>A16:0-22:6<br>P16:0-22:5<br>P18:2-20:3<br>A16:1-22:5<br>A18:2-20:4 | 1.36±0.09 | 1.84±0.10* |
| | D16:1-22:6<br>D18:2-20:5 | 1.20±0.06 | 1.57±0.06* |
| | D16:0-22:6<br>D18:1-20:5<br>D18:2-20:4 | 13.90±1.00 | 13.72±1.05 |
| | D18:1-20:4<br>D18:0-20:5<br>D16:0-22:5 | 1.46±0.07 | 1.88±0.13* |
| | D18:0-20:4<br>D18:1-20:3<br>D16:0-22:4 | 0.80±0.06 | 1.01±0.07 |
| | P18:1-22:6<br>A18:2-22:6<br>P18:2-22:5 | 2.07±0.08 | 1.41±0.04* |

| | | |
|---|---|---|
| P18:0-22:6<br>P18:1-22:5<br>A18:1-22:6<br>A18:2-22:5 | 1.32±0.06 | 1.41±0.11 |
| D18:1-22:6<br>D18:2-22:5 | 12.57±0.50 | 8.95±0.67* |
| D18:0-22:6<br>D18:1-22:5 | 6.86±0.40 | 5.39±0.48* |
| Total phosphate (mmol $l^{-1}$) | 18.07±1.61 | 11.38±0.89* |

Values are expressed as means ± s.e.m. *N*=6. Only phospholipids present in amounts 1 mol% for at least one of the species are shown. When all phospholipid species are included, the total abundance equals 100 mol%. CL, cardiolipin; MPC, major phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine. D, phosphatidyl (ester linked); A, plasmanyl (alkyl ether linked); and P, plasmenyl (vinyl ether linked). Significant differences in abundance of individual phospholipids between species are indicated with asterisks. $P<0.05$.