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## RESEARCH ARTICLE

# Exposure to critical thermal maxima increases oxidative stress in hearts of white- but not red-blooded Antarctic notothenioid fishes

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## SUMMARY

**Antarctic icefishes have a significantly lower critical thermal maximum ($CT_{max}$) compared with most red-blooded notothenioid fishes. We hypothesized that the lower thermal tolerance of icefishes compared with red-blooded notothenioids may stem from a greater vulnerability to oxidative stress as temperature increases. Oxidative muscles of icefishes have high volume densities of mitochondria, rich in polyunsaturated fatty acids, which can promote the production of reactive oxygen species (ROS). Moreover, icefishes have lower levels of antioxidants compared with red-blooded species. To test our hypothesis, we measured levels of oxidized proteins and lipids, and transcript levels and maximal activities of antioxidants in heart ventricle and oxidative pectoral adductor muscle of icefishes and red-blooded notothenioids held at 0°C and exposed to their $CT_{max}$. Levels of oxidized proteins and lipids increased in heart ventricle of some icefishes but not in red-blooded species in response to warming, and not in pectoral adductor muscle of any species. Thus, increases in oxidative damage in heart ventricles may contribute to the reduced thermal tolerance of icefishes. Despite an increase in oxidative damage in hearts of icefishes, neither transcript levels nor activities of antioxidants increased, nor did they increase in any tissue of any species in response to exposure to $CT_{max}$. Rather, transcript levels of the enzyme superoxide dismutase (SOD) decreased in hearts of icefishes and the activity of SOD decreased in hearts of the red-blooded species *Gobionotothen gibberifrons*. These data suggest that notothenioids may have lost the ability to elevate levels of antioxidants in response to heat stress.**

Key words: Antarctic fish, oxidative stress, temperature.

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## INTRODUCTION

Antarctic icefishes, lacking the oxygen-binding protein hemoglobin (Hb) (Ruud, 1954), display the lowest critical thermal maximum ($CT_{max}$) among notothenioid fishes inhabiting the Western Antarctic Peninsula region (Beers and Sidell, 2011). The $CT_{max}$ of the icefishes *Chionodraco rastrospinosus* and *Chaenocephalus aceratus* is 13.3 and 13.9°C, respectively, which is significantly lower than the $CT_{max}$ of the red-blooded species *Gobionotothen gibberifrons* (15.5°C) and *Notothenia coriiceps* (17.1°C) (Beers and Sidell, 2011). There are likely multiple factors that contribute to the lower thermal tolerance of icefishes compared with red-blooded notothenioids. A recent study showed that the thermal tolerance of Antarctic notothenioids is correlated with hematocrit, suggesting that the loss of Hb might contribute to the lower thermal tolerance of icefishes compared with red-blooded species (Beers and Sidell, 2011). The loss of Hb reduces the oxygen-carrying capacity of icefish blood to only 10% that of red-blooded notothenioids. As metabolic rate increases with temperature, icefishes are more likely to experience a mismatch between oxygen supply and demand at lower temperatures than red-blooded notothenioids.

Icefishes may also be more vulnerable to oxidative stress as temperature increases than red-blooded species. Although rates of production of reactive oxygen species (ROS) are similar in mitochondria isolated from red- and white-blooded notothenioids, the density of unsaturated mitochondrial phospholipids, which can promote the formation of ROS, is higher in aerobic muscles of icefishes compared with red-blooded fishes (Mueller et al., 2011; O'Brien and Sidell, 2000; O'Brien et al., 2003). Unsaturated fatty acids are easily oxidized, and once oxidized can promote oxidative damage of other phospholipids *via* the lipid peroxidation cycle (reviewed in Girotti, 1998). ROS, such as hydrogen peroxide, abstract allylic hydrogen from unsaturated fatty acids, converting them into lipid radicals. These radicals react with oxygen, forming lipid peroxyl radicals, which in turn abstract allylic hydrogen from other unsaturated fatty acids, initiating a self-perpetuating and potentially deleterious cycle (reviewed in Girotti, 1998). Moreover, when the respiratory chain is disrupted, which occurs during hypoxic events, mitochondria from icefishes produce more ROS than those from red-blooded fishes (Mueller et al., 2011). Compounding the effect, icefishes have lower levels of antioxidants compared with red-blooded species. Maximal activities of the antioxidants superoxide dismutase (SOD) and catalase (CAT) are between 1.4- and 6.0-times lower in liver, heart and muscle of the icefish *Chionodraco hamatus* compared with the red-blooded notothenioid *Trematomus bernacchii*

(Cassini et al., 1993). Similarly, maximal activity of SOD is 6.5-times lower in livers of the icefishes *C. aceratus* and *Pseudochaenichthys georgianus* compared to the red-blooded species *G. gibberifrons* and *N. coriiceps* (Witas et al., 1984). Furthermore, levels of vitamin E and its derivatives, which protect membranes from peroxidation, are up to 13.0-times lower in liver and 1.7-times lower in the pectoral adductor muscle of *C. aceratus* compared with *G. gibberifrons* (Dunlap et al., 2002). Together, these findings suggest that icefishes may be more likely to experience oxidative stress during warming, which might contribute to their lower $CT_{max}$ compared with red-blooded notothenioids.

We tested the hypothesis that icefishes are more vulnerable to oxidative stress compared with red-blooded notothenioids during exposure to $CT_{max}$. The $CT_{max}$ method is commonly used to assess thermal tolerance in organisms because a large number of animals can be surveyed within a short period of time (Beitinger and Bennett, 2000; Beitinger et al., 2000; Lutterschmidt and Hutchinson, 1997). Using this approach, animals are heated at a rate of between 0.5 and 1.5°C $min^{-1}$ and the $CT_{max}$ is defined as the temperature at which an organism's movement becomes disorganized and they lose the ability to escape death (Cowles and Bogert, 1944). In fish, this is frequently defined as the temperature at which an animal loses the ability to right itself (Lutterschmidt and Hutchinson, 1997). Although measuring $CT_{max}$ exposes animals to a very rapid increase in temperature, not likely experienced in their natural habitat, this approach is useful for revealing differences in physiological responses to elevations in temperature. Thus, we quantified levels of oxidized proteins and lipids, as well as transcript levels and activities of the antioxidants SOD and CAT in heart ventricles and oxidative pectoral adductor muscle of icefishes (*C. aceratus*, *C. rastrospinosus*) and red-blooded notothenioids (*G. gibberifrons*, *N. coriiceps*) maintained at ambient temperature and exposed to their $CT_{max}$. Our results indicate that icefishes, but not red-blooded notothenioids, experience an increase in oxidative stress during exposure to $CT_{max}$, which might contribute to their reduced thermal tolerance.

## MATERIALS AND METHODS

### Animal and tissue collection

*Chaenocephalus aceratus* (Lönnberg 1906), *C. rastrospinosus* (DeWitt and Hureau 1979), *G. gibberifrons* (Lönnberg 1905) and *N. coriiceps* (Richardson 1844) were caught during the austral autumn of 2009 in Dallmann Bay (64°08′S, 62°40′W), Antarctica. Animals were captured using an otter trawl or baited traps deployed from the ARSV *Laurence M. Gould* and transferred to the US Antarctic research station, Palmer Station, where animals were maintained in circulating seawater tanks at 0±0.5°C. The $CT_{max}$ was measured (*C. aceratus*, 13.9±0.4°C; *C. rastrospinosus*, 13.3±0.2°C; *G. gibberifrons*, 15.5±0.2°C; *N. coriiceps*, 17.1±0.2°C) as described previously (Beers and Sidell, 2011). Briefly, two or three individuals were transferred into insulated, 700 l experimental tanks containing seawater at ambient temperature. The $CT_{max}$ was then determined by increasing the water temperature in the tank by 3.6°C $h^{-1}$ until the fish lost the ability to right themselves. Control animals were maintained in flow-through seawater tanks at 0°C. Animals were anesthetized in MS-222 (1:7500 in seawater) and killed by transection of the spinal cord. Tissues were quickly excised, frozen in liquid nitrogen and stored at –80°C until further use. All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (134774-2).

### Protein carbonylation

Levels of protein carbonyls were quantified in ventricle and pectoral adductor muscle of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* as described elsewhere (Levine et al., 2000). Briefly, tissues (21–49 mg) were homogenized in 9 volumes (v/w) of ice-cold 0.5 mol $l^{-1}$ potassium phosphate buffer pH 7.8 and incubated with 70 µl of 10% streptomycin in 50 mmol $l^{-1}$ HEPES, pH 7.2 for 15 min at room temperature to precipitate nucleic acids. Homogenates were then centrifuged for 10 min at 9300 ***g*** at 4°C. Supernatant was decanted and incubated with 1 ml of ice-cold acetone for 30 min at –20°C to precipitate proteins. Proteins were then pelleted by centrifugation (15 min, 16,100 ***g***, 4°C) and washed with 1 ml of ice-cold 80% acetone. The final protein pellet was resuspended in 100 µl of 6 mol $l^{-1}$ guanidinium HCl, pH 2.5 and divided into four equal aliquots. Two aliquots were incubated with 35 µl of 6 mol $l^{-1}$ guanidinium HCl, pH 2.5 and two aliquots were incubated with 35 µl of 10 mmol $l^{-1}$ dinitrophenylhydrazine (DNPH) in 6 mol $l^{-1}$ guanidinium HCl, pH 2.5 for 30 min at room temperature immediately prior to protein separation using HPLC. DNPH reacts with protein carbonyls, forming hydrazones, which were detected at 366 nm using Waters 1525 HPLC equipped with a Waters 2296 Photodiode Array Detector (Waters, Milford, MA, USA). Proteins were separated on Zorbax 450 and Zorbax 250 gel filtration columns (Waters) arranged in series and eluted with 6 mol $l^{-1}$ guanidinium HCl, pH 2.5 at a flow-rate of 1 ml $min^{-1}$. All measurements were made in duplicate in 7–11 individuals per species and temperature treatment. Levels of protein carbonyls were quantified by integrating the area under the curve detected at 366 nm and normalized to total protein content, which was quantified by integrating the area under the curve detected at 280 nm. Background was measured in aliquots that were not treated with DNPH and subtracted from protein carbonyls detected in DNPH-treated aliquots to obtain the final protein carbonyl level (mmol carbonyls $mol^{-1}$ protein) as follows:

$$\text{Protein carbonyl level} = \frac{C_1 \times A_{366}}{C_2 (A_{280} - 0.43 A_{366})} - \frac{C_1 \times A_{366}}{C_2 \times A_{280}} \times 1000 , \quad (1)$$

where $A_{366}$ represents the integrated area of peaks detected at 366 nm, $A_{280}$ represents the integrated area of peaks detected at 280 nm, $C_1$ represents the extinction coefficient of average proteins ($\varepsilon$=50,000 l $mol^{-1}$ $cm^{-1}$) and $C_2$ represents the extinction coefficient of hydrazones ($\varepsilon$=22,000 l $mol^{-1}$ $cm^{-1}$).

### Lipid peroxidation

Thiobarbituric acid-reactive substances (TBARS), expressed as levels of malondialdehyde (MDA), were measured in heart ventricle and pectoral adductor muscle of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* as described elsewhere (Uchiyama and Mihara, 1978). Tissues were diced in ice-cold 1.15% KCl (10% w/v) and then homogenized with five short bursts using a Tekmar Tissuemizer (Teledyne Tekmar, Cincinnati, OH, USA). Further homogenization was done with three passes in Ten-Broeck ground glass homogenizers (Wheaton, Milliville, NJ, USA). After addition of 1% phosphoric acid and 0.6% thiobarbituric acid, sample homogenates were heated to 95°C for 45 min (1.4 ml total volume). Samples were subsequently cooled to room temperature before 1.1 ml butanol was added. Samples were vortexed for 5 s then centrifuged at 1000 ***g*** for 10 min, top layers were decanted, and absorbance of samples was measured at 535 and 520 nm (with the difference between the two indicating the MDA content) using a

Hewlett Packard 8453 spectrophotometer (Hewlett Packard, Palo Alto, CA, USA). All measurements were made in at least duplicate in 7–14 individuals per species and temperature treatment. An MDA standard curve was prepared and run alongside samples to quantify MDA content in samples.

Lipids were extracted from tissue homogenates using HPLC grade reagents to quantify lipid content (Bligh and Dyer, 1959). Volumes were chosen to maintain a final extraction ratio of 2:2:1.8 ($CHCl_3$:methanol:$H_2O$). After extractions were complete, samples were centrifuged to generate two phases. The bottom (organic) phase was collected *via* Pasteur pipette and transferred to pre-weighed 12×75 mm culture tubes. The organic phase was then dried in an SC 110 Speedvac (Savant, Thermo Fisher Scientific Inc., Waltham, MA, USA). The mass of each tube was recorded again and the mass of lipid extracted calculated. All measurements were made in duplicate in 7–8 individuals per species and temperature treatment.

Levels of MDA and total lipid content obtained from animals held at 0°C were combined and used for correlation analysis.

### Isolating RNA

Total RNA was isolated from heart ventricles of *C. aceratus*, *C. rastrospinosus*, *N. coriiceps* and *G. gibberifrons* using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) with minor modifications to the manufacturer's protocol. The tissue homogenate was treated twice with DNase I at room temperature for 25 and 20 min, respectively. The quality of the RNA was verified with a Nanodrop ND-1000 spectrophotometer (ThermoScientific, Fisher, Pittsburgh, PA, USA). Samples having a 260/230 nm absorbance ratio of >1.6, and a 260/280 nm absorbance ratio of >1.8 were used for further analyses. The integrity of the RNA was verified by separation on a 2% agarose gel stained with ethidium bromide.

RNA was transcribed into cDNA using TaqMan Reverse Transcriptase Reagents (Applied Biosystems, Carlsbad, CA, USA). Reverse transcriptase was omitted in control reactions to ensure that genomic DNA was not amplified during quantitative real-time PCR (qRT-PCR).

### Cloning and sequencing of SOD1, SOD2, CAT and EF-1α

Degenerate primers were used to amplify partial cDNA sequences of superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), CAT and elongation factor 1α (EF-1α) (Table 1). Partial sequences of SOD1, SOD2 and CAT were amplified in all four species. A partial sequence of EF-1α was obtained only from *G. gibberifrons* because we had previously obtained partial sequences of EF-1α from the other species (GenBank, EU857824, EU857825, EU857826) (Urschel and O'Brien, 2008). Degenerate primers were designed over conserved regions using CODEHOP (Rose et al., 2003) (Table 1). SOD1, SOD2, CAT and EF-1α sequences were amplified with an iCycler (Bio-Rad, Hercules, CA, USA) using a touchdown protocol with annealing temperatures between 65 and 55°C. PCR products were separated on 2% agarose gels stained with ethidium bromide. cDNA fragments of appropriate size were excised, isolated using the QIAquick Gel Extraction Kit (Qiagen), and cloned into *Escherichia coli* using TOPO TA Cloning Kit with pCR2.1-TOPO vector and TOP10 chemically competent cells (Invitrogen, Carlsbad, CA, USA). Transformed colonies were selected based on ampicillin resistance and blue/white screening on LB plates (10 mg ml$^{-1}$ bactotryptone, 5 mg ml$^{-1}$ yeast extract, 5 mg ml$^{-1}$ NaCl, 1 mg ml$^{-1}$ glucose, 15 mg ml$^{-1}$ agar, 50 μg ml$^{-1}$ ampicillin) supplemented with 64 μg ml$^{-1}$ 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Transformed *E. coli* were grown in LB media (10 mg ml$^{-1}$ bactotryptone, 5 mg ml$^{-1}$ yeast extract, 5 mg ml$^{-1}$ NaCl, 1 mg ml$^{-1}$ glucose) supplemented with 50 μg ml$^{-1}$ ampicillin at 37°C in a shaking water bath overnight. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and prepared for sequencing with the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). Products were purified with Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA) packed with Sephadex G-50 gel (Sigma-Aldrich, St Louis, MO, USA) and sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequence identity was determined by searching for homologous sequences in the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov).

### Quantitative real-time PCR

Gene-specific primers were designed for qRT-PCR using Primer Express v2.0 software (Applied Biosystems) (Table 1). Either the forward or the reverse primer of each primer set annealed over a splice site to ensure that genomic DNA was not amplified. Primers for amplifying EF-1α were determined previously (Urschel and O'Brien, 2008). Transcript levels of SOD1, SOD2, CAT and EF-

Table 1. Degenerate and gene-specific primers used for quantification of SOD1, SOD2, CAT and EF-1α transcript levels

| Gene | Type | Primer | Amplicon |
|---|---|---|---|
| *SOD1* | Degenerate | F 5′ CGTGCACGCCTTCGGNGAYAAYAC 3′<br>R 5′ GGCCGCCGGCGTTNCCNGTYTT 3′ | 291 bp |
| *SOD1* | Specific | F 5′ CAAACGGGTGCATCAGTGC 3′<br>R 5′ CACATTCCCCAGGTCTCCAA 3′ | 101 bp |
| *SOD2* | Degenerate | F 5′ GCAGCTGCACCACTCCAARCAYCAYGC 3′<br>R 5′ TCCTTGTTGTATCCCAGCCANCCCCANCC 3′ | 300 bp |
| *SOD2* | Specific | F 5′ GAGGAGAGCCACAGGGGG 3′<br>R 5′ TCTGGAAGGAGCCAAAGTCCC 3′ | 60 bp |
| *CAT* | Degenerate | F 5′ GAAGTTCTACACCGAGGAGGGNAAYTGGGA 3′<br>R 5′ GCCCTGGTTGTCGTGCATRCACATNGG 3′ | 798 bp |
| *CAT* | Specific | F 5′ CATGAAAGACCCCGACATGG 3′<br>R 5′ GTCGCTGAACAAGAAAGACACCT 3′ | 82 bp |
| *EF-1α* | Degenerate | F 5′ CGACATCGCCCTGTGGAARTTYGARAC 3′<br>R 5′ GATGGCCGCCGATCTTGTANACRTCYTG 3′ | 561 bp |
| *EF-1α* | Specific | F 5′ CTGGAAGCCAGTGAAAAGATGAC 3′<br>R 5′ ACGCTCAACCTTCCATCCC 3′ | 51 bp |

SOD, superoxide dismutase; CAT, catalase; EF-1α, elongation factor 1α. For primers, F indicates forward, R indicates reverse.
Degenerate nucleotides are indicated by N, R and Y (N=A or C or G or T; R=A or G; Y=C or T).
Degenerate and gene-specific primers for EF-1α were published previously (Urschel and O'Brien, 2008).

1α were quantified using qRT-PCR and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as described previously (Orczewska et al., 2010). Each reaction contained 1× Power SYBR Green PCR Master Mix, 300 μmol $l^{-1}$ forward primer, 300 μmol $l^{-1}$ reverse primer and 7.5 ng cDNA. All measurements were made in triplicate in 6–8 individuals per species and treatment. A standard curve, generated by serially diluting cDNA pooled from all species, was used to determine relative transcript levels of SOD1, SOD2, CAT and EF-1α. The relative transcript levels of SOD1, SOD2 and CAT were normalized to transcript levels of EF-1α, which was identified as a suitable housekeeping gene using BestKeeper v1 software (Pfaffl et al., 2004) as described previously (Orczewska et al., 2010).

### Activity of SOD and CAT

Maximal activities of SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) were quantified in ventricle and pectoral adductor muscle of *C. aceratus*, *C. rastrospinosus*, *N. coriiceps* and *G. gibberifrons* held at 0°C and exposed to their $CT_{max}$ using a PerkinElmer Lambda 25 spectrophotometer (PerkinElmer, Waltham, MA, USA) equipped with a refrigerated, circulating water bath.

Maximal activity of SOD was quantified at 5±0.5°C by monitoring the reduction of cytochrome *c* at 550 nm (Crapo et al., 1978; McCord and Fridovich, 1969). Briefly, tissues were homogenized in 9 volumes (v/w) of ice-cold 50 mmol $l^{-1}$ potassium phosphate, 0.1 mmol $l^{-1}$ EDTA, pH 7.8. The reduction of 0.01 mmol $l^{-1}$ acetylated cytochrome *c* was measured in the presence of 0.05 mmol $l^{-1}$ xanthine, 0.01 mmol $l^{-1}$ KCN and xanthine oxidase. The final concentration of xanthine oxidase was determined each day to obtain a reduction rate of cytochrome *c* of 0.02 OD $min^{-1}$. One unit of SOD activity is defined as the amount of SOD needed to achieve 50% inhibition of the reduction rate of cytochrome *c*. Homogenates were diluted until a reduction rate of 0.01±0.0002 OD $min^{-1}$ was achieved. All measurements were made in duplicate in 6 individuals per species and temperature treatment. Activity of SOD was expressed as units $g^{-1}$ wet tissue.

Maximal activity of CAT was quantified at 5±0.5°C by monitoring the decomposition of hydrogen peroxide at 240 nm as described elsewhere (Beers and Sizer, 1952). Briefly, tissues were homogenized in 9 volumes (v/w) of 50 mmol $l^{-1}$ phosphate buffer, pH 7.8. Background rates were determined for 2 min by monitoring rates of hydrogen peroxide decomposition in a reaction mixture containing 10 or 25 μl tissue homogenate in 50 mmol $l^{-1}$ phosphate buffer, pH 7.8 in a final volume of 1 ml. Enzyme reactions were initiated by adding a final concentration of 11 mmol $l^{-1}$ hydrogen peroxide to the reaction mixture. All measurements were made in triplicate in 6–8 individuals per species and temperature treatment. Activity of CAT was expressed as μmol $min^{-1}$ $g^{-1}$ wet tissue.

### Statistical analysis

Significant differences in levels of protein and lipid oxidation, transcript levels of SOD1, SOD2 and CAT, and enzyme activity of SOD and CAT between species and at a common temperature were determined using an ANOVA followed by a *post hoc* Tukey–Kramer honestly significant difference (HSD) test. Data were log transformed as necessary to maintain assumptions of normality. Data not meeting the assumption of normality after log transformation were compared using a Wilcoxon test followed by a *post hoc* Tukey–Kramer HSD test. Significant differences in levels of protein and lipid oxidation, transcript levels of SOD1, SOD2 and CAT, and enzyme activity of SOD and CAT between temperature treatments within a species, and between tissues within a species were determined using a Student's *t*-test and the software JMP7 (SAS, Cary, NC, USA). Correlation analysis between levels of MDA and total lipid content was done using a Pearson product moment correlation in SigmaPlot 11.0 (Systat Software, San Jose, CA, USA). Significance was set at $P<0.05$. All data are expressed as means ± s.e.m.

## RESULTS

### Levels of protein oxidation

Levels of protein carbonyls increased 28-times in ventricles of *C. aceratus* exposed to their $CT_{max}$ ($P<0.05$, Fig. 1A) but remained constant in all other species ($P>0.05$, Fig. 1A). In animals held at ambient temperature, levels of protein carbonyls were lowest in ventricles of *C. aceratus* (0.34±0.33 mmol carbonyl $mol^{-1}$ protein), intermediate in *C. rastrospinosus* (8.8±2.1 mmol carbonyl $mol^{-1}$ protein) and *G. gibberifrons* (9.4±3.0 mmol carbonyl $mol^{-1}$ protein) and highest in *N. coriiceps* (27.5±5.0 mmol carbonyl $mol^{-1}$ protein) ($P<0.05$, Fig. 1A).

Levels of protein carbonyls did not increase in response to exposure to $CT_{max}$ in pectoral adductor muscle of any species and were similar among all four species when held at 0°C ($P>0.05$,

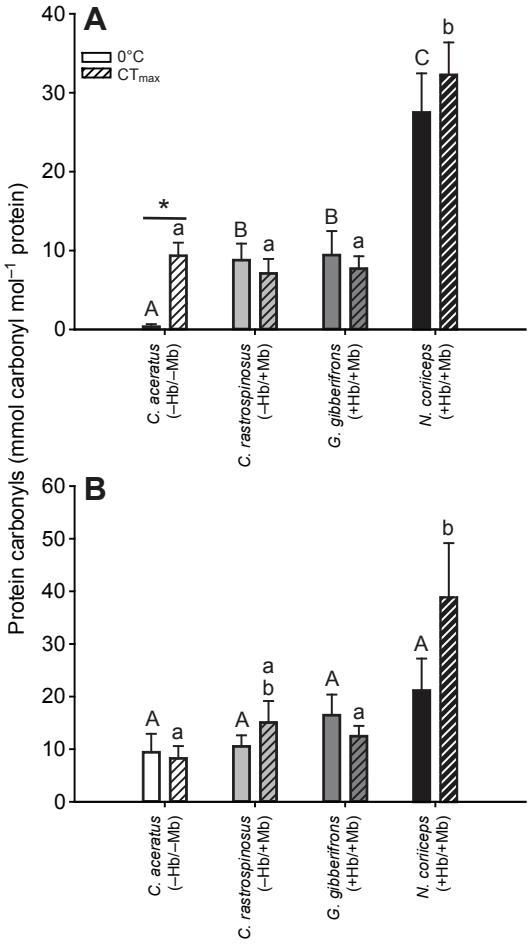


Fig. 1. Levels of oxidized proteins in ventricle (A) and pectoral adductor muscle (B) of *Chaenocephalus aceratus*, *Chionodraco rastrospinosus*, *Gobionotothen gibberifrons* and *Notothenia coriiceps*. Animals were held at 0°C (solid bars) or exposed to their $CT_{max}$ (hatched bars). $N$=7–11. Differences between species at a common temperature are indicated by different capital (0°C) or lowercase ($CT_{max}$) letters. Significant differences between temperature treatments within a species are indicated by asterisks. $P<0.05$. Hb, hemoglobin; Mb, myoglobin.

Fig. 1B). Levels of carbonyls ranged between 9.4±3.5 mmol carbonyl mol$^{-1}$ protein (*C. aceratus*) and 21.2±6.1 mmol carbonyl mol$^{-1}$ protein (*N. coriiceps*).

Levels of carbonyls were significantly higher in pectoral adductor muscle of *C. aceratus* than in heart ventricle at 0°C ($P$<0.05), but were equivalent between ventricles and pectoral adductor muscles in all other species held at ambient temperature ($P$>0.05).

### Levels of oxidized lipids

Levels of MDA increased 1.4-times in hearts of both *C. aceratus* and *C. rastrospinosus* in response to exposure to $CT_{max}$ ($P$<0.05) but did not increase significantly in hearts of red-blooded species ($P$>0.05, Fig. 2A). Levels of MDA were similar between heart ventricles of red- and white-blooded species held at 0°C and ranged from 8.8±1.0 nmol MDA g$^{-1}$ wet tissue (*N. coriiceps*) to 12.9±0.9 nmol MDA g$^{-1}$ wet tissue (*C. rastrospinosus*) ($P$>0.05, Fig. 2A).

Levels of MDA in pectoral adductor muscles were not affected by exposing animals to their $CT_{max}$ ($P$>0.05, Fig. 2B). Levels of MDA were similar in pectoral adductor muscle of *C. aceratus*, *C. rastrospinosus* and *G. gibberifrons*, ranging between 16.5±0.9 and 22.9±2.5 nmol MDA g$^{-1}$ wet tissue ($P$>0.05), but were up to 2.8-times higher in *N. coriiceps* ($P$<0.05, Fig. 2B).

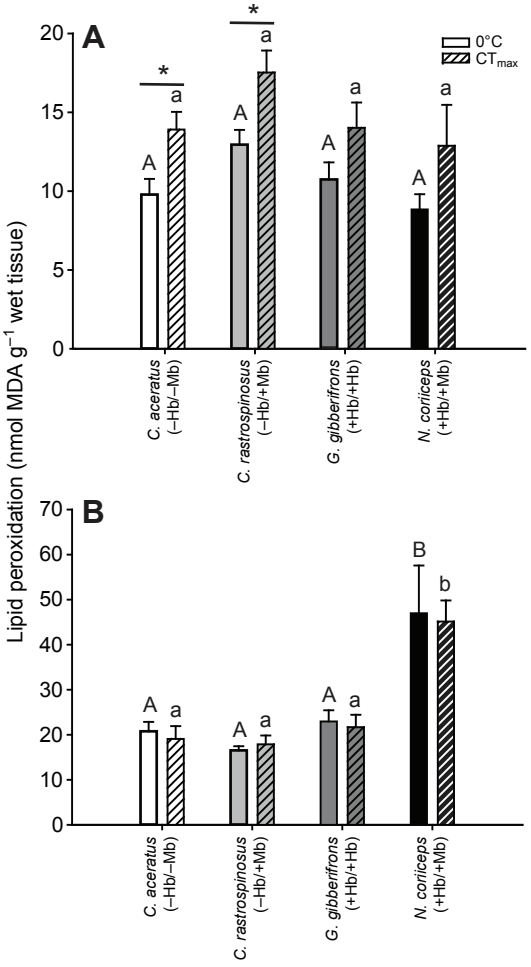


Fig. 2. Levels of oxidized lipids (MDA, malondialdehyde) in ventricle (A) and pectoral adductor muscle (B) of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Animals were held at 0°C (solid bars) or exposed to their $CT_{max}$ (hatched bars). $N$=6–14. Differences between species at a common temperature are indicated by different capital (0°C) or lowercase ($CT_{max}$) letters. Significant differences between temperature treatments within a species are indicated by asterisks. $P$<0.05.

Levels of MDA were significantly higher in the pectoral adductor muscle of all four species compared with their heart ventricles at 0°C ($P$<0.05). Levels of MDA measured in heart ventricle and pectoral muscle from animals held at ambient temperature were positively correlated with total lipid content per gram tissue in Antarctic fishes (Fig. 3, Pearson correlation factor=0.48, $P$<0.05). Total lipid content was similar in the heart ventricle of icefishes and red-blooded species, ranging between 23.6±2.3 mg lipid g$^{-1}$ wet tissue (*G. gibberifrons*) and 31.5±1.7 mg lipid g$^{-1}$ wet tissue (*C. aceratus*) ($P$>0.05, Fig. 4A). In contrast, total lipid content was 49.2±5.0 and 63.1±8.0 mg lipid g$^{-1}$ wet tissue in pectoral adductor muscle of the red-blooded species *G. gibberifrons* and *N. coriiceps*, respectively, which was 2.1- to 2.8-times higher than the total lipid content in the two icefish species ($P$<0.05, Fig. 4B).

### Transcript level of antioxidants

Transcript levels of SOD did not increase in any species or tissue but, rather, SOD1 decreased in hearts of both *C. aceratus* and *C. rastrospinosus*. Transcript levels of SOD2 also decreased in hearts of *C. aceratus* in response to exposure to $CT_{max}$ ($P$<0.05, Fig. 5A,B).

In general, transcript levels of antioxidants were similar among hearts of red-blooded species, and similar among hearts of icefishes, yet higher in red-blooded species than in white-blooded ones. Transcript levels of SOD1 were 1.7- to 2.3-times higher in ventricle of *G. gibberifrons* and *N. coriiceps* compared with *C. aceratus* and *C. rastrospinosus* ($P$<0.05, Fig. 5A). Similarly, transcript levels of SOD2 were 2.8- to 4.5-times higher in ventricles of *G. gibberifrons* and *N. coriiceps* compared with ventricles of *C. aceratus* and *C. rastrospinosus* ($P$<0.05, Fig. 5B). Transcript levels of CAT were 4.8- to 5.6-times higher in ventricles of the two red-blooded species compared with ventricles of *C. aceratus*, and 1.8-times higher compared with the ventricle of *C. rastrospinosus* ($P$<0.05, Fig. 5C). Similar to SOD1 and SOD2, transcript levels of CAT were not significantly different between the two red-blooded fishes, *N. coriiceps* and *G. gibberifrons* ($P$>0.05, Fig. 5C).

### Activity of enzymatic antioxidants

Similar to transcript levels, the activities of antioxidants did not increase in response to exposure to $CT_{max}$, and tended to be higher in red-blooded fishes than in icefishes. Maximal activity of SOD

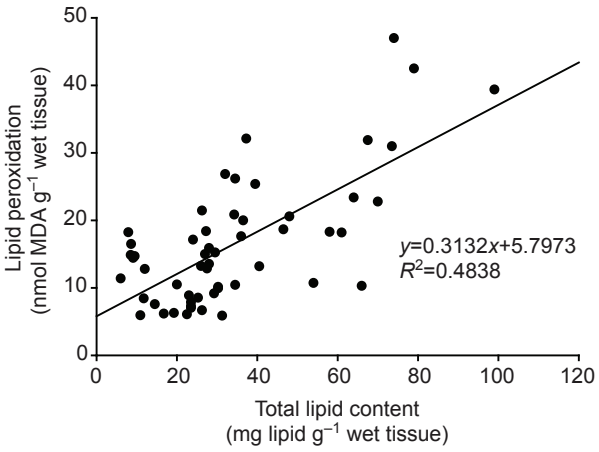


Fig. 3. Levels of oxidized phospholipids in oxidative muscle of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* held at 0°C are correlated with total lipid content of the tissue. $N$=55, $P$<0.05.

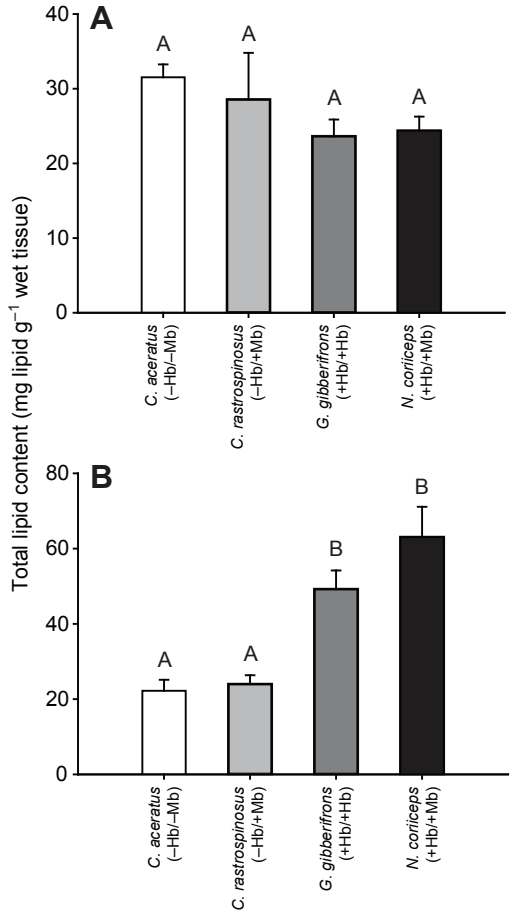


Fig. 4. Total lipid content in heart ventricle (A) and pectoral adductor muscle (B) of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Total lipid content within a species and tissue were pooled for animals exposed to 0°C or their $CT_{max}$. *N*=13–16. Differences between species are indicated by different capital letters. *P*<0.05.

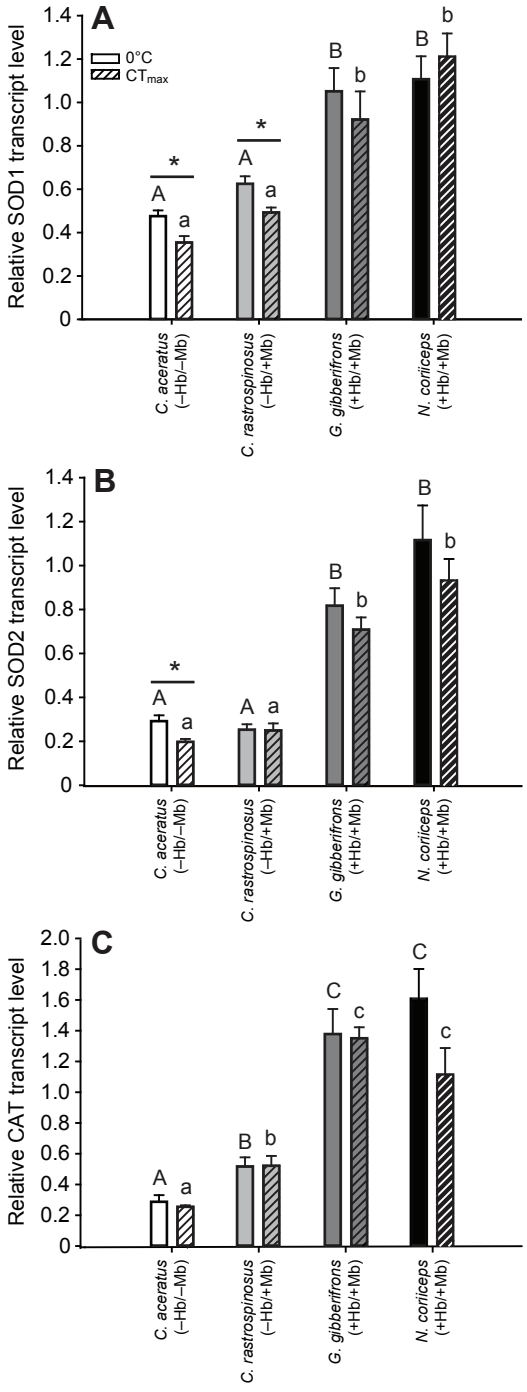


Fig. 5. Relative transcript levels of superoxide dismutase 1 (SOD1, A), SOD2 (B) and catalase (CAT, C) in ventricles of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Animals were held at 0°C (solid bars) or exposed to their $CT_{max}$ (hatched bars). Transcript levels of SOD1, SOD2 and CAT were normalized to transcript levels of elongation factor 1α (EF-1α). *N*=6–8. Differences between species at a common temperature are indicated by different capital (0°C) or lowercase ($CT_{max}$) letters. Significant differences between temperature treatments within a species are indicated by asterisks. *P*<0.05.

and CAT did not change in heart ventricle of the two icefish species or the red-blooded notothenioid *N. coriiceps* in response to exposure to $CT_{max}$ (*P*>0.05, Fig. 6A,B), but decreased 1.2-times in the heart ventricle of *G. gibberifrons* in response to exposure to $CT_{max}$ (*P*<0.05, Fig. 6A). Maximal activity of SOD was $1.70(\pm0.07)\times10^3$ and $1.78(\pm0.05)\times10^3$ U g$^{-1}$ wet tissue in the heart ventricle of the two icefish species *C. aceratus* and C. *rastrospinosus*, respectively, which was significantly lower than in the red-blooded species *G. gibberifrons* and *N. coriiceps* [$2.69(\pm0.05)\times10^3$ and $3.00(\pm0.10)\times10^3$ U g$^{-1}$ wet tissue, respectively] (*P*<0.05, Fig. 6A). Maximal activity of CAT was 135±15 μmol min$^{-1}$ g$^{-1}$ wet tissue in ventricles of *C. aceratus*, which was 2.0-times lower than that of *C. rastrospinosus* and 2.9- to 4.0-times lower compared with the red-blooded species at 0°C (*P*<0.05, Fig. 6B). CAT activity was not significantly different between ventricles of the two red-blooded species (*P*>0.05, Fig. 6B).

Maximal activity of SOD and CAT in pectoral adductor muscle was also not affected by exposure to $CT_{max}$ in any species (*P*>0.05, Fig. 6C,D). Similar to heart ventricles, however, maximal activity of SOD was up to 2.1-times lower in pectoral adductor muscle of icefishes compared with red-blooded species (*P*<0.05, Fig. 6C). Maximal activity of CAT was 1.6- to 1.7-times lower in pectoral adductor muscle of *C. aceratus* compared with the red-blooded species (*P*<0.05, Fig. 6D), but not was significantly different between the two icefish species or the two red-blooded species (*P*>0.05, Fig. 6D).

While maximal activity of SOD was always significantly higher in pectoral adductor muscle than in heart ventricle in all species

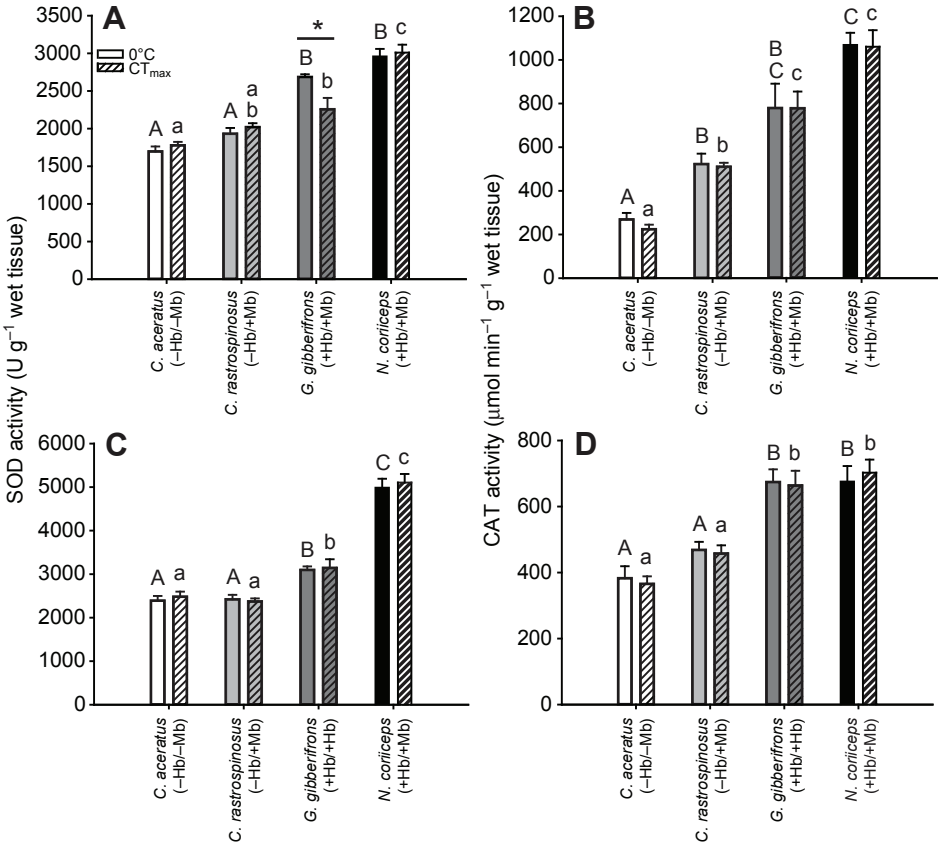


Fig. 6. Activity of SOD (A,C) and CAT (B,D) in ventricle (A,B) and pectoral adductor muscle (C,D) of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Animals were either held at 0°C (solid bars) or exposed to their $CT_{max}$ (hatched bars). $N$=6–8. Differences between species at a common temperature are indicated by different capital (0°C) or lowercase ($CT_{max}$) letters. Significant differences between temperature treatments within a species are indicated by asterisks. $P$<0.05.

($P$<0.05, Fig. 6A,C), maximal activity of CAT did not show a consistent trend between tissues. Maximal activity of CAT was higher in pectoral adductor muscle of *C. aceratus* than in heart ventricle, yet lower in pectoral adductor muscle of *N. coriiceps* compared with heart ventricle, and similar between heart and pectoral adductor muscle in *C. rastrospinosus* and *G. gibberifrons* (Fig. 6B,D).

## DISCUSSION

This is the first study to quantify changes in oxidative stress in response to warming in Antarctic notothenioid fishes. We determined that levels of protein carbonyls, a marker for oxidized proteins, and/or MDA, a marker for peroxidized lipids, increased only in hearts of icefishes, and not in red-blooded notothenioids in response to exposure to their $CT_{max}$. Thus, our results suggest that increases in levels of oxidized macromolecules may contribute to the lower thermal tolerance of icefishes compared with red-blooded species. We also determined that despite a rise in levels of oxidized proteins and lipids in some notothenioids exposed to their $CT_{max}$, neither transcript levels nor maximal activities of antioxidants increased in any heat-stressed notothenioids.

### Levels of oxidized macromolecules

Levels of oxidized macromolecules increased in hearts of icefishes in response to exposure to $CT_{max}$ but absolute levels of oxidized proteins were always higher in the heart ventricle of the red-blooded *N. coriiceps* compared with the two icefishes. The presence of oxidized macromolecules is not necessarily harmful *per se*. Controlled protein and lipid oxidation, balanced by antioxidants, contributes to the oxidant–antioxidant balance, or redox tone (reviewed in Crockett, 2008; Dröge, 2002), which differs among tissues and species. Short-term changes in redox status due to minor elevations in ROS production regulate signaling pathways and the activity of signaling molecules including MAPK, NFκB, protein tyrosine phosphatases, Src family kinases and JNK kinases (reviewed in Dröge, 2002). However, prolonged, extreme shifts in redox tone damage macromolecules, causing oxidative stress (reviewed in Dröge, 2002). Thus, higher levels of oxidized macromolecules in muscles of red-blooded notothenioids might not be deleterious but the temperature-induced increases in levels of oxidized proteins and lipids in hearts of icefishes might shift redox status and impair function of specific proteins and lipids. Mammalian studies have shown that sarcomeric myofibrillar proteins, which are abundant in contractile tissues, are especially vulnerable to oxidation (reviewed in Bayeva and Ardehali, 2010). Increased oxidation of myofibrilar proteins impacts the function of the contractile system, which might eventually lead to heart failure (reviewed in Bayeva and Ardehali, 2010). In addition, even modest changes in the redox status of membranes are associated with alterations in the molecular organization of the lipid bilayer, affecting the function of integral membrane proteins and disrupting membrane integrity (Mason et al., 1997). Consequently, increases in levels of oxidized proteins and/or lipids in heart ventricles of icefishes in response to exposure to $CT_{max}$, rather than differences in absolute levels between species, might be problematic for icefishes and contribute to their lower thermal tolerance compared with red-blooded species.

The increase in oxidized macromolecules in hearts of icefishes but not in red-blooded species in response to exposure to $CT_{max}$ may be due to differences in rates of production of ROS and/or levels of antioxidant defenses. Rates of ROS production are similar in mitochondria isolated from heart ventricles of icefishes and red-blooded notothenioids at 2 and 10°C (Mueller et al., 2011). However, *in situ*, ROS production may be amplified in oxidative muscles of icefishes by their high density of mitochondrial

membranes, rich in polyunsaturated fatty acids. Mitochondrial volume density is 2.0- to 2.3-times higher in hearts of *C. aceratus* and 1.1- to 1.3-times higher in hearts of *C. rastrospinosus* compared with those of *G. gibberifrons* and *N. coriiceps* (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). Furthermore, mitochondrial membranes of Antarctic notothenioids are rich in polyunsaturated fatty acids (PUFAs) (Mueller et al., 2011), which can undergo lipid peroxidation. Once oxidized, PUFAs may further promote oxidative damage *via* a self-propagating lipid peroxidation cycle (Cosgrove et al., 1987; Girotti, 1998).

Hypoxia may also promote the formation of ROS during exposure to $CT_{max}$. Hearts of fishes often become hypoxic as temperature increases because most lack a coronary circulation (Farrell, 2002). Like most teleosts, Antarctic fishes have a type I spongy heart, oxygenated by venous blood (Agnisola and Tota, 1994; Zummo et al., 1995). Hearts of icefishes may be especially vulnerable to hypoxia during exposure to $CT_{max}$ because of their reduced blood oxygen-carrying capacity compared with red-blooded species. Hypoxia can promote the production of ROS because it prolongs the reduction state of electron carriers within the electron transport chain, particularly at complex III. This increases the likelihood of interactions between ubisemiquinone and molecular oxygen, which produces ROS (reviewed in Guzy and Schumacker, 2006). The effect of hypoxia can be mimicked *in vitro* by the addition of antimycin A (reviewed in Guzy and Schumacker, 2006), and previously we determined that rates of ROS production are up to 6-times higher in mitochondria isolated from hearts of icefishes treated with antimycin A compared with untreated ones (Mueller et al., 2011). In addition, ROS production was up to 4.0-times higher in mitochondria of icefishes treated with antimycin A compared with treated mitochondria from red-blooded fishes (Mueller et al., 2011). Thus, if hearts of notothenioids become hypoxic during exposure to $CT_{max}$, icefishes are likely to produce more ROS than red-blooded species.

Icefishes have lower levels of enzymatic antioxidant defenses against ROS compared with red-blooded notothenioids, which may also contribute to oxidative stress during exposure to $CT_{max}$. The maximal activity of SOD, the only antioxidant that detoxifies superoxide anions, is up to 1.7-times lower in heart ventricles of the icefishes *C. aceratus* and *C. rastrospinosus* compared with the red-blooded fish species *G. gibberifrons* and *N. coriiceps*. Similarly, the maximal activity of CAT, one of several enzymes that degrades hydrogen peroxide, is up to 4.0-times lower in heart ventricles of *C. aceratus* and tended to be lower in *C. rastrospinosus* compared with the two red-blooded notothenioids. These findings are in agreement with a previous study which determined that levels of SOD are 6.0-times lower and levels of CAT are 4.0-times lower in heart ventricles of the icefish *C. hamatus* compared with the red-blooded notothenioid *T. bernacchii* (Cassini et al., 1993).

Levels of oxidized macromolecules did not increase in pectoral adductor muscle of any species in response to exposure to $CT_{max}$. This may be due to differences in antioxidant levels and/or rates of ROS production between hearts and pectoral muscles. The maximal activity of SOD in the pectoral adductor muscle was 1.4-times higher in *C. aceratus* and 1.3-times higher in *C. rastrospinosus* compared with heart ventricles. Additionally, maximal activity of CAT was 1.4-times higher in pectoral adductor muscle of *C. aceratus* compared with the heart ventricle. Typically, levels of antioxidant defenses are correlated with aerobic metabolic capacity in notothenioid fishes (Crockett, 2011) but this trend is not apparent in hearts and pectoral muscles of icefishes. In fact, maximal activity of cytochrome *c* oxidase per gram of tissue is lower in pectoral muscle of the icefishes *C. aceratus* and *C. rastrospinosus* than in heart ventricle (O'Brien and Sidell, 2000; O'Brien et al., 2003) while SOD activity is higher, suggesting SOD may be protecting against sources of ROS, in addition to those produced by the respiratory chain, in pectoral adductor muscles of icefishes.

Rates of ROS production have only been measured in mitochondria isolated from the heart ventricle and not in oxidative pectoral adductor muscle of Antarctic notothenioids (Mueller et al., 2011). As a result, we do not know whether rates of ROS production are lower in pectoral muscle compared with hearts. However, if hypoxia contributes to ROS production, the pectoral adductor muscle is likely to be less affected than hearts. In contrast to the heart, which is supplied by venous blood, the pectoral adductor muscle is supplied with freshly oxygenated blood *via* a hypobranchial shunt, branching directly from the first three gill arches (Egginton and Rankin, 1998).

### Thermal plasticity of Antarctic notothenioids

Despite an increase in oxidized proteins and/or lipids in hearts of the icefishes *C. aceratus* and *C. rastrospinosus* during exposure to $CT_{max}$, neither species increased mRNA levels or activities of antioxidants. Thus, the ability to induce the expression of antioxidants in response to a rise in temperature may have been lost during their evolution in the constantly cold environment of the Southern Ocean, similar to the loss of the heat shock response (Carpenter and Hofmann, 2000; Hofmann et al., 2000; Hofmann and Place, 2005). Consistent with our results, a previous study using microarrays to quantify changes in the expression of 9502 genes in gills of *Trematomus bernacchii* exposed to 4°C for 4 h showed that while the transcript level of 262 genes changed in response to an increase in temperature, none of these were antioxidants (Buckley and Somero, 2008). We cannot, however, rule out the possibility that antioxidant levels might increase in response to prolonged exposure to elevated temperature.

Recent studies have shown that some notothenioids acclimate to warmer temperatures. Both cardiac function and metabolism were altered in response to acclimation to 4°C for 4–5 weeks in the red-blooded notothenioid *Pagothenia borchgevinki* (Franklin et al., 2007; Robinson and Davison, 2008; Seebacher et al., 2005). In addition, previous studies have shown that the $CT_{max}$ of notothenioids is influenced by thermal history and significantly increased by warm acclimation (Bilyk and Devries, 2011; Bilyk and Devries, 2012). The $CT_{max}$ of notothenioids captured in McMurdo Sound, where temperatures are –1.8°C year round, is significantly lower than the $CT_{max}$ of notothenioids captured in the warmer Western Antarctic Peninsula region, where temperatures fluctuate seasonally between –1.8°C and +2°C (Bilyk and Devries, 2011; Eastman, 1993; Hofmann and Klinck, 1998; Jacobs et al., 2002). In addition, warm acclimation of *P. borchgevinki*, *Trematomus hansoni*, *T. bernacchii*, *G. gibberifrons* and *N. coriiceps* to 4°C for 1–3 weeks increased their $CT_{max}$ by as much as 3°C (Bilyk and Devries, 2011) and acclimation of *N. coriiceps* to 10°C increased $CT_{max}$ by more than 5°C (Bilyk and Devries, 2012). Notably, however, these studies were conducted on red-blooded notothenioids and the degree of thermal plasticity may vary among families within the suborder and even among species within a family.

### Inter-relationship between the expression of oxygen-binding proteins, oxidized proteins and antioxidants

There is a positive correlation between the expression of oxygen-binding proteins, levels of oxidized proteins and levels of

antioxidants among notothenioids, with levels of oxidized proteins and antioxidants tending to be highest in fishes expressing the oxygen-binding proteins Hb and myoglobin (Mb). Mb is expressed in the heart ventricle of the two red-blooded species and the icefish *C. rastrospinosus* but is absent in the heart ventricle of the icefish *C. aceratus* (Moylan and Sidell, 2000; Sidell et al., 1997; Vayda et al., 1997). Hb and Mb, like other heme-containing proteins, exhibit peroxidase activity. Oxidation of a ferrous heme protein by superoxide radicals or hydrogen peroxide produces ferryl Mb/Hb and protein-based radicals, which are powerful oxidants capable of damaging proteins and lipids (reviewed in Reeder and Wilson, 2005). In addition, when $Fe^{2+}$ is released from damaged heme proteins, it participates in the Fenton reaction, producing highly reactive hydroxyl radicals from hydrogen peroxide, which not only damage macromolecules but also initiate the lipid peroxidation cycle (reviewed in Girotti, 1998). The higher levels of antioxidants in red-blooded notothenioids compared with icefishes may be necessary to counter the oxidative reactions originating from the heme-based proteins Hb and Mb.

Higher rates of protein synthesis in red-blooded species are consistent with higher rates of damage because oxidatively damaged proteins are degraded by 20S proteasome complexes and must be replaced with newly synthesized ones (reviewed in Davies, 2001). Previous studies have shown that rates of $^{14}$C-phenylalanine incorporation are 3.1-times lower in heart ventricles and 4.3-times lower in pectoral muscles of *C. aceratus* compared with *N. coriiceps* at 2°C (Haschemeyer, 1983).

The loss of Hb and Mb is considered a ‘disaptation’ – a neutral mutation that has persisted in the population because it does not negatively impact fitness (Montgomery and Clements, 2000; Sidell and O’Brien, 2006). However, if the presence of Hb and Mb promotes oxidative damage, elevates rates of protein synthesis and warrants higher levels of antioxidants, then the loss of these oxygen-binding proteins may offer an energetic advantage. Protein synthesis is an energetically costly process, with approximately 15–22% of an organism’s standard metabolic rate being allocated to the synthesis of new proteins (Rolfe and Brown, 1997). Future studies will address the question of whether the loss of expression of oxygen-binding proteins might protect against damaging oxidative reactions.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| CAT | catalase |
| $CT_{max}$ | critical thermal maximum |
| EF-1α | elongation factor 1α |
| Hb | hemoglobin |
| Mb | myoglobin |
| MDA | malondialdehyde |
| ROS | reactive oxygen species |
| SOD | superoxide dismutase |

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