© 2018. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2018) 221, jeb162503. doi:10.1242/jeb.162503

RESEARCH ARTICLE

The loss of hemoglobin and myoglobin does not minimize oxidative stress in Antarctic icefishes

Kristin M. O'Brien[1,*], Elizabeth L. Crockett[2], Jacques Philip[3], Corey A. Oldham[1], Megan Hoffman[1], Donald E. Kuhn[2], Ronald Barry[4] and Jessica McLaughlin[1]

ABSTRACT

The unusual pattern of expression of hemoglobin (Hb) and myoglobin (Mb) among Antarctic notothenioid fishes provides an exceptional model system for assessing the impact of these proteins on oxidative stress. We tested the hypothesis that the lack of oxygen-binding proteins may reduce oxidative stress. Levels and activity of pro-oxidants and small-molecule and enzymatic antioxidants, and levels of oxidized lipids and proteins in the liver, oxidative skeletal muscle and heart ventricle were quantified in five species of notothenioid fishes differing in the expression of Hb and Mb. Levels of ubiquitinated proteins and rates of protein degradation by the 20S proteasome were also quantified. Although levels of oxidized proteins and lipids, ubiquitinated proteins, and antioxidants were higher in red-blooded fishes than in Hb-less icefishes in some tissues, this pattern did not persist across all tissues. Expression of Mb was not associated with oxidative damage in the heart ventricle, whereas the activity of citrate synthase and the contents of heme were positively correlated with oxidative damage in most tissues. Despite some tissue differences in levels of protein carbonyls among species, rates of degradation by the 20S proteasome were not markedly different, suggesting either alternative pathways for eliminating oxidized proteins or that redox tone varies among species. Together, our data indicate that the loss of Hb and Mb does not correspond with a clear pattern of either reduced oxidative defense or oxidative damage.

KEY WORDS: Antarctic fish, Antioxidants, Oxygen-binding proteins, Oxidative damage

INTRODUCTION

Antarctic notothenioid fishes provide a natural experimental model in which questions pertaining to the risks and prevention of oxidative stress at very low temperatures can be probed. A life cycle spent entirely at very low body temperatures may, at first glance, suggest a means to avoid oxidative injury as the rates of production of reactive oxygen species (ROS) are significantly retarded at cold temperature (Abele et al., 2002). Given their habitat and their cellular architecture, however, these animals may be predisposed to an imbalance of ROS production and ROS degradation (i.e. oxidative stress). First, oxygen solubility is inversely proportional to temperature, and the low temperature of the Southern Ocean, in conjunction with substantial vertical mixing, results in highly oxygenated water (Eastman, 1993). Second, in Antarctic fishes, the tissues with particularly high energy demands, including oxidative skeletal muscle and cardiac ventricles, are endowed with a large complement of mitochondria (Johnston et al., 1998; Londraville and Sidell, 1990; O'Brien and Sidell, 2000), the organelle responsible for the vast majority of ROS production in eukaryotic cells (Andreyev et al., 2005; Murphy, 2009). Third, biological membranes of Antarctic fishes are enriched with phospholipids with a high degree of unsaturation (i.e. polyunsaturated fatty acids or PUFA) (Logue et al., 2000; O'Brien and Mueller, 2010). Because fatty acids with high numbers of double bonds are particularly susceptible to oxidation by ROS (Cosgrove et al., 1987), the membranes of Antarctic fishes may be especially vulnerable to lipid peroxidation (Crockett, 2008). Finally, Antarctic notothenioids possess a unique twist in the typical expression of Hb among vertebrates. The family Channichthyidae (icefishes) is characterized by the absence of Hb, and six of the 16 species of icefishes also lack expression of Mb in the heart ventricle (Moylan and Sidell, 2000; Sidell et al., 1997). In addition, oxidative skeletal muscle in all species of Antarctic notothenioids is devoid of Mb (Moylan and Sidell, 2000).

One of our earlier studies indicated that the susceptibility of proteins and lipids to oxidation may, in fact, be related to the expression of Hb and Mb in notothenioid fishes. We found significantly lower levels of secondary products of lipid peroxidation in the oxidative muscle of two icefishes (−Hb), *Chionodraco rastrospinosus* and *Chaenocephalus aceratus*, than in those of the red-blooded (+Hb) species *Notothenia coriiceps* (Mueller et al., 2012). Furthermore, levels of oxidized proteins were lower in the heart of the −Mb icefish *C. aceratus* compared with the +Mb heart of *C. rastrospinosus*, and were the highest in the heart of *N. coriiceps* (Mueller et al., 2012). In addition, we (Mueller et al., 2012) and others (Cassini et al., 1993; Witas et al., 1984) have found that the capacities for central antioxidants in selected tissues are lower in white-blooded (−Hb) than in red-blooded (+Hb) fishes, suggesting that the loss of Hb and Mb may permit reduced antioxidant defenses.

The mechanism(s) responsible for elevated levels of oxidized proteins and lipids in red-blooded and red-hearted notothenioids are unclear. As iron (Fe)-centered proteins, Hb and Mb may facilitate the production of ROS (King et al., 1964; Tappel, 1955). Any unbound (i.e. excess) Fe may promote the formation of hydroxyl radicals ($^{\bullet}OH$) via the Fenton reaction (Halliwell and Gutteridge, 1986), and these radicals are sufficiently potent that they can oxidize biological molecules including proteins and the lipids that constitute the matrices of biological membranes (Dikalov, 2011; Lee et al., 2012). Autoxidation of Hb [with Fe in the ferrous (Fe^{2+}) state] to methemoglobin (metHb) with Fe in the ferric (Fe^{3+}) state leads to

[1]Institute of Arctic Biology, University of Alaska, Fairbanks, Alaska, 99775, USA. [2]Department of Biological Sciences, Ohio University, Athens, Ohio, 45701, USA. [3]Center for Alaska Native Health Research, University of Alaska, Fairbanks, Alaska, 99775, USA. [4]Department of Mathematics and Statistics, University of Alaska, Fairbanks, Alaska, 99775, USA.

*Author for correspondence (kmobrien@alaska.edu)

K.M.O., 0000-0002-3311-0690

Received 8 May 2017; Accepted 11 December 2017

Journal of Experimental Biology (2018) 221, jeb162503. doi:10.1242/jeb.162503

List of abbreviations

CAT	catalase
CS	citrate synthase
ETC	electron transport chain
GPx1	glutathione peroxidase 1
GPx4	glutathione peroxidase 4
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione *S*-transferase
Hb	hemoglobin
Mb	myoglobin
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SOD	superoxide dismutase
TAP	total antioxidant potential

the production of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) when metHb reacts with O_2 (Winterbourn, 1990). Heme in the Fe^{3+} state also reacts with H_2O_2 to form two oxidants, ferryl iron (Fe^{4+}=O_2^-) and a protein-bound free radical on the globin (Reeder and Wilson, 2005). However, most cells, particularly red blood cells, have an abundance of low molecular weight antioxidants and antioxidant enzymes that detoxify ROS and maintain iron in the ferrous state (Cohen and Hochstein, 1963; Johnson et al., 2005; Lopez-Torres et al., 1993; Low et al., 2008; Perez-Campo et al., 1993; van Zwieten et al., 2014). The central antioxidants superoxide dismutase (SOD) and catalase (CAT) are able to detoxify two common ROS, $O_2^{\bullet-}$ and H_2O_2, respectively (Finkel and Holbrook, 2000). H_2O_2 may also be detoxified by several antioxidant enzymes that require reduced glutathione (GSH), including the glutathione peroxidases (GPx), with glutathione reductase (GR) having the important role of recycling the GSH (Brigelius-Flohé, 1999). Another family of enzymes, the glutathione *S*-transferases (GST), also depends on GSH for the catalytic conjugation of a variety of potentially deleterious electrophilic compounds and xenobiotics prior to excretion (Hayes and Pulford, 1995). Additionally, any extracellular Hb should be bound by haptoglobin (Alayash et al., 2013), and free heme by hemopexin (Smith and McCulloh, 2015), minimizing Fe-mediated ROS formation. An alternative explanation for the high levels of oxidized macromolecules in red-blooded fishes is that they are caused by high rates of aerobic metabolism, supported by the expression of oxygen-binding proteins, which can enhance ROS formation, rather than by Fe-mediated ROS formation via Hb and Mb.

Lower levels of oxidized proteins in icefishes compared with red-blooded species may reduce rates of protein degradation. Oxidized proteins are marked for degradation by the ATP-dependent covalent conjugation of ubiquitin (Dudek et al., 2005; Shang et al., 1997). Ubiquitinated proteins are degraded by the 26S proteasome in another ATP-dependent process (Davies, 2001; Shringarpure et al., 2003). Oxidized proteins may also be degraded by the 20S proteasome in a ubiquitin- and ATP-independent process (Davies, 2001; Reinheckel et al., 2000). Although both the 26S and 20S proteasome degrade oxidatively modified proteins (Shang and Taylor, 2011), the 20S proteasome does not require proteins to be ubiquitinated to degrade them and can, therefore, degrade oxidatively damaged proteins in cells with compromised ubiquitin conjugation systems (Davies, 2001; Shringarpure et al., 2003). Additionally, the 26S proteasome is more susceptible to oxidative damage than the 20S proteasome, suggesting that the 20S proteasome may play a more prominent role in the degradation of oxidized proteins (Reinheckel et al., 2000).

We took a comprehensive look at relationships among pro-oxidants, oxidative damage, antioxidant defenses, and protein ubiquitination and degradation in both white- and red-blooded Antarctic notothenioids in order to test the hypothesis that loss of circulating Hb, and of Mb in the cardiac muscle of these fishes is accompanied by lower levels of oxidized proteins and lipids, a reduced arsenal of antioxidants and lower rates of protein degradation.

MATERIALS AND METHODS

Animal collection

The following species were used for this study [animal masses (means±s.e.m.) are shown in parentheses]: *Chaenocephalus aceratus* (Lönnberg 1906) (1675±169 g; *n*=18), *Champsocephalus gunnari* Lönnberg 1905 (581±36 g; *n*=16), *Pseudochaenichthys georgianus* Norman 1937 (1528±228 g; *n*=8), *Chionodraco rastrospinosus* DeWitt & Hureau 1979 (349±30 g; *n*=14), *Gobionotothen gibberifrons* (Lönnberg 1905) (formerly *Notothenia gibberifrons*; 860±38 g; *n*=33) and *Notothenia coriiceps* Richardson 1844 (1847±94 g; *n*=18). Animals were collected off the southwestern shore of Low Island (63°25′S, 62°10′W) and in Dallmann Bay (64°10′S, 62°35′W) using a benthic otter trawl and fish pots between April and June, 2011 and 2013. Animals collected in 2013 were used for quantifying levels of ubiquitinated proteins and the activity of the 20S proteasome. All other measurements were completed using animals collected in 2011. Fish were held in recirculating seawater tanks on board the US ARSV *Laurence M. Gould* and then transferred to the aquarium at the US Antarctic Research Station, Palmer Station, where they were held in tanks with recirculating seawater at 0.1±0.5°C. Animals were killed with a sharp blow to the head followed by cervical transection. Samples of the heart ventricle, pectoral adductor muscle and liver were harvested, flash frozen in liquid nitrogen and stored at −80°C. Icefishes and *G. gibberifrons* were harvested within 2 weeks of capture. *Notothenia coriiceps* were fed a diet of fish muscle approximately every 3 days and were harvested within approximately 3 weeks of capture. All protocols were approved by the University of Alaska Fairbanks Institutional Care and Use Committee (1374774-2).

Hematocrit, hemoglobin and heme quantification

Hematocrit (Hct) measurements were obtained by drawing blood into heparinized capillary tubes and centrifuging for 5 min in an Hct centrifuge. Each individual was measured in triplicate.

Whole blood was mixed with 3.2% sodium citrate (9:1 for red-blooded species and 4:1 for icefishes) to prevent clotting. Hb concentration was determined by mixing 20 µl of citrated blood with 5 ml Drabkin's reagent (Sigma-Aldrich, St. Louis, MO, USA), incubating the mixture at room temperature for 30 min and measuring absorbance at 540 nm. Samples and a standard curve with concentrations between 0 and 180 mg ml^{-1} of Hb were measured in triplicate.

Heme concentration in blood diluted with sodium citrate (as described above) was determined using the Quantichrome Heme Assay Kit according to the manufacturer's instructions (BioAssay Systems, Hayward, CA, USA). Samples were measured in duplicate.

Myoglobin quantification

Heart ventricles were homogenized (10% w/v) in 20 mmol l^{-1} Hepes buffer (pH 7.8 at 4°C) using a Tenbroeck ground-glass

Journal of Experimental Biology (2018) 221, jeb162503. doi:10.1242/jeb.162503

homogenizer (Wheaton, Millville, NJ, USA) on ice. Homogenates were centrifuged at 10,000 ***g*** for 10 min at 4°C, the supernatant collected and protein concentration determined using the bicinchoninic acid assay (Sigma-Aldrich) with bovine serum albumin (BSA) as a standard (Smith et al., 1985). The supernatant was mixed with loading buffer (150 mmol l^{-1} Tris–HCl, 4% SDS, 12% glycerol, 100 mmol l^{-1} dithiothreitol, 0.01% Bromophenol Blue, pH 6.8) and heated at 98°C for 10 min. Proteins were separated on 12% Tris-Tricine gels as described previously (Moylan and Sidell, 2000). Each gel included a standard curve of known amounts of purified Mb from *N. coriiceps*. Proteins from each individual were run in triplicate on two separate gels. Gels were stained in 0.10% (w/v) Coomassie Brilliant Blue R-250 stain (50% methanol, 10% acetic acid), destained in 5% methanol and 7% acetic acid and scanned with an ImageScanner (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Images were quantified using ImageQuant (GE Healthcare Bio-Sciences) and the concentration of Mb was calculated from the standard curve.

Citrate synthase activity

The maximal activity of citrate synthase (EC 2.3.3.1) was measured at 5°C±0.5°C using a Lambda 25 spectrophotometer (PerkinElmer, Waltham, MA, USA) and a modification of the protocol described by Srere et al. (1963). Frozen tissues were finely chopped on an ice-cold stage and then homogenized in 19 volumes of ice-cold buffer (75 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 2 mmol l^{-1} $MgCl_2$, pH 8.2 at 5°C) using Tenbroeck ground-glass homogenizers. The final reaction mixture contained 0.25 mmol l^{-1} 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 0.40 mmol l^{-1} acetyl coenzyme A (CoA), 0.5 mmol l^{-1} oxaloacetate, 75 mmol l^{-1} Tris-HCl, pH 8.2, with 0.025% tissue. Background activity was measured for 5 min in the absence of the initiating substrate oxaloacetate. The progress of the reaction was monitored by following the reduction of DTNB at 412 nm for 5 min following the addition of oxaloacetate. Activity is expressed as µmol product min^{-1} g^{-1} wet mass.

Protein carbonylation

Protein carbonylation was quantified using an immunochemical dot blot method as described by Wehr and Levine (2012). A carbonylated aconitase standard was prepared by resuspending aconitase (Sigma-Aldrich) at a final concentration of 5 mg ml^{-1} in buffer (10 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} Hepes, 100 mmol l^{-1} KCl, pH 7.2) and dialyzed against 25 mmol l^{-1} ascorbate and 0.1 mmol l^{-1} $FeCl_3$ for 24 h at room temperature using a Pur-A-Lyzer Maxi 12000 Dialysis Kit (Sigma-Aldrich) as described by Rivett and Levine (1990). On day two, the buffer was replaced with one containing 1 mmol l^{-1} EDTA to stop the carbonylation reaction and then placed at 4°C with stirring. On day three, the buffer was replaced with the original dialysis buffer to remove EDTA. All buffers were changed three times daily. Protein concentration of the aconitase carbonyl standard was determined using the Bradford Assay with BSA as the standard (Bradford, 1976). Absorbance at 595 nm was measured using a Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The aconitase standard was then divided into 50 µg aliquots, brought to a final volume of 100 µl with MQ H_2O, dried in a vacuum centrifuge (CentriVap Concentrator, Labconco Corporation, Kansas City, MO, USA) at room temperature for 2 h and stored at −80°C until use.

Levels of protein carbonylation in the aconitase standard were determined using high performance liquid chromatography (HPLC) with a Waters 1525 Binary HPLC equipped with an in-line degasser and photodiode array detector (Waters Corporation, Milford, MA, USA). Proteins were separated using TSKgel Guard SW_{XL} and QC–PAK GFC 200 columns (TOSOH Bioscience, King of Prussia, PA, USA) and a running buffer of 6 mol l^{-1} guanidinium hydrochloride, pH 2.5. Spectra were analyzed with Waters Empower Pro Analysis software (Waters Corporation). From the aconitase standard, 100 µg was resuspended to a final concentration of 300 ng protein $µl^{-1}$ in 50 mmol l^{-1} KH_2PO_4 (pH 7.8); 300 µl of the standard was mixed with 70 µl of 10% streptomycin sulfate and incubated at room temperature for 15 min to precipitate DNA. The samples were then centrifuged at 10,000 ***g*** for 10 min at 4°C, the supernatant was collected and 1200 µl of ice-cold 100% acetone was added. The samples were then vortexed and incubated for 30 min at −20°C to precipitate protein. Samples were centrifuged at 16,000 ***g*** for 15 min at 4°C and rinsed with ice-cold 80% acetone. The pellet was rinsed in acetone and centrifuged at 16,000 ***g*** for 15 min at 4°C. Acetone was discarded and the pellet was resuspended in 100 µl of running buffer. The aconitase standard was divided into four 25 µl aliquots. Two aliquots per sample were incubated with 35 µl of 10 mmol l^{-1} 2,4-dinitrophenylhydrazine (DNPH) in running buffer and two in running buffer alone. Absorption was measured at 280 nm for total protein and 366 nm for the carbonyl hydrozone. Carbonyl concentration was determined using the equations described in Levine et al. (2000).

Tissues were homogenized in 50 mmol l^{-1} KH_2PO_4 pH 7.8 and centrifuged at 4°C for 10 min at 9000 ***g***. Bradford assays, as described above, were used to determine the protein concentration of the supernatant; 50 µg of protein was brought to a final volume of 100 µl with MQ H_2O and dried in a vacuum centrifuge (CentriVap Concentrator, Labconco Corporation, Kansas City, MO, USA) for 2 h at room temperature. Samples and 100 µg of aconitase standard were resuspended to a final concentration of 300 ng $µl^{-1}$ in a derivitization solution consisting of 20 mmol l^{-1} DNPH, 0.5% trifluoroacetic acid (TFA) and 92.5% dimethyl sulfoxide (DMSO). Samples were vortexed at room temperature for 45–60 min before being spotted onto a PVDF membrane. A standard curve of aconitase containing 0–300 ng $µl^{-1}$ protein was loaded onto each membrane in triplicate. The samples and aconitase were diluted in derivitization solution followed by phosphate-buffered saline (PBS) to a minimum volume of 200 µl. The final concentration of each sample and standard was determined using a Bradford assay as described above but with BSA resuspended in derivitization solution as the standard. All samples were prepared and loaded in triplicate.

PVDF membranes (GE Healthcare Bio-Sciences) were wetted using 100% methanol and soaked for at least 10 min in transfer buffer (25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, 0.1% SDS, 20% MeOH) before loading samples using a vacuum-drawn slot blotter (Bio-Dot SF Cell, Bio-Rad, Hercules, CA, USA). A total of 200 µl of transfer buffer was pipetted into each slot and drawn across the membrane using a vacuum, followed by samples, and then another 200 µl of transfer buffer. Blots were dried for 15 min, washed with glacial acetic acid, twice for 2 min with 100% acetic acid, and once for 2 min in 5 ml acetic acid with a sufficient amount of MQ H_2O added to cover the membrane (approximately 60–70 ml total volume), and then once for 5 min in MQ H_2O, dried and then stored at −80°C for up to 2 days before developing.

Membranes were blocked with 5% non-fat dry milk in 0.1% PBS-Tween for 1 h and then washed with 0.1% PBS-Tween (twice quick, once for 15 min, and twice for 5 min). Membranes were incubated with the primary antibody goat anti-DNPH (Bethyl Laboratories, Montgomery, TX, USA), diluted 1:5000 in 5% non-fat dry milk in 0.1% PBS-Tween for 2 h, and then washed as described above.

Membranes were then incubated with the secondary antibody of donkey anti-goat HRP (SC2020; Santa Cruz Biotechnology, Dallas, TX, USA), diluted 1:10,000 in 5% non-fat dry milk in 0.1% PBS-Tween for 1 h and washed as described above. Membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences), viewed using AlphaImager 3300 Imaging System (Protein Simple, San Jose, CA, USA) and analyzed with ImageQuant TL (GE Healthcare Bio-Sciences).

Lipid peroxidation

Tissue homogenates were used to measure malondialdehyde, a thiobarbituric acid-reactive substance (TBARS). Tissues were processed and analyzed according to the method of Mihara and Uchiyama (1978) and as described previously by our group (Mueller et al., 2012). For the purpose of normalizing malondialdehyde to lipid content, total lipids were determined gravimetrically. Lipids were extracted from tissues using methanol:chloroform:water as described by Bligh and Dyer (1959). After lipids were extracted, a five decimal place balance (Mettler H54AR, Columbus, OH, USA) was used to quantify the final lipid mass of each sample.

Glutathione

Tissue contents of glutathione were measured using an assay based on the 2-vinylpyridine (2VP) method described by Griffith (1980). Total glutathione (GST_{tot}=GSH+GSSG) was measured, along with oxidized glutathione (GSSG) following the elimination of GSH using 2VP. Tissues were homogenized in five volumes of ice-cold 5% 5′-sulfosalicylic acid in 100 mmol l^{-1} potassium phosphate and 1 mmol l^{-1} EDTA (pH 7.0) using a BioHomogenizer tissue homogenizer (BioSpec Products, Bartlesville, OK, USA). Following centrifugation at 10,000 ***g*** for 5 min at 4°C, supernatants were collected and used immediately or stored at −70°C. Supernatants were added to a buffered 2VP solution (0.3 mol l^{-1} 2VP final concentration) or buffer alone and incubated at room temperature for 10–15 min. An assay solution containing 100 mmol l^{-1} K-phosphate, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} DTNB and 0.164 units ml^{-1} glutathione reductase (400 units ml^{-1}) (pH 7.0) was added (150 µl) to the wells of a 96-well microplate. Ten microliters of diluted sample (typically 20-fold) or standard was added to each well in duplicate and the reaction was initiated by adding 50 µl of an NADPH solution (0.05 mmol l^{-1} final concentration). Absorbance readings at 412 nm were begun immediately thereafter and taken every 20 s for 3 min using a SpectraMax M2e microplate reader (Molecular Devices) and the accompanying SoftMax Pro software kinetic program.

Enzymatic antioxidants

SOD (EC 1.15.1.1) activity was measured using a kit from Sigma Life Sciences (catalog no. 19160) by following the extent of inhibition of the reduction of xanthine by xanthine oxidase, coupled to the reduction of the chromogen 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) at 440 nm. One unit of activity was defined as the amount of SOD necessary to inhibit the reduction of xanthine by 50%. Tissues were homogenized (10%, w/v) in ice-cold 50 mmol l^{-1} potassium phosphate buffer (pH 7.2) as described above, followed by probe sonication (three pulses of 4 s duration, 2 mm probe, output at 20% of maximum). Aliquots of each sample were removed and centrifuged at 2000 ***g*** for 10 min at 4°C. Supernatants were then diluted as necessary in homogenization buffer for use in the assay. All samples were measured at 5°C in triplicate using a SpectraMax M2e microplate spectrophotometer (Molecular Devices).

CAT (EC 1.11.1.6) activity was measured at 5°C using a Beckman DU-640 spectrophotometer (Brea, CA, USA) according to the method of Beers and Sizer (1952).

Glutathione reductase (EC 1.8.1.7) activities in tissue supernatants were measured at 5°C using a slightly modified version of a protocol described by Sigma-Aldrich (GRSA Technical Bulletin). Tissues were homogenized in assay buffer (125 mmol l^{-1} potassium phosphate, 1.25 mmol l^{-1} EDTA, pH 7.5) using a BulletBlender Storm24 from Next Advance (Averill, NY, USA) at a 2:1 (w/w) bead to tissue ratio. The resulting homogenates were centrifuged at 21,000 ***g*** for 15 min. Supernatants were collected and, if not used immediately, stored at –70°C. The assay was performed in assay buffer at a final volume of 200 µl containing 0.15 mmol l^{-1} DTNB, 1.5 mmol l^{-1} GSSG and 0.215 mmol l^{-1} NADPH. Ten microliters of tissue supernatants were used for each sample. Reactions were initiated by the addition of NADPH and absorbance values were recorded every 2 min over a 20 min time period. Samples run in the absence of GSSG served as controls.

Enzymatic activities of both glutathione peroxidase (EC 1.11.1.9) and GST (EC 2.5.1.18) were quantified as previously described (Grim et al., 2013), with the following exceptions: (1) all assays were conducted at 5°C and in 96-well microplates and (2) the concentration of 1-chloro-2,4-dinitrobenzene (CDNB) used in the GST assay was 0.83 mmol l^{-1}.

Levels of glutathione peroxidase 4 (GPx4) (EC 1.11.1.12) were quantified using western blot analysis. Tissue samples were homogenized at 10% (w/v) in an ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mmol l^{-1} NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 50 mmol l^{-1} Tris, pH 8.0) using a Tenbroeck ground-glass homogenizer. Homogenates were centrifuged at 21,000 ***g*** for 15 min at 4°C. The resulting supernatants were used immediately or stored at −80°C for future use. Protein concentrations of samples were determined using the bicinchoninic acid method (Smith et al., 1985). For each sample, 40 µg protein was diluted with RIPA buffer to a volume of 10 µl. Laemmli buffer (5 µl) containing 5% β-mercaptoethanol was added and the sample mixture was heated at 96°C for 10 min. Ten microliters of each sample, or molecular weight standard (Precision Plus Protein), was added to one of each lanes in a 10-well Mini-Protean TGX precast gel and proteins were separated at 200 V. The running solution was a standard SDS-PAGE buffer (192 mmol l^{-1} glycine, 0.1% w/v SDS, 25 mmol l^{-1} Tris, pH 8.3). Following electrophoresis, gels were rinsed several times and transferred to PVDF membranes using a transfer buffer (SDS-PAGE buffer described above containing 20% methanol). Proteins were transferred using a mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories) at 1.3A/1 mini gel or 2.5A/2 mini gels and a maximum of 25V for 7 min. PVDF membranes were rinsed several times and then incubated for 1 h at room temperature (or overnight at 4°C) in blocking buffer (0.5 mmol l^{-1} NaCl, 1% casein, 20 mmol l^{-1} Tris, pH 7.4). Blocked membranes were rinsed briefly and then incubated for 1 h at room temperature in an antibody solution (20-fold dilution of the blocking buffer described above) containing a 1:1000 dilution of primary antibody (ab40993; Abcam, Cambridge, MA, USA). This solution was removed and the membranes rinsed briefly, after which the membranes were washed four times for 5 min each in blocking buffer. Washed membranes were next incubated for 1 h at room temperature in an antibody solution containing a 1:3000 dilution of secondary antibody (ab6721; Abcam). The secondary antibody solution was removed and the membranes rinsed and washed as described above. Antibody binding was visualized using a horseradish peroxidase

(HRP) detection kit (Pierce ECL2 Western Blotting Substrate, ThermoFisher Scientific, Waltham, MA, USA). Two milliliters of final HRP substrate solution were added to cover each membrane and the membrane was immediately placed in a Bio-Rad ImageDoc photo imager. The signal was read every 20 s for 3 min. GPx4-specific bands were analyzed using GelAnalyzer 2010 after removing the non-specific background with ImageJ. Individual samples from all species were run on a single gel for comparison purposes and each sample was run in duplicate. Proportions of sample GPx4 were quantified as a percent of total GPx4 from each gel.

Total antioxidant power

Total antioxidant power was measured using a colorimetric microplate assay kit from Oxford Biomedical Research (TA02; Oxford, MI, USA) according to the manufacturer's instructions. Values for total antioxidant power are expressed as Trolox equivalents (μmol g^{-1} wet mass of tissue).

Levels of ubiquitinated proteins

Levels of ubiquitinated protein were measured based on the methods used by Hofmann and Somero (1995) and modified by Todgham et al. (2007). Frozen tissues were finely chopped on an ice-cold stage and then homogenized in five volumes of ice-cold homogenization buffer [4% SDS (w/v), 1 mmol l^{-1} EDTA, 50 mmol l^{-1} Tris-HCl, pH 6.8] supplemented with protease inhibitors (c*O*mplete Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN, USA) using a Tissuemizer homogenizer (Tekmar, Cincinnati, OH, USA). Homogenization was completed using Tenbroeck ground-glass tissue homogenizers. Tissue homogenates were boiled for 5 min to denature proteins. Homogenates were then centrifuged at 12,000 ***g*** for 15 min at room temperature and the supernatant was retained. Protein content of the supernatant was determined using a Bradford protein assay (1976) with BSA used for the standard curve. Supernatants were stored at −80°C.

Samples were diluted with Tris-buffered saline solution (TBS) (20 mmol l^{-1} Tris-HCl, 140 mmol l^{-1} NaCl, pH 7.6) to a concentration of 0.5 μg μl^{-1} for ventricle and pectoral adductor samples, and to 0.25 μg μl^{-1} for liver samples. One μl of each sample was pipetted onto a 12×10 cm sheet of 0.2 μm nitrocellulose membrane (GE Healthcare Bio-Sciences) in triplicate. The protein was heat-fixed to the membrane at 65°C for 20 min. The membrane was then blocked with 5% non-fat milk powder dissolved in Tween-20 Tris-buffered saline solution (TTBS) (20 mmol l^{-1} Tris-HCl, 140 mmol l^{-1} NaCl, 0.01% Tween-20, pH 7.6, room temperature) for 1 h. After blocking, the membranes were rinsed twice briefly with TTBS and then three times for 5 min each with TTBS. The membranes were incubated at 4°C with the ubiquitin conjugate primary antibody (mono- and poly-ubiquitinylated conjugates monoclonal antibody produced in mice; Enzo Life Sciences, BML-PW8810, Farmdale, NY, USA) diluted 1:5000 in 5% non-fat milk powder dissolved in TTBS. Incubation times were 12.5 h for ventricle, 15 h for pectoral adductor and 2 h for liver samples. The membranes were then rinsed briefly twice with TTBS and then rinsed three times for 5 min each with TTBS. The membranes were incubated at room temperature with the secondary antibody (rabbit anti-Mouse IgG peroxidase antibody A9044; Sigma-Aldrich) diluted 1:10,000 in 5% non-fat dry milk powder in TTBS. Membranes with ventricle samples were incubated for 1.5 h whereas those with pectoral adductor samples and liver samples were incubated for 2 and 2.25 h, respectively. The membranes were then rinsed briefly twice with TTBS and then washed three times for 5 min each in TTBS and developed using a chemiluminescence kit (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare Bio-Sciences) according to the manufacturer's specifications. Chemiluminescence was detected for 15 min using an AlphaImager 3300 Imaging System (Protein Simple, San Jose, CA, USA) and quantified using ImageQuant TL software (GE Healthcare Bio-Sciences). Levels of ubiquitinated proteins were normalized to levels in the heart ventricles of *N. coriiceps* (n=6) and were blotted in triplicate on membranes for measurements in the heart ventricle and in duplicate on membranes for measurements in the pectoral adductor and liver.

20S proteasome activity

Activity of the 20S proteasome was measured based on the method developed by Shibatani and Ward (1995) and adapted for use in fish by Dobly et al. (2004). Frozen tissues were finely chopped on an ice-cold stage and then homogenized in five volumes of ice-cold lysis buffer (50 mmol l^{-1} Tris-HCl, 0.1 mmol l^{-1} EDTA, 1.0 mmol l^{-1} β-mercaptoethanol, pH 8.0) using a Tissuemizer homogenizer (Tekmar) and Tenbroeck ground-glass tissue homogenizers. The homogenate was centrifuged at 20,000 ***g*** for 1 h at 4°C and the supernatant was retained. Protein content of the supernatant was determined using a Bradford protein assay (1976) with BSA used for the standard curve.

Proteasome activity was measured using the proteasome-specific fluorogenic substrate LLVY-7-amino-4-methlycoumarin (LLVY-AMC; Enzo). The substrate was dissolved in DMSO (5.71 mmol l^{-1}), then aliquoted and stored at −80°C until use. Although maximal activity of the 20S proteasome has been shown to require SDS (Shibatani and Ward, 1995), we found, after testing for activity in SDS concentrations ranging from 0 to 0.025%, that the maximal activity of the 20S proteasome was obtained in Tris buffer lacking SDS and, thus, we omitted it from the reaction mixture. Activity was measured by incubating 50 μg protein from the supernatant with 40 μmol l^{-1} LLVY-AMC in 22.5 μl 100 mmol l^{-1} Tris-HCl (pH 8.0) for 60 min at 5°C. The reaction was determined to be linear for 90 min. The reaction was stopped by adding 225 μl of 0.1 mol l^{-1} sodium borate (pH 9.1) and 65 μl 1% SDS. Fluorescence of AMC was determined at excitation or emission wavelengths of 380 and 460 nm, respectively, on a Gemini EM Microplate Reader (Molecular Devices). Parallel samples were prepared by adding the proteasome inhibitor MG-132 (133 μmol l^{-1}; Enzo) prior to incubation. Activity was calculated by determining the concentration of AMC in the samples using the standard curve minus the activity in the presence of MG-132. Activity is expressed as pmol AMC h^{-1} 50 μg^{-1} protein. The standard curve was prepared using eight AMC concentrations between 3.3 and 44 μmol l^{-1}. The samples and standard curve were measured in triplicate.

Statistical analysis

Differences in the mean values of antioxidants, pro-oxidants, ubiquitinated proteins and 20S proteasome activity among species and within each tissue were determined using a one-way ANOVA followed by a Tukey's *post hoc* test. Equal variance was confirmed and normality tested using a Shapiro–Wilk test. A Kruskal–Wallis test followed by a Dunn's test on ranked data was used to determine differences among species for data that failed to meet the assumptions of the ANOVA. Outliers were identified by calculating quantiles and samples with values outside the third quantile were removed from the analysis of proteasome activity and levels of ubiquitinated proteins. Simple linear regressions were used to assess correlations between pro-oxidants and oxidative damage

Table 1. Parameters measured in the blood of notothenioids

	Chaenocephalus aceratus (–Hb/–Mb)	*Champsocephalus gunnari* (–Hb/–Mb)	*Chionodraco rastrospinosus* (–Hb/+Mb)	*Notothenia coriiceps* (+Hb/+Mb)	*Gobionotothen gibberifrons* (+Hb/+Mb)
Heme (mmol l^{-1})	0.04±0.01^{a} (6)	0.25±0.01^{a,b} (6)	0.09±0.05^{a} (6)	5.53±0.55^{b} (9)	5.55±0.25^{b} (16)
Hb (mg ml^{-1})				119.81±11.03* (8)	95.54±2.81 (16)
Hct (%)				32.91±1.78 (8)	37.02±1.16 (16)

Hb, hemoglobin; Hct, hematocrit; Mb, myoglobin. Values are means±s.e.m. Different superscript letters indicate significant differences as determined by a Dunn's *post hoc* test (P<0.05). The asterisk indicates a significant difference based on a Student's *t*-test (P<0.05). The number of individuals (n) is indicated in parentheses.

(levels of TBARS and carbonyls) in each tissue. Significance was set at P<0.05. ANOVA tests and regression analyses were conducted using Sigma Plot 11.0 (Systat Software, San Jose, CA, USA) and JMP 7 (SAS Institute, Cary, NC, USA); all other analyses were conducted using R (www.r-project.org).

To characterize the clustering of antioxidants among species for each tissue, we conducted a principal component analysis (PCA) after imputing missing values (Josse and Husson, 2013) followed by a hierarchical clustering on principal components (HCPC) using the PCA and HCPC functions from the FactoMineR R package, respectively. The first five components of the PCA were used as input for the HCPC, and the number of clusters retained was determined by looking at how species clustered on the PCA plots. Using only the first five components allows us to separate the noise from the structure of the data before performing the HCPC. A χ^2 test was performed between species and groups (clusters) of individuals. Finally, for each group, the group and overall mean value of each antioxidant were compared by calculating a statistic corresponding to their standardized difference, which follows a standard normal distribution under the null hypothesis that the means are equal. Individuals that were missing more than four of the nine antioxidant measurements were excluded, and individuals missing values for antioxidants (17 for liver, 14 for adductor muscle and heart ventricle) were imputed using the regularized iterative PCA algorithm (Josse and Husson, 2013), resulting in a sample size of 38 for each tissue.

RESULTS

Pro-oxidants

Levels of heme were significantly higher in the blood of red-blooded fishes than in the blood of most icefishes, except for the icefish species *C. gunnari* (Table 1). Mb levels were 3.4-fold higher in the heart ventricles of red-blooded *N. coriiceps* than in red-blooded *G. gibberifrons* and white-blooded *C. rastrospinosus* (Table 2). Although Hct levels were similar in the two red-blooded species, Hb levels were higher in *N. coriiceps* than in *G. gibberifrons* (Table 1).

Maximal activity of CS, a measure of flux through the citric acid cycle, was lowest in the heart of white-blooded *C. aceratus* (Table 2). However, low activity of CS in the *C. aceratus* ventricle was not associated with the lack of both oxygen-binding proteins, Hb and Mb, because CS activity in the hearts of the icefish *C. gunnari*, a species which lacks Mb, was similar to that in the hearts of red-blooded notothenioids that express Mb (Table 2). In the pectoral adductor muscle, CS activity was highest in the two red-blooded fishes and in the icefish *C. gunnari* (Table 3). CS activity in the liver was highest in *C. gunnari* (Table 4).

Oxidative damage

The ANOVA indicated that differences in levels of both oxidized proteins (carbonyls) and lipids (TBARS normalized to total lipid) did not always correspond with the presence or absence of Hb and Mb (Tables 2–4). In the heart ventricle, TBARS were lower in red-blooded fishes compared with icefishes, whereas protein carbonyls were lowest in the icefish *C. aceratus* (Table 2). Levels of protein carbonyls and TBARS were higher in the tissues of red-blooded fishes compared with icefishes only in oxidative skeletal muscle tissue (Table 3). In the liver, TBARS were highest in *C. gunnari* and *G. gibberifrons*, and protein carbonyls were highest in *C. gunnari* and *C. aceratus* (Table 4).

Although neither the presence nor the absence of Hb and Mb corresponded with levels of oxidative damage, regression analyses

Table 2. Levels of pro-oxidants, antioxidants and oxidized macromolecules in the heart ventricles of notothenioids

	C. aceratus (–Hb/–Mb)	*C. gunnari* (–Hb/–Mb)	*C. rastrospinosus* (–Hb/+Mb)	*N. coriiceps* (+Hb/+Mb)	*G. gibberifrons* (+Hb/+Mb)
Pro-oxidants					
CS (U g^{-1} wet mass)	9.32±0.17^{a} (5)	12.93±0.92^{b} (5)	9.72±0.63^{a,c} (4)	12.58±0.75^{b,c} (5)	10.75±0.62^{a,b,c} (5)
Mb (mg g^{-1})			0.95±0.20^{a} (6)	3.57±0.30^{b} (6)	0.96±0.12^{a} (5)
Antioxidants					
SOD (U g^{-1})	1836.63±105.77^{a} (8)	1692.25±86.56^{a} (8)	1811.83±150.92^{a} (6)	2278.75±99.37^{b} (8)	2708.53±80.14^{c} (8)
CAT (μmol min^{-1})	142.69±15.20^{a} (8)	260.86±9.40^{a,b} (8)	269.23±16.10^{a,b} (6)	461.66±42.15^{b} (9)	243.27±43.20^{a,b} (7)
GST (U g^{-1})	1.13±0.10^{a} (8)	0.85±0.07^{a} (8)	0.67±0.10^{b} (6)	1.10±0.12^{a,b} (9)	1.13±0.10^{a} (8)
TAP (μmol g^{-1})	6.65±0.19^{a,c} (8)	6.42±0.35^{a} (8)	5.99±0.12^{a} (6)	8.26±0.27^{b} (9)	7.59±0.29^{b,c} (8)
GPx1 (U g^{-1})	0.84±0.07^{a,b} (8)	1.06±0.10^{a} (8)	0.55±0.12^{b} (6)	0.96±0.04^{a,b} (8)	0.83±0.10^{a,b} (7)
GPx4 (% of total)	19.04±3.83 (6)	16.74±1.77 (6)	18.46±5.71 (6)	21.65±2.54 (6)	18.86±1.73 (6)
GR (mU g^{-1})	280.89±15.74^{a} (8)	177.93±13.65^{b} (8)	235.75±14.73^{a,b} (6)	297.95±22.07^{a} (8)	461.06±17.57^{c} (8)
2GSSG:GSH	0.097±0.008^{a} (8)	0.148±0.009^{b} (8)	0.119±0.007^{a,b} (6)	0.112±0.016^{a} (8)	0.109±0.006^{a,b} (8)
Oxidative damage					
TBARS (nmol g^{-1} lipid)	132.67±20.40^{a} (8)	167.05±10.69^{a} (8)	142.80±16.71^{a} (6)	81.82±7.40^{b} (8)	76.93±4.40^{b} (9)
Carbonyls (mmol mol^{-1})	17.44±5.15^{a} (7)	85.56±17.02^{b} (8)	43.40±10.54^{a,b} (6)	33.50±3.67^{a,b} (7)	73.38±12.77^{b} (7)

Values are means±s.e.m. Different superscript letters indicate significant differences as determined by a one-way ANOVA or Kruskal–Wallis test followed by a Tukey's or Dunn's *post hoc* test (P<0.05). The number of individuals (n) is indicated in parentheses.

Table 3. Levels of pro-oxidants, antioxidants and oxidized macromolecules in the pectoral adductor muscle of notothenioids

	C. aceratus (–Hb/–Mb)	*C. gunnari* (–Hb/–Mb)	*C. rastrospinosus* (–Hb/+Mb)	*N. coriiceps* (+Hb/+Mb)	*G. gibberifrons* (+Hb/+Mb)
Pro-oxidants					
CS (U g^{-1} wet mass)	13.40±1.69^{a} (6)	23.13±1.18^{b} (4)	15.44±1.40^{a} (4)	26.13±1.67^{b} (5)	22.30±1.85^{b} (5)
Antioxidants					
SOD (U g^{-1})	1513.13±170.99^{a} (8)	2500.36±127.71^{b} (8)	1810.50±203.29^{c} (6)	2031.88±107.19^{a,b,c} (8)	2271.35±108.32^{b,c} (8)
CAT (μmol min^{-1})	131.31±13.34^{a} (8)	243.04±8.42^{b} (8)	324.54±22.27^{c} (6)	409.24±19.90^{d} (8)	218.46±17.67^{b} (7)
GST (U g^{-1})	0.97±0.13 (8)	1.34±0.17 (8)	1.11±0.19 (6)	1.57±0.12 (8)	1.54±0.27 (8)
TAP (μmol g^{-1})	5.37±0.39 (8)	4.61±0.24 (8)	5.60±0.48 (6)	4.59±0.60 (8)	5.66±0.49 (8)
GPx1 (U g^{-1})	0.55±0.07^{a,b} (8)	0.85±0.06^{c} (8)	0.29±0.06^{a} (6)	0.64±0.10^{b,c} (8)	0.66±0.06^{b,c} (8)
GPx4 (% of total)	8.89±0.49^{a} (6)	20.64±2.32^{b} (6)	15.48±0.99^{b} (6)	27.70±1.58^{c} (6)	28.09±1.71^{c} (6)
GR (mU g^{-1})	127.29±10.52^{a} (8)	134.67±10.48^{a,b,c} (8)	111.16±6.58^{b} (6)	286.48±17.15^{d} (8)	177.37±7.19^{c} (8)
2GSSG:GSH	0.131±0.007^{a} (8)	0.172±0.008^{a,b} (8)	0.181±0.020^{b} (6)	0.138±0.008^{a,b} (8)	0.143±0.010^{a,b} (8)
Oxidative damage					
TBARS (nmol g^{-1} lipid)	50.79±4.40^{a} (8)	75.52±6.33^{a,b} (8)	74.38±12.73^{a,b} (6)	240.19±42.19^{b,c} (8)	279.22±15.95^{c} (8)
Carbonyls (mmol mol^{-1})	4.77±0.88^{a} (7)	5.70±1.21^{a,c} (6)	7.15±1.70^{a,b,c} (5)	41.98±9.99^{b} (7)	25.38±3.53^{b,c} (6)

Values are means±s.e.m. Different superscript letters indicate significant differences as determined by a one-way ANOVA or Kruskal–Wallis test followed by a Tukey's or Dunn's *post hoc* test (P<0.05). The number of individuals (n) is indicated in parentheses.

indicated that maximal activity of CS was significantly and positively correlated with levels of protein carbonyls and TBARS in both oxidative skeletal muscle (Fig. S2A,B) and the liver (Fig. S3A,B) but not in the heart ventricle (Fig. S1A,B). Heme was also positively correlated with TBARS and carbonyls in oxidative skeletal muscle (Fig. S2C,D).

Antioxidants

The ANOVA indicated that antioxidants (capacities or levels) were higher in red-blooded fishes than in icefishes (Tables 2–4). However, in the pectoral adductor muscle, activities of SOD and GPx1 were higher in *C. gunnari* than in other icefishes, and equivalent to those in the two red-blooded species (Table 3). Also, in the liver, activities of SOD and CAT were higher in *C. gunnari* than in other icefishes and similar to those in *G. gibberifrons* (SOD) (Table 4).

We used PCA followed by hierarchical clustering analysis to identify differences in antioxidant levels among species. The χ^2 analysis indicated that there was a significant difference in antioxidant levels among the species in all tissues (P<0.001; Tables S1–S3). Components 1 and 2 of the PCA analysis explained most of the variation among the species in antioxidant levels (37–40% and 16–17%, respectively) (Fig. 1). The PCA plot for the pectoral muscle is shown for components 1 and 3 because this aligned most closely with the results from the hierarchical clustering. There was a small difference (4%) between the variation explained by components 2 and 3 in this tissue (Fig. 1B). Only in the heart ventricle were levels of antioxidants similar in the three species of icefishes and lower than in the two red-blooded species (Fig. 1A, Table S1). In the pectoral muscle and liver, antioxidant levels in the two icefishes *C. aceratus* and *C. rastrospinosus* were similar to each other (Fig. 1B,C) and lower than in the two red-blooded species (Tables S2, S3). However, in the liver and pectoral muscle of the icefish *C. gunnari*, antioxidant levels were different from those in the other icefishes as well as those in the red-blooded species, with some antioxidants higher than the overall mean and some lower (Fig. 1B,C, Tables S2, S3). Notably, SOD activities in the *C. gunnari* pectoral muscle and liver were higher than the overall mean (Tables S2, S3), which corresponds with the high activity of CS in these tissues (Tables 3, 4).

Levels of ubiquitinated proteins

Levels of ubiquitinated proteins were higher in the heart ventricles of the red-blooded fishes *N. coriiceps* and *G. gibberifrons* than in those of icefishes, and levels were higher in the heart of *N. coriiceps* than in that of *G. gibberifrons* (Fig. 2A). There was no difference between levels of ubiquitinated proteins in the heart ventricles of icefishes that express Mb compared with those that do not. In the

Table 4. Levels of pro-oxidants antioxidants and oxidized macromolecules in the livers of notothenioids

	C. aceratus (–Hb/–Mb)	*C. gunnari* (–Hb/–Mb)	*C. rastrospinosus* (–Hb/+Mb)	*N. coriiceps* (+Hb/+Mb)	*G. gibberifrons* (+Hb/+Mb)
Pro-oxidants					
CS (U g^{-1} wet mass)	0.29±0.01^{a} (5)	0.79±0.11^{b} (5)	0.40±0.05^{a} (5)	0.42±0.03^{a} (5)	0.38±0.03^{a} (5)
Antioxidants					
SOD (U g^{-1})	4492.63±376.14^{a} (8)	9542.63±338.19^{b} (8)	6090.67±246.47^{c} (6)	6847.75±357.20^{c} (8)	8713.80±168.19^{b} (8)
CAT (μmol min^{-1})	1563.06±195.11^{a} (7)	3880.22±488.35^{b} (6)	1806.98±308.02^{a} (6)	5460.75±342.61^{c} (9)	5526.70±327.11^{c} (7)
GST (U g^{-1})	13.20±2.45^{a} (8)	15.92±1.88^{a,b} (8)	13.41±2.03^{a,b} (6)	20.68±1.67^{a,b,c} (8)	29.97±1.54^{c} (8)
TAP (μmol g^{-1})	4.87±0.27^{a} (8)	4.82±0.69^{a,b} (8)	4.06±1.40^{a,b} (6)	7.84±0.54^{b} (8)	6.89±0.67^{a,b} (8)
GPx1 (U g^{-1})	0.48±0.11 (8)	0.46±0.06 (8)	0.60±0.10 (6)	0.70±0.06 (8)	0.66±0.05 (8)
GPx4 (% of total)	19.78±1.60 (6)	21.75±1.94 (6)	18.30±2.19 (6)	19.67±1.50 (6)	20.49±2.32 (6)
GR (mU g^{-1})	241.16±31.06^{a} (8)	116.66±18.77^{b} (8)	245.45±26.55^{a} (6)	276.97±31.10^{a} (8)	397.83±31.53^{a} (8)
2GSSG:GSH	0.192±0.020^{a} (8)	0.171±0.017^{a} (8)	0.211±0.024^{a,b} (6)	0.285±0.030^{b} (8)	0.203±0.028^{a,b} (8)
Oxidative damage					
TBARS (nmol g^{-1} lipid)	117.66±13.80^{a} (8)	245.38±18.78^{b} (8)	94.93±28.91^{a} (6)	114.13±27.09^{a} (8)	149.98±9.86^{a,b} (8)
Carbonyls (mmol mol^{-1})	19.78±4.20^{a,b} (6)	30.14±7.81^{a} (8)	4.43±1.19^{c} (6)	5.61±1.24^{b,c} (6)	8.04±3.46^{b,c} (6)

Values are means±s.e.m. Different superscript letters indicate significant differences as determined by a one-way ANOVA or Kruskal–Wallis test followed by a Tukey's or Dunn's *post hoc* test (P<0.05). The number of individuals (n) is indicated in parentheses.

Journal of Experimental Biology (2018) 221, jeb162503. doi:10.1242/jeb.162503

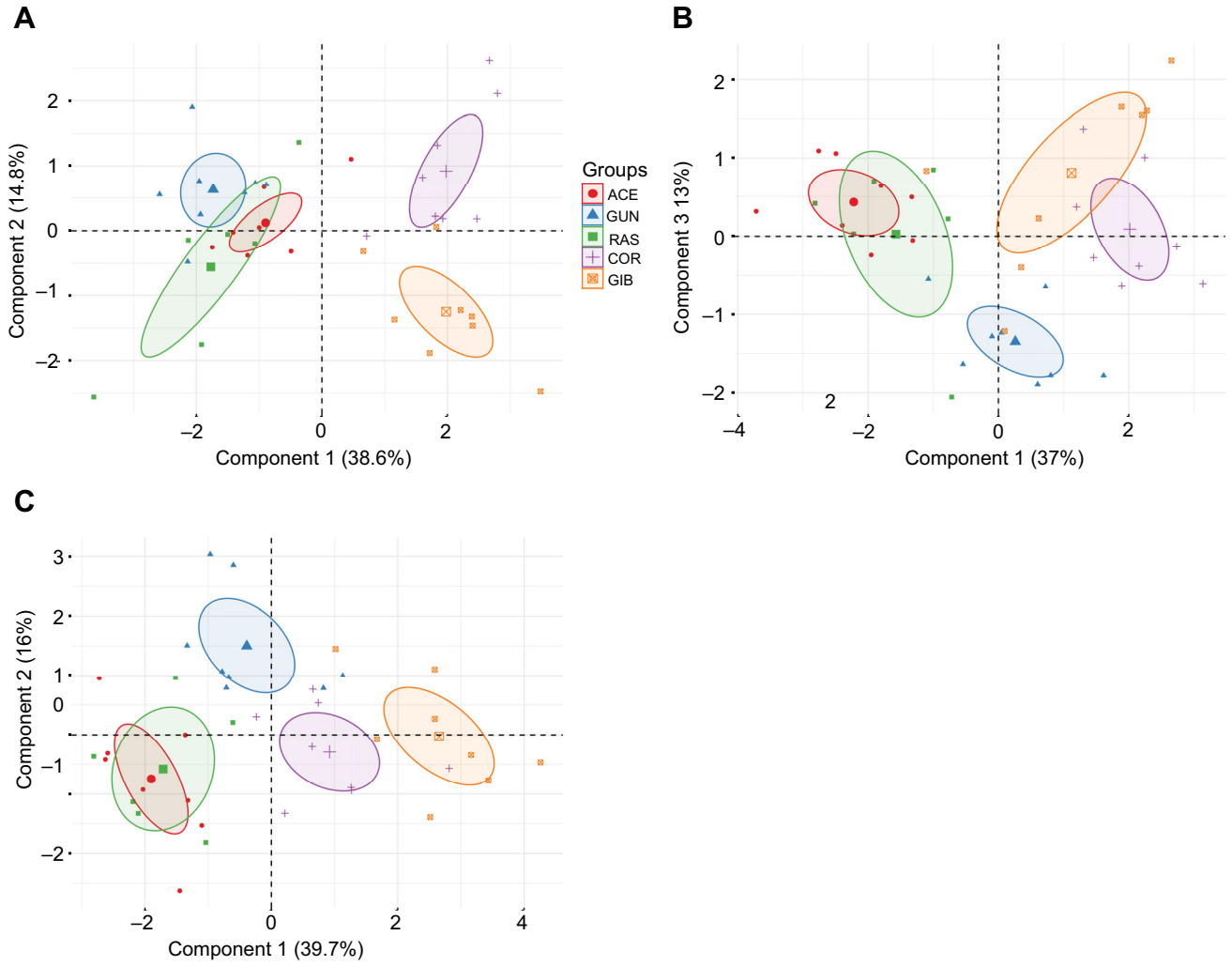

Fig. 1. Antioxidants in the heart ventricle, pectoral adductor and liver of notothenioids. Distribution of antioxidant levels of individual fish by species (ACE: *Chaenocephalus aceratus*, GUN: *Champsocephalus gunnari*, RAS: *Chionodraco rastrospinosus*, COR: *Notothenia coriiceps*, GIB: *Gobionotothen gibberifrons*), over principal components 1 and 2 for (A) heart ventricle and (C) liver, and 1 and 3 for (B) pectoral adductor muscle. The clustering of antioxidants is visualized by 0.95 confidence ellipses around the barycenter for each species. Individuals that were missing more than four of the nine antioxidant measurements were excluded, and individual missing values for antioxidants (17 for liver, 14 for adductor muscle and heart ventricle) were imputed using the regularized iterative PCA algorithm (Josse and Husson, 2013), resulting in a sample size of 38 for each tissue.

pectoral adductor muscle, levels of ubiquitinated proteins were highest in *N. coriiceps* (1.6–2.4-fold higher than in other species). Levels of ubiquitinated proteins were mostly similar in the other species, with the exception that levels were lower in *C. rastrospinosus* than in *C. aceratus* (Fig. 2B). Similar to the heart ventricle, levels of ubiquitinated proteins were higher in the livers of the two red-blooded species than in those of the icefishes except *C. aceratus* (Fig. 2C).

20S proteasome activity

In the heart ventricle, the chymotrypsin-like activity of the 20S proteasome did not show a clear trend associated with the expression of oxygen-binding proteins. The only significant differences in proteasome activity were between the two red-blooded species and the icefish *C. aceratus*, with the activity being on average 1.6-fold higher in the hearts of the red-blooded fishes (Fig. 3A). In the pectoral muscle, 20S proteasome activity was mostly similar in all species, although activity was higher in *C. aceratus* than in *N. coriiceps* (Fig. 3B). In the liver, 20S proteasome activity was highest in *C. gunnari* and was 1.7–2.9-fold higher than in the other species (Fig. 3C). There was no significant difference in 20S proteasome activity in the liver between red-blooded notothenioids and the icefishes *C. aceratus*, *P. georgianus* and *C. rastrospinosus* (Fig. 3C).

DISCUSSION

We had originally predicted that the loss of the iron-centered oxygen-binding proteins, Hb and Mb, would result in lower levels of oxidized biological macromolecules, capacities of antioxidants and rates of protein degradation by the 20S proteasome. Our data demonstrate that levels of oxidized proteins and lipids and capacities for antioxidant activity are indeed lower in some tissues of Hb-less species, yet this pattern is not observed in all tissues, nor does the activity of the 20S proteasome show a trend with the expression of Hb and Mb. Neither Hb nor Mb is associated with oxidative damage across tissues. However, the activity of CS is positively correlated with levels of oxidized proteins and lipids in both the pectoral adductor muscle and the liver (Figs S2–S3).

Journal of Experimental Biology (2018) 221, jeb162503. doi:10.1242/jeb.162503

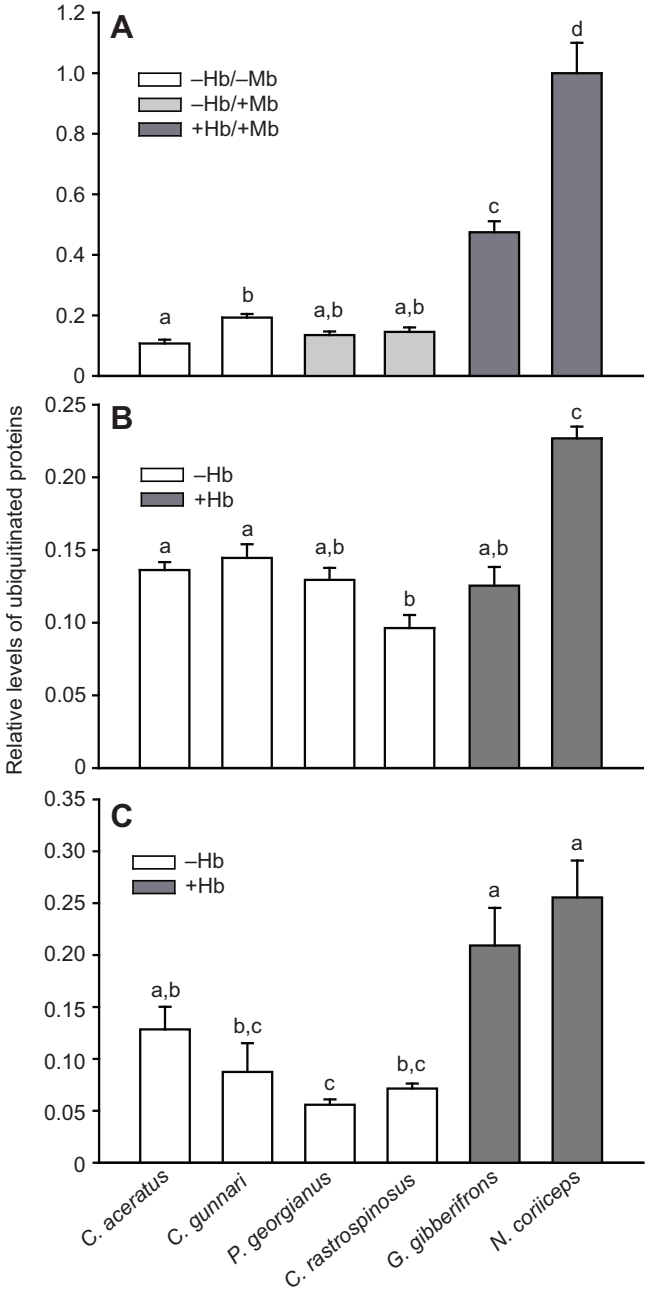

Fig. 2. Levels of ubiquitinated proteins in tissues of notothenioids that vary in the expression of hemoglobin (Hb) and myoglobin (Mb). Relative levels of ubiquitinated proteins in the (A) heart ventricle, (B) pectoral adductor muscle and (C) liver of six species of notothenioids. Levels of ubiquitinated proteins were normalized to levels in the heart ventricle of *N. coriiceps*. Values are means±s.e.m. (n=6). Significant differences among species and within each tissue are indicated by different letters (P<0.05).

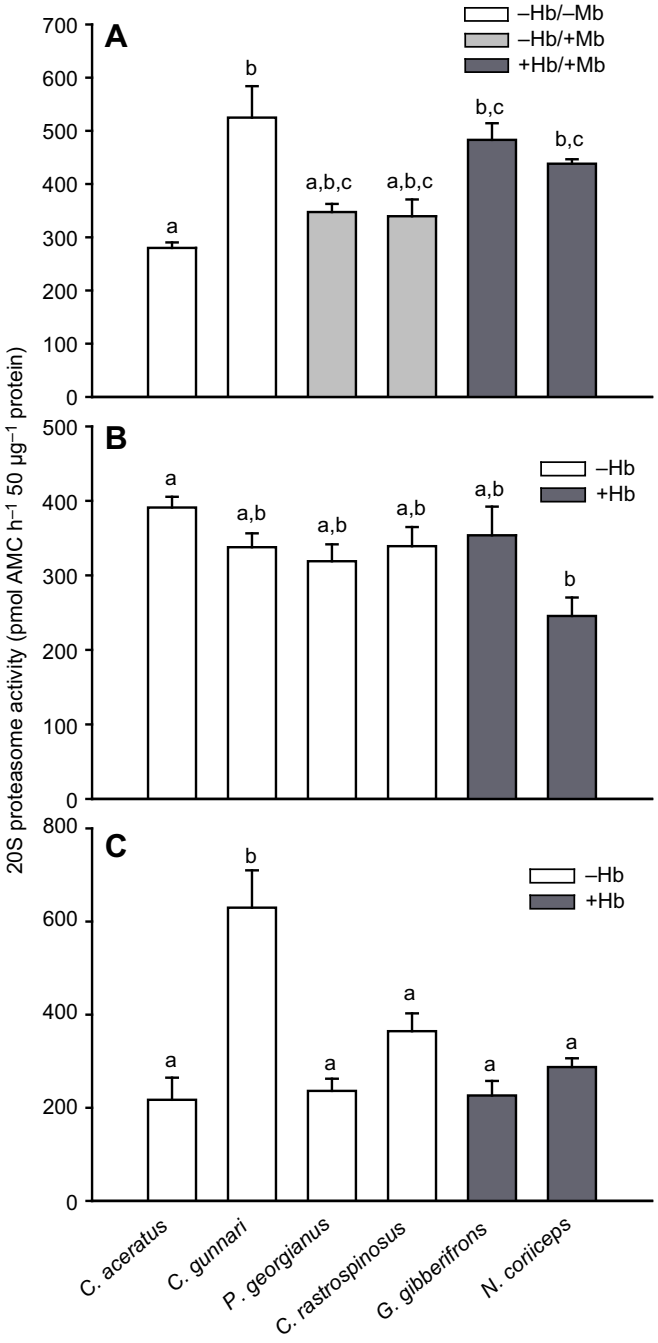

Fig. 3. Activity of the 20S proteasome in tissues of six species of notothenioids that vary in the expression of Hb and Mb. (A) Heart ventricle, (B) pectoral adductor muscle and (C) liver. Values are means±s.e.m. (n=6–8). Significant differences among species and within each tissue are indicated by different letters (P<0.05). AMC, 7-amino-4-methylcoumarin.

Heart ventricle: evidence that Mb does not amplify oxidative damage

Mb stores intracellular oxygen and may facilitate its transport to mitochondria (Wittenberg, 1970). The importance of cardiac Mb under normoxic conditions in teleost fishes is equivocal, as evidenced by *in vitro* studies (Canty and Driedzic, 1987) and the widespread loss of Mb among teleost species (Grove and Sidell, 2002; Macqueen et al., 2014). Under hypoxic conditions, Mb is clearly advantageous and has been shown to be essential for maintaining cardiac performance (Bailey and Driedzic, 1992; Driedzic, 1983). The loss of Mb expression has occurred four times during the radiation of the Channichthyidae family, suggesting weakened selective pressure maintaining the Mb gene (Moylan and Sidell, 2000). The oxygen-rich environment of the Southern Ocean is likely to have relaxed selective pressure to maintain Mb expression, making the loss of Mb a neutral mutation, and yet *in vitro* studies have shown that Mb enhances cardiac performance in notothenioids at high afterload pressures (Acierno et al., 1997). Alternatively, if Mb contributes to ROS formation, perhaps through the reaction of H_2O_2 with MetMb, forming a ferryl

species and tyrosine peroxyl radical (Newman et al., 1991), loss of Mb may be beneficial, thereby reducing selective pressure to maintain the protein. Heart ventricles of *N. coriiceps* have a Mb content 3.4-fold greater than those of *G. gibberifrons* and *C. rastrospinosus*; however, levels of oxidized and ubiquitinated proteins are similar among red- and white-blooded species, and levels of TBARS are lower in the two red-blooded species compared with icefishes, arguing against a redox advantage conferred by the loss of Mb. As additional support for this, the PCA and hierarchical clustering analyses show that the presence of Mb in the heart of the icefish *C. rastrospinosus* is not associated with higher levels of antioxidants compared with icefishes that lack the protein (Fig. 1A, Table S1).

Oxidative damage in skeletal muscle reflects activity of CS, Hb and heme levels

Maximal activities of CS, a regulatory enzyme of the Krebs cycle, is often used as a marker for oxidative capacity per gram of tissue and mitochondrial volume density (e.g. Lucassen et al., 2003; Orczewska et al., 2010) and, as the majority of ROS are produced by mitochondria (Boveris and Chance, 1973), CS activity can also be used to infer capacity for ROS production. Levels of oxidized proteins and lipids are threefold to eightfold higher in the pectoral adductor muscle of red-blooded notothenioids compared with icefishes, and correspond with the higher activities of CS in red-blooded species compared with most icefishes (Table 3). The approximately twofold higher maximal activity of CS in oxidative skeletal muscle of red-blooded fishes compared with most icefishes makes it tempting to conclude that elevated capacity for oxidative metabolism is a factor contributing to the high levels of oxidative damage in this particular tissue.

We suggest, however, that this is probably not the case because the icefish *C. gunnari* displays CS activity similar to that of the red-blooded species but maintains low levels of oxidized proteins. We posit that a more probable explanation is that ROS produced by the electron transport chain (ETC) are amplified in reactions mediated by iron. This is supported by the observation that heme levels are associated with oxidative damage in the tissues of some species (e.g. pectoral adductor muscle for *G. gibberifrons* and *N. coriiceps*) (Table 3; Fig. S2). The mechanism by which heme potentiates the formation of intracellular ROS is unclear, especially given that transcript levels of haptoglobin, ferritin, serotransferrin and hepcidin, which are involved in Fe transport and sequestration, are higher in the Antarctic fish *Dissostichus mawsoni* than in temperate species (Chen et al., 2008). Also, in our previous work, we found that tissue levels of the proteins ferritin and ceruloplasmin were generally higher in red-blooded species than in icefishes, although transferrin levels were no different among species (Kuhn et al. 2016).

Despite significantly higher levels of some antioxidants (GR and GPx4) in the pectoral adductor muscle of *N. coriiceps* and *G. gibberifrons* compared with most icefishes, it appears that antioxidant levels are insufficient to prevent oxidative damage, given that levels of both protein carbonyls and peroxidized lipids are up to eightfold higher in red-blooded species compared with icefishes (Table 3). The high levels of carbonylated proteins in the pectoral adductor muscle of *N. coriiceps* are also associated with higher levels of ubiquitinated proteins compared with other species (Fig. 2B).

It is clear from our results that the high levels of oxidized proteins in the pectoral adductor of red-blooded notothenioids cannot be attributed to either heme or Hb alone. As high levels of oxidized proteins were also observed in other tissues (e.g. the liver and the heart) from animals lacking Hb, these data indicate that products of oxidative stress can also arise from other processes (Tables 1–4). Two factors that could potentially contribute to higher levels of oxidized proteins in skeletal muscle compared with both the liver and the heart, and to the differences among species, are rates of ROS formation and protein turnover. As described above, although rates of ROS formation may correlate with maximal CS activity, ROS generation is also affected by the degree of coupling between the activity of the ETC and ATP synthesis, which cannot be inferred from CS activity. Mitochondria from the liver, in general, have a lower respiratory control ratio than those from muscle (e.g. Jørgensen et al., 2012), and mitochondria from skeletal muscle have a lower RCR than those from cardiac muscle (Park et al., 2014). Thus, high levels of oxidized macromolecules in the pectoral adductor may be driven, in part, by greater rates of mitochondrial ROS production compared with those in the liver.

Rates of protein turnover may also impact steady state levels of oxidized proteins. Rates of protein synthesis in Antarctic notothenioids are approximately fivefold lower in the pectoral adductor than in the heart ventricle and approximately 20-fold lower compared with rates in the liver, consistent with the higher metabolic cost of protein synthesis in the liver compared with that in cardiac myocytes (Lewis et al., 2015; J. M. Lewis and K.M.O., unpublished data). Despite higher levels of ubiquitinated proteins in the pectoral muscle of red-blooded species compared with icefishes (Fig. 2B), rates of degradation by the 20S proteasome are lowest in *N. coriiceps* and similar in all other species (Fig. 3B). If the rates of oxidative damage are similar in tissues, lower rates of protein turnover in the pectoral muscle could result in higher levels of oxidized proteins.

Absence of Hb is not accompanied by reduction in oxidative stress in notothenioid livers

The particularly high level of oxidative damage in the liver of the icefish *C. gunnari* provides compelling evidence that the absence of oxygen-binding proteins does not confer an advantage for white-blooded notothenioids by reducing oxidative stress in hepatic tissue (Table 4). In fact, CS activity appears to be the best predictor of levels of oxidative damage in the livers of all species, with the liver of *C. gunnari* possessing the highest levels or activities of CS, protein carbonyls, TBARS and the 20S proteasome among hepatic tissues of all the species studied (Table 4, Fig. 3C).

Several antioxidants (SOD, CAT and GST) are highest in the liver, compared with other tissues, among all notothenioid tissues studied and yet the liver displays the lowest maximal activity of CS (Table 4). These data indicate that in notothenioid fishes, as in other organisms, ROS can be generated by both mitochondrial and non-mitochondrial sources. The liver contains a large complement of peroxisomes, an organelle with various oxidase enzymes, which produce a significant portion of the total cellular H_2O_2 (Boveris et al., 1972; Schrader and Fahimi, 2006). Red-blooded *G. gibberifrons* has exceptionally high capacities for peroxisomal β-oxidation (Crockett and Sidell, 1993), and the first step in this pathway is catalyzed by acyl CoA oxidase, which, like other peroxisomal oxidases, generates H_2O_2 (Lazarow and De Duve, 1976). In addition, high levels of cytochrome P450s, enzymes that detoxify xenobiotics and generate ROS as a by-product (Hrycay and Bandiera, 2012), may warrant high levels of antioxidants.

Rates of protein degradation by the 20S proteasome are not related to the expression of Hb or Mb

There is no clear trend in 20S proteasome activity with regard to expression of Hb and Mb, despite the higher levels of ubiquitinated proteins (suggesting higher levels of damaged proteins) in red-blooded notothenioids compared with icefishes (Fig. 3). For example, 20S proteasome activity in the heart ventricle of the red-blooded and red-hearted species *N. coriiceps* and *G. gibberifrons* is similar to that in the icefishes *C. gunnari*, *C. rastrospinosus* and *P. georgianus* but is lower in the pectoral adductor muscle of *N. coriiceps* than in all other species (Fig. 3A,B). In the liver, 20S proteasome activity in the icefish *C. gunnari* is 1.7–2.9-fold higher than in other notothenioids, in which proteasome activity is similar (Fig. 3C). Together, these data suggest two possibilities: either proteins are not degraded at a higher rate in red-blooded species or oxidatively modified proteins (e.g. in pectoral muscle) are degraded by enzymes other than the 20S proteasome. Rates of protein synthesis are similar in the hearts of red- and white-blooded notothenioids, and the metabolic costs of protein synthesis are similar in both red- and white-blooded species in cardiomyocytes and hepatocytes, suggesting that the rates of protein degradation do not differ either (Lewis et al., 2015). However, there are multiple pathways by which oxidized proteins may be eliminated.

Rather than being degraded by the 20S proteasome, some oxidized proteins are ubiquitinated by the combined activities of the E1, E2 and E3 ubiquitin conjugating enzymes and are targeted for degradation by the 26S proteasome (Pajares et al., 2015). Additionally, molecular chaperones may repair the unfolding and aggregation of moderately oxidized proteins (Hawkins, 1991; Wickner et al., 1999). Chaperones such as heat shock proteins 70 (Hsp70) and 90 (Hsp90) recognize and repair damaged or denatured proteins as well as non-native protein aggregates (Feder and Hofmann, 1999). It has been suggested that cold temperature denatures proteins (Todgham et al., 2007) and, consistent with this, red-blooded notothenioids from McMurdo Sound have higher constitutive levels of Hsp/Hsc70 than temperate notothenioids (Buckley et al., 2004; Hofmann et al., 2000; Place and Hofmann, 2005; Place et al., 2004). This may be an adaptation to life at cold body temperatures but perhaps also to high levels of protein oxidation in red-blooded fishes. Studies of levels of chaperones in icefishes would address this question. More recently, a novel isoform of acylpeptide hydrolase (APEH-2) has been shown to effectively hydrolyze oxidized proteins (Gogliettino et al., 2014). The relative expression and activity of APEH-2 is higher in erythrocytes of the red-blooded notothenioid *T. bernacchii* than in the erythrocyte-like cells of the icefish *Chionodraco hamatus*, suggesting that this pathway may be important for eliminating oxidized proteins in red-blooded notothenioids (Riccio et al., 2015).

Champsocephalus gunnari stands out among icefishes

The PCA and hierarchical clustering analyses of antioxidant levels clearly illustrate that redox balance in notothenioids is not attributable solely to the presence of Hb and Mb. Antioxidant levels in *C. gunnari* are dissimilar from those of both icefishes and red-blooded species in the pectoral adductor muscle and the liver, with the levels of some antioxidants higher than the overall mean, and some lower (Fig. 1B,C; Tables S2, S3). This is in contrast to the other two species of icefishes, whose antioxidant levels are consistently lower than the overall mean. Notably, the activity of SOD, the first and only line of defense against superoxide, is similar to (in pectoral muscle) or higher than (in the liver) SOD activity in red-blooded species. The majority of superoxide is produced during cellular respiration (Boveris, 1977) and, consistent with this, CS activity shows the same pattern of expression in *C. gunnari* as SOD in these two tissues. The high activity of CS in the liver and pectoral adductor muscle in *C. gunnari* most likely reflects their more active lifestyle compared with other icefish species. *Champsocephalus gunnari* is semipelagic and undergoes diurnal migrations, feeding almost exclusively on krill (Flores et al., 2004). In contrast, the two other species of icefish studied in this report are less active, benthic ambush predators, feeding on both crustaceans and other benthic fishes (Flores et al., 2004; Takahashi and Iwami, 1997).

Conclusions and perspectives

Our results suggest that the loss of Hb and Mb in Antarctic icefishes does not minimize oxidative stress. In some tissues, expression of heme and Hb is associated with high levels of oxidative damage and antioxidants, and yet, in others, the species that lacks both Hb and Mb display the highest levels of oxidatively modified macromolecules. In addition, we observe no significant differences in the rates of protein degradation (this study) or synthesis between red- and white-blooded fishes (Lewis et al., 2015). Although protein and lipid oxidation may reflect pathology in some cases, oxidation of biological molecules can also play a pivotal role in regulating protein function and cell signaling, mediating apoptosis, metabolism, cellular immunity and gene transcription (Li et al., 2016; Niki, 2009; Shadel and Horvath, 2015). At this time, it is unclear whether elevated levels of oxidized macromolecules in certain tissues of particular notothenioid species represent a deleterious condition or a variation in steady-state levels of redox mediators for cellular processes. Current studies aimed at identifying oxidatively modified proteins will shed light on this question.

Acknowledgements

This work could not have been completed without the outstanding support from the Masters and crew of ARSV *Laurence M. Gould* and the support staff at the US Antarctic Research Station, Palmer Station. Thanks to undergraduate students Elizabeth Evans (Ohio University), Erin Atkins (Ohio University) and Autumn Fish (University of Alaska) for their assistance with some of the assays. We thank an anonymous reviewer, whose suggestions markedly improved the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.M.O., E.L.C.; Methodology: K.M.O., E.L.C., J.P., M.H., R.B.; Validation: K.M.O., E.L.C.; Formal analysis: K.M.O., E.L.C., J.P., C.A.O., M.H., D.K., R.B., J.M.; Investigation: K.M.O., E.L.C., C.A.O., M.H., D.K., J.M.; Resources: K.M.O., E.L.C.; Data curation: K.M.O., E.L.C., M.H., D.K.; Writing - original draft: K.M.O., E.L.C., J.P., C.A.O., M.H., D.K., J.M.; Writing - review & editing: K.M.O., E.L.C., J.P.; Visualization: K.M.O., E.L.C., J.P., C.A.O., M.H.; Supervision: K.M.O., E.L.C.; Project administration: K.M.O., E.L.C.; Funding acquisition: K.M.O., E.L.C.

Funding

This research was supported by grants from the National Science Foundation (PLR 1043781 to K.M.O. and PLR 1043576 to E.L.C.). J.P. was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health (NIH) (P20GM103395) and by NIH/NIGMS award 1P30GM103325 (B. Boyer, P.I.). The content is solely the responsibility of the authors and does not necessarily reflect the official views of the NIH. Deposited in PMC for release after 12 months.

Data availability

Data are available from the Dryad Digital Repository (O'Brien et al., 2017): https://doi.org/10.5061/dryad.5228g

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.162503.supplemental

References

Abele, D., Heise, K., Pörtner, H.-O. and Puntarulo, S. (2002). Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam Mya arenaria. *J. Exp. Biol.* **205**, 1831-1841.

Acierno, R., Agnisola, C., Tota, B. and Sidell, B. D. (1997). Myoglobin enhances cardiac performance in antarctic icefish species that express the protein. *Am. J. Physiol.* **273**, R100-R106.

Alayash, A. I., Andersen, C. B., Moestrup, S. K. and Bulow, L. (2013). Haptoglobin: the hemoglobin detoxifier in plasma. *Trends Biotechnol.* **31**, 2-3.

Andreyev, A. Y., Kushnareva, Y. E. and Starkov, A. A. (2005). Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc.)* **70**, 200-214.

Bailey, J. R. and Driedzic, W. R. (1992). Myoglobin supported oxygen consumption in isolated rat hearts under dysoxic conditions. *J. Mol. Cell. Cardiol.* **24**, 799-807.

Beers, R. F., Jr. and Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195**, 133-140.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917.

Boveris, A. (1977). Mitochondrial production of superoxide radical and hydrogen peroxide. *Adv. Exp. Med. Biol.* **78**, 67-82.

Boveris, A. and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* **134**, 707-716.

Boveris, A., Oshino, N. and Chance, B. (1972). The cellular production of hydrogen peroxide. *Biochem. J.* **128**, 617-630.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brigelius-Flohé, R. (1999). Tissue-specific functions of individual glutathione peroxidases. *Free Radic. Biol. Med.* **27**, 951-965.

Buckley, B. A., Place, S. P. and Hofmann, G. E. (2004). Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, Trematomus bernacchii. *J. Exp. Biol.* **207**, 3649-3656.

Canty, A. A. and Driedzic, W. R. (1987). Evidence that myoglobin does not support heart performance at maximal levels of oxygen demand. *J. Exp. Biol.* **128**, 469-473.

Cassini, A., Favero, M. and Albergoni, V. (1993). Comparative studies of antioxidant enzymes in red-blooded and white-blooded antarctic teleost fish. pagothenia bernacchii and Chionodraco hamatus. *Comp. Biochem. Physiol. C* **106**, 333-336.

Chen, Z., Cheng, C.-H. C., Zhang, J., Cao, L., Chen, L., Zhou, L., Jin, Y., Ye, H., Deng, C., Dai, Z. et al. (2008). Transcriptomic and genomic evolution under constant cold in Antarctic notothenioid fish. *Proc. Natl. Acad. Sci. USA* **105**, 12944-12949.

Cohen, G. and Hochstein, P. (1963). Glutathione peroxidase: the primary agent for the elimination of hydrogen peroxide in erythrocytes. *Biochemistry* **2**, 1420-1428.

Cosgrove, J. P., Church, D. F. and Pryor, W. A. (1987). The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids* **22**, 299-304.

Crockett, E. L. (2008). The cold but not hard fats in ectotherms: consequences of lipid restructuring on susceptibility of biological membranes to peroxidation, a review. *J. Comp. Physiol. B* **178**, 795-809.

Crockett, E. L. and Sidell, B. D. (1993). Substrate selectivities differ for hepatic mitochondrial and peroxisomal beta-oxidation in an Antarctic fish, Notothenia gibberifrons. *Biochem. J.* **289**, 427-433.

Davies, K. J. A. (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie* **83**, 301-310.

Dikalov, S. (2011). Cross talk between mitochondria and NADPH oxidases. *Free Radic. Biol. Med.* **51**, 1289-1301.

Dobly, A., Martin, S. A. M., Blaney, S. C. and Houlihan, D. F. (2004). Protein growth rate in rainbow trout (Oncorhynchus mykiss) is negatively correlated to liver 20S proteasome activity. *Comp. Biochem. Physiol. A* **137**, 75-85.

Driedzic, W. R. (1983). The fish heart as a model system for the study of myoglobin. *Comp. Biochem. Physiol. A* **76**, 487-493.

Dudek, E., Shang, F., Valverde, P., Liu, Q., Hobbs, M. and Taylor, A. (2005). Selectivity of the ubiquitin pathway for oxidatively modified proteins: relevance to protein precipitation diseases. *FASEB J.* **19**, 1707-1709.

Eastman, J. T. (1993). *Antarctic Fish Biology: Evolution in a Unique Environment*. San Diego: Academic Press.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and Ecological Physiology. *Ann. Rev. Physiol.* **61**, 243-282.

Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.

Flores, H., Kock, K.-H., Wilhelms, S. and Jones, C. D. (2004). Diet of two icefish species from the South Shetland Islands and Elephant Island, *Champsocephalus gunnari* and *Chaenocephalus aceratus*. *Polar Biol.* **27**, 119-129.

Gogliettino, M., Riccio, A., Balestrieri, M., Cocca, E., Facchiano, A., D'Arco, T. M., Tesoro, C., Rossi, M. and Palmieri, G. (2014). A novel class of bifunctional acylpeptide hydrolases–potential role in the antioxidant defense systems of the Antarctic fish Trematomus bernacchii. *FEBS J.* **281**, 401-415.

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **106**, 207-212.

Grim, J. M., Simonik, E. A., Semones, M. C., Kuhn, D. E. and Crockett, E. L. (2013). The glutathione-dependent system of antioxidant defense is not modulated by temperature acclimation in muscle tissues from striped bass, Morone saxatilis. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **164**, 383-390.

Grove, T. J. and Sidell, B. D. (2002). Myoglobin deficiency in the hearts of phylogenetically diverse temperate-zone fish species. *Can. J. Zool.* **80**, 893-901.

Halliwell, B. and Gutteridge, J. M. C. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **246**, 501-514.

Hawkins, A. J. S. (1991). Protein turnover: a functional appraisal. *Funct. Ecol.* **5**, 222-233.

Hayes, J. D. and Pulford, D. J. (1995). The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.* **30**, 445-600.

Hofmann, G. E. and Somero, G. N. (1995). Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel Mytilus trossulus. *J. Exp. Biol.* **198**, 1509-1518.

Hofmann, G. E., Buckley, B. A., Airaksinen, S., Keen, J. E. and Somero, G. N. (2000). Heat-shock protein expression is absent in the Antarctic fish Trematomus bernacchii (family Nototheniidae). *J. Exp. Biol.* **203**, 2331-2339.

Hrycay, E. G. and Bandiera, S. M. (2012). The monooxygenase, peroxidase, and peroxygenase properties of cytochrome P450. *Arch. Biochem. Biophys.* **522**, 71-89.

Johnston, I. I., Calvo, J. and Guderley, Y. H. (1998). Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. *J. Exp. Biol.* **201**, 1-12.

Johnson, R. M., Goyette, G., Jr., Ravindranath, Y. and Ho, Y. S. (2005). Hemoglobin autoxidation and regulation of endogenous H2O2 levels in erythrocytes. *Free Radic. Biol. Med.* **39**, 1407-1417.

Jørgensen, W., Jelnes, P., Rud, K. A., Hansen, L. L., Grunnet, N. and Quistorff, B. (2012). Progression of type 2 diabetes in GK rats affects muscle and liver mitochondria differently: pronounced reduction of complex II flux is observed in liver only. *Am. J. Physiol. Endocrinol. Metab.* **303**, E515-E523.

Josse, J. and Husson, F. (2013). Handling missing values in exploratory multivariate data analysis methods. *J. de la SFdS* **153**, 79-99.

King, N. K., Looney, F. D. and Winfield, M. E. (1964). Myoglobin free-radicals. *Biochim. Biophys. Acta* **88**, 235-236.

Kuhn, D., O'Brien, K. M. and Crockett, E. L. (2016). Expansion of capacities for iron transport and sequestration reflects plasma volumes and heart mass among white-blooded notothenioids. *Am. J. Physiol.* **311**, R649-R657.

Lazarow, P. B. and De Duve, C. (1976). A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. *Proc. Natl. Acad. Sci. USA* **73**, 2043-2046.

Lee, J., Giordano, S. and Zhang, J. (2012). Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *Biochem. J.* **441**, 523-540.

Levine, R. L., Wehr, N., Williams, J. A., Stadtman, E. R. and Shacter, E. (2000). Determination of carbonyl groups in oxidized proteins. *Methods Mol. Biol.* **99**, 15-24.

Lewis, J. M., Grove, T. J. and O'Brien, K. M. (2015). Energetic costs of protein synthesis do not differ between red- and white-blooded Antarctic notothenioid fishes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **187**, 177-183.

Li, Z., Xu, X., Leng, X., He, M., Wang, J., Cheng, S. and Wu, H. (2016). Roles of reactive oxygen species in cell signaling pathways and immune responses to viral infections. *Arch. Virol.* **162**, 603-610.

Logue, J. A., De Vries, A., Fodor, E. and Cossins, A. R. (2000). Lipid compositional correlates of temperature-adaptive interspecific differences in membrane physical structure. *J. Exp. Biol.* **203**, 2105-2115.

Londraville, R. L. and Sidell, B. D. (1990). Maximal diffusion-distance within skeletal muscle can be estimated from mitochondrial distributions. *Respir. Physiol.* **81**, 291-301.

Lopez-Torres, M., Pérez-Campo, R., Cadenas, S., Rojas, C. and Barja, G. (1993). A comparative study of free radicals in vertebrates—II. Non-enzymatic antioxidants and oxidative stress. *Comp. Biochem. Physiol. B* **105**, 757-763.

Low, F. M., Hampton, M. B. and Winterbourn, C. C. (2008). Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. *Antioxid Redox Signal.* **10**, 1621-1630.

Lucassen, M., Schmidt, A., Eckerle, L. G. and Portner, H. O. (2003). Mitochondrial proliferation in the permanent vs. temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **285**, R1410-R1420.

Macqueen, D. J., Garcia de la Serrana, D. and Johnston, I. A. (2014). Cardiac myoglobin deficit has evolved repeatedly in teleost fishes. *Biol. Lett.* **10**.

Mihara, M. and Uchiyama, M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* **86**, 271-278.

Moylan, T. J. and Sidell, B. D. (2000). Concentrations of myoglobin and myoglobin mRNA in heart ventricles from Antarctic fishes. *J. Exp. Biol.* **203**, 1277-1286.

Mueller, I. A., Devor, D. P., Grim, J. M., Beers, J. M., Crockett, E. L. and O'Brien, K. M. (2012). Exposure to critical thermal maxima causes oxidative stress in

hearts of white- but not red-blooded Antarctic notothenioid fishes. *J. Exp. Biol.* **215**, 3655-3664.
Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem. J.* **417**, 1-13.
Newman, E. S. R., Rice-Evans, C. A. and Davies, M. J. (1991). Identification of initiating agents in myoglobin-induced lipid peroxidation. *Biochem. Biophys. Res. Commun.* **179**, 1414-1419.
Niki, E. (2009). Lipid peroxidation: physiological levels and dual biological effects. *Free Radic. Biol. Med.* **47**, 469-484.
O'Brien, K. M. and Mueller, I. A. (2010). The unique mitochondrial form and function of Antarctic channichthyid icefishes. *Integ. Comp. Biol.* **50**, 993-1008.
O'Brien, K. M. and Sidell, B. D. (2000). The interplay among cardiac ultrastructure, metabolism and the expression of oxygen-binding proteins in Antarctic fishes. *J. Exp. Biol.* **203**, 1287-1297.
O'Brien, K. M., Crockett, E. L., Philip, J., Oldham, C. A., Hoffman, M., Kuhn, D. E., Barry, R., McLaughlin, J. (2017). Data from: The loss of hemoglobin and myoglobin does not minimize oxidative stress in Antarctic icefishes. *Dryad Digital Repository*. https://doi.org/10.5061/dryad.5228g
Orczewska, J. I., Hartleben, G. and O'Brien, K. M. (2010). The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **299**, 352-364.
Pajares, M., Jiménez-Moreno, N., Dias, I. H. K., Debelec, B., Vucetic, M., Fladmark, K. E., Basaga, H., Ribaric, S., Milisav, I. and Cuadrado, A. (2015). Redox control of protein degradation. *Redox Biol.* **6**, 409-420.
Park, S.-Y., Gifford, J. R., Andtbacka, R. H. I., Trinity, J. D., Hyngstrom, J. R., Garten, R. S., Diakos, N. A., Ives, S. J., Dela, F., Larsen, S. et al. (2014). Cardiac, skeletal, and smooth muscle mitochondrial respiration: are all mitochondria created equal? *Am. J. Physiol. Heart Circ. Physiol.* **307**, H346-H352.
Pérez-Campo, R., López-Torres, M., Rojas, C., Cadenas, S. and Barja, G. (1993). A comparative study of free radicals in vertebrates—I. Antioxidant enzymes. *Comp. Biochem. Physiol. B* **105**, 749-755.
Place, S. P. and Hofmann, G. E. (2005). Constitutive expression of a stress-inducible heat shock protein gene, hsp70, in phylogenetically distant Antarctic fish. *Polar Biol.* **28**, 261-267.
Place, S. P., Zippay, M. L. and Hofmann, G. E. (2004). Constitutive roles for inducible genes: evidence for the alteration in expression of the inducible hsp70 gene in Antarctic notothenioid fishes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **287**, R429-R436.
Reeder, B. J. and Wilson, M. T. (2005). Hemoglobin and myoglobin associated oxidative stress: from molecular mechanisms to disease States. *Curr. Med. Chem.* **12**, 2741-2751.
Reinheckel, T., Ullrich, O., Sitte, N. and Grune, T. (2000). Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. *Arch. Biochem. Biophys.* **377**, 65-68.
Riccio, A., Gogliettino, M., Palmieri, G., Balestrieri, M., Facchiano, A., Rossi, M., Palumbo, S., Monti, G. and Cocca, E. (2015). A new APEH cluster with antioxidant functions in the antarctic hemoglobinless icefish chionodraco hamatus. *PloS ONE* **10**, e0125594.
Rivett, A. J. and Levine, R. L. (1990). Metal-catalyzed oxidation of Escherichia coli glutamine synthetase: correlation of structural and functional changes. *Arch. Biochem. Biophys.* **278**, 26-34.
Schrader, M. and Fahimi, H. D. (2006). Peroxisomes and oxidative stress. *Biochim. Biophys. Acta* **1763**, 1755-1766.
Shadel, G. S. and Horvath, T. L. (2015). Mitochondrial ROS signaling in organismal homeostasis. *Cell* **163**, 560-569.
Shang, F. and Taylor, A. (2011). Ubiquitin–proteasome pathway and cellular responses to oxidative stress. *Free Radic. Biol. Med.* **51**, 5-16.
Shang, F., Gong, X. and Taylor, A. (1997). Activity of ubiquitin-dependent pathway in response to oxidative stress ubiquitin-activating enzymes is transiently up-regulated. *J. Biol. Chem.* **272**, 23086-23093.
Shibatani, T. and Ward, W. F. (1995). Sodium dodecyl sulfate (SDS) activation of the 20S proteasome in rat liver. *Arch. Biochem. Biophys.* **321**, 160-166.
Shringarpure, R., Grune, T., Mehlhase, J. and Davies, K. J. A. (2003). Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J. Biol. Chem.* **278**, 311-318.
Sidell, B. D., Vayda, M. E., Small, D. J., Moylan, T. J., Londraville, R. L., Yuan, M.-L., Rodnick, K. J., Eppley, Z. A. and Costello, L. (1997). Variable expression of myoglobin among the hemoglobinless Antarctic icefishes. *Proc. Natl. Acad. Sci. USA* **94**, 3420-3424.
Smith, A. and McCulloh, R. J. (2015). Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. *Front. Physiol.* **6**, 187.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.
Srere, P. A., Brazil, A. and Gonen, L. (1963). The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. *Acta Chem. Scand.* **17**, S219-S234.
Takahashi, M. and Iwami, T. (1997). The summer diet of demersal fish at the South Shetland Islands. *Ant. Sci.* **9**, 407-413.
Tappel, A. D. (1955). Unsaturated lipide oxidation catalyzed by hematin compounds. *J. Biol. Chem.* **217**, 721-734.
Todgham, A. E., Hoaglund, E. A. and Hofmann, G. E. (2007). Is cold the new hot? Elevated ubiquitin-conjugated protein levels in tissues of Antarctic fish as evidence for cold-denaturation of proteins in vivo. *J. Comp. Physiol. B* **177**, 857-866.
van Zwieten, R., Verhoeven, A. J. and Roos, D. (2014). Inborn defects in the antioxidant systems of human red blood cells. *Free Radic. Biol. Med.* **67**, 377-386.
Wehr, N. B. and Levine, R. L. (2012). Quantitation of protein carbonylation by dot blot. *Anal. Biochem.* **423**, 241-245.
Wickner, S., Maurizi, M. R. and Gottesman, S. (1999). Posttranslational quality control: folding, refolding, and degrading proteins. *Science* **286**, 1888-1893.
Winterbourn, C. C. (1990). Oxidative reactions of hemoglobin. *Methods Enzymol.* **186**, 265-272.
Witas, H., Gabryelak, T. and Matkovics, B. (1984). Comparative studies on superoxide dismutase and catalase activities in livers of fish and other Antarctic vertebrates. *Comp. Biochem. Physiol. C* **77**, 409-411.
Wittenberg, J. B. (1970). Myoglobin-facilitated oxygen diffusion: role of myoglobin in oxygen entry into muscle. *Physiol. Rev.* **50**, 559-636.