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

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Key words: oxidative stress, hemoglobin, myoglobin, Antarctic fish

ABBREVIATIONS

CAT   catalase

CS   citrate synthase

ETC   electron transport chain

GPx1   glutathione peroxidase 1

GPx4   glutathione peroxidase 4

GSSG   oxidized glutathione

GSH   reduced glutathione

GST   glutathione *S*-transferase

Hb   hemoglobin

Mb   myoglobin

PUFA   polyunsaturated fatty acid

ROS   reactive oxygen species

SOD   superoxide dismutase

TAP   total antioxidant potential

SUMMARY

The loss of iron-centered oxygen-binding proteins in Antarctic fishes does not correspond with an overall reduction in levels of oxidized macromolecules, antioxidants, or rates of protein degradation thereby minimizing oxidative stress.

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, small-molecule and enzymatic antioxidants, and levels of oxidized lipids and proteins in 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 are higher in red-blooded fishes than in Hb-less icefishes in some tissues, this pattern does not persist across all tissues. Expression of Mb is not associated with oxidative damage in heart ventricle, whereas the activity of citrate synthase and contents of heme are 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 are not markedly different, suggesting either alternative pathways for eliminating oxidized proteins or 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.

INTRODUCTION

Antarctic notothenioid fishes provide a natural experiment to probe questions pertaining to the risks and prevention of oxidative stress at very low temperatures. A life cycle spent entirely at very low body temperatures may, at first glance, suggest a means to avoid oxidative injury, since 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 ventricle, 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 hemoglobin (Hb) among vertebrates. The family Channichthyidae (the "icefishes") is characterized by the absence of Hb, and six of the sixteen species of icefishes also lack expression of myoglobin (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 oxidative muscle of the two icefishes (-Hb), *Chionodraco rastrospinosus* and *Chaenocephalus aceratus* compared to the red-blooded (+Hb) species, *Notothenia*

coriiceps, (Mueller et al., 2012). Furthermore, levels of oxidized proteins are lower in hearts of the -Mb icefish *C. aceratus* compared to the +Mb hearts of *C. rastrospinosus,* and highest in hearts 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 are lower in selected tissues of white-blooded (-Hb) than red-blooded (+Hb) fishes, suggesting that 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 is(are) unclear. As iron-centered proteins, Hb and Mb may facilitate the production of reactive oxygen species (ROS) (King et al., 1964; Tappel, 1955). Any unbound (i.e., excess) Fe may promote the formation of hydroxyl radicals ($\cdot$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 when Fe is in the ferrous state (Fe^{2+}), to methemoglobin (metHb) with Fe in the ferric state (Fe^{3+}), produces superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) when MetHb reacts with O_2 (Winterbourn, 1990). Heme in the ferric (Fe^{3+}) state also reacts with H_2O_2 to form two oxidants, ferryl iron ($Fe^{4+}=0_2^-$) 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 poised to destroy two common ROS, superoxide (O_2^-) and hydrogen peroxide (H_2O_2), respectively (Finkel and Holbrook, 2000). Hydrogen peroxide may also be detoxified by several antioxidant enzymes requiring participation by reduced glutathione (GSH), including the glutathione peroxidases (GPx), with the important role of recycling GSH catalyzed by glutathione reductase (GR) (Brigelius-Flohe, 1999). Another family of enzymes, the glutathione-*S*-transferases (GST) also depend 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, that can enhance ROS formation, rather than by Fe-mediated ROS formation via Hb and Mb.

Lower levels of oxidized proteins in icefishes compared to 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, another ATP-dependent process (Davies, 2001; Shringarpure et al., 2003). Oxidized proteins may also be degraded by the 20S proteasome, in an ubiquitin- and ATP-independent process (Davies, 2001; Reinheckel et al., 2000). While 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 take 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 Mb in 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.

METHODS

Animal collection

The following species were used for this study with animal masses ± s.e.m shown in parenthesis: *Chaenocephalus aceratus* (1675 ± 169 g; n=18) (Lönnberg 1906), *Champsocephalus gunnari* (581 ± 36 g; n=16) (Lönnberg 1905), *Pseudochaenichthys georgianus* (1528 ± 228 g; n=8) (Norman 1937), *Chionodraco rastrospinosus* (349 ± 30 g; n=14) (DeWitt & Hureau 1979), *Gobionotothen gibberifrons* (860 ± 38 g; n=33) (Lönnberg 1905), *and Notothenia coriiceps* (1847 ± 94 g; n=18) (Richardson 1844). Animals were collected off the south western 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 onboard 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 euthanized with a sharp blow to the head followed by cervical transection. Samples of heart ventricle, pectoral adductor muscle, and liver were harvested and flash frozen in liquid nitrogen and stored at -80°C. Icefishes and *G. gibberifrons* were harvested within 2 weeks of capture. *N. coriiceps* were fed every ~ 3 days a diet of fish muscle and were harvested within ~ 3 weeks of capture. All protocols were approved by the UAF 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 a hematocrit 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), 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 manufacturer's instructions (BioAssay Systems, Hayward, CA). Samples were measured in duplicate.

Myoglobin quantification

Heart ventricles were homogenized (10% w/v) in 20 mM HEPES buffer (pH 7.8 at 4 °C) using a Tenbroeck homogenizer on ice. Homogenates were centrifuged at 10,000 g for 10 min at 4 °C, supernatant collected and protein concentration determined using the bicinchoninic acid assay (Sigma-Aldrich) with bovine serum albumin (BSA) as a standard. The supernatant was mixed with loading buffer (150 mM Tris – HCl, 4% SDS, 12% glycerol, 100 mM 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, 7% acetic acid, and scanned with an ImageScanner (GE Healthcare Bio-Sciences, Pittsburgh, PA). Images were quantified using ImageQuant (GE Healthcare Bio-Sciences) and the concentration of Mb calculated from the standard curve.

Citrate synthase activity

The maximal activity of citrate synthase (EC 2.3.3.1) was measured at 5ºC ± 0.5°C using a Lambda 25 spectrophotometer (PerkinElmer, Waltham, MA) 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 mM Tris-HCl, 1 mM EDTA, 2 mM MgCl$_2$, pH 8.2 at 5°C) using Tenbroeck ground-glass homogenizers. The final reaction mixture contained 0.25 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 0.40 mM acetyl CoA, 0.5 mM oxaloacetate, 75 mM 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 mM MgCl$_2$, 50 mM Hepes, 100 mM KCl, pH 7.2) and dialyzed against 25 mM ascorbate and 0.1 mM 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 mM 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. Absorbance at 595 nm was measured using a Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). The aconitase standard was then divided into 50 µg aliquots, brought to a final volume of 100 µl with MQ H$_2$O, dried in a vacuum centrifuge (CentriVap Concentrator, Labconco Corporation, Kansas City, MO) at room temperature for two 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). Proteins were separated using (TSKgel Guard SW$_{XL}$ and QC-PAK GFC 200 columns (TOSOH Bioscience, King of Prussia, PA) and a running buffer of 6M

guanidinium hydrochloride, pH 2.5. Spectra were analyzed with Waters Empower Pro Analysis software (Waters Corporation). 100 µg of the aconitase standard was resuspended to a final concentration of 300 ng protein μl^{-1} in 50 mM 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 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 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 mM 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 mM KH_2PO_4 pH 7.8 and centrifuged at 4°C for 10 min at 9,000 g. Bradford assays, as described above, were used to determine protein concentration of the supernatant. 50 µg of protein was brought to a final volume of 100 µl with MQH_2O and dried in a vacuum centrifugation (CentriVap Concentrator, Labconco Corporation) 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 derivitization solution consisting of 20 mM DNPH, 0.5% triflouroacetic acid (TFA) and 92.5% DMSO. Samples were vortexed at room temperature for 45-60 min before spotting onto a PVDF membrane. A standard curve of aconitase containing 0 – 300 ng μl^{-1} protein was loaded on each membrane in triplicate. The samples and aconitase were diluted in derivitization solution followed by 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 mM Tris, 192 mM glycine, 0.1% SDS, 20% MeOH) before loading samples using a vacuum-drawn slot-blotter (Bio-Dot SF Cell, Bio-Rad, Hercules, CA). 200 μl of transfer buffer was pipetted into each slot and drawn across the membrane using a vacuum, followed by samples, and then 200 μl transfer buffer per slot. 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 MQH_2O added to cover the membrane (approximately 60-70 ml total volume), and then once for 5 min in MQH_2O, dried and then stored at -80 °C for up to two days before developing.

Membranes were blocked with 5% non-fat dry milk in 0.1% PBS-Tween for one 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) diluted 1:5000 in 5% non-fat dry milk in 0.1% PBS-Tween for two 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) 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) and 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 5-decimal place balance (Mettler H54AR) was used to quantify the final lipid weight 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 (GSTtot = GSH + GSSG) was measured, along with oxidized glutathione (GSSG) following the elimination of reduced glutathione (GSH) using 2VP. Tissues were homogenized in 5 volumes of ice-cold 5% 5'-sulfosalicylic acid in 100 mM potassium phosphate, 1mM EDTA (pH 7.0) using a BioHomogenizer tissue homogenizer (BioSpec Products, Bartlesville, OK). 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 M 2VP final concentration) or buffer alone, and incubated at room temperature for 10-15 min. An assay solution containing 100 mM K-phosphate, 1mM EDTA, 0.1 mM 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 initiated by adding 50 μl of an NADPH solution (0.05 mM final concentration). Absorbance readings at 412 nm were begun immediately thereafter and taken every 20 seconds for 3 min using a SpectraMax M2e microplate reader (Molecular Devices) and the accompanying SoftMax Pro software kinetic program.

Enzymatic antioxidants

Superoxide dismutase (EC 1.15.1.1) activity was measured using a kit from Sigma Life Sciences (#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 mM potassium phosphate buffer (pH 7.2) as described above, followed by probe sonication (3 pulses of 4 seconds 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).

Catalase (EC 1.11.1.6) activity was measured at 5°C using a Beckman DU-640 spectrophotometer 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 slight modifications of a protocol described by Sigma-Aldrich (GRSA Technical Bulletin). Tissues were homogenized in assay buffer (125 mM potassium phosphate, 1.25 mM EDTA, pH 7.5) using a BulletBlender Storm24 from Next Advance (Averill, NY) 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 mM DTNB, 1.5 mM GSSG and 0.215 mM 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 glutathione S-transferase (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 mM.

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 ice-cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris; pH 8.0) using a Ten-Broeck ground-glass homogenizer. Homogenates were centrifuged at 21,000 g for 15 min at 4°C. 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 heated at 96°C for 10 min. Ten

microliters of each sample, or molecular weight standard (Precision Plus Protein), were added to one of each lanes in a 10 well Mini-Protean TGX precast gel and proteins separated at 200 V. The running solution was a standard SDS-PAGE buffer (192 mM glycine, 0.1% w/v SDS, 25 mM 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, Hercules, CA) 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 mM NaCl, 1% casein, 20 mM 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). This solution was removed and the membranes rinsed briefly, after which the membranes were washed 4 times for 5 min each in blocking buffer. Washed membranes were next incubated for 1 h at room temperature in antibody solution containing a 1:3000 dilution of secondary antibody (ab6721; Abcam, Cambridge, MA). 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). Two milliliters of final HRP substrate solution were added to cover each membrane and the membrane immediately placed in a Bio-Rad ImageDoc photo imager and the signal read every 20 seconds for 3 min. GPx4 specific bands were analyzed using GelAnalyzer 2010 after removing non-specific background with Image J. 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 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) according to the manufacturer's instructions. Values for total antioxidant power are expressed as Trolox Equivalents (μmoles g^{-1} wet weight 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 mM EDTA, 50 mM Tris-HCl, pH 6.8) supplemented with protease inhibitors (c*O*mplete Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN) using a Tissuemizer homogenizer (Tekmar, Cincinnati, OH, USA). Homogenization was completed using Tenbroeck ground-glass tissue homogenizers. Tissue homogenates were boiled 5 min to denature proteins. Homogenates were then centrifuged at 12,000 *g* for 15 min at room temperature and the supernatant 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 mM Tris-HCl, 140 mM NaCl, pH 7.6) to a concentration of 0.5 µg µl^{-1} for ventricle and pectoral adductor samples, and 0.25 µg µl^{-1} for liver. One µl of each sample was pipetted onto a 12 cm x 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% nonfat milk powder dissolved in Tween-20 Tris-buffered saline solution (TTBS) (20 mM Tris-HCl, 140 mM 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 3 times for 5 min each with TTBS. The membranes were incubated at 4°C with the ubiquitin conjugate primary antibody (mono- and polyubiquitinylated conjugates mAb produced in mice, Enzo Life Sciences, BML-PW8810, Farmdale, NY) diluted 1:5000 in 5% nonfat milk powder dissolved in TTBS. Incubation times were 12.5

h for ventricle, 15 h for pectoral adductor, and 2 h for liver. The membranes were then rinsed briefly twice with TTBS and then rinsed 3 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% nonfat dry milk powder in TTBS. Membranes with ventricle samples were incubated 1.5 h, pectoral adductor samples for 2 h, and liver samples for 2.25 h. The membranes were then rinsed briefly twice with TTBS and then washed 3 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 manufacturer's specifications. Chemiluminescence was detected for 15 min using AlphaImager 3300 Imaging System (Protein Simple, San Jose, CA) and quantified using ImageQuant TL software (GE Healthcare Bio-Sciences). Levels of ubiquitinated proteins were normalized to levels in heart ventricles of *N. coriiceps* (n=6), blotted in triplicate on membranes for measurements in heart ventricle and in duplicate on membranes for measurements in 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 on 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 mM Tris-HCl, 0.1 mM EDTA, 1.0 mM 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 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-AMC (Enzo). The substrate was dissolved in DMSO (5.71 mM), 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), in our hands after testing in SDS concentrations ranging from 0% to 0.025%, maximal activity of the 20S proteasome was obtained in Tris buffer lacking SDS and so we omitted it from the reaction mixture.

Activity was measured by incubating 50 µg protein from the supernatant with 40 µM LLVY-AMC in 22.5 µl 100 mM 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 0.1 M sodium borate (pH 9.1) and 65 µl 1% SDS. Fluorescence of AMC was determined at excitation/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 µM) (Enzo) prior to incubation. Activity was calculated by determining the concentration of AMC in the samples using the standard curve minus 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 8 AMC concentrations between 3.3 µM and 44 µM. The samples and standard curve were measured in triplicate.

Statistical analysis

Differences in 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 analysis of proteasome activity and levels of ubiquitinated proteins. Simple linear regressions were used to assess correlations between pro-oxidants and oxidative damage (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) and JMP 7 (SAS Institute, Cary, NC); all other analyses were conducted using R (2013).

To characterize the clustering of antioxidants among species for each tissue, we first conducted a Principal Components Analysis (PCA) after imputing missing values (Josse and Husson, 2013), then a Hierarchical Clustering on Principle Components (HCPC), using the PCA and HCPC functions from the FactoMineR R package. The first 5 components of the PCA were used as input of the HCPC and the number of clusters

retained was determined by looking how species clustered on the PCA plots. Using only the first 5 components allows us to separate the noise from the structure of the data before performing the HCPC. A chi square test was performed between species and group (cluster) 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 4 of the 9 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.

RESULTS

Pro-oxidants

Levels of heme are significantly higher in the blood of red-blooded fishes compared to the blood in most icefishes, except for the icefish species *C. gunnari* (Table 1). The contents of the intracellular oxygen-binding protein Mb are 3.4-fold higher in heart ventricles of red-blooded *N. coriiceps* compared to red-blooded *G. gibberifrons* and white-blooded *C. rastrospinosus* (Table 2). While Hct is similar between the two red-blooded species, Hb levels are higher in *N. coriiceps* than *G. gibberifrons* (Table 1).

Maximal activity of CS, a measure of flux through the citric acid cycle, tends to be lowest in hearts of white-blooded *C. aceratus* (Table 2). However, low activity of CS in *C. aceratus* ventricle is not associated with the lack of both oxygen-binding proteins, Hb and Mb, because CS activity is equivalent between hearts of the icefish *C. gunnari*, a species which also lacks Mb, and hearts of those notothenioid species (i.e., both white- and red-blooded) that express Mb (Table 2). In pectoral adductor muscle, CS activity is highest in the two red-blooded fishes and in the icefish, *C. gunnari* (Table 3). In liver, CS activity is highest in *C. gunnari* compared to all other species (Table 4).

Oxidative damage

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

While neither the presence nor absence of Hb and Mb correspond with levels of oxidative damage, regression analyses indicate that maximal activity of CS is significantly and positively correlated with levels of protein carbonyls and TBARS in both oxidative skeletal muscle (Fig. S2 A,B) and liver (Fig. S3 A,B) but not heart ventricle (Fig. S1 A,B). Heme is also positively correlated with TBARS and carbonyls in oxidative skeletal muscle (Fig. S2 C,D).

Antioxidants

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

We used PCA followed by hierarchical clustering analysis to identify differences in antioxidant levels among species. The chi-square analysis indicates there is 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 explain most of the variation among the species in antioxidant levels (37-40% and 16-17%, respectively) (Fig. 1). The PCA plot for pectoral muscle is shown for components 1 and 3 because this aligned most closely

with the results from the hierarchical clustering and there is a small difference (4%) between the variation explained by component 2 and 3 in this tissue (Fig. 1B). Only in the heart ventricle are levels of antioxidants similar among the three species of icefishes and lower than the two red-blooded species (Fig. 1A, Table S1). In pectoral muscle and liver, antioxidant levels in the two icefishes, *C. aceratus* and *C. rastrospinosus*, are similar to each other (Fig. 1 B,C) and lower than the two red-blooded species (Tables S2-S3). However, in liver and pectoral muscle of the icefish, *C. gunnari*, antioxidant levels are different from both of the other icefishes and red-blooded species with some antioxidants higher than the overall mean and some lower (Figs. 1 B,C, Tables S2-S3). Notably, SOD activities in *C. gunnari* pectoral muscle and liver are higher than the overall mean (Tables S2-S3), which corresponds with their high activity of CS in these tissues (Table 3-4).

Levels of ubiquitinated proteins

Levels of ubiquitinated proteins are higher in the heart ventricle of the red-blooded fishes *N. coriiceps* and *G. gibberifrons* compared to icefishes, and levels are higher in the hearts of *N. coriiceps* than in *G. gibberifrons* (Fig. 2A). There is no difference between levels of ubiquitinated proteins in the heart ventricle of icefishes that express Mb and those that do not. In the pectoral adductor muscle, levels of ubiquitinated proteins are highest in *N. coriiceps*, with levels 1.6- to 2.4-fold higher than other species. Levels of ubiquitinated proteins are mostly similar among the other species, with the exception that levels are lower in *C. rastrospinosus* than *C. aceratus* (Fig. 2B). Similar to the heart ventricle, levels of ubiquitinated proteins are higher in the liver of the two red-blooded species compared to the icefishes except *C. aceratus* (Fig. 2C).

20S proteasome activity

In heart ventricle, the chymotrypsin-like activity of the 20S proteasome does not show a clear trend associated with expression of oxygen-binding proteins. The only significant differences in proteasome activity are between the two red-blooded species and icefish *C. aceratus*, where activity is on average 1.6-fold higher in hearts of the red-blooded fishes (Fig. 3A). In pectoral muscle, 20S proteasome activity is mostly similar among all

species, although activity is higher in *C. aceratus* than *N. coriiceps* (Fig. 3B). In the liver, 20S proteasome activity is highest in *C. gunnari* and is 1.7- to 2.9-fold higher than in the other species (Fig. 3C). There is 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 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 compared to species expressing the proteins. 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 trend with the expression of Hb and Mb. Neither Hb nor Mb are associated with oxidative damage across tissues. However, the activity of CS is positively correlated with levels of oxidized proteins and lipids in both pectoral adductor muscle and liver (Figs. S2-S3).

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 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* possess a Mb content 3.4-fold greater compared to *G. gibberifrons* and *C. rastrospinosus* and yet, 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 to 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 hearts of the icefish *C. rastrospinosus* is not associated with higher levels of antioxidants compared to 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 g tissue and mitochondrial volume density (e.g., Lucassen et al., 2003; Orczewska et al., 2010), and since 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 3-8 fold higher in pectoral adductor muscle of red-blooded notothenioids compared to icefishes, and parallel the higher activities of CS in red-blooded species compared to most icefishes (Table 3). The approximately two-fold higher maximal activity of CS in oxidative skeletal muscle from red-blooded fishes compared to 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 not likely the case since the icefish *C. gunnari* displays CS activity similar to that of the red-blooded species while maintaining low levels of oxidized proteins. We posit that a more likely scenario 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 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*, compared to temperate species (Chen et al., 2008), and higher in red-blooded species than icefishes (Kuhn et al., 2016).

Despite significantly higher levels of some antioxidants (GR and GPx4) in pectoral adductor muscle of *N. coriiceps* and *G. gibberifrons* compared to 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 8-fold higher in red-blooded species compared to icefishes (Table 3). The high levels of carbonylated proteins in pectoral adductor muscle of *N. coriiceps* are also associated with higher levels of ubiquitinated proteins compared to other species (Fig. 2B).

It is clear from our results that the high levels of oxidized proteins in pectoral adductor of the red-blooded notothenioids cannot be attributed to either heme or Hb alone. Since high levels of oxidized proteins were also observed in other tissues (e.g. liver and 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 to both liver and heart, and 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 electron transport chain and ATP synthesis, which cannot be inferred from CS activity. Mitochondria from liver, in general, have a lower RCR than muscle (e.g., Jorgensen 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 pectoral adductor may be driven in part, by greater rates of mitochondrial ROS production compared to liver.

Rates of protein turnover may also impact steady-state levels of oxidized proteins. Rates of protein synthesis are ~ 5-fold lower in pectoral adductor compared to heart ventricle and ~ 20-fold lower compared to liver, consistent with the higher metabolic cost of protein synthesis in liver compared to cardiac myocytes (Lewis et al., 2015 and

unpublished data). Despite higher levels of ubiquitinated proteins in pectoral muscle of red-blooded species compared to icefishes (Fig. 2B), rates of degradation by the 20S proteasome are lowest in *N. coriiceps* and similar among all other species (Fig. 3B). If rates of oxidative damage are equivalent among tissues, lower rates of protein turnover in 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 livers 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). CS activity appears, in fact, to be the best predictor of levels of oxidative damage in livers of all species, with the liver of *C. gunnari* possessing highest levels or activities of CS, protein carbonyls, TBARS, and the 20S proteasome among hepatic tissues of all 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* (formerly *Notothenia 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 byproduct (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 to icefishes (Fig. 3). For example, 20S proteasome activity is equivalent in the heart ventricle of the red-blooded and red-hearted species *N. coriiceps* and *G. gibberifrons* to that of the icefishes *C. gunnari*, *C. rastrospinosus* and *P. georgianus* but lower in the pectoral adductor muscle of *N. coriiceps* compared to all other species (Fig. 3A,B). In liver, 20S proteasome activity in the icefish *C. gunnari* is 1.7- to 2.9-fold higher than other notothenioids, in which proteasome activity is equivalent (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 equivalent among hearts of red- and white-blooded notothenioids, and the metabolic costs of protein synthesis are equivalent between red- and white- blooded species in cardiomyocytes and hepatocytes, suggesting 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 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 protein 70 (Hsp70) and 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* compared to erythrocyte-like cells of the icefish *Chionodraco hamatus*, suggesting this pathway may be important for eliminating oxidized proteins in red-blooded notothenioids (Riccio et al., 2015).

C. 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 both icefish and red-blooded species in pectoral adductor muscle and liver, with levels of some antioxidants higher than the overall mean and some lower (Fig 1B,C; Table 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 equivalent to (in pectoral muscle) or higher (in liver) than 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 liver and pectoral adductor muscle in *C. gunnari* most likely reflects their more active lifestyle than other icefish species. *C. gunnari* is semipelagic and undergoes diurnal migrations, feeding almost exclusively on krill (Flores et al., 2004). In contrast, the other two 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 losses of Hb and Mb in Antarctic icefishes do 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 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 pivotal roles 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 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.

COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

K.M.O. and E.L.C conceived and designed the research. K.M.O., E.L.C, C.A.O, M.H., D.E.K., and J.M. conducted experiments and analyzed data. J.P and R.B. conducted statistical analyses. K.M.O., E.L.C., J.P., C.A.O., M.H., and D.E.K. drafted the manuscript and prepared figures. K.M.O, E.L.C, and J.P. approved the final version of the manuscript.

FUNDING

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**, R100-6.

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. (2005). Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Moscow)* **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**, 133-40.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911-7.

Boulesteix, A. L. and Strimmer, K. (2007). Partial least squares: a versatile tool for the analysis of high-dimensional genomic data. *Briefings in Bioinformatics* **8**, 32-44.

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**, 707-16.

Boveris, A., Oshino, N. and Chance, B. (1972). The cellular production of hydrogen peroxide. *Biochem J* **128**, 617-30.

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-Flohe, R. (1999). Tissue-specific functions of individual glutathione peroxidases. *Free Radic Biol Med* **27**, 951-65.

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**, 469-73.

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., 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 U S A* **105**, 12944-9.

Cohen, G. and Hochstein, P. (1963). Glutathione Peroxidase: The Primary Agent for the Elimination of Hydrogen Peroxide in Erythrocytes. *Biochemistry* **2**, 1420-8.

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** (**Pt 2**), 427-33.

Davies, K. J. (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie* **83**, 301-10.

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

Dobly, A., Martin, S. A., 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**, 487-93.

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. *The FASEB Journal* **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**, 207-12.

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**, 383-90.

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. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch Biochem Biophys* **246**, 501-14.

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., 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.

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.

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

Janssens, B. J., Childress, J. J., Baguet, F. and Rees, J. F. (2000). Reduced enzymatic antioxidative defense in deep-sea fish. *J Exp Biol* **203**, 3717-25.

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**, 1407-17.

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 (Pt 1)**, 1-12.

Jorgensen, 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**, E515-23.

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

Kammer, A. R., Orczewska, J. I. and O'Brien, K. M. (2011). Oxidative stress is transient and tissue specific during cold acclimation of threespine stickleback. *J Exp Biol* **214**, 1248-56.

King, N. K., Looney, F. D. and Winfield, M. E. (1964). Myoglobin free-radicals. *Biochimica et Biophysica 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 U S A* **73**, 2043-6.

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**, 177-83.

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. *Resp Physiol* **81**, 291-301.

Lopez-Torres, M., Perez-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**, 1621-30.

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**, R1410-20.

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**, 271-8.

Moylan, T. J. and Sidell, B. D. (2000). Concentrations of myoglobin and myoglobin mRNA in heart ventricles from Antarctic fishes. *J Exp Biol* **203**, 1277-86.

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*.

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

Newman, E. S., Rice-Evans, C. A. and Davies, M. J. (1991). Identification of initiating agents in myoglobin-induced lipid peroxidation. *Biochem Biophys Res Commun* **179**, 1414-9.

Niki, E. (2009). Lipid peroxidation: physiological levels and dual biological effects. *Free Radic Biol Med* **47**, 469-84.

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.

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**, 352-64.

Pajares, M., Jimenez-Moreno, N., Dias, I. H., 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**, 409-20.

Park, S. Y., Gifford, J. R., Andtbacka, R. H., 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**, H346-52.

Perez-Campo, R., Lopez-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-Reg, Integ 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**, 2741-51.

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**.

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**, 1755-66.

Shadel, G. S. and Horvath, T. L. (2015). Mitochondrial ROS Signaling in Organismal Homeostasis. *Cell* **163**, 560-9.

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.

Shang, F. and Taylor, A. (2011). Ubiquitin–proteasome pathway and cellular responses to oxidative stress. *Free Radic Biol Med* **51**, 5-16.

Shibatani, T. and Ward, W. F. (1995). Sodium dodecyl sulfate (SDS) activation of the 20S proteasome in rat liver. *Arch Biochem Biophys* **321**, 160-6.

Shringarpure, R., Grune, T., Mehlhase, J. and Davies, K. J. (2003). Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J Biol Chem* **278**, 311-8.

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 U S A* **94**, 3420-4.

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**, 407-13.

Tappel, A. d. (1955). Unsaturated lipide oxidation catalyzed by hematin compounds. *J Biol Chem* **217**, 721-734.

Team, R. C. (2013). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

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**, 857-66.

Toime, L. J. and Brand, M. D. (2010). Uncoupling protein-3 lowers reactive oxygen species production in isolated mitochondria. *Free Radic Biol Med* **49**, 606-11.

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**, 377-86.

Wehr, N. B. and Levine, R. L. (2012). Quantitation of protein carbonylation by dot blot. *Anal Biochem* **423**, 241-5.

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**, 265-72.

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.

Wold, S., Sjöström, M. and Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems* **58**, 109-130.

Figures
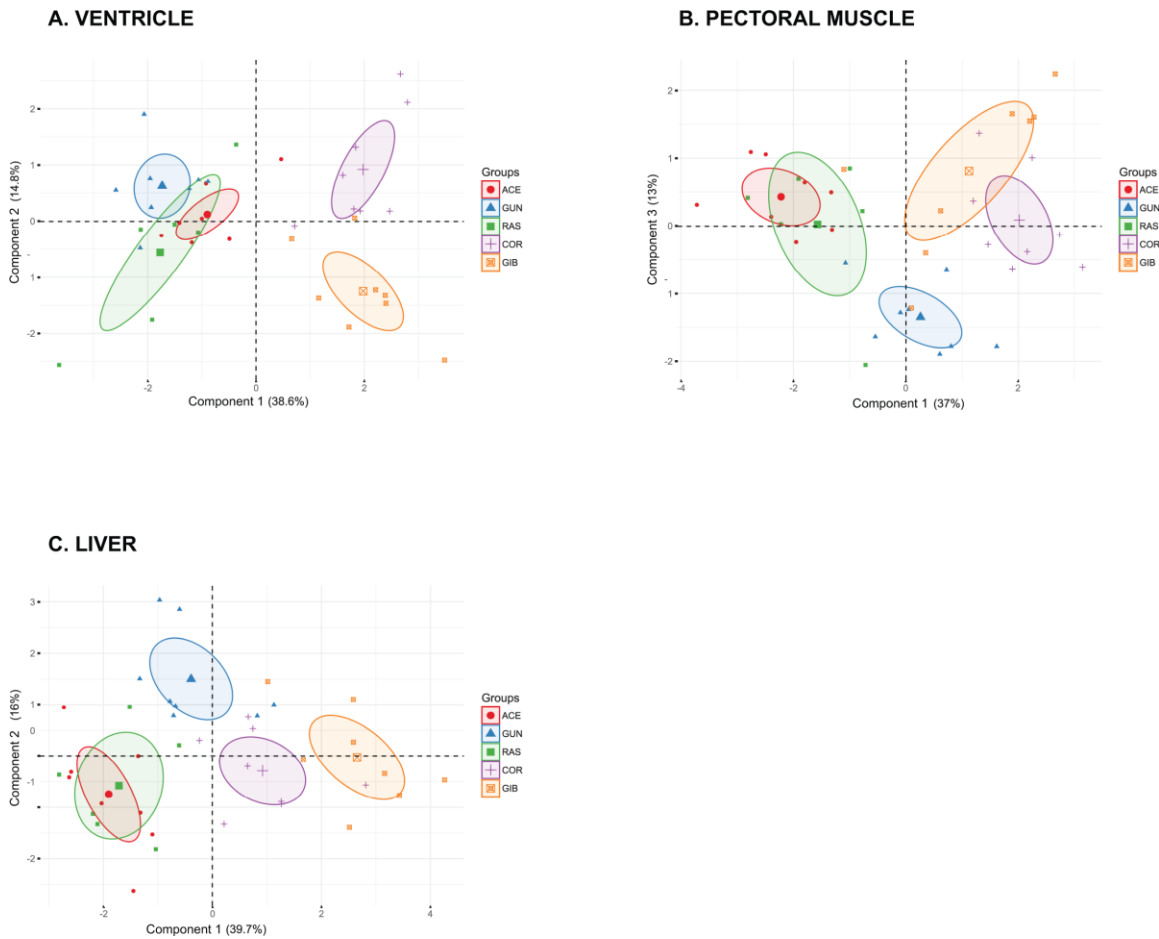
Figure 1. Distribution of individual fish by species, over antioxidants principal components 1 and 2 for heart ventricle (A) and liver (C), and 1 and 3 for pectoral adductor muscle (B). The clustering of antioxidants is visualized by 0.95 confidence ellipses around the barycenter for each species. Individuals that were missing more than 4 of the 9 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.
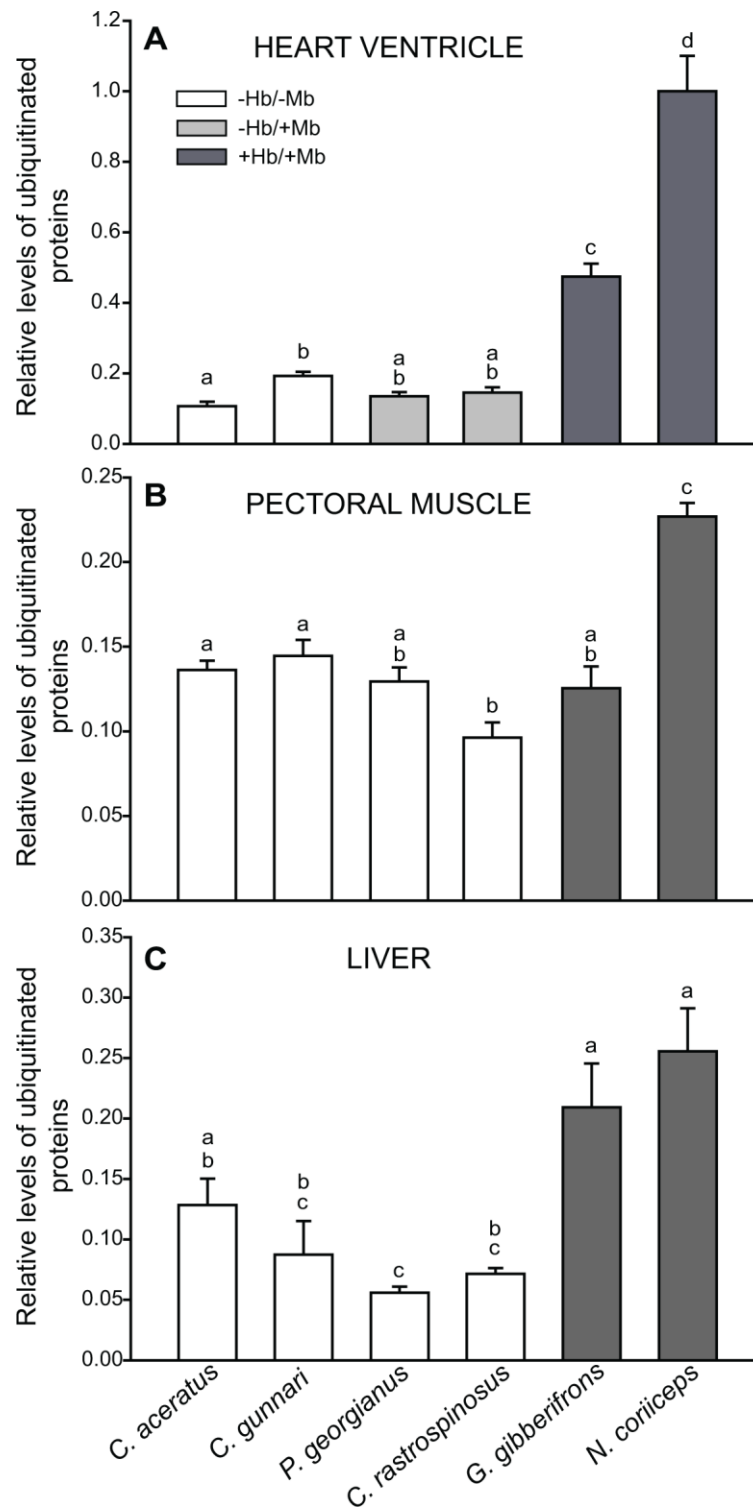
Figure 2. Levels of ubiquitinated proteins in tissues of notothenioids that vary in the expression of hemoglobin (Hb) and myoglobin (Mb). Relative levels of ubiquitinated proteins in heart ventricle (A), pectoral adductor muscle (B), and liver (C) of 6 species of

notothenioids. Levels of ubiquitinated proteins were normalized to levels in 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 ($P < 0.05$).

Figure 3. Activity of the 20S proteasome in tissues of notothenioids that vary in the expression of hemoglobin (Hb). Activity of the 20S proteasome in heart ventricle (A), pectoral adductor muscle (B), and liver (C) of 6 species of notothenioids that vary in the

expression of hemoglobin (Hb). 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).
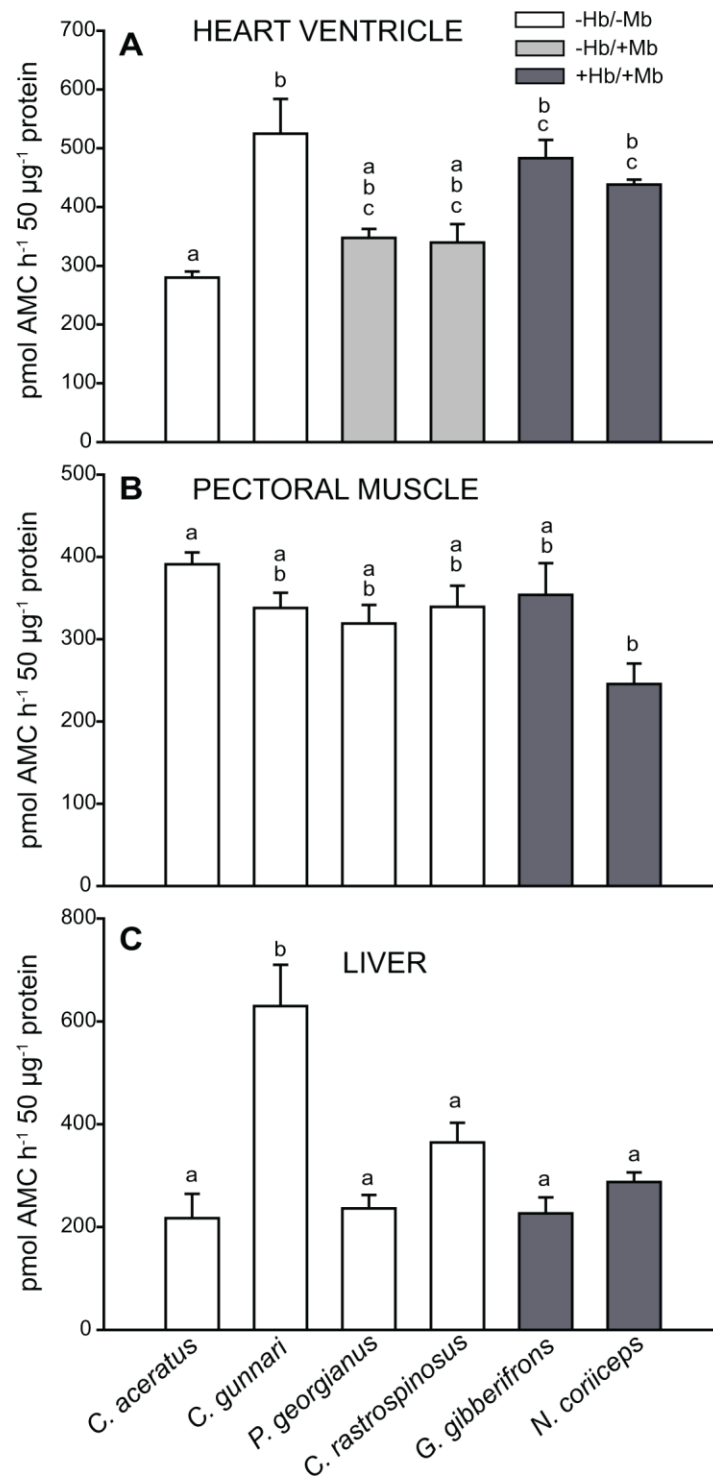
Table 1. Parameters measured in the blood of notothenioids.

	C. aceratus (-Hb/-Mb)	*C. gunnari* (-Hb/-Mb)	*C. rastrospinosus* (-Hb/+Mb)	*N. coriiceps* (+Hb/+Mb)	*G. gibberifrons* (+Hb/+Mb)
Heme (mM)	0.04±0.01^A (6)	0.25±0.01^{AB} (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)

Values are mean ± s.e.m. Different letters indicate significant differences as determined by a Dunn's post-hoc test (*P* < 0.05). Asterisk indicates a significant difference based on a Students t-test *(P* < 0.05). The number of individuals (n) is indicated in parentheses.

Table 2. Levels of pro-oxidants, antioxidants, and oxidized macromolecules in 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 wet mass^{-1})	9.32±0.17^A (5)	12.93±0.92^B (5)	9.72±0.63^{AC} (4)	12.58±0.75^{BC} (5)	10.75±0.62^{ABC} (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^{AB} (8)	269.23±16.10^{AB} (6)	461.66±42.15^B (9)	243.27±43.20^{AB} (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^{AB} (9)	1.13±0.10^A (8)
TAP (µmol g^{-1})	6.65±0.19^{AC} (8)	6.42±0.35^A (8)	5.99±0.12^A (6)	8.26±0.27^B (9)	7.59±0.29^{BC} (8)
GPx1 (U g^{-1})	0.84±0.07^{AB} (8)	1.06±0.10^A (8)	0.55±0.12^B (6)	0.96±0.04^{AB} (8)	0.83±0.10^{AB} (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^{AB} (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^{AB} (6)	0.112±0.016^A (8)	0.109±0.006^{AB} (8)
Oxidative damage					
TBARS (nmol g^{-1})	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^{AB} (6)	33.50±3.67^{AB} (7)	73.38±12.77^B (7)

Values are mean ± s.e.m. Different 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 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 wet mass^{-1})	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^{ABC} (8)	2271.35±108.32^{BC} (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^{AB} (8)	0.85±0.06^C (8)	0.29±0.06^A (6)	0.64±0.10^{BC} (8)	0.66±0.06^{BC} (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^{ABC} (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^{AB} (8)	0.181±0.020^B (6)	0.138±0.008^{AB} (8)	0.143±0.010^{AB} (8)
Oxidative damage					
TBARS (nmol g^{-1})	50.79±4.40^A (8)	75.52±6.33^{AB} (8)	74.38±12.73^{AB} (6)	240.19±42.19^{BC} (8)	279.22±15.95^C (8)
Carbonyls (mmol mol^{-1})	4.77±0.88^A (7)	5.70±1.21^{AC} (6)	7.15±1.70^{ABC} 5)	41.98±9.99^B (7)	25.38±3.53^{BC} (6)

Values are mean ± s.e.m. Different 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.
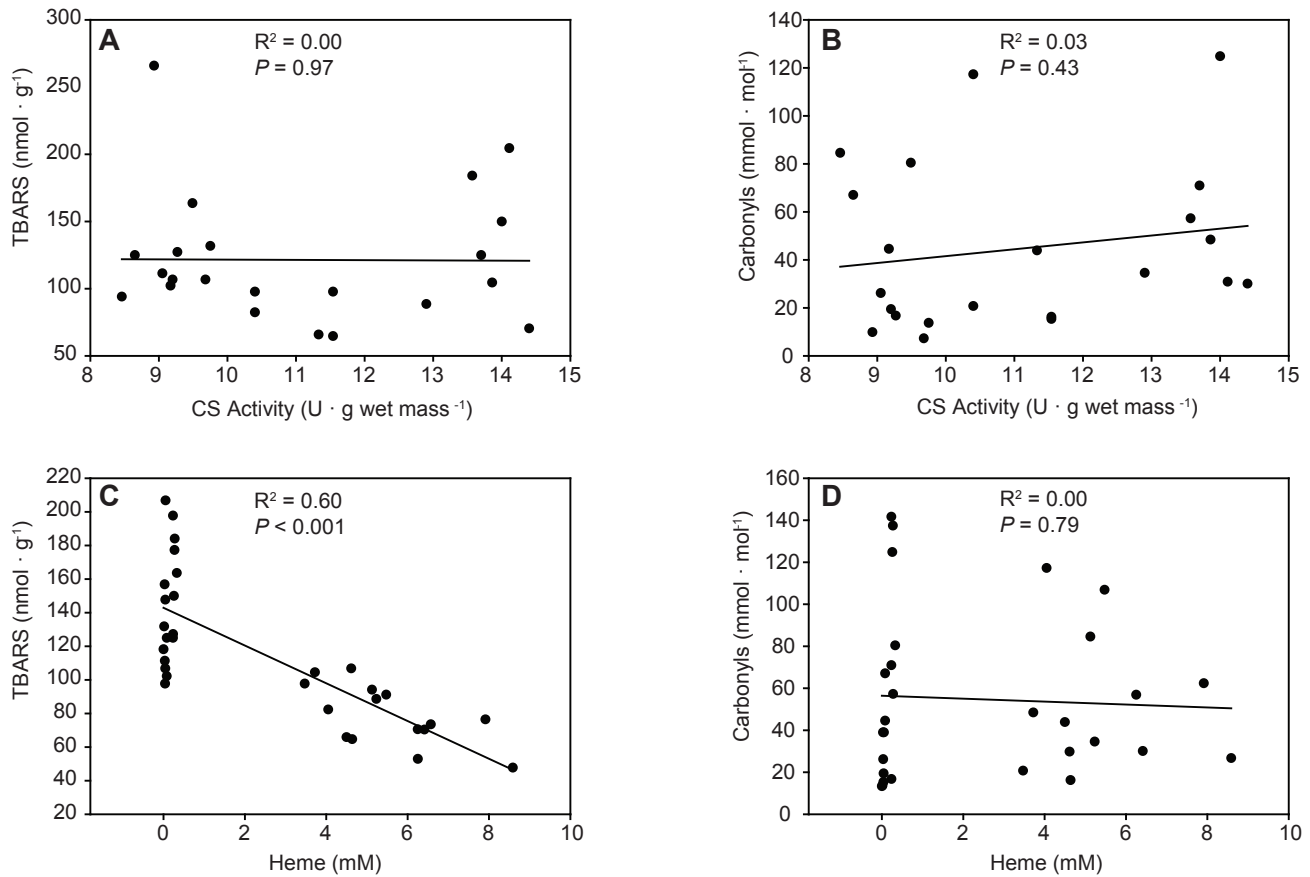
Table 4. Levels of pro-oxidants antioxidants, and oxidized macromolecules in livers of notothenioids.

	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 wet mass^{-1})	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^{AB} (8)	13.41±2.03^{AB} (6)	20.68±1.67^{ABC} (8)	29.97±1.54^C (8)
TAP (µmol g^{-1})	4.87±0.27^A (8)	4.82±0.69^{AB} (8)	4.06±1.40^{AB} (6)	7.84±0.54^B (8)	6.89±0.67 ^{AB} (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^{AB} (6)	0.285±0.030^B (8)	0.203±0.028^{AB} (8)
Oxidative damage					
TBARS (nmol g^{-1})	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^{AB} (8)

| **Carbonyls (mmol mol⁻¹)** | 19.78 ± 4.20^{AB} (6) | 30.14 ± 7.81^{A} (8) | 4.43 ± 1.19^{C} (6) | 5.61 ± 1.24^{BC} (6) | 8.04 ± 3.46^{BC} (6) |

Values are mean ± s.e.m. Different 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 parenthese

HEART VENTRICLE

Supplementary Figure 1. Linear regression of pro-oxidants and levels of oxidized lipids (TBARS; 1A,C) and proteins (carbonyls; 1B,D) in heart ventricles of 5 species of notothenioids. n=22-32.
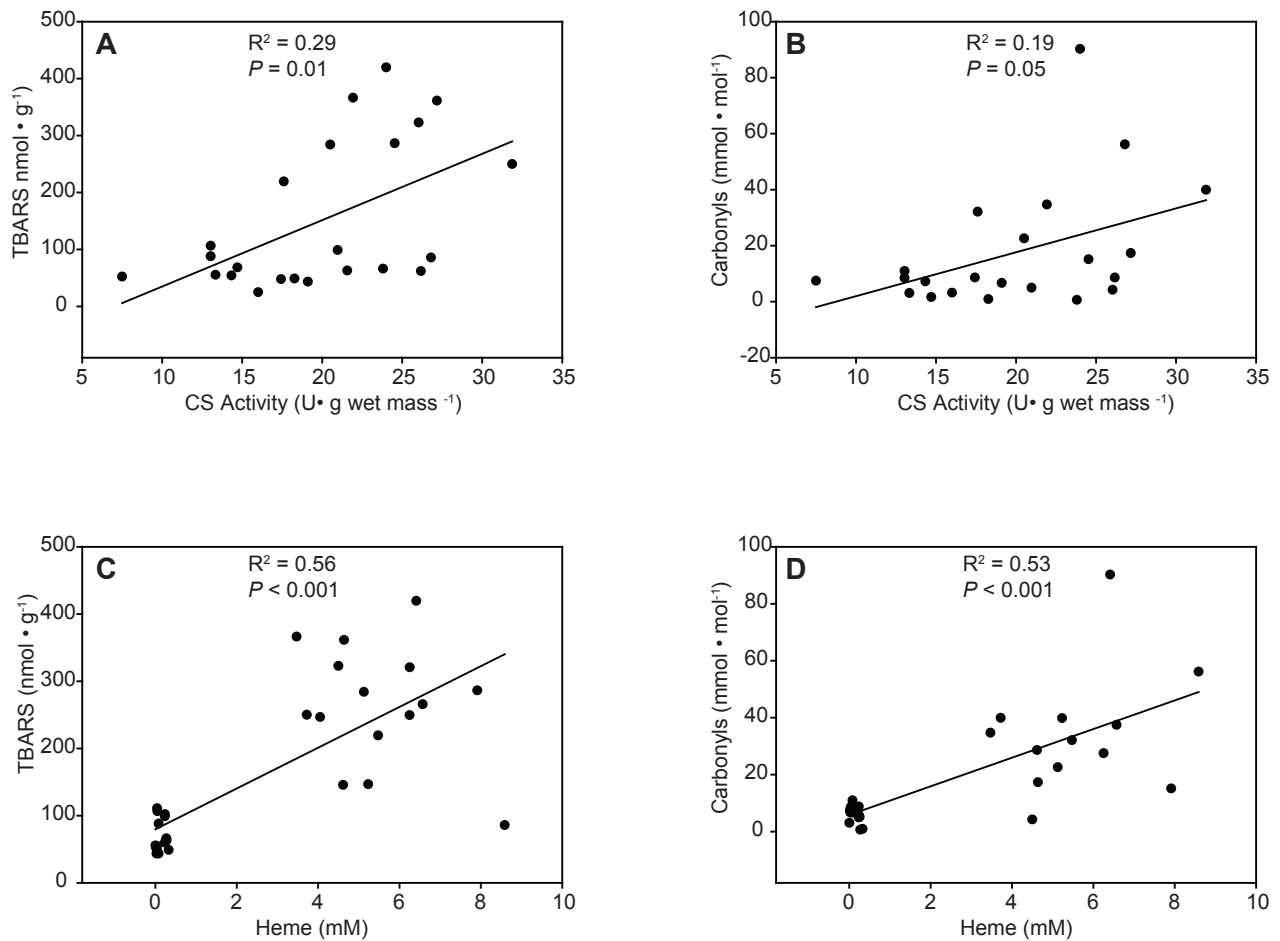
PECTORAL ADDUCTOR

Supplementary Figure 2. Linear regression of pro-oxidants and levels of oxidized lipids (TBARS; 2A,C) and proteins (carbonyls; 2B,D) in oxidative pectoral adductor of 5 species of notothenioids. n=23-32.

LIVER

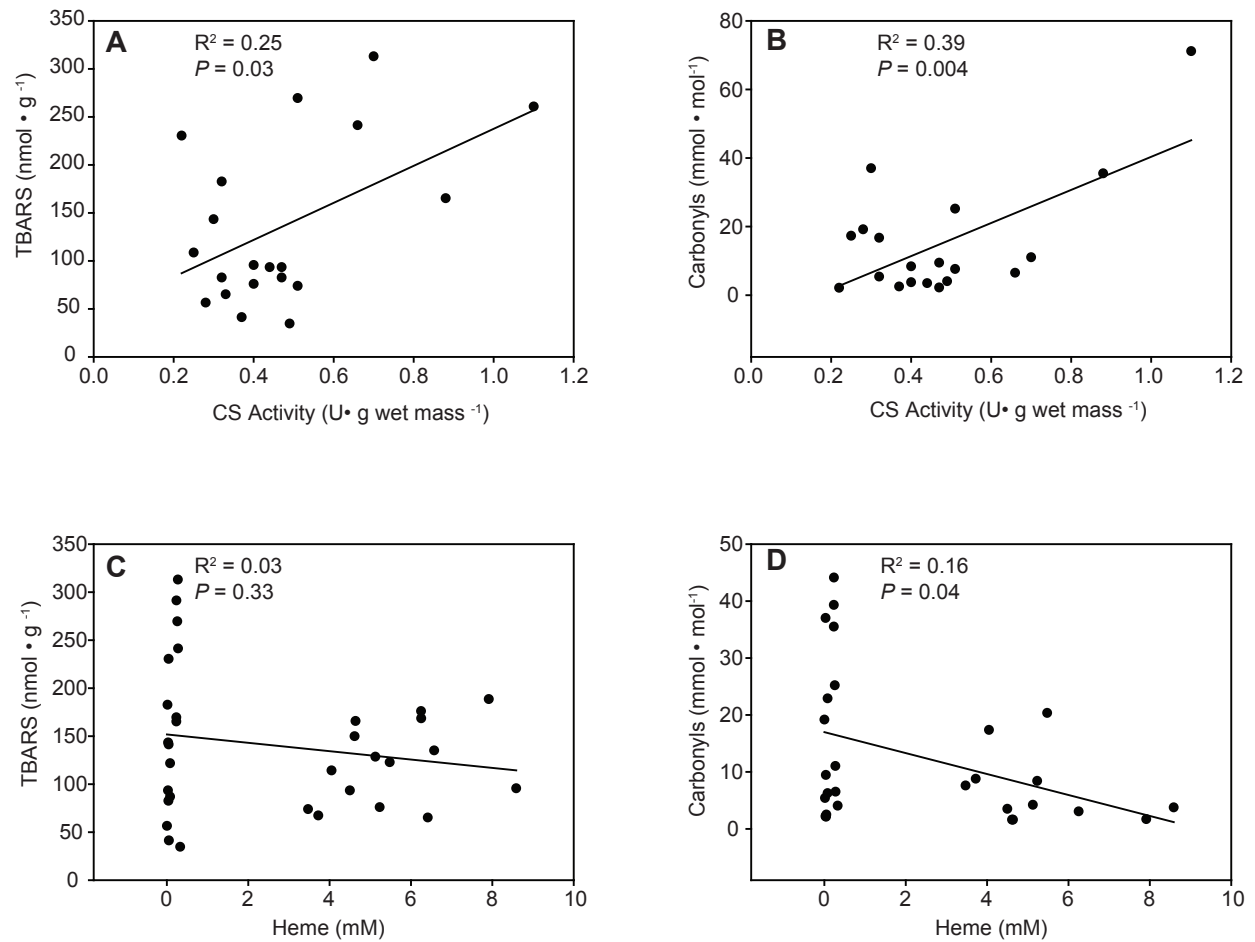**Supplementary Figure 3.** Linear regression of pro-oxidants and levels of oxidized lipids (TBARS; 3A,C) and proteins (carbonyls; 3B,D) in livers of 5 species of notothenioids. n=20-31.

Supplemental Table 1. Hierarchical clustering for heart ventricle

Antioxidant	V statistic*	Group Mean ± S.D.	Overall Mean ± S.D.	*P* value
Group 1 (*C. gunnari, C. aceratus, C. rastrospinosus*)				
GSH	-3.60	177.25 ± 51.97	218.99 ± 82.69	< 0.001
CAT	-3.76	220.17 ± 67.90	284.13 ± 121.35	< 0.001
Ferritin	-4.04	14.48 ± 10.90	46.37 ± 56.34	< 0.001
GR	-4.20	231.14 ± 57.76	293.61 ± 106.16	< 0.001
SOD	-4.45	1777.36 ± 287.82	2055.84 ± 446.23	< 0.001
TAP	-4.64	6.40 ± 0.72	7.11 ± 1.09	< 0.001
Group 2 (*G. gibberifrons*)				
GR	4.84	435.09 ± 67.98	293.61 ± 106.16	<0.001
Ferritin	4.61	117.87 ± 64.56	46.37 ± 56.34	<0.001
SOD	3.64	2502.90 ± 371.13	2055.84 ± 446.23	<0.001
TAP	2.33	7.81 ± 0.51	7.11 ± 1.09	0.020
Group 3 (*N. coriiceps*)				
CAT	4.63	497.48 ± 94.06	284.13 ± 121.35	< 0.001
GSH	4.24	352.12 ± 91.78	218.99 ± 82.69	< 0.001
TAP	3.47	8.54 ± 0.70	7.11 ± 1.09	< 0.001

Overall significance of species for group belonging: X^2 (8, n=38), *P* <0.001

* The standardized difference between the group and overall means, which follows a standard normal distribution under Ho. Ferritin levels from Kuhn et al., 2016).

Supplemental Table 2. Hierarchical clustering for pectoral adductor

Antioxidant	V statistic*	Group Mean ± S.D.	Overall Mean ± S.D.	*P* value
Group 1 **(*C. aceratus, C. rastrospinosus*)**				
CAT	-2.67	203.86 ± 100.10	261.09 ± 105.27	0.010
GPX1	-2.69	0.48 ± 0.23	0.61 ± 0.25	0.010
GR	-2.93	128.47 ± 32.1	170.33 ± 70.22	<0.001
Ferritin	-3.45	6.69 ± 5.98	24.42 ± 25.27	<0.001
GST	-3.51	0.94 ±0.35	1.32 ± 0.53	<0.001
SOD	-4.09	1614.47 ± 428.28	2036.74 ±506.55	<0.001
GPX4	-5.11	12.39 ± 3.18	20.26 ± 7.57	<0.001
Group 2 **(*C. gunnari*)**				
SOD	3.50	2493.27 ± 256.72	2036.74 ± 506.55	<0.001
GPX1	2.40	0.77 ± 0.19	0.61 ± 0.25	0.020
TAP	-2.33	4.37 ± 0.76	5.08 ± 1.19	0.020
Group 3 **(*N. coriiceps*)**				
GR	5.20	286.48 ± 45.37	170.33 ± 70.22	<0.001
CAT	4.42	409.24 ± 52.66	261.09 ± 105.27	<0.001
GPX4	2.99	27.45 ± 3.09	20.26 ± 7.57	<0.001
Ferritin	2.06	41.02 ± 24.11	24.42 ± 25.27	0.040
Group 4 **(*G. gibberifrons*)**				
Ferritin	4.09	73.94 ± 15.43	24.42 ± 25.27	<0.001
GST	3.30	2.16 ± 0.36	1.32 ± 0.53	<0.001
TAP	2.65	6.60 ± 0.48	5.08 ± 1.19	0.010
GPX4	2.17	28.14 ± 3.84	20.26 ± 7.57	0.030
GSH	2.08	197.72 ± 20.59	161.47 ± 36.37	0.040

Overall significance of species for group belonging: X^2 (12, n=38), *P* <0.001
* The standardized difference between the group and overall means, which follows a standard normal distribution under Ho.

Supplemental Table 3. Hierarchical clustering for liver

Antioxidant	V statistic*	Group Mean ± S.D.	Overall Mean ± S.D.	*P* value
Group 1 (*C. aceratus, C. rastrospinosus*)				
TAP	-2.66	4.42 ± 2.22	5.85 ± 2.37	0.010
Ferritin	-3.35	9.73 ± 6.03	39.94 ± 39.51	<0.001
GST	-3.43	12.60 ± 5.37	18.91 ± 8.05	<0.001
SOD	-4.71	5030.46 ± 1058.52	7192.55 ± 2013.93	<0.001
CAT	-4.86	1631.33 ± 604.50	3703.37 ± 1871.42	<0.001
Group 2 (*C. gunnari*)				
SOD	3.67	9542.63 ± 894.76	7192.55 ± 2013.93	< 0.001
GR	-3.72	116.66 ± 49.66	256.15 ± 117.76	< 0.001
Group 3 (*N. coriiceps*)				
TAP	2.60	7.54 ± 1.47	5.85 ± 2.37	0.010
CAT	2.53	5007.53 ± 1415.39	3703.37 ± 1871.42	0.010
Group 4 (*G. gibberifrons*)				
GSH	4.50	281.40 ± 58.54	170.33 ± 71.34	<0.001
GST	3.99	30.02 ± 4.34	18.91 ± 8.05	<0.001
GR	3.48	398.07 ± 89.17	256.15 ± 117.76	<0.001
Ferritin	3.19	83.49 ± 39.91	39.94 ± 39.51	<0.001
CAT	2.85	5547.79 ± 780.98	3703.37 ± 1871.42	<0.001
SOD	2.14	8684.14 ± 468.28	7192.55 ± 2013.93	0.030

Overall significance of species for group belonging: X^2 (12, n=38), P <0.001
* The standardized difference between the group and overall means, which follows a standard normal distribution under Ho.
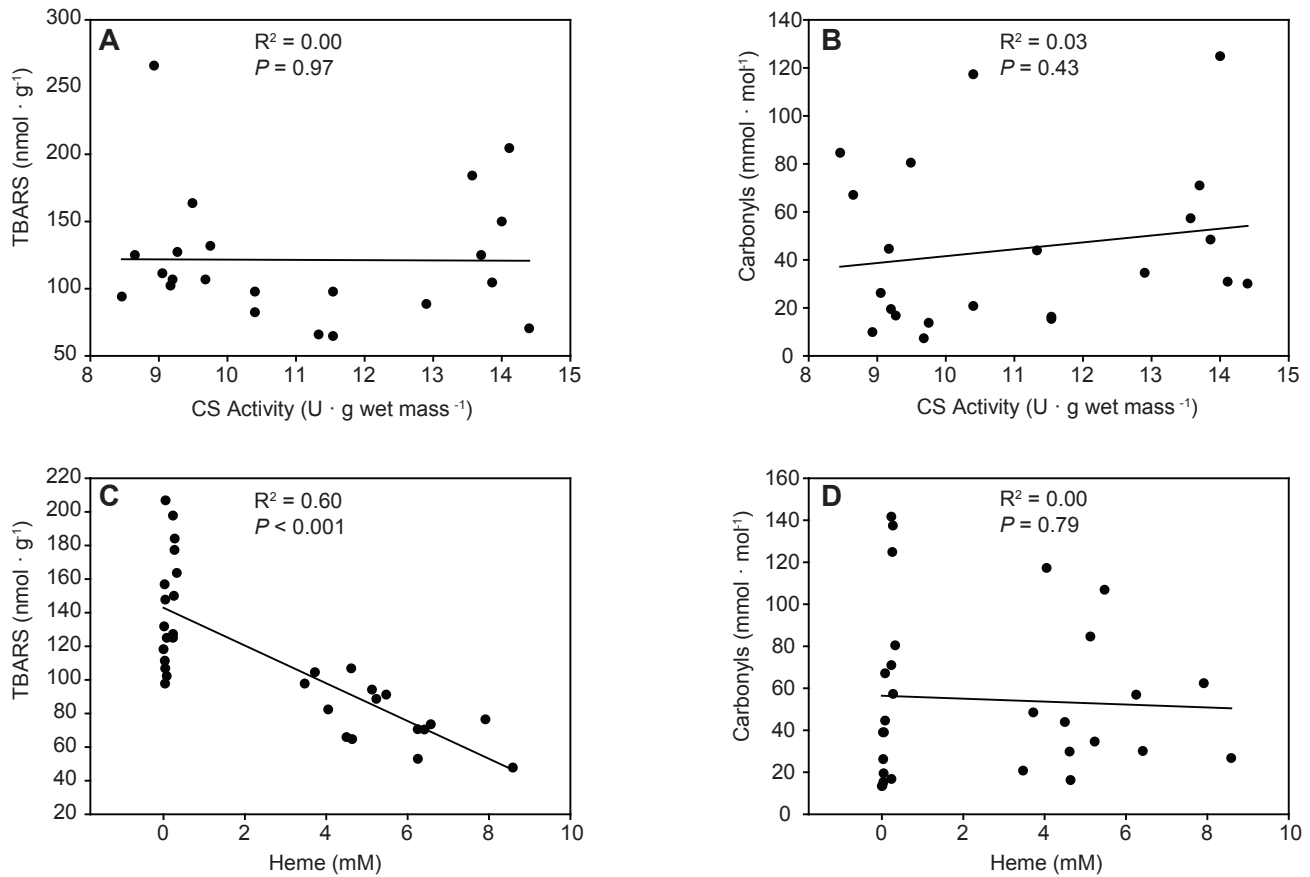
HEART VENTRICLE

Fig. S1. Linear regression of pro-oxidants and levels of oxidized lipids (TBARS; 1A,C) and proteins (carbonyls; 1B,D) in heart ventricles of 5 species of notothenioids. n=22-32.
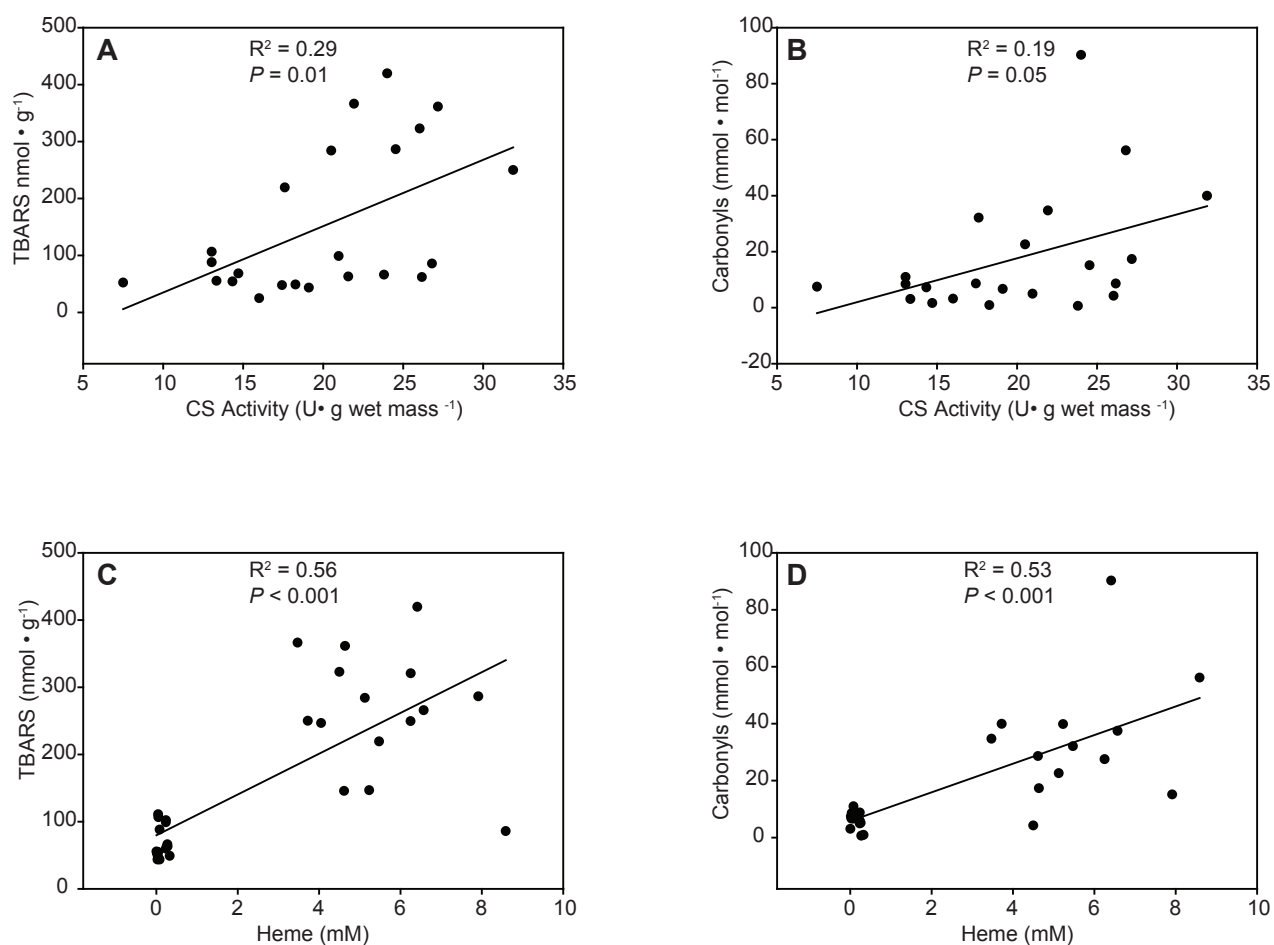
PECTORAL ADDUCTOR

Fig. S2. Linear regression of pro-oxidants and levels of oxidized lipids (TBARS; 2A,C) and proteins (carbonyls; 2B,D) in oxidative pectoral adductor of 5 species of notothenioids. n=23-32.
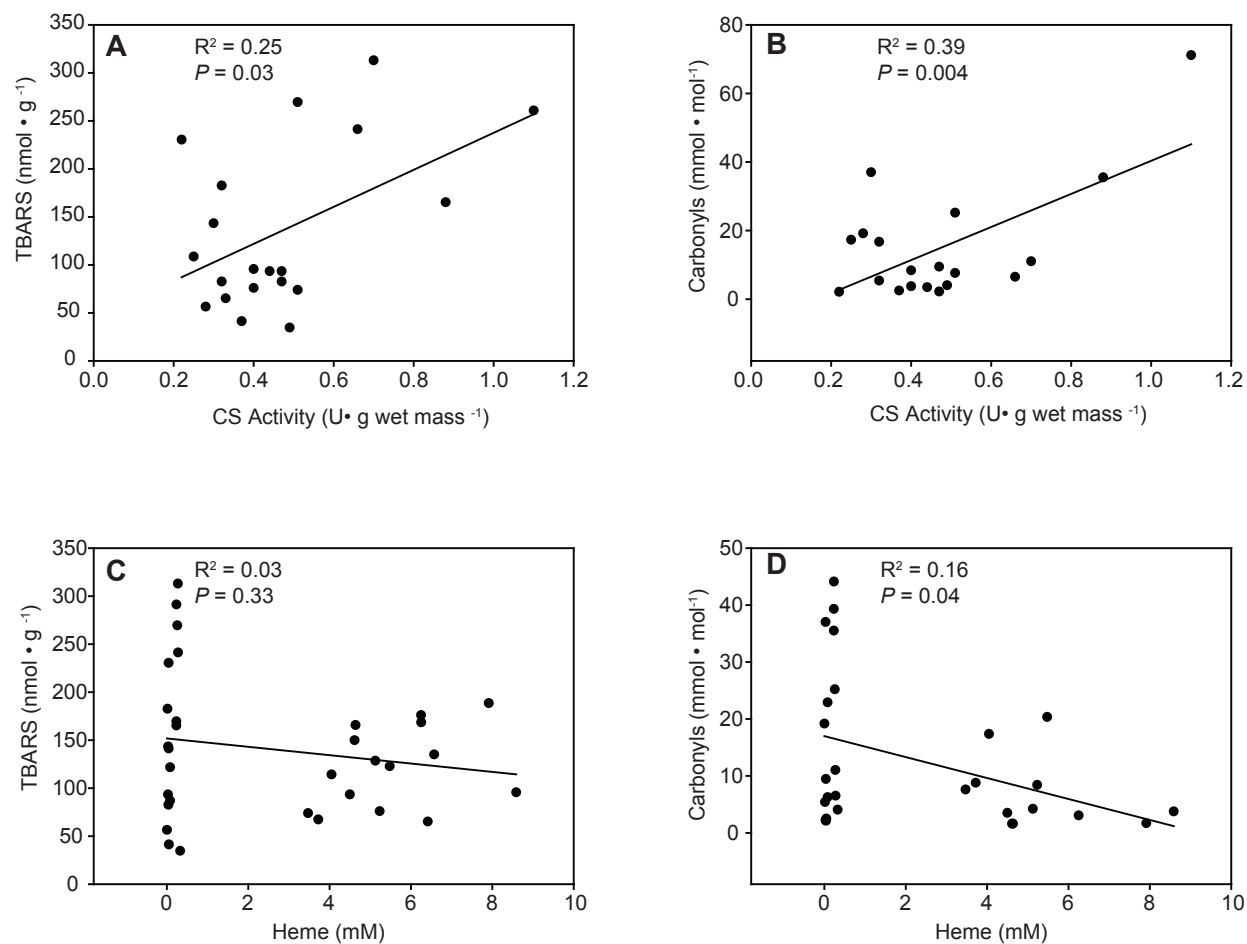
LIVER

Fig. S3. Linear regression of pro-oxidants and levels of oxidized lipids (TBARS; 3A,C) and proteins (carbonyls; 3B,D) in livers of 5 species of notothenioids. n=20-31.

Table S1. Hierarchical clustering for heart ventricle

Antioxidant	V statistic*	Group Mean ± S.D.	Overall Mean ± S.D.	*P* value
Group 1 (*C. gunnari, C. aceratus, C. rastrospinosus*)				
GSH	-3.60	177.25 ± 51.97	218.99 ± 82.69	< 0.001
CAT	-3.76	220.17 ± 67.90	284.13 ± 121.35	< 0.001
Ferritin	-4.04	14.48 ± 10.90	46.37 ± 56.34	< 0.001
GR	-4.20	231.14 ± 57.76	293.61 ± 106.16	< 0.001
SOD	-4.45	1777.36 ± 287.82	2055.84 ± 446.23	< 0.001
TAP	-4.64	6.40 ± 0.72	7.11 ± 1.09	< 0.001
Group 2 (*G. gibberifrons*)				
GR	4.84	435.09 ± 67.98	293.61 ± 106.16	<0.001
Ferritin	4.61	117.87 ± 64.56	46.37 ± 56.34	<0.001
SOD	3.64	2502.90 ± 371.13	2055.84 ± 446.23	<0.001
TAP	2.33	7.81 ± 0.51	7.11 ± 1.09	0.020
Group 3 (*N. coriiceps*)				
CAT	4.63	497.48 ± 94.06	284.13 ± 121.35	< 0.001
GSH	4.24	352.12 ± 91.78	218.99 ± 82.69	< 0.001
TAP	3.47	8.54 ± 0.70	7.11 ± 1.09	< 0.001

Overall significance of species for group belonging: X^2 (8, n=38), *P* <0.001
* The standardized difference between the group and overall means, which follows a standard normal distribution under Ho. Ferritin levels from Kuhn et al., 2016).

Table S2. Hierarchical clustering for pectoral adductor

Antioxidant	V statistic*	Group Mean ± S.D.	Overall Mean ± S.D.	*P* value
Group 1 **(*C. aceratus, C. rastrospinosus*)**				
CAT	-2.67	203.86 ± 100.10	261.09 ± 105.27	0.010
GPX1	-2.69	0.48 ± 0.23	0.61 ± 0.25	0.010
GR	-2.93	128.47 ± 32.1	170.33 ± 70.22	<0.001
Ferritin	-3.45	6.69 ± 5.98	24.42 ± 25.27	<0.001
GST	-3.51	0.94 ±0.35	1.32 ± 0.53	<0.001
SOD	-4.09	1614.47 ± 428.28	2036.74 ±506.55	<0.001
GPX4	-5.11	12.39 ± 3.18	20.26 ± 7.57	<0.001
Group 2 **(*C. gunnari*)**				
SOD	3.50	2493.27 ± 256.72	2036.74 ± 506.55	<0.001
GPX1	2.40	0.77 ± 0.19	0.61 ± 0.25	0.020
TAP	-2.33	4.37 ± 0.76	5.08 ± 1.19	0.020
Group 3 **(*N. coriiceps*)**				
GR	5.20	286.48 ± 45.37	170.33 ± 70.22	<0.001
CAT	4.42	409.24 ± 52.66	261.09 ± 105.27	<0.001
GPX4	2.99	27.45 ± 3.09	20.26 ± 7.57	<0.001
Ferritin	2.06	41.02 ± 24.11	24.42 ± 25.27	0.040
Group 4 **(*G. gibberifrons*)**				
Ferritin	4.09	73.94 ± 15.43	24.42 ± 25.27	<0.001
GST	3.30	2.16 ± 0.36	1.32 ± 0.53	<0.001
TAP	2.65	6.60 ± 0.48	5.08 ± 1.19	0.010
GPX4	2.17	28.14 ± 3.84	20.26 ± 7.57	0.030
GSH	2.08	197.72 ± 20.59	161.47 ± 36.37	0.040

Overall significance of species for group belonging: X^2 (12, n=38), *P* <0.001
* The standardized difference between the group and overall means, which follows a standard normal distribution under Ho.

Table S3. Hierarchical clustering for liver

Antioxidant	V statistic*	Group Mean ± S.D.	Overall Mean ± S.D.	*P* value
Group 1 **(*C. aceratus, C. rastrospinosus*)**				
TAP	-2.66	4.42 ± 2.22	5.85 ± 2.37	0.010
Ferritin	-3.35	9.73 ± 6.03	39.94 ± 39.51	<0.001
GST	-3.43	12.60 ± 5.37	18.91 ± 8.05	<0.001
SOD	-4.71	5030.46 ± 1058.52	7192.55 ± 2013.93	<0.001
CAT	-4.86	1631.33 ± 604.50	3703.37 ± 1871.42	<0.001
Group 2 **(*C. gunnari*)**				
SOD	3.67	9542.63 ± 894.76	7192.55 ± 2013.93	< 0.001
GR	-3.72	116.66 ± 49.66	256.15 ± 117.76	< 0.001
Group 3 **(*N. coriiceps*)**				
TAP	2.60	7.54 ± 1.47	5.85 ± 2.37	0.010
CAT	2.53	5007.53 ± 1415.39	3703.37 ± 1871.42	0.010
Group 4 **(*G. gibberifrons*)**				
GSH	4.50	281.40 ± 58.54	170.33 ± 71.34	<0.001
GST	3.99	30.02 ± 4.34	18.91 ± 8.05	<0.001
GR	3.48	398.07 ± 89.17	256.15 ± 117.76	<0.001
Ferritin	3.19	83.49 ± 39.91	39.94 ± 39.51	<0.001
CAT	2.85	5547.79 ± 780.98	3703.37 ± 1871.42	<0.001
SOD	2.14	8684.14 ± 468.28	7192.55 ± 2013.93	0.030

Overall significance of species for group belonging: X^2 (12, n=38), P <0.001

* The standardized difference between the group and overall means, which follows a standard normal distribution under Ho.