Integrative and Comparative Biology

Integrative and Comparative Biology, volume 56, number 4, pp. 531–541
doi:10.1093/icb/icw062

Society for Integrative and Comparative Biology

SYMPOSIUM

New Lessons from an Old Fish: What Antarctic Icefishes May Reveal about the Functions of Oxygen-Binding Proteins

Kristin M. O'Brien[1]

University of Alaska Fairbanks, Institute of Arctic Biology, Department of Biology and Wildlife, Fairbanks, AK 99775, USA

From the symposium "Life on the Edge: the Biology of Organisms Inhabiting Extreme Environments" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2016 at Portland, Oregon.

[1]E-mail: kmobrien@alaska.edu

Synopsis The loss of expression of the oxygen-binding protein hemoglobin (Hb) in the family Channichthyidae (suborder Notothenioidei) of Antarctic fishes is considered a disaptation that has persisted because of the unusual conditions prevailing in the Southern Ocean during the evolution of the family. The loss of expression of the intracellular oxygen-binding protein myoglobin (Mb) in heart ventricles is more of a conundrum because it occurred at four points during the radiation of the family, suggesting weakened selective pressure maintaining expression of the protein. Yet, studies have shown that when present, Mb enhances function. Here, I discuss potential reasons for weakened selective pressure maintaining Mb expression in light of the multiple functions proposed for Mb. Additionally, I discuss results from recent studies exploring the possibility that the loss of Hb and Mb may be advantageous because it reduces the production of reactive oxygen species, levels of oxidized proteins, and the energetic costs associated with replacing oxidatively damaged proteins.

Introduction

Antarctic fishes are unparalleled model organisms for studying novel traits that may arise during millions of years of evolution in a stable, cold environment. One of the most curious traits among the Antarctic fish fauna is the lack of hemoglobin (Hb) in the family Channichthyidae (icefishes), evidenced by their stark white gills and milky-white blood. One of the earliest records of an Antarctic icefish is from 1928 when the young Norwegian biologist Ditlef Rustad captured the icefish *Chaenocephalus aceratus* while fishing offshore of Bouvet Island during the voyage of the *Norvegia* (1927–1928). Twenty-five years later, while participating in a whale marking expedition near South Georgia, and just in time for Christmas, fellow Norwegian and physiologist Johan Ruud obtained three specimens of *C. aceratus* (Ruud 1965). Although known to whalers in South Georgia for over half a century, the first description of these "anemic" fish was published in the scientific literature by Ruud (1954). Since then, icefishes have captured the imagination and fascination of physiologists worldwide. Icefishes are the only vertebrates that lack Hb as adults and as Ruud astutely observed, could only survive in the frigid waters of the Southern Ocean. The icy cold and oxygen-rich waters of the Southern Ocean, depauperate of predators, was key to their survival without Hb. There have been several excellent reviews on the evolution of the Antarctic fish fauna in recent years (Cheng and Detrich 2007; Beers and Jayasundara 2015). I direct you to these to learn more about the extraordinary evolutionary history of notothenioids. My focus here is on the icefish family, the patterns of loss of oxygen-binding proteins in the family, the consequences of these losses, and what the patterns of loss might reveal about the multiple functions of oxygen-binding proteins.

Loss of Hb expression

The mutations leading to the loss of Hb occurred approximately 8.5 MYA (Near 2004). By this time,

Advanced Access publication June 1, 2016

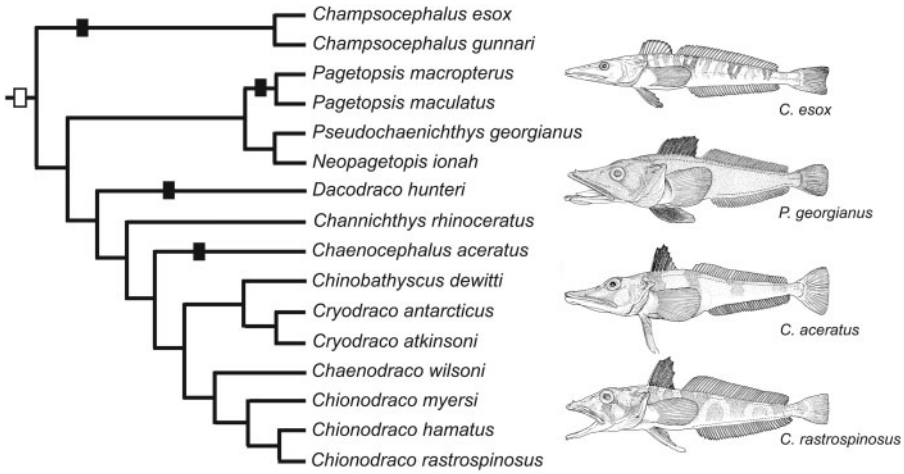

Fig. 1 Phylogenetic tree of the Channichthyidae family. The mutational event leading to the loss of Hb is indicated by an open square, whereas the mutations leading to the loss of Mb are indicated by filled squares. The figure is from O'Brien and Mueller (2010).

the Southern Ocean had cooled to less than 5°C and today ranges between −1.9°C and 1.5°C depending on season and sector (Knox 1994). The extensive extinctions in the Antarctic fish fauna that coincided with the cooling of the Southern Ocean likely contributed to the survival of icefishes by relaxing competition and expanding available habitat (Eastman 1993, 2005). The structure of the Hb genes in the derived icefish, *Neopagetopsis ionah* (Fig. 1), indicates that the loss of Hb expression was a progressive process. *N. ionah* contains two β-globin pseudo genes and an intact α-globin gene (Near et al. 2006). In contrast, 15 of the 16 species of icefishes lack the entire β-globin gene and possess only one of the three exons encoding the α-globin gene. In *N. ionah*, one β-globin gene contains part of exon 3 and the 3' untranslated region. The second β-globin gene is intact but has a 10 bp deletion at the splice junction between intron 1 and exon 2. This intermediate state in the derived *N. ionah* is thought to have arisen as a result of retention of ancestral polymorphism and lack of coalescence among ancestral icefish α and β globin alleles (Near et al. 2006).

Adaptations to compensate for the loss of Hb

How do icefishes accomplish the seemingly impossible task of adequately delivering oxygen to tissues with a blood oxygen-carrying capacity only one-tenth of red-blooded species (Holeton 1970) and a body mass that can be upwards of one kg? The icy-cold and oxygen-rich waters of the Southern Ocean were likely imperative for the survival of the ancestral icefish. Indeed, red-blooded notothenioids can survive in these waters when Hb is poisoned by carbon monoxide (di Prisco et al. 1992) or when hematocrit and is reduced by over 90% and Hb levels by more than 70% following treatment with the hemolytic agent, phenylhydrazine (Borley et al. 2010). Nevertheless, the lack of Hb is also correlated with extensive remodeling of the cardiovascular system to enhance oxygen delivery to tissues—modifications potentially stimulated by an increase in circulating levels of nitric oxide (NO) in the absence of Hb (Sidell and O'Brien 2006; Borley et al. 2010). Blood volumes are on average four times greater than that of red-blooded fishes (Hemmingsen and Douglas 1970; Fitch et al. 1984; Acierno et al. 1995) and the diameter of blood vessels are two-to-three times greater than most teleosts (Fitch et al. 1984). Blood is pumped at a high velocity, which maintains a high PO_2 gradient between capillaries and tissue. The heart-to-body mass ratio of icefishes is greater than red-blooded fishes, resulting in a five-fold greater cardiac output (Hemmingsen et al. 1972), and capillary density is higher in some tissues compared with red-blooded fishes (Wujcik et al. 2007). Icefishes have a trabeculated heart, constructed entirely of spongiosa and lacking a compact myocardial layer and coronary circulation (Tota et al. 1997). To enhance oxygen diffusion, hearts of icefishes have a greater surface area compared with red-blooded species, which shortens the diffusion distance for oxygen between the lumen of the heart and mitochondria (O'Brien et al. 2000). Icefish hearts also have a greater capacity for anaerobic metabolism compared with red-blooded species (Bacila et al. 1989; O'Brien and Sidell 2000). Nevertheless, their aerobic metabolic capacity is not compromised by the lack of Hb, as indicated by similar maximal activities of citrate synthase and cytochrome c oxidase per g wet cardiac mass compared with red-blooded fishes (O'Brien and

Sidell 2000). Additionally, maximal activities of glycolytic and aerobic metabolic enzymes are similar between red-and white-blooded species in oxidative and glycolytic muscles (Dunn and Johnston 1986; Johnston 1987). Like red-blooded notothenioids, icefishes rely predominantly on their lipid-rich diet and stores to fuel metabolic fires (Crockett and Sidell 1990; Sidell et al. 1995; O'Brien and Sidell 2000).

The renovated cardiovascular system of icefishes ensures adequate oxygen delivery under ambient conditions but is likely insufficient in the face of rising temperatures. The critical thermal maximum (CTMax) of icefishes, as determined by the temperature at which equilibrium is lost during a rapid heat ramp, is 2–4°C lower than that of red-blooded fishes (Beers and Sidell 2011). Although adding supplemental oxygen does not extend CTMax (Devor et al. 2016), suggesting that oxygen *per se* is not limiting thermal tolerance, our recent studies indicate that cardiac failure contributes to the low CTMax of icefishes (Egginton and Farrell, unpublished data). The cardiovascular system of icefishes operates close to its maximal capacity under ambient conditions and can only modestly increase cardiac output in response to elevations in temperature (Egginton and Farrell, unpublished data). Behavioral differences between red- and white-blooded fishes in response to warming reflect these differences in aerobic scope. Red-blooded species exhibit a strong escape response, while icefishes remain the eternal optimists, waiting and hoping for a change in the weather, likely due to the limitations of their cardiovascular system (unpublished observations).

Loss of Mb expression

Not only are icefishes challenged by the absence of Hb, but 6 of the 16 members of the family also lack the intracellular oxygen-binding protein myoglobin (Mb) in cardiac muscle (Sidell et al. 1997; Moylan and Sidell 2000). Curiously, neither red nor white-blooded notothenioids express Mb in oxidative skeletal muscle (Moylan and Sidell 2000). The loss of Mb expression occurred four times during the radiation of the Channichthyidae family and by multiple molecular mechanisms (Sidell et al. 1997; Fig. 1). The loss of Mb expression in *Champsocephalus esox* and *Champsocephalus gunnari* is due to a 5 bp insertion causing a frame shift and insertion of a premature stop codon (Grove et al. 2004). *Pagetopsis macropterus* and *Pagetopsis maculatus* have intact gene sequences but a mutation in the polyadenylation signal, potentially leading to premature degradation of the mRNA and lack of the protein (Vayda et al. 1997). The mutation leading to the loss of Mb in *C. aceratus* was once thought to be due to a 15 bp insertion 600 bp upstream from the transcriptional start site, duplicating the TATA box and potentially inhibiting binding of RNA polymerase (Small et al. 2003). More recently however, it has been shown that this insertion is present not only in *C. aceratus* but also in 14 of the 16 species icefishes, including those expressing Mb, discounting its role in restricting Mb expression (Borley and Sidell 2011). The mutations causing the loss of Mb in *Dacodraco hunteri* and *C. aceratus* are yet to be elucidated.

Lack of cardiac Mb is not restricted to channichthyids but rather, is widespread among teleosts inhabiting a wide variety of ecological niches (Macqueen et al. 2014). Fishes lacking Mb include species that are air and water breathers, active and sedentary, temperate and tropical, making it unclear what conditions reduce selective pressure on Mb expression (Macqueen et al. 2014). Studies in notothenioids have shown that hearts of icefishes expressing the protein are capable of withstanding greater afterload pressures than those that do not (Acierno et al. 1997) and Mb functions at the cold body temperature of notothenioids (Cashon et al. 1997). Loss of Mb in hearts of icefishes is correlated with an expansion of the mitochondrial compartment, and in mammals, when Mb is knocked out, hearts compensate by increasing capillary density and Hb concentration, thereby steepening the PO_2 gradient between capillaries and mitochondria (Godecke et al. 1999; O'Brien and Sidell 2000). Together, these studies show that when present, Mb is beneficial as an oxygen-binding and storage protein and when absent, the cardiovascular system compensates to ensure adequate oxygen delivery to mitochondria.

Multiple functions of Mb

Perhaps to better understand the widespread loss of Mb in teleosts, we have to consider its functions other than that as an oxygen-binding and storage protein. Mb plays an important role in the metabolism of NO, which regulates a plethora of physiological functions, including vasodilation, mitochondrial biogenesis, cardiac contractility, and cellular respiration (Kerwin et al. 1995). Under normoxic conditions, oxygenated Mb (and Hb) displays NO dioxygenase activity, metabolizing NO to nitrate (Equation (1)). This is crucial for eliminating NO, which impairs cellular respiration by binding to, and inhibiting, the activity of cytochrome c oxidase (Cleeter et al. 1994). Additionally, when subunit 3

(ND3) of complex I is nitrosylated, the rate of electron transfer is reduced (Piantadosi 2012). Related to this, Mb has recently been localized to the mitochondrion where it enhances the activity of cytochrome c oxidase (Yamada et al. 2015). Whether this is due to Mb's NO dioxygenase activity is unclear. Under hypoxic conditions, deoxygenated Mb displays nitrite reductase activity, producing NO (Equation (2); Flogel et al. 2001; Rassaf et al. 2007), thereby slowing cellular respiration and minimizing production of reactive oxygen species (ROS; Hendgen-Cotta et al. 2008).

$$Mb(Fe^{2+})O_2 + NO \rightarrow Mb(Fe^{3+}) + NO^{3-} \quad (1)$$

$$NO_2^- + Mb(Fe^{2+}) + H^+ \rightarrow NO + Mb(Fe^{3+}) + OH^- \quad (2)$$

NO is produced by the enzyme nitric oxide synthase (NOS), and in many vertebrates there are three isoforms of the enzyme: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS; Kerwin et al. 1995). Although the cardiovascular system of icefishes is responsive to NO, as NO dilates the branchial vasculature and increases cardiac stroke volume and power output (Pellegrino et al. 2003; Pellegrino et al. 2004), cardiac and oxidative skeletal muscles of both red- and white-blooded notothenioids have nearly undetectable levels of NOS (Beers et al. 2010). Notably, eNOS, present in hearts of mammals, is absent from the genome of teleosts (Mueller and O'Brien 2011; Syeda et al. 2013). The lack of NOS and of substantial NO production in hearts of notothenioids, and presumably other teleosts as well, may have reduced selective pressure maintaining the expression of Mb as an NO dioxygenase (Beers et al. 2010). Additionally, the cold, oxygen-rich environment of the Southern Ocean may minimize hypoxic episodes in notothenioid hearts and reduce selective pressure on Mb as a nitrite reductase. Nevertheless, the lack of Mb in hearts of the icefish *C. aceratus* may explain why levels of oxidatively damaged proteins increase in their hearts in response to exposure to CTMax (and associated hypoxia), but not in hearts of Mb-expressing notothenioids (Mueller et al. 2012). Additional studies of relationships between NOS activity and Mb may help to elucidate the pattern of Mb expression in teleost hearts.

Despite of the loss of Hb and Mb, neuroglobin (Ngb), a monomeric globin similar in structure to Mb, is found in the brains of icefishes, as well as in those of red-blooded notothenioids (Cheng et al. 2009). Ngb has been ascribed several putative functions, including oxygen delivery to mitochondria, and detoxifying NO, reactive nitrogen species and/or ROS (Burmester et al. 2000; Sun et al. 2001; Burmester and Hankeln 2009; Beers et al. 2010). Correlated with the expression of Ngb, NOS activity is high in brains of nototothenioids, including icefishes (Beers et al. 2010), supporting Ngb's putative role in metabolizing NO, and suggesting that although selective pressure may be insufficient to maintain expression of all oxygen-binding proteins in notothenioids, selective pressure has maintained expression of oxygen-binding proteins for detoxifying NO in NO-producing tissues.

Recent studies have shown that oxygenated Mb binds both palmitate and oleic acid, and in the deoxygenated state, releases the fatty acids, suggesting that Mb may effectively deliver fatty acids from the sarcolemma (high PO_2) to the mitochondrion (low PO_2; Shih et al. 2014, 2015). Unlike all other vertebrates, Antarctic notothenioids express two isoforms of fatty acid binding protein (FABP) in hearts—a heart-type and adipose-type protein (Vayda et al. 1998). The FABP-endowed hearts of notothenioids may have reduced selective pressure on Mb if it indeed is important for transporting fatty acids to the mitochondrion, the preferred substrate for fueling cardiac work (Sidell et al. 1995). Few studies have investigated levels or isoforms of FABP in teleost hearts to determine how widespread the expression of the two isoforms are in fish hearts or if there is a relationship between cardiac FABP levels and Mb.

Another possibility to explain the loss of oxygen-binding proteins in notothenioids is that perhaps rather than a neutral mutation, the loss of Hb and/or Mb is advantageous. The associated decrease in blood viscosity accompanying the loss of Hb and red-blood cells (Wells et al. 1990) might suggest an energetic advantage to the loss of Hb as a result of reduced cardiac work, but the large blood volume of icefishes nearly doubles the cardiac work per unit time of icefishes compared with red-blooded species, suggesting that there is no energetic advantage to the lack of the protein (Sidell and O'Brien 2006). However, recent observations by my colleague, Dr. Elizabeth Crockett, and I suggested that the loss of Hb and Mb might impart an energetic advantage to icefishes by reducing oxidative stress. Our current studies are exploring this possibility.

Hb and Mb as pro-oxidants

Oxygen is often described as a Janus-faced molecule because although essential for most living organisms, it is a powerful oxidant, capable of wrecking havoc on biological macromolecules when it gains electrons and forms the ROS superoxide ($O_2\bullet^-$), hydrogen peroxide (H_2O_2) or the hydroxyl radical ($OH\bullet$;

Halliwell and Gutteridge 1999). When oxygen binds the heme prosthetic group of Hb or Mb, oxygen can promote the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), resulting in the formation of $O_2\bullet^-$ (Equation (3); Reeder and Wilson 2005). Additionally, H_2O_2 reacts with ferrous iron in Hb and Mb, generating the ferryl iron (Fe^{4+}), which quickly decays to ferric iron, which can then react with H_2O_2 and form a protein-based radical (Equation (4); Reeder and Wilson 2005). The protein-based radical and ferryl species can oxidatively damage proteins, DNA, and lipids (Wilson and Reeder 2008)

$$P - Fe^{2+} - O_2 \rightarrow P - Fe^{3+} + O_2\bullet^- \quad (3)$$

$$P - Fe^{3+} + H_2O_2 \rightarrow P\bullet^+ - Fe^{4+} = O_2\bullet^- + H_2O \quad (4)$$

ROS may sever the peptide backbone of proteins, crosslink proteins, and/or oxidatively modify amino acids (Stadtman and Levine 2003). Oxidation of the sulfur-containing amino acids, methionine and cysteine, is reversible and so these amino acids are often found as constituents of redox-sensing proteins (Garcia-Santamarina et al. 2014). In contrast, oxidation of lysine, arginine, proline and threonine side chains, results in the formation of carbonyl derivatives, a frequently measured index of oxidative stress, which until recently, was thought to be irreversible (Levine et al. 1994; Wong et al. 2013).

The first clue that suggested to us that the loss of oxygen-binding proteins might be advantageous for icefishes was the discovery that hearts of the red-blooded notothenioid, *Notothenia coriiceps*, have higher levels of carbonylated proteins compared with the icefish *C. aceratus*, and levels of carbonyls are intermediate in hearts of the Mb-expressing icefish, *Chionodraco rastrospinosus* (Mueller et al. 2012; Fig. 2). Additionally, there is a significant positive correlation between levels of Mb and levels of protein carbonyls in two species of icefishes and two red-blooded notothenioids (Fig. 3). Consistent with this trend, maximal activities of the antioxidants superoxide dismutase and catalase are higher in hearts of red-blooded notothenioids compared with icefishes (Table 1). Together, this suggested that iron-centered oxygen-binding proteins may promote the formation of ROS and increase oxidative stress in species that express the proteins.

Fate of oxidized proteins

Higher levels of oxidized proteins in red-blooded notothenioids may warrant higher rates of protein degradation compared with icefishes. Proteins are degraded by lysosomal enzymes, calcium-dependent calpains, and the proteasome, but the vast majority of oxidatively-damaged proteins are degraded by the ubiquitin-proteasome pathway (UPP; Knecht et al. 2009; Pajares et al. 2015). Proteins are directed to the 26S proteasome for degradation by the addition of polyubiquitin to a lysine residue by the concerted activity of the E1, E2, and E3 ubiquitin conjugating enzymes (Komander 2009). The 26S proteasome includes a 20S multi-subunit core protease composed of four-heptameric-rings, and an associated regulatory element, referred to as the PA700 or the 19S regulatory particle (Demartino and Gillette 2007). PA700 includes six Adenosine triphosphatases (ATPases) that are involved in deubiquitinating and unfolding proteins prior to degradation. Additionally, many other proteins may associate with the 20S core and regulate its activity (Schmidt et al. 2005). Unlike the 26S proteasome that degrades ubiquitinated proteins, the 20S proteasome, lacking PA700, does not require ubiquitinated substrates or ATP. In mammals, the majority of oxidatively-modified proteins are degraded by the 20S proteasome (Grune et al. 1995; Grune et al. 1996, 1997; Grune et al. 2003), although some oxidized proteins are degraded by the UPP and the activity of the ubiquitin conjugating system increases following exposure to oxidative stress (Shang et al. 1997; Dudek et al. 2005). However, the 26S proteasome is more susceptible to oxidative damage than the 20S proteasome and protein oxidation is thought to denature proteins sufficiently to expose hydrophobic regions necessary for recognition by the 20S proteasome and entry into the catalytic core (Grune et al. 1997; Reinheckel et al. 1998). Proteins that are highly oxidized or cross-linked are resistant to degradation by the proteasome and the accumulation of oxidatively damaged proteins is associated with several disease states and aging (Shringarpure and Davies 2002; Grune et al. 2003; Pajares et al. 2015).

Oxidatively damaged and degraded proteins must be replaced in the energetically expensive process of protein synthesis. Between approximately 11 and 42% of an organism's energy budget is devoted to synthesizing proteins (Lyndon and Houlihan 1998). We hypothesized that the loss of Hb and Mb might be advantageous for icefishes by reducing energetic costs associated with degrading oxidatively modified proteins and synthesizing new proteins.

We measured rates of protein synthesis in hearts of notothenioids differing in the expression of Hb and Mb using a flooding dose of radiolabeled phenylalanine as described by Garlick (Garlick et al. 1980; Lewis et al. 2015). We also measured the

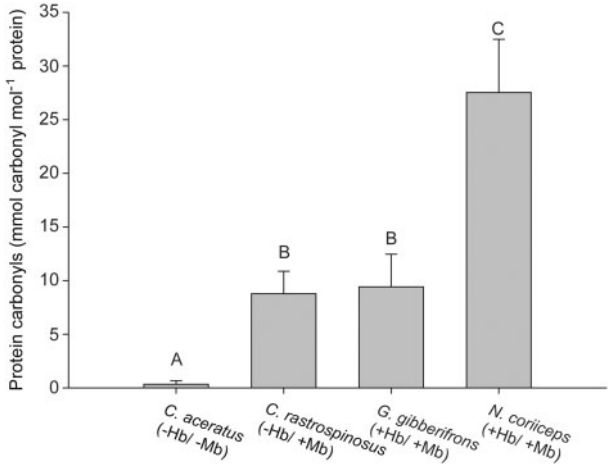

Fig. 2 Levels of protein carbonyls in heart ventricles of notothenioids. Significant differences are indicated by different letters ($P < 0.05$). The figure is adapted from Mueller et al. (2012).

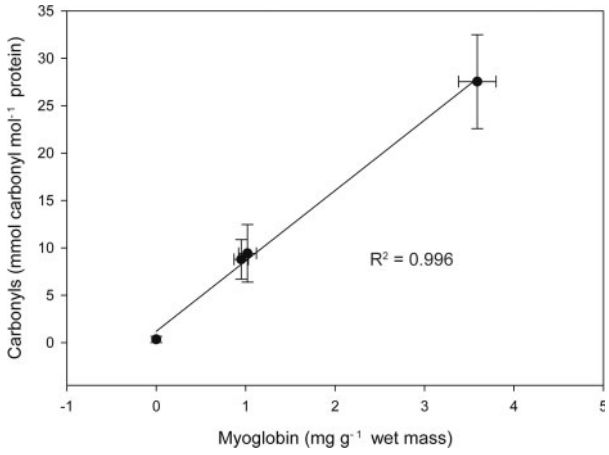

Fig. 3 Levels of protein carbonyls are positively correlated with levels of Mb in heart ventricles of notothenioids. Values are based on the average measurements in 8–11 individuals for protein carbonyls and 5–6 individuals for Mb concentration. $F = 0.002$.

energetic costs of protein synthesis by measuring cellular respiration rates in the presence and absence of the protein synthesis inhibitor, cyclohexamide (Lewis et al. 2015). We determined that despite higher levels of oxidized proteins in hearts of species expression Mb and/or Hb, rates of protein synthesis are equivalent between red- and white-blooded fishes and ranged between 0.10 and 0.15 nmol phe mg protein^{-1} h^{-1}. These rates are similar to those in hearts of temperate fishes (Sephton and Driedzic 1995; Lewis and Driedzic 2007), indicating that the protein synthesis machinery is cold adapted in notothenioids. We also found the energetic costs of protein synthesis are equivalent in cardiac myocytes and hepatocytes between red- and white-blooded fishes (Fig. 4). The metabolic cost of protein synthesis in hearts ranges between 1.8 and 4.1% of total oxygen consumption and is similar to rainbow trout (Houlihan et al. 1988). In hepatocytes, 17.5–20.3% of oxygen consumed is devoted to producing ATP for protein synthesis, which is similar to other notothenioids (Mark et al. 2005). Recently, we completed measurements of the maximal activity of the 20S proteasome and determined it is similar between hearts of red- and white-blooded fishes (unpublished). In summary, our results indicate that the loss of Hb and Mb does not minimize rates of protein turnover, ruling out an energetic cost savings in heart and liver tissues of icefishes. However, we cannot discount the possibility that rates of protein turnover may be higher in red-blooded species in tissues other than the liver and heart, particularly in the anterior kidney, where red-blood cells are produced.

Consequences of oxidized proteins

The higher levels of oxidized proteins in hearts of red-blooded fishes raise the intriguing question of how do these species persist with higher levels of oxidized proteins compared with icefishes? The effects of ROS are dependent on the redox state of the environment and the concentration and type of ROS. While elevated levels of ROS may severely damage biological macromolecules, low levels of ROS are vital second messengers (Schieber and Chandel 2014; Shadel and Horvath 2015). For example, transcription and translation of the mitochondrial genome are regulated by ROS, providing a conduit for crosstalk between the nuclear and mitochondrial genome that is responsive to metabolic state (Allen 2015). Inhibitors such as antimycin, which promote ROS formation, increase mitochondrial transcription and translation, whereas cyanide, which blocks complex cytochrome c oxidase, represses transcription and translation (Wilson et al. 1996; Zubo et al. 2014).

Studies of the mammalian heart beautifully illustrate how redox balance regulates function. In cardiac muscle, many of the myofibrillar proteins, including troponin, tropomyosin, titan, actin, and the myosin light and heavy chains, as well as proteins involved in calcium homeostasis, are regulated by their redox state (Steinberg 2013; Zima and Blatter 2006). While protein oxidation often decreases cardiac contractility, redox regulation of protein kinases and phosphatases may enhance it. For example, oxidation of cys^{190} in tropomyosin, induces disulfide

Table 1 Activity of antioxidants in hearts of notothenioids differing in the expression of Hb and Mb

Species	SOD activity ± SEM (U g^{-1} wet mass)	CAT activity ± SEM (μmol min^{-1} g^{-1} wet mass)
C. aceratus (−Hb/−Mb)	1696.50 ± 66.36^{A}	269.04 ± 29.73^{A}
C. rastrospinosus (−Hb/+Mb)	1931.53 ± 76.95^{A}	522.74 ± 47.49^{B}
G. gibberifrons (+Hb/+Mb)	2685.57 ± 36.13^{B}	777.68 ±111.67^{BC}
N. coriiceps (+Hb/+Mb)	2958.25 ± 100.74^{B}	1068.55 ± 55.51^{C}

Note: Significant differences are indicated with different letters ($P < 0.05$). $N = 6$–8. SOD, superoxide dismutase; CAT, catalase. Table is adapted from Mueller et al. (2012).

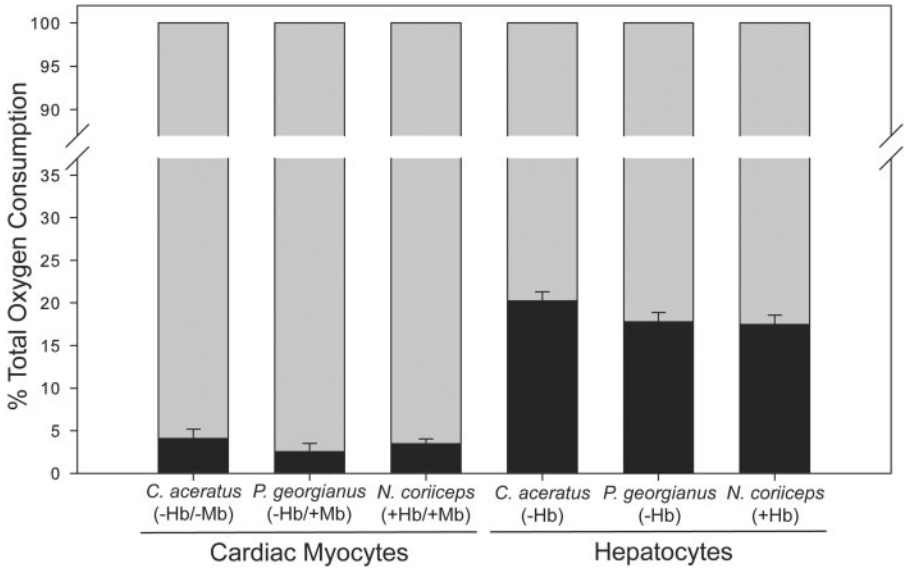

Fig. 4 Energetic costs of protein synthesis in notothenioids differing in the expression of Hb and Mb. The dark shaded region of each box represents the percentage of total oxygen consumption inhibited by cyclohexamide. Values represent the mean ± SEM ($N = 5$–7). The figure is adapted from Lewis et al. (2015).

cross-bridge formation that reduces contractility (Canton et al. 2006). Oxidation of met^{394} in myosin II decreases its ATPase activity but is reversible by methionine sulfoxide reductase (Moen et al. 2014). In contrast, oxidation of protein kinase A and disulfide bond formation stimulates its activity and phosphorylation of its substrates, including troponin I, which increases contractility (Brennan et al. 2006). Calcium homeostasis is also regulated by redox control of calcium channels and ATPases (Zima and Blatter 2006). Calcium release by the ryanodine receptor (RyR) in the sarcoplasmic reticulum (SR) is activated by oxidation of thiol groups (Eager et al. 1997). Oxidation of the RyR also decreases affinity of calmodulin binding, stimulating its activity (Balshaw et al. 2001). In contrast, oxidation of the sarcoplasmic Ca^{2+}ATPase (SERCA), responsible for Ca^{2+} uptake into the SR, is inhibited by oxidation of thiol groups, prolonging Ca^{2+}-activated muscle contraction (Scherer and Deamer 1986). Together, a picture emerges in which small increases in ROS that may arise as hearts approach maximum rates of oxygen consumption (VO_2 max; Cortassa et al. 2014), stimulate cardiac contraction, whereas high ROS levels lead to dysfunction. Although our measurements of oxidized proteins have focused on protein carbonyls, they likely reflect the oxidation state of thiol groups as well. The higher levels of oxidized proteins in hearts of red-blooded notothenioids compared with icefishes are likely not pathological but rather, may be essential to maintain contractile function and higher heart rates compared with icefishes. Our current studies are aimed at identifying the redox proteome to better understand the functional consequences of protein oxidation in hearts of notothenioids.

Conclusions

The loss of Hb in Antarctic channichthyids is considered a disaptation—a trait inferior to the ancestral

state (Montgomery and Clements 2000), whereas the loss of Mb expression remains a conundrum. The widespread loss of Mb expression in hearts of teleosts may be due to relaxed selective pressure on Mb as a NO dioxygenase, nitrite reductase, and/or FABP. When present, Mb likely enhances cardiac function and aerobic scope by maintaining oxygen delivery to mitochondria, as well as perhaps by potentiating the production of ROS necessary for increasing contractility. The loss of expression of Mb and Hb reflect single traits within complex organisms. The persistence of these traits in notothenioids (−Hb and −Mb) and temperate teleosts (−Mb) likely reflects trade-offs, providing physiological benefits at some points during development and/or under some environmental conditions and incurring costs in others.

Acknowledgements

First and foremost, I would like to thank my good friend and collaborator, Dr. Elizabeth Crockett, for many stimulating conversations about the biology of Antarctic fishes. Many of the ideas presented in this manuscript were conceived and developed during our discussions and her thoughtful comments on an earlier draft markedly improved this final version. Thanks also to Dr. Theresa Grove for conducting the experiments on measuring the metabolic costs of protein synthesis and to Dr. Johanne Lewis for leading the experiments measuring rates of protein synthesis. I would also like to acknowledge the excellent support from the Masters and Crew of the ARSV Laurence M. Gould and the support staff at the US Antarctic Research Station, Palmer Station.

Funding

Funding was provided by the National Science Foundation (PLR-1043781 and PLR-1341663).

References

Acierno R, Agnisola C, Tota B, Sidell BD. 1997. Myoglobin enhances cardiac performance in antarctic icefish species that express the protein. Am J Physiol 273:R100–6.

Acierno R, MacDonald JA, Agnisola C, Tota B. 1995. Blood volume in the hemoglobinless Antarctic teleost *Chionodraco hamatus* (Lonnberg). J Exp Zool 272:407–9.

Allen JF. 2015. Why chloroplasts and mitochondria retain their own genomes and genetic systems: Colocation for redox regulation of gene expression. Proc Natl Acad Sci USA 112:10231–8.

Bacila M, Rosa R, Rodrigues E, Lucchiari PH, Rosa CD. 1989. Tissue metabolism of the ice-fish Chaenocephalus aceratus Loenberg. Comp Biochem Physiol 92B:313–8.

Balshaw DM, Xu L, Yamaguchi N, Pasek DA, Meissner G. 2001. Calmodulin binding and inhibition of cardiac muscle calcium release channel (ryanodine receptor). J Biol Chem 276:20144–53.

Beers JM, Borley KA, Sidell BD. 2010. Relationship among circulating hemoglobin, nitric oxide synthase activities and angiogenic poise in red- and white-blooded Antarctic notothenioid fishes. Comp Biochem Physiol 156:422–9.

Beers JM, Jayasundara N. 2015. Antarctic notothenioid fish: what are the future consequences of ''losses'' and ''gains'' acquired during long-term evolution at cold and stable temperatures? J Exp Biol 218:1834–45.

Beers JM, Sidell BD. 2011. Thermal tolerance of Antarctic notothenioid fishes correlates with level of circulating hemoglobin. Physiol Biochem Zool 84:353–62.

Borley KA, Beers JM, Sidell BD. 2010. Phenylhydrazine-induced anemia causes nitric-oxide-mediated upregulation of the angiogenic pathway in *Notothenia coriiceps*. J Exp Biol 213:2865–72.

Borley KA, Sidell BD. 2011. Evolution of the myoglobin gene in Antarctic icefishes (Channichthyidae). Polar Biol 34:659–65.

Brennan JP, Bardswell SC, Burgoyne JR, Fuller W, Schroder E, Wait R, Begum S, Kentish JC, Eaton P. 2006. Oxidant-induced activation of type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation. J Biol Chem 281:21827–36.

Burmester T, Hankeln T. 2009. What is the function of neuroglobin? J Exp Biol 212:1423–8.

Burmester T, Weich B, Reinhardt S, Hankeln T. 2000. A vertebrate globin expressed in the brain. Nature 407:520–3.

Canton M, Skyschally A, Menabo R, Boengler K, Gres P, Schulz R, Haude M, Erbel R, Di Lisa F, Heusch G. 2006. Oxidative modification of tropomyosin and myocardial dysfunction following coronary microembolization. Eur Heart J 27:875–81.

Cashon RE, Vayda ME, Sidell BD. 1997. Kinetic characterization of myoglobins from vertebrates with vastly different body temperatures. Comp Biochem Physiol B Biochem Mol Biol 117:613–20.

Cheng C-HC, Detrich WW. 2007. Molecular ecophysiology of Antarctic notothenioid fishes. Philos Trans R Soc Lond B Biol Sci 362: 2215–32.

Cheng CH, di Prisco G, Verde C. 2009. Cold-adapted Antarctic fish: the discovery of neuroglobin in the dominant suborder Notothenioidei. Gene 433:100–1.

Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH. 1994. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. FEBS Lett 345:50–4.

Cortassa S, O'Rourke B, Aon MA. 2014. Redox-optimized ROS balance and the relationship between mitochondrial respiration and ROS. Biochim Biophys Acta 1837:287–95.

Crockett E, Sidell B. 1990. Some pathways of energy metabolism are cold adapted in Antarctic fishes. Physiol Zool 63:472–88.

Demartino GN, Gillette TG. 2007. Proteasomes: machines for all reasons. Cell 129:659–62.

Devor DP, Kuhn DE, O'Brien KM, Crockett EL. 2016. Hyperoxia does not extend critical thermal maxima

(CTmax) in white- or red-blooded Antarctic notothenioid fishes. Physiol Biochem Zool 89:1–9.

di Prisco G, Macdonald JA, Brunori M. 1992. Antarctic fishes survive exposure to carbon-monoxide. Experientia 48:473–5.

Dudek EJ, Shang F, Valverde P, Liu Q, Hobbs M, Taylor A. 2005. Selectivity of the ubiquitin pathway for oxidatively modified proteins: relevance to protein precipitation diseases. FASEB J 19:1707–9.

Dunn JF, Johnston IA. 1986. Metabolic constraints on burst swimming in the Antarctic teleost *Notothenia neglecta*. Mar Biol 91:433–40.

Eager KR, Roden LD, Dulhunty AF. 1997. Actions of sulfhydryl reagents on single ryanodine receptor Ca(2+)-release channels from sheep myocardium. Am J Physiol 272:C1908–18.

Eastman JT. 1993. Antarctic fish biology: Evolution in a unique environment. San Diego: Academic Press.

Eastman JT. 2005. The nature of the diversity of Antarctic fishes. Polar Biology 28:93–107.

Fitch NA, Johnston IA, Wood RE. 1984. Skeletal muscle capillary supply in a fish that lacks respiratory pigments. Respir Physiol 57:201–11.

Flogel U, Merx MW, Godecke A, Decking UK, Schrader J. 2001. Myoglobin: A scavenger of bioactive NO. Proc Natl Acad Sci USA 98:735–40.

Garcia-Santamarina S, Boronat S, Hidalgo E. 2014. Reversible cysteine oxidation in hydrogen peroxide sensing and signal transduction. Biochemistry 53:2560–80.

Garlick PJ, McNurlan MA, Preedy VR. 1980. A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [3H]phenylalanine. Biochem J 192:719–23.

Godecke A, Flogel U, Zanger K, Ding Z, Hirchenhain J, Decking UK, Schrader J. 1999. Disruption of myoglobin in mice induces multiple compensatory mechanisms. Proc Natl Acad Sci USA 96:10495–500.

Grove TJ, Hendrickson JW, Sidell BD. 2004. Two species of Antarctic icefishes (Genus Champsocephalus) share a common genetic lesion leading to the loss of myoglobin expression. Polar Biol 27:579–85.

Grune T, Merker K, Sandig G, Davies KJ. 2003. Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Commun 305:709–18.

Grune T, Reinheckel T, Davies KJ. 1996. Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. J Biol Chem 271:15504–9.

Grune T, Reinheckel T, Davies KJ. 1997. Degradation of oxidized proteins in mammalian cells. FASEB J 11:526–34.

Grune T, Reinheckel T, Joshi M, Davies KJ. 1995. Proteolysis in cultured liver epithelial cells during oxidative stress. Role of the multicatalytic proteinase complex, proteasome. J Biol Chem 270:2344–51.

Halliwell B, Gutteridge JMC. 1999. Free radicals in biology and medicine. Oxford, UK: Oxford University Press.

Hemmingsen EA, Douglas EL. 1970. Respiratory characteristics of the hemoglobin-free fish Chaenocephalus aceratus. Comp Biochem Physiol 33:733–44.

Hemmingsen EA, Douglas EL, Johansen K, Millard RW. 1972. Aortic blood flow and cardiac output in the hemoglobin-free fish Chaenocephalus aceratus. Comp Biochem Physiol A Comp Physiol 43:1045–51.

Hendgen-Cotta UB, Merx MW, Shiva S, Schmitz J, Becher S, Klare JP, Steinhoff HJ, Goedecke A, Schrader J, Gladwin MT et al.. 2008. Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. Proc Natl Acad Sci USA 105:10256–61.

Holeton GF. 1970. Oxygen uptake and circulation by a hemoglobinless Antarctic fish (Chaenocephalus aceratus lonnberg) compared with three red-blooded Antartic fish. Comp Biochem Physiol 34:457–71.

Houlihan DF, Agnisola C, Lyndon AR, Gray C, Hamilton NM. 1988. Protein synthesis in a fish heart: responses to increased power output. J Exp Biol 137:565–87.

Johnston IA. 1987. Respiratory characteristics of muscle fibres in a fish (Chaenocephalus aceratus) that lacks haem pigments. J Exp Biol 133:415–28.

Kerwin JF, Jr, Lancaster JR Jr, Feldman PL. 1995. Nitric oxide: a new paradigm for second messengers. J Med Chem 38:4343–62.

Knecht E, Aguado C, Carcel J, Esteban I, Esteve JM, Ghislat G, Moruno JF, Vidal JM, Saez R. 2009. Intracellular protein degradation in mammalian cells: recent developments. Cell Mol Life Sci 66:2427–43.

Knox GA. 1994. The biology of the Southern Ocean. Cambridge University Press.

Komander D. 2009. The emerging complexity of protein ubiquitination. Biochem Soc Trans 37:937–53.

Levine RL, Williams JA, Stadtman ER, Shacter E. 1994. Carbonyl assays for determination of oxidatively modified proteins. Meth Enzymol 233:346–57.

Lewis JM, Driedzic WR. 2007. Tissue-specific changes in protein synthesis associated with seasonal metabolic depression and recovery in the north temperate labrid, Tautogolabrus adspersus. Am J Physiol Regul Integr Comp Physiol 293:R474–81.

Lewis JM, Grove TJ, O'Brien KM. 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.

Lyndon AR, Houlihan DF. 1998. Gill protein turnover: costs of adaptation. Comp Biochem Physiol A Mol Integr Physiol 119:27–34.

Macqueen DJ, Garcia de la Serrana D, Johnston IA. 2014. Cardiac myoglobin deficit has evolved repeatedly in teleost fishes. Biol Lett 10.

Mark FC, Hirse T, Portner HO. 2005. Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes. Polar Biol 28:805–14.

Moen RJ, Cornea S, Oseid DE, Binder BP, Klein JC, Thomas DD. 2014. Redox-sensitive residue in the actin-binding interface of myosin. Biochem Biophys Res Commun 453:345–9.

Montgomery J, Clements K. 2000. Disaptation and recovery in the evolution of Antarctic fishes. Trends Ecol E 15:267–71.

Moylan TJ, Sidell BD. 2000. Concentrations of myoglobin and myoglobin mRNA in heart ventricles from Antarctic fishes. J Exp Biol 203:1277–86.

Mueller IA, Devor DP, Grim JM, Beers JM, Crockett EL, O'Brien KM. 2012. Exposure to critical thermal maxima causes oxidative stress in hearts of white- but not red-blooded Antarctic notothenioid fishes. J Exp Biol 215: 3655–64.

Mueller IA, O'Brien KM. 2011. Nitric oxide synthase is not expressed, nor up-regulated in response to cold acclimation in liver or muscle of threespine stickleback (Gasterosteus aculeatus). Nit Oxi 25:416–22.

Near TJ. 2004. Estimating divergence times of notothenioid fishes using a fossil-calibrated molecular clock. Antar Sci 16:37–44.

Near TJ, Parker SK, Detrich HW. 2006. A genomic fossil reveals key steps in hemoglobin loss by the antarctic icefishes. Mol Biol E 23:2008–16.

O'Brien KM, Mueller IA. 2010. The unique mitochondrial form and function of Antarctic channichthyid icefishes. Integr Comp Biol 50:993–1008.

O'Brien KM, Sidell BD. 2000. The interplay among cardiac ultrastructure, metabolism and the expression of oxygen-binding proteins in Antarctic fishes. J Exp Biol 203:1287–97.

O'Brien KM, Xue H, Sidell BD. 2000. Quantification of diffusion distance within the spongy myocardium of hearts from antarctic fishes. Respir Physiol 122:71–80.

Pajares M, Jimenez-Moreno N, Dias IH, Debelec B, Vucetic M, Fladmark KE, Basaga H, Ribaric S, Milisav I, Cuadrado A. 2015. Redox control of protein degradation. Redox Biol 6:409–20.

Pellegrino D, Acierno R, Tota B. 2003. Control of cardiovascular function in the icefish *Chionodraco hamatus*: involvement of serotonin and nitric oxide. Comp Biochem Physiol A Mol Integr Physiol 134:471–80.

Pellegrino D, Palmerini CA, Tota B. 2004. No hemoglobin but NO: the icefish (*Chionodraco hamatus*) heart as a paradigm. J Exp Biol 207:3855–64.

Piantadosi CA. 2012. Regulation of mitochondrial processes by protein S-nitrosylation. Biochim Biophys Acta 1820:712–21.

Rassaf T, Flogel U, Drexhage C, Hendgen-Cotta U, Kelm M, Schrader J. 2007. Nitrite reductase function of deoxymyoglobin: oxygen sensor and regulator of cardiac energetics and function. Circ Res 100:1749–54.

Reeder BJ, Wilson MT. 2005. Hemoglobin and myoglobin associated oxidative stress: from molecular mechanisms to disease States. Curr Med Chem 12:2741–51.

Reinheckel T, Sitte N, Ullrich O, Kuckelkorn U, Davies KJ, Grune T. 1998. Comparative resistance of the 20S and 26S proteasome to oxidative stress. Biochem J 335:637–42.

Ruud JT. 1954. Vertebrates without erythrocytes and blood pigment. Nature 173:848–50.

Ruud JT. 1965. The ice fish. Sci Am 213:108–15.

Scherer NM, Deamer DW. 1986. Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca2+-ATPase. Arch Biochem Biophys 246:589–601.

Schieber M, Chandel NS. 2014. ROS function in redox signaling and oxidative stress. Curr Biol 24:R453–62.

Schmidt M, Hanna J, Elsasser S, Finley D. 2005. Proteasome-associated proteins: regulation of a proteolytic machine. Biol Chem 386:725–37.

Sephton DH, Driedzic WR. 1995. Low temperature acclimation decreases rates of protein synthesis in rainbow trout (Oncorhynchus mykiss) heart. Fish Physiol Biochem 14:63–9.

Shadel GS, Horvath TL. 2015. Mitochondrial ROS signaling in organismal homeostasis. Cell 163:560–9.

Shang F, Gong X, Taylor A. 1997. Activity of ubiquitin-dependent pathway in response to oxidative stress. Ubiquitin-activating enzyme is transiently up-regulated. J Biol Chem 272:23086–93.

Shih L, Chung Y, Sriram R, Jue T. 2014. Palmitate interaction with physiological states of myoglobin. Biochim Biophys Acta 1840:656–66.

Shih L, Chung Y, Sriram R, Jue T. 2015. Interaction of myoglobin with oleic acid. Chem Phys Lipids 191:115–22.

Shringarpure R, Davies KJ. 2002. Protein turnover by the proteasome in aging and disease. Free Radic Biol Med 32:1084–9.

Sidell BD, Crockett EL, Driedzic WR. 1995. Antarctic fish tissues preferentially catabolize monoenoic fatty acids. J Exp Zool 271:73–81.

Sidell BD, O'Brien KM. 2006. When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes. J Exp Biol 209:1791–802.

Sidell BD, Vayda ME, Small DJ, Moylan TJ, Londraville RL, Yuan ML, Rodnick KJ, Eppley ZA, Costello L. 1997. Variable expression of myoglobin among the hemoglobinless Antarctic icefishes. Proc Natl Acad Sci USA 94:3420–4.

Small DJ, Moylan T, Vayda ME, Sidell BD. 2003. The myoglobin gene of the Antarctic icefish, Chaenocephalus aceratus, contains a duplicated TATAAAA sequence that interferes with transcription. J Exp Biol 206:131–9.

Stadtman ER, Levine RL. 2003. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Ami Acids 25:207–18.

Steinberg SF. 2013. Oxidative stress and sarcomeric proteins. Circ Res 112:393–405.

Sun Y, Jin K, Mao XO, Zhu Y, Greenberg DA. 2001. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. Proc Natl Acad Sci USA 98:15306–11.

Syeda F, Hauton D, Young S, Egginton S. 2013. How ubiquitous is endothelial NOS? Comp Biochem Physiol A Mol Integr Physiol 166:207–14.

Tota B, Cerra MC, Mazza R, Pellegrino D, Icardo J. 1997. The heart of the Antarctic icefish as paradigm of cold adaptation. J Them Biol 22:409–17.

Vayda ME, Londraville RL, Cashon RE, Costello L, Sidell BD. 1998. Two distinct types of fatty acid-binding protein are expressed in heart ventricle of Antarctic teleost fishes. Biochem J 330:375–82.

Vayda ME, Small DJ, Yuan ML, Costello L, Sidell BD. 1997. Conservation of the myoglobin gene among Antarctic notothenioid fishes. Mol Mar Biol Biotechnol 6:207–16.

Wells RMG, Macdonald JA, DiPrisco G. 1990. Thin-blooded Antarctic fishes: a rheological comparison of the haemoglobin-free icefishes *Chionodraco kathleenae* and *Cryodraco antarcticus* with a red-blooded nototheniid, *Pagothenia bernacchii.* J Fish Biol 36:595–609.

Wilson MT, Reeder BJ. 2008. Oxygen-binding haem proteins. Exp Physiol 93:128–32.

Wilson SB, Davidson GS, Thomson LM, Pearson CK. 1996. Redox control of RNA synthesis in potato mitochondria. Eur J Biochem 242:81–5.

Wong CM, Marcocci L, Das D, Wang X, Luo H, Zungu-Edmondson M, Suzuki YJ. 2013. Mechanism of protein decarbonylation. Free Radic Biol Med 65:1126–33.

Wujcik JM, Wang G, Eastman JT, Sidell BD. 2007. Morphometry of retinal vasculature in Antarctic fishes is dependent upon the level of hemoglobin in circulation. J Exp Biol 210:815–24.

Yamada T, Takakura H, Jue T, Hashimoto T, Ishizawa R, Furuichi Y, Kato Y, Iwanaka N, Masuda K. 2015. Myoglobin and the Regulation of Mitochondrial Respiratory Chain Complex IV. J Physiol.

Zima AV, Blatter LA. 2006. Redox regulation of cardiac calcium channels and transporters. Cardiovasc Res 71:310–21.

Zubo YO, Potapova TV, Yamburenko MV, Tarasenko VI, Konstantinov YM, Borner T. 2014. Inhibition of the electron transport strongly affects transcription and transcript levels in Arabidopsis mitochondria. Mitochondrion 19:222–30.