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Commentary

When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes

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Summary

The Antarctic icefishes (Family Channichthyidae) provide excellent examples of unique traits that can arise in a chronically cold and isolated environment. Their loss of hemoglobin (Hb) expression, and in some cases, loss of myoglobin (Mb) expression, has taught us much about the function of these proteins. Although absences of the proteins are fixed traits in icefishes, the losses do not appear to be of adaptive value. Contrary to some suggestions, loss of Hb has led to higher energetic costs for circulating blood, and losses of Mb have reduced cardiac performance. Moreover, losses of Hb and Mb have resulted in extensive modifications to the cardiovascular system to ensure adequate oxygen delivery to working muscles. Recent studies suggest that losses of Hb and Mb, and their associated nitric oxide (NO)-oxygenase activities, may have accelerated the development and evolution of these cardiovascular modifications. The high levels of NO that should occur in the absence of Hb and Mb have been shown in other animal groups to lead to an increase in tissue vascularization, an increase in the lumenal diameter of blood vessels, and an increase in mitochondrial densities. These characteristics are all hallmark traits of Antarctic icefishes. Homeostatic feedback mechanisms thus may have accelerated evolution of the pronounced cardiovascular traits of Antarctic icefishes.

Key words: hemoglobin, myoglobin, Antarctic icefish, nitric oxide, heart, circulation.

Introduction

Comparative physiologists instinctively are drawn to organisms that display superlative physiological characteristics. This really is a corollary to the famous 'August Krogh Principle' that states: "*For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied*" (Krogh, 1929). Although we may take issue with the grammar of this sentence, the concept is unassailable. In the realm of comparative cardiovascular physiology, few groups of animals can rival the Antarctic icefishes in meeting the criterion described by Krogh. It is upon this group that we will focus our Commentary.

The Antarctic icefishes (Family Channichthyidae) are one of eight families of the single perciform suborder, Notothenioidei, which dominate the fish fauna surrounding Antarctica (for excellent reviews, see Eastman, 2005; Kock, 2005). They occupy the coldest, most thermally stable marine environment on earth. Sea temperatures near the Ross Ice Shelf at McMurdo Station, Antarctica, are nearly constant at –1.9°C (Littlepage, 1965) and even those in the more northerly Antarctic Peninsula range only between summer temperatures of +1.5°C to winter temperatures of –1.8°C (DeWitt, 1971). The water column south of the Antarctic Polar Front is exceedingly well mixed vertically, and all depths are close to complete oxygen saturation. Because oxygen solubility in seawater is inversely proportional to temperature, the cold Antarctic seas thus are an exceptionally oxygen-rich aquatic habitat.

Notothenioids account for approximately 35% of fish species and 90% of fish biomass found south of the Antarctic Polar Front (Ekau, 1990). Radiation of closely related notothenioid species has occurred rapidly (within the last 12 million years, MY) (Bargelloni et al., 1994) and under a very unusual set of conditions. First, notothenioids have evolved in relative oceanographic isolation from other faunas due to circumpolar currents and deep ocean trenches surrounding the continent. Second, the Southern Ocean has been characterized by severely cold water temperatures for the last 10–14 MY (Kennett, 1977). Finally, evolution of these fishes has progressed under conditions of very low levels of niche competition because a dramatic crash in fish diversity occurred in the Southern ocean sometime between the mid-Tertiary and present (see Eastman, 1993; Eastman, 2005). These features

make Antarctic notothenioid fishes an uniquely attractive group for the study of physiological and biochemical adaptations to cold body temperature. Today's notothenioids are arguably the end result of an extraordinary natural experiment. They provide a window into the exceptional physiological characteristics that can arise in animals living at chronically cold body temperatures. Some of these characteristics clearly are adaptive (e.g. development of antifreeze glycoproteins). Others would be deleterious, if not lethal, in warmer and more competitive environments.

Patterns of oxygen-binding protein loss among the Channichthyidae

One of the most unusual and fascinating physiological characteristics of Antarctic notothenioids is the complete loss of hemoglobin (Hb) in the family Channichthyidae. These peculiar looking fishes were appropriately named ice fish, by early British whalers and were first described physiologically in 1954 (Ruud, 1954).

Icefishes are the only known vertebrate animals to lack Hb as adults (Fig. 1). Oxygen is found solely in physical solution in icefish blood, which has an oxygen carrying capacity of <10% of that seen in red-blooded notothenioid fishes (Holeton, 1970). Several fairly draconian modifications of the cardiovascular system of icefishes compensate for their lack of a circulating oxygen-carrier. Icefishes possess very large hearts compared to red-blooded fishes of equivalent body size, resulting in a weight-specific cardiac output that is four- to fivefold greater than that of red-blooded species (Hemmingsen et al., 1972; Fig. 2). The blood volumes of icefishes are up to fourfold those of red-blooded teleosts, and the diameter of their capillaries is unusually large (Fitch et al., 1984). These features collectively permit a large volume of blood to circulate throughout the bodies of icefishes at high flow rate, yet, at low vascular pressure because of decreased peripheral resistance. Combined with the very high oxygen content of Antarctic waters and relatively low absolute metabolic rates, these unusual cardiovascular attributes ensure that adequate oxygen is delivered to tissues to support the obligately aerobic mode of metabolism of these animals (Hemmingsen, 1991).

The intracellular oxygen-binding protein, myoglobin (Mb), is also not uniformly expressed in species of Family Channichthyidae. Myoglobin is widely distributed in aerobically poised tissues of animals, and has been ascribed critical roles in both intracellular storage and diffusion of oxygen (Wittenberg and Wittenberg, 2003). Indeed, *The Journal of Experimental Biology* recently carried a Commentary (Ordway and Garry, 2004), the title of which indicated that myoglobin was an 'essential hemoprotein in striated muscle'. It would appear that the channichthyid icefishes confirm the old adage that there are exceptions to every rule. Among the 16 known species of the family, ten icefish species do express Mb in their heart muscle, while six others do not produce the protein (Grove et al., 2004).

The very close phylogenetic relationship among families of Hb-expressing and Hb-less notothenioids and among Mb-expressing and Mb-lacking icefishes presents an unique matrix of 'naturally occurring genetic knockouts' that can be exploited to probe and understand the myriad of processes that regulate

Fig. 1. Lack of circulating hemoglobin and red cells is the signature characteristic of Antarctic icefishes. These two tubes contain freshly drawn blood from a hemoglobin-expressing notothenioid fish (*Notothenia coriiceps*) on the left and a hemoglobinless Antarctic icefish (*Chaenocephalus aceratus*) on the right.

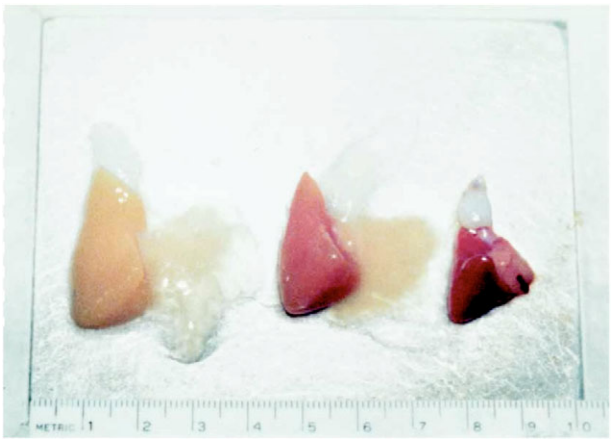

Fig. 2. Hearts from three species of notothenioid fishes. The channichthyid icefish *Chaenocephalus aceratus* has a pale yellow ventricle (far left) and lacks myoglobin (Mb) protein expression. The channichthyid icefish *Chionodraco rastrospinosus* expresses myoglobin protein and displays a rose-colored ventricle (middle). The related notothenioid species *Notothenia coriiceps* has a characteristically red ventricle (far right) associated with the presence of myoglobin protein. Note that both channichthyid hearts are considerably larger than that from the red blooded species despite all having been dissected from animals of equivalent body mass. (Figure is from Moylan and Sidell, 2000.)

both oxygen delivery and utilization in aerobic tissues. Because these 'knockouts' have withstood the tests of real-world biology, they offer advantages over experimentally produced genetic knockouts for Mb expression in mice (Garry et al., 1998; Gödecke et al., 1999).

The pattern of Hb and Mb expression in icefishes leads us to a series of intriguing questions. How and when did the losses of expression of these important oxygen-binding proteins come about? Was loss of expression of either hemoglobin or myoglobin of adaptive value? If 'yes', what advantage was conferred? If 'no', why have the traits persisted in populations of these animals? How has the suite of physiological characteristics, which appear to be aimed at compensating for loss of each of these proteins, come about? Obviously, many of the 'answers' to these questions must, of necessity, fall within the realm of speculation. However, recent findings are pointing toward a series of provocative explanations.

When and how were hemoglobin and myoglobin lost in the icefishes?

Because the hemoglobinless condition is synapomorphic among the entire icefish family (i.e. a derived or specialized character shared by two or more groups that originated in their last common ancestor), it is clear that the original mutation that ablated Hb expression occurred at, or near the point of divergence of channichthyids from their notothenioid ancestors. Ruud recognized that the stably cold and well-oxygenated conditions of the modern Southern Ocean are undoubtedly environmental characteristics required for survival of the group (Ruud, 1954; Ruud, 1965). These conditions are thought to have been attained ca. 10–14 MYA (Kennett, 1977). This timing is consistent with the emergence of most notothenioid lineages, which is thought to have occurred 12–5 MYA, based on genetic distances (Bargelloni et al., 2000). These same genetic data suggest that the channichthyid icefishes diverged only 5.5–2 MYA. By this time, the Southern Ocean had become a stable, cold environment, favoring the survival of this group. Despite their descent from an ancestral red-blooded stock, genomic DNA of today's icefishes completely lacks any detectable vestiges of the gene for β-globin and contains only remnants of the gene encoding α-globin, the two subunits of which Hb is composed (Cocca et al., 1995; Cocca et al., 1997).

The pattern of Mb loss among the channichthyid icefishes is more puzzling. We have been unable to detect expression of Mb in oxidative skeletal muscle of any icefish or red-blooded notothenioid species that we have examined to date, suggesting that this phenotype is even more ancient than absence of Hb (Moylan and Sidell, 2000). A 'myoglobin-like' protein has been detected in glycolytic skeletal muscle of icefishes based upon immunochemical methods (Morlá et al., 2003). This observation seems perplexing, given that Mb expression is typically restricted to aerobically poised oxidative muscle, and is not expressed in anaerobically poised glycolytic, skeletal muscles; we have been unable to duplicate their results.

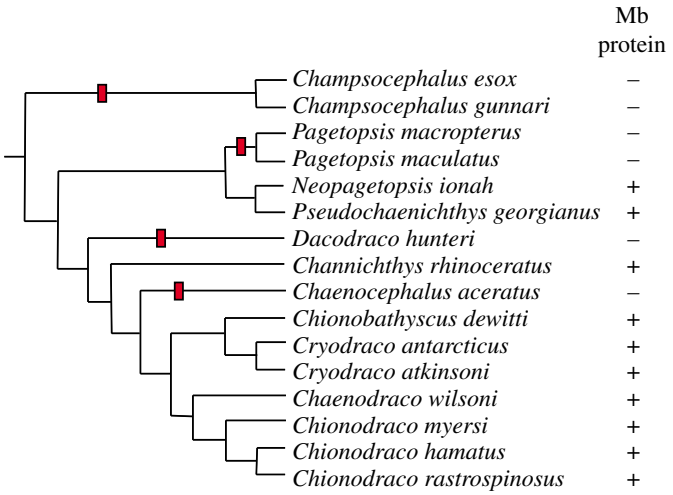

Fig. 3. Several independent mutational events have led to loss of myoglobin (Mb) expression during evolution of the icefish family. Expression of Mb is mapped on the consensus phylogeny of the Family Channichthyidae. Vertical red bars indicate points of independent mutational events leading to loss of Mb expression. The figure is modeled after that from Grove et al. (Grove et al., 2004) and based upon a cladogram originally presented by Near et al. (Near et al., 2003).

As mentioned earlier, Mb is expressed in heart ventricle of 10 of the 16 known species of icefishes, while six others lack the protein. By mapping loss of Mb expression on the consensus phylogeny of the icefishes, it is clear that ability to produce Mb has been lost at several discrete times during evolution of the family (Fig. 3). We also have identified at least three entirely different mutational mechanisms that account for loss of Mb expression in the different clades of non-expressers (Table 1; Small et al., 1998; Small et al., 2003). The most parsimonious interpretation of this pattern is that Mb is nonfunctional at the very cold body temperature of these animals and its loss, by whatever mechanism, might be advantageous, or selectively neutral at worst (Sidell et al., 1997). All available data, however, point toward rejection of this hypothesis.

Was loss of Hb and/or Mb of adaptive value?

The absence of Hb and red cells is such a startling characteristic that it seems reasonable to assume that its persistence must confer some adaptive advantage to the organism. Indeed, this has been the somewhat sanguine (pun intended) assumption of many investigators. (Such an assumption does not, however, address the question of why an even larger number of sympatrically occurring notothenioids are red-blooded.) Logically, the viscosity of blood lacking Hb and red cells will be less than that of blood from typical teleost fishes with hematocrits of 25–40%. Hematocrits of temperate zone species decrease during winter months (e.g. Powers, 1974) and polar fishes generally display lower hematocrits than warmer-bodied fishes (e.g. Scholander and Van Dam, 1957;

Table 1. *Distinctly different genetic mutations have caused losses of myoglobin expression during the evolution of the Antarctic icefishes*

Species	Nature of genetic lesion (Reference)
Champsocephalus spp.	A 5-nt insertion causes a shift in reading frame downstream of aa 91 and premature termination after residue 103 (Grove et al., 2004)
Pagetopsis macropterus	A mutation in the polyadenylation signal, 57-nt downstream of the stop codon, may interfere with efficient polyadenylation (Small et al., 1998)
Chaenocephalus aceratus	A 15-nt insertion, 647 nt upstream of the start codon, duplicates the muscle-specific TATA box and is capable of binding factors that prevent normal start of transcription (Small et al., 2003)

nt, nucleotide; aa, amino acid.

Wells, 1990). These observations have prompted some to conclude that the hemoglobinless condition of channichthyids is at the extreme end of a general trend toward a reduction in hematocrit at cold body temperature (e.g. di Prisco et al., 1991). In fact, Egginton reports that hematocrit values of red-blooded notothenioids show considerable overlap with the range of values observed in fishes from warmer waters (Egginton, 1996) and that the major difference between these groups is a lower mean corpuscular hemoglobin concentration (MCHC) in red-blooded notothenioids, not lower hematocrit. These arguments further highlight the divergent mechanisms that underlie differences in Hb expression between icefishes and cold-bodied but red-blooded fishes. The loss of Hb expression in icefishes is the result of a wholesale gene deletion. In contrast, the decrease in Hb content that occurs in response to a decrease in temperature in red-blooded fishes, is brought about by the downregulation of an existing gene. Clearly, these are fundamentally different processes. Many physiologists, nonetheless, have deduced that loss of Hb and red cells in icefishes results in an energetic advantage because of reduction in the intrinsic viscosity of blood (e.g. see di Prisco et al., 1991; Zhao et al., 1998; Cocca et al., 1997). These arguments are sound only if one considers the work necessary to pump an identical volume of blood. However, energetic arguments ultimately must work at the organismal level, and there are vast differences in heart sizes, blood volumes and cardiac outputs between red-blooded and white-blooded notothenioids.

When taking into account published mean ventral aortic pressures and cardiac output values normalized to body mass, it is possible to calculate the cardiac power development necessary to support an identical body mass of fish for both Hb-containing notothenioids and Hb-lacking icefishes (Table 2). [Calculation of actual cardiac work would require subtraction of venous return pressure to the heart from mean ventral aortic pressure. Few estimates of the former are available, but the consensus is that pressures in the sinus venosus of these animals are very low, approaching zero. For comparative purposes, body weight-specific cardiac power output thus is an excellent proxy for actual cardiac work.] The results are both instructive and initially surprising. On average, icefishes expend approximately twice the cardiac energy per unit time than do red-blooded notothenioids of equivalent body mass. Although each ml of blood can be moved by icefish at lower energetic cost, they pump a far greater volume per unit time to support an equivalent body mass. This emphasis on high volume circulation in icefishes prompted Tota and Gattuso to cite the very large hearts of icefishes as exemplifying pumps with Type I or spongy cardiac morphology (Tota and Gattuso, 1996), which achieve a high throughput of fluid predominantly by high stroke volume, despite being capable of attaining only modest output pressures (ca. 3 kPa). [For an excellent overview of the functional morphology of fish hearts, see Tota et al. (Tota et al., 1991).] As a consequence, loss of Hb and red cells ultimately is

Table 2. *Loss of hemoglobin expression is not energetically advantageous*[†]

Species	Hemoglobin expression	Cardiac output (ml kg^{-1} min^{-1})	Ventral aortic pressure (kPa)	Cardiac power development (mW kg^{-1})
Trematomus bernacchii[a]	+	17.6	3.09	0.905
Pagothenia borchgrevinki[a]	+	29.6	3.60	1.76
Pseudochaenichthys georgianus[b]	–	80.5	1.87	2.51
Chaenocephalus aceratus[b]	–	77.0	2.30	2.94

[†]Cardiac output is expressed as kg^{-1} body mass of fish to permit assessment of cardiac power development necessary to support an equivalent mass of whole organism. To ensure comparability, values given are those calculated from *in situ* measurements of intact animals. Axelsson et al. give a more complete comparison of estimates of body weight-specific cardiac power development in fishes from a variety of environments, including those determined using isolated, perfused heart preparations from polar fishes (Axelsson et al., 1998).

[a]Axelsson et al., 1992; [b]Hemmingsen and Douglas, 1977.

correlated with a substantially higher expenditure of cardiac energy at the organismal level, and definitely does not result in energetic savings! In fact, it has been estimated that 22% of resting metabolic rate in icefishes is devoted to cardiac work (Hemmingsen and Douglas, 1977). Cardiac work thus represents a far greater fraction of total energetic expenditure in icefishes than the range of 0.5% to 5.0% of total metabolism reported for temperate zone fishes and 2.3% of total metabolism for even such athletic fishes as skipjack tuna (Farrell and Jones, 1992). In contrast to hearts of icefish, tuna hearts have been cited as clear examples of mammalian-like pumps, possessing a well developed compact epicardium and predominantly relying upon pressure development to elevate cardiac work (Tota and Gattuso, 1996). In light of these energetic considerations and the rather draconian compensatory alterations in cardiovascular anatomy and physiology seen in icefishes, it seems reasonable to conclude, as suggested originally (Wells, 1990), that loss of Hb and red cells did not confer an adaptive advantage to the channichthyids.

The multiple occurrences of Mb loss observed among the icefishes are even more perplexing. This is particularly evident when one realizes that each clade characterized by lack of Mb expression is more closely related to clades that produce the protein than to those that do not (cf. Fig. 3). The ability to produce Mb has thus been lost through several completely independent mutational events. This pattern suggests that Mb might be a 'vestigial' protein that may not work well at the severely cold body temperature of icefishes. We have pursued several independent lines of investigation to evaluate this possibility.

We used stopped-flow spectroscopy to establish that oxygen binds and dissociates from icefish Mb more rapidly than from mammalian Mbs at all temperatures (Cashon et al., 1997). When measurements are compared at respective physiological temperatures, however, Mbs of these two groups show very similar kinetics of binding and dissociation. In short, icefish Mb appears to function at 0°C as well as mammalian Mb does at 37°C. Our results also showed that cold-temperature function of Mb is not unique to icefishes, but is a trait shared with other teleosts. The enhanced activity of teleost Mbs at cold temperature appears to be due to the replacement of the common D-helix found in mammalian Mbs with a random-coil. This substitution undoubtedly confers greater conformational flexibility to the protein, enhancing oxygen's entry and exit from the heme binding-pocket (Cashon et al., 1997). Oxygen-binding kinetics, therefore, demonstrate that icefish Mb is functional at cold temperature.

Even more compelling evidence of Mb function in icefishes has been obtained from isolated, perfused heart studies (Acierno et al., 1997). Hearts from icefish species that possess Mb can maintain cardiac output at higher afterload pressures than closely related species that lack Mb (Fig. 4). We were further able to substantiate that these differences in cardiac performance were unequivocally due to the presence or absence of Mb, by using sodium nitrite, a selective poison of

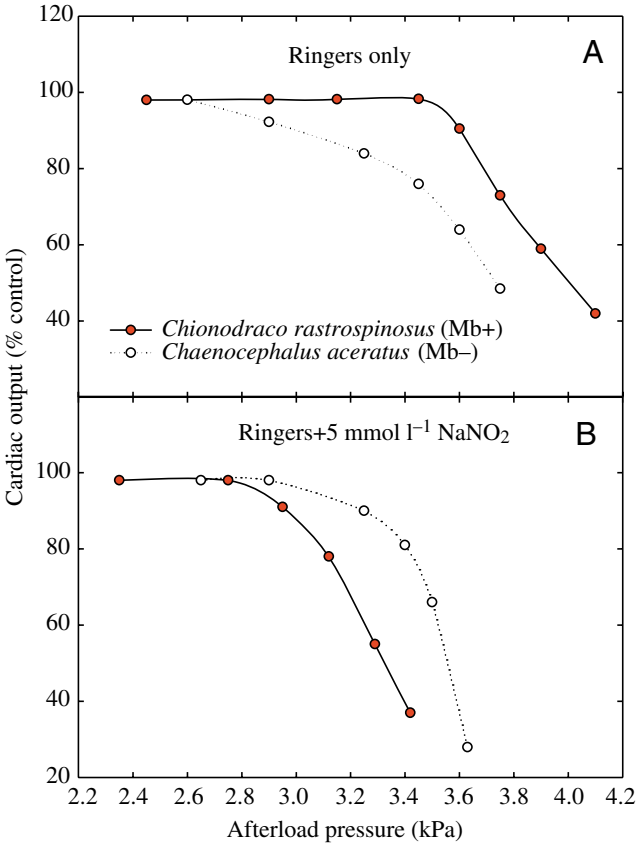

Fig. 4. Myoglobin enhances the performance of icefish hearts. Cardiac output was measured in hearts from Mb-containing *Chionodraco rastrospinosus* and Mb-lacking *Chaenocephalus aceratus* at their inherent rhythms. The afterload against which the heart pumped perfusate was incrementally increased to describe the decay in heart performance as a function of pressure challenge. (A) When pumping oxygenated Ringers solution, Mb-containing hearts (Mb+, filled circles) were able to maintain cardiac output to greater afterload challenges than could Mb-lacking hearts (Mb–, open circles). (B) When 5 mmol l^{-1} $NaNO_2$, which selectively poisons Mb function, was incorporated into the perfusate, mechanical performance of Mb-containing hearts was decremented so dramatically that they were outperformed by Mb-lacking hearts, which were refractory to the treatment. (Adapted from Acierno et al., 1997.)

Mb oxygen-binding function. When 5 mmol l^{-1} $NaNO_2$ is incorporated into the perfusate, mechanical performance of hearts from species that express the protein is significantly impaired, while those naturally lacking the protein are refractory to this treatment (Fig. 4). The unexpected result of these experiments, however, was that hearts that naturally lack Mb performed better than Mb-expressing hearts in which the function of the protein had been chemically ablated. This result suggested that physiological features have developed to compensate for the lack of Mb in those hearts that normally do not express the protein. Several lines of evidence indicate that $NaNO_2$ is an Mb-specific inhibitor and does not release NO into the perfusate, which would confound these conclusions. This point is essential because nitric oxide can affect cardiac

function in several ways. First, at high concentrations, NO inhibits the activity of cytochrome *c* oxidase (COX) (Antunes et al., 2004). This does not appear to occur in the experiments. We have previously shown that the activity of cytochrome *c* oxidase per g ventricular tissue is equivalent between *C. aceratus* and *C. rastrospinosus* (O'Brien and Sidell, 2000). Thus, if NO is present at sufficient levels to inhibit cytochrome oxidase, it should inhibit COX activity to the same extent in hearts from both species. This is clearly not the case; hearts from *C. aceratus* are refractory to the treatment of $NaNO_2$, whereas cardiac output in hearts from *C. rastrospinosus* significantly declines. Second, NO has a positive inotropic effect on hearts of icefish (Pellegrino et al., 2004). Thus, if NO were being released from $NaNO_2$, one would anticipate an increase in cardiac output, particularly in hearts of *C. aceratus* lacking Mb, and not a decrease, as was seen in hearts from *C. rastrospinosus*.

All of the evidence cited above strongly indicates that the losses of the ability to express Hb and Mb were not advantageous. Indeed, available information clearly suggests that each of these losses must have resulted in a decrement in physiological performance of the fishes. Under the nomenclature of Baum and Larsen, this would qualify both of these traits as 'disaptations' (Baum and Larsen, 1991). Indeed, multiple losses of myoglobin during evolution of the icefishes have been cited as a prime example of a disaptation among Antarctic fishes (Montgomery and Clements, 2000). Such a conclusion appears at odds with modern evolutionary theory, which suggests that selective pressure should lead to the retention of Hb and Mb expression, and that mutations causing their loss should be subject to negative selection and eliminated from the population. Regardless of the specific nomenclature employed to describe them, persistence of these traits appears to be a conundrum.

Why have the hemoglobinless and myoglobinless traits persisted in icefishes?

Like virtually all such evolutionary questions, there is no means of arriving at a definitive answer to the question above. The abundance and obvious success of icefishes in the Southern Ocean, however, begs us at least to speculate on a possible explanation. To do so, we must marshal information from areas as diverse as geology, climatology, oceanography and biology. In combination, these features underscore the truly unique set of conditions under which the notothenioid fishes of Antarctica have evolved.

Both environmental and physiological characteristics help explain why Hb and Mb losses are not lethal at the level of the individual organism. We have seen that the very cold temperature and extensive vertical mixing of the Southern Ocean results in highly oxygenated water. Moreover, the absolute metabolic rates of Antarctic fishes are relatively low because of cold body temperature and their modest locomotory activity, as a result of their descent from a common sluggish demersal ancestor. Convergence of these features likely ensured sufficient tissue oxygenation to sustain life in early channichthyids, despite the loss of oxygen-binding proteins. Although providing an explanation of why losses of oxygen-binding proteins may not have been lethal, this line of reasoning still does not address the more difficult question of why such apparently 'disadvantageous' traits were maintained at the population level.

It is essential to remember that, if a trait is sublethal, then it is 'disadvantageous' only within the context of competition with other organisms. In other words, if competition is relaxed sufficiently and environmental resources (e.g. caloric resources) are not limiting, sublethal traits may have no real effect on the fitness of organisms. The unusual evolutionary history of the Antarctic fish fauna suggests that they may have radiated under conditions of little or no niche competition. The massive crash of species diversity among fishes in the Southern Ocean occurring between the mid-Tertiary and present left an ancestral stock of demersal notothenioids to colonize approximately 10% of the world's ocean volume. The prevailing view is that this event explains the ultimate dominance of notothenioid species in Antarctic seas (Eastman, 1993).

The final piece of the puzzle comes with recognition that the climatic cooling of Antarctica during the last 25 MY has not been smoothly monotonic. Indeed, evidence exists to suggest that deep (>100 m) ice-free marine embayments developed during periodic recessions of the glacial shield of the continent on several occasions during even the last 5 MY (e.g. see Webb, 1990). Periodic availability of these deep fjords to colonization by sparsely distributed notothenioids provides the final ingredient of refugia that may have contributed to both the exceptionally rapid radiation of notothenioid species in general, and the fixation of the unusual Hb-less and Mb-less traits of some icefish species.

What other apparently compensatory mechanisms for oxygen delivery are expressed in icefishes and how have they come about?

If the relaxed-competition interpretation above is correct, then we are confronted with another pressing question. In the absence of significant competition, what has driven development of the many physiological characteristics that can be viewed as compensations for the loss of expression of Hb and/or Mb? These compensatory characteristics are manifold and observed at all levels of biological organization. In addition to the striking differences in cardiovascular physiology already described, the channichthyids also display two other notable features.

Enhanced vascular densities

Reflecting their very large blood volumes, Antarctic icefish display large lumenal diameters of the microvasculature (Fitch et al., 1984). Although we have long known that the capillary bore of Hb-less icefishes is two- to threefold greater than that of red-blooded relatives (Fitch et al., 1984), the majority of

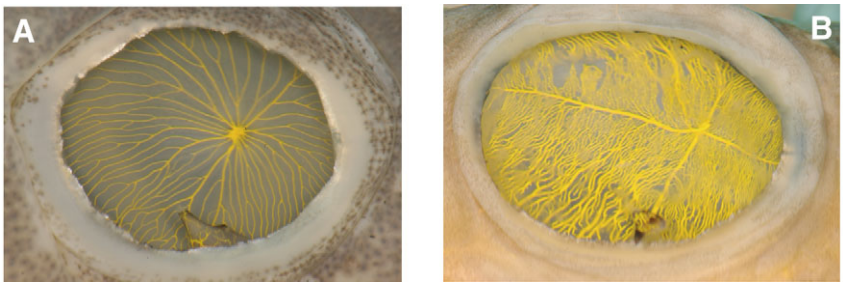

Fig. 5. Retinal vasculature of: (A) *Pagothenia borchgrevinki*, an Hb-expressing notothenid (reproduced with permission from Eastman and Lannoo, 2004). (B) *Chaenocephalus aceratus*, a Hb-lacking icefish (J. Eastman and B. D. Sidell, unpublished). Lenses and vitreous bodies have been removed from the eyes to allow a clear view of the blood vessels. Yellow coloration is from perfusion and filling of the vasculature with *Microfil*™ silicone rubber compound.

vascular adaptations that have been described for these fish are at the gross systemic level. In collaboration with Dr Joseph Eastman of Ohio University, we recently performed a series of preliminary vascular perfusions of Hb-producing and Hb-lacking Antarctic notothenioids that reveal stunning differences in the vascular densities of a highly aerobic tissue, the retina of the eye (Fig. 5). Vascular densities of the Hb-less icefish are dramatically greater than those of Hb-expressing notothenioid species. This has the effect of reducing the diffusion distance for oxygen and ensuring greater oxygenation of the highly aerobic retinal tissue in animals whose blood has an oxygen carrying capacity per unit volume far below that of their red-blooded counterparts.

Altered structural and ultrastructural features of heart muscle

We have compared structural features of hearts from three species of Antarctic notothenioid fishes that differ in expression of oxygen-binding proteins: *Gobionotothen gibberifrons* (a red-blooded species whose heart also contains Mb), *Chionodraco rastrospinosus* (an Hb-lacking icefish that does express cardiac Mb) and, *Chaenocephalus aceratus* (an Hb- and Mb-lacking icefish). At the tissue-level, we found that hearts of both Hb-lacking icefishes were more spongy (i.e. the average diffusion distance that oxygen would have to traverse between lumenal blood and the tissue was shorter) than were hearts from red-blooded species (O'Brien et al., 2000) (Table 3). Hearts of both Mb-expressing and Mb-lacking icefishes, however, showed no significant difference in this feature. It appears that icefish hearts have developed a more pervasive system of blood-filled lacunae within their spongy myocardium to ensure adequate oxygen delivery from their comparatively oxygen deficient blood.

At the level of fine cellular structure, mitochondrial densities in the oxidative muscle of Antarctic fishes are also correlated with the presence or absence of Hb and Mb (O'Brien and Sidell, 2000). Mitchondria (1) account for approximately 16% of cell volume in hearts of red-blooded *Gobionotothen gibberifrons*, which also contain Mb; (2) account for ~20% of cell volume of cardiomyocytes in *Chionodraco rastrospinosus*, which lack Hb but express Mb, and (3) displace ~36% of cell volume in cardiac myocytes from *Chaenocephalus aceratus,* which are devoid of both Hb and Mb (Table 3; O'Brien and Sidell, 2000). Thus, loss of Hb results in only a modest (~4%) expansion of the mitochondrial population in heart muscle, as long as the tissue continues to express Mb. However, when both Hb and Mb are absent, expansion of the mitochondrial population is dramatic (i.e. a further 16% increase in cell volume). Surprisingly, the high densities of mitochondria in the hearts of fishes lacking Hb and Mb do not increase aerobic metabolic capacity. In fact, the activity of aerobically poised enzymes (per g cardiac tissue) such as cytochrome oxidase and citrate synthase, are equivalent among all three species, despite the dramatic differences in mitochondrial number. The high densities of enlarged mitochondria in icefishes lacking Hb and Mb, result in the formation of an interwoven network of membranes. This lipid highway likely serves as an important pathway for oxygen, enhancing its delivery in the absence of oxygen-binding proteins (Sidell, 1998).

The suite of anatomical and physiological characteristics of icefishes that appear to be linked to the loss of Hb and Mb expression is extensive. How then could these apparently adaptive traits have evolved under conditions of relaxed competition? One possibility is that the loss of Hb and Mb triggered immediate, ameliorating modifications in icefish

Table 3. *Structural and ultrastructural features of heart muscle from Antarctic notothenioid fishes*

Species	Hb/Mb	O_2 diffusion distance through tissue (μm)[a]	Mitochondrial volume density [$V_{v,mit}$, f %)][b]
Gobionotothen gibberifrons	+/+	9.82±1.37	15.87±0.74
Chionodraco rastrospinosus	–/+	6.20±0.86	20.10±0.74
Chaenocephalus aceratus	–/–	6.23±0.41	36.53±2.07

[a]Data from O'Brien et al., 2000; [b]data from O'Brien and Sidell, 2000.

Oxygen diffusion distance through tissue is the maximum distance that O_2 originating in the ventricular lumen of the heart (which lacks a coronary circulation) would need to diffuse to penetrate heart tissue. From a conceptual standpoint, the shorter the diffusion distance, the 'spongier' the tissue.

physiology, which became fixed traits over time. In other words, the initial loss of Hb and Mb accelerated the evolution of secondary cardiovascular traits. This idea is supported by recent studies illuminating the novel role of Hb and Mb in the metabolism of the potent signaling molecule, nitric oxide.

Hemoglobin and myoglobin as nitric oxide-oxygenases

Fish physiologists studying the channicthyid icefish have thus far focused on questions related to the economy of oxygen delivery and consumption. Logically, these were the most pertinent and primary problems to address, given our understanding of hemoglobin and myoglobin as critical oxygen storage and transport proteins. However, recent studies on the evolution of globin proteins have revealed an additional function for these proteins. We now know that vertebrate hemoglobins are derived from the more primitive globins of bacteria, nematodes and yeast, which all metabolize nitric oxide (NO) (Liu et al., 2000; Minning et al., 1999; Gardner et al., 1998). These ancestral globins function as NO oxygenases, using oxygen to convert NO to nitrate. Studies of Hb–NO oxygenase activity in unicellular organisms provided precedence for later work on vertebrate hemoglobins, which showed that in addition to binding oxygen, both Hb and Mb also break down NO to nitrate (Gardner, 2005; Flögel et al., 2001). Moreover, this function is integral to an animal's physiology. The absence of Mb in the much-studied myoglobinless mice leads to enhanced sensitivity to NO and the activation of downstream pathways regulated by NO (Flögel et al., 2001; Grange et al., 2001).

These findings prompt us to widen the scope of our questions related to the physiology of icefish and ask: *What are the potential effects of the loss of Hb and Mb as NO-oxygenases?* The answers are enticing, and suggest that the loss of NO-oxygenase activity, and subsequent elevation of NO levels, could explain many, if not all, of the unique cardiovascular and physiological traits that have evolved in icefishes.

The production of nitric oxide

NO is a small, highly diffusible molecule that regulates a wide array of physiological processes (reviewed by Kerwin, Jr et al., 1995). In higher vertebrates, NO is the product of a reaction catalyzed by three distinct isoforms of the enzyme nitric oxide synthase (NOS; E.C. 1.14.13.39): endothelial NOS (eNOS, NOS-III), neuronal NOS (nNOS, ncNOS, NOS-I) and inducible NOS (iNOS, mNOS, NOS-II). All NOS isoforms catalyze a 5-electron oxidation of arginine to produce NO and L-citrulline using NADPH as an electron source (Moncada and Higgs, 1993). Two of the isoforms, eNOS and nNOS, are constitutively expressed in mammals, while iNOS is inducible by cytokines and other stimuli, including hypoxia (Kerwin, Jr et al., 1995). Even the constitutively expressed NOS isoforms, however, are upregulated in response to physiological stimuli such as hypoxia and hemodynamic shear stress (Shaul, 2002).

All three isoforms of NOS are present in fish. Endothelial NOS (eNOS) has been reported in vascular endothelium and heart muscle of developing zebrafish (Fritsche et al., 2000), and the presence of nNOS and iNOS clearly has been established in a variety of tissues from several fish species (e.g. Holmqvist et al., 1998; Holmqvist et al., 2004; Morlá et al., 2003, Pellegrino et al., 2002; Pellegrino et al., 2004). A recent study shows that nNOS is expressed at a higher level in skeletal muscle from icefishes than in the tissue from red-blooded species (Morlá et al., 2003). Both eNOS and iNOS have been identified in ventricular cardiomyocytes of white and red-blooded nototheniods, and eNOS is also found in the endothelium and epicardium in heart ventricles from these fishes (Tota et al., 2005). NO has been shown to regulate cardiovascular activities in icefish, including dilation of branchial vasculature, cardiac stroke volume and power output (Pellegrino et al., 2003). Interestingly, NO has a positive inotropic effect on cardiovascular function in icefish, whereas in other fish and mammals, NO has a negative inotropic effect (Tota et al., 2005). At this point it is unknown if this difference is related to the absence of hemoglobin, or some other species-specific difference in the expression of signaling molecules operating downstream of NO. Regardless, it seems likely that if NO is present at sufficient levels to control these processes, then it likely also contributes to the regulation of additional features of the cardiovascular and muscular system.

What pathways may be upregulated in response to high levels of nitric oxide?

Nitric oxide and angiogenesis

Nitric oxide is best known for its function as a vasodilator that enhances blood flow and thus oxygen delivery to tissues (Palmer et al., 1987). In addition, NO-mediated pathways have been implicated in promoting the growth of capillary networks (angiogenesis) (reviewed by Conway et al., 2001). Angiogenesis involves the expansion, growth and remodeling of blood vessels into a mature network. This process occurs *via* sprouting of new vessels from the ends and sides of existing vessels or by longitudinal splitting of existing vessels. NO induces upregulation of one of the most potent factors influencing blood vessel proliferation, vascular endothelial growth factor (VEGF) (Kimura et al., 2000). Nitric oxide appears to play a role in both hypoxia- and exercise-induced stimulation of VEGF and angiogenesis in muscle (Milkiewicz et al., 2005; Kimura and Esumi, 2003). It is also noteworthy that VEGF, along with Angiopoietin-1 (Ang-1), promote enlargement of the lumenal diameter of the microvasculature (Suri et al., 1998).

Nitric oxide and mitochondrial biogenesis

Only within the last couple of years we have learned that another very important role of NO is to stimulate and maintain high densities of mitochondria in a variety of tissues (Nisoli et al., 2003; Nisoli et al., 2004). NO induces mitochondrial

biogenesis *via* a guanylate cyclase and cGMP-dependent pathway (Fig. 6). It also plays a role in maintaining constitutive levels of mitochondrial densities. Null-mutant mice, lacking eNOS, have lower levels of mtDNA, as well as mRNA levels of subunit IV of cytochrome oxidase (COXIV) and cytochrome *c*, compared to wild-type mice in brain, liver and heart tissue (Nisoli et al., 2003).

Nitric oxide activates mitochondrial biogenesis through the transcriptional co-activator, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (Wu et al., 1999). PGC-1α is a member of the PPARγ family of transcriptional coactivators, which modulate the activity of transcription factors through protein–protein interactions with transcription factors, proteins with histone acetyl transferase activity, and RNA processing proteins (reviewed in Puigserver and Spiegelman, 2003). PGC-1α stimulates and modulates the expression of two nuclear transcription factors, nuclear respiratory factors 1 and 2 (NRF-1, -2) (Wu et al., 1999). Together, these transcription factors regulate the expression of a number of nuclear-encoded mitochondrial genes, including cytochrome *c*, subunits of both ATP synthase and cytochrome *c* oxidase, and enzymes of heme biosynthesis (Scarpulla, 1997). NRFs also induce expression of mitochondrial transcriptional factor-A (mtTFA), which translocates to the mitochondrion and controls mtDNA transcription (Shadel and Clayton, 1993; Wu et al., 1999). In concert, these factors coordinate the upregulation of genes required for mitochondrial biogenesis.

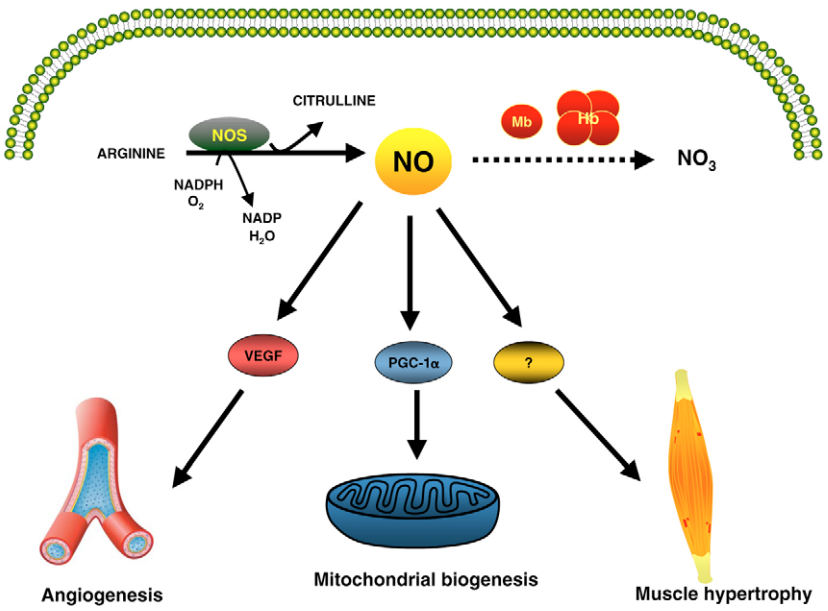

Fig. 6. Nitric oxide (NO) regulates many of the physiological processes that are characteristic traits of icefish. NO stimulates angiogenesis through the activation of vascular endothelial growth factor (VEGF). Mitochondrial biogenesis is induced by NO *via* peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1). NO has also been shown to induce muscle hypertrophy, although this molecular pathway has yet to be defined. The authors acknowledge the use of Scienceslides (Visiscience Corp.) in creating this figure.

Nitric oxide and muscle hypertrophy

High levels of NO can induce cardiac hypertrophy in animals lacking myoglobin. The heart-to-body-mass index increases by 33% in myoglobinless mice over-expressing iNOS, compared to wild-type animals (Gödecke et al., 2003). The details of the pathway regulating these changes are unknown.

Nitric oxide also induces muscle hypertrophy and activates satellite cells in the skeletal muscle of mammals (Smith et al., 2002; Anderson, 2000). Neuronal nitric oxide synthase is part of the dystrophin glycoprotein protein complex, which links actin to components of the basal lamina. Neuronal NOS is activated by both mechanical force and pressure. The subsequent production of NO induces the expression of the two cytoskeletal proteins, talin and vinculin, resulting in muscle hypertrophy (Tidball et al., 1999). NO produced by nNOS also activates muscle satellite cells, although the molecular components of this pathway are unknown (Anderson, 2000).

Nitric oxide as a unifying trigger for traits of icefishes

Clearly, nitric oxide is an important signaling molecule that regulates a myriad of biological processes. Many of these are phenotypic characteristics that have long been associated with icefishes. Traditionally, we have thought that these characteristics evolved over time exclusively due to selective pressures during the radiation of the Family Channichthyidae. However, the recent revelation of the role of globin proteins in NO metabolism has suggested that these characteristics may have first appeared as a direct result of the loss of expression of Hb and Mb.

When mutations leading to the loss of expression of Hb and Mb occurred during icefish evolution, the primary degradative pathways for NO were eliminated. NO constitutively produced by the NOS system in these animals would then have a much longer biological half-life and the end result should have been elevation of steady-state levels of NO in icefish tissues compared to those of Hb- and Mb-expressers. In fact, preliminary data suggest that circulating levels of NO in icefish are >2-fold those observed in red-blooded notothenioids (B.D.S., unpublished). We would predict that elevated levels of NO would lead to virtually all of the hallmark characteristics that we have described for icefishes. Elevated vascular densities in retinal tissue, and increased lumenal diameters of blood vessels in the oxidative muscle of icefishes, are consistent with a marked upregulation of angiogenic processes, that would be expected in response to constitutively higher NO

levels. Likewise, elevated mitochondrial densities and modified mitochondrial morphologies are exactly the patterns that we would predict if NO levels regulate densities of mitochondria in the tissues. In fact, NO may also play a role in regulating the size difference in mitochondria among species that vary in their expression of oxygen-binding proteins. We find that mitochondria from Mb/Hb-less fishes are significantly larger than those from red-blooded fishes (O'Brien and Sidell, 2000). Higher NO levels in icefish tissue would be consistent with findings that show a decrease in NO production reduces mitochondrial size (Nisoli et al., 2004). Increases in nitric oxide could also account for the enlarged heart size of icefishes. We find that the heart-to-body-mass of notothenioids correlates with Mb and Hb expression, with values from red-blooded species being the smallest, those from icefish expressing Mb intermediate, and from icefishes lacking Mb, the largest. These observations are in accordance with the role of NO in inducing cardiac hypertrophy in myoglobinless mice.

Intriguingly, elevated NO concentration may even help clarify the unknown origin of another striking feature of muscles in Antarctic fishes. Muscle fiber size of fishes generally increases as body temperature decreases (Egginton and Johnston, 1984; Egginton and Sidell, 1989; Rodnick and Sidell, 1997; Johnston et al., 1998). This trend correlates with the inverse relationship between hematocrit and body temperature mentioned previously. Muscle fibers from Antarctic fishes are even larger than those of temperate species and fibers of Antarctic icefish lie at the extreme of this continuum, with oxidative muscle fibers that are approximately twofold greater in cross-sectional area compared to red-blooded Antarctic species (O'Brien et al., 2003; Egginton et al., 2002). All oxidative pectoral muscle from Antarctic species lack Mb. Thus, higher NO levels as a result of the loss of Mb and Hb, may also influence maintenance of unusually large fiber size in the pectoral muscle of icefish.

Conclusion

The extraordinary biology of channichthyid icefish was first recognized more than 50 years ago. Today we continue to learn much about basic biological processes by studying these unusual animals. The icefishes have provided a tremendous opportunity to learn more about the function of the oxygen-binding proteins hemoglobin and myoglobin, and the unique physiologies that arise in the absence of these proteins. Indeed, icefishes were around several million years before the development of the much-studied myoglobinless mice.

The recent discovery of Hb and Mb as NO-oxygenases prompts us to return to the icefishes and examine their physiology from a new perspective. We now have the opportunity to ask: *What are the potential adaptations that might occur in the absence of NO-oxygenase activity?* The answers are exciting. Current research in mammalian systems suggests that nearly all of the hallmark traits of channichthyid may be a result of high levels of nitric oxide, an outcome that one would expect in the absence of Hb and Mb. These results, coupled with molecular biological techniques, will help us to uncover the genetic underpinnings that have led to establishment of many of the distinctive traits in icefish including: high vascular and mitochondrial densities, large capillary lumenal diameters and even, perhaps, enlarged muscle fibers.

The novel globin proteins, cytoglobin and neuroglobin, have recently been identified in several vertebrate species (Pesce et al., 2002). This latter protein is expressed in brain and neuronal tissue, and has been implicated in combating nitrosative stress, caused by high levels of nitric oxide (Herold et al., 2004). Intriguingly, we have observed a distinct reddish tint in the retinal tissue *C. aceratus*, hinting that this may be a heme-containing globin protein that is expressed in this animal. The channichthyids, or 'blodlaus fisk' (Ruud, 1954) will undoubtedly help us to learn more about the function of these proteins in the future, and provide us with at least another 50 years of fruitful studies on the fascinating physiology of these animals.

List of abbreviations

Ang-1	angiopoietin-1
COX	cytochrome *c* oxidase
COXIV	subunit IV of COX
e-, i-, n-NOS	endothelial, inducible and neuronal NO synthase
Hb	hemoglobin
Mb	myoglobin
MCHC	mean corpuscular Hb concentration
MYA	million years ago
NO	nitric oxide
NRF	nuclear respiratory factor
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α
TFA	transcriptional factor A
VEGF	vascular endothelial growth factor

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