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SYMPOSIUM

The Unique Mitochondrial Form and Function of Antarctic Channichthyid Icefishes

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Synopsis Antarctic icefishes of the family Channichthyidae are the only vertebrate animals that as adults do not express the circulating oxygen-binding protein hemoglobin (Hb). Six of the 16 family members also lack the intracellular oxygen-binding protein myoglobin (Mb) in the ventricle of their hearts and all lack Mb in oxidative skeletal muscle. The loss of Hb has led to substantial remodeling in the cardiovascular system of icefishes to facilitate adequate oxygenation of tissues. One of the more curious adaptations to the loss of Hb and Mb is an increase in mitochondrial density in cardiac myocytes and oxidative skeletal muscle fibers. The proliferation of mitochondria in the aerobic musculature of icefishes does not arise through a canonical pathway of mitochondrial biogenesis. Rather, the biosynthesis of mitochondrial phospholipids is up-regulated independently of the synthesis of proteins and mitochondrial DNA, and newly-synthesized phospholipids are targeted primarily to the outer-mitochondrial membrane. Consequently, icefish mitochondria have a higher lipid-to-protein ratio compared to those from red-blooded species. Elevated levels of nitric oxide in the blood plasma of icefishes, compared to red-blooded notothenioids, may mediate alterations in mitochondrial density and architecture. Modifications in mitochondrial structure minimally impact state III respiration rates but may significantly enhance intracellular diffusion of oxygen. The rate of oxygen diffusion is greater within the hydrocarbon core of membrane lipids compared to the aqueous cytosol and impeded only by proteins within the lipid bilayer. Thus, the proliferation of icefish's mitochondrial membranes provides an optimal conduit for the intracellular diffusion of oxygen and compensates for the loss of Hb and Mb. Currently little is known about how mitochondrial phospholipid synthesis is regulated and integrated into mitochondrial biogenesis. The unique architecture of the oxidative muscle cells of icefishes highlights the need for further studies in this area.

Introduction

Antarctic icefishes of the family Channichthyidae represent a rare physiological phenomenon. They are the only vertebrate animals that, as adults, lack the circulating oxygen-binding protein hemoglobin (Hb) (Ruud 1954). Moreover, not all members of the family express the intracellular oxygen-binding protein myoglobin (Mb) in their cardiac ventricular muscle and all lack Mb in oxidative skeletal muscle (Sidell et al. 1997; Moylan and Sidell 2000; Grove et al. 2004). Physiologists, long intrigued by these remarkable animals, have characterized multiple modifications in their cardiovascular system, which enhance delivery of oxygen (Holeton 1970; Hemmingsen et al. 1972; Fitch et al. 1984; Tota et al. 1991; Wujcik et al. 2007). One of the more curious adaptations of icefishes is the high mitochondrial density found in aerobic muscle cells (Archer and Johnston 1991; O'Brien and Sidell 2000; O'Brien et al. 2003). We will focus our review on this unique trait of icefishes and examine the structure, function and molecular basis of high mitochondrial densities in oxidative muscle.

The evolution of the Hb-less condition in Antarctic icefishes

Antarctic icefishes (family Channichthyidae) were well known as *blodlaus-fisk* to Norwegian whalers working in South Georgia at the turn of the 20th century. It was not until 1954 that their

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extraordinary physiological characteristics were revealed to the scientific community by Prof. Johan Ruud (Ruud 1954). Ruud detailed the biology of *Chaenocephalus aceratus*, describing its nearly transparent blood, devoid of erythrocytes and with an oxygen-carrying capacity only 1/10th that of the red-blooded species *Notothenia rossi* (Ruud 1954). In closing, Ruud pondered the surprising existence of a fish lacking an oxygen-binding blood pigment. He astutely surmised that such a large vertebrate fish could only have survived without Hb in the constantly cold and oxygen-rich environment of the polar seas. Today, having a more comprehensive knowledge of the geological and climactic history of Antarctica, we can more fully appreciate the accuracy of Ruud's insight.

The Channichthyidae family is one of eight families within the perciform suborder Notothenioidei, the predominant fish clade in the Southern Ocean. This suborder comprises 45% of the benthic fish species and 91% of the fish biomass on the Antarctic shelf (Eastman 2005). There are 16 members of this family (Near et al. 2003). Only one, *Champsocephalus esox*, is found north of the Polar Front on the southern Patagonian shelf and in the Strait of Magellan (Iwami and Kock 1990). The remaining species are found in both high-Antarctic and low-Antarctic regions (Iwami and Kock 1990). Members of the icefish family exhibit a range of activity levels. Some are sedentary and benthic such as *C. aceratus*, while others are semipelagic or pelagic, and migrate up and down through the water column to feed (reviewed by Eastman 1993; Kock 2005).

All icefishes lack Hb, suggesting that the trait arose prior to the radiation of the family, and at or near the time of divergence from the notothenioid lineage (Fig. 1). Phylogenetic analyses using mitochondrial DNA sequences and molecular clock methodology suggest that icefishes diverged from the notothenioid lineage between 5.5 and 2 million years ago (MYA) (Bargelloni et al. 2000). This was long after the formation of the Antarctic Polar Front (25–22 MYA) and by 12 MYA, the temperature of the Southern Ocean was lower than 5°C (Kennett 1977; Eastman 1993). Temperatures today in most regions of the Southern Ocean are lower than 0°C and vary little on a seasonal basis (Eastman 1993). In McMurdo Sound (78°S), water temperature is a nearly constant −1.86°C. Even in the more northerly regions of the West Antarctic Peninsula, water temperatures rarely exceed 2°C (Eastman 1993; Clarke et al. 2007). The low temperature of the Southern Ocean sustains an oxygen-rich environment, as oxygen solubility is inversely related to temperature. Thus, Ruud's assessment was correct: Optimal conditions were in place for the survival of an ancestral notothenioid suffering a mutation leading to the loss of Hb.

The loss of Hb by channichthyids was not due to a single mutational event (Near et al. 2006). Fifteen of the 16 members of the icefish family lack the β-globin gene and have retained only a fragment of the 3′ region of the α-globin gene (Cocca et al. 1995; Zhao et al. 1998). Interestingly though, the derived icefish species, *Neopagetopsis ionah*, possesses a complex globin gene structure with intact, although non-functional α- and β-globin genes (Near et al. 2006). The major mutation in *N. ionah* lies between the α- and β-globin genes. In red-blooded notothenioids, this intergenic region contains the regulatory sequences controlling the transcription of both globin genes (Lau et al. 2001). In *N. ionah*, these regulatory sequences have been disrupted by the insertion of a segment of a second β-globin gene (Near et al. 2006). The nearly-intact globin gene structure in *N. ionah* is thought to represent an intermediate step during the evolutionary loss of Hb in the icefish family (Near et al. 2006; Cheng and Detrich 2007).

Multiple cardiovascular modifications compensate for the loss of Hb

The loss of Hb expression in icefishes is correlated with substantial remodeling of their cardiovascular system in ways that maintain adequate oxygenation of tissues. Oxygen is carried throughout the circulatory system dissolved in the blood plasma. In icefishes, oxygen-carrying capacity is elevated by a large blood volume, two to four times greater than that of red-blooded teleosts (Hemmingsen and Douglas 1970). Cardiac output is high and maintained by a large, slowly beating heart, which functions as a volume pump rather than a pressure pump (Hemmingsen et al. 1972; Holeton 1970; Harrison et al. 1991; Zummo et al. 1995). Most notothenioids have a type I heart, characterized by a spongy myocardium and lack of coronary circulation (Feller et al. 1985; Zummo et al. 1995). The trabeculated nature of the heart provides a large surface area for the diffusion of oxygen between the venous blood bathing the lumen and cardiomyocytes. The extent of trabeculation is greater in icefishes compared to red-blooded species (O'Brien et al. 2000). High capillary densities also enhance oxygen delivery to some aerobic tissues such as the retina (Wujcik et al. 2007). Blood pressure and work of the heart are minimized by large-bore vessels (two to three times larger than in red-blooded species) (Fitch et al. 1984;

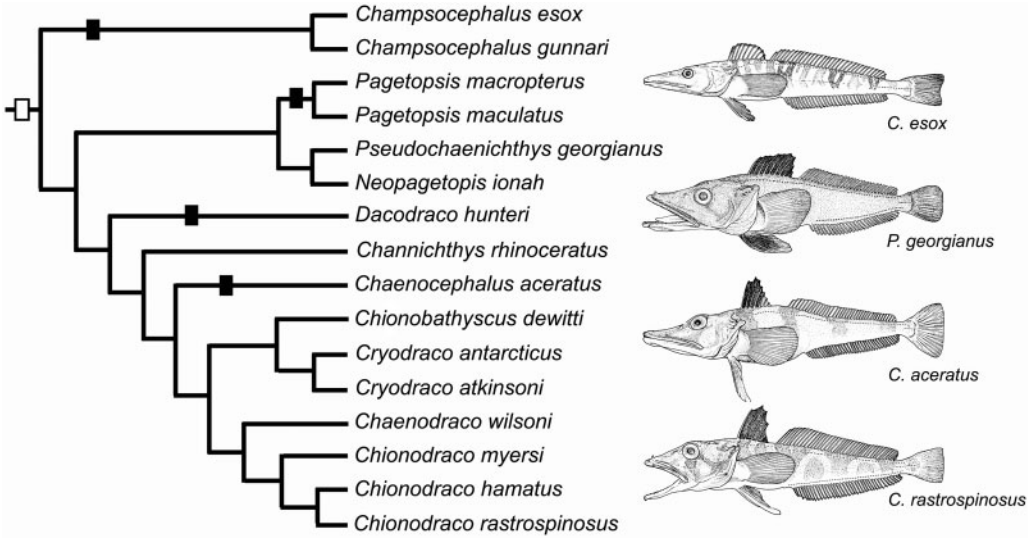

Fig. 1 Pattern of Hb and Mb expression in the family Channichthyidae. This consensus phylogeny of the icefish family is based on mtDNA sequences and morphological characteristics by Near et al. (2003). The mutational event leading to the loss of Hb expression is represented by an open rectangle, whereas the mutational events leading to the loss of Mb are represented by filled rectangles. The representative icefishes shown are from Gon and Heemstra (1990). The figure is adapted from Sidell and O'Brien (2006).

Wujcik et al. 2007). Large-bore vessels allow for blood transport at a high velocity, which maintains a steep oxygen gradient between the vessels and tissues, thereby facilitating diffusion of oxygen to the tissues (Holeton 1970). Although the loss of Hb reduces blood viscosity, cardiac work is not reduced relative to red-blooded species. In fact, the body weight-specific cardiac power output is 1.4–3.2 times higher in icefishes compared to red-blooded species (Sidell and O'Brien 2006). This provides compelling evidence that the loss of Hb is not advantageous but rather, an example of disadaptation (Montgomery and Clements 2000; Sidell and O'Brien 2006; Garofalo et al. 2009b).

All of the adjustments to the cardiovascular system of icefishes ensure adequate delivery of oxygen to the tissues. Yet, the final destination of oxygen is the mitochondrion, where it is reduced to water in the process of oxidative phosphorylation that produces ATP. Cellular respiration is the primary means of energy production in oxidative muscle and is particularly important in Antarctic fishes for which anaerobic metabolic capacity is reduced compared to temperate teleosts (Crockett and Sidell 1990). Mb is an intracellular oxygen-binding protein found in abundance in cardiac myocytes and oxidative skeletal muscle fibers where it stores oxygen and facilitates the delivery of oxygen from the sarcolemma to the mitochondria (Wittenberg 1970). It is somewhat surprising that Mb is lacking in the oxidative muscles of some icefishes, already challenged by the loss of Hb (Sidell et al. 1997).

Some icefishes also lack the intracellular oxygen-binding protein Mb

Both red-blooded and white-blooded notothenioids lack Mb in the aerobic pectoral adductor profundus muscle used to power their labrifrom style of swimming (Moylan and Sidell 2000). The lack of Mb within this tissue, in species having an intact Mb gene is likely due to its expression being repressed at some point during development.

Mb expression in the ventricle of the heart of icefishes is more complex. Of the 16 species of icefishes, six do not express Mb in their ventricle (Sidell et al. 1997; Moylan and Sidell 2000). Mapping Mb expression onto the phylogeny of channichthyids indicates that Mb expression was lost at four distinct points during the radiation of the family (Fig.1; Grove et al. 2004; Sidell and O'Brien 2006). DNA sequences reveal that the lack of Mb expression is caused by at least three different genetic lesions (Small et al. 1998, 2003; Grove et al. 2004). Although this irregular pattern of Mb expression might lead one to believe that the protein is non-functional, all evidence points to the contrary. The binding kinetics of notothenioid Mb are similar to that of other teleosts, and the protein functions as well at 0°C as mammalian Mb does at 37°C (Cashon et al. 1997). Moreover, hearts of icefishes expressing Mb maintain cardiac output at higher afterload pressures than do those lacking the protein (Acierno et al. 1997). This difference is eliminated when Mb is poisoned, and in fact under these conditions, hearts naturally lacking

Mb perform better than those expressing the protein, suggesting that adaptations have occurred in the hearts of Mb-less icefishes which compensate for the lack of Mb (Acierno et al. 1997). Indeed, examination of the ultrastructure of both cardiac myocytes and the aerobic pectoral adductor profundus muscle of icefishes shows a significant expansion of the mitochondrial compartment which may compensate for the lack of oxygen-binding proteins.

Oxidative muscles of icefishes are characterized by high mitochondrial densities

The percentage of cell volume displaced by mitochondria is higher in both the cardiac myocytes and oxidative muscle fibers of Mb-less icefishes compared to red-blooded species (Table 1). Although mitochondrial surface densities and volume densities [Sv(mit,my) and Vv(mit,my)] are slightly higher in hearts of icefishes expressing Mb compared to some red-blooded notothenioids, the difference is modest (on average ~1%) (Table 1). More substantial increases in mitochondrial volume density of between 15 and 19% occur in response to the loss of both Hb and Mb in aerobic muscle cells. The mitochondrial volume density recorded in icefishes such as *C. aceratus*, is extraordinarily high considering *C. aceratus* is a sedentary, demersal fish. The mitochondrial volume densities of icefish muscles are on par with those measured in the highly aerobic flight muscles of hummingbirds (35%) and greater than densities observed in the oxidative swimming muscles of active fishes such as the tuna, *Katsuwonus pelamis* (29%) (Mathieu-Costello et al. 1992, 1996). These high mitochondrial densities may approach the maximum values attainable without impeding muscle function, particularly in oxidative skeletal muscle where the volume density of mitochondria is ~50% of the total cell volume and greater than the myofibril volume [Vv(myf,f)].

The stunningly high mitochondrial densities in the aerobic musculature of icefishes were first recorded by Ian Johnston and colleagues over 25 years ago (Johnston et al. 1983). The question remains: How

Table 1 Ultrastructural characteristics of myocytes from oxidative muscles of notothenioid fishes

Species	O_2-Binding Proteins	Vv(mit,my) (%)	Vv(myf,my) (%)	Sv(mit,my) (μm^{-1})	Sv(imm,mit) (μm^{-1})	Sv(imm,m) ($m^2 g^{-1}$)
Cardiac myocytes						
Gobionotothen gibberifrons[a]	(+Hb/+Mb)	15.87 ± 0.74	40.12 ± 0.91	1.19 ± 0.05	29.63 ± 1.62	4.46 ± 0.31
Notothenia rossii[b]	(+Hb/+Mb)	22.8 ± 3.5	44.9 ± 2.2			
Notothenia coriiceps[c]	(+Hb/+Mb)	25.0 ± 1.0	47.0 ± 2.2			
Chionodraco rastrospinosus[a]	(−Hb/+Mb)	20.10 ± 0.74	24.50 ± 1.26	1.34 ± 0.05	21.52 ± 0.69	4.11 ± 0.24
Channicthys rhinoceratus[b]	(−Hb/+Mb)	24.4 ± 3.3	43.7 ± 6.6			
Chaenocephalus aceratus[a]	(−Hb/−Mb)	36.53 ± 2.07	25.07 ± 1.64	1.63 ± 0.05	20.04 ± 0.79	6.91 ± 0.39
Pectoral adductor muscle fibers						
Gobionotothen gibberifrons	(+Hb/−Mb)	24.9 ± 0.7[d]	54.9 ± 2.8[d]		37.68 ± 3.62[e]	8.89 ± 0.85[e]
Notothenia coriiceps[e]	(+Hb/−Mb)	28.7 ± 2.9			33.60 ± 3.02	9.14 ± 0.88
Lepidonotothen nudifrons[f]	(+Hb/−Mb)	29 ± 2.4	40 ± 2.1			
Trematomus newnesi[f]	(+Hb/−Mb)	31 ± 1.9	36 ± 1.9		35.8 ± 8.2	
Chionodraco rastrospinosus[e]	(−Hb/−Mb)	39.0 ± 3.0			25.47 ± 1.81	9.08 ± 0.53
Chaenocephalus aceratus[e]	(−Hb/−Mb)	52.5 ± 2.5			20.79 ± 1.60	10.91 ± 0.65
Champsocephalus gunnari[g]	(−Hb/−Mb)	49 ± 1	40 ± 1	2.01 ± 0.08	25.2 ± 1.5	11.70
Champsocephalus esox[f]	(−Hb/−Mb)	51 ± 2.6	38 ± 2.6		43.9 ± 2.1	21.22

Vv(mit,my), volume density of mitochondria per volume myocyte; Vv(myf,my), volume density of myofibrils per volume myocyte; Sv(mit,my), surface density of mitochondria per volume myocyte; Sv(imm,mit) surface density of inner-mitochondrial membranes per volume mitochondria; Sv(imm,m), surface density of mitochondria per gram muscle, as reported in the literature or calculated using a value for muscle density of 1.055 g cm^{-3} (Webb, 1990).

[a]O'Brien and Sidell (2000)
[b]Feller et al. (1985)
[c]Johnston and Harrison (1987)
[d]Londraville and Sidell (1990)
[e]O'Brien et al. (2003)
[f]Johnston et al. (1998)
[g]Archer and Johnston (1991)

did this remarkable phenotype come about? The answer might be: The multi-faceted molecule, nitric oxide (NO) (reviewed by Sidell and O'Brien 2006).

Molecular drivers of muscle remodeling in Antarctic icefishes: Is NO the answer?

NO is a highly reactive, gaseous molecule that mediates multiple pathways of inter- and intra-cellular communication, including those associated with several hallmark traits of icefishes: Large-diameter blood vessels, high vascular densities, and mitochondrial-rich muscle cells. NO has long been known as a potent vasodilator, relaxing smooth muscle and expanding the diameter of blood vessels (Furchgott and Zawadzki 1980; Ignarro et al. 1987; Palmer et al. 1987). NO induces angiogenesis, the growth of new blood vessels from pre-existing ones (Fukumura et al. 2006; Hudlicka and Brown 2009; Sessa 2009). In addition, NO induces the proliferation of mitochondria through the process of mitochondrial biogenesis (Nisoli et al. 2003). Thus, high circulating levels of NO in icefishes could explain how many, if not all, of the distinguishing characteristics of icefishes arose.

Several lines of evidence suggests that NO levels may have become elevated when the ancestral notothenioid incurred the loss of Hb expression, thereby sustaining delivery of oxygen in the face of this life-threatening mutation. First, NO is produced in notothenioid fishes. The primary source of NO in vertebrates is the enzyme nitric oxide synthase (NOS), which catalyzes the oxidation of L-arginine to L-citrulline in a reaction requiring O_2 and NADPH. There are three isoforms of NOS expressed in mammals: Neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3), and inducible NOS (iNOS, NOS2) (Moncada et al. 1991). Despite the nomenclature, all three isoforms are found in multiple cell types (reviewed by Mungrue et al. 2003). Both nNOS and eNOS are constitutively expressed and their activity is calcium-dependent, whereas the activity of iNOS is calcium-independent. A mitochondrial isoform of NOS has also been described for mammals, although its existence is controversial (Tatoyan and Giulivi 1998; Brookes 2004; Lacza et al. 2006). If present, it may regulate cellular respiration as a competitive inhibitor of cytochrome *c* oxidase (COX) (Giulivi 1998).

Neuronal NOS, eNOS and iNOS have been detected in many species of fish and in nearly all cell types, including oxidative muscle. The majority of fishes express nNOS and/or iNOS, which have been detected by immunocytochemistry as well as by gene cloning and sequencing (Laing et al. 1996, 1999; Holmqvist et al. 2000; Oyan et al. 2000; Saeij et al. 2000; Cox et al. 2001; Bordieri et al. 2005; Masini et al. 2005; Hyndman et al. 2006; Lepiller et al. 2009; Zhou et al. 2009). Although there is no genomic evidence for the presence of the eNOS isoform in fishes, eNOS has been detected using immunocytochemistry and mammalian-derived antibodies in zebrafish and Antarctic notothenioids (Fritsche et al. 2000; Garofalo et al. 2009a). Both eNOS and iNOS have been detected by immunostaining in the ventricle of the Antarctic fishes *Chionodraco hamatus*, *C. aceratus* and *Trematomus bernacchii* (Pellegrino et al. 2004; Amelio et al. 2006; Garofalo et al. 2009). The neuronal isoform of NOS has been detected in the brains of both red- and white-blooded notothenioids and in the glycolytic skeletal muscle of icefishes (Morla et al. 2003; Masini et al. 2005). In addition, studies with isolated perfused hearts from notothenioids reveal that, as in mammals, NO regulates several aspects of cardiac function, including heart rate, stroke volume and power output (reviewed by Garofalo et al. 2009b). This provides compelling evidence that NOS is expressed in Antarctic fishes and involved in regulating the cardiovascular system.

The emerging role of globin proteins in NO metabolism provides additional evidence that NO may play a role in shaping several of the cardiovascular features of icefishes (Gardner 2005). The oxygen-binding proteins Hb and Mb represent the major pathway by which NO is detoxified in organisms expressing the proteins (Gardner 2005). The oxy-form of both Hb and Mb react with NO, yielding nitrate and ferric Hb (Met-Hb) or ferric Mb (Met-Mb), which are reduced by their respective reductases (Brunori 2001; Flogel et al. 2001; Gardner 2005). One would anticipate NO levels to be elevated in organisms lacking Hb and Mb and indeed, empirical evidence supports this conjecture.

NO is an unstable free radical and thus the more stable metabolites, nitrite and nitrate, are frequently assayed as an indirect measure of NO (Nussler et al. 2002). Circulating levels of nitrite and nitrate were quantified in the blood plasma of four species of Antarctic notothenioid fishes differing in the expression of Hb: *C. aceratus* (−Hb), *C. rastrospinosus* (−Hb), *Gobionotothen gibberifrons* (+Hb), and *Notothenia coriiceps* (+Hb). Levels of nitrite plus nitrate were significantly higher in the blood plasma of the two icefishes compared to the two red-blooded species (Beers et al. 2010). Moreover, treatment of the red-blooded species, *N. coriiceps*, with the potent hemolytic agent, phenylhydrazine, dramatically reduced hematocrit and significantly increased levels

of NO circulating in the blood plasma (K.A. Borley et al., in preparation). Together, these results indicate that Hb is indeed a major pathway by which NO is degraded in notothenioid fishes. Consequently, icefishes lacking Hb have higher circulating levels of NO compared to red-blooded notothenioids. Next, we sought to determine if high levels of NO in icefishes maintain high densities of mitochondria in oxidative muscles through the process of mitochondrial biogenesis.

NO and mitochondrial biogenesis in notothenioid fishes

Mitochondrial biogenesis is complex because mitochondria contain proteins encoded in both the nuclear and mitochondrial genomes. There are an estimated 1500–2000 proteins in the mitochondrial proteome, only 13 of which are encoded in the mitochondrial genome (Taylor et al. 2003). Although small in number, these mitochondrially-encoded proteins are essential for mitochondrial function; all are components of the electron transport chain. The activity of the two genomes is coordinated by nuclear-encoded transcriptional activators and co-activators (reviewed by Hock and Kralli 2009). The best characterized of these is the co-transcriptional activator, peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1α) (Puigserver et al. 1998; Wu et al. 1999). PGC-1α binds to several transcription factors, including peroxisome proliferator-activated receptors (PPARs), estrogen-related receptors (ERRs), nuclear respiratory factor-1 and factor-2 (NRF-1 and NRF-2), Yin yang 1(YY-1), cAMP response element-binding protein (CREB), and c-Myc (reviewed by Hock and Kralli 2009). Together, these proteins transactivate the expression of nuclear-encoded genes destined for the mitochondrion, including mitochondrial transcription factor A (TFAM). Following its translation in the cytosol, TFAM is imported into the mitochondrion where it regulates the expression of mitochondrially-encoded genes involved in oxidative phosphorylation, as well as the replication of the mitochondrial genome (reviewed by Scarpulla 2008).

Previous studies have shown that NO stimulates mitochondrial biogenesis through a cGMP-dependent pathway in a variety of cell types (Nisoli et al. 2003, 2004; Wadley and McConell 2007). These studies showed that exogenous treatment with NO-donors stimulates mitochondrial biogenesis and that steady-state levels of NO maintain mitochondrial densities. Knocking out eNOS in mice reduced mitochondrial DNA (mtDNA) copy number and mRNA levels of cytochrome *c* (CYC) and subunit IV of cytochrome *c* oxidase (COXIV) in several tissues, including heart (Nisoli et al. 2003). Similarly, we hypothesized that elevated levels of NO in the blood plasma of icefishes would result in higher transcript levels of PGC-1α, NRF-1 and downstream components of the mitochondrial biogenic pathway.

We quantified the transcript abundance of several key factors involved in mitochondrial biogenesis, and surprisingly, mRNA levels of PGC-1α and NRF-1 were equivalent between hearts of red-blooded and white-blooded notothenioid fishes, despite having significantly different mitochondrial densities and circulating levels of NO (Fig. 2; Urschel and O'Brien 2008). The copy number of mtDNA and mRNA levels of citrate synthase (CS) also did not differ among these species (Urschel and O'Brien 2008). Moreover, while treatment of *N. coriiceps* with the hemolytic agent phenylhydrazine increased circulating levels of NO, it had no effect on the expression level of PGC-1α, NRF-1, or COXIV in pectoral adductor muscle (Fig. 3). At first glance, our data suggested that our original hypothesis was flawed; NO was not involved in maintaining high mitochondrial densities in hearts of icefishes. However, upon further scrutiny of the literature, we identified a major gap in our knowledge of mitochondrial biogenesis that might explain these puzzling results: All of the known activators and co-activators of mitochondrial biogenesis regulate

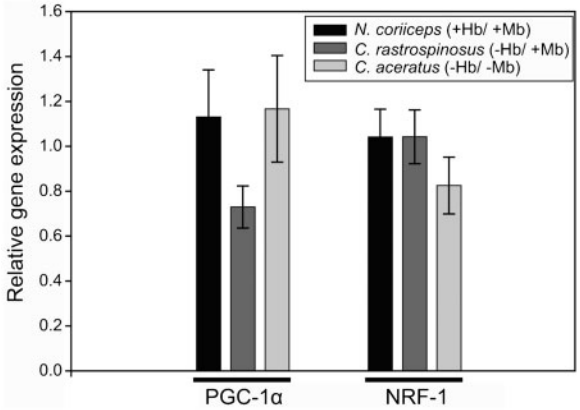

Fig. 2 Transcript levels of mitochondrial biogenic factors in hearts of notothenioid fishes differing in the expression of oxygen-binding proteins. The mRNA levels of the co-transcriptional activator PGC-1α and activator NRF-1 were quantified using quantitative real-time PCR in the heart ventricle of three species of Antarctic fishes differing in the expression of Hb and Mb. Transcript levels were normalized to levels of 18S rRNA. $N = 8$, $P > 0.05$. Modified from Urschel and O'Brien (2008).

the transcription of mitochondrial proteins and the replication of the mitochondrial genome. To date, nothing is known about how mitochondrial membrane biosynthesis is integrated into the pathway of mitochondrial biogenesis. Clearly, to build more mitochondria requires an increase in the biosynthesis of phospholipids. The prevailing wisdom in the literature is that mitochondrial biogenesis is synonymous with the assembly of respiratory chain complexes. Our data suggest otherwise. A closer examination of the architecture of mitochondria from red-blooded and white-blooded notothenioids suggests membrane biogenesis is up-regulated independently of protein synthesis and the replication of the mitochondrial genome in the oxidative muscle of icefishes.

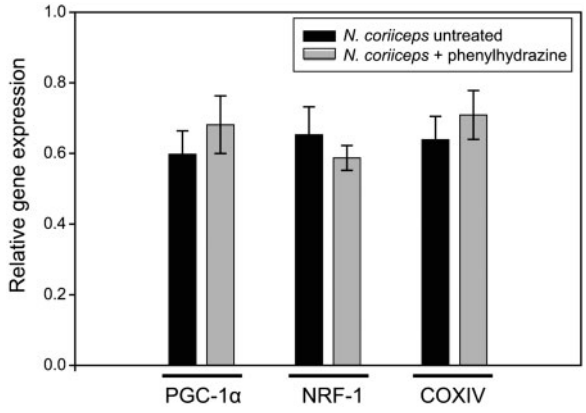

Fig. 3 Transcript levels of mitochondrial biogenic factors in the red-blooded notothenioid, *Notothenia coriiceps*, in response to treatment with phenylhydrazine. The mRNA levels of PGC-1α and NRF-1 were quantified using quantitative real-time PCR in the oxidative pectoral adductor profundus muscle of *N. coriiceps* treated or untreated with the hemolytic chemical, phenylhydrazine for 10 days. $N = 8$, $P > 0.05$. Transcript levels were normalized to the geometric mean of 18S rRNA and EF-1α mRNA levels.

Icefish mitochondria are rich in lipids

Not only do mitochondria occupy a larger fraction of myocyte volume in icefishes compared to red-blooded species, but the mitochondria are also structurally different between the two groups of fishes, slightly modifying their function (Fig. 4). Mitochondrial volume density is typically positively correlated with aerobic metabolic capacity per gram mass of tissue. The notable exception is the aerobic muscle of icefishes (O'Brien and Sidell 2000; O'Brien et al. 2003). Despite having significantly higher mitochondrial densities compared to red-blooded notothenioids, maximal activities of aerobically-poised enzymes such as CS and COX are similar, or in some cases, lower in icefishes compared to red-blooded species (Johnston and Harrison 1985; O'Brien and Sidell 2000; O'Brien et al. 2003). This apparent incongruence can be reconciled when mitochondrial ultrastructure is considered.

The mitochondria of icefish are larger than those of red-blooded species. The surface-to-volume ratio of mitochondria from the icefish *C. aceratus* is 1.9-fold lower compared to those of the red-blooded species *N. coriiceps* (Urschel and O'Brien 2008). These enlarged mitochondria of icefishes are sparsely populated with inner-mitochondrial membranes (cristae). The surface density of cristae [Sv(imm,mit)] is on average, 1.3- to 1.5-fold lower in icefishes compared to that of red-blooded species (Table 1). One exception is in the oxidative skeletal muscle of the sub-Antarctic icefish, *C. esox*, which has mitochondrial cristae surface densities similar to that of red-blooded species (Johnston et al. 1998).

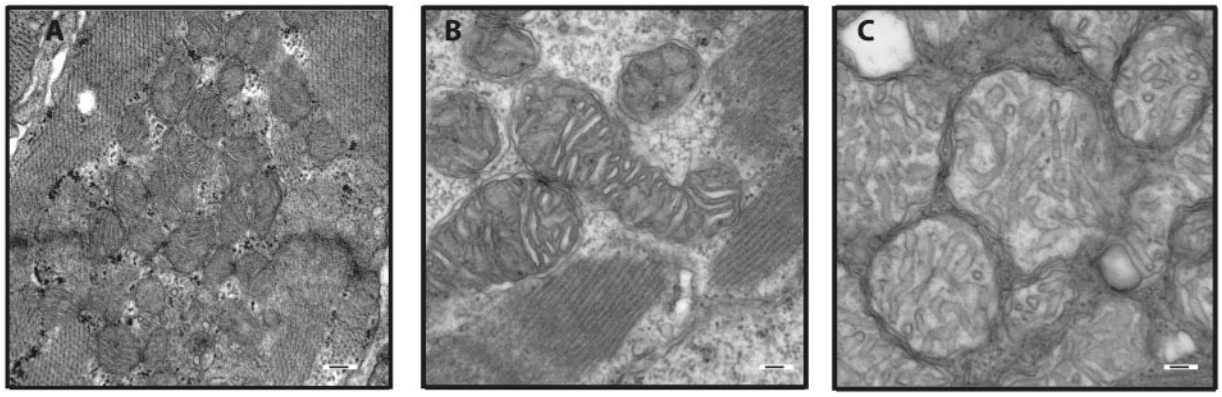

Fig. 4 Alterations in mitochondrial ultrastructure associated with the loss of Hb and Mb in notothenioid fishes. Transmission electron micrographs of mitochondria from the heart ventricles of (**A**) *N. coriiceps* (+Hb/+Mb), (**B**) *C. rastrospinosus* (−Hb/+Mb), and (**C**) *C. aceratus* (−Hb/−Mb). Samples were prepared for transmission electron microscopy as described in O'Brien and Sidell (2000). Scale bar = 124 nm.

The mitochondrial cristae surface density per g tissue [Sv(imm,m)] is a more accurate estimate of aerobic metabolic capacity per gram wet mass tissue than mitochondrial volume density because the density of components of the electron transport chain per μm^2 of cristae is nearly constant (Schwerzmann et al. 1989). Although surface densities of cristae per volume of mitochondria are lower in icefishes compared to those of red-blooded species, mitochondrial volume densities are higher. Consequently, the surface density of cristae per gram muscle tissue is nearly equivalent between red-blooded and white-blooded species (Table 1). As mentioned above, the maximal activity per gram mass of tissue of mitochondrial enzymes, such as COX, are equivalent between red-blooded and white-blooded fishes. Together, these data indicate that the density of respiratory-chain proteins per gram tissue of oxidative muscle is essentially equivalent between red-blooded and white-blooded fishes; the respiratory chains are simply spread out over a larger network of mitochondrial membranes within the myocytes of icefishes.

Analysis of the composition of the phospholipids of mitochondrial membranes supports this line of reasoning. Levels of the two major classes of mitochondrial phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), are 1.3- to 1.4-fold higher per mg mitochondrial protein in the icefish *C. aceratus* than in the red-blooded species, *N. coriiceps* (Fig. 5). In contrast, levels of the phospholipid, cardiolipin are equivalent between the two species (Fig. 5). Cardiolipin is a tetra-acyl phospholipid found almost exclusively within the inner mitochondrial membrane where it stabilizes the activity of enzymes of the electron-transport chain, as well as ATP synthase and the adenine nucleotide transporter (reviewed by Chicco and Sparagna 2007).

These data suggest two things. First, that the biosynthesis of mitochondrial membranes is up-regulated independently of the synthesis of proteins and the replication of mtDNA in the oxidative muscle of channichthyids. How this is accomplished is unclear. This is the only example of which we know that the three components of mitochondrial biogenesis are not coordinately regulated (Fig. 6). Secondly, these data indicate that mitochondrial phospholipids are not equally distributed between the two mitochondrial membranes in icefish. Rather, newly-synthesized phospholipids are predominantly targeted to the outer-mitochondrial membrane. The synthesis and/or distribution of phospholipids may be mediated by NO, thereby explaining the differences in mitochondrial density and

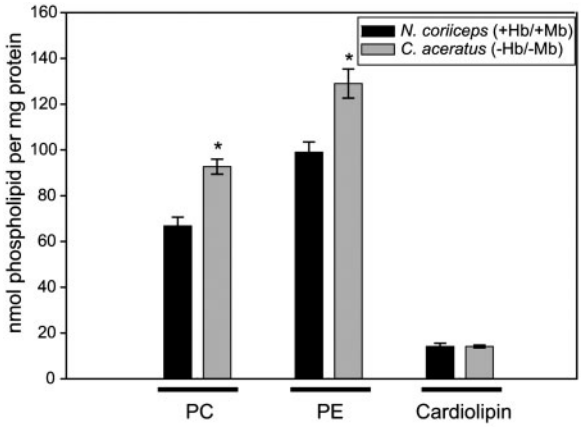

Fig. 5 Composition of mitochondrial membranes in red- and white-blooded notothenioid fishes. PC = phosphatidylcholine; PE = phosphatidylethanolamine. The asterisk indicates a significant difference in phospholipid concentration between species ($P < 0.05$). $N = 6$ for *C. aceratus* and $N = 5$ for *N. coriiceps*. Samples were analyzed as described in Han and Gross (2005) and Yang et al. (2009).

architecture between red-blooded and white-blooded notothenioids. Addressing this question will require a better understanding of both processes, and studies from mammals provide some insight.

Mitochondrial phospholipids are synthesized in the endoplasmic reticulum (ER) and mitochondria (Ellis and Reid 1994). The two most abundant mitochondrial phospholipids are PC and PE; phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin are found in lower abundance (Ellis and Reid 1994). PS, PC, and PI all must be imported from the ER, whereas PE and cardiolipin are synthesized in the mitochondrion (Voelker 1991, 2003). PS is synthesized in regions of the ER closely associated with mitochondria, called the mitochondrial-associated membranes (MAM). PS is then imported into the mitochondrial outer, and then inner membrane, where it is decarboxylated to PE (Lebiedzinska et al. 2009). Excess PE is exported from mitochondria to other cellular compartments. Recent studies suggest that the juxtaposition of donor and acceptor membranes is critical for efficient membrane transport (Lebiedzinska et al. 2009). These contact sites are likely stabilized, at least transiently, through protein–protein interactions (Voelker 2003; Lebiedzinska et al. 2009). The ratio of PE to PC is similar between mitochondria from *C. aceratus* and *N. coriiceps*, suggesting that one or more enzymes upstream of the synthesis of PE and PC are up-regulated in icefishes. More likely, one or more enzymes operating upstream of the synthesis of PE and PC are up-regulated in icefishes. A likely candidate is

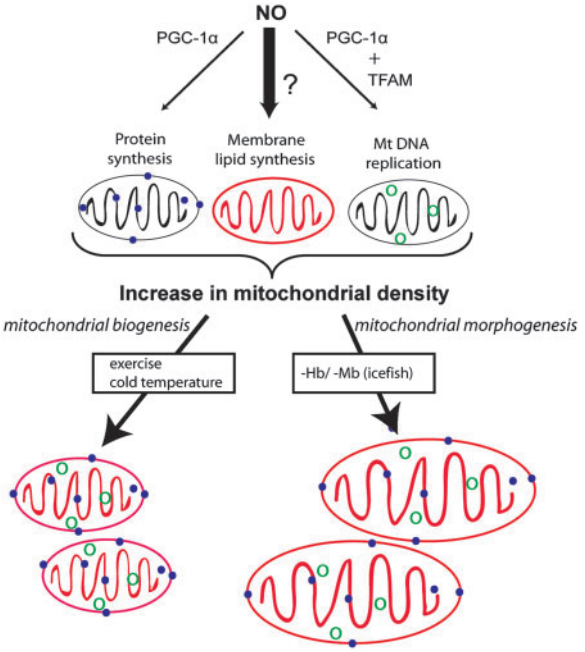

Fig. 6 Pathways that increase mitochondrial content in cells. The canonical mitochondrial biogenic pathway involves the synthesis of mitochondrial proteins (blue dots) and phospholipids, and the replication of the mitochondrial genome (green circles). In response to stimuli such as low temperature, and exercise in mammals, these three components of mitochondrial biogenesis are coordinately upregulated, resulting in an increase in mitochondrial density. In contrast, in response to the loss of Hb and Mb in the oxidative muscle of icefishes, the biosynthesis of phospholipids is increased independently of the synthesis of mitochondrial proteins and of the replication of mitochondrial DNA (mtDNA), resulting in an increase in mitochondrial size. We refer to this process as mitochondrial morphogenesis. Studies in mammals have determined that NO stimulates the canonical mitochondrial biogenic pathway, leading to an up-regulation of mitochondrial proteins regulated by PGC-1α, and replication of mtDNA regulated by PGC-1α and TFAM. Nothing is known about how the biosynthesis of mitochondrial phospholipids is integrated into the process of mitochondrial biogenesis, so it is unknown if components of this pathway might also be regulated by NO and induced by high circulating levels of NO in icefishes (dark arrow).

glycerol-3-phosphate acyl transferase (GPAT), which catalyzes the first committed and presumed rate-limiting step in glycerophospholipid biosynthesis (Kent 1995). We are currently investigating whether the activity of GPAT differs between red-blooded and white-blooded fishes and if it is regulated by NO.

Little is known about how phospholipids are distributed between the two mitochondrial membranes. Recent studies of mammals have shown that the mitochondrial isoforms of creatine kinase (MtCK) and nucleoside disphosphate kinase (NDPK-D) maintain both energy homeostasis and mitochondrial architecture (Epand et al. 2007; Schlattner et al. 2009). Both enzymes are localized to the intermembrane space where they associate with anionic phospholipids of the inner- and outer-mitochondrial membranes, stabilizing contact sites between the two membranes (Speer et al. 2005). Both MtCk and NDPK-D facilitate the transfer of lipids between liposomes *in vitro* and *in vivo* between the inner-mitochondrial and outer-mitochondrial membranes, although the exact mechanism is unknown (Epand et al. 2007). Interestingly, the cardiac muscle of knockout mice lacking mtCK display enlarged mitochondria similar in architecture to those of icefishes. The increase in mitochondrial size is thought to be important for decreasing the diffusion distance for ATP between mitochondria and myofibrils, thereby compensating for the lack of CK activity and lower levels of creatine phosphate (Kaasik et al. 2001). We are currently investigating whether differences in mtCK expression may account for differences in the distribution of mitochondrial phospholipids and in mitochondrial size between red-blooded and white-blooded fishes and if mtCK is regulated by NO.

High mitochondrial densities maintain oxygen flux

The high mitochondrial densities in the oxidative muscle of icefishes do not increase aerobic metabolic capacity but their unusual architecture provides an excellent conduit for the diffusion of oxygen and compensates for the loss of oxygen-binding proteins (reviewed in Sidell 1998).

Mb has two functions in aerobic muscle cells: It stores oxygen, buffering intracellular PO_2 levels, and it facilitates the diffusion of oxygen (reviewed by Wittenberg 1970). Both of these functions can also be accomplished by mitochondrial phospholipid membranes (reviewed in Sidell 1998). The solubility of oxygen in phospholipids is approximately four-times higher compared to its solubility in water, based on *in-vitro* measurements using vesicles composed of PC, one of the major components of mitochondrial membranes (Gennis 1989; Smotkin et al. 1991). Thus, mitochondrial membranes carry out the first function of Mb; they store substantial amounts of oxygen.

The diffusive flux of oxygen in muscle is enhanced by several characteristics of mitochondria. The rate of oxygen diffusion is described by the one-dimensional equation (Mahler et al. 1985):

$$\frac{\delta O_2}{\delta t} = DO_2 \cdot \alpha O_2 \cdot A \cdot \left(\frac{\delta PO_2}{X}\right) \quad (1)$$

where DO_2 is the diffusion coefficient for oxygen, αO_2 is the solubility constant for oxygen, A is the area through which diffusion takes place, δPO_2 is the partial pressure gradient of oxygen across the length of the diffusion path of X. The higher solubility coefficient for oxygen in lipid compared to water results in a larger αO_2 in mitochondrial-rich cells. In addition, high mitochondrial densities decrease the diffusion distance that oxygen must traverse between the capillary or ventricular lumen and the mitochondrion, reducing the value for "X" in Equation (1). Although not described by this one-dimensional equation, the architecture of membranes enhances oxygen diffusion because it constrains it to two-dimensions, rather than permitting random diffusion in three (McCabe et al. 2003). Studies have shown that the hydrocarbon core of membranes, sandwiched between the polar head groups, provides the pathway of least resistance for oxygen transport (Subczynski et al. 2009). Not only does this channel oxygen transport within the cell, but it also directs it to the primary consumer of oxygen in the cell: COX, which is located within the inner mitochondrial membrane.

The loss of both Hb and Mb results in a substantial proliferation of mitochondria that maintains oxygen flux in muscle, yet the loss of Mb alone in the pectoral adductor muscle of red-blooded notothenioids does not (Table 1). Admittedly, we do not know this for certain, given that all notothenioids sampled to date lack Mb in this muscle. The loss of Mb expression in the pectoral adductor muscle of red-blooded notothenioids may not significantly constrain oxygen flux to mitochondria, and therefore may not necessitate extensive remodeling of muscle. Hb increases oxygen-carrying capacity and maintains a steep PO_2 gradient [δPO_2 in Equation (1)] between the capillary and mitochondria. This maintains oxygen diffusion between the capillary and muscle fiber, even during periods of activity. In contrast, blood PO_2 is likely to decline during intense activity in icefishes, requiring an intracellular storehouse of oxygen, in the form of Mb or lipid that maintains oxygen delivery to mitochondria and sustains aerobic respiration. Similarly, mitochondrial densities are not altered in hearts of Mb-less mice, but several modifications in the cardiovascular system compensate for the lack of Mb, including an increase in Hb concentration (Godecke et al. 1999).

Interrelationship between mitochondrial form and function

Mitochondrial form is inextricably linked to mitochondrial function. Hackenbrock (1966) was the first to note that even under normal physiological conditions mitochondria undergo reversible, morphological changes dependent on their respiratory state. Even more compelling are the myriad of diseases characterized by altered mitochondrial form and loss of function including: Alzheimer's, Parkinson's and Huntington's diseases, cancer, and Barth and Leigh syndromes (Kurabayashi et al. 1988; Baloyannis et al. 2004; Stichel et al. 2007; Hastings et al. 2009; Kucharczyk et al. 2009; Reddy et al. 2009).

Studies in our laboratory are aimed at determining if differences in mitochondrial structure between red-blooded and white-blooded notothenioids are correlated with differences in function. To date, results indicate that the striking differences in mitochondrial morphology have little impact on function. There are minimal differences in the state III respiration rates of isolated mitochondria between red-blooded and white-blooded notothenioids (Urschel and O'Brien, 2009). At 2°C, state III respiration rates are 1.2-fold higher in the red-blooded species *G. gibberifrons*, compared to that in the icefish *C. aceratus*, but there is no difference is state III rates between *G. gibberifrons* or *C. aceratus* and the icefish *C. rastrospinosus* (Table 2). These small differences disappear at higher temperatures and there is no difference in the Arrhenius break point (the temperature at which function fails) between the three species (Urschel and O'Brien 2009). We are investigating whether other aspects of mitochondrial

Table 2 Mitochondrial state III respiration rates of notothenioid fishes

	Gobionotothen gibberifrons (+Hb/+Mb)	*Chionodraco rastrospinosus* (−Hb/+Mb)	*Chaenocephalus aceratus* (−Hb/−Mb)
State III respiration rate (nmol $O_2\,mg^{-1}$ protein min^{-1})	46.7 ± 0.9^{a}	$42.0 \pm 1.3^{a,b}$	38.5 ± 2.4^{b}

Measurements were made at 2°C in mitochondria isolated from heart ventricle by differential centrifugation. Values are presented as means ± SEM. ($N = 6$ for *G. gibberifrons* and *C. aceratus*; $N = 4$ for *C. rastrospinosus*). Letters denote significant differences among the species ($P < 0.05$). Adapted from Urschel and O'Brien (2009).

function might differ between red-blooded and white-blooded fishes, including rates of proton leak and the formation of reactive oxygen species.

Overall, the striking alterations in the mitochondrial architecture of icefishes appear solely directed at enhancing oxygen diffusion. Mitochondria are versatile organelles that govern multiple cellular activities including energy production, calcium homeostasis, thermogenesis, apoptosis, and nuclear-gene expression (Scheffler 1999). Studies from icefishes reveal that we can attribute yet two more functions to the multi-faceted mitochondrion: Storage and transport of oxygen.

The fate of icefishes as temperatures rise in the Southern Ocean

The survival of icefishes is at risk if temperatures in the Southern Ocean continue to rise. The West Antarctic Peninsula (WAP), home to over one-half of the channichthyid species, is one of the three regions in the world currently experiencing rapid warming (Iwami and Kock 1990; Vaughan et al. 2003). Since 1951, the WAP atmospheric temperatures have risen 3°C, while sea surface temperatures in summer have increased by more than 1°C, resulting in significant changes in salinity, sea ice and marine populations (Atkinson et al. 2004; Meredith and King 2005; Clarke et al. 2007). Studies have shown that Antarctic fishes are extremely stenothermic animals with little capacity to acclimate to elevations in temperature (Hofmann et al. 2000; Podrabsky and Somero 2006; Buckley and Somero 2009). The upper incipient lethal temperature of red-blooded species is ~6°C (Somero and DeVries 1967). This value is probably lower for icefishes, having a reduced oxygen-carrying capacity. Early studies by Holeton (1970) showed that while red-blooded notothenioids survive at oxygen tensions as low as 15 mmHg with little sign of distress, the icefish *C. aceratus* died at oxygen tensions below 50 mmHg.

The phospholipid-rich muscle cells of icefishes may also contribute to their reduced thermal tolerance. The mitochondrial respiratory chain is the primary source of reactive oxygen species (ROS) which when left unchecked, damage proteins, lipids, and DNA (Scandalios 2002). Many factors increase the rate of production of mitochondrial ROS, including a rise in temperature (Heise et al. 2003). Moreover, mitochondrial membranes of Antarctic fishes are rich in polyunsaturated fatty acids, which accelerate the formation of ROS through an auto-catalytic mechanism (Girotti 1985). This effect may be magnified in the mitochondrial-rich oxidative muscles of icefishes, resulting in an increased sensitivity to elevations in temperature compared to that of red-blooded notothenioids. Our current studies are aimed at testing this hypothesis. It may be that the very characteristics that make icefishes so unique also imperil their survival.

Summary

The loss of Hb and Mb has resulted in stunning increases in mitochondrial density in the oxidative muscle of icefishes, which are brought about through an unusual pathway of mitochondrial biogenesis (Fig. 6). Biosynthesis of mitochondrial phospholipids is up-regulated independently of protein and mtDNA synthesis, resulting in a lipid-rich mitochondrial reticulum that enhances the trans-cellular movement of oxygen. The only barriers to oxygen diffusion within membranes are the polar head groups and proteins. These impediments are minimized in icefishes by the proliferation of lipid-rich outer-mitochondrial membranes.

The unique architecture of icefishes' oxidative muscle and mitochondria highlights our dearth of knowledge of the biogenesis of mitochondrial membranes. Until now, the prevailing wisdom in the literature has been, "build the proteins and the lipids will follow". Icefishes expose this oversimplification. The remarkable physiology of Antarctic channichthyids has taught us much about the biology of oxygen-binding proteins during the past 55 years since their discovery. We anticipate that future studies of icefishes will yield similarly invaluable contributions to the field of mitochondrial biology.

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