

## Mitochondrial biogenesis in cold-bodied fishes

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### Summary

**Mitochondrial biogenesis is induced in response to cold temperature in many organisms. The effect is particularly pronounced in ectotherms such as fishes, where acclimation to cold temperature increases mitochondrial density. Some polar fishes also have exceptionally high densities of mitochondria. The net effect of increasing mitochondrial density is threefold. First, it increases the concentration of aerobic metabolic enzymes per gram of tissue, maintaining ATP production. Second, it elevates the density of mitochondrial membrane phospholipids, enhancing rates of intracellular oxygen diffusion. Third, it reduces the diffusion distance for oxygen and metabolites between capillaries and mitochondria. Although cold-induced mitochondrial biogenesis has been well documented in fishes, little is known about the molecular pathway governing it. In mammals, the co-transcriptional activator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 $\alpha$ ) is thought to coordinate the three components of mitochondrial biogenesis: the synthesis of mitochondrial proteins, the synthesis of phospholipids and the replication of mitochondrial DNA. Some components of the mitochondrial biogenic pathway are conserved between fishes and mammals, yet the pathway appears more versatile in fishes. In some tissues of cold-acclimated fishes, the synthesis of mitochondrial proteins increases in the absence of an increase in phospholipids, whereas in some polar fishes, densities of mitochondrial phospholipids increase in the absence of an increase in proteins. The ability of cold-bodied fishes to fine-tune the mitochondrial biogenic pathway may allow them to modify mitochondrial characteristics to meet the specific needs of the cell, whether it is to increase ATP production or enhance oxygen diffusion.**

Key words: mitochondria, fishes, aerobic metabolism.

### Introduction

Temperature has a profound effect on all levels of organismal biology, from the activity of the transcriptional and translational machinery to whole-body metabolic rates (Johnston and Dunn, 1987; Podrabsky and Somero, 2004). As ectotherms, fishes are particularly affected by changes in temperature. As temperature declines, one of the greatest challenges is maintaining the production of ATP because the rate of biochemical reactions is acutely sensitive to temperature. The ratio of reaction rates measured at temperatures 10°C apart is expressed as the  $Q_{10}$ , or temperature coefficient, which is typically between 2 and 3 when measured within a species' physiological temperature range (Hochachka and Somero, 2002). Despite the hardships imposed by cold temperature, fishes thrive in the icy-cold waters of the polar regions and even at lower latitudes many fishes endure seasonally induced decreases in temperature, sometimes as great as 20°C.

Comparative physiologists and biochemists, long intrigued by the thermal tolerance of fishes, have identified several strategies employed to maintain aerobic metabolic capacity at cold temperatures (Moerland, 1995; Somero, 2004). One of the most common strategies is to increase the concentration of aerobic metabolic enzymes per gram of tissue through an increase in transcription, translation and/or protein stability. Aerobic metabolic enzymes, including those involved in oxidative phosphorylation, the Krebs cycle and fatty acid oxidation, reside within the mitochondrion. Consequently, increases in the concentration of these enzymes often coincide with increases in the percentage of cell volume displaced by mitochondria. Increases in mitochondrial volume density have been documented in response to cold acclimation in the oxidative muscle fibers of eel (*Anguilla anguilla*),

striped bass (*Morone saxatilis*), carp (*Carassius carassius*), goldfish (*Carassius auratus*) and stickleback (*Gasterosteus aculeatus*) (Egginton and Johnston, 1984; Egginton and Sidell, 1989; Johnston and Maitland, 1980; Orczewska et al., 2010; Tyler and Sidell, 1984) (Table 1). Despite the prevalence of this phenomenon, little is known about the molecular pathway governing metabolic restructuring in response to cold acclimation in fishes. This review focuses on our current understanding of this complex process.

### Molecular regulation of mitochondrial biogenesis

In mammals, increases in aerobic metabolic capacity occur through the transcriptionally regulated process of mitochondrial biogenesis. This process is complicated because the mitochondrion houses proteins encoded in both the mitochondrial and nuclear genomes. As a result, assembling new mitochondria requires crosstalk and coordination between the two genomes. Studies in the late 1990s by Spiegelman and colleagues revealed that the PGC-1 family of co-transcriptional activators synchronizes the transcriptional activities of the two genomes, and now PGC-1 co-regulators are considered the master regulators of mitochondrial biogenesis (e.g. Puigserver et al., 1998). PGC-1 family members interact with and co-activate a handful of transcription factors that regulate the expression of nuclear-encoded genes destined for the mitochondrion. These transcription factors include two families of nuclear receptors, peroxisome proliferator-activated receptors (PPARs) and estrogen-related receptors (ERRs); nuclear respiratory factor-1 and nuclear respiratory 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). PGC-1 family members enhance gene expression by recruiting histone deacetylases and the Mediator

complex to the transcriptional start site. PGC-1 co-regulators also interface with the environment; their activity is rapidly increased by post-translational modifications including phosphorylation by AMP kinase and MAP kinase (Jager et al., 2007), methylation by protein arginine methyltransferase 1 (Teyssier et al., 2005) and deacetylation by sirtuin 1 (Gerhart-Hines et al., 2007). The PGC-1 family of proteins controls the activity of the mitochondrial genome by regulating the expression of nuclear-encoded genes that govern mitochondrial DNA transcription and translation. These include mitochondrial transcription factor A (TFAM), two homologs of mitochondrial transcription factor B (TFB1M and TFB2M), mitochondrial DNA-directed RNA polymerase (POLRMT), mtDNA polymerase  $\gamma$  (POL  $\gamma$ ) and mitochondrial helicase (TWINKLE) (reviewed by Falkenberg et al., 2007). NRF-1 and NRF-2 binding sites have been identified within the upstream regulatory regions of most of these genes and PGC-1 $\alpha$  is a well-known regulator of TFAM (reviewed by Scarpulla, 2008).

It is unknown if all elements of the mitochondrial biogenic pathway are conserved between fishes and mammals, nor is it known what stimulates aerobic metabolic reprogramming in response to cold acclimation in fishes. In mammals, mitochondrial biogenesis is induced in response to a change in the energy status of the cell, relayed through multiple signaling molecules including AMP-activated protein kinase and sirtuin-1 (Jager et al., 2007; Lagouge et al., 2006). Calcium released through muscle contraction also stimulates mitochondrial biogenesis (Wu et al., 2002), and mitochondrial biogenesis is induced when mitochondrial function is disrupted by oxidative stress, nitric oxide or carbon monoxide (Kang et al., 2009; Nisoli et al., 2003; Suliman et al., 2007). The thread uniting all of these stimuli is that they lead to an increased demand for energy production. Cold acclimation also elevates the demand for ATP in fishes because energy production declines. However, the effects of cold temperature on fishes are more pervasive compared with the metabolic stresses experienced by mammals. Cold temperature not only compromises the ability to produce ATP, but also reduces membrane fluidity, depresses the rate of protein synthesis and degradation, and decreases the diffusion

rate of oxygen and metabolites (Desaulniers et al., 1996; Hazel, 1984; Sidell, 1977). All of these have the potential to directly or indirectly impact metabolism and metabolic remodeling. Studies in our laboratory and in others have revealed two differences between how metabolic remodeling is regulated in fishes, responding to cold acclimation, and in mammals, responding to an increased demand for ATP. First, not all changes in aerobic metabolic capacity are transcriptionally regulated in fishes (Battersby and Moyes, 1998; Lucassen et al., 2003; Lucassen et al., 2006; Orczewska et al., 2010; Sidell, 1977). Second, increases in aerobic metabolic capacity do not always coincide with mitochondrial biogenesis in fishes (Orczewska et al., 2010).

#### Metabolic remodeling in fishes in response to cold acclimation

We are investigating the molecular basis of aerobic metabolic remodeling using the threespine stickleback, *Gasterosteus aculeatus* L. Sticklebacks are eurythermic fishes, widely distributed throughout the Northern Hemisphere where they inhabit both freshwater and marine environments (Wooten, 1984). They are the only eurythermic fish whose genome has been sequenced, making molecular biological studies more straightforward compared with other fishes commonly used for studies of cold acclimation, e.g. killifish (*Fundulus heteroclitus*) and goldfish (*Carassius auratus*).

Measurements of the maximal activity of aerobically poised enzymes are frequently used to evaluate changes in aerobic metabolic capacity during cold acclimation in fishes. We measured the maximal activity of citrate synthase (CS), a key enzyme in the Krebs cycle, and cytochrome *c* oxidase (COX), the terminal electron acceptor in the electron transport chain, in the oxidative pectoral adductor muscle and liver of sticklebacks during cold acclimation (Orczewska et al., 2010). Similar to other studies of cold acclimation in fishes, we determined that the activity of CS and COX increased during cold acclimation in liver and oxidative muscle (Table 1, Figs 1, 2). Notably, the time frame in which these changes occurred differed for each enzyme and tissue, suggesting different modes of regulation.

Table 1. Aerobic metabolic remodeling in response to cold acclimation in fishes

Species	Acclimation regime	Mitochondrial volume density (C:W)	COX activity (C:W)	CS activity (C:W)	Reference
<b>Oxidative muscle</b>					
<i>Carassius carassius</i>	28°C → 2°C	2.13	ND	ND	Johnston and Maitland, 1980
<i>Carassius auratus</i>	25°C → 5°C	3.59 <sup>a</sup>	2.83 <sup>b</sup>	ND	<sup>a</sup> Tyler and Sidell, 1984; <sup>b</sup> Freed, 1965 (in muscle)
<i>Morone saxatilis</i>	25°C → 5°C	1.6 <sup>a</sup>	1.97 <sup>b</sup>	1.57 <sup>b</sup>	<sup>a</sup> Egginton and Sidell, 1989; <sup>b</sup> Jones and Sidell, 1982
<i>Gasterosteus aculeatus</i>	20°C → 8°C	1.9	1.86	1.92	Orczewska et al., 2010
<i>Anguilla anguilla</i>	29°C → 10°C	1.4			Egginton and Johnston, 1984
<i>Cyprinus carpio</i>	23°C → 7°C	ND	1.58	2.25	Johnston et al., 1985
<i>Esox niger</i>	25°C → 5°C	ND	1.63	1.59	Kleckner and Sidell, 1985
<i>Oncorhynchus mykiss</i>	18°C → 4°C	ND	1.37	1.40	Battersby and Moyes, 1998
<b>Liver</b>					
<i>Blennius pholis</i>	20°C → 10°C	1.71*	ND	ND	Campbell and Davies, 1978
<i>Fundulus heteroclitus</i>	25°C → 5°C	ND	NS (N)	1.50 (N)	Fangue et al., 2009
			NS (S)	1.25 (S)	
<i>Esox niger</i>	25°C → 5°C	ND	NS	1.46	Kleckner and Sidell, 1985
<i>Gasterosteus aculeatus</i>	20°C → 8°C	NS	1.49	2.02	Orczewska et al., 2010
<i>Lepomis cyanellus</i>	25°C → 5°C	ND	3.89	ND	Shaklee et al., 1977
<i>Zoarces viviparus</i>	18°C → 0°C	ND	NS	ND	Hardewig et al., 1999
	10°C → 3.5°C	ND	NS	1.86	Lucassen et al., 2003

\*Mitochondrial number per 1000  $\mu\text{m}^2$  liver.

C:W: ratio of measurement in cold-acclimated fishes:warm-acclimated fishes; (N): northern population of *F. heteroclitus*; ND: no data available; NS: not significantly different ( $P > 0.05$ ); (S): southern population of *F. heteroclitus*.

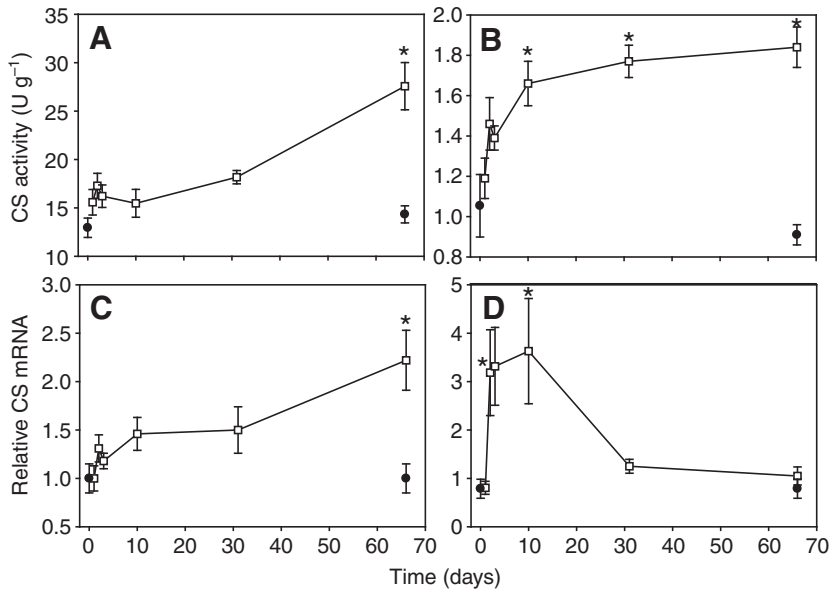


Fig. 1. Changes in the activity and mRNA level of citrate synthase (CS) in threespine sticklebacks during cold acclimation. Fish were held at 20°C for 12 weeks and then acclimated to cold temperature (8°C) by decreasing the temperature over 3 days. Cold-acclimated fish (open squares) were then held at 8°C for 9 weeks. Warm-acclimated fish (closed circles) were maintained at 20°C and harvested at the start and end of the experimental period. The maximal activity of CS was measured at 10°C in oxidative muscle (A) and liver tissue (B) during cold acclimation. Transcript levels of CS were quantified in oxidative muscle (C) and liver (D) using quantitative real-time PCR. mRNA levels were normalized to elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) in oxidative muscle and 18S rRNA in liver. Values are presented as means  $\pm$  s.e.m.;  $N=4-6$  for measurements of enzyme activity and  $N=5-8$  for measurements of transcript levels. Asterisks indicate a significant difference between warm- and cold-acclimated fish ( $P<0.05$ ). Adapted from Orczewska et al. (Orczewska et al., 2010).

The activity of CS increased at day 66 of cold acclimation in oxidative muscle and by day 10 in liver (Fig. 1A,B). We measured the mRNA levels of CS to determine whether increases in enzyme activity were driven by increases in gene transcription. In oxidative muscle, increases in mRNA levels of CS coincided with increases in CS activity; in liver, mRNA levels increased 8 days prior to an increase in CS activity, suggesting that the increase in activity of CS was transcriptionally regulated in both tissues (Fig. 1C,D). CS mRNA levels decreased by day 31 of cold acclimation in liver, yet CS activity remained elevated, suggesting that CS activity is maintained by post-transcriptional mechanisms. A similar trend was observed in livers of cold-acclimated eelpout (*Zoarces viviparus*), where mRNA levels of CS initially increased on day 4 of cold acclimation and decreased on day 7, yet the maximal activity of CS activity did not become significantly elevated until day 9 and remained elevated throughout the remainder of 25-day cold acclimation period (Lucassen et al., 2003). The activity of CS may be maintained during cold acclimation by a decrease in the turnover rate of the enzyme. Sidell showed that levels of cytochrome *c* increased in the skeletal muscle of green sunfish (*Lepomis cyanellus*) in response to cold acclimation because of a decrease in the rate of protein degradation (Sidell, 1977). Protein synthesis was also depressed at cold temperature, but the rate of protein degradation was reduced to a greater extent, resulting in a net increase in the concentration of protein. Similar mechanisms may be an important for maintaining CS activity during cold acclimation after mRNA levels have declined.

The time course for changes in COX activity differed from that of CS during cold acclimation of sticklebacks. The activity of COX increased by day 3 of cold acclimation in oxidative muscle, but in liver it did not become significantly elevated until day 66 (Fig. 2A,B). The activity of COX increased in both tissues by non-transcriptional mechanisms. Transcript levels of the nuclear-encoded subunit COXIV did not increase until day 66 of cold acclimation in oxidative muscle. This was 9 weeks after the activity of COX increased (Fig. 2A,C), and in liver, there was no change in transcript levels of COXIV (Fig. 2B,D). Similar results were found in arctic cod (*Gadus morhua*), where cold acclimation led to an increase in the activity of COX in glycolytic muscle but no change in transcript levels of COXII (Lucassen et al., 2006).

Also, the activity of COX increased in both oxidative and glycolytic muscles of rainbow trout (*Oncorhynchus mykiss*) during cold acclimation, whereas mRNA levels of COXI remained constant (Battersby and Moyes, 1998).

Alterations in the lipid milieu of COX may lead to increases in COX activity in the absence of increases in gene transcription. Membrane structure is acutely affected by temperature (reviewed in Hazel, 1984). Decreases in temperature restrict the mobility of fatty acyl chains, resulting in a decrease in membrane fluidity. Membrane function is maintained at cold temperatures by modifying membrane composition, typically by increasing the proportion of polyunsaturated fatty acids relative to saturated fatty acids and increasing levels of phosphatidylethanolamine (PE) relative to phosphatidylcholine (PC) (Hazel, 1984). Membrane remodeling is initiated rapidly in response to cold acclimation and affects the activity of membrane-associated metabolic enzymes. Caldwell first reported in 1969 that the activity of COX, succinate dehydrogenase and NADH dehydrogenase increased in gills of goldfish in response to cold acclimation, independently of an increase in cytochrome concentration but correlated with changes in the composition of mitochondrial membranes (Caldwell, 1969). Similar results were found in the oxidative muscle of carp (Wodtke, 1981a; Wodtke, 1981b), where COX activity increased in response to cold acclimation, independently of an increase in cytochrome levels, but correlated with an increase in degree of unsaturation of phosphatidylcholine in mitochondrial membranes. In goldfish, the activity of succinate dehydrogenase was higher in phospholipids extracted from mitochondria of cold-acclimated fish compared with warm-acclimated ones (Hazel, 1972). Changes in membrane composition occur very rapidly in response to cold acclimation, some within the first 24 h of cold acclimation, making it a probable mechanism by which COX activity rapidly increases in fishes during cold acclimation (Hazel and Landrey, 1988; Trueman et al., 2000; Williams and Hazel, 1995).

Membrane remodeling may also drive qualitative changes in mitochondrial function during cold acclimation. Mitochondria isolated from the short-horned sculpin (*Myoxocephalus scorpius*) acclimated to 5°C displayed higher state III respiration rates per mg mitochondrial protein compared with mitochondria from fish acclimated to 15°C at all assay temperatures between 2.5 and 20°C

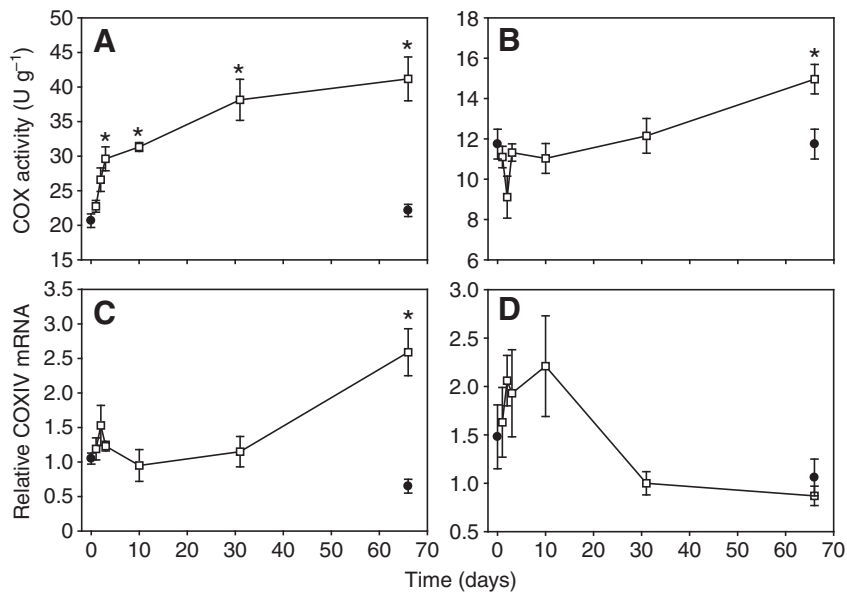


Fig. 2. Changes in the activity and mRNA level of cytochrome *c* oxidase (COX) in threespine sticklebacks during cold acclimation. Details as in Fig. 1.

(Guderley and Johnston, 1996). Seasonal acclimatization of rainbow trout (*Oncorhynchus mykiss*) to winter temperatures also resulted in an increase in state III respiration rates in isolated mitochondria, which was correlated with changes in mitochondrial membrane composition (Guderley et al., 1997). Additionally, both acclimation and acclimatization of trout to cold temperatures altered ADP affinity of isolated mitochondria (Guderley and St Pierre, 1999; Kraffe et al., 2007). In general, ADP affinity of mitochondria isolated from red muscle decreased as seasonal temperatures decreased (Guderley and St Pierre, 1999), and the ADP affinity of mitochondria from cold-acclimated trout was lower compared with warm-acclimated trout when measured at a common temperature (Kraffe et al., 2007). These changes have been attributed to alterations in mitochondrial membrane composition, affecting both the activity of enzymes involved in oxidative phosphorylation and the adenine nucleotide transporter (Bouchard and Guderley, 2003; Guderley et al., 1997; Guderley and St Pierre, 1999; Kraffe et al., 2007).

One notable difference between the response of liver and oxidative muscle to cold acclimation in sticklebacks was that increases in aerobic metabolic capacity coincided with mitochondrial biogenesis in oxidative muscle but not liver (Orzewska et al., 2010). Less is known about ultrastructural changes in liver in response to cold acclimation compared with those in muscle. Only one other study (Campbell and Davies, 1978) has quantified changes in mitochondrial mass in liver in response to cold acclimation in fishes. In that study, it was determined that cold acclimation of blennies (*Blennius pholis*) to 10°C resulted in a 1.7-fold increase in the number of mitochondria per  $\mu\text{m}^2$  of liver compared with fish held at 20°C (Table 1). All other studies of cold acclimation in liver have reported an increase in the maximal activity of CS as a metric for changes in aerobic metabolic capacity and, in most cases, the activity of CS increased in the absence of an increase in COX activity, or to a greater extent than COX activity (Table 1). By contrast, increases in mitochondrial density and in the activity of COX and CS have been well documented in the oxidative muscle of a large number of fish species (Table 1). These results elicit two questions: how are the differing responses of oxidative muscle and liver regulated at the molecular level, and why does cold acclimation trigger different responses between these two tissues?

Few studies have evaluated the potential role of PGC-1 family members in regulating increases in aerobic metabolic capacity in response to cold acclimation in fishes. Transcript levels of PGC-1 $\alpha$  were unchanged during cold acclimation in the oxidative muscle, glycolytic muscle and liver of goldfish, glycolytic muscle of zebrafish, and oxidative muscle and liver of sticklebacks (LeMoine et al., 2008; McClelland et al., 2006; Orzewska et al., 2010). However, mRNA levels of PGC-1 $\beta$  increased in the liver and oxidative muscle of goldfish and in the liver of sticklebacks in response to cold acclimation (LeMoine et al., 2008; Orzewska et al., 2010). Also, mRNA levels of NRF-1 increased in the liver and muscles of zebrafish, goldfish and sticklebacks in response to cold acclimation (LeMoine et al., 2008; McClelland et al., 2006; Orzewska et al., 2010). Together, these results indicate that NRF-1 probably regulates increases in aerobic metabolic capacity in response to cold acclimation in fishes and may be co-activated by PGC-1 $\beta$  in liver. The ability to detect an increase in PGC-1 $\alpha$  may be hampered by the high degree of variability in the expression of this gene. Alternatively, the activity or protein level of PGC-1 $\alpha$  may increase during cold acclimation. The activity of PGC-1 $\alpha$  is regulated by several post-translational modifications (see above), as well as by localization to the nucleus (Wright et al., 2007). Further studies will be required to address this question.

The differing response to cold acclimation between liver and oxidative muscle may be due to their differing metabolic demands. Anabolic processes, requiring intermediates of the Krebs cycle, may increase in the liver during cold acclimation. For example, increases in the activity of CS in liver have been attributed to increased lipid biosynthesis (Lucassen et al., 2003). Alternatively, these differences may be due to differences in cell architecture, considered within the context of the benefits of mitochondrial biogenesis.

#### Versatility of mitochondrial biogenesis in fishes

Mitochondrial biogenesis imparts three benefits at cold temperatures. First, increasing mitochondrial volume density increases the concentration of aerobic metabolic enzymes per gram of tissue, compensating for the  $Q_{10}$  effect. Second, expanding mitochondrial populations increases the density of intracellular lipids, enhancing the diffusion rate of oxygen because oxygen is approximately four times more soluble in lipid than water (Battino et al., 1968; Smotkin



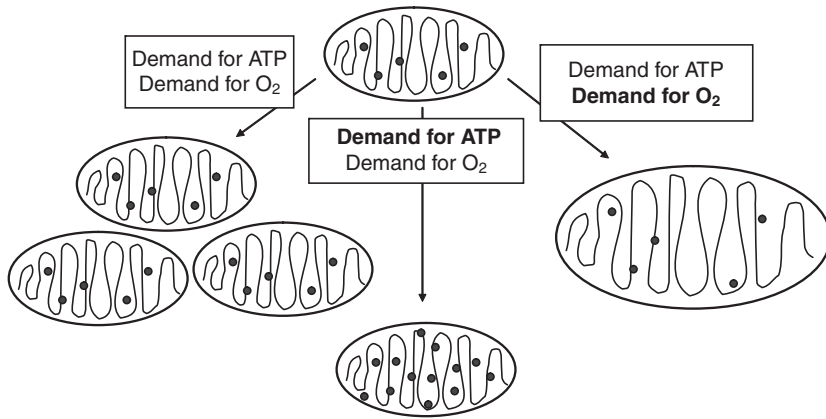


Fig. 3. Multiple pathways of mitochondrial biogenesis. The synthesis of mitochondrial proteins and phospholipids is coordinately upregulated when cells require both an increase in the production of ATP and an increase in the diffusion rate of oxygen (and/or metabolites) (left). In some cases, cells may only need to increase production of ATP, resulting in an increase in protein synthesis but not phospholipids (center). Alternatively, only oxygen flux may need to increase and not the production of ATP, resulting in an increase in the synthesis of mitochondrial phospholipids but not proteins (right).

et al., 1991). Third, increasing mitochondrial densities decreases the diffusion distance that oxygen and metabolites must traverse between the capillary and mitochondrion.

These benefits of mitochondrial biogenesis are brought about by different components of the mitochondria biogenic pathway. ATP production is maintained by an increase in protein synthesis, whereas oxygen diffusion is enhanced and diffusion distances are decreased by the proliferation of mitochondrial membranes. Each of these components of mitochondrial biogenesis may be regulated independently, depending on the demands of the cell, which in turn may be influenced by cellular architecture.

Two differences in cell structure between oxidative muscle fibers and hepatocytes of sticklebacks are size and lipid density. The cross-sectional area of oxidative muscle fibers is 4.5 times greater than that of hepatocytes, and neutral lipid droplets occupy <1% of the volume of oxidative muscle fibers compared with 59% of the volume of hepatocytes (Orzewska et al., 2010). During cold acclimation, both the supply of ATP and oxygen diffusion may be constrained in oxidative muscle fibers, requiring an upregulation of both protein and lipid biosynthesis (Fig. 3). In liver, cold temperatures may increase the demand for ATP, requiring an increase in the synthesis of aerobic enzymes, but the small size and high lipid density of hepatocytes may maintain oxygen diffusion in the cold, circumventing the need to proliferate mitochondrial membranes during mitochondrial biogenesis (Fig. 3). Under some circumstances, oxygen diffusion may be limited but not the supply of ATP, requiring an upregulation of lipid but not protein synthesis. Studies in polar fishes provide an example of this latter pathway (Fig. 3).

#### High mitochondrial densities are a characteristic of Antarctic icefishes

The oxidative muscles of Antarctic channichthyid icefishes (suborder Notothenioidei) possess extraordinarily high densities of mitochondria. Mitochondria occupy over 50% of myocyte volume in some species (reviewed by O'Brien and Mueller, 2010). This is a remarkable trait, considering that these animals are demersal,

sedentary or moderately active fishes, known for their lack of hemoglobin (Hb) expression (Ruud, 1954). There are 16 members of the icefish family and six of these species also lack the intracellular oxygen-binding protein, myoglobin (Mb), in the ventricle of their heart (Moylan and Sidell, 2000; Sidell et al., 1997). There is an interesting relationship between the expression of oxygen-binding proteins and mitochondrial content in ventricular myocytes (Fig. 4) (O'Brien and Sidell, 2000). The mitochondrial volume density of cardiomyocytes from red-blooded nototheniid species expressing both Hb and Mb is 16–25% (Johnston and Harrison, 1987; O'Brien and Sidell, 2000). Mitochondrial densities are at the higher end of this range in hearts of icefishes expressing Mb (20–24%) and are highest in hearts of icefishes lacking both Hb and Mb (37%) (Feller et al., 1985; O'Brien and Sidell, 2000).

Typically, the percentage of cell volume displaced by mitochondria is positively correlated with aerobic metabolic capacity and the activity of CS and COX per gram of tissue. Icefishes are one exception to this paradigm. The maximal activities of CS and COX per gram of tissue are equivalent among hearts of nototheniid fishes differing in the expression of oxygen-binding proteins and in mitochondrial density (O'Brien and Sidell, 2000). Although mitochondrial volume density is higher in hearts of icefishes compared with red-blooded fishes, the surface density of inner-mitochondrial membranes (cristae) is lower (O'Brien and Sidell, 2000). The net effect is that the cristae surface density per gram of ventricular tissue is essentially equivalent between red- and white-blooded fishes, but components of the respiratory chain are spread out over a larger network of mitochondrial membranes within hearts of icefishes. Consistent with this, we find higher levels of the two most abundant mitochondrial phospholipids, PE and PC, in mitochondria from *Chaenocephalus aceratus* (–Hb/–Mb) compared with those from the red-blooded nototheniid *Notothenia coriiceps* (Table 2). Rather than increasing aerobic metabolic capacity, high mitochondrial densities in hearts of icefishes lacking Mb are likely crucial for maintaining oxygen diffusion because as described above, the phospholipid bilayer provides an excellent conduit for the diffusion of oxygen (Sidell, 1998). Together, these data suggest

Table 2. Phospholipid composition of mitochondrial membranes from red- and white-blooded nototheniid fishes

Species	PE (nmol mg <sup>-1</sup> protein)	PC (nmol mg <sup>-1</sup> protein)	CL (nmol mg <sup>-1</sup> protein)
<i>Notothenia coriiceps</i> (+Hb/+Mb)	98.93±4.56	66.80±3.86	14.17±1.33
<i>Chaenocephalus aceratus</i> (–Hb/–Mb)	129.07±6.39*	92.68±3.29*	14.09±0.56

CL, cardiolipin; Hb, hemoglobin; Mb, myoglobin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; \*, significant difference between the two species ( $P < 0.05$ ).

Adapted from O'Brien and Mueller (O'Brien and Mueller, 2010).

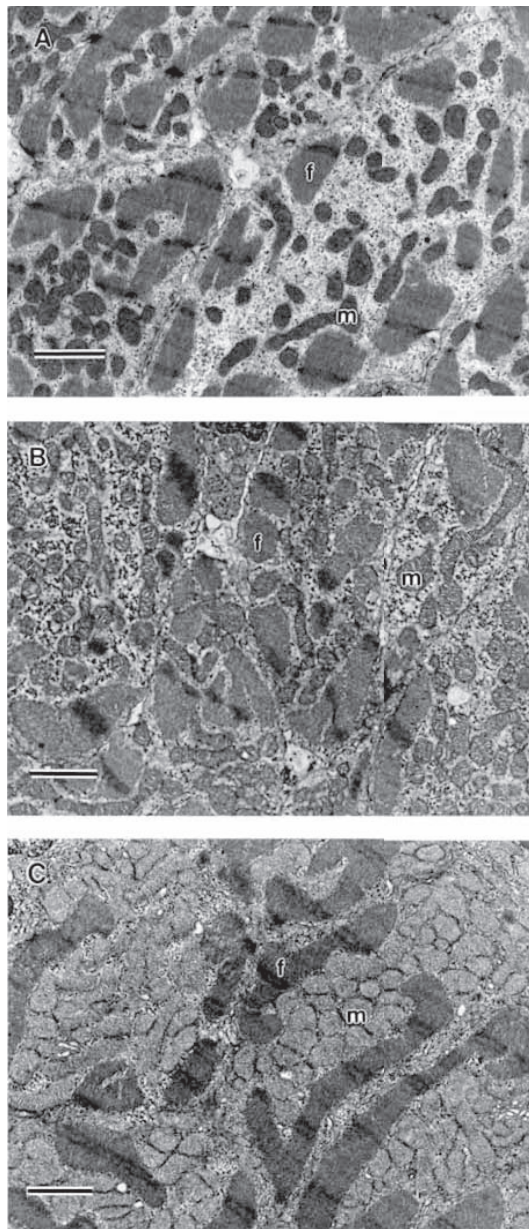


Fig. 4. Mitochondrial volume density varies with expression of oxygen-binding proteins in Antarctic fishes. Mitochondria occupy (A) 16% of cardiac myocyte volume in the red-blooded notothenioid *Gobionotothen gibberifrons* (+Hb/+Mb), (B) 20% of myocyte volume in the red-hearted icefish *Chionodraco rastrospinosus* (–Hb/+Mb) and (C) 37% of myocyte volume in the icefish *Chaenocephalus aceratus* (–Hb/–Mb). f, myofibrils; m, mitochondrion. Scale bars: 2  $\mu$ m. Figure from O'Brien and Sidell (O'Brien and Sidell, 2000).

that mitochondrial phospholipid biosynthesis is upregulated independently of the synthesis of mitochondrial proteins in the hearts of icefishes. How this is regulated at the molecular level is unknown; in general, little is known about how the synthesis of phospholipids is integrated into mitochondrial biogenesis.

#### The synthesis of mitochondrial membranes must increase during mitochondrial biogenesis

Studies in our laboratory indicate that PGC-1 $\alpha$  and NRF-1 do not directly regulate the synthesis of PC and PE. The mRNA levels of

both PGC-1 $\alpha$  and NRF-1 are equivalent between hearts of red- and white-blooded notothenioids despite their differing levels of PC and PE (Urschel and O'Brien, 2008). This is somewhat surprising given that PGC-1 family members are considered the master regulators of mitochondrial biogenesis and suggests that other proteins, lying downstream of PGC-1, likely regulate the synthesis of phospholipids destined for the mitochondrion.

Interestingly, levels of cardiolipin (CL) per mg mitochondrial protein are equivalent between mitochondria from *C. aceratus* and *N. coriiceps* (Table 2). CL is a tetra-acyl phospholipid localized predominantly to the inner-mitochondrial membrane and to contact points between the inner- and outer-mitochondrial membranes (Ardail et al., 1990; Chicco and Sparagna, 2007). The constant proportion of CL:COX between red- and white-blooded fishes suggests that the synthesis of inner-mitochondrial membrane proteins and lipids is coordinately regulated, possibly by PGC-1 $\alpha$ . Recent studies in yeast have identified translocator and maintenance protein 41 (Tam41) and prohibitin as potential candidates for coordinating the assembly of inner-mitochondrial membrane proteins and phospholipids (Kutik et al., 2008; Osman et al., 2009a).

PC and PE comprise the majority of phospholipids within mitochondrial membranes. Phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA) and CL are found in lower abundance, although the inner-mitochondrial membrane is rich in CL (Zambrano et al., 1975). PC, PI, PS and PA are synthesized and imported from the endoplasmic reticulum (ER) whereas PE, PG and CL are synthesized 'in house', within the mitochondrion (Fig. 5) (Daum and Vance, 1997). PS is imported into the mitochondrion and is decarboxylated to form PE. PA is also imported and serves as the precursor for the synthesis of PG and CL. In yeast, the mitochondrial protein Tam41 regulates the synthesis of PG, CL and the assembly of protein complexes localized to the inner-mitochondrial membrane (Kutik et al., 2008). Knocking out Tam41 resulted in a depletion of both CL and PG, and an increase in PA, the precursor for synthesizing both of these lipids. This suggests that Tam41 regulates the expression of CDP-diacylglycerol synthase (CDS), one of the enzymes involved in synthesizing PG and CL from PA. Lack of Tam41 also resulted in the loss of mitochondrial membrane potential, and a decline in protein import and assembly of supercomplexes of the respiratory chain, likely due to the loss of CL.

Studies in yeast have shown that prohibitin-1 (Phb1) and prohibitin-2 (Phb2) are also involved in regulating the synthesis of PE and CL (Osman et al., 2009a). Prohibitins are highly conserved proteins found in the cytosol and nucleus, as well as at the inner-mitochondrial membrane, where they form large, ringed structures of  $\sim$ 1.2 MDa (Artal-Sanz and Tavernarakis, 2009). Prohibitins regulate several processes within mitochondria, including mitochondrial protein degradation, stability of the mitochondrial genome and cristae ultrastructure (Osman et al., 2009b). Osman et al. identified 35 genetic interactors of prohibitins (GEPs), which were lethal when deleted from prohibitin-deficient cells (Osman et al., 2009a). The majority of these proteins are involved in regulating the assembly of the respiratory chain and mitochondrial morphology. Notably, some are involved in the synthesis of phospholipids, including cardiolipin synthase (CLS) and phosphatidylserine decarboxylase (PSD), which decarboxylates PS to form PE. Another GEP, Ups1, was determined to regulate CL levels, and GEP1 was determined to be required for the stability of PE.



PGC-1 $\alpha$  might regulate the expression or activity of Tam41 and/or prohibitin, explaining how the biogenesis of the inner-mitochondrial membrane is integrated into the assembly of new mitochondria. However, we still lack a clear understanding of how phospholipid biosynthesis within the ER is regulated and integrated into mitochondrial biogenesis (Fig. 5). The synthesis of PA is one potential site of regulation as it is required to synthesize CDP-diacylglycerol (CDP-DAG), which is the precursor for synthesizing all mitochondrial phospholipids (Fig. 5). PA is synthesized from glycerol-3 phosphate by two sequential reactions catalyzed by glycerol-3-phosphate acyltransferase (GPAT) and 1-acylglycerol-3-phosphate acyltransferase (AGAT), respectively (reviewed by Daum and Vance, 1997). The first reaction, catalyzed by GPAT, is considered to be the rate-limiting step in the synthesis of phospholipids, and may be the enzyme upregulated in the icefishes (Dircks and Sul, 1997). The regulation of expression and/or activity of GPAT are likely integrated into the mitochondrial biogenic pathway to increase the density of mitochondrial membranes, yet we still have much to learn about this important aspect of mitochondrial biogenesis.

#### Signaling molecules that might stimulate changes in mitochondria in cold-bodied fishes

The trigger stimulating mitochondrial biogenesis in fishes has not yet been identified but, given its flexibility (Fig. 3), there are likely multiple signaling pathways involved. I'll briefly discuss the potential role of five candidates: sirtuins, AMP kinase, thyroid hormone, reactive oxygen species (ROS) and nitric oxide.

In mammals, mitochondrial biogenesis is most often triggered by an energy deficit, which is relayed by the activity of sirtuins (SIRT) or AMP kinase (AMPK). SIRT1 is an NAD<sup>+</sup>-dependent deacetylase, which deacetylates lysine residues within PGC-1 $\alpha$ , increasing its activity in response to caloric restriction (Nemoto et al., 2005; Rodgers et al., 2008). AMPK is another cellular energy sensor, sensitive to the ratio of AMP:ATP. As AMP levels increase during endurance exercise or caloric restriction, the activity of AMPK increases, stimulating mitochondrial biogenesis (de Lange et al., 2007; Zong et al., 2002). There are multiple targets of AMPK, including PGC-1 $\alpha$  (Jager et al., 2007). AMPK also increases NAD<sup>+</sup> levels, activating SIRT1, suggesting a convergence of these two pathways (Canto et al., 2009). It is conceivable that a deficiency in energy production triggers changes in mitochondria in response to cold acclimation, particularly in oxidative muscle where mitochondrial biogenesis occurs. If so, our data would suggest that such a deficit does not occur until between weeks 4 and 9 of cold acclimation in sticklebacks, when the expression of aerobic metabolic genes and NRF-1 are induced (Orczewska et al., 2010). Prior to that, ATP demand may be met by non-genomic increases in the activity of aerobic metabolic enzymes.

The thyroid hormones (THs) 3',3,5-triiodo-L-triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) regulate growth and development in both endotherms and ectotherms, including fishes, and TH has long been known to stimulate metabolism in mammals (Goglia et al., 2002; Oetting and Yen, 2007; Power et al., 2001). THs increase aerobic metabolic capacity through both non-genomic and genomic mechanisms. T<sub>3</sub> stimulates mitochondrial biogenesis by binding to TH receptors, inducing the expression of both mitochondrial- and nuclear-encoded aerobic metabolic genes (Fernandez-Vizarra et al., 2008; Sheehan et al., 2004). Studies suggest that this is mediated by an increase in the activity of PGC-1 $\alpha$  (Irrcher et al., 2003; Irrcher et al., 2008). A third TH, 3,5-diiodo-L-thyronine (T<sub>2</sub>), increases the activity of COX by binding to subunit Va and relieving inhibition

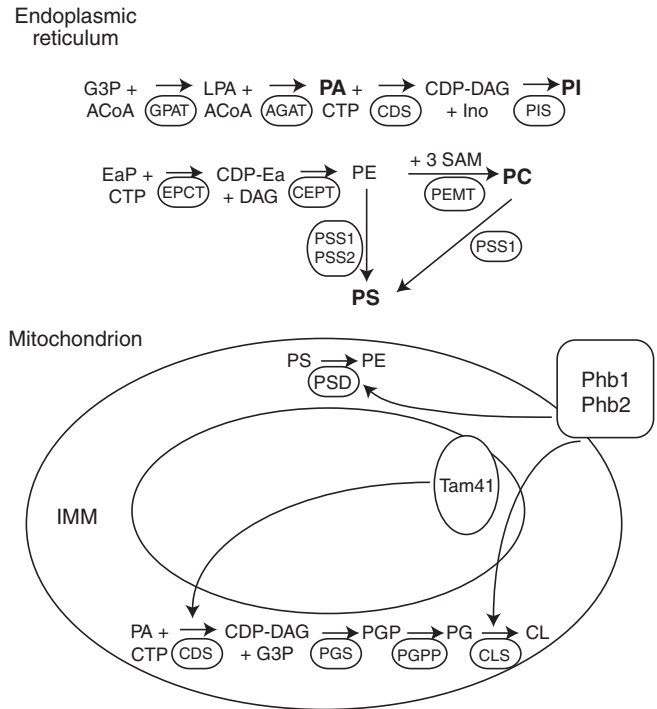


Fig. 5. Synthesis and regulation of phospholipids that reside in the mitochondrion. PA, PI, PC and PS are synthesized in the endoplasmic reticulum (ER) and must be imported to the mitochondrion, whereas PE, PG and CL are synthesized by enzymes localized within the inner-mitochondrial membrane (IMM). The first step in the synthesis of PA, catalyzed by GPAT, is considered the rate-limiting step in the synthesis of phospholipids. The synthesis of PA is crucial because PA is used to synthesize CDP-DAG, which is required for synthesizing PI, and CDP-DAG is required for synthesizing DAG, which is used to produce all other phospholipids synthesized in the ER, including PE, PC and PS. It is currently unknown how the synthesis of phospholipids within the ER is integrated into mitochondrial biogenesis. By contrast, recent studies have identified some proteins involved in regulating phospholipid biosynthesis within the mitochondrion. Tam41 likely regulates the activity of CDS, whereas prohibitins (Phb1 and Phb2) interact with several proteins, including PSD and CLS, controlling the synthesis of PE and CL (reviewed by Gohil and Greenberg, 2009). Both Tam41 and prohibitins are associated with the IMM. It has not yet been determined whether Tam 41 and/or prohibitins are regulated by members of the PGC-1 family or other mediators of the mitochondrial biogenic pathway. Metabolites: ACoA, acyl coenzyme A; CDP-Ea, CDP-ethanolamine; CDP-DAG, CDP-diacylglycerol; CL, cardiolipin; CTP, cytidyltransferase; DAG, diacylglycerol; EaP, ethanolaminephosphate; G3P, glycerol-3-phosphate; Ino, inositol; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PI, phosphatidylinositol; PS, phosphatidylserine; SAM, S-adenosyl methionine. Enzymes: AGAT, 1-acylglycerol-3-phosphate acyltransferase; CDS, CDP-diacylglycerol synthase; CEPT, CDP-ethanolamine (1,2 diacylglycerol ethanolaminephosphotransferase); CLS, cardiolipin synthase; CDS, CDP-diacylglycerol synthase; EPCT, ethanolaminephosphate cytidyltransferase; GPAT, glycerol-3-phosphate acyltransferase; PEMT, phosphatidylethanolamine N-methyltransferase; PGPP, phosphatidylglycerolphosphate phosphatase; PGS, phosphatidylglycerolphosphate synthase; PIS, phosphatidylinositol synthase; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase. Based on Daum and Vance (Daum and Vance, 1997) and Gohil and Greenberg (Gohil and Greenberg, 2009).

by ATP (Arnold et al., 1998; Lombardi et al., 1998). THs also induce changes in the composition of mitochondrial membranes, affecting

the activity of the electron transport chain (Brand et al., 1992; Hulbert et al., 1976; Schlame and Hostetler, 1997). Increases in TH levels in response to cold acclimation may drive rapid increases in the activity of aerobic metabolic enzymes, as well as increases that occur over a longer time scale, requiring changes in gene expression. The effects of TH are tissue specific (Fernandez-Vizcarra et al., 2008; Sheehan et al., 2004), making it possible for THs to mediate the dissimilar responses of liver and muscle to cold acclimation. There is some evidence that TH increases in response to cold acclimation in fishes. Levels of both T<sub>3</sub> and T<sub>4</sub> were significantly higher in sea bream (*Sparus sarba*) acclimated to 12°C compared with those warm-acclimated to 25°C. In rainbow trout (*Oncorhynchus mykiss*), T<sub>3</sub> levels in plasma were higher in fish acclimated to 8°C compared with those acclimated to 16°C (Deane and Woo, 2005; Gabillard et al., 2003). However, there is conflicting evidence in the literature as to whether TH influences metabolism in fishes. In some fishes, exposure to TH increased oxygen consumption (Pandey and Munshi, 1976), whereas in others, it had no effect (van Ginneken et al., 2007). To my knowledge, no studies to date have examined the role of TH in mediating changes in metabolism during cold acclimation in fishes. Evidence suggests that such studies might be worthwhile pursuing.

ROS might also induce increases in aerobic metabolism in response to cold temperature in fishes. Studies have shown that the production of ROS increases in fishes in response to cold acclimation and acclimatization (Heise et al., 2007; Ibarz et al., 2010; Malek et al., 2004) and in mammals, ROS stimulate mitochondrial biogenesis (Irrcher et al., 2009; Kang et al., 2009; Perez-de-Arce et al., 2005; Suliman et al., 2004). ROS levels may increase through several potential mechanisms in response to a decline in temperature. Decreases in temperature reduce membrane fluidity, which may interrupt electron transfer among components of the mitochondrial respiratory chain, increasing the rate of ROS formation (Clement et al., 2009; Clement et al., 2010). Membrane remodeling, which leads to an increase in levels of polyunsaturated fatty acids, restores membrane fluidity but may also promote ROS production (reviewed by Crockett, 2008). Lastly, the increase in cellular P<sub>O<sub>2</sub></sub> accompanying a decrease in temperature may increase oxidative stress in fishes (Lushchak et al., 2005). In liver, the increase in CS activity has been attributed to an increase in lipid biosynthesis at cold temperatures (Lucassen et al., 2003). However, CS activity may also increase to provide intermediates of the Krebs cycle for the synthesis of proteins and nucleic acids damaged by ROS. Several studies have detected an increase in the activity of enzymes of the pentose phosphate pathway (or hexose monophosphate shunt, HMS) in response to cold acclimation in fishes (Campbell and Davies, 1978; Hochachka and Hayes, 1962; Stone and Sidell, 1981), which has been attributed to an increased demand for NADPH for biosynthetic pathways. However, absolute rates of synthesis are generally reduced during cold acclimation (Stone and Sidell, 1981), so the question remains, why does the activity of the pentose phosphate pathway increase? A possible explanation is to increase production of NADPH, a cofactor for the antioxidant glutathione reductase. This might be critical if ROS production increases during cold acclimation, and although potentially damaging, ROS may also stimulate metabolic remodeling.

Nitric oxide (NO) is an interesting candidate for stimulating mitochondrial biogenesis in response to cold temperature. In mammals, NO induces an increase in mitochondrial mass and is required for maintaining basal densities of mitochondria in several cell types (Momken et al., 2002; Nisoli et al., 2003). NO is produced by three isoforms of the enzyme nitric oxide synthase

(NOS): inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS) (Moncada et al., 1991). There is immunohistochemical evidence for the presence of all three isoforms in fishes, and genomic evidence for iNOS and nNOS, but not eNOS (Amelio et al., 2006; Bordieri et al., 2005; Cox et al., 2001; Fritsche et al., 2000; Holmqvist et al., 2000; Laing et al., 1999; Masini et al., 2005; Saeij et al., 2000). It is unknown if NO increases in response to cold acclimation in fishes, but we do know that circulating levels of NO are higher in icefishes than in red-blooded fishes because of their lack of Hb (Beers et al., 2010). Both Hb and Mb have NO dioxygenase activity. When bound to oxygen, both reduce NO to nitrate and are the major pathways by which NO is degraded (Gardner, 2005). Previously we have shown that higher levels of circulating NO in icefishes do not lead to an increase in PGC-1 $\alpha$ , NRF-1 or the copy number of mtDNA in icefishes compared with red-blooded nototheniid fishes (Urschel and O'Brien, 2008). However, we cannot rule out the possibility that NO stimulates proliferation of mitochondrial membranes in icefishes. Interestingly, mitochondria from brown adipocytes treated with the NO donor *S*-nitrosoacetyl penicillamine (SNAP) appeared larger compared with untreated cells (Nisoli et al., 2003). However, it was not determined in this study whether SNAP increased the biosynthesis of phospholipids. The potential role of NO in stimulating the synthesis of phospholipids would be consistent with its well-known role in enhancing oxygen delivery by inducing angiogenesis and vasodilation (Ignarro et al., 1987; Palmer et al., 1987; Sessa, 2009). It's enticing to consider that NO may also enhance the intracellular flux of oxygen by stimulating mitochondrial membrane proliferation.

### Conclusions

The molecular basis of mitochondrial biogenesis has been an area of intense research, particularly in the last 20 years, as the central role of mitochondria in human health has emerged (Wallace, 1999). Much of this research has been conducted using mammalian model organisms. Studying non-model organisms, exposed to different stresses that provoke mitochondrial biogenesis, allows us to expand our understanding of this complex process. For example, studies in fishes have shown that the role of PGC-1 family members as master regulators of mitochondrial biogenesis may be oversimplified. It has yet to be determined whether PGC-1 regulates the synthesis of mitochondrial phospholipids, an essential element of mitochondrial biogenesis. It is not surprising that the components of the mitochondrial biogenic pathway are not always coordinately regulated. Mitochondria are multi-faceted organelles. They not only produce energy, but also regulate apoptosis, calcium levels and the expression of some nuclear genes, and are the site of heme and lipid biosynthesis (Scheffler, 1999). The demands of mitochondria are tissue specific, which is reflected in the diversity of mitochondrial proteomes (Mootha et al., 2003). It is unknown how these tissue-specific mitochondrial proteomes are generated and then propagated through mitochondrial biogenesis. Using non-model organisms and comparative approaches may lead us toward the answers.

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### List of abbreviations

AGAT	1-acylglycerol-3-phosphate acyltransferase
AMPK	AMP kinase



CDP-DAG	CDP-diacylglycerol
CDS	CDP-diacylglycerol synthase
CL	cardiolipin
CLS	cardiolipin synthase
COX	cytochrome <i>c</i> oxidase
COXIV	cytochrome <i>c</i> oxidase subunit IV
CREB	cAMP response element-binding protein
CS	citrate synthase
eNOS	endothelial NOS
ER	endoplasmic reticulum
ERR	estrogen-related receptor
GEP	genetic interactors of prohibitins
GPAT	glycerol-3-phosphate acyltransferase
Hb	hemoglobin
iNOS	inducible NOS
Mb	myoglobin
mtDNA	mitochondrial DNA
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
NRF-1	nuclear respiratory factor-1
NRF-2	nuclear respiratory factor-2
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGC-1 $\alpha$	peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PGC-1 $\beta$	peroxisome proliferator-activated receptor gamma coactivator 1 beta
Phb1	prohibitin 1
Phb2	prohibitin 2
PI	phosphatidylinositol
POL $\gamma$	mtDNA polymerase $\gamma$
POLRMT	mitochondrial DNA-directed RNA polymerase
PPAR	peroxisome proliferator-activated receptor
PS	phosphatidylserine
PSD	phosphatidylserine decarboxylase
ROS	reactive oxygen species
SIRT	sirtuin
SNAP	S-nitrosoacetyl penicillamine
T <sub>2</sub>	3,5-diiodo-L-tyronine
T <sub>3</sub>	3',3,5-triiodo-L-triiodothyronine
T <sub>4</sub>	thyroxine
Tam41	translocator and maintenance protein 41
TFAM	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factor B 1
TFB2M	mitochondrial transcription factor B 2
TH	thyroid hormone
TWINKLE	mitochondrial helicase
YY-1	Yin yang 1

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