

High mitochondrial densities in the hearts of Antarctic icefishes are maintained by an increase in mitochondrial size rather than mitochondrial biogenesis

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SUMMARY

We investigated the molecular mechanisms regulating differences in mitochondrial volume density between heart ventricles of Antarctic notothenioids that vary in the expression of hemoglobin (Hb) and myoglobin (Mb). In mammals, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) and nuclear respiratory factor 1 (NRF-1) stimulate mitochondrial biogenesis and maintain mitochondrial density in muscle tissues. We hypothesized that these factors would also maintain mitochondrial density in the hearts of Antarctic notothenioids. The percent cell volume occupied by mitochondria is significantly lower in hearts of the red-blooded notothenioid *Notothenia coriiceps* (18.18 \pm 0.69%) in comparison with those of the icefish *Chaenocephalus aceratus* (36.53 \pm 2.07%), which lacks both Hb and cardiac Mb. Mitochondrial densities are not different between hearts of *N. coriiceps* and *Chionodraco rastrospinosus*, which lacks Hb, but whose heart expresses Mb. Despite differences in mitochondrial volume density between hearts of *N. coriiceps* and *C. aceratus*, the levels of transcripts of the genes encoding PGC-1 α , NRF-1 and citrate synthase, and the copy number of mitochondrial DNA do not differ. Our results indicate that the high mitochondrial densities in hearts of *C. aceratus* may result from an increase in organelle size. The surface-to-volume ratio of mitochondria from *N. coriiceps* is 1.9-fold greater than that of mitochondria from *C. aceratus*. In addition, the levels of PGC-1 α correlate with mitochondrial density in muscle tissues of notothenioids possessing mitochondria of similar size and morphology. Finally, the levels of PGC-1 α are 4.6-fold higher in the aerobic pectoral adductor muscle in comparison with the glycolytic skeletal muscle of *N. coriiceps*. The potential physiological significance of an increase in mitochondrial size in hearts of Antarctic icefishes is discussed.

Key words: mitochondria, Antarctic fish, muscle, *Notothenia coriiceps*, *Chaenocephalus aceratus*, *Chionodraco rastrospinosus*.

INTRODUCTION

The startling lack of hemoglobin (Hb) expression in Antarctic icefishes (family Channichthyidae, suborder Notothenioidae) was first described over a half century ago (Ruud, 1954). Some 40 years later, close examination of heart ventricles from species within the channichthyid family determined that six of the 16 members do not express the intracellular oxygen-binding protein myoglobin (Mb) (Grove et al., 2004; Moylan and Sidell, 2000; Sidell et al., 1997). Since then, many studies have revealed how these unique animals thrive despite the loss of, what were once thought to be, essential oxygen-binding proteins.

The loss of Hb is associated with several modifications in the cardiovascular system of icefishes. These animals have enlarged hearts in comparison with those of red-blooded teleosts (Hemmingsen et al., 1972; Johnston and Harrison, 1987) and circulate a blood volume equivalent to 7.6% of their body mass, which is significantly greater than red-blooded teleosts, with blood volumes only 2–3% of body mass (Hemmingsen and Douglas, 1970). The work of the heart is minimized by large-bore capillaries, with cross-sectional areas approximately two-times larger than those of red-blooded teleosts (Fitch et al., 1984). Nevertheless, icefishes still expend more cardiac energy per unit time than red-blooded notothenioids, indicating that, although the loss of circulating red blood cells reduces blood viscosity, this strategy is not energetically favorable (Sidell and O'Brien, 2006). Oxygen delivery is also enhanced in some highly aerobic tissues, such as retinae, by an increase in vascular density (Wujcik et al., 2007). In summary, these

adaptations ensure adequate tissue oxygenation in icefishes possessing a 9- to 10-times lower oxygen-carrying capacity compared with that of red-blooded notothenioids (Ruud, 1954).

One of the more paradoxical adaptations to the loss of Hb and Mb expression is an increase in mitochondrial volume density in heart ventricular tissue. Typically, mitochondrial volume density correlates with aerobic metabolic capacity (Hoppeler and Lindstedt, 1985). However, icefishes do not conform to this paradigm. There is an interesting relationship between the expression of Hb and Mb and the percentage cell volume occupied by mitochondria in heart ventricles. Mitochondria comprise 16% of the cell volume in hearts of species that express both Hb and Mb (O'Brien and Sidell, 2000). The percentage cell volume occupied by mitochondria increases to 20% in hearts of icefishes expressing Mb, and, in icefishes that lack both Hb and Mb, mitochondria occupy a stunning 36% of the cell volume (O'Brien and Sidell, 2000). Surprisingly, high mitochondrial densities in icefish hearts do not enhance aerobic metabolic capacity. The activity per gram wet mass of tissue of aerobically poised enzymes is similar between species that vary in expression of Hb and Mb (Johnston and Harrison, 1987; O'Brien and Sidell, 2000). Although the high densities of mitochondria in icefish hearts do not increase the aerobic metabolic capacity, they may provide a favorable avenue for intracellular oxygen diffusion and compensate for the lack of oxygen-binding proteins (O'Brien and Sidell, 2000; Sidell, 1998).

Although the impressive remodeling of cardiac myocytes in response to the loss of oxygen-binding proteins has been well

established, the molecular underpinnings maintaining the high mitochondrial densities in hearts of icefishes are unknown. In mammals, mitochondrial proliferation is driven by the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α , encoded by the *PPARGC1A* gene). Together with nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), PGC-1 α transactivates the expression of nuclear-encoded genes involved in mitochondrial oxidative phosphorylation (Puigserver and Spiegelman, 2003). PGC-1 α and NRFs also stimulate the expression of mitochondrial transcription factor A (TFAM), which translocates to the mitochondrion and activates the transcription and replication of the mitochondrial genome (Kang and Hamasaki, 2005).

We hypothesized that high mitochondrial densities in the hearts of icefishes would be maintained by the same mitochondrial biogenic pathway observed in mammals. Mitochondrial volume density was quantified in hearts of the red-blooded notothenioid *N. coriiceps* using stereological point-counting methods. Values were compared with previous measurements made of mitochondrial densities in the hearts of *C. aceratus* (–Hb/–Mb) and *C. rastrispinosus* (–Hb/+Mb) (O'Brien and Sidell, 2000). We also measured the expression of PGC-1 α and NRF-1 in heart ventricles of these three species, as well as two additional indices of mitochondrial density: the expression of the aerobic metabolic gene encoding citrate synthase, and the copy number of mitochondrial DNA (mtDNA). Our results indicate that the high mitochondrial densities in Mb-deficient icefish hearts are not maintained through a canonical mitochondrial biogenic pathway. Instead, they appear to be brought about through an expansion of the outer mitochondrial membrane that occurs independently of an increase in mitochondrial protein expression and replication of mtDNA.

MATERIALS AND METHODS

Tissue collection

Notothenia coriiceps (Richardson), *Chaenocephalus aceratus* (Lönnerberg) and *Chionodraco rastrispinosus* (Dewitt and Hureau) were captured in Dallman Bay near the Astrolabe Needle (64°10'S, 62°35'W) at approximately 150 m depth. The fish were caught by using an otter trawl and baited traps deployed from the ARSV *Lawrence M. Gould* during the austral autumns of 2005 and 2007. Animals were maintained in circulating seawater tanks while on board ship and transferred to aquaria at the U.S. Antarctic Research Station, Palmer Station on Anvers Island. Here, they were maintained unfed in covered, circulating seawater tanks at 0 \pm 0.5°C.

Animals were anesthetized in 1:7500 (w/v) MS-222 (tricaine methanesulfonate) and then killed by brain pithing. Heart, aerobic pectoral adductor muscle and glycolytic skeletal muscle were quickly excised, frozen in liquid nitrogen and stored at –80°C. Portions of each tissue type were also diced into 1-mm-sized blocks on an ice-cold stage, immersed in nine volumes of RNAlater (Ambion, Austin, TX, USA), stored overnight at 4°C and frozen at –80°C.

Preparation of heart ventricles from *N. coriiceps* for electron microscopy

Hearts were excised from *N. coriiceps* and placed into an ice-cold isotonic Ringer solution (260 mmol l⁻¹ NaCl, 2.5 mmol l⁻¹ MgCl₂, 5 mmol l⁻¹ KCl, 2.5 mmol l⁻¹ NaHCO₃, 5 mmol l⁻¹ NaH₂PO₄, pH 8.0) in which they were allowed to contract several times to clear blood from the heart lumen. Hearts were then transferred into an ice-cold fixative solution (3% glutaraldehyde, 100 mmol l⁻¹ sodium cacodylate, 110 mmol l⁻¹ sucrose, 2 mmol l⁻¹ CaCl₂, pH 7.4) and perfused through the atria at a rate of approximately 1 ml min⁻¹ for

10 min. Tissues were placed in fresh fixative and stored at 4°C for 4–6 hours and then transferred again into fresh fixative for an additional 2–4 hours. Hearts were stored in Trumps buffer (1% glutaraldehyde, 4% formaldehyde, 0.1 mol l⁻¹ sodium cacodylate, 0.11 mol l⁻¹ sucrose, 2 mmol l⁻¹ CaCl₂, pH 7.4) at 4°C and transported back to the University of Alaska, Fairbanks for further processing.

Transmural sections spanning from the epicardium to the endocardium were excised from the heart ventricles. Epicardial tissue was removed, and the remaining endomyocardial tissue was cut into 1-mm-sized blocks. The blocks were rinsed overnight at 4°C in 0.1 mol l⁻¹ sodium cacodylate, 0.11 mol l⁻¹ sucrose, 2 mmol l⁻¹ CaCl₂, pH 7.4. The following day, blocks were post-fixed in an ice-cold solution of 1% osmium tetroxide, 0.1 mol l⁻¹ sodium cacodylate, 0.11 mol l⁻¹ sucrose, 2 mmol l⁻¹ CaCl₂, pH 7.4, for 2 h. The blocks were rinsed briefly three times with distilled water and then dehydrated through a series of acetone washes. The blocks were embedded in a mixture of Epon and Araldite resin [24% Eponate, 15% Araldite, 58% DDSA, 3% BDMA (v/v)] by first incubating for 30 min in a 2:1 acetone:resin mixture, followed by 30 min in a 1:1 acetone:resin mixture and then placed in a 1:2 acetone:resin mixture overnight, with caps slightly ajar to allow the acetone to evaporate slowly. The following day, the blocks were transferred into fresh resin, placed under vacuum for 30 min, transferred to fresh resin and placed under vacuum at 60°C for 30 min. The blocks were cured in resin for 24–36 h at 60°C.

Tissue blocks were sectioned using a Sorvall MT2 ultramicrotome and placed on 200-mesh copper grids. Sections were stained with 2% uranyl acetate, followed by 0.5% lead citrate and viewed with a JEOL JEM-1200EX transmission electron microscope equipped with a digital camera. Between seven and 10 micrographs were obtained from one section per individual (four individuals in total) for measuring mitochondrial volume and surface densities using the aligned-systematic-quadrats-subsampling method (Cruz-Orive and Weibel, 1981). Micrographs were taken at a magnification of \times 7500 and viewed with Adobe Photoshop 6.0 at a final magnification of \times 13 100. Images were overlaid with a digital square lattice test pattern, with spacing equal to 0.53 μ m on the micrograph. The mitochondrial volume density was quantified using point-counting methods, and the mitochondrial surface density was measured using the line-intercept technique (Weibel, 1979). The extracellular matrix, luminal spaces and epithelial cells were excluded from measurements.

Isolation of RNA and DNA

Total RNA was isolated using either TRIZOL reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) or an RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA). DNA was isolated using the DNeasy Tissue Kit (Qiagen). The concentration and quality of nucleic acids were assessed with a Nanodrop ND-1000 spectrophotometer or a PerkinElmer Lambda 25 UV/VIS spectrometer. Only samples with a 260 nm to 280 nm ratio of 1.8–2.0 and a 260 nm to 230 nm ratio of 1.6–2.0 were used for further analysis. The integrity of the RNA was verified by visualizing samples on a 1% agarose gel stained with ethidium bromide. RNA was stored at –80°C and DNA at –20°C.

Sequencing genes of interest for qRT-PCR

Partial cDNA sequences from the genes of interest were obtained to design gene-specific primers for quantitative real-time PCR (qRT-PCR). Homologous amino acid sequences from at least four species were aligned, and degenerate primers were designed using CODEHOP (<http://bioinformatics.weizmann.ac.il/blocks/codehop.html>) (Table 1). When possible, primers were selected to

Table 1. Degenerate primers used to obtain partial gene sequences

Gene	Primer (5'–3')	PCR product size (bp)
<i>CS</i>	Forward: GGCGTGCACAAGACCAARTAYTGGGA Reverse: GGACACGTTGCCGCCYTCRTGRTC	255
<i>EF-1A</i>	Forward: CGACATCGCCCTGTGGAARTTYGARAC Reverse: GATGGCCGCGGATCTGTANACRTCYTG	561
<i>18S</i>	Forward: ACTGTGGYAATYCYAGAGCTAATACATGC Reverse: TRYRCTCATTCCRATTACAGGGCC	400
<i>NRF-1</i>	Forward: CAGACCGAGCACATGACCACNATHGARGC Reverse: TGTCCCACTCGGGTGGTRTAYTCRTC	370
<i>PGC-1α</i>	Forward: GGCACTGCACCGAGCTGYTNAARTAYYT Reverse: GCAGGGGTGGCCGAARTGYTTRTT	1200
<i>TBP</i>	Forward: GGAGGAGCAGCAGCGACARCARCA Reverse: GGATCCACCATGTTCTGGATYTTAARTC	516

N, R and Y indicate degenerate nucleotides (N=A+C+G+T; R=A+G; Y=C+T).

Table 2. Primers used for quantitative real-time PCR

Gene	Primer (5'–3')	PCR product size (bp)
<i>CS</i>	Forward: CCATCGACTCCAACCTGGAC Reverse: ATCCCAGCATGTTGCTGAAGT	51
<i>EF-1A</i>	Forward: CTGGAAGCCAGTGAAAAGATGAC Reverse: ACGCTCAACCTTCCATCCC	51
<i>18S</i>	Forward: ACCACATCCAAGGAAGGCAG Reverse: CCGAGTCGGGAGTGGGTAAT	51
<i>ND2</i>	Forward: TAATAGCACAAAATCACCACC Reverse: GCAAAAAGTAGTACGGCG	91
<i>NRF-1</i>	Forward: TCCAGCAGGTTTCAGGTACATGT Reverse: TCATCATGGACGCTTCGGTAT	51
<i>PGC-1α</i>	Forward: CTCCAATGACCACAAGGGATC Reverse: CGTGGTAGGTGGTGTACAGAC	101
<i>16S</i>	Forward: GAACGCTGAAAGAGAAATGA Reverse: TGTCACTCTACTCAAAGCTC	216
<i>TBP</i>	Forward: CGGGAGCCAAGAGTGAGGA Reverse: GAGCGTATTTCTGGCAGCTAAC	51

amplify regions that contained two or more splice sites within homologous sequences.

Complementary DNA was synthesized using either Superscript III reverse transcriptase (Invitrogen) or MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamers.

Sequences of interest were amplified using an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) programmed with a touchdown protocol with annealing temperatures between 55°C and 65°C. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. Products corresponding to the expected size were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Products were cloned using a TOPO TA Cloning Kit (Invitrogen). Transformed *Escherichia coli* were identified by blue–white screening on LB-agar plates (15 mg ml⁻¹ agar, 10 mg ml⁻¹ Bacto-tryptone, 5 mg ml⁻¹ NaCl, 5 mg ml⁻¹ yeast extract, 1 mg ml⁻¹ glucose, 12.5 μ g ml⁻¹ ampicillin) supplemented with 0.2 mg ml⁻¹ X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Transformed *E. coli* were grown in 5 ml LB media (10 mg ml⁻¹ Bacto-tryptone, 5 mg ml⁻¹ NaCl, 5 mg ml⁻¹ yeast extract, 1 mg ml⁻¹ glucose) supplemented with 0.25 mg ml⁻¹ ampicillin overnight in an OLS200 shaking water bath (Grant Instruments, Cambridge, UK) at 200 r.p.m. and 37°C. Plasmids were isolated using the QIAprep Miniprep kit (Qiagen), labeled for sequencing with the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) and purified with Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA) packed with Sephadex G-50 gel (Sigma-Aldrich, St Louis,

MO, USA). Genes of interest were sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

The 5' region of the gene encoding NRF-1 was obtained by rapid amplification of cDNA ends (RACE) PCR using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Sparks, MD, USA). Gene-specific primers for amplifying both the 5' and 3' ends of the gene were designed from sequence obtained with degenerate primers. PCR reactions were carried out using an iCycler (Bio-Rad Laboratories) programmed with a touchdown protocol and annealing temperatures between 68°C and 70°C. The RACE products were cloned and sequenced as described above.

Quantitative real-time PCR

RNA was treated twice with DNase I to remove genomic DNA – once for 25 min at 25°C and a second time for 20 min at 25°C. Gene-specific primers were designed from partial cDNA sequences using Primer Express Software v2.0 (Applied Biosystems). Primers were designed within regions of cDNA that are conserved between all three species of interest (Table 2) and in all cases, except citrate synthase, such that at least one primer from each set spanned a splice site to ensure that genomic DNA was not amplified.

Quantitative RT-PCR was carried out with an ABI PRISM 7900HT Sequence Detection System with Power SYBR Green PCR Master Mix (Applied Biosystems). Template cDNA of all target and housekeeping genes, except 18S rRNA, was diluted by a factor of 20 to a final concentration of 1 ng μ l⁻¹. Complementary DNA used for measuring the expression of 18S rRNA was diluted by a factor of 2000 to a final concentration of 0.01 ng μ l⁻¹. This was done to compensate for the greater abundance of 18S rRNA compared with that of the target genes. DNA used for measuring the ratio of mitochondrial-to-nuclear DNA was diluted to a final concentration of 5 ng μ l⁻¹. Complementary DNA or genomic DNA was pooled from all individuals and serially diluted to generate a standard curve to determine the reaction efficiency. The reactions were carried out in triplicate, and two types of controls were used. The first control lacked template cDNA (or DNA for measurements of mitochondrial DNA copy number), and the second (for measuring gene expression) contained RNA in which the reverse transcriptase was omitted during cDNA synthesis. Both of these controls ensured that genomic or contaminating DNA was not amplified. Dissociation curves were analyzed to verify that only a single product was amplified in each reaction mixture. The expression of target genes was normalized to the expression of 18S rRNA using the comparative critical threshold ($\Delta\Delta C_t$) method corrected for primer efficiency (Pfaffl, 2001). The expression level of all target genes was expressed relative to levels in *N. coriiceps*, except for measurements in oxidative and glycolytic muscle of *N. coriiceps*. In these experiments, the expression of target genes in oxidative muscle was expressed relative to the levels in glycolytic muscle.

The ratio of mitochondrial-to-nuclear DNA in all species was normalized to the ratio in *N. coriiceps*.

Evaluation of housekeeping genes

The expression of three genes (encoding EF-1A, TBP, 18S rRNA) was evaluated as potential housekeeping genes (HKGs) for normalizing the expression of the target genes. The expression level of these genes was measured using qRT-PCR in the heart ventricle of the three species of interest. The results were analyzed using the Excel-based program BestKeeper (Pfaffl, 2004).

BestKeeper uses a combination of descriptive statistics and regression analysis of Ct values to identify the most stably expressed HKGs. The Ct values measured from all individuals are pooled for each gene. Genes with Ct values having standard deviations greater than 1 are considered unstable. Two types of linear regression analysis are also performed. First, a regression analysis is carried out between all possible combinations of all genes. Second, the average Ct values of all genes with s.d. of <1 from each individual are pooled to create the bestkeeper index. A linear regression is then carried out between the bestkeeper index and each gene. Suitable HKGs are those with the highest correlation coefficient (*r*) when compared with the bestkeeper index and a s.d. of <1.

Statistics

Significant differences in gene expression were determined using a Student's *t*-test or an ANOVA followed by a *post-hoc* Tukey's Honestly Significantly Different (HSD) test. Differences were considered significant at *P*<0.05. The results are presented as the means ± s.e.m.

RESULTS

Mitochondrial volume density

We measured mitochondrial volume density in the heart ventricles of *N. coriiceps* (+Hb/+Mb) and compared it with measurements made previously in the hearts of the *C. aceratus* (-Hb/-Mb) and *C. rastrispinosus* (-Hb/+Mb) (O'Brien and Sidell, 2000). Mitochondrial densities are twofold lower in hearts of *N. coriiceps* compared with hearts of *C. aceratus* (Table 3). However, there is no significant difference in the percentage cell volume occupied by mitochondria between *N. coriiceps* and *C. rastrispinosus* (*P*=0.11) (Table 3).

Analysis of housekeeping genes

The Excel-based software tool BestKeeper (Pfaffl et al., 2004) was used to analyze the expression of three commonly used HKGs for studies of gene expression. These HKGs included the TATA-box-binding protein (TBP), elongation factor 1A (EF-1A) and 18S ribosomal RNA (18S) (de Kok et al., 2005; McClelland et al., 2006; Olsvik et al., 2005). The expression of TBP is highly variable (s.d.>1), indicating that this gene is not a suitable HKG for measurements of gene expression in hearts of notothenioids (Table 4). Measurements of the expression of the genes encoding both 18S and EF-1A (with s.d. <1) are correlated with the bestkeeper index (Table 4), and thus both are appropriate HKGs. We normalized the expression of all target genes to the expression of 18S rRNA. Normalization to EF-1A gave similar results (data not shown).

Expression of mitochondrial biogenic genes

PGC-1 α and NRF-1 play central roles in regulating the density of mitochondria in a variety of mammalian tissue types (Puigserver

Table 3. Mitochondrial volume densities in the heart ventricle of notothenioid fishes

	<i>N. coriiceps</i> (+Hb/+Mb)	<i>C. rastrispinosus</i> ^a (-Hb/+Mb)	<i>C. aceratus</i> ^a (-Hb/-Mb)
Volume density (%)	18.18±0.69 ^A	20.10±0.74 ^A	36.53±2.07 ^B

Values are means ± s.e.m. (*N*=6 for *C. aceratus* and *C. rastrispinosus*; *N*=4 for *N. coriiceps*). ^A, ^B denote significant differences between the three species (*P*<0.05).
^aData from (O'Brien and Sidell, 2000).

and Spiegelman, 2003). Despite significant differences in mitochondrial volume densities between hearts of the red-blooded *N. coriiceps* and the icefish *C. aceratus*, there is no significant difference in the expression of either PGC-1 α or NRF-1 (Fig. 1). The amount of RNA per gram of tissue is equivalent between the hearts of *C. aceratus*, *C. rastrispinosus* and *N. coriiceps* (Table 5). Therefore, the relative expression of PGC-1 α and NRF-1 per gram of tissue is also equivalent between the hearts of these three species.

Mitochondrial DNA copy number

The copy number of mtDNA typically correlates with mitochondrial volume density (Wu et al., 1999). We quantified the mtDNA copy number relative to the abundance of nuclear DNA (nDNA) using qRT-PCR. The copy numbers of the mitochondrially encoded genes NADH dehydrogenase subunit 2 (ND2) and 16S rRNA (16S) were compared with the copy number of the nuclear gene encoding citrate synthase (CS). No significant differences were detected in the ratio of mtDNA-to-nDNA between the heart ventricles of *N. coriiceps*, *C. rastrispinosus* and *C. aceratus* (Fig. 2). In addition, the amount of DNA per gram of tissue does not vary between the hearts of the three species (Table 5). As a result, there is no significant difference in the copy number of mtDNA per gram wet mass of tissue.

Citrate synthase expression

The activity and expression of aerobic metabolic enzymes typically correlates with mitochondrial density in aerobic tissues. We measured transcript levels of CS in the heart ventricle of *N. coriiceps*, *C. rastrispinosus* and *C. aceratus* and determined that there is no significant difference in the level of mRNA encoding CS in the hearts of these three animals, despite the much higher density of mitochondria in the hearts of *C. aceratus* compared with that of the other two species (Fig. 3).

Levels of mitochondrial biogenic genes in oxidative and glycolytic muscle of *N. coriiceps*

To verify that PGC-1 α and NRF-1 regulate mitochondrial biogenesis in notothenioids, we measured the expression of these factors in the pectoral adductor and glycolytic skeletal muscles of *N. coriiceps*, which differ in mitochondrial density but not in mitochondrial morphology. The expression level of PGC-1 α is 4.6-fold higher in pectoral adductor muscle compared with glycolytic skeletal muscle in *N. coriiceps* (Fig. 4A). The expression level of NRF-1 tends to

Table 4. Analysis of housekeeping genes using BestKeeper[®]

Gene	s.d.	<i>r</i>
EF-1A	0.77	0.89
18S	0.58	0.81
TBP	1.08	0.88

N=24; eight individuals per species. *r*, Pearson correlation coefficient with Bestkeeper index.

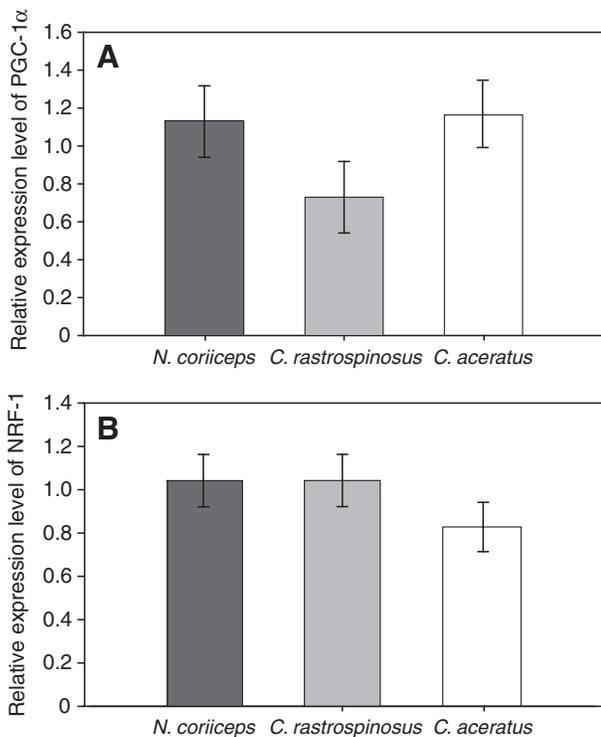


Fig. 1. Expression level of genes mediating mitochondrial biogenesis in heart ventricles of notothenioid fishes that vary in expression of Hb and Mb. The relative expression levels of (A) PGC-1 α and (B) NRF-1 were quantified using qRT-PCR. Values are means \pm s.e.m. ($N=8$).

be higher in pectoral adductor muscle, compared with glycolytic skeletal muscle, although this difference is not significant (Fig. 4B; $P=0.12$).

The amount of RNA per gram wet mass of muscle does not vary between oxidative ($0.36\pm 0.08\text{ mg g}^{-1}$) and glycolytic (0.28 mg g^{-1}) muscles ($P>0.05$). As a result, the expression of PGC-1 α per gram of tissue is significantly higher in oxidative muscle compared with glycolytic muscle of *N. coriiceps*.

Mitochondrial size

Mitochondria from hearts of *C. aceratus* are larger than those from *N. coriiceps*. The mitochondrial surface-to-volume ratio is $4.52\pm 0.27\mu\text{m}^{-1}$ in *C. aceratus* (O'Brien and Sidell, 2000), compared with $8.69\pm 0.40\mu\text{m}^{-1}$ in hearts of *N. coriiceps* ($P<0.05$).

DISCUSSION

Antarctic icefishes possess many well-characterized adaptations to the loss of Hb and Mb, yet little is known about the underlying molecular mechanisms that maintain these physiological traits. We attempted to characterize the molecular pathway maintaining the high density of mitochondria in the hearts of Antarctic icefishes.

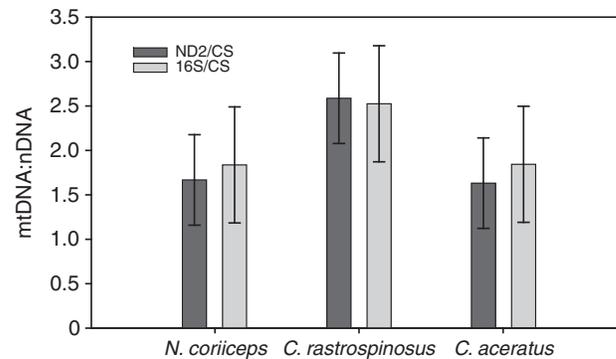


Fig. 2. Quantification of mitochondrial DNA copy number in heart ventricles of Antarctic notothenioids that vary in the expression of Hb and Mb. The mitochondrial-to-nuclear DNA ratio (mtDNA:nDNA) was quantified using qRT-PCR. The copy numbers of NADH dehydrogenase subunit 2 (ND2) and 16S ribosomal RNA (16S) were measured as indices of mtDNA copy number. Nuclear DNA copy number was quantified by measuring the copy number of citrate synthase (CS). Values were normalized to the mtDNA:nDNA ratio in *N. coriiceps*. Values are means \pm s.e.m. ($N=6$).

Much to our surprise, measurements of several indices of mitochondrial biogenesis that are typically correlated with mitochondrial density do not differ between hearts of red- and white-blooded notothenioids. Our results suggest that the high densities of mitochondria in the hearts of icefishes result from an increase in organelle size rather than from mitochondrial biogenesis.

Mitochondrial volume densities are significantly higher in hearts of icefishes lacking Mb compared with red-blooded notothenioids

Consistent with previous studies, we determined that the percentage of cell volume occupied by mitochondria is significantly lower in the hearts of the red-blooded notothenioid *N. coriiceps* compared with the icefish *C. aceratus*, which does not express Hb or cardiac Mb (Table 3). This difference has been observed not only in the heart ventricle tissue between red- and white-blooded notothenioids but also in the aerobic pectoral adductor muscle (Johnston, 1987; O'Brien and Sidell, 2000; O'Brien et al., 2003).

We did not find significant differences in mitochondrial volume densities between the hearts of *N. coriiceps* and the icefish *C. rastrospinosus*, which does express cardiac Mb. This is in contrast to a previous study (O'Brien and Sidell, 2000) that determined that mitochondrial densities are 4% higher in hearts of *C. rastrospinosus* compared with the red-blooded *Gobionotothen gibberifrons*. *N. coriiceps* are more active compared with *G. gibberifrons*, which may account for the higher mitochondrial density in hearts of *N. coriiceps*. Overall, these data suggest that the loss of both Hb and Mb leads to a much larger expansion (18–20%) of mitochondrial volume density in icefish hearts compared with the expansion upon loss of Hb alone ($\leq 4\%$).

The high mitochondrial densities in hearts of *C. aceratus* are not maintained by a conventional pathway of mitochondrial biogenesis

PGC-1 α and NRF-1 play pivotal roles in controlling mitochondrial density in mammalian muscle tissues (Evans and Scarpulla, 1990; Lehman et al., 2000;

Table 5. Amount of RNA and DNA in heart ventricles of Antarctic notothenioids

	<i>N. coriiceps</i> (+Hb/+Mb)	<i>C. rastrospinosus</i> (-Hb/+Mb)	<i>C. aceratus</i> (-Hb/-Mb)
Total RNA (mg g^{-1} wet mass)	1.01 ± 0.16	0.67 ± 0.08	1.13 ± 0.14
Total DNA (mg g^{-1} wet mass)	0.33 ± 0.03	0.24 ± 0.02	0.26 ± 0.02

Values are means \pm s.e.m. ($N=8$ for RNA measurements; $N=6$ for DNA measurements).

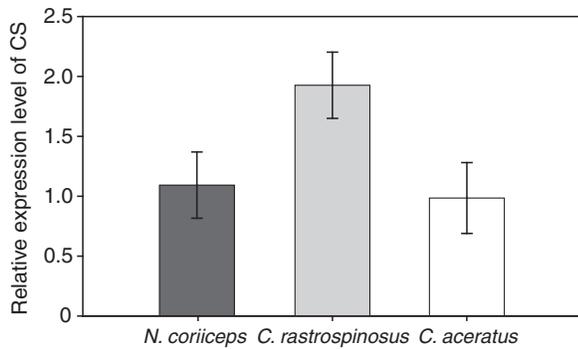


Fig. 3. The expression level of citrate synthase (CS) in heart ventricles of Antarctic notothenioids that vary in the expression of Hb and Mb. The relative expression level of CS was measured using qRT-PCR. Values are means \pm s.e.m. ($N=8$).

Puigserver et al., 1998; Wu et al., 1999). These factors stimulate the expression of genes required for increasing mitochondrial density in response to an increase in ATP demand, such as exercise or cold temperature (Baar, 2004; Wu et al., 1999). In addition, the levels of PGC-1 α and NRF-1 correlate with the oxidative capacities of tissues, suggesting that they are required for maintaining steady-state levels of mitochondrial densities (Lin et al., 2002; Puigserver et al., 1998; Terada and Tabata, 2004). Surprisingly, despite the twofold difference in mitochondrial volume density between hearts of *N. coriiceps* and *C. aceratus*, we do not detect a significant difference in the expression of PGC-1 α or NRF-1 in these animals.

The lack of difference between the two fish in their expression of PGC-1 α and NRF-1 suggests three possibilities. First, these factors may be regulated by post-transcriptional processes. Previous work has shown that phosphorylation of serine and threonine residues by p38 MAP kinase, as well as deacetylation at multiple sites by the NAD-dependent deacetylase SIRT1, regulates the DNA binding activity of PGC-1 α (Gerhart-Hines et al., 2007; Puigserver et al., 2001). Second, PGC-1 α and NRF-1 may not regulate mitochondrial biogenesis in notothenioids. Little is known about the molecular mechanisms of mitochondrial biogenesis in fish. A recent study in goldfish indicates that another member of the PGC-1 family of transcriptional co-activators, PGC-1 β , may be more important than PGC-1 α in controlling mitochondrial biogenesis in fish (Lemoine et al., 2008). Finally, the lack of difference in PGC-1 α and NRF-1 between hearts of *N. coriiceps* and *C. aceratus* may indicate that the expression of mitochondrial proteins does not differ between these two species. The expression of mitochondrial proteins and replication of mtDNA are clearly regulated by PGC-1 α in mammals. However, it is not known whether PGC-1 α also controls mitochondrial membrane biogenesis (Scarpulla, 2006). If not, the high mitochondrial densities in the hearts of Mb-deficient icefishes may arise through a novel, PGC-1 α -independent pathway, in which membrane, but not protein, biosynthesis is elevated. Previous work, as well as our own observations, supports this hypothesis. The surface-to-volume ratio of mitochondria from hearts of *C. aceratus* is significantly lower compared with that of mitochondria from hearts of the red-blooded species *G. gibberifrons* (O'Brien and Sidell, 2000) and *N. coriiceps* (Fig. 5).

To eliminate the possibility that PGC-1 α and NRF-1 do not maintain mitochondrial density in Antarctic notothenioids, we measured the expression of these factors in oxidative pectoral adductor muscle and glycolytic skeletal muscle from *N. coriiceps*.

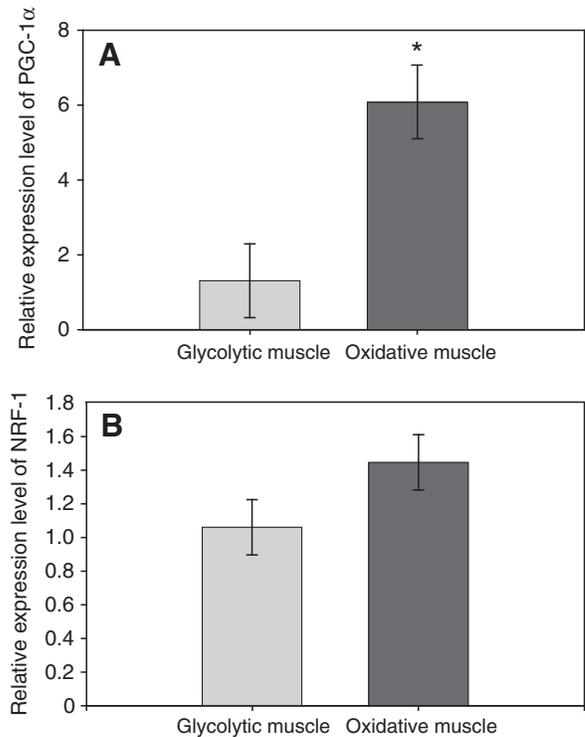


Fig. 4. The expression level of genes mediating mitochondrial biogenesis in pectoral adductor and glycolytic skeletal muscles of *N. coriiceps*. The relative expression levels of (A) PGC-1 α and (B) NRF-1 were quantified using qRT-PCR. The asterisk denotes a significant difference in expression between the two tissue types ($P<0.05$). Values are means \pm s.e.m. $N=4$ for PGC-1 α ; $N=8$ for NRF-1.

These two tissues differ in mitochondrial density but not mitochondrial morphology. Mitochondria occupy 34.3% of the cell volume in pectoral adductor muscle of *N. coriiceps* compared with only 1.4% in glycolytic skeletal muscle fibers (Johnston, 1989). In addition, the maximal activity of aerobically poised enzymes is typically 10-fold greater in oxidative muscle tissue compared with glycolytic muscle of notothenioids (Crockett and Sidell, 1990). We found that the levels of PGC-1 α correlate with mitochondrial densities in muscle tissues of Antarctic fishes when differences in organelle ultrastructure are eliminated. The expression of PGC-1 α is 4.6-fold higher in pectoral adductor muscle compared with glycolytic skeletal muscle of *N. coriiceps*. The levels of NRF-1 tend to be higher in oxidative muscle compared with glycolytic muscle, although this difference is not significant.

Together, these results indicate that the differences in mitochondrial density between hearts of *C. aceratus* and *N. coriiceps* are not maintained by PGC-1 α and NRF-1. To confirm this, we measured the expression of two additional components of the mitochondrial biogenic pathway: mtDNA copy number and expression of CS. PGC-1 α , together with NRF-1 and NRF-2, activates the expression of the mitochondrial transcription factor TFAM, which controls both the transcription and replication of the mitochondrial genome (Virbasius and Scarpulla, 1994). Consistent with our measurements of PGC-1 α and NRF-1, there is no significant difference in the copy number of mtDNA between hearts of *N. coriiceps* and *C. aceratus*. In addition, there is no difference in the expression of CS between the hearts of these animals. This agrees with previous measurements, which found no difference in the activity of CS per gram of tissue between hearts

of the red-blooded notothenioid *G. gibberifrons* and the icefish *C. aceratus* (O'Brien and Sidell, 2000).

These results, along with ultrastructural analysis of mitochondria, strongly suggest that the high mitochondrial densities in hearts of *C. aceratus* are not brought about through mitochondrial biogenesis. The unique architecture of mitochondria in hearts of *C. aceratus* suggests that each mitochondrion is enlarged through a proliferation of the outer mitochondrial membrane without a corresponding increase in inner membrane surface density, protein synthesis or mtDNA replication. To our knowledge, this is the only example in any organism in which an increase in mitochondrial density is brought about in this fashion.

Nothing is known about how mitochondrial membrane synthesis is integrated into mitochondrial biogenesis. However, our results suggest that PGC-1 α , considered the 'master regulator of mitochondrial biogenesis', may not control this process (Kelly and Scarpulla, 2004; Scarpulla, 2006). Unlike mtDNA and most mitochondrial proteins, which are localized solely to the mitochondrion, mitochondrial membrane phospholipids are part of a general pool of phospholipids synthesized in the endoplasmic reticulum (ER) (Daum and Vance, 1997). From the ER, they are directed to multiple locations within the cell. Only cardiolipin is synthesized exclusively within mitochondria, where it is localized within the inner membrane and at contact points between the inner and outer membranes (Ardail et al., 1990; Chen et al., 2006). Currently, little is known about how the ER directs phospholipids to specific cellular compartments. Because the ER synthesizes the majority of membrane phospholipids, it must be capable of regulating phospholipid biosynthesis independently of mitochondrial biogenesis. In addition, during mitochondrial biogenesis, the ER must direct newly synthesized phospholipids specifically to the mitochondrion to maintain a constant ratio between mitochondrial proteins and phospholipids. How these dual functions are accomplished is unclear. However, it appears that, in the hearts of Mb-deficient icefish, the former pathway is upregulated.

Potential advantages to an increase in mitochondrial size in the hearts of icefish lacking Mb

The unusual ultrastructure of mitochondria in the hearts of *C. aceratus* raises the issue of whether there is a physiological advantage to mitochondrial remodeling in the hearts of fishes that lack Hb and Mb. The enlargement of mitochondria in the hearts of icefishes results in two alterations to the architecture of cardiac myocytes. First, it leads to a higher surface density of mitochondrial membranes per g ventricular tissue relative to those of red-blooded fish species. Second, the surface-to-volume ratio of individual mitochondria is decreased relative to that of red-blooded notothenioids. Each of these modifications might have a distinct advantage to the cardiovascular system of icefishes.

As previous studies have suggested, the increase in surface density of mitochondrial membranes in icefishes may enhance oxygen delivery (i.e. O'Brien and Sidell, 2000). Oxygen is more than four times more soluble in nonpolar solvents than it is in water (Battino et al., 1968). As a result, the hydrocarbon core of mitochondrial membranes serves as a storehouse for oxygen, as well as acting as

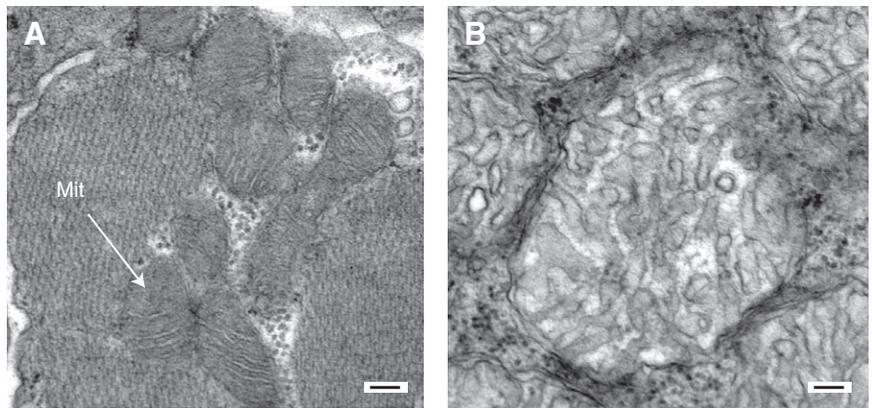


Fig. 5. Transmission electron micrographs of mitochondria within heart ventricular tissue of red- and white-blooded Antarctic notothenioids. The mitochondria are significantly smaller in hearts of (A) *N. coriiceps* (+Hb/+Mb) compared with (B) *C. aceratus* (-Hb/-Mb). Micrographs of ventricular tissue were taken at a final magnification of $\times 75\,700$. Bar, 100 nm; Mit, mitochondria.

an effective conduit for the diffusion of oxygen (Desaulniers et al., 1996; Sidell, 1998). In addition, the proliferation of mitochondrial membranes decreases the diffusion distance for oxygen between the lumen of the heart and mitochondria within cardiac myocytes (O'Brien et al., 2000).

Enhanced oxygen delivery is one important advantage of the unusual mitochondrial morphology in the hearts of *C. aceratus*, but there may be other advantages. Mitochondria are complex organelles containing more than 1500 proteins and are involved in a broad array of cellular functions (McDonald and Van Eyk, 2003). In addition to producing ATP through oxidative phosphorylation, mitochondria also store calcium, regulate apoptosis, produce nitric oxide and oxygen radicals and are sites for the biosynthesis of amino acids and heme (Scheffler, 1999). One or more of these processes might be altered by changes in mitochondrial morphology. For example, oxidative phosphorylation, apoptosis and proton leakage are all affected by mitochondrial architecture (Bach et al., 2003; Brand et al., 1994; Brooks et al., 2007; Mannella, 2006). Future studies should address not only the molecular mechanisms that bring about changes in mitochondrial structure in response to the loss of Hb and Mb but also the physiological impact of these modifications.

The potential role of NO in mediating high mitochondrial densities in the hearts of Antarctic icefishes

The stimulus inducing mitochondrial membrane proliferation in the hearts of Mb-deficient icefish is unknown but appears to be related to the expression of oxygen-binding proteins. Nitric oxide (NO) is a potent signaling molecule that may play a role in this pathway (Sidell and O'Brien, 2006). Two lines of evidence support this hypothesis. First, the hearts of both red- and white-blooded Antarctic notothenioids possess the enzyme nitric oxide synthase, which produces NO (Amelio et al., 2006). Second, NO levels are tightly regulated by both Hb and Mb, which are potent NO dioxygenases that metabolize NO to nitrate (Gardner, 2005). Thus, the hearts of icefishes have the capacity to produce NO but not metabolize it, leading to elevated levels of NO compared with those of red-blooded species. Consistent with this, recent work has found higher levels of the metabolic byproducts of NO – nitrite and nitrate – in the blood plasma of icefishes compared with red-blooded notothenioids (K. Borley and B. Sidell, personal communication).

Observations of the pectoral adductor muscle of *C. rastrispinosus* provide further evidence that mitochondrial membrane biosynthesis is sensitive to the presence of oxygen-binding proteins. The pectoral adductor muscles of all notothenioids examined to date do not express Mb (Sidell et al., 1997). Mitochondria in the pectoral adductor muscle of *C. rastrispinosus*, lacking both Hb and Mb, are enlarged compared with those in the heart ventricle, where Mb is expressed (O'Brien and Sidell, 2000; O'Brien et al., 2003). In fact, mitochondria in *C. rastrispinosus* pectoral muscles look strikingly similar to those found in the pectoral adductor and heart ventricle of *C. aceratus*, which lack Hb and Mb (O'Brien et al., 2003). This suggests that mitochondrial morphology is not a genetically fixed trait in Antarctic icefish but, rather, is influenced directly by the expression of Hb and Mb or indirectly by NO.

Although previous studies in mammals have determined that NO stimulates PGC-1 α expression and mitochondrial biogenesis (Nisoli et al., 2003; Nisoli et al., 2004), our data indicate that this pathway does not maintain high densities of mitochondria in the hearts of Antarctic icefishes. The mitochondrial biogenic pathway may be refractory to NO in icefishes if levels of NO-sensitive intermediates, such as guanylyl cyclase, are expressed at a lower level compared to red-blooded species. Nevertheless, NO may induce the expression of genes governing the biosynthesis of mitochondrial membranes, and future studies will address this question.

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