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RESEARCH ARTICLE

Coordination of cytochrome *c* oxidase gene expression in the remodelling of skeletal muscle

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SUMMARY

Many fish species respond to low temperature by inducing mitochondrial biogenesis, reflected in an increase in activity of the mitochondrial enzyme cytochrome *c* oxidase (COX). COX is composed of 13 subunits, three encoded by mitochondrial (mt)DNA and 10 encoded by nuclear genes. We used real-time PCR to measure mRNA levels for the 10 nuclear-encoded genes that are highly expressed in muscle. We measured mRNA levels in white muscle of three minnow species, each at two temperatures: zebrafish (*Danio rerio*) acclimated to 11 and 30°C, goldfish (*Carassius auratus*) acclimated to 4 and 35°C, and northern redbelly dace (*Chrosomus eos*) collected in winter and summer. We hypothesized that temperature-induced changes in COX activity would be paralleled by COX nuclear-encoded subunit transcript abundance. However, we found mRNA for COX subunits showed pronounced differences in thermal responses. Though zebrafish COX activity did not change in the cold, the transcript levels of four subunits decreased significantly (*COX5A1*, 60% decrease; *COX6A2*, 70% decrease; *COX6C*, 50% decrease; *COX7B*, 55% decrease). Treatments induced changes in COX activity in both dace (2.9 times in winter fish) and goldfish (2.5 times in cold fish), but the response in transcript levels was highly variable. Some subunits failed to increase in one (goldfish *COX7A2*, dace *COX6A2*) or both (*COX7B*, *COX6B2*) species. Other transcripts increased 1.7–100 times. The most cold-responsive subunits were *COX4-1* (7 and 21.3 times higher in dace and goldfish, respectively), *COX5A1* (13.9 and 5 times higher), *COX6B1* (6 and 10 times higher), *COX6C* (11 and 4 times higher) and *COX7C* (13.3 and 100 times higher). The subunits that most closely paralleled COX increases in the cold were *COX5B2* (dace 2.5 times, goldfish 1.7 times) and *COX6A2* (dace 4.1 times, goldfish 1.7 times). Collectively, these studies suggest that *COX* gene expression is not tightly coordinated during cold-induced mitochondrial remodelling in fish muscle. Further, they caution against arguments about the importance of transcriptional regulation based on measurement of mRNA levels of select subunits of multimeric proteins.

Key words: cytochrome oxidase, environmental physiology, mitochondria, muscle, temperature.

INTRODUCTION

One mechanism by which cells fine-tune ATP production in response to persistent changes in energy demand is through changes in mitochondrial content. There is a general impression that cells possess master controllers of mitochondrial biogenesis to ensure that the diverse genes are regulated in a coordinated manner (Dhar et al., 2008; Puigserver and Spiegelman, 2003; Scarpulla, 2006). For example, biosynthesis of cytochrome *c* oxidase (COX) requires the coordination of COX genes in two genomes: 10 nuclear-encoded subunits (many of which have paralogues) and three subunits encoded by mitochondrial DNA (mtDNA). In addition to these gene regulatory steps, the whole process is influenced by diverse assembly factors that ensure metals, hemes and subunits are incorporated in a stepwise manner (Nijtmans et al., 1998; Ugalde et al., 2002). The regulation of mitochondrial biogenesis is best understood in mammals, where a central role has been argued for a few DNA-binding proteins and coactivators considered to be master regulators (Hock and Kralli, 2009). These include nuclear respiratory factor 1 (NRF-1), NRF-2 and the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Chau et al., 1992; Evans and Scarpulla, 1989; Wu et al., 1999). Their importance in coordination is illustrated in studies where small hairpin (sh)RNA is used to interrupt synthesis of the transcription factor. Inhibition of NRF-1 signalling results in a parallel 50–80% reduction in all nuclear-encoded COX gene expression (Dhar et al., 2008). Likewise, shRNA inhibition of the NRF-2α subunit of NRF-2 results in a parallel 40–70% decrease in all nuclear-encoded COX gene expression (Ongwijitwat et al., 2006).

These transcriptional regulators are present in all vertebrates examined and it would be reasonable to expect that their roles are ubiquitous. However, recent studies have questioned the importance of PGC-1α in coordinating gene expression in fish (LeMoine et al., 2008). This coactivator has experienced an elevated rate of evolution in fish, and has incurred multiple insertional mutations in the domain that interacts with NRF-1 (LeMoine et al., 2010a). In contrast to mammals, several studies on fish have shown that PGC-1α transcript levels decline or fail to change under conditions that stimulate mitochondrial biogenesis (LeMoine et al., 2010b; McClelland et al., 2006; Orczewska et al., 2010). Thus, we asked whether fish might display a loss of the exquisite coordination of mitochondrial genes that typifies the mammalian response to conditions that induce mitochondrial biogenesis.

The model we used to induce mitochondrial biogenesis was cold acclimation. It has been recognized for decades that cold acclimation causes mitochondrial proliferation in skeletal muscles of fish (Egginton and Sidell, 1989; Johnston, 1982; Tyler and Sidell, 1984).

It typically induces an increase in mitochondrial volume (Egginton and Sidell, 1989; Johnston, 1982; Tyler and Sidell, 1984), mitochondrial enzyme activity and transcript levels (Battersby and Moyes, 1998; Itoi et al., 2003; Kikuchi et al., 1999; Kondo et al., 2010). We focused on COX because of its critical role in oxidative phosphorylation (OXPHOS) (Villani and Attardi, 2000), the wealth of knowledge about the function of its individual subunits, and the distinctions between the repertoire of isoforms in fish and mammals (Little et al., 2010). Furthermore, our previous studies have shown that transcripts for COX subunits are present at considerably different levels in muscle (Little et al., 2010), presenting the possibility that a subset of subunits may exert greater control over the rate of synthesis of COX. We assessed transcript levels in three species of minnow (Cyprinidae) exposed to thermal challenges: zebrafish, goldfish and northern redbelly dace. Cold-induced mitochondrial biogenesis is known to occur in temperate cyprinids, so with goldfish and dace we had two independent models that were suited to exploring links between mRNA and COX activity. Furthermore, a previous study suggested that cold acclimation of zebrafish also increases mitochondrial enzyme activity (McClelland et al., 2006). In assaying three species, we hoped to better distinguish between spurious and ubiquitous patterns in subunit mRNA profiles during thermal remodelling of muscle mitochondria.

MATERIALS AND METHODS

Animals and tissue collection

Zebrafish (*Danio rerio*, Hamilton) were obtained from a local pet store in Kingston, ON (Pet Paradise). They were held in the Animal Care aquatic facility at Queen's University in a 140 l aquarium with dechlorinated water at 26°C for at least 2 weeks to acclimate before any experimental manipulations began. The fish were fed daily (Tetramin Tropical Crisps, Tetra, Germany) *ad libitum* and were kept under a 12 h:12 h light:dark photoperiod. Zebrafish were randomly separated into 80 l tanks in groups of 20 and the temperature was adjusted by approximately 1°C daily until reaching a final temperature of either 11 or 30°C. Fish were then held at this temperature for at least 3 weeks. We did not conduct a time course that would enable us to demonstrate that the fish had reached a fully acclimated state.

Dace (northern redbelly dace, *Chrosomus eos*, Cope) were captured from the Queen's University Biological Station in the outflow of Beaver Marsh using minnow traps. This is a shallow waterway where temperatures vary spatially and daily. The winter samples were caught in April 2009 just after ice-out, with 4–6°C water temperature at the time of capture. Summer fish were caught in late August 2009, when water temperature at the time of capture was 25–27°C.

Goldfish (*Carrasius auratus*, Linnaeus) were acclimated to 4 and 35°C, as described in a previous study (LeMoine et al., 2008). COX enzyme activity has previously been reported for these treatment groups (LeMoine et al., 2008). Archived cDNA from that study was used for the RNA analyses in the present study.

Dace were killed by severing the spinal column; zebrafish and goldfish were killed in a solution of 0.4 g l^{-1} tricaine methanesulphonate and 0.8 g l^{-1} sodium bicarbonate, then decapitated. Fish were subsequently dissected and white muscle tissue was flash frozen in liquid nitrogen, powdered under liquid nitrogen and stored at –80°C until needed.

COX enzyme assays

Samples (~50–150 mg) of the powdered white muscle were homogenized on ice with glass homogenizers in 20 volumes of ice-cold extraction buffer (25 mmol l^{-1} potassium phosphate, 1 mmol l^{-1} EDTA, 0.6 mmol l^{-1} lauryl maltoside). Homogenates were diluted 10× in assay buffer (25 mmol l^{-1} potassium phosphate, 0.6 mmol l^{-1} lauryl maltoside) and added to a well of a 96-well plate containing assay buffer and reduced 0.05 mmol l^{-1} cytochrome *c* at 25°C. Absorbance (550 nm) was followed for 3 min in a Molecular Devices Spectromax spectrophotometer (Sunnyvale, CA, USA).

Reduced cytochrome *c* was prepared by dissolving solid horse heart cytochrome *c* in 10 mmol l^{-1} Tris pH 8.0 and reducing it with an equal mass of ascorbic acid. The mixture was placed in dialysis tubing (5000 M_r cut-off) and transferred to 4 l (200–400 volumes) of Tris buffer (10 mmol l^{-1} at pH 8.0) at 4°C. After 8–12 h, the tubing was transferred to fresh Tris buffer (3 times). The reduced cytochrome *c* was stored in aliquots at –80°C.

RNA extraction and cDNA synthesis

RNA was extracted from flash frozen white muscle samples with TRIzol (Invitrogen, Carlsbad, CA, USA) using 1 ml of TRIzol reagent per 50 mg of tissue. The samples were homogenized in TRIzol using a polytron and centrifuged at 1100 ***g*** for 20 min at 4°C. The supernatant was then collected and mixed with 0.2 ml of chloroform per 1 ml of TRIzol. After a brief room temperature incubation to allow the phases to separate, the samples were centrifuged at 1100 ***g*** for 30 min at 4°C. Following centrifugation, the aqueous layer was collected and mixed with 0.5 ml of isopropanol for every 1 ml of TRIzol to precipitate the RNA. RNA was collected by centrifuging at 1100 ***g*** for 3 min at 4°C. The RNA pellet was then washed with 75% ethanol in a volume equal to that of the original TRIzol and re-suspended in RNase-free water. The quality and quantity of RNA present in each extraction were determined using a spectrophotometer.

RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) with 1 μg of RNA in a 20 μl reaction as per the manufacturer's instructions.

Quantitative PCR

Primers were designed for amplicons of 50–200 bp (Table 1). Zebrafish primers were designed from sequence data available from Ensembl. Dace and goldfish primers were designed from consensus sequences of published sequence data of zebrafish and other teleosts. Large fragments were amplified and real-time primers designed from the resulting sequence. We were unable to develop effective primers for *COX8B* in dace or goldfish. All newly designed primers were tested by PCR using an Eppendorf Mastercycler Gradient thermocycler. All reactions were carried out using 2.5 μl of 10× PCR buffer, 0.5 μl of 2 mmol l^{-1} $MgCl_2$ and 0.15 μl of Taq DNA polymerase (Qiagen), with 0.5 μl of 0.01 mmol l^{-1} dNTPs (Promega, Madison, WI, USA), 1.0 μl of each of the primers (diluted to 7.25 μmol l^{-1}), 50 ng of cDNA template and enough sterile double-distilled H_2O to bring the total reaction volume to 25 μl. The PCR runs involved an initial 3 min denaturation at 94°C, followed by 30 cycles of 15 s at 94°C, 30 s at the appropriate annealing temperature and 30 s at 72°C, followed by a final hold of 10 min at 72°C. The resulting PCR products were visualized on 2% agarose gels with ethidium bromide. DNA bands of the appropriate size were excised and extracted from the gel, ligated into a pDrive cloning vector (Qiagen) and transformed into DH5α competent cells (Invitrogen), which were grown on agar plates containing 0.05 mmol l^{-1} IPTG, 0.2 mmol l^{-1} X-gal and 0.130 mg l^{-1} ampicillin. Colonies positive for inserts were grown in LB Broth (Lennox) (Bishop, Burlington, Canada) with ampicillin (50 ng μl^{-1}). Plasmids were isolated with a QIAprep Spin Miniprep Kit (Qiagen), quantified at 260 nm and

Table 1. Primers used for real time PCR quantification of target mRNA in northern redbellied dace, goldfish and zebrafish

Gene	Species	Forward (5′–3′)	Reverse (5′–3′)	(°C)	Source
COX4-1	RD	AGCTTCGCTGAAATGGAAAA	CCTCATGTCCAGCATCCTCT	56	1
	GF	AGGGATCCTGGCTGCACT	TCAAAGGTATGGGGGACATC	59	2
	ZF	CAAGTTTGTGCAGCAGCTG	CAAAGAAGAAGATTCCTGCAAC	61	3
COX5A1	RD	CTTGAAAGCCTGTCGGAGAC	CAAGCTCACTGAGGGTAGGC	56	1
	GF	GGACAAATCTGGCCCACAC	CAATGCCAAGCTCTTCAGGT	59	1
	ZF	AAGCATAGATGTCTACGATTGTGAG	AGGCCAATTAAATAGAACACAAACAC	65	3
COX5B2	RD and GF	TTCCCACAGATGATGAGCAG	CCGCAGTGTTGTCTTCTTCA	59	1
	ZF	GCCCAGCATCAATAACAAACG	AAGCGCTGTACATGGCAGAA	63	3
COX6A2	RD and GF	CTGGAAGATCCTGTCCTTCG	AATGCGGAGGTGTGGATATG	59	1
	ZF	GGCAAACGTTTACCTGAAGATG	TCAGTGATGAGGGCCTTCA	63	3
COX6B1	RD and GF	GAAGATNAAGAACTACAGGACGGC	CTCTGTAGACYCTCTGGTACCA	59	1
	ZF	CACATCACACAGGGAGCGTCA	GCTGTATCTACACCTTTGGCATC	61	3
COX6B2	RD and GF	ATGCCAAAAAGCTCTGGATG	CAGGACAGAGGCAGAGAGA	56 (RD), 59 (GF)	1
	ZF	GAGCCTGTGTCCAATTAGCTG	TGACCTTTCATCCTTCAGCAG	63	3
COX6C	RD and GF	ATGTCACTTGCAAAGCCAGCAATGAG	TTTCCTGGGSTCTGWTACTGTGAACTTGAA	59	1
	ZF	CAAGCGCTTGAGGTTTCAI	GCTGCTTTACTCTCCAGAAGG	59	3
COX7A2	RD and GF	GAACCGTATGAGGAGCCAAA	GGGTTCGTGGACTGTCTGAT	56 (RD), 59 (GF)	1
	ZF	TAGTGCACGCAGACAGCTG	CTGCGTCGAACAATGTGTACA	65	3
COX7B	RD and GF	GATTCTGAGCCACATTCT	ATGCAATGCCTGTTGAAGTG	59	1
	ZF	CAAGGGTATTAGCACGCACA	AACAGCTGTGCAGAATGTGG	59	3
COX7C	RD	GCAGAACCTGCCTTTCTCAG	GACAACGATGAAGGGGAAAG	56	1
	GF	ACCAGGAAAGAACCTGCC	AATCCACTGCCGAAGAACA	50	1
	ZF	GTCGCACAGATCCAACACTTC	CACCATCATGCCAAGAAGC	59	3
COX8B	ZF	TCTTTCACCCTCCTGAGGG	ACGGTGCTTGTAGTCCTCCA	61	3
TBP	RD and GF	AGTGGTGCAGAAGTTGGGTTTTCC	ATGTGTAAGCACCAGGCCCTCTAA	61 (RD), 59 (GF)	1
	ZF	AGCCAAAAGTGAGGAACAGTC	AAATAACTCCGGTTCATAGCTG	59	3
β-*Actin*	RD and GF	TCCAGGCTGTGCTGTCCCTGTA	GTCAGGATCTTCATGAGGTAGTC	61 (RD), 59 (GF)	1
	ZF	CGAGCTGTCTTCCCATCCA	TCACCAACGTAGCTGTCTTCTG	59	4
18S	All	GGCGGCGTTATTCCCATGACC	GGTGGTGCCCTTCCGTCAATTC	61(RD) or 59	5
RPL13α	RD and ZF	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	59	4

RD, northern redbelly dace; GF, goldfish; ZF, zebrafish.
COX, cytochrome *c* oxidase; TBP, TATA-binding protein; RPL13α, ribosomal protein L13α.
Source: [1]this study; [2]LeMoine et al. (2008); [3]Little et al. (2010); [4]Tang et al. (2007); [5]Braun et al. (2009).

then sequenced (Robarts Research Institute, London, Canada). Sequences derived from these plasmids were compared with GenBank and Ensembl databases to ensure the products were from the correct gene product.

Real-time quantitative PCR was conducted on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using FastStart Universal SYBR Green Master Rox (Roche Applied Science, Penzberg, Bavaria, Germany). Reactions were carried out with 50 ng of cDNA template, 0.58 μmol l^{-1} of forward and reverse primer and 12.5 μl of FastStart SYBR master mix in a final volume of 25 μl. Real-time runs consisted of an initial 15 min denaturation at 95°C, then 40 cycles of 15 s at 95°C, 15 s at the appropriate annealing temperature and 36 s at 72°C, followed by a final 15 s at 95°C, 1 min at 60°C and another 15 s at 95°C. All cDNA samples were run in duplicate and compared with a no-template control.

Data analysis

Each primer–template combination was evaluated for amplification efficiency using a range of cDNAs to ensure equivalent efficiencies. The cycle threshold (Ct) values of the real-time data were corrected for any differences in cDNA synthesis efficiency by adjusting the results based on the amplification of housekeeping genes. Housekeeping genes for zebrafish were *TATA-binding protein* (*TBP*), *18S*, β-*actin* and *ribosomal protein L13α* (*RPL13α*). For dace, *TBP* was excluded because it appeared to show a direct response to temperature, and for goldfish, *RPL13α* was excluded. A geometric mean of all housekeeping genes of each sample served as a correction factor for all target genes (Pfaffl et al., 2004). That is, the experimental genes were normalized against housekeeping genes, which should be present in equal amounts across all samples, to ensure that observed differences in Ct values were the result of differing gene expression in relation to the environment as opposed to differing efficiencies in the reverse transcription reaction. The resulting data sets were scaled relative to warm/summer data a value of 1 and analysed using Student's *t*-test ($P<0.05$). Enzyme data were analysed as absolute rates using Student's *t*-test ($P<0.05$).

To assess which subunit mRNAs changed in parallel with COX, we used a bootstrap approach. For each subunit, we resampled the observed data to generate the potential distribution of individual responses derived from the ratio of each mRNA in cold fish to each mRNA in warm fish. This ratio (log transformed) was compared with the effects of temperature on COX activity (mean cold COX activity/mean warm COX activity, log transformed). Values were then compared with the expected value of 1, which would arise if mRNA and COX changed stoichiometrically.

RESULTS AND DISCUSSION

The mitochondrial proliferation seen in fish in response to cold temperature is an intriguing phenomenon that raises questions about the underlying nature of the cellular signalling mechanisms that communicate energy state to the nucleus, and the mechanisms by which transcriptional responses are coordinated. The goal of this study was to assess the extent to which expression of nuclear-encoded *COX* genes was coordinated during mitochondrial remodelling. Our observations were confined to measuring transcript levels. Though this does not equate to gene expression (i.e. mRNA synthesis) or subunit synthesis (i.e. monomer levels), measurement of steady-state mRNA allows us to survey how gene regulation

responds to physiological or environmental change. In addition to assessing the extent of genetic coordination in fish, the data also contribute to the development of hypotheses about the relative importance of individual subunits in determining the rate of COX synthesis. Finally, the heterogeneity we see also provides a cautionary note about using mRNA measurements of select gene products to assess the impact of transcriptional processes on the synthesis of multimeric proteins.

Changes in COX activity

We used three different species of minnow (Cyprinidae) to assess how *COX* genes responded to temperatures that approach the lower thermal limits of the fish (Fig. 1). In a previous study, zebrafish acclimated to 18°C showed a trend towards increased mitochondrial biogenesis (McClelland et al., 2006), so we thought that even lower temperatures would create a more pronounced response. We found that thermal acclimation did not affect COX activity in zebrafish; however, the data serve to illustrate how much COX subunit transcripts can vary without consequence to COX activity. Cold acclimation led to an increase in COX activity in both dace (2.9 times, $P<0.05$) and goldfish (2.5 times, $P<0.05$), consistent with the mitochondrial enzyme changes seen in other species including rainbow trout (Milanesi and Bird, 1972; Battersby and Moyes, 1998) and common carp (Cai and Adelman, 1990). Our assays for COX measured catalytic activity, which we think reflects differences in COX content; the presence of 0.6 mmol l^{-1} lauryl maltoside in the extraction and assay buffers would mitigate the potential impact of differences in membrane lipids. The actual *in vivo* activities may differ as a result of thermal-induced changes in membrane lipids (see Guderley and St-Pierre, 2002) but the assay we employed should reflect differences in COX holoenzyme levels between treatment groups.

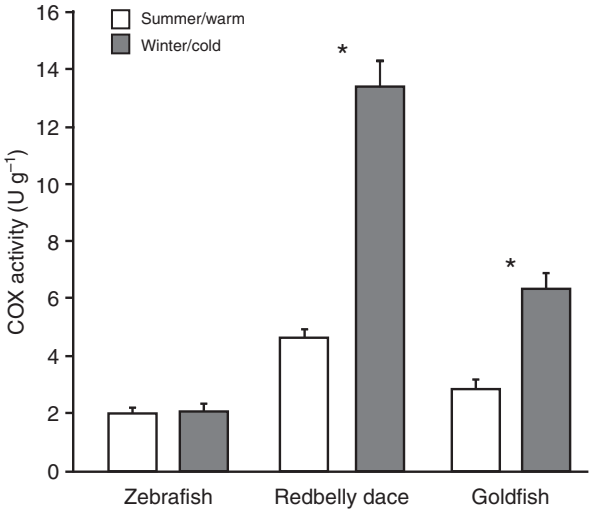

Fig. 1. Relative COX enzyme activity in white muscle of warm- and cold-treated fish. Zebrafish were acclimated to warm (30°C) or cold (11°C) temperatures. Dace were collected from the field in August (summer) and April (winter). Goldfish were acclimated to warm (30°C) or cold (5°C) temperatures. Significant differences between acclimation states of a species (Student's *t*-test, $P<0.05$) are indicated by an asterisk. $N=7$ (warm) or 8 (cold) for each zebrafish temperature condition; $N=7$ for each redbelly dace sampling season; $N=6$ for goldfish [from LeMoine et al. (LeMoine et al., 2008)]. Data are presented as means + s.e.m.

Transcript levels for nuclear-encoded COX subunits in each species

In a previous study on zebrafish (Little et al., 2010), we measured the transcript levels for all nuclear-encoded COX subunits in muscles (white muscle, heart), liver, brain and gills. In the present study on muscle, we focused on the single dominant subunit for each isoform, with the exception of COX6B, where transcripts of two paralogues (*COX6B1* and *COX6B2*) were present at near-equivalent levels in warm zebrafish.

In zebrafish, COX activity was unaffected by temperature but transcript levels for several COX subunits differed between treatment groups (Fig. 2). *COX5A1*, *COX6A2*, *COX6C* and *COX7B* transcript levels were all significantly lower in the cold ($P<0.05$). Transcript levels for *COX6B1* and *COX7A2* appeared to be 1.5–2 times higher in cold fish, though the differences were not significant.

Though winter-acclimatized dace demonstrated 2.9 times higher COX activity, transcript levels of *COX6B2* were reduced by about half and *COX7B* mRNA tended to decrease but not significantly (Fig. 2). Other COX transcripts increased in abundance in winter fish but to markedly different extents. The mRNA in winter fish was more than 10 times greater for *COX4-1* (21.3 times) *COX5A1* (13.9 times), *COX6C* (11 times) and *COX7C* (13.3 times). The remaining subunits increased significantly by 6 times or less: *COX5B2* (2.5 times), *COX6A2* (4.1 times), *COX6B1* (6 times) and *COX7A2* (1.7 times).

In goldfish, cold acclimation led to an increase in COX activity (2.3 times) and increases in transcript levels of at least 5 times for the following transcripts: *COX4-1* (7 times), *COX5A1* (5 times), *COX6B1* (10 times) and *COX7C* (100 times). Other subunits significantly increased but to a lesser extent: *COX5B2* (1.7 times) and *COX6C* (4 times). There was no significant change in the levels of transcripts for *COX6A2*, *COX6B2*, *COX7A2* and *COX7B*.

Apart from assessing which gene products showed a significant change, we were also interested in determining which showed patterns that best reflected the enzyme response. In other words, which subunits experienced a change in mRNA level that paralleled the change in enzyme activity? The bootstrap analysis compared each mRNA value in cold/winter fish with each mRNA value in warm/summer fish. The resulting values were then compared with the change seen in COX activity, generating a potential distribution of ratios of mRNA and enzyme. (A ratio of 1 means that the subunit changed to the same magnitude as the enzyme.) The bootstrap analysis (Fig. 3) identified two genes, *COX5B2* and *COX6A2*, as showing a transcript pattern that in both species closely reflected changes in COX activity.

Coordination of of *COX* gene expression

The complexity of the organelle requires that changes in mitochondrial content involve the coordinated regulation of membrane lipid synthesis, mtDNA replication and protein synthesis in both the cytoplasm and the mitochondria (Moyes and Hood, 2003). Even when restricting discussion of mitochondrial biogenesis to synthesis of an enzyme such as COX, the pathways are inherently complex (Herrmann and Funes, 2005; Nijtmans et al., 1998; Ugalde et al., 2002). Coordination of transcription is enabled by proteins that regulate promoter activity and chromatin remodelling. For the genes encoding proteins of OXPHOS complexes, including COX, two DNA-binding proteins (NRF-1, NRF-2) and the coactivator PGC-1α are paramount in coordinating expression (Czubryt et al., 2003; Puigserver and Spiegelman, 2003; Wu et al., 1999). However, the extent to which these genes are tightly coordinated remains untested. Studies with shRNA have shown that disruption of either

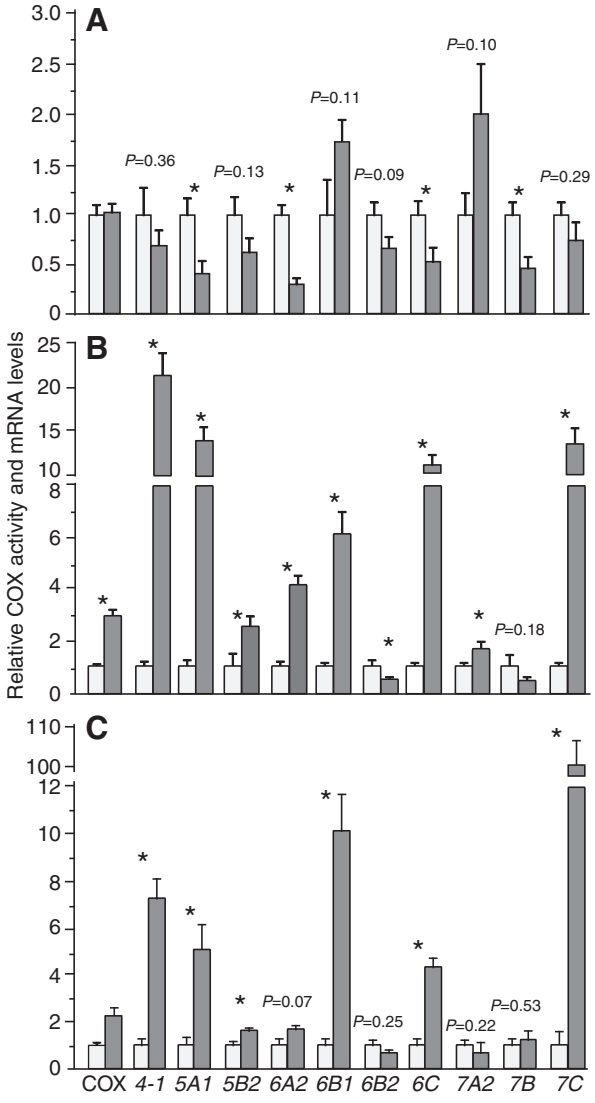

Fig. 2. Transcript levels for nuclear-encoded *COX* genes in white muscle in relation to environmental temperature for (A) zebrafish, (B) dace and (C) goldfish; COX enzyme activity is included for reference. Values are expressed relative to warm water fish (open bars). Significant differences between acclimation states (Student's *t*-test, $P<0.05$) are indicated by an asterisk. $N=6$ for each temperature condition. Data are presented as means + s.e.m.

NRF-1 or NRF-2 synthesis results in near-parallel reductions in transcript levels for all nuclear-encoded COX subunits (Dhar et al., 2008; Ongwijiwat et al., 2006). Consistent with these loss-of-function treatments, treatments that increase COX activity are also accompanied by near-parallel increases in COX subunit mRNA (e.g. Kim et al., 1995). However, there has not been a study where the synthesis of each subunit is incrementally increased to assess the effects on COX activity. Thus, it is not clear whether a coordinated increase in all *COX* genes is required to increase steady-state levels of COX.

We expected that the coordination typified in mammalian models would also be seen in these fish models; we expected to see no changes in zebrafish subunit mRNA where COX activity did not change, and parallel increases in transcripts and COX activity in cold-acclimated goldfish and winter-acclimatized dace. Instead, we saw very poor coordination between COX activity and *COX* transcripts, and remarkable variation in transcript patterns between subunits. In zebrafish, where cold acclimation did not lead to a change in COX activity, four transcripts declined in abundance by as much as 70%. In the two species that showed cold-induced mitochondrial enzyme increases, several genes showed no significant increase in transcript levels (dace: *COX6B2* and *COX7B*; goldfish: *COX6A2*, *COX6B2*, *COX7A2* and *COX7B*). Of the genes with transcripts that increased in the cold, most increased much more dramatically than did COX activity: *COX4-1* increased 7–21 times, *COX6B1* increased 6–10 times and *COX7C* increased 14–100 times.

It is possible that *COX* gene expression, which is well coordinated in mammals, is simply less coordinated in fish. The generally poor coordination amongst *COX* genes may be a function of a fundamental difference in the roles of the transcriptional master regulators in fish. In mammals, PGC-1α is thought to play the role of master regulator of mitochondrial biogenesis in tissues (Puigserver and Spiegleman, 2003). PGC-1α transcript levels have been measured in a number of fish studies assessing its role as a master regulator of mitochondrial differences arising between tissues, such as muscle fibre types in goldfish (LeMoine et al., 2008), and physiological states, such as cold acclimation in goldfish (LeMoine et al., 2008) and three-spine stickleback (Orczewska et al., 2010), and exercise training in zebrafish (McClelland et al., 2006). There is some evidence that the *PGC-1α* gene in fish experienced mutations that may have altered its capacity to interact with NRF-1. The NRF-1 binding domain of *PGC-1α* displays a high rate of evolution and possesses large insertional mutations (LeMoine et al., 2010a). However, there is better support for a role for NRF-1 as a master regulator in fish. Previous studies have shown a fairly close relationship between *NRF-1* mRNA level and mitochondrial changes (LeMoine et al., 2008; Orczewska et al., 2010).

Potential determinants of COX synthesis

By any standard, COX is a complex enzyme. The 13 subunits are encoded by two genomes (three mitochondrial, 10 nuclear). The expression of the genes involves the coordination of expression of individual genes and genome-wide structural regulation (Dhar et al., 2009). Once subunits are translated and imported, they are assembled into a holoenzyme that includes haem units (a, a_3), and metals (Cu_A, Cu_B). The assembly process is mediated by a series of chaperones that aid in the delivery of building blocks and assembly into the holoenzyme (Fontanesi et al., 2006). The assembly process consists of four steps yielding the subcomplexes S1–S4 (Nijtmans et al., 1998; Ugalde et al., 2002). S1 consists solely of COX1. S2 is formed upon the addition of COX4, two haem molecules and Cu_B. S3 is formed after the addition of the remaining catalytic subunits COX2 and COX3 as well as most of the remaining nuclear-encoded subunits. The assembly of subunits COX6A and COX7B completes the COX monomer (S4) and it need only dimerize to become active. Though it is clear that each of the subunits is required for proper COX assembly and function (unless replaced by alternative isoforms), it is not entirely obvious whether one or more steps of the process may limit the rate of COX synthesis. It is generally held that the mtDNA-encoded subunits are present in excess, and that COX synthesis is regulated by the nucleus, which can integrate cellular signals to control gene expression (Kadenbach et al., 2000). Whether one or more of the genes exerts greater control over the process is not yet clear.

COX synthesis and assembly are also controlled by factors that do not form part of the final structure. There are numerous accessory proteins implicated in COX synthesis and function (Fontanesi et

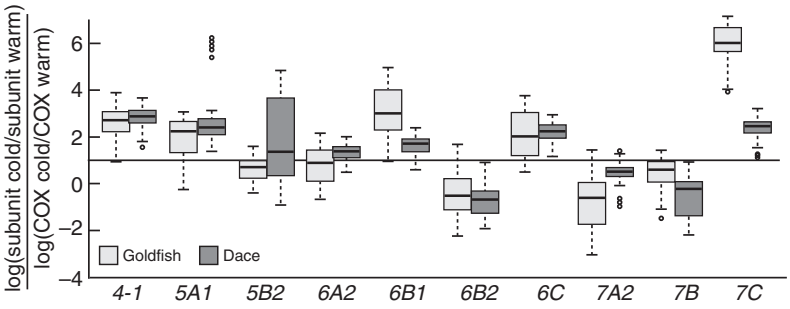

Fig. 3. Boxplots of the bootstrap response of COX subunit mRNA compared with observed changes in COX activity. Bars show the mean observed ratios, with boxes defining the 25th and 75th percentiles and whiskers spanning the lowest and highest data points within 1.5 interquartiles of the lower and upper quartiles. Circles show individual points that fall outside this range. The data sets include 36 ratios (6×6) for goldfish and 49 (7×7) for dace.

al., 2006). COX10 and COX15 are not part of the final COX holoenzyme but are crucial to the synthesis of the haem molecules (Tzagoloff et al., 1993). Surf1 and its homologue Shy1 are crucial for the translation of COX1 (Mick et al., 2007). Several proteins, notably SCO1 and SCO2, are involved in the delivery of copper ions to the COX complex (Leary et al., 2004; Leary et al., 2007). COX synthesis is also influenced by cytochrome *c* (Pearce and Sherman, 1995) and F_1F_O-ATPase (Rak et al., 2007). COX assembly intermediates can accumulate under some conditions, suggesting that the assembly process can stall if subunit availability is compromised (Fornuskova et al., 2010). There is also some capacity to respond to subunit mismatches and subcomplex accumulation through feedback mechanisms. Unassembled COX1 interacts with Mss51 and COX14; the presence of this aggregation appears to halt the translation of new COX1 until the existing protein has been assembled with other subcomplexes in the path to holoenzyme formation (Barrientos et al., 2004).

In any multistep process, some steps will be more important in determining the overall rate. In the case of metabolic pathways, quantitative control analyses can assign a step a control coefficient, or, in more qualitative terms, some enzymes are considered to be 'rate limiting' or 'rate determining'. These steps are often the slowest, and are typically subject to other forms of metabolic regulation. It is reasonable to speculate that some COX subunits may be more limiting than others and, if so, may have a greater role in determining changes in COX activity. By analogy with metabolic pathways, rate-determining subunits might be those where changes in mRNA level best paralleled COX activity. Many studies have used molecular genetic interventions to impose a reduction in the synthesis of individual subunits to assess the effects on COX biosynthesis. For example, dramatic reductions in COX arise when *COX5A* is disrupted using morpholinos in zebrafish (Baden et al., 2007) and RNAi in *Caenorhabditis elegans* (Suthammarak et al., 2009). Likewise, loss of COX activity is seen when COX6A synthesis is disrupted in a mouse knockout (Radford et al., 2002), *Drosophila* mutants (Liu et al., 2007) and human cell line knockouts (Fornuskova et al., 2010). Reductions in COX4 lead to a decrease in COX content in mammalian lines (Li et al., 2006; Fornuskova et al., 2010) and *C. elegans* (Suthammarak et al., 2009). In some cases, this intervention leads to the accumulation of assembly intermediates and enzymatic abnormalities (Fornuskova et al., 2010). Such studies are used to assess the importance of subunits in assembly or function but they are not intended to explore constraints on the rate of synthesis during adaptive remodelling. The question of whether any of these genes exert disproportionate control over COX synthesis has not been addressed experimentally. This study gives an indication of the sensitivity of COX synthesis to variation in subunit expression. Thus, we consider these data to provide some insight into which of the subunits is most likely to determine the patterns in COX activity.

For a gene to play a crucial role in determining the rate of synthesis of a subunit, we would expect that (i) decreases in mRNA would result in reduced COX activity, and (ii) increases in mRNA would accompany parallel increases in enzyme activity. One gene failed these criteria in all three species; *COX7B* displayed significantly lower transcript levels in cold zebrafish (where COX activity did not change) and failed to increase in either goldfish or dace, where COX activity increased 2.5–2.9 times. Transcript abundance for one gene failed to increase in the two species that showed an increase in COX activity in the cold: *COX6B2* mRNA levels decreased significantly in dace and did not change in goldfish. One gene, *COX7A2*, showed a minor (60%) increase in transcript abundance in cold-acclimated dace but no change in goldfish. Thus, for these three subunits, there is little support for the argument that they play a disproportionate role in determining COX biosynthesis.

Of the remaining seven subunits we studied, some appear to better correlate with COX activity patterns than others. Five subunits showed the same general pattern, though they differed quantitatively. *COX4-1*, *COX5A*, *COX6B1*, *COX6C* and *COX7C* transcripts each showed an increase in dace and goldfish that markedly exceeded the change in COX activity. COX4 and COX5A are among the first of the nuclear-encoded subunits to be assembled into the COX enzyme complex, producing S2 (Nijtmans et al., 1998; Ugalde et al., 2002). The other three subunits are folded into the holoenzyme in the second step, forming S3 (Nijtmans et al., 1998; Ugalde et al., 2002). Of these five gene products, four are amongst the least abundant COX subunit transcripts. *COX5A* is one of the most abundant transcripts amongst the COX subunits in mammals (Nijtmans et al., 1998; Ugalde et al., 2002) and zebrafish (Little et al., 2010). Transcripts for *COX4-1*, *COX6B1*, *COX6C* and *COX7C* are among the least abundant of the subunits in zebrafish muscle (Little et al., 2010).

Both of the remaining subunits, *COX5B2* and *COX6A2*, showed a pattern that very closely reflected the changes in COX activity (Figs 2 and 3). COX5B is incorporated into the COX assembly pathway prior to the formation of S3 and COX6A is incorporated into the holoenzyme in the final step of assembly, resulting in the formation of S4 (Nijtmans et al., 1998; Ugalde et al., 2002). For dace, COX activity increased 2.9 times, and transcript level increased for both *COX5B2* (2.5 times) and *COX6A2* (5 times). For goldfish, COX activity increased 2.3 times, and transcript level increased for both *COX5B2* (2.5 times) and *COX6A2* (1.7 times, P=0.07). The close relationship between these transcripts and enzyme abundance could suggest that they exert greater control over the synthesis of COX. In zebrafish, *COX5B2* transcript level did not change but there was a 70% decrease in *COX6A2* transcript level without consequence

to COX enzyme activity, suggesting the mRNA for this subunit is in at least modest excess of demands.

Temperature-sensitive changes in transcript levels

In addition to generating hypotheses about potentially rate-limiting subunits, the data can also be used to identify genes that might be responsive to temperature. Though temperature has pervasive effects on metabolic, biochemical and genetic processes, the mechanisms by which temperature exerts specific effects on individual genes is far from clear. In this study, we identified genes that demonstrate a dramatic response to decreased temperature, particularly *COX4-1* (7–21 times increase) and *COX7C* (12–100 times increase). Furthermore, the COX6B paralogues appear to be reciprocally sensitive to temperature. Low temperature caused an increase in *COX6B1* and a decrease in *COX6B2* in all three species. This subunit forms a bridge between COX monomers, binding with the cytosolic domains of COX2 and COX3 in the adjacent monomer (Tsukihara et al., 1996). In mammals, the two isoforms appear to differ in their effects on cytochrome *c* binding, influencing the turnover rate of the enzyme. The mammalian paralogues are tissue-specific variants, with a testes-specific subunit that may be specialized to work with a testes-specific cytochrome *c* (Hüttemann et al., 2001; Weishaupt and Kadenbach, 1992). The evolutionary relationships between the two fish paralogues and the two mammalian paralogues are not clear (Little et al., 2010). If the fish variants differentially affect COX efficiency, as is the case with the mammalian variants, this might have important ramifications for metabolism in the thermal remodelling of muscle.

Caveats for measuring mRNA levels for multimeric proteins

Many studies use mRNA analyses to explore the relative importance of transcriptional regulation in the adaptive remodelling of tissues in response to challenges and stressors. However, it is important to acknowledge that the conclusions of mRNA analyses may be complicated by many factors, particularly for multimeric proteins such as COX.

It is widely appreciated that measurement of mRNA levels is not synonymous with gene expression, which refers specifically to mRNA synthesis. The levels of mRNA can change independently of synthesis rates through effects on degradative processes. Translation is usually a stochastic process where a mRNA collects ribosomes in a manner that reflects its concentration. However, the propensity for protein synthesis can be affected by signalling pathways that generally increase or decrease translation initiation. A specific mRNA can be rendered impotent through changes in secondary structure or through interactions with proteins that prevent translation. Thus, there are many situations where mRNA levels can change independent of gene expression and with unpredictable effects on protein synthesis.

Superimposed on these caveats are additional issues that affect the interpretation of transcriptional control of multisubunit enzymes. Had we measured only *COX6B2* or *COX7B* transcript, we would have been at a loss to explain how COX activity increased. Had we measured only *COX4-1* or *COX6B1* transcripts, we would have had to explain why COX activity changed less than expected. Only upon measurement of mRNA for many subunits does the complexity of the process emerge. Our approach neglected measurement of the mtDNA-encoded subunits and we made no attempt to measure the potential influence of the numerous chaperones and assembly factors that are crucial for COX biosynthesis. With these caveats in mind, the collective data point to the importance of transcriptional regulation in the control of COX synthesis; however, the mechanisms by which these processes are orchestrated in fish appear significantly different from the picture emerging from mammalian studies.

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