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RESEARCH ARTICLE

mRNA degradation: an underestimated factor in steady-state transcript levels of cytochrome *c* oxidase subunits?

Katharina Bremer* and Christopher D. Moyes

ABSTRACT

Steady-state mRNA levels are determined by synthesis and degradation; however, changes in mRNA levels are usually attributed to transcription. For cytochrome *c* oxidase (COX), cold acclimation typically leads to an increase in COX activity while transcript levels for the nuclear-encoded subunits change non-stoichiometrically. Whether those patterns are caused by differences in subunit transcription rates, decay rates or both was not known. We assessed decay rates of transcripts for COX subunits, including representatives that decreased, increased in parallel with COX or increased in excess of COX. Low temperature reduced the decay rate of all transcripts; however, COX subunits displayed higher thermal sensitivity than housekeeping genes. The lower decay rates for COX transcripts might explain some of their increase in response to cold acclimation. The reason for the exaggerated transcript response of two subunits (COX6B-1 and COX7A-2) may be due to decreased decay. However, decay rate differences could not explain the patterns seen with another subunit that did not change in mRNA level with thermal acclimation (COX6A-2). Further, the decay patterns differed between two thermal acclimation experiments, which may explain some of the heterogeneity seen in fish studies. The differences in decay rates suggest that the lack of stoichiometry in mRNA levels is exacerbated by post-transcriptional mechanisms. Collectively, these results suggest that temperature-induced differences in COX subunit mRNA levels and deviations from stoichiometry between them may partially arise from subunit-specific sensitivities to degradation. We suggest that all subunits are controlled by transcription, and that exaggerated responses of some subunits are due to reduced decay rates.

KEY WORDS: COX activity, Fish, Post-transcriptional control, Thermal acclimation

INTRODUCTION

Measurements of mRNA levels are a widely used approach to investigate gene expression in a variety of experimental contexts, from mechanistic molecular genetics to ecological and evolutionary frameworks. Although steady-state transcript levels within a cell are determined by rates of synthesis (transcription) and degradation, changes in mRNA levels are usually inferred to arise from changes in synthesis.

Transcription rates change when promoter activity is altered because of chromatin remodeling or binding of transcriptional regulators (DNA-binding proteins and co-regulators), which collectively affect the recruitment of the general transcriptional machinery and initiation of transcription. Once the precursor mRNA is made, it must be processed (i.e. splicing, polyadenylation, capping) and exported to the cytoplasm. There, many post-transcriptional factors influence whether a specific mRNA enters translation. Such factors are mRNA surveillance mechanisms (e.g. nonsense mediated mRNA decay, nonstop mediated mRNA decay and no-go decay), various decay pathways (i.e. exo- or endoribonucleases) and stability controls [e.g. adenylate-uridylate (AU)-rich elements, poly(A)-binding proteins] (see Garneau et al., 2007).

Apart from the factors that affect mRNA levels, there are other post-transcriptional controls that alter the ability of specific transcripts to be translated. For example, in the pathway of RNA interference, some microRNAs (miRNA) function as gene silencers by binding target mRNAs and preventing translation or initiating degradation (see Valencia-Sanchez et al., 2006). Thus, the level of a specific mRNA may remain high but result in little translation because of the binding of regulators. Such mechanisms help explain the apparent loss of stoichiometry when mRNA and protein levels change incongruently (see Suarez and Moyes, 2012).

For many applications, the underlying cause of a change in transcript level is of less interest than the fact that the change has occurred. Conversely, transcript levels are often used to infer a change in gene regulation, and thus changes in transcript levels are also attributed to changes in transcription. Even with single genes, it is difficult to quantitatively link the degree of gene activation (i.e. mRNA synthesis) to mRNA accumulation, and thus these two parameters are often discussed in qualitative terms. However, when profiling transcripts of multimeric proteins and complex pathways, there is an underlying assumption that transcript levels should change in parallel to transcription. During mitochondrial biogenesis, described in mammals under exercise (Hawley and Holloszy, 2009), electrical stimulation (Baar et al., 2002) and cold exposure (Puigserver et al., 1998; Wu et al., 1999), a network of genetic master regulators is thought to coordinate the transcription of genes encoding the five complexes (I–V) of the electron transport chain (ETC) (Hock and Kralli, 2009).

When mitochondrial biogenesis is induced in fish through cold acclimation, the mRNA levels of the various subunits of complex IV [cytochrome *c* oxidase (COX)] of the ETC lack any stoichiometry (Duggan et al., 2011). Under conditions that caused an increase in COX activities, mRNA levels for some subunits did not change at all (e.g. COX6B-2), while others changed in parallel with COX activity (COX5B-2 and COX6A-2), and several showed an exaggerated response (e.g. COX4-1 and COX7C). The potential influence of subunit-specific degradation rates on COX transcript profiles has not been well studied, and not in the context of thermal acclimation in an ectothermic animal. To date in mammals there is some evidence for a role of mRNA degradation in the control of COX4 (Zhang and Wong-Riley, 2000), and a role for miRNA in the control of nuclear-encoded COX genes [miRNA-338 (Aschrafi et al., 2012)], mitochondrial-encoded COX genes [miRNA-181c (Das

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et al., 2012)] and COX assembly [miRNA-210 (Colleoni et al., 2013)].

In this study, we investigate (1) the impact of thermal acclimation on mRNA degradation, and how degradation might contribute to (2) changes in steady-state transcript levels with temperature and (3) loss of mRNA stoichiometry between subunits. We use a paradigm of thermal acclimation of goldfish [*Carassius auratus* (Linnaeus)] to assess the role of transcript-specific mRNA degradation as a potential explanation for the nonstoichiometric changes in the mRNA of the various subunits. The results of this study not only elucidate the transcriptional control of COX in fish but also shed light on mRNA control in ectotherms.

RESULTS

This study contrasts patterns seen in two thermal acclimation experiments: 32°C versus 4°C (32vs4) and 20°C versus 4°C (20vs4). The goal was to assess whether the differences seen in stoichiometries between COX activity and transcript levels can be attributed in part to differences in mRNA degradation between gene products or between experiments.

Steady-state enzyme activities and transcript levels

The first question of this study was whether temperature-induced changes in COX activity correlated with changes in COX subunit mRNA. In the 32vs4 experiment, COX activity was 4.5-fold higher in the cold-acclimated fish (t_{10}=4.53, P=0.001; Fig. 1A). The mRNA for half of the 14 subunits statistically paralleled COX activity (COX1, COX2, COX3, COX4-1, COX5B-2, COX6B-2 and COX6C; Table 1). Of the remaining seven subunits, four showed a thermal response greater than that shown by COX (COX5A-1: 9.5-fold higher in the cold; COX6B-1: 10.6-fold; COX7A-2: 15.6-fold; and COX7C: 9.2-fold). The changes in mRNA for three subunits were significantly lower than that seen for COX activity and were not affected by temperature (COX4-2, COX6A-2 and COX7B; Table 1).

In the 20vs4 experiment, COX activity was not different between the two acclimation groups (t_{10}=0.93, P=0.374; Fig. 1B). The numerical explanation for the discrepancy in the responses of COX activities between the two experiments is that in the 20v4 experiment the warm-acclimated fish had twofold higher COX activity than the 32°C-acclimated fish (1.74±0.45 versus 0.86±0.26 U g^{-1} tissue, mean ± s.d.) and the cold-acclimated fish of the 20vs4 experiment had a 60% lower COX activity than the cold-acclimated fish of the 32vs4 experiment (1.50±0.21 versus 3.79±1.24 U g^{-1} tissue). The physiological explanation for these different responses in the two experiments is not known, but this study represents an opportunity to explore the potential role of mRNA decay in explaining unexpected patterns.

In the 20vs4 experiment, a subset of four COX subunits (COX4-1, COX5B-2, COX6A-2 and COX7C) was investigated. This selection reflects subunits that paralleled COX activity (COX4-1 and COX5B-2), did not change with temperature (COX6A-2) or showed an exaggerated response (COX7C) in their fold change in mRNA in the 32vs4 experiment. However, in the 20vs4 experiment, the fold change in mRNA for each of these subunits paralleled COX activity (which did not change) and as such did not display a significant temperature response (Fig. 1B, Table 1). However, there was a trend apparent with COX4-1, COX5B-2 and COX7C, each tending to increase 1.5- to 2.0-fold in the cold-acclimated compared with the warm-acclimated fish (Table 1), an effect that was significant before correcting for multiple comparisons using false discovery rate (FDR).

Table 1. Results of unpaired *t*-tests for differences between steady-state mRNA level ratios and cytochrome *c* oxidase (COX) activity ratios of cold over warm values

Gene	d.f.	*t*	*P*	Q_{BH}
32°C versus 4°C				
COX1	15	0.84	0.412	0.536
COX2	15	1.78	0.096	0.178
COX3	15	0.28	0.783	0.848
COX4-1	18	1.11	0.280	0.404
COX4-2	15	3.86	0.002	0.013
COX5A-1	15	2.66	0.018	0.039
COX5B-2	15	0.02	0.985	0.985
COX6A-2	n.a.	n.a.	n.a.	n.a.
COX6B-1	15	3.51	0.003	0.013
COX6B-2	15	1.57	0.137	0.223
COX6C	15	0.49	0.629	0.743
COX7A-2	15	3.60	0.003	0.013
COX7B	15	2.72	0.016	0.039
COX7C	15	2.90	0.011	0.036
20°C versus 4°C				
COX4-1	12	2.41	0.033	0.065
COX5B-2	12	2.18	0.049	0.065
COX6A-2	12	1.38	0.192	0.192
COX7C	12	2.73	0.018	0.065

Results are given for both acclimation experiments (32°C versus 4°C and 20°C versus 4°C). n.a., not applicable.

Thermal sensitivity of mRNA decay rates

Based on the mRNA patterns seen in the 32vs4 experiment, we selected a group of COX subunits to investigate the impact of degradation on their steady-state transcript levels. We chose one subunit that did not respond to temperature (COX6A-2), two that appeared to change in parallel with COX activity (COX4-1 and COX5B-2) and three that responded to the low temperature in excess of the change seen in COX activity (COX6B-1, COX7A-2 and COX7C). We included β-actin and elongation factor 1α (EF-1α), the two housekeeping genes used for determining the steady-state levels of mRNA for the COX subunits.

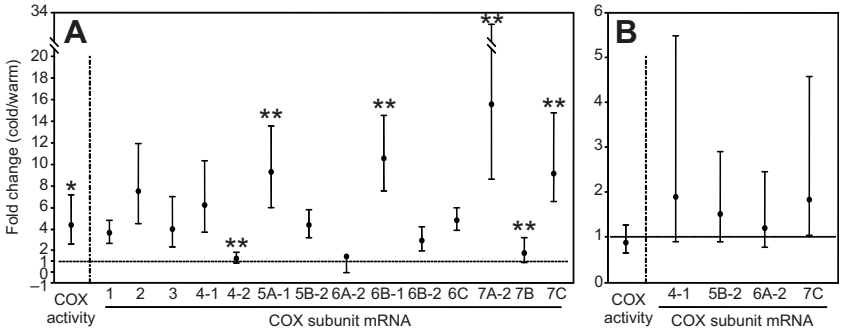

Fig. 1. Steady-state cytochrome *c* oxidase (COX) activity ratios and transcript level ratios of COX subunits in white muscle after thermal acclimation. Goldfish were acclimated to 32°C and 4°C (A) and 20°C and 4°C (B). Error bars represent 95% confidence intervals (CI). Single asterisk indicates a significant difference (*P≤0.05) of the COX activity ratio from 1. Double asterisks indicate significant differences [false discovery rate (FDR)≤5%] of mRNA ratios from COX activity. COX activities and COX4-1 transcript levels of the 32vs4 experiment are obtained from a previous study (Bremer et al., 2012).

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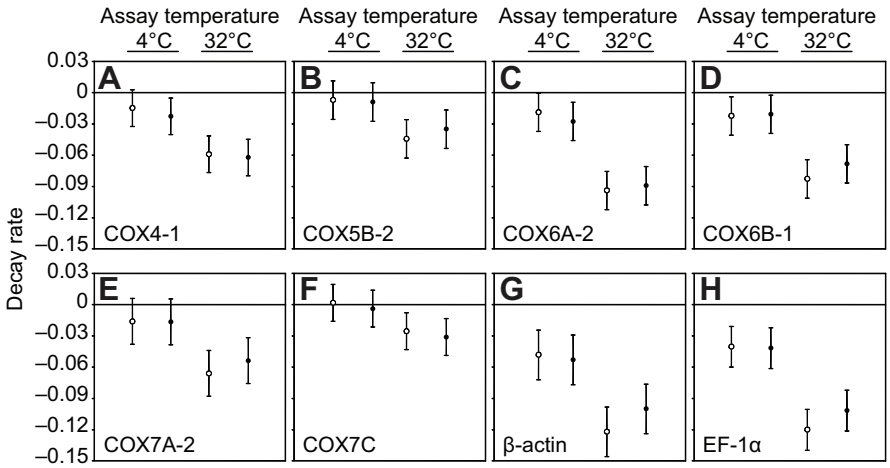

Fig. 2. Average decay rates measured at 32°C and 4°C in 32°C- and 4°C-acclimated fish. Decay rates of 32°C-acclimated fish (closed circles) and 4°C-acclimated fish (open circles) were measured for six COX subunits (A–F), and two housekeeping genes (G,H). Error bars represent approximate 95% CI. The horizontal line indicates a zero decay rate.

When considering the impact of temperature on fish *in vivo*, there is the potential for mRNA decay kinetics to be affected by both holding temperature and thermal history. In other words, the decay rates seen in cold- and warm-acclimated fish *in vivo* would be affected by both thermodynamic effects on decay pathways and changes in the machinery that controls mRNA decay as part of acclimation-dependent remodelling. Experimentally, the thermodynamic effects can be assessed by assaying each acclimation group at both temperatures. Changes in the machinery arising from acclimation would be reflected in differences in the thermal sensitivities of each group. The combination of these thermodynamic and acclimation effects would be reflected in a comparison of decay rates of each fish at its respective acclimation temperature: warm-acclimated fish assayed at 32°C (or 20°C) versus cold-acclimated fish assayed at 4°C.

When investigating mRNA degradation rates, the change in total RNA should be taken into account as a potential decrease in total RNA per gram tissue would underestimate the decay rates for each target gene. In our experiment, total RNA decreased by up to 20% over the duration of the experiment. Because we are using a fixed amount of RNA in the reverse transcription reaction (see Materials and methods), the measurement of RNA level must be adjusted to compensate for the loss of RNA per gram tissue in order to correctly express the changes in target RNA per gram tissue. Failure to make this correction would lead to an underestimation of RNA decay rates.

In this experiment, decay rates were higher (i.e. larger negative values) at the 32°C assay temperature than at the 4°C assay temperature (Fig. 2, Table 2). This assay temperature effect was the same for both acclimation groups (assay temperature × acclimation temperature) and this was consistently observed across all genes (assay temperature × acclimation temperature × gene) (Table 2). These findings suggest that the acclimation history of the fish did not have an impact on the degradation machinery for all genes. The magnitude of the difference (i.e. a Q_{10} value) was not meaningful for many of the genes because some of the rates at cold temperature were extremely low and generated nonsensical Q_{10} values.

We focused on the most biologically relevant comparison of fish assayed at their respective acclimation temperatures. The warm fish had a higher decay rate than the cold fish for β-actin (2.1-fold) and EF-1α (2.5-fold), corresponding to a Q_{10} of 1.3 and 1.4, respectively. The COX genes appeared more sensitive to temperature than the housekeeping genes, estimated as the ratio of decay rate in the warm over decay rate in the cold (Fig. 3, Table 3). COX5B-2 and COX6A-2 each showed the greatest thermal sensitivity on average, with 4.8-fold higher decay rates in the 32°C fish than the 4°C fish (Q_{10} of 1.8). COX4-1 decay was 4.2-fold higher (Q_{10} of 1.7) in the warm-acclimated fish than in the cold-acclimated fish, COX7A-2 was 3.4-fold higher (Q_{10} of 1.5), and the lowest sensitivity was observed for subunit COX6B-1, with a 3.2-fold difference (Q_{10} of 1.5).

Table 2. Results of the mixed model analysis on decay rates for two acclimation experiments

Term	d.f.	d.d.f.	*F*	*P*
32°C versus 4°C				
Acclimation temperature	1	10.0	0.0	0.969
Assay temperature	1	10.0	30.5	<0.001
Gene	7	51.1	101.5	<0.001
Acclimation temperature × assay temperature	1	10.0	0.1	0.747
Acclimation temperature × gene	7	51.1	2.3	0.038
Assay temperature × gene	7	28.6	41.0	<0.001
Acclimation temperature × assay temperature: gene	7	28.6	1.9	0.100
20°C versus 4°C				
Acclimation temperature	1	10.0	23.1	<0.001
Assay temperature	1	9.9	19.2	0.001
Gene	5	35.5	17.8	<0.001
Acclimation temperature × assay temperature	1	9.9	2.8	0.128
Acclimation temperature × gene	5	35.6	0.5	0.738
Assay temperature × gene	5	19.5	1.7	0.186
Acclimation temperature × assay temperature × gene	5	19.5	3.7	0.015

Acclimation temperatures, assay temperatures and genes were taken as fixed effects, and fish, fish × assay temperature and fish × gene were taken as random effects for both acclimation experiments (32°C versus 4°C and 20°C versus 4°C). d.d.f., denominator degrees of freedom.

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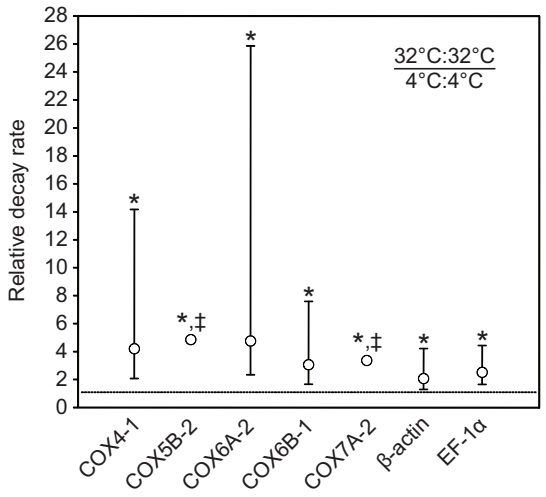

Fig. 3. Relative decay rates of cold- and warm-acclimated fish assayed at their respective holding temperatures (4°C or 32°C). Error bars represent the 95% CI. Asterisks indicate significant differences (*P≤0.05) of ratios from 1. Double daggers indicate unbounded variances.

One question we asked was whether the thermal sensitivity of decay rates differed between housekeeping (β-actin and EF-1α) and COX genes. We could only analyze COX4-1, COX6A-2 and COX6B-1 because the other subunits showed unbounded variances (infinite boundaries), making Q_{10} calculations unrealistically high. When analyzed as ratios, none of the three subunits significantly differed in their responsiveness from the housekeeping genes (Fig. 3, Table 4). Nonetheless, the overall trend amongst all COX genes was that their decay rates were more strongly temperature-responsive than were the housekeeping genes. Two subunits (COX5B-2 and COX6A-2) showed thermal sensitivity of decay rates twice that of the housekeeping genes. In general, the effect size thermal sensitivity of decay rates was at least 1.2 times greater for all COX subunits tested than the housekeeping genes.

There were some fundamental differences in the mRNA decay patterns between the two thermal acclimation experiments. In contrast to the 32vs4 experiment, acclimation in the 20vs4 experiment significantly altered the temperature-responsiveness of decay rates, and this response differed among genes (i.e. a significant three-way interaction of assay temperature × acclimation temperature × gene; Fig. 4A–F, Table 2). Mechanistically, it appears that the thermal acclimation history altered the degradation machinery, and statistically this required separate assessments of the assay temperatures with respect to both acclimation temperatures and separately for each gene. Interestingly, the absolute decay rates for each gene measured at 4°C were similar in both experiments.

For the 4°C-acclimated fish, the decay rates were temperature responsive in all genes, with higher decay rates at 20°C relative to the 4°C assay temperature; although the effect of assay temperature was non-significant with β-actin, the effect size was similar to that of EF-1α. The most pronounced effect was detected in subunit COX5B-2 (7.1-fold, Q_{10} of 3.4), followed by COX6A-2 (6.0-fold, Q_{10} of 3.1), COX4-1 (5.7-fold, Q_{10} of 3.0), and the two housekeeping genes EF-1α (1.9-fold, Q_{10} of 1.5) and β-actin (1.5-fold, Q_{10} of 1.3) (Fig. 4G). However, in the 20°C-acclimated fish, the response to assay temperature was muted and there was no significant difference in decay rates between assay temperatures (Table 5).

In the biologically relevant context (i.e. rates measured at temperatures corresponding to acclimation temperature), COX6A-2 was the only subunit that displayed a higher decay rate (3.2-fold, Q_{10} of 2.1) in the warm-acclimated fish compared with the cold-acclimated fish (Fig. 5, Table 3). However, because of its unbounded variances, we were unable to statistically test whether COX6A-2 differed in its response to temperature from β-actin or EF-1α (Table 4). The same statistical caveat applied to COX5B-2. COX4-1, with its 2.2-fold higher decay rate in the warm-acclimated fish compared with the cold-acclimated fish, however, was not significantly different from the two housekeeping genes (Table 4).

Table 3. Results of *t*-tests for the ratio of means of independent samples for two acclimation experiments

Gene	d.f.	*t*	*P*
32°C versus 4°C			
COX4-1	6.9	4.4	0.003
COX5B-2	10.0	2.6	0.028
COX6A-2	7.7	5.3	<0.001
COX6B-1	8.0	4.1	0.003
COX7A-2	9.6	3.4	0.007
β-actin	9.7	4.0	0.003
EF-1α	9.7	5.5	<0.001
20°C versus 4°C			
COX4-1	7.6	1.5	0.173
COX5B-2	8.4	1.0	0.332
COX6A-2	8.0	2.4	0.042
β-actin	10.0	0.1	0.889
EF-1α	8.3	0.4	0.685

The ratios tested are the relative decay rates of cold- and warm-acclimated fish assayed at their respective holding temperatures (4, 32 or 20°C).

Table 4. Results of independent *t*-tests testing for differences in thermal sensitivity of decay rates between genes

Gene	d.f.	*t*	*P*
32°C versus 4°C			
COX4-1 versus β-actin	10	1.8	0.099
COX4-1 versus EF-1a	10	1.5	0.160
COX6A-2 versus β-actin	10	1.8	0.105
COX6A-2 versus EF-1a	10	1.9	0.092
COX6B-1 versus β-actin	10	0.5	0.596
COX6B-1 versus EF-1a	10	0.6	0.596
20°C versus 4°C			
COX4-1 versus β-actin	10	1.6	0.151
COX4-1 versus EF-1a	10	1.5	0.177

The ratios reflect the relative decay rates of cold- and warm-acclimated fish assayed at their respective holding temperatures (4, 32 or 20°C). Unbounded ratios had to be omitted from this test.

DISCUSSION

Many fish species compensate for the kinetically unfavourable conditions of low temperatures on multiple organismal levels (see Bullock, 1955; Somero, 2004). For many species, though not all (Bremer and Moyes, 2011), cold acclimation/winter acclimatization leads to an increase in mitochondrial gene expression and increases in mitochondrial enzyme content (e.g. Egginton et al., 2000; Hardewig et al., 1999; O'Brien, 2011). In the present study, we compared two acclimation experiments, one where thermal compensation in COX activity was seen (32vs4) and one where no change occurred (20vs4). Our main goal was to explore the potential role of post-transcriptional control of COX subunits in fish under thermal acclimation and how the process of degradation might

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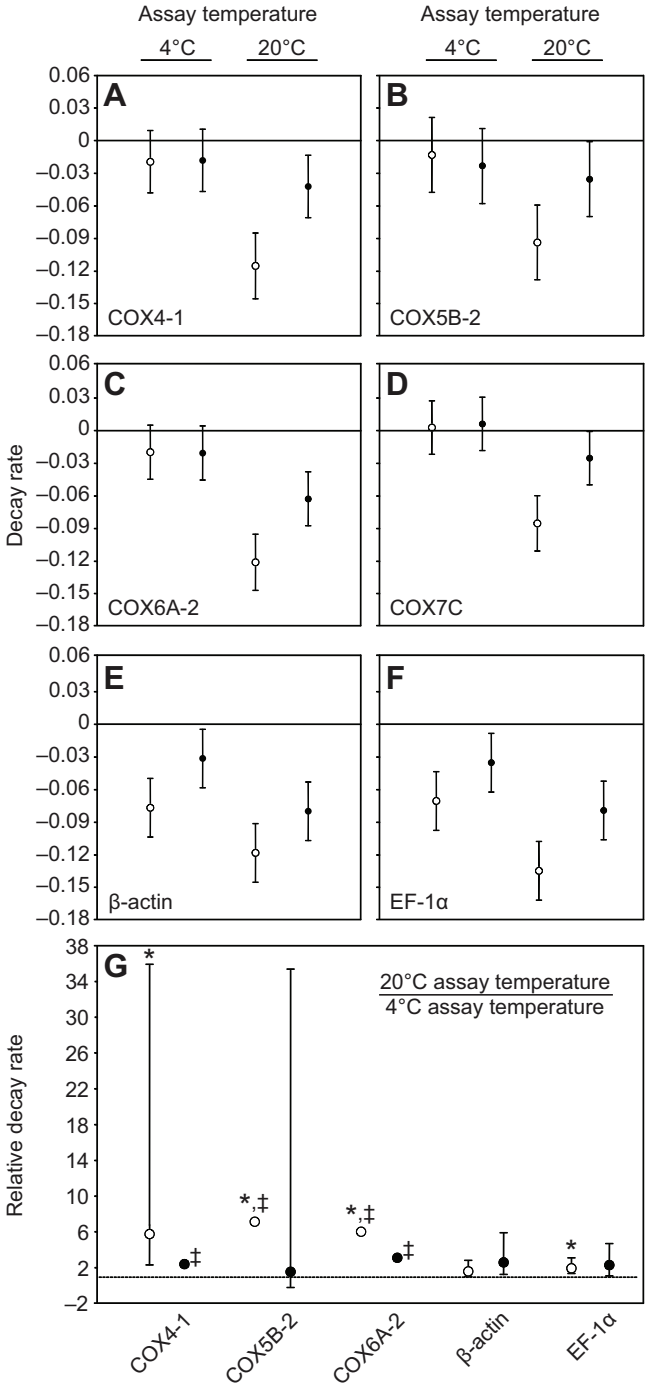

Fig. 4. Average decay rates measured at 20°C and 4°C in 20°C- and 4°C-acclimated fish. Decay rates of 20°C- (closed circles) and 4°C-acclimated fish (open circles) were estimated by mixed models for each of the two assay temperatures, four COX subunits (A–D) and two housekeeping genes (E,F). (G) Summary of relative decay rates (20°C assay temperature over 4°C assay temperature) of 4°C- (open circles) and 20°C-acclimated fish (closed circles). Error bars represent the approximate 95% CI. Asterisks indicate significant differences (*P≤0.05) of ratios from 1. Double daggers indicate unbounded variances. The horizontal line indicates a zero decay rate in A–F and a ratio of 1 in G.

impact steady-state transcript levels, with implications for transcription rates. However, this study may also provide insight into why different mitochondrial compensatory responses are seen across studies and even species.

Table 5. Results of paired *t*-tests of decay rates between two assay temperatures (20°C and 4°C) for each acclimation group (20°C and 4°C)

Acclimation group	Gene	d.f.	*t*	*P*
4°C	COX4-1	4	3.0	0.039
	COX5B-2	5	2.6	0.048
	COX6A-2	4	6.4	0.003
	COX7C	4	4.4	0.012
	β-actin	5	2.5	0.054
	EF-1α	5	5.0	0.004
20°C	COX4-1	5	1.1	0.341
	COX5B-2	5	0.5	0.619
	COX6A-2	5	1.7	0.142
	COX7C	5	1.4	0.234
	β-actin	5	2.5	0.053
	EF-1α	5	2.3	0.072

COX activities and the uncoordinated stoichiometry of COX subunit mRNAs

The high temperature responsiveness of COX activity in white muscle seen in the 32vs4 experiment (4.5-fold) is in agreement with a multitude of previous studies that showed pronounced increases in mitochondrial enzyme activities in the cold (Caldwell, 1969; Freed, 1965; Heap et al., 1985; Orczewska et al., 2010; Vézina and Guderley, 1991). A common explanation for such a remodelling of muscle bioenergetics is to ensure sufficient energy production at low temperature. Thus, it is surprising that a similar response in mitochondrial enzymes was not seen in the second (20vs4) experiment. It is unlikely that the difference between the two experiments was due to the difference in the upper temperature chosen because a previous study showed little difference between fish acclimated to 20°C versus 35°C, and both thermal conditions yielded COX activities that were significantly lower than those seen in fish acclimated to 4°C (LeMoine et al., 2008). When comparing our two experiments, COX activities in the cold-acclimated fish were lower in the 20vs4 experiment and the activities in the warm-acclimated fish were higher in the 20v4 experiment. While we

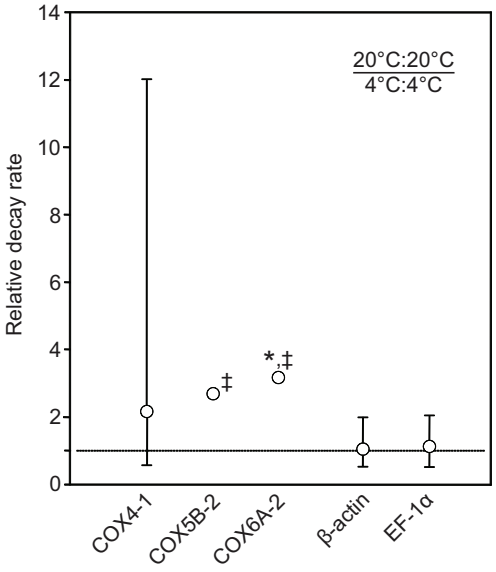

Fig. 5. Relative decay rates of cold- and warm-acclimated fish assayed at their respective holding temperatures (4°C or 20°C). Error bars represent the 95% CI. Asterisk indicates a significant difference (*P≤0.05) of the ratio from 1. Double daggers indicate unbounded variances.

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cannot rule out some aspect of the uncertain physiological history of the fish, it is noteworthy that the fish used for the 20vs4 experiment were three times smaller (22.6±3.0 g and 10.2±0.7 cm, versus 60.2±15.8 g and 13.2±1.0 cm in the 32vs4 experiment). The lack of response in COX activity in these smaller fish may be related to the phenomenon of size-related winter mortality in fish (Hurst, 2007). The 4°C fish of the 20vs4 experiment might have had too little energy reserves to invest in mitochondrial remodelling, leading to a lack in thermal compensation. Despite the unexpected pattern in COX response, our study creates an opportunity to explore the determinants of COX synthesis.

As shown in previous studies (Duggan et al., 2011), thermal acclimation in the 32vs4 experiment led to changes in transcripts of COX subunits that did not universally parallel COX activity nor each other. Where COX activity increased 4.5-fold, some subunits failed to increase (COX4-2, COX6A-2 and COX7B), some changed in parallel with COX activity (COX1, COX2, COX3, COX4-1, COX5B-2, COX6B-2 and COX6C) and others changed considerably more than did COX activity (COX5A-1, COX6B-1, COX7A-2 and COX7C). These data raise a number of questions and issues. First, in experiments where researchers measure mRNA levels of a single subunit, it is ill-advised to assume that the enzyme changes in parallel. Second, it is possible that some subunits are hyper-responsive to cold, and thus may be expected to increase even when there is no change in COX activity. In the 20v4 experiment, three subunits (COX4-1, COX5B-2 and COX7C) increased approximately 2-fold in the cold-acclimated fish, though the difference was non-significant (based upon the Q_{BH} values).

To synthesize a multimeric enzyme, equal amounts of protein for each subunit are required, and it is reasonable to expect that mRNA levels for each subunit might be similar. However, this does not seem to be the case with ETC complex subunits in mammals (Duborjal et al., 2002) or in COX subunits in fish (Little et al., 2010). Another question is whether changes in levels of a multimeric enzyme require parallel changes in the synthesis of protein and mRNA for each subunit. In mammals, it is generally held that changes in COX activity are accompanied by coordinated changes in COX mRNA levels through the use of master regulators of transcription (Dhar et al., 2008; Ongwijitwat et al., 2006). However, in fish studies it is commonplace to see a lack of stoichiometry in COX subunit mRNA levels when changes in COX activity are observed in remodelling (Duggan et al., 2011). One explanation for the observed lack of stoichiometry may be that mRNAs can be translated with different efficiencies, meaning that different steady-state mRNA levels may be needed to produce the sufficient number of proteins for COX biosynthesis. Also, it may be unwise to make the assumption that the entire pathway from a gene to its final product as a protein has evolved in ways that produce exactly enough transcript in any given circumstance. However, an important aspect in this story is whether the observed changes in mRNA levels are entirely due to changes in mRNA synthesis or whether changes in mRNA decay also have important effects. In other words, these COX genes may be transcribed in a coordinated way, but non-stoichiometric patterns in steady-state levels arise through post-transcriptional processes.

Can COX mRNA decay rates explain their steady-state pattern?

Though many enzymes and processes have been studied in relation to acclimation, this is the first study to look at how mRNA decay rates may change with temperature, and to assess the impact of differential changes in target versus housekeeping genes. In our first experiment (32vs4), where COX activity changed, we saw no acclimation effect on the thermal response of decay rate for any of the subunits. This means all subunits responded to the two assay temperatures similarly in both the warm- and cold-acclimated fish. This result suggests that the degradation machinery itself was not modified over the course of acclimation in a way that changes its turnover at high or low temperatures. In contrast, the 20vs4 experiment, where COX activity unexpectedly did not change, marked acclimation effects on decay rates were seen. Though mRNA decay rates in cold-acclimated fish showed the expected response to assay temperature, similar to that seen in the 32vs4 experiment, decay rates in warm-acclimated fish appeared much less temperature sensitive. Thus, acclimation appeared to affect some aspects of the general mRNA decay pathway, such as the amount and/or efficiency of ribonucleases or poly(A)-binding proteins (PABPs).

Apart from questions about acclimation effects, we also investigated whether thermal sensitivities of decay rates differ between genes. This approach helps resolve whether the observed differences in steady-state COX subunit mRNA stoichiometry are related to subunit-specific decay rates.

Overall, it appeared that the decay rates for the COX subunits had a higher thermal sensitivity than the decay of the two housekeeping genes. Thus, if effects of temperature on transcription were the same in COX and housekeeping genes, one would expect to see a greater effect on steady-state mRNA levels for COX subunits because of the RNA decay kinetics.

To put these differences in context, consider the influence of decay on the mRNA levels of COX4-1 and the two housekeeping genes, β-actin and EF-1α, each assayed in fish at their respective holding temperatures. The COX4-1 decay rate was twice as temperature sensitive as the decay rates for β-actin or EF-1α. The origin of gene-specific differences in the stability and decay of mRNA species is not known, though some scenarios are possible. Assuming that the housekeeping genes are ‘typical’, the question is why the influence of temperature on decay of COX4-1, for example, is greater than that on housekeeping genes. It is possible that COX4-1 mRNA could have sequence-specific motifs that bind stabilizing proteins, which could account for lower decay rates at low temperatures. For example, COX4-1 may bind more of the stabilizing RNA binding proteins, such as PABPs or AU-rich binding proteins associated with the 3′-poly(A) tail or AU-rich elements, respectively (see Garneau et al., 2007). However, it is not known whether such factors have a temperature sensitivity that could explain why degradation dynamics of COX subunits differ from those of housekeeping genes. Similarly, the thermal sensitivity of endoribonucleases is not known, which may be important in genes that differ in sequences in ways that alter their vulnerability to endoribonuclease attack. The degradation through endoribonucleases is an important factor in the control of transcripts that underlie extracellular stimuli (Tourrière et al., 2002) and as such may play a role in the control of COX subunits. The possibilities for increased stability/reduced decay rate have been mentioned above with an emphasis on the binding of stabilizing proteins to mRNA species. One example specific for COX subunit mRNAs is the cytochrome *c* oxidase L-form transcript-binding protein. It has been identified as a tissue- and subunit-specific binding protein impacting the expression of COX subunits in bovines (Preiss and Lightowlers, 1993).

Although we did not measure the rate of transcription, we assume it to be equal to the overall decay rate at the point of steady state at which we measured all the gene-specific decay rates. For

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housekeeping genes, the assumption is that mRNA levels do not change as a function of any experimental treatment. Given that in this study the decay rates for the housekeeping genes decreased by ~40% for EF-1α and ~50% for β-actin in the cold relative to the warm assay temperature, this would suggest that to keep steady-state mRNA levels constant, the housekeeping gene transcription rate must have also declined by 40–50% in response to temperature (Fig. 6A,C). For target genes, such as our COX subunits, we here present a mathematical model that describes a potential scenario leading to an increase in their steady-state mRNA levels. The model presented in Fig. 6B explores a situation where a cell requires (for compensatory reasons) a 10-fold increase in the levels of an mRNA of interest, under the combined influence of differential (relative to housekeeping genes) effects of temperature on gene-specific degradation and synthesis rates. The effects on gene transcription are instantaneous, though the effects on total mRNA levels are delayed. Likewise, the change in per-molecule rate of mRNA degradation (i.e. the mRNA-level-independent proportional reduction per unit time) by change in temperature is instantaneous; however, the global rate of mRNA degradation (the absolute number of molecules processed) also depends on the mRNA level: it increases with increasing mRNA levels (Fig. 6B). This process continues until the rate of degradation equals the rate of synthesis, elevating mRNA levels to a new steady state.

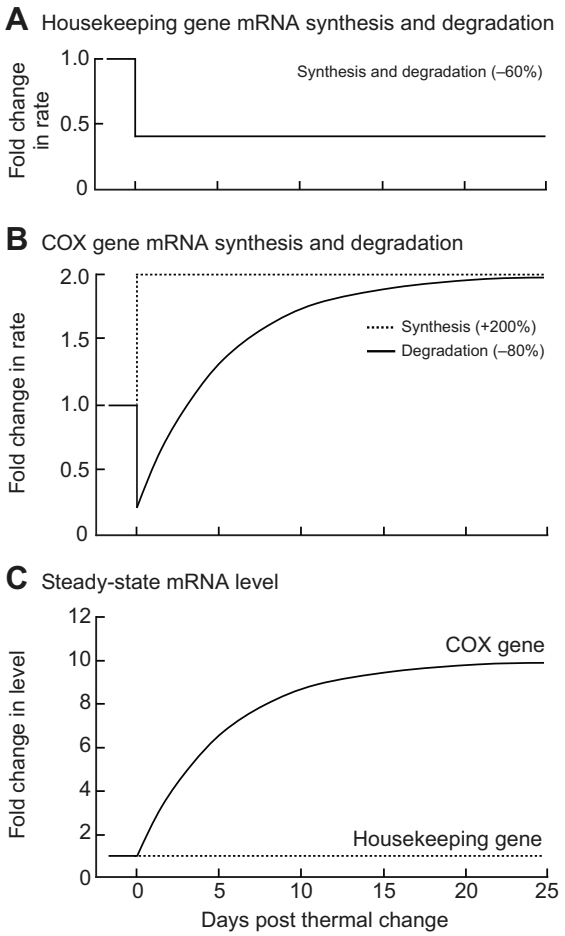

Fig. 6. Mathematical model describing the relationship between mRNA synthesis and degradation and steady-state mRNA levels. The rate of change of [mRNA] is the rate of synthesis minus the rate of degradation: $dm/dt=S-\mu m$, where m is the [RNA] at time t, S is the rate of mRNA synthesis, μ is the per-molecule rate of mRNA degradation, and μm is the total (global) rate of mRNA degradation. Thus, at equilibrium, the rate of synthesis and degradation are equal (A) and $dm/dt=0$, describing the equilibrium concentration m^* as S/μ or, in terms of half-life (τ) as $S\tau/\ln 2$ as $\mu=\ln 2/\tau$. With changing rates of synthesis and degradation (B), the relative change of mRNA (C) is calculated as:

$$m(t) = m_0 \frac{1}{2^{t/\tau}} + \left(1 - \frac{1}{2^{t/\tau}}\right)\frac{S\tau}{\ln 2}.$$

We measured the proportional reduction of mRNA levels per unit time at steady-state level where the global mRNA degradation (the absolute number of molecules processed) and transcription rates are equal (Fig. 6B at 25 days). In contrast to the proportional decay rate, the transcription rate is independent of the amount of mRNA present and a proportional transcription rate likely does not exist. Unfortunately, we did not measure the absolute number of mRNA molecules in each sample, which would have made it possible to calculate the global rate of mRNA degradation, and which would have allowed us to make inferences about the corresponding transcription rate.

Superimposed on these thermodynamic effects on degradation and synthesis are mechanisms by which mRNA levels can increase without changes in transcription. In this case they would be pulled out of the pool of degradable mRNA. In an intact cell, this would manifest as a reduced decay rate, but it is not clear whether such mRNA would be protected from decay in our *in vitro* assay. In some scenarios, mRNA can be stalled in translation and accumulate in so-called stress granules or P-bodies, also known as RNA interference (Balagopal and Parker, 2009). The role for miRNA in the control of nuclear-encoded COX genes (Aschrafi et al., 2012), COX assembly (Colleoni et al., 2013) and mitochondrial-encoded COX mRNA (Das et al., 2012) has not been evaluated in the context of thermal remodelling of mitochondrial metabolism. This process of gene silencing would allow an mRNA species to accumulate, and re-enter translation when needed. Such a mechanism could help reconcile the differences in subunit stoichiometry, explaining the apparent lack of coordination of COX genes.

Conclusions

Our study adds an important quantitative perspective to the interpretation of steady-state transcript levels of multimeric proteins, and in particular to the regulation of COX subunits in the context of thermal acclimation in fish. The lack in stoichiometry seen in COX subunits can partially be explained by the differences in the subunit-specific decay rates. The impact of decay rates seems to correlate inversely with the thermal responsiveness of mRNA levels. This means that the more a subunit responds to low temperatures with increases in mRNA, the more of this increase is due to a decrease in this particular mRNA decay rate. Thus, taking into account different decay rates among subunits tends to reduce the magnitude of deviations from stoichiometric changes in thermal acclimation. In summary, caution is warranted when trying to describe gene expression based on mRNA levels. Superimposed on the pathways that regulate COX levels via protein-dependent pathways are other cellular mechanisms that have the potential to alter COX specific activity, such as membrane environment, and allosteric and covalent regulators.

MATERIALS AND METHODS

Fish and experimental setup

Goldfish for both experiments were obtained from the pet trade (Aleong's International, Mississauga, ON, Canada) and kept in a 750-l round (diameter: 132 cm; height: 75 cm), blue plastic tank set up as a flow-through system in the animal care aquatic facility at Queen's University, Kingston,

ON, Canada. The fish were fed commercial pellets (Wardley brand Premium Goldfish Medium) *ad libitum* and maintained under a 12 h:12 h light:dark photoperiod at ~20°C for 6 weeks before the experiment. The experiments were approved by the Queen's University Animal Care Committee. This study involves two thermal acclimation experiments that differed in their upper acclimation temperature and outcome, and are distinguished as '32vs4' and '20vs4'.

The details of the first of the two experiments (32vs4) have been published previously (Bremer et al., 2012). In brief, fish were acclimated (33 days) to 32±2°C or 4±1°C. In the second experiment (20vs4), fish were acclimated for 58 days to 22±1°C or 4±1°C. For this experiment, fish were exposed to decreasing water temperatures (1°C day^{-1}) until the acclimation temperature of 4°C was reached using a chiller (Frigid Units, Toledo, OH, USA) along with sparse cold (~13°C) water inflow to maintain a flow-through system.

Fish were euthanized in a 2 l solution of 0.4 g l^{-1} tricaine methane sulphonate (MS-222, Syndel Laboratories, Qualicum Beach, Canada) and 0.8 g l^{-1} $NaHCO_3$. Morphometric data, including masses and fork lengths of the fish, were taken prior to sampling to calculate Fulton condition factors ($K=W/L^3$, where W is fish mass in g and L is fish length in cm) (Ricker, 1975). For body comparisons between acclimation groups, the Bonferroni-corrected significance level $P\leq0.0167$ (0.05/3) was used, as mass, length and condition factor are correlates of body metrics. The morphometric data for the 32vs4 experiment have been reported earlier in this paper and in a previous publication (Bremer et al., 2012). For the 20vs4 experiment, the mass and fork length of warm-acclimated fish were not significantly different than those from the cold-acclimated fish, at 23.8±3.5 and 22.6±3.0 g (Mann–Whitney U test, U=29, P=0.753), and 9.7±0.5 and 10.2±0.7 cm (Mann–Whitney U test, U=19, P=0.172), respectively. The condition of the 4°C acclimation group, however, was significantly lower (0.022±0.002) than that of the 20°C acclimation group (0.026±0.002) (Mann–Whitney U test, U=2, P=0.002).

After each of the two experiments, white muscle for the 32vs4 and 20vs4 experiments were immediately dissected from the epaxial muscle below the dorsal fin, but above the lateral line, flash frozen in liquid nitrogen and stored at −80°C.

COX activities

For the COX extraction, white muscle samples (n=10 for each acclimation group of the 32vs4 experiment; n=8 for each acclimation group for the 20vs4 experiment) were powdered under liquid nitrogen. The subsequent steps followed the protocol for COX activity as described previously (Bremer et al., 2012). All samples were measured in triplicate. COX activities for the 32vs4 experiment have been published previously (Bremer et al., 2012).

Decay assay and RNA extraction

For each acclimation group, six samples were randomly chosen for the decay assay. Frozen white muscle tissue (350–400 mg) was homogenized in 15 ml of cold non-denaturing stability assay buffer (50 mmol l^{-1} Tris-HCl, pH 7.6, 150 mmol l^{-1} NaCl, 1% Triton X 100) and divided into two 7.5 ml volumes. One half was then incubated at 4°C and the other at 20°C for the 20vs4 experiment, or 32°C for the 32vs4 experiment. Subsamples of 1 ml were then taken after 1, 2, 4, 8, 16, 20 and 30 min after the start of the experiment. Immediately after sampling, we proceeded to the RNA extraction according to the TRIzol® Reagent (Invitrogen Corporation, ON, Canada) protocol with few modifications. For all steady-state transcript levels, the RNA of the samples was extracted using a slight modification of the single-step method by guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 2006). The purified RNA pellet was dissolved in nuclease-free water and photospectrometrically quantified at 260 nm prior to storage at −80°C. Reverse transcription of RNA and the removal of genomic DNA were carried out using the QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions using 1 ng of total RNA per reaction.

Real-time PCR

All real-time PCR analyses were performed on an ABI 7500 Real Time PCR System (Foster City, CA, USA) using the following protocol: 10 min at 95°C, 40 cycles of 15 s at 95°C, 15 s at annealing temperature (supplementary material Table S1), 34 s at 72°C. The efficiency of each primer set was determined in real-time PCR with an appropriate dilution series of cDNA prior to the sample analyses. Based upon the result, an appropriate cDNA concentration for each primer pair was chosen. Samples were then assayed in duplicate in 25 µl total reaction volume containing 5 µl cDNA (ng of cDNA per reaction differed between target genes) 12.5 µl FastStart Universal SYBR Green Master (Roche Applied Science, Penzberg, Bavaria, Germany), 3.5 µl doubly distilled H_2O and 2 µl each of forward and reverse primer (final concentration, 0.58 µmol l^{-1}). Controls were run with water instead of cDNA to ensure the absence of contamination. Results for the steady-state mRNA levels were analyzed according to the ΔC_t method using β-actin and EF-1α as housekeeping genes with their calculated geometric mean for each sample as standardized C_t [i.e. $2(C_{t,HK}-C_{t,target})^{-1}$ (Pfaffl et al., 2004)]. Specific primers were used to amplify single products of 81-201 bp length for the steady-state mRNA levels, and 3′ end-specific primer sets of each gene were designed for the mRNA decay analysis (supplementary material Table S1).

Data analysis

All statistical analyses were performed using R (Version 2.14.2, R Development Core Team, 2012). For steady-state COX activities and mRNA levels of both acclimation experiments (32vs4 and 20vs4), ratios of values from cold-acclimated fish over warm-acclimated fish and corresponding 95% confidence intervals (CI) were calculated according to Fieller's theorem (Fieller, 1954) using the R package mratios (Dilba et al., 2012). The same method was used to calculate all ratios in the decay experiments. The advantage of using Fieller's method for the calculation of ratios of two means is that it allows for unbounded variances to avoid arbitrarily large deviations from the expected confidence levels, which is a major problem in almost all other methods of ratio calculation (Franz, 2007). However, ratios with unbounded variances do not permit further statistical analyses. In those cases we only discussed the means.

For COX activities, a Fieller ratio ± 95% CI was regarded as significant thermal response when excluding 1 using the function t.test.ratio implemented in the mratios package. Differences between steady-state mRNA ratios and COX activities were tested using unpaired t-tests. As this involved multiple comparisons for both experiments, we controlled for the FDR by adjusting P-values after Benjamini and Hochberg (Benjamini and Hochberg, 1995).

Decay rates of mRNA for each sample were calculated as follows. First, all relative C_t values were ln-transformed (C_t values accounted for amplification efficiency but were not corrected for housekeeping genes). Decay rates (i.e. change in transcript concentration over time) were then estimated as the slope of the linear regression of the ln-transformed relative C_t values against time, so that the decay rate represents the instantaneous decay rate, i.e. a fixed proportion of the total mRNA amount decayed per unit time. Please note that this decay rate is influenced only by temperature, and not by absolute mRNA concentration.

For the analyses of decay rates as the response for the two assay temperatures, the two acclimation temperatures and the seven- (32vs4) or five-gene (20vs4) linear mixed models were used. Non-independence among data from the same fish individuals was accounted for in these models. In particular, non-independence between data because of repeated measurements on every fish at both assay temperatures (every fish was assayed at two temperatures) and for several genes (every fish was assayed for several genes) was accounted for by including the random effects factor 'fish' in the model. Similarly, to account for non-independence between data for testing the acclimation temperature × assay temperature interaction, we included the random effects factor 'fish × assay temperature'. Lastly, to account for the same non-independence between data for testing acclimation temperature × gene interactions, assay temperature × gene interactions, and the three-way interactions of these factors (every fish of an acclimation group was tested for multiple genes within each assay temperature), we included the random effects factor 'fish × gene' in the model. Furthermore, we tested for heteroscedasticity of residual variance among genes using likelihood ratio tests between models with homogeneous and heterogeneous variance for all genes. Fitting heterogeneous residual variance improved the

model for the 32vs4 comparison (χ^2_7=53.9, P<0.001) and the model for the 20vs4 comparison (χ^2_5=12.2, P=0.032). Significance of fixed model terms was tested by F-tests for which the denominator degrees of freedom were approximated according to Kenward and Roger (Kenward and Roger, 1997). Models were fit using the ASREML-R package (Butler et al., 2009).

For the decay rate analyses testing the difference between the two assay temperatures within each acclimation group for the 20vs4 experiment, we used paired sample t-tests. This accounts for the non-independence caused by repeated measurements for each fish individual at both assay temperatures. This test was only necessary for the 20vs4 experiment as there was a significant three-way interaction (acclimation temperature × assay temperature × gene) only in this experiment based on the mixed model results.

To test for differences in thermal sensitivity of decay rates between genes, unpaired t-tests were used.

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Competing interests
The authors declare no competing financial interests.

Author contributions
Both authors contributed to the conception and design of the study, and writing of the manuscript. K.B. performed the experiments and analyzed the data.

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