

# Adaptive expression of uncoupling protein 1 in the carp liver and kidney in response to changes in ambient temperature

Masaru Murakami<sup>1\*</sup>, Masahiro Ohi<sup>1</sup>, Shoko Ishikawa<sup>1</sup>, Mitsuyuki Shirai<sup>2</sup>, Hiroki Horiguchi<sup>1</sup>, Yoshii Nishino<sup>3</sup> and Masayuki Funaba<sup>4\*</sup>

<sup>1</sup>Laboratory of Molecular Biology, Azabu University School of Veterinary Medicine, Sagamihara 252-5201, Japan

<sup>2</sup>Laboratory of Veterinary Pharmacology, Azabu University School of Veterinary Medicine, Sagamihara 252-5201, Japan

<sup>3</sup>Department of Animal Medical Sciences, Faculty of Life Sciences, Kyoto Sangyo University, Kyoto 603-8555, Japan

<sup>4</sup>Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

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\*:correspondence

Masaru Murakami (murakami@azabu-u.ac.jp) or Masayuki Funaba (mfunaba@kais.kyoto-u.ac.jp)

## Abstract

The expression of uncoupling protein (UCP1) is up-regulated in mammalian brown adipocytes during cold exposure. However, a previous study revealed that UCP1 was highly expressed in the liver of common carps, and that the hepatic expression of UCP1 was down-regulated during cold exposure. The present study examined the effects of temperature on the recovery of UCP1 expression levels and the expression of genes involved in UCP1 transcription in the livers and kidneys of common carps. The hepatic and renal expression of UCP1 was decreased by acclimation from 22°C to 8°C, and a subsequent increase in the water temperature from 8°C to 28°C recovered the renal, but not hepatic expression of UCP1. Changes in the expression of peroxisome proliferator-activator receptor (PPAR)  $\gamma$ , retinoid X receptor (RXR)  $\alpha$  and PPAR $\gamma$  co-activator (PGC)-1 $\alpha$ , genes that are involved in the expression of UCP1 in mammals, with ambient temperature indicated that the expressions of PPAR $\gamma$  and RXR $\alpha$ , but not expression of PGC-1 $\alpha$  was decreased in response to cold exposure; the hepatic and renal expressions of PPAR $\gamma$  and RXR $\alpha$  recovered to basal levels with the cessation of cold exposure, although this was not complete for hepatic expression of PPAR $\gamma$ . Cold exposure increased the expressions of PPAR $\beta/\delta$  and NRF1 both in the liver and kidney, as well as renal PGC-1 $\alpha$  expression, and subsequent increases in the water temperature from 8°C to 28°C decreased these expression levels. The results of the present study indicate that a unique regulatory mechanism is responsible for the hepatic and renal expression of carp UCP1 during cold exposure and subsequent reacclimation, and is distinct from that in murine brown adipocytes.

## Introduction

Uncoupling protein (UCP) 1 increases proton leakage from the mitochondrial inner membrane, resulting in a decreased proton motive force, which leads to increased oxygen consumption and heat generation without the concomitant generation of ATP. The restricted expression of UCP1 in mammalian brown adipocytes located in brown and white fat renders these cells responsible for non-shivering heat production (Cannon and Nedergaard, 2004).

The expression of UCP1 and its orthologs has been detected not only in placental mammals, but also in ectothermic vertebrates (Gesta et al., 2007). UCP1 was identified in the common carp (*Cyprinus carpio*), an ectothermic vertebrate, based on the conserved synteny within the mammalian lineage (Jastroch et al., 2005). It was found to be highly expressed in the liver and to a lesser extent in the kidney. The expression of UCP1 could not be detected in carp adipose tissues, which suggested that the role of UCP1 in energy expenditure in fish differed from that in placental mammals (Jastroch et al., 2005).

The expression of UCP1 in murine brown fat was previously shown to be up-regulated in response to cold exposure (Puigserver et al., 1998; Barbatelli et al., 2010), whereas hepatic UCP1 transcript levels were lower in the common carp acclimated at 8°C for 4 weeks than in those kept at 20°C (Jastroch et al., 2005). Similar responses were observed in gilthead sea bream (*Sparus aurata*); expression levels of hepatic UCP1 were lower in winter than in summer and fall (Bermejo-Nogales et al., 2010). The mechanism underlying the regulation of fish UCP1 expression currently remains unknown. In addition, changes in UCP1 expression upon recovery from cold exposure have not experimentally been examined. Furthermore, studies are needed to elucidate the relationship between the expression of UCP1 and non-shivering heat production in

ectothermic vertebrates.

In murine brown fat, the transcription of UCP1 is regulated by several key molecules including the tissue-restricted transcription factors peroxisome proliferator-activator receptor (PPAR)  $\alpha$ ,  $\beta/\delta$  and  $\gamma$ , thyroid hormone receptor (TR)  $\alpha$ , and the transcriptional co-activator PPAR $\gamma$  co-activator (PGC)-1 $\alpha$  (Puigserver et al., 1998; Barbera et al., 2001; Cannon and Nedergaard, 2004; Cao et al., 2004; Komatsu et al., 2010; Seebacher and Glanville, 2010). In addition, PGC-1 $\beta$ , a molecule structurally related to PGC-1 $\alpha$ , is known to be highly expressed in murine brown fat (Lin et al., 2002; Seale et al., 2007). PPAR and TR heterodimerize with retinoid X receptor (RXR), and the formed complex regulates transcription of target genes including UCP1 and PGC-1 (Cannon and Nedergaard, 2004; Evans and Mangelsdorf, 2014). Furthermore, a transcription factor, cAMP responsive element binding protein (CREB) is phosphorylated and activated by protein kinase A, which leads to stimulation of UCP1 transcription (Cannon and Nedergaard, 2004). The expression levels of nuclear respiratory factor 1 (NRF1), which stimulates mitochondrial biogenesis by interacting with PGC-1 $\alpha$  (Scarpulla, 2008), have been closely related to those of UCP1, PPAR $\delta$  and PGC-1 $\alpha$  during cold exposure in murine brown fat (Seebacher and Glanville, 2010).

We hypothesized that regulation of carp UCP1 expression is similar to that established in murine UCP1 expression, although response to cold exposure is different between common carps and mice. As the first step to clarify regulation of fish UCP1 expression, we isolated not only 5' flanking region of carp UCP1 gene but also coding region of carp mRNA for several molecules involved in regulation of murine UCP1 expression partially, because the information has not yet been available yet. By use of the information, we examined the effects of ambient temperature on expression levels of

UCP1 and the candidates to regulate UCP1 expression in common carps.

## Materials and methods

### Animals

A total of 30 common carps aged 7 months were used. These carps were fed commercial pellets *ad libitum* with a 12-h light/12-h dark cycle. They were kept in a temperature-controlled recirculating water system maintained at 22°C for at least 2 weeks (day 0). The water temperature was gradually lowered to 8°C over 3 days. After 14 days at 8°C (days 3-17), the water temperature was gradually returned to 28°C over 3-5 days and kept at 28°C for 14 days (days 21-35). Carps were sacrificed on days 0, 17, and 35, and the livers and kidneys were removed.

### RNA isolation and RT-quantitative real-time PCR

RNA isolation and reverse transcription (RT)-quantitative real-time PCR (qPCR) analyses were performed as described previously (Murakami et al., 2008; Asai et al., 2014). Total RNA was isolated from the livers and kidneys of carps using QuickGene RNA tissue kit S (Wako, Osaka, Japan) in QuickGene-810 (Wako, Osaka, Japan), an automatic nucleic acid extraction system, according to the manufacturer's protocol. The concentration of RNA was determined from absorbance at 260 nm. In RT-qPCR analyses, cDNA was synthesized using the high capacity cDNA reverse transcription kit with an RNase inhibitor (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. The cDNA corresponding to 5 ng of total RNA was used as a template of qPCR; the qPCR was performed using KAPA SYBR FAST Universal qPCR Master Mix (Kapa Biosystems, Boston, MA, USA) in Thermal Cycler Dice Real Time System TP800 (Takara, Otsu, Japan), according to the manufacturer's protocol. The qPCR profile was as follows: after denaturing for 30 s at 95 °C, 40 cycles consisted of 5

s at 95 °C and 30 s at 60 °C. The oligonucleotide primers for qPCR are shown in Table 1. After 40 cycles of RT-qPCR, the dissociation (melting) curve of the products was examined by changes in the ramp temperature from 60°C to 95°C. Each sample showed a single peak, suggesting the expected PCR products. The mRNA levels were expressed relative to EF1 $\alpha$  mRNA levels, and the expression level in the liver of control carps was set at 100.

#### Isolation of 5' flanking region of UCP1 gene from carps

The 5' flanking region of carp UCP1 gene was isolated by two rounds of PCR; we designed 4 PCR primers. Because of lack of information on 5' flanking region of carp UCP1 gene, forward primers were designed on the basis of information of zebrafish UCP1 gene; 5'-gtttagttttggtttattacacaagg-3' named as primer A that corresponds to nt -4500 to nt -4474 of zebrafish UCP1 gene (GenBank accession number NC\_007112) and 5'-taaagtctgctgcagtggaacag-3' as primer B that is nt -3000 to nt -2976 of zebrafish UCP1 gene. The reverse primers were designed based on the carp UCP1 gene (AY461434), i.e., 5'-gatggagtgcaggatgatgcctctg-3' (nt +45 to nt +19) and 5'-ctgacagctgtgattgagttcctctg-3' (nt +74 to nt +49) as primer C and primer D, respectively. The first round PCR was performed using genomic DNA, which was isolated from carp muscle cells, as a template DNA as well as primers A and D; PCR was conducted in a total volume of 50  $\mu$ L by use of PrimeSTAR GXL DNA Polymerase that is a high fidelity polymerase (TaKaRa). The PCR profile consisted of 35 cycles of denature for 10 sec at 98°C, annealing for 15 sec at 55°C and extension for 4 min at 68°C. The second round of PCR re-amplified using one-fiftieth of the PCR products as the template, and primers B and C with the same PCR profile. The products of the nested-PCR were electrophoresed in agarose gels, followed by ethidium bromide staining and visualization under ultraviolet light. A significant band was detected at ~2800 bp, which was within the range of expected size. The band was excised from the

gels, and the nucleotide sequence was determined by direct sequencing and deposited in GenBank (LC003596). To examine the validity of sequence, PCR was performed by use of genomic DNA as a template DNA and PCR primers spanning nt -434 to nt -412 (5'-tttctgagctcctttaatgcatc-3') and nt +930 to nt +908 (5'-gcggtgtccagaggggaaggtgac-3'), and the nucleotide sequence of PCR product was confirmed by direct sequencing. The 5' flanking region of carp, human, mouse, rat and zebrafish UCP gene was compared by use of Pairwise Sequence Alignment of EMBOSS (<http://emboss.open-bio.org/>).

#### Isolation of PGC-1 $\alpha$ , PGC-1 $\beta$ , PPAR $\beta/\delta$ , TR $\alpha$ , RXR $\alpha$ , CREB and NRF1 mRNA from carp livers

To isolate PGC-1 $\alpha$ , PGC-1 $\beta$ , PPAR $\beta/\delta$ , TR $\alpha$ , RXR $\alpha$ , CREB and NRF1 cDNA from carp livers, 1  $\mu$ g of the recovered RNA was treated with RNase-free DNase I (Invitrogen) to remove residual DNA. RNA was subsequently reverse-transcribed in a 21  $\mu$ l volume reaction using the oligo(dT) primer and SuperscriptIII First-Strand Synthesis System for RT-PCR (Invitrogen) to generate first-strand cDNA (Murakami et al., 2008).

Based on the nucleotide sequence of the PGC-1 $\alpha$  mRNAs of goldfish (*Carassius auratus*, FJ710611 and EU426842), zebrafish (*Danio rerio*, FJ710604), grass carp (*Ctenopharyngodon idella*, JN195739), and golden shiner (*Notemigonus crysoleucas*, FJ710606), the following PCR primers were designed to isolate PGC-1 $\alpha$  cDNA: 5'-atggcgtgggacaggtgtaac-3' and 5'-tcaaaggaagtggcaagatggt-3'. To isolate PGC-1 $\beta$  mRNA, the following PCR primers were designed based on the nucleotide sequence of zebrafish PGC-1 $\beta$  (XM\_003199895): 5'-atggcggactgcgcttcactgttagatg-3' and 5'-agacccccgactccgtcctcaacaat-3'. To isolate PPAR $\beta/\delta$  mRNA, the PCR primers, 5'-cagtgcaccacagtggaaactg-3' and 5'-gaatgccatcctgaatctgctc-3', were designed based on the nucleotide sequence of zebrafish PPAR $\delta$ b (NM\_131468 and XM\_683192). The

mRNA of TR $\alpha$  was isolated on the bases of nucleotide sequences of goldfish TR $\alpha$ 1 (AY973629) and TR $\alpha$ 2 (DQ172902) and zebrafish TR $\alpha$ a (NM\_131396) by use of 5'-gcatcacatgtgagggtgcaa-3' and 5'-tctccatacagcagccttcag-3' as PCR primers. The mRNA of RXR $\alpha$  was isolated on the bases of nucleotide sequences of goldfish RXR $\alpha$  (AY197562) and zebrafish RXR $\alpha$ a (NM\_001161551 and XM\_001923838) by use of 5'-cagaagtgttgccatgggc-3' and 5'-gcaaacctccaggtgttcag-3' as PCR primers. The mRNA of CREB was isolated on the bases of nucleotide sequences of goldfish CREB1 (AM886438) and zebrafish CREB1 (NM\_001161551 and XM\_001923838) by use of 5'-caggagcagatgccagcagg-3' and 5'-gcatactgcaggatggtggtg-3' as PCR primers. As for NRF1 mRNA, the following PCR primers were designed based on the nucleotide sequence of zebrafish NRF1 (XM\_005164709) and Japanese pufferfish (*Takifugu rubripes*) NRF1-like isoform 1 (XM\_003972802): 5'-caggcccagctccgagcgttcac-3' and 5'-ggccacagcctgcgtggcctct-3'. Using these primers, cDNA was isolated from common carp livers, and agarose gel electrophoresis of the PCR products indicated bands with an expected size of ~920 bp for PGC-1 $\alpha$ , ~350 bp for PGC-1 $\beta$ , ~270 bp for PPAR $\beta/\delta$ , ~440 bp for TR $\alpha$ , ~450 bp for RXR $\alpha$ , ~440 bp for CREB and ~560 bp for NRF1. The nucleotide sequence of the PCR product was determined by direct sequencing.

### Statistical analyses

Data are expressed as the mean  $\pm$  SE. Data were log-transformed to provide an approximation of a normal distribution before analysis. Differences between groups in each tissue were analyzed by Tukey's multiple comparison test.  $P < 0.05$  was considered significant.

### Results

The expression of UCP1 was evaluated in the livers and kidneys of common carps

acclimatized at various temperatures (Fig. 1). A decrease in the water temperature from 22°C to 8°C significantly reduced the expression of UCP1 in the liver and kidney within 14 days. Although increasing the water temperature from 8°C to 28°C did not change the hepatic expression of UCP1, the renal expression of UCP1 returned to basal levels. Consistent with previous findings (Jastroch et al., 2005), the expression level of UCP1 was higher in the liver than in the kidney.

Expression of mammalian UCP1 gene is transcriptionally regulated (Cannon and Nedergaard, 2004). We determined nucleotide sequence of 5' flanking region of carp UCP1 gene, because it has not yet been available. Whole genome sequence of common carps is not known, and, therefore, we isolated 2634 bases upstream sequence of the putative transcriptional initiation site of carp UCP1 through the nested-PCR strategy in reference to the nucleotide sequence of 5' flanking region of zebrafish UCP1 gene (Fig. 2A). When genomic DNA from three carps was individually used as the template DNA, comparable results were obtained (data not shown). The validity of the isolated nucleotide sequence was also determined by the expected amplification of genomic DNA using PCR primers designed within the region of determined nucleotide (data not shown).

The nucleotide sequence indicated the lack of the first 18 bases (5'-tggatccaaagaattcgg-3') within 5' untranslated region of carp UCP1 mRNA in GenBank accession number AY461434, and three nucleotide substitutions, T, C and T instead of A, G and G of the 20th, 22nd and 25th nucleotide, respectively. We performed RT-PCR using liver cDNA as the template DNA and the first 22 bases of AY461434 (primer E: 5'-tggatccaaagaattcggcag-3') as the forward primer and primer for the RT-qPCR as the reverse primer, and found no band (Fig. 2B, lanes 1-3). In contrast, when the first 22 bases overlapping to AY461434 determined in this study (primer F:

5'-ctccagtcacatcatcctgtcact-3'), i.e., corresponding region spanning the 19th nucleotide to the 40th nucleotide of AY461434, were used as the forward primer, we detected the PCR product with the expected size (Fig. 2B, lanes 5-7). Therefore, we conclude that the 19th nucleotide of AY461434 is the putative transcriptional initiation site in common carps used in this study, and designated as nt +1; the difference of strain may be related to the difference of nucleotide sequence near 5' untranslated region of carp UCP mRNA. The nucleotide sequence has limited homology with that of 5' flanking region of human, mouse and rat UCP1 gene (Fig. 2C). The less homology of the nucleotide sequence was also detected between carps and zebrafish.

Previous studies identified and characterized the brown adipose tissue (BAT)-specific enhancer region (~ 220 bp) around nt -2500 upstream of murine UCP1 transcriptional initiation site (Kozak et al., 1994; Sim and Kozak, 2002). The region plays a critical role in the regulation of UCP1 that includes responsive element to PPAR, TR and CREB (Cannon and Nedergaard, 2004). Homology search indicated no similarity between the murine BAT-specific enhancer and the carp UCP1 promoter (data not shown). In addition, there was no consensus responsive element to PPAR, TR and CREB within the carp UCP1 promoter, i.e., direct repeats (DR) 1 and 2 (core sequence 5'-agg(a/t)ca-3' separated by one and two base-pairs, respectively) for PPAR (Evans and Mangelsdorf, 2014), DR-4 for TR (Evans and Mangelsdorf, 2014) and cAMP response element (5'-tgacgtca-3') for CREB (Montminy, 1997). We also searched specific sequence motifs present in the murine BAT-specific enhancer region, and found two half-site consensus sequence of CRE (5'-cgtca-3' and its complementary sequence 5'-tgacg-3', Fig. 2A, *underline*), two contiguous TTCC motifs within brown adipocyte-regulatory element 1 (BRE-1, Fig. 2A, *box*), and two possible NF-E2 sites (Fig. 2A, *double underline*).

The expression of genes involved in the expression of UCP1 in mammals (Puigserver et al., 1998; Barbera et al., 2001; Cannon and Nedergaard, 2004; Cao et al., 2004; Komatsu et al., 2010; Seebacher and Glanville, 2010) was examined next. Sequence information for common carp PGC-1 $\alpha$ , PGC-1 $\beta$ , PPAR $\beta/\delta$ , TR $\alpha$ , RXR $\alpha$ , CREB and NRF1 cDNAs was unavailable; thus, we isolated gene transcripts from the liver of the common carp. The partial amino acid sequences deduced from the nucleotide sequences exhibited sequence similarity to fish PGC-1 $\alpha$ , PGC-1 $\beta$ , PPAR $\beta/\delta$ , TR $\alpha$ , RXR $\alpha$ , CREB and NRF1, respectively (Fig. 3), which suggested that these are orthologs of respective genes in common carps. Since fish PGC-1 $\alpha$ , PGC-1 $\beta$ , PPAR $\beta/\delta$ , TR $\alpha$ , RXR $\alpha$ , CREB and NRF1 consist of ~878, ~495, ~517, ~421, ~343, ~318 and ~514 amino acids, respectively, the gene transcripts clarified in the present study are expected to correspond to approximately one-fifth of PGC-1 $\beta$  and PPAR $\beta/\delta$ , one-third of PGC-1 $\alpha$ , TR $\alpha$  and NRF1, and half of RXR $\alpha$  and CREB of the coding regions.

Unlike the hepatic and renal expression of UCP1, the expression level of PGC-1 $\alpha$  did not decrease with a reduction in the water temperature from 22°C to 8°C; the renal expression of PGC-1 $\alpha$  was increased by cold exposure (Fig. 4A). The hepatic expression of PGC-1 $\alpha$  remained unchanged by increases in the water temperature from 8°C to 28°C, whereas the cold-induced expression of renal PGC-1 $\alpha$  returned to basal levels with increases in the water temperature from 8°C to 28°C. Decreasing the water temperature from 22°C to 8°C decreased the expression of PGC-1 $\beta$  in the kidney, but not significantly in the liver (Fig. 4B). The renal expression of PGC-1 $\beta$  tended to return to basal levels with increases in the water temperature from 8°C to 28°C.

The hepatic expression of PPAR $\alpha$  was unaffected by cold exposure, but decreased with increases in the water temperature from 8°C to 28°C (Fig. 5A). In contrast, changes in the water temperature did not affect the renal expression of PPAR $\alpha$ . Expression of

hepatic and renal PPAR $\beta/\delta$  was increased by cold exposure, and the increase in water temperature from 8°C to 28°C decreased the expressions; hepatic expression levels at 28°C were lower than those at 22°C (Fig. 5B). Both the hepatic and renal expression of PPAR $\gamma$  was markedly reduced with decreasing water temperature from 22°C to 8°C, and the cessation of cold exposure returned to these expression levels back to basal levels; however, hepatic expression was not completely recovered (Fig. 5C). Expression of renal TR $\alpha$  was decreased during the cold exposure, and the cessation of cold exposure further decreased the expression; hepatic expression of TR $\alpha$  was also decreased by the increase in water temperature from 8°C to 28°C (Fig. 5D). Similar to the regulatory expression of PPAR $\gamma$ , RXR $\alpha$  expressions in the liver and kidney were significantly decreased during cold exposure, and the re-warming resulted in return to the basal expression levels (Fig. 5E).

Cold exposure did not affect expression levels of hepatic and renal expression of CREB, but the increase in water temperature from 8°C to 28°C significantly decreased renal CREB expression (Fig. 6A). The cold exposure increased the hepatic and renal expression levels of NRF1, and an increase in the water temperature from 8°C to 28°C decreased expression levels; expression levels at 28°C were lower than those at 22°C (Fig. 6B).

## Discussion

This study revealed six aspects of the regulation of the hepatic and renal expression of UCP1 in carps in response to changes in ambient temperature 1) both the hepatic and renal expression of UCP1 was down-regulated by cold exposure, 2) expression levels in the kidney, but not in the liver recovered with the cessation of cold exposure, 3) water temperature-related changes in the renal expression of UCP1 paralleled those in the

renal expression of PPAR $\gamma$  and RXR $\alpha$ , 4) the cold-induced down-regulation of hepatic PPAR $\gamma$  partially recovered with the cessation of cold exposure, 5) contrary to hepatic PPAR $\gamma$  and RXR $\alpha$  expressions, expressions of hepatic and renal PPAR $\beta/\delta$  and NRF1 were up-regulated during cold exposure, and the cessation of cold exposure returned the expression, and 6) the expression of hepatic and renal PGC-1 $\alpha$  was not decreased by cold exposure. Of these, the down-regulation observed in the hepatic expression of UCP1 during cold exposure was consistent with previous findings (Jastroch et al., 2005); considering that the common carp is an ectothermic animal, down-regulation of UCP1, a drive engine of non-shivering thermogenesis, during cold exposure is reasonable. The other 5 points were not evaluated in the study by Jastroch et al. (2005). Our results confirm that regulatory expression of carp UCP1 during cold exposure and subsequent recovery from cold exposure is distinct from that of mammalian UCP1 expression, but suggest that expression level of carp UCP1 is partly but not completely related to that of molecules involved in regulation of mammalian UCP1 expression. The present study has provided a novel insight into the regulatory expression of carp UCP1.

Although expression levels of UCP1 were closely related to those of molecules regulating UCP1 expression in murine brown adipocytes during the changes in ambient temperature in common carps, the detailed regulation was distinct between murine brown adipocytes and carp tissues such as liver and kidney. In murine brown adipocytes, the cold-induced stimulation of the sympathetic nervous system and accompanying  $\beta$ 3 adrenergic receptor activation induce the transcription of PGC-1 $\alpha$ , which in turn stimulates UCP1 transcription through complex formation with PPAR $\gamma$  (Oberkofler et al., 2002; Cao et al., 2004). The expression level of PGC-1 $\alpha$ , but not PPAR $\gamma$  was shown to increase during cold exposure in murine brown fat (Seale et al., 2007; Karamitri et al., 2009; Komatsu et al., 2010). The present study indicates that renal expression of UCP1 is closely related to that of PPAR $\gamma$  rather than PGC-1 $\alpha$ ; water temperature-related

changes in the renal expression of UCP1 paralleled those in the expression of PPAR $\gamma$ , but not PGC-1 $\alpha$ . Cold exposure also decreased hepatic expressions of UCP1 and PPAR $\gamma$ , but the expression level of hepatic PGC-1 $\alpha$  was unaffected.

Consistent with previous findings on the effects of cold on the expression of PPAR $\alpha$  in murine brown fat (Komatsu et al., 2010), the hepatic and renal expression levels of PPAR $\alpha$  in common carps were unaffected by cold exposure. In addition, hepatic and renal expressions of PPAR $\beta/\delta$  were rather increased during cold exposure. Taking the expression level of PPARs and PGC-1 $\alpha$  together, the down-regulation of PPAR $\gamma$  expression during cold exposure may be responsible for the down-regulation of UCP1 expression in common carps. Changes in expression levels of RXR $\alpha$ , a PPAR $\gamma$  partner (Evans and Mangelsdorf, 2014), basically paralleled to those of PPAR $\gamma$ . Therefore, expression levels of RXR $\alpha$  may be also involved in regulation of UCP1 expression by PPAR $\gamma$  during cold exposure.

However, the regulatory expressions of PPAR $\gamma$ /RXR $\alpha$  do not explain UCP1 expression in liver of common carps during the water temperature from 8°C to 28°C; the cessation of cold exposure increased hepatic expressions of PPAR $\gamma$  and RXR $\alpha$  but not UCP1. It is possible that inhibitory factor(s) on UCP1 expression is induced in carp livers during the recovery period from cold exposure.

We isolated 2634 bases upstream to putative transcription initiation site of UCP1 gene. The nucleotide sequence has low homology to corresponding region of humans, mice, rats and even zebrafish, although some sequence motifs in the murine BAT-specific enhancer are present scattered widely. The enhancer region of human UCP1 gene, which includes responsive elements to PPAR, TR and CREB, is located around nt -3800 (Rim and Kozak, 2002; Shore et al., 2013). It is possible that the transcriptional

enhancer is located more distal region of UCP1 promoter. In addition, the sequence motifs in fish may not match to those suggested in mammals. These should be evaluated in future studies.

We also evaluated the expression of NRF1, because NRF1 has been shown to regulate energy metabolism in concert with PGC-1 $\alpha$  in mammals (Scarpulla, 2008). A previous study revealed that changes in NRF1 expression paralleled those in UCP1, PGC-1 $\alpha$  and PPAR $\delta$  expression during cold exposure and physical activity in murine brown fat (Seebacher and Glanville, 2010). Cold exposure increased the renal expression of PGC-1 $\alpha$ , PPAR $\beta/\delta$  and NRF1, and the cessation of cold exposure decreased the expression of these genes; similar changes were also detected in hepatic expressions of PPAR $\beta/\delta$  and NRF1. However, water temperature-related changes in the expression pattern of PGC-1 $\alpha$ , PPAR $\beta/\delta$  and NRF1 differed from those in UCP1 expression. PGC-1 $\alpha$ , PPAR $\beta/\delta$  and NRF1 may cooperatively function through their up-regulation during cold exposure, and this is distinct from the regulation of energy metabolism through the modulation of UCP1 expression.

A previous study showed that the hepatic expressions of PGC-1 $\alpha$ , PGC-1 $\beta$ , and PPAR $\beta$  and NRF1 were higher in goldfish acclimated at 4°C for 3 weeks than in those acclimated at 20°C, and PPAR $\alpha$  expression was lower during cold exposure (LeMoine et al., 2008). Bremer et al. (2012) observed that cold exposure significantly increased expression of PGC-1 $\beta$  and NRF1, decreased expression of PGC-1 $\alpha$ , PPAR $\alpha$  and TR $\alpha$ , and did not affect expression of PPAR $\beta/\delta$  and RXR $\alpha$  in white muscle of goldfish. The temperature-related changes in gene expression were partly but not completely consistent with the previous results. At present, it is difficult to understand relationships among expression levels of these genes comprehensively; effects of cold exposure are also different among tissues in gold fish (LeMoine et al., 2008), and there may be

species differences on cold-related changes in gene expression.

Although carp UCP1 may be a functional uncoupling protein, similar to its mammalian counterpart (Jastroch et al., 2007), a correlation between the expression of UCP1 and non-shivering heat production has yet to be established in fish. Future studies are needed to determine the role of UCP1 expression, the physiological significance of regulatory changes in the hepatic and renal expression of UCP1, and the molecular mechanisms underlying altered expression levels of genes during cold exposure and subsequent recovery from cold exposure in common carps. Also, aging, diet, feed restriction and hypoxia are known to affect expression levels of UCP2 and UCP3, structurally related molecules to UCP1, in gilthead sea bream (Bermejo-Nogales et al., 2010, 2014). To understand uncoupling-related regulation of energy metabolism in ectothermic animals fully, it is needed to explore regulatory expressions of UCP2 and UCP3 in addition to UCP1.

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## Figure legends

Fig. 1. UCP1 expression in the livers and kidneys of common carps acclimated to various temperatures

Common carps acclimated at 22°C were subjected to cold exposure for 14 days at 8°C. They were then acclimated at 28°C for 14 days. The gene transcript levels of UCP1 were determined by RT-qPCR and are expressed relative to the expression of EF1 $\alpha$ , with the hepatic level determined on day 0 (control) set to 100. Data shown are the mean  $\pm$  SE (n=10). A, B and a, b:  $P < 0.05$ .

Fig. 2. Nucleotide sequence of the 5' flanking region of the carp UCP1 gene

(A) Nucleotide sequence of the 5' flanking region of the carp UCP1 gene. The nt +1 is defined as putative transcriptional-initiation site. Possible CRE, BRE-1 and NF-E2 recognition sequences are shown with underline, box and double underline, respectively. (B) RT-PCR analyses were performed using cDNA prepared from kidney (K: lanes 1 and 5) and liver (L: lanes 2, 3, 6 and 7) as the template. The cDNA was prepared by Superscript III First-Strand Synthesis System (S) to isolate full-length of carp UCP1 mRNA (lanes 1, 2, 5 and 6) or by high capacity cDNA reverse transcription kit (H: lanes 3 and 7). Lanes 1-3 and 5-7 indicate PCR products using primer E and the reverse primer for RT-qPCR, and primer F and the reverse primer for RT-qPCR, respectively. Lanes 4 and 8: negative control and 100-bp DNA ladder, respectively. (C) Homology between nucleotide sequences spanning nt -2634 to nt -1 of UCP1 gene of various animal species.

Fig. 3. Comparison of the amino acid sequence deduced from the nucleotide sequence of PGC-1, PPAR $\beta/\delta$ , TR $\alpha$ , RXR $\alpha$ , CREB and NRF1

The partial gene transcripts of PGC-1 $\alpha$ , PGC-1 $\beta$ , PPAR $\beta/\delta$ , TR $\alpha$ , RXR $\alpha$ , CREB and

NRF1 were isolated and sequenced. The deduced amino acid sequences of PGC-1 $\alpha$  (A), PGC-1 $\beta$  (B), PPAR $\beta/\delta$  (C), TR $\alpha$  (D), RXR $\alpha$  (E) CREB (F) and NRF1 (G) were compared to that of fish PGC-1 $\alpha$ , PGC-1 $\beta$ , PPAR $\beta/\delta$ , TR $\alpha$ , RXR $\alpha$ , CREB and NRF1, respectively. The dot stands for the same amino acid as that in Cca. Cca: *Cyprinus carpio*, Cau: *Carassius auratus*, Dre: *Danio rerio*, Cid: *Ctenopharyngodon idella*, Spr: *Schizothorax prenanti*, Ncr: *Notemigus crysoleucas*, Ssa: *Salmo salar*, Caca: *Carassius carassius*, Omy: *Oncorhynchus mykiss*, Pma: *Pagrus major*, Ola: *Oryzias latipes*.  $\beta/\delta$ ,  $\beta$ ,  $\beta 2$  and  $\delta b$ : PPAR $\beta/\delta$ , PPAR $\beta$ , PPAR $\beta 2$  and PPAR $\delta b$ , respectively.  $\alpha 1$ ,  $\alpha 2$  and  $\alpha A$ : TR $\alpha 1$ , TR $\alpha 2$  and TR $\alpha A$ , respectively.

**Fig. 4. PGC-1 expression in the livers and kidneys of common carps acclimated to various temperatures**

Common carps acclimated at 22°C were subjected to cold exposure for 14 days at 8°C. They were then acclimated at 28°C for 14 days. Transcript levels of PGC-1 $\alpha$  (A) and PGC-1 $\beta$  (B) were determined by RT-qPCR and are expressed relative to the expression of EF1 $\alpha$ , with the hepatic level determined on day 0 (control) set to 100. Data shown are the mean  $\pm$  SE (n=10). a, b:  $P < 0.05$ .

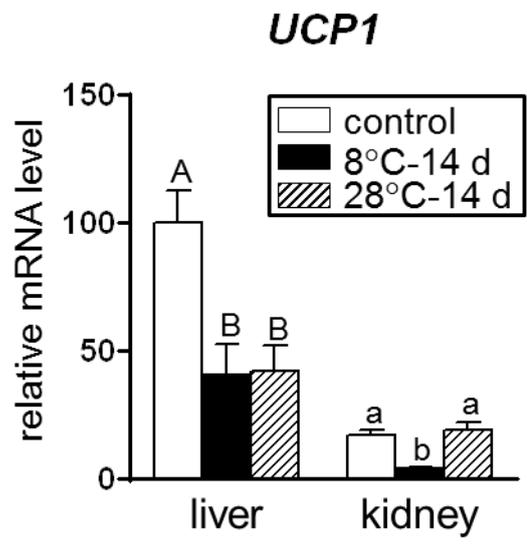
**Fig. 5. Expression of PPAR, TR $\alpha$  and RXR $\alpha$  in the livers and kidneys of common carps acclimated to various temperatures**

Common carps acclimated at 22°C were subjected to cold exposure for 14 days at 8°C. They were then acclimated at 28°C for 14 days. Transcript levels of PPAR $\alpha$  (A), PPAR $\beta/\delta$  (B) PPAR $\gamma$  (C), TR $\alpha$  (D) and RXR $\alpha$  (E) were determined by RT-qPCR and are expressed relative to the expression of EF1 $\alpha$ , with the hepatic level determined on day 0 (control) set to 100. Data shown are the mean  $\pm$  SE (n=10). A, B, C and a, b, c:  $P < 0.05$ .

Fig. 6. Expression of CREB and NRF1 in the livers and kidneys of common carps acclimated to various temperatures

Common carps acclimated at 22°C were subjected to cold exposure for 14 days at 8°C. They were then acclimated at 28°C for 14 days. Transcript levels of CREB (A) and NRF1 (B) were determined by RT-qPCR and are expressed relative to the expression of EF1 $\alpha$ , with the hepatic level determined on day 0 (control) set to 100. Data shown are the mean  $\pm$  SE (n=10). A, B, C and a, b, c:  $P < 0.05$ .

Fig. 1



A

Fig. 2A

-2634 CTACCATGG ATCATCTGGT TGGARTGAGA AGTAGAGTTG AGTGTGATAG GAAAGCCAT GCTTCTGART GACGGATCCT AAGATGACA TGATGATGGA  
 -2534 GAGTAAAGT GGTCCARGCA CTGAGCCCTG AGGARCCCCA GTAGCTTAGG TGGTAAACAC GCCTGGATGT GTTATTTATT TATTTATTTA TTATTATTAT  
 -2434 TATTATTATG ATTGTGGACA AGGCTTTTGA GATCGGAAAA ACCAGTTAGA ATTCARTGTT CTTTAAAGTA AGTCTTAGTC TCTGCTCTCA TTGGCATATC  
 -2334 TGGCCTGATT TTCCCTTGAT AAGTGTTC CCGGATCCAG TCGTGGTTCA GAGCGGGCTG TAGGACAGTC AGCAGATGAC TGAAGATAAT GCTCCAGCTG CTGCTGGTGT  
 -2234 CTGTTGCATA AACCTGTACA GTTCAGGCCT GTTGTARATC CTCATGAAAC TTCAGTCGAA ACGTCARCCC CTTCCATCC TAATGACTAA CTCAGATAAA  
 -2134 CATTAGACCA GTACTAGACC ACTTCACAGT CCGGATCCAG TCCTTCTGAG AGAGATCAGA CTATCAGTGA AGCTGAAATC TTCATATTAG ATTGACAAC  
 -2034 AGATCTTACT TTACAGCATT GAGGATATAC AGTATATTGA TTTTGGACAG TAGCTCGGCT GTTTTCAGAC TGTAGCAAAA GATAGTAAA CATGACAAA  
 -1934 TTATACACTG CATTTGTTAA GCAARTGCAA ATGAACATG GCCAGCTGGA ATATCCCTTCCTCATCT GTTCGGTTCG GTGACTGAT TCTGGTGGT  
 -1834 TGTTCAGGA AAGCAGTCTG TACATGCGGT TCATTTCACT GCACTGATCC GCTGATTCAT GAGATATATC TCATTTCTAG ACAARTAGAG TTTTACTGCT  
 -1734 GCTTTTATGT AGTAAATGT TTAGTTACAT GATATTARTA ATCACAATTC AGTATTATTT TGGGTTCTTT TGGATAAAGG TGTATGTTTG GGTAAAGAT  
 -1634 TAATGCTGTG CTGTCACTCT GGCTGAGATC ATTGTTTGTG TGCCATAAAA ATACATGTTT TTAARAATAC ATATAATCTG ACTATTATCT AACGATATT  
 -1534 GCTCTTTTAA ATGGCATAGG TTTAGTTTGC TACTTTAAAA AAAAAATGT AGCTCTGTTT GTTTGAGAAT TCTGACACAG CATCAGTGGC TCACCAATT  
 -1434 ACGTGAGATT GGGCGGGAC TGCTTTTTT TTTGCCAAT CGCGGATGGG GGAGTGTTC GGAACCTTGT TTAARAACAA ACTTTATTTT TGCAGTCCAA  
 -1334 CTTGAGGAT CACATACTTC AACTTTAACA GTTCTTTTAT ATTCAATTCA ATTTAGACT ATATCTGTCC GAAGATTAT TACCATTGCT ATTATTACTA  
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 -934 ATATTARTGG TTCARTTAG AATCATGTCT GTCTGACGT CCATATTATT ATTAATTATT TTAARTTTT ATACATTTAA TTTAATATA AATTTAGCAT  
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 -534 TGTATTGG CTATATTTT ACCGTTTCCC ACCCTTCCAA GAGGCCAATC ATCTTTCAGA CCCTGCAGGA CTGTCCTGCT GCTGCTATTG GCTGACAGCT  
 -434 TTTCTGAGCT CCTTARTGC ATCTGCACTT TCATTAGTAG CCAATTTAA AACATCTGCA GTCATGTTGC AGTGACATT TATTTATTA GCATTGGTAC  
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 -234 CTGTTAATGA CAGCAGCATC GTGAAGTGT TTCAAAAGCT TTGTGTTCC TAGTTATCTA AGCGCTTGT TATCTGGATG TGGATGTTT TGACTGGAC  
 -134 TCTGTGCTGG AAGGTGGACT TTGTGTCGGC CAGTARTCTT TCCTGAGAA ACACCTCCCT GAGCGGAGGA GGAAGAACCC CTCTGGATA ATGATATATA  
 -34 AAGAAAGACA GAGCATGCC TCCTCCCTCC AGAGCTCCAG TCATCATCCT GCACTCCAT CAGACAGAG AACTCAATCA CAGGTCAGTT CACCATGTTT

**B**

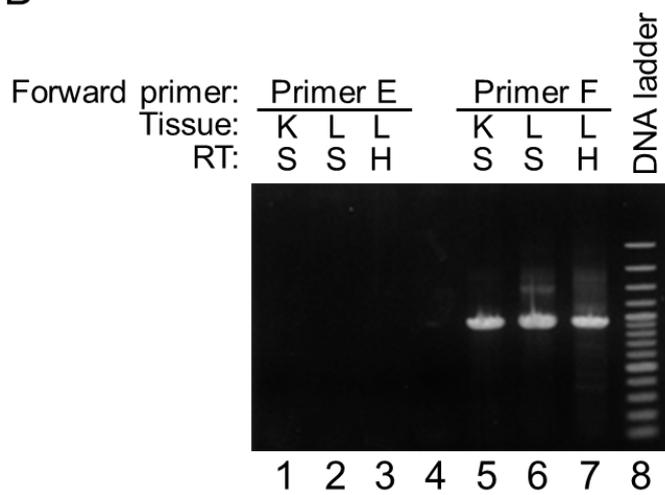


Fig. 2B, C

**C**

	Human	Mouse	Rat	Zebrafish	Carp
Human	100	45	44	39	36
Mouse		100	70	39	41
Rat			100	41	40
Zebrafish				100	46
Carp					100

Fig. 3A-C

A

Cca	1	LECAALVGEDQPLCPDLPELDLSELDVSDLDADSFLLGGLKWYSDQSEIISQYGNETSNFLFEKIDEENEANLLAVLTETLDSIPVDEDGLPSFEALADGD	100
Cau	1	.....I.....I.....	100
Dre	1	.....A.....	100
Cid	1	.....A.....	100
Spr	1	.....A.....	100
Ncr	1	.....T.....A.....	100
Cca	101	VTNASDQSCPSTPDGSPRTPEPEEPSLLKLLLLAPANSQLSYNQYPGGKAQNHAASNLRIRPTPAVAKTENPWNTKPRGSCPNRSMRRPCTELLKYLTS	200
Cau	101	.....A.....V.H.....	200
Dre	101	.....Q...A.....S...A.....	200
Cid	101	.....Q.....S...A.....	200
Spr	101	.....H.....Q.....S.....	200
Ncr	101	.....Q.....S.T.A.....	200
Cca	201	DEAFQTKAREAKSTWTGCSKDRGGA-CTSSCSSSSSPSSSSTSSFSSLS CSSSSTAKKKKTSSASPSQQQQQ	272
Cau	201	.....GI...G.....-.....P.....E	271
Dre	201	.....G.....G.....-IL.....L	271
Cid	201	.....G.....-.....	272
Spr	201	.....V.....G.....-.....	272
Ncr	201	.....PL.....	272

B

Cca	1	ELSSFVFNFLTENSGSQYGEVEVCSDRLDADFPDFDLSQLDASDFDSVNCLSELHWCNEHSDHSPAISQYSGGNPELFE-E-ENALLAALTDSLDG	95
Cau	1	.....C.....R.....D.....-.....	77
Dre	1	.....Q.....A.D.....-.....	95
Ssa	1	.....T...I.....DQ..I.A.....T.D..F..I.D.....	97

C

Cca β/δ	1	PGFVDLFLNDQVTLTKYGVHEAIFAMLPMLNKDGLLVANGKGFVTREFLRSLRKPFGIMEPKFEFAVKFNAELDDSDLALFVAAILC	91
Cau β	1	.....S.....	59
Dre β2	1	.....S.....	91
Dre δb	1	.....S.....	91
Cid β	1	.....S.....	75
Omy β	1	.....R.S.....	91
Pma β	1	.....S.....	91

**D**

Cca	1	SCKYDNCCIIDKITRNQCQLCRFKKCSVGMAMDLVLDSSKRVAKRRRIEENRERKKKEEMVKTLEHNRPEPTVSEWELIRMVTEAHRHTNAQGPHWKQKR	100
Cau α1	1	.....S.....M.....T.....	100
Cau α2	1	.....S.....M.....T.....	100
Dre αA	1	.....S.....R.....KR.....I.....T.....	100
Ssa	1	.....G.....A.....VKR.....Q.....G.....L.....S.....	99
Omy	1	.....G.....A.....KR.....Q.....G.....L.....S.....	100
Cca	101	KFLPEDIGQSP-APTSDNDKVDLEAFSEFTKIITPAITRVVDFAKKLP	147
Cau α1	101	.....-.....	147
Cau α2	101	.....-.....	147
Dre αA	101	.....-.....	147
Ssa	100	.....VV.....G.....	147
Omy	101	.....V.....G.....	148

**E**

Cca	1	VEPKTETYIETNPMPSPNDPVTNICQAADKQLFTLVWAKRIPHFSELPLDDQVILLRAGWNELLIASFSHRSIIVKDGILLATGLHVHRNSAHSAG	100
Cau	1	.....	100
Dre	1	.....	100
Ola	1	.....LSV.....D.....	100
Cca	101	VGAIFDRVLTSLVSKMRDMQMDKTELGLRAIVLFNPDSKGLSNPGEVE	149
Cau	101	.....	149
Dre	101	.....	149
Ola	101	.....	149

**F**

Cca	1	SETDTQQIATLAQVSMAAAQASATAPTITVTLVQLPNGQTVQVHGVIQAAQPSVVIQSPQVTVQISTIAESDDSQESVDSVSDSQKRREILSRRPSYRKILN	100
Dre	1	.....T.....	100
Caca	1	.....G.....T.....	100
Cid	1	QQLTQA.....T.....GH.....G.....V.....E.....T.....	100
Cca	101	DLSSDAPAVPRIIEERSEEDSTPAITTVTMPTPIYQTSSGQYIAIT	146
Dre	101	.....K.....V.....	146
Caca	101	...F...QS.....K.....V.....	146
Cid	101	.....G.....K.....TA.....V.....	146

**G**

Cca	1	PEMLKYSTGRGKPGWGKESCKPIWVPEDIPWANVRSVDRTEEQQRVSWTQALRTIVKNCYKQHGREDLLYAFEDQITTOQVATTTTHSIAHLVPSQTVV	100
Dre	1	.....V.....A.....	99
Cid	1	.....Y.....V.....A.....	100
Cca	101	QTISNPDGTVSLIQVGTGATVATLADASELPGTVTVAAQVNYSTVTDGEVEQNWATLQGGEMTIQTTP	168
Dre	100	.....A.....	167
Cid	101	.....T.....-----	160

Fig. 4

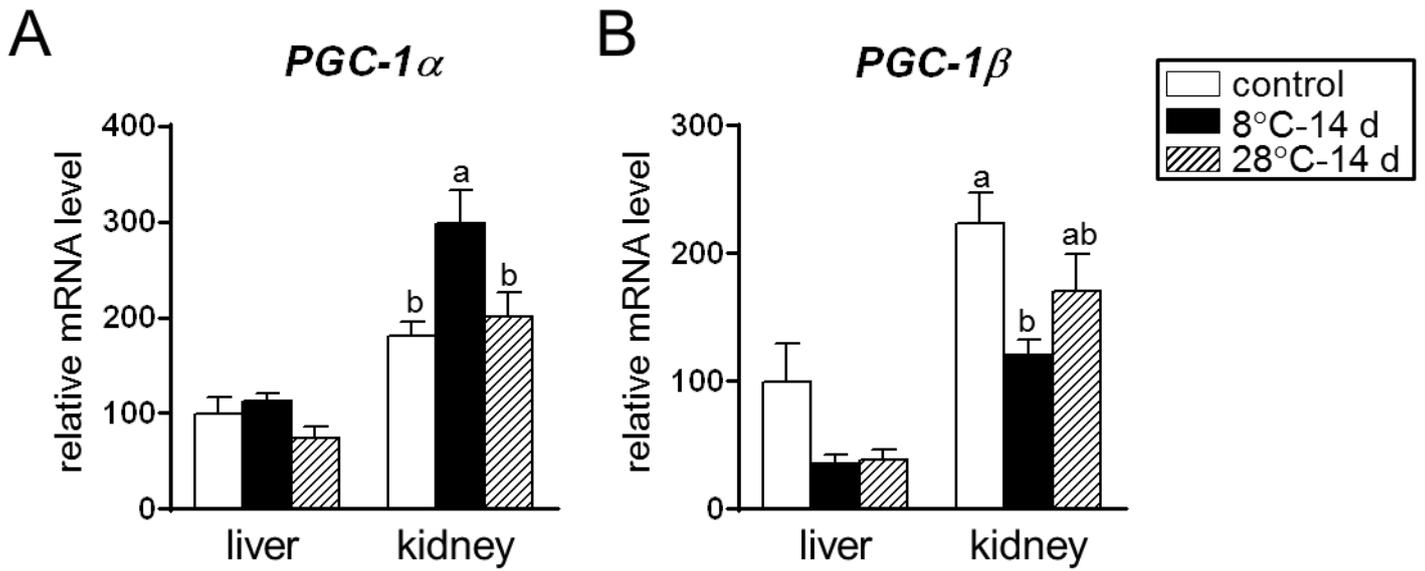


Fig. 5

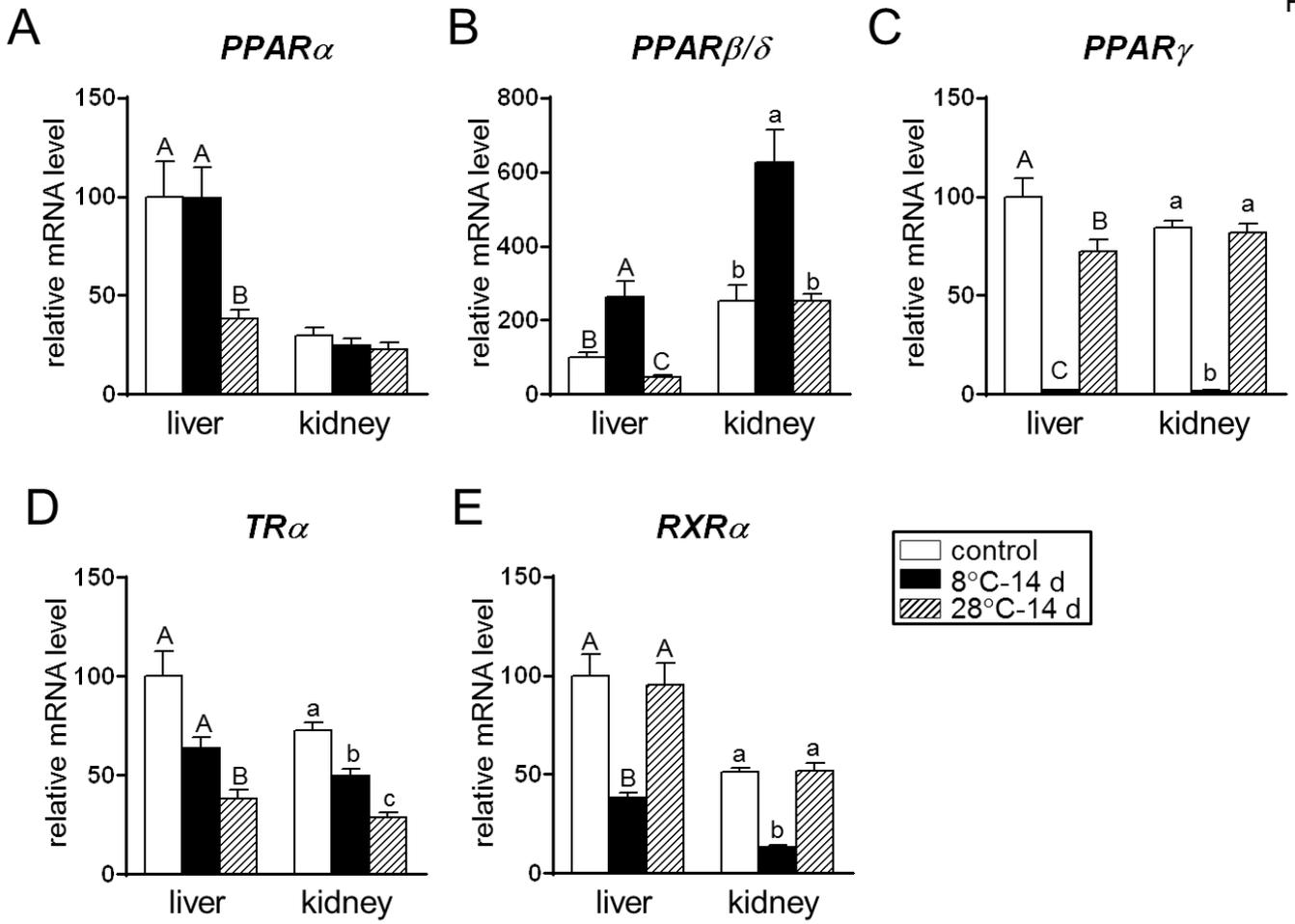


Fig. 6

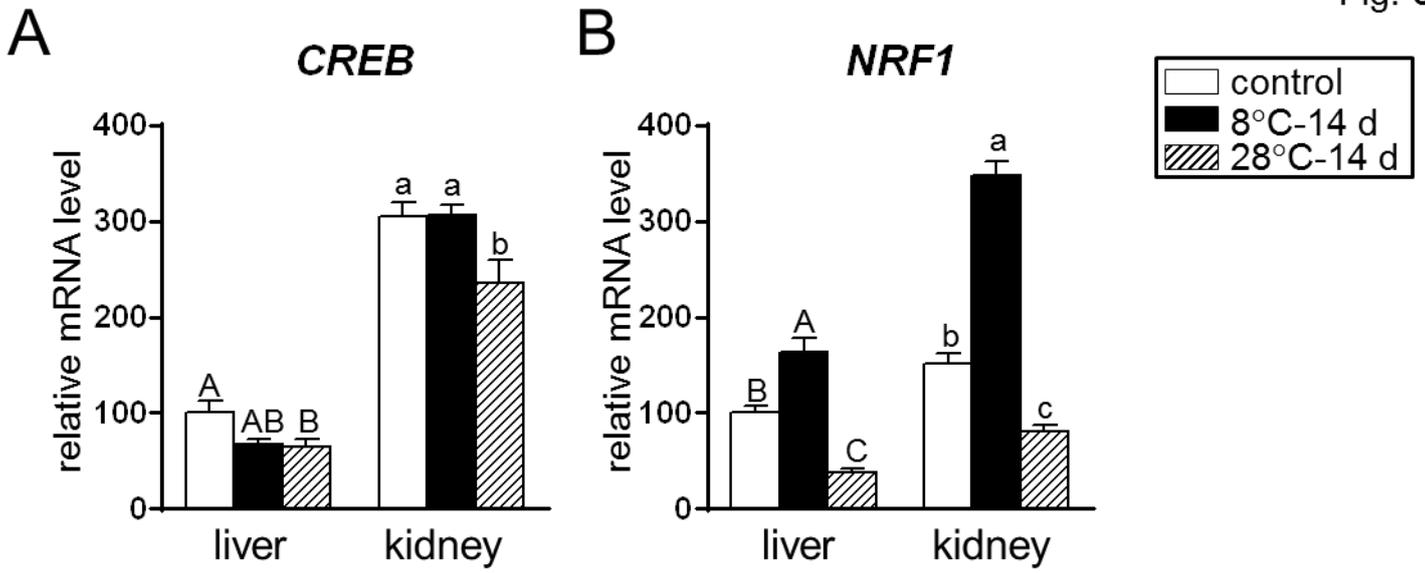
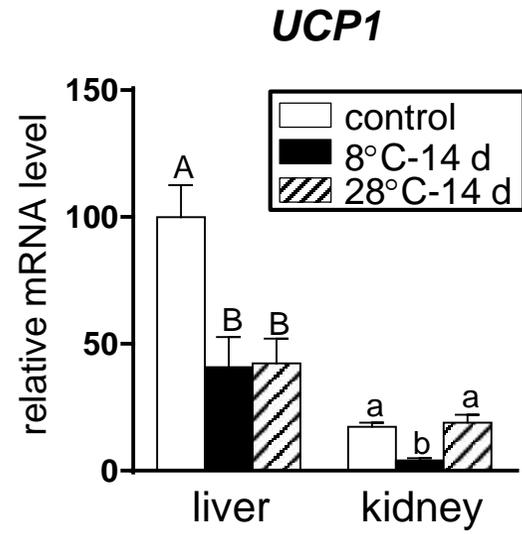


Table 1. Nucleotide sequence of primers used in RT-qPCR analyses

Gene	Forward primer	Reverse primer	GenBank accession number
CREB	5'-ctcagcagattgccaccttg-3'	5'-gggcagctgaactaaggcac-3'	LC000680 <sup>1</sup>
EF1 $\alpha$	5'-atgcggtggaatcgacaa-3'	5'-cagagagcaatgtcaatggtg-3'	AF485331
NRF1	5'-aagccctgaggactattgtt-3'	5'-gctcctgtgccaacctgtat-3'	AB924641 <sup>1</sup>
PGC-1 $\alpha$	5'-tgcctgagcttgacctct-3'	5'-cgttccatccactgggatac-3'	AB767302 <sup>1</sup>
PGC-1 $\beta$	5'-tggggaagaggaggctctgc-3'	5'-ccgtccaggctgtctgtg-3'	AB767303 <sup>1</sup>
PPAR $\alpha$	5'-gggaaagagcagcacgagtc-3'	5'-ggtaggcttcatgcatctgc-3'	FJ849065
PPAR $\beta/\delta$	5'-tggctttgtggatctctcc-3'	5'-gatctcgccgaaaggttgc-3'	LC000683 <sup>1</sup>
PPAR $\gamma$	5'-aggcaactctacgagctctatct-3'	5'-agttgatcatctgctgccttcc-3'	FJ849064
RXR $\alpha$	5'-cacccaatgatccagtcacaaaca-3'	5'-agctcattccatcctgctcgtaga-3'	LC000682 <sup>1</sup>
TR $\alpha$	5'-aatcaccgcaaccagtgccag-3'	5'-tcgatcagacgcctcttgcc-3'	LC000681 <sup>1</sup>
UCP1	5'-cgcttctacaaaggttgcg-3'	5'-cgaatgacacgaacatcacc-3'	AY461434

<sup>1</sup>Nucleotide sequence was determined in this study.

Fig. 1



A

-2634 CTACCATTGG ATCATCTGGT TGGAAATGAGA AGTAGAGTTG AGTGTGATAG GAAAGGCCAT GCTTCTGAAT GACGGATCCT AAGATGACA TGTAGATGGA  
 -2534 GAGTAARAGT GGTCCARAGCA CTGAGCCCTG AGGARACCCCA GTAGCTTAGG TGGTARACAC GCCTGGATGT GTTATTTATT TATTTATTTA TTATTATTAT  
 -2434 TATTATTATG ATTGTGGACA AGGCTTTTGA GATCGGAAAA ACCAGTTAGA ATTCARTGTT CTTTAAAGTA AGTCTTAGTC TCTGCTCTCA TTGGCATATC  
 -2334 TGGCCTGATT TTCCTTGAT AAGTGTTC TGTGTTCA GGAGCGGCTG TAGGACAGTC AGCAGATGAC TGAGATATAT GCTCCAGCTG CTGCTGGTGT  
 -2234 CTGTTGCATA AACCTGTACA GTTCAGGCCT GTTGTAGTC CTCCATGAAC TTCAGTCGAA ACGTCAACCC CTTACATCC TAATGACTAA CTCAGATAAA  
 -2134 CATTAGACCA GTACTAGACC ACTTCACAGT CCGGATCCAG TCCTTCTGAG AGAGATCAGA CTATCAGTGA AGCTGAGTC TTCATATTAG ATTGACAAC  
 -2034 AGATCTTACT TTACAGCATT GAGGATATAC AGTATATTGA TTTTGGACAG TAGCTCGGCT GTTTTCAGAC TGTAGCAAAA GATAGTAAAA CATGACAAAA  
 -1934 TTATACACTG CATTTGTTAA GCAARTGCAA ATGAACATG GCCAGCTGGA ATATTCCTTCCTTCARTCT GTTCGGTTCG GTGAATGAT TCTGGTGGT  
 -1834 TGTTTCAGGA AAGCAGTCTG TACATGCGGT TCATTTCACT GCACTGATCC GCTGATTCAT GAGATAATC TCATTTCATG ACAATAGAG TTTTACTGCT  
 -1734 GCTTTTATGT AGTAAATGT TTAGTTACAT GATATTATA ATCACATTC AGTATTATTT TGGGTCTTT TGGATAAAGG TGTATGTTG GGTAAAGAT  
 -1634 TAATGCTGTG CTGTCACTCT GGCTGAGATC ATTGTTTGTG TGCCATAAAA ATACATGTTT TTAARAATAC ATATAATCTG ACTATTATCT AACGATATT  
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 -1334 CTTCAGGAT CACATACTTC AACTTTAACA GTTCTTTTAT ATTCARTCA ATTTAGAACT ATATCTGTCC GAGAATTAT TACCATTGCT ATTATTACTA  
 -1234 TTAATATAA TAATACCATA ACACATGTA ATACTGTAC TATTATCATT ACTATTATTA TTAATTATTC TTTCAATTTAT TATTACATT CATAGTTTA  
 -1134 ATGTAARTAA ATATGTATA TTTATATTAA ATTATATATT TTTGGCGATG CAGTGGCAAA GAATCATAT AGTTCARCTT TATTGTTTTT GGATTGTGTT  
 -1034 CATGGTTCAA TTTAGAACCA TCTGTCTGAA GAGGATTAT TACATAATA RAATAGATGA TTACATTART TACATTAAAT GTAATTTAG TTGTTCTTTT  
 -934 ATATTARTGG TTCAATTAAG AATCATGTCT GTCTGACGT CCATATTATT ATTAATTATT TTAATTTTT ATACATTTAA TTTAATATA AATTTAGCAT  
 -834 TTAATTTTT TTGGTATTC ATTGGTGGAG AATCACATG GTTCACTTT AATGGTCTT TGATTATATT AATGGTTTCA TTTAGAGCA TATCTGTCTA  
 -734 AACACCCCTT AAGGTCTTG ACTTGTATG AGTATTATTT AATTTTTTT TAGTTTTAAT GAGCAGACA TGTGTTGTA GGATCATCGA GTTTGTGTTG  
 -634 CATTTGAGG CTACAGTAT TGATGCTTCA TGATTCAGCT GAACTCCTGC GTGTTTTCT CCATCTGTTA AACAGAACT CATATACATT CTTAGAGCTT  
 -534 TGTTATTTGG CTCATATTT ACCGTTTCCC ACCCTTCCAA GAGGCCATC ATCTTTCAGA CCCTGCAGGA CTGTCCCTGC GCTGCTATTG GCTGACAGCT  
 -434 TTTCTGAGCT CCTTTAATGC ATCTGCACTT TCATTAGTAG CCAATTTAA AACATCTGCA GTCATGTTGC AGTGAACTT TATTTATTAA GCATTGGTAC  
 -334 TTTAATGTAT TGCTTAAGC TGAATGTTTT ACACACATC CCATTGGTTG GTGATTAGC ATGCATGAGT CATTTGTTGA TGCTGTGCAT GTTAATGTGC  
 -234 CTGTTAATGA CAGCAGCATC GTGAAGTGT TTCAAAGCT TTGTGTTTAC TAGTTATCTA AGCGCTTGT TATCTGGATG TGGGATGTTT TGTACTGGAC  
 -134 TCTGTCTGG AAGGTGGACT TTGTGTCGGC CAGTATCTT TCCCTGAGAA ACACCTCCCT GAGCGGAGGA GGAGAACCC CTCTTGATA ATGATATATA  
 -34 AAGAAAGACA GAGCATGCC TCGTCCCTCC AGAGCTCCAG TCATCATCT GCACTCCAT CAGACAGAG AACTCAATCA CAGGTCAGTT CACCATGTTT

B

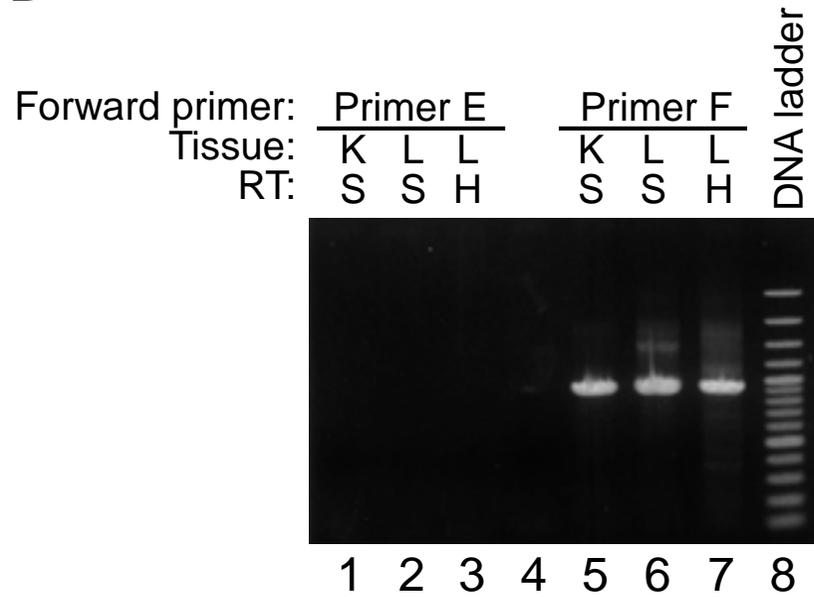


Fig. 2B, C

C

	Human	Mouse	Rat	Zebrafish	Carp
Human	100	45	44	39	36
Mouse		100	70	39	41
Rat			100	41	40
Zebrafish				100	46
Carp					100

A

Cca	1	LECAALVGEDQPLCPDLPELDLSELDVSDLDADSFLLGGLKWYSDQSEIISQYGNETSNFLFEKIDEENEANLLAVLTETLDSIPVDEDGLPSFEALADGD	100
Cau	1	.....I.....I.....	100
Dre	1	.....A.....	100
Cid	1	.....A.....	100
Spr	1	.....A.....	100
Ncr	1	.....T.....A.....	100
Cca	101	VTNASDQSCPSTPDGSPRTPEPEEPSLLKLLLLAPANSQLSYNQYPGGKAQNHAASNLRIRPTPAVAKTENPWNTKPRGSCPNRSMRRPCTELLKYLTS	200
Cau	101	.....A.....V.H.....	200
Dre	101	.....Q..A.....S..A.....	200
Cid	101	.....Q.....S..A.....	200
Spr	101	.....H.....Q.....S.....	200
Ncr	101	.....Q.....S.T..A.....	200
Cca	201	DEAFQTKAREAKSTWTGCSKDRGGA-CTSSCSSSSPSSSSTSSFSSLSSCSSSTASKKKTSSASPSSQQQQQ	272
Cau	201	.....GI...G.....-.....P.....E	271
Dre	201	.....G.....G.....-IL.....L	271
Cid	201	.....G.....-.....	272
Spr	201	.....V.....G.....-.....	272
Ncr	201	.....PL.....	272

B

Cca	1	ELSSFVFNLYLTENSGSQYGEEVCSDRLDADFPDFDLSQLDASDFDSVNCLSELHWCNEHSDHSPASIQYSGGNPELFE-E-ENAALLAALTDSLDG	95
Cau	1	-----C.....R.....D.....-.....	77
Dre	1	.....Q.....A.D.....-.....	95
Ssa	1	.....T...I.....DQ..I.A.....T.D..F..I.D.....	97

C

Cca	$\beta/\delta$	1	PGFVDLFLNDQVTLTKYGVHEAIFAMLPMLMNDGLLVANGKGFVTREFLRSLRKPFGGEIMEPKFEFAVKFNALELDDSDLALFVAAILC	91
Cau	$\beta$	1	-----S.-----	59
Dre	$\beta 2$	1	.....S.....	91
Dre	$\delta b$	1	.....S.....	91
Cid	$\beta$	1	.....S.....S.....	75
Omy	$\beta$	1	.....R..S.....	91
Pma	$\beta$	1	.....S.....	91

D

Cca	1	SCKYDNCCIIDKITRNQCQLCRFKKCSVGMAMDVLDDSKRVAKRRLIEENRERKKKEEMVKTLHNRPEPTVSEWELIRMVTEAHRHTNAQGPHWKQKR	100
Cau α1	1	.....S.....M.....T.....	100
Cau α2	1	.....S.....M.....T.....	100
Dre αA	1	.....S.....R.....KR.....I.....	100
Ssa	1	.....G.....A.....VKR.....Q.....G.....L.....S.....	99
Omy	1	.....G.....A.....KR.....Q.....G.....L.....S.....	100
Cca	101	KFLPEDIGQSP-APTSNDKVDLEAFSEFTKIITPAITRVVDFAKKLP	147
Cau α1	101	.....-.....	147
Cau α2	101	.....-.....	147
Dre αA	101	.....-.....	147
Ssa	100	.....VV.....G.....	147
Omy	101	.....V.....G.....	148

E

Cca	1	VEPKTETYIETNVPMPSPNDPVTNICQAADKQLFTLVEWAKRIPHFSELPLDDQVILLRAGWNELLIASFSHRSIIVKDGILLATGLHVHRNSAHSAG	100
Cau	1	.....	100
Dre	1	.....	100
Ola	1	.....LSV.....D.....	100
Cca	101	VGAIFDRVLTSLVSKMRDMQMDKTELGCLRAIVLFNPDASKGLSNPGEVE	149
Cau	101	.....	149
Dre	101	.....	149
Ola	101	.....	149

F

Cca	1	SETDTQIATLAQVSMAAAQASATAPVTLVQLPNGQTVQVHGVIQAAQPSVIQSPQVQTVQISTIAESDDSQESVDSVSDSQKRREILSRRPSYRKILN	100
Dre	1	.....T.....	100
Caca	1	.....G.....T.....	100
Cid	1	QQLTQA.....T...GH...G.....V...E.....T.....	100
Cca	101	DLSSDAPAVPRIIEERSEEDSTPAITTVTMPTPIYQTSSGQYIAIT	146
Dre	101	.....K.....V.....	146
Caca	101	...F..QS.....K.....V.....	146
Cid	101	.....G.....K...TA.....V.....	146

G

Cca	1	PEMLKYSTGRGKPGWGKESCKPIWWPEDIPWANVRSQVTEEQQRVSWTQALRTIVKNCYKQHGREDLLYAFEDQITQVATTTTHSIAHLVPSQTVV	100
Dre	1	.....V.....A.....	99
Cid	1	.....Y.....V.....A.....	100
Cca	101	QTISNPDGTVSLIQVGTGATVATLADASELPGTVTVAQVNYSTVTDGEVEQMWATLQGGEMTIQTTQP	168
Dre	100	.....A.....	167
Cid	101	.....T.....	160

Fig. 4

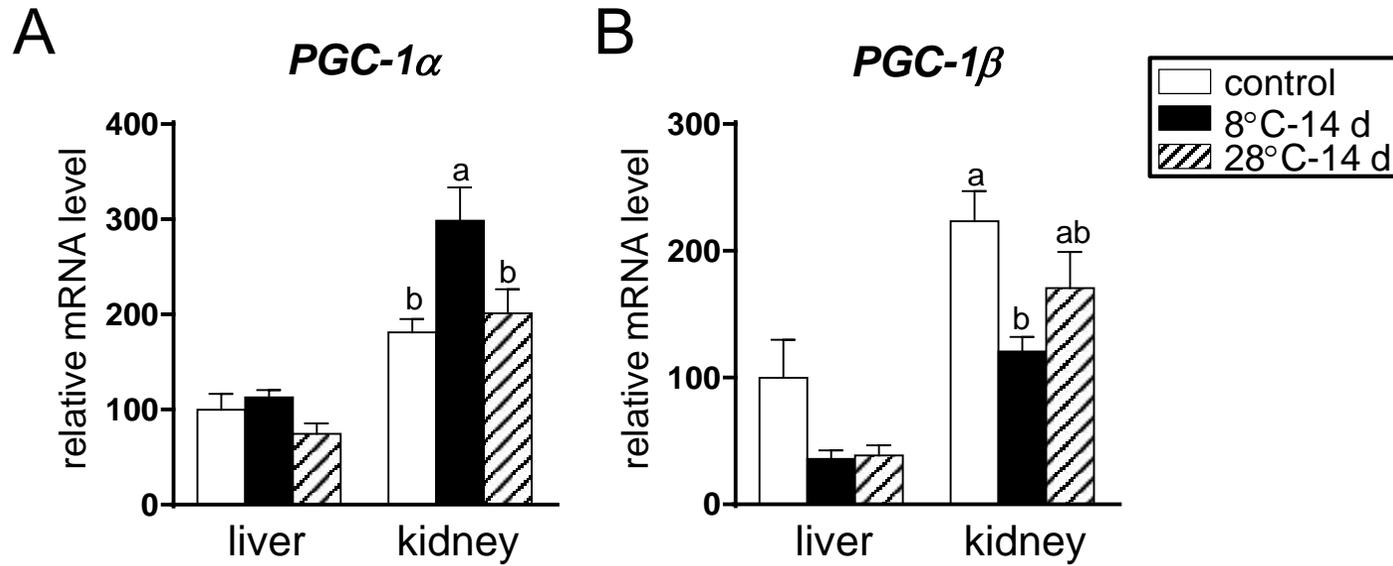


Fig. 5

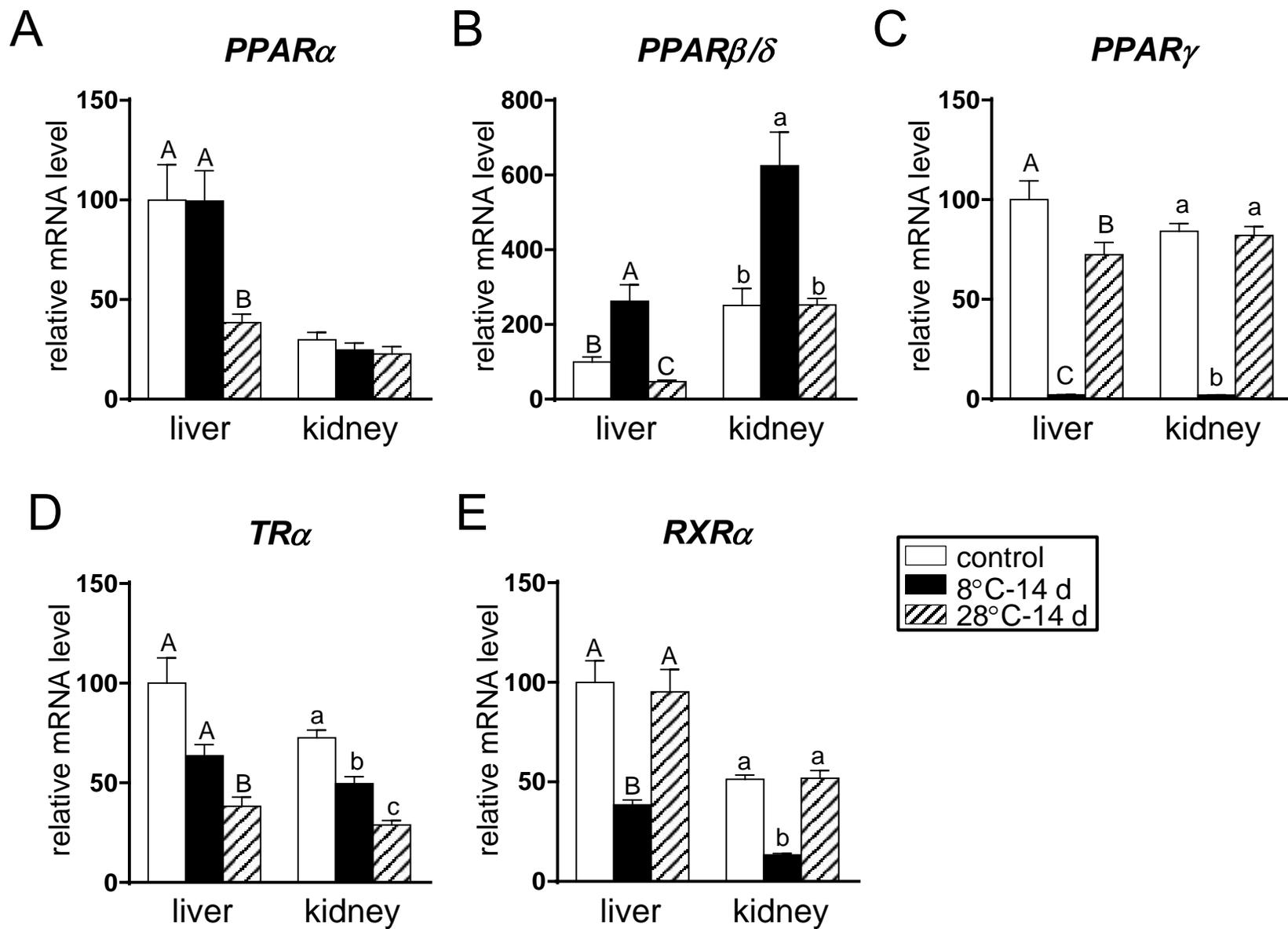


Fig. 6

