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RESEARCH PAPER

# Browning plasticity of white adipose tissue in tree shrew during cold acclimation and rewarming

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**Abstract.** This study investigated the browning plasticity of white adipose tissue (WAT) in *Tupaia belangeri* during cold acclimation and rewarming in order to demonstrate the adaptation mechanism of tree shrews to environmental change. The experimental group was transferred to a cold temperature, 5 ± 1 °C, acclimated for 28 d, and then returned to 25 ± 1 °C for 28 d, while the control group was maintained at the acclimation temperature, 25 ± 1 °C, for 56 d. Body mass, food intake, resting metabolic rate (RMR), WAT mass, morphology and related gene expression in male *T. belangeri* were measured. The results showed that body mass, food intake and RMR increased significantly under cold acclimation. There was also a significant increase in WAT mass and expression of peroxisome proliferation receptor $\alpha$ (PPAR$\alpha$), peroxisome proliferator-activated receptor $\gamma$ coactivator-1$\alpha$ (PGC-1$\alpha$), cyclooxygenaseII (COXII), bone morphogenetic protein 7 (BMP7) and the PR domain containing 16 (PRDM16), all of which decreased to control levels after rewarming. Further, WAT cells showed more multilocule adipocytes during cold acclimation, which returned to control levels after rewarming. These results suggest that browning may appear in the WAT of *T. belangeri* during cold acclimation. The return to control levels of WAT cell characteristics and expression of the genes involved in WAT browning after rewarming demonstrates strong browning plasticity.

**Key words:** *Tupaia belangeri*, adipose transcriptional regulator, morphology, mammals

## Introduction

Adipose tissue plays an extremely important role in the regulation of energy homeostasis in animals (Elsen et al. 2014). In mammals, adipose tissue can be divided into white adipose tissue (WAT) and brown adipose tissue (BAT; Gburcik et al. 2012). WAT stores energy mainly in the form of triglycerides, while BAT responds to low temperatures through burning fat for thermogenesis (Fisher et al. 2012). Increasing BAT activity and promoting WAT browning can be considered anti-obesity strategies, and this is becoming a key area in research on the treatment of obesity and related diseases in mammals (Wang et al. 2014).

The first report on the browning phenomenon showed that when mice adapt to cold temperatures, the region of uterine adipose tissue induces brown adipose characteristics (Young et al. 1984). Brown

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fat-like cells expressing uncoupling protein 1 (UCP1) positive in most WAT appeared after prolonged lower-temperature stimulation or activation of β-adrenergic receptors (Stephens et al. 2011), which were named beige or "brite" cells (Jones et al. 2002). Transformations of white adipocytes to beige cells were significantly enhanced during low temperature adaptation (Wu et al. 2012). Scans using the 18fluorabeled 2-deoxy-glucose imaging technique suggested that adults had larger amounts of active brown fat deposits, which were more similar to "brite" cells (Sharp et al. 2012). Beige cells had similar characteristics to BAT activation: the emergence of polyatrial lipid droplets, higher mitochondrial content, and expression of UCP1 (Harms & Seale 2013). Another study found that obesogenic mouse strains had a lower heat-producing potential compared with obesity-resistant strains (Shabalina et al. 2013). PR domains containing 16 (PRDM16) and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) were key transcriptional regulators in the cell lineage, which, if induced in mice, could produce a classic accumulation of brown fat (Seale et al. 2011). In WAT, brown fat cells in the process of raising UCP1 induction depends on the activity of cyclooxygenaseII (COXII; Vegiopoulos et al. 2010). Peroxisome proliferation receptor α (PPARα) mediated lipid catabolism and thermogenesis by sensing PGC-1α and PRDM16 expression as a key component of brown fat thermogenesis (Hondares et al. 2011). Based on studies of mouse cells, bone morphogenetic proteins played an important role in regulating the formation of white and brown fat, among which bone morphogenetic protein 7 (BMP7) promoted the formation of brown fat (Elsen et al. 2014). In recent years, most studies on browning have been carried out in model animals, while few studies have been conducted on small-sized free-living mammals. *Tupaia belangeri* is a member of Tupaiidae of Scandentia, which is the only representative of Tupaiidae in China. As the metabolic system and anatomical structure of *T. belangeri* are closer to humans than mice, they are widely used in biomedical research as a new experimental animal model (Wu et al. 2013). Previous studies have shown that the browning of tree shrews WAT could induced during cold temperature (Zhu et al. 2017). However, WAT browning plasticity of tree shrews during cold acclimation and rewarming has not been reported. The present study observed the browning process and plasticity of WAT in tree shrews during cold acclimation and rewarming, providing data for the basic physiological study of tree shrews. We hypothesized that browning plasticity of WAT would be observed in *T. belangeri* as an adaptation to cold acclimation and rewarming.

## Material and methods

### Samples

Tree shrews, *T. belangeri*, were wild-captured from farmland and shrub near Luquan County (25°26′-25°62′ N, 102°13′-102°57′ E, altitude 1,650-1,700 m) in Yunnan Province, China and brought back to the animal room of the College of Life Sciences of Yunnan Normal University. All individuals were males. All procedures were licensed under the Animal Care and Use Committee of the School of Life Science, Yunnan Normal University (approval ref. 13-0901-011).

### Experiment 1

*Effects of cold acclimation and rewarming on body mass, RMR and food intake*

Twenty healthy adult tree shrews of similar body mass were acclimated for 28 d at 25 ± 1 °C before the experiment and then randomly divided into two groups. The experimental group was transferred to 5 ± 1 °C for 28 d, and then returned to 25 ± 1 °C for a further 28 d. The control group was maintained 25 ± 1 °C throughout. All animals were maintained in a photoperiod of 12L:12D with food and water provided *ad libitum*. Body mass and food intake were measured every two days and RMR every three days.

### Experiment 2

*Effects of cold acclimation and rewarming on WAT mass, morphology and gene expressions*

Sixty healthy adult tree shrews with similar body mass were acclimated for 28 d at 25 ± 1 °C before being randomly divided into two groups. The experimental group was transferred to 5 ± 1 °C for 28 d, and returned to 25 ± 1 °C for a further 28 d. The control group was maintained at 25 ± 1 °C for 56 d, and individuals were killed at 0 d, 28 d, 56 d, respectively and samples of groin WAT preserved at –80 °C.

### Histomorphological analysis

The WAT samples were washed with PBS, and fixed in 4% paraformaldehyde. They were then dehydrated in ethanol and xylene, embedded in wax, sectioned and mounted on numbered slides. The sections were dewaxed in ethanol and xylene, stained in haematoxylin for 10-30 min and

**Table 1.** gDNA removal reaction system.

| Composition | Application amount |
|---|---|
| 5XgDNA Buffer | 2 μL |
| Total RNA | According to 2 g |
| RNase-Free dd$H_2O$ | Make up to 10 μL |

**Table 2.** Reverse transcription reaction system.

| Composition | Application amount |
|---|---|
| 10xFast RT Buffer | 2 μL |
| RT Enzyme Mix | 1 μL |
| FQ-RT Primer Mix | 2 μL |
| RNase-Free dd$H_2O$ | 5 μL |

**Table 3.** Primer amplification of *Tupaia belangeri*.

| Name | Forward Primer | Reverse Primer |
|---|---|---|
| PRDM16 | 5′-CTGTCACAGCCTGGAGCAGCACAT-3′ | 5′-GGTAGGGTGTCTGTGCACGTTACC-3′ |
| BMP7 | 5′-ACATCACAGCCACCAGCAA-3′ | 5′-CACCATCCAGCGTCTCCA-3′ |
| PPAR$\alpha$ | 5′-GCTCCGATGGGTCTGTTAT-3′ | 5′-GGTGAGGATTTCCGCTTT-3′ |
| COXII | 5′-CGACTCATACATAATCCCAACA-3′ | 5′-TTCAGAACACTGCCCATAAA-3′ |
| PGC-1$\alpha$ | 5′-GTGTCACCACCCAAATCCTTA-3′ | 5′-ATTCTTCCCTCTTCAGCCTCT-3′ |

washed with distilled water to remove excess dye. They were differentiated in 1% hydrochloric acid ethanol solution, rinsed with distilled water and dehydrated in ethanol. After staining for 1-3 min, excess red dye was washed off with 95% ethanol, dehydrated with anhydrous ethanol and rinsed in xylene until transparent. Finally, the neutral resin seal was observed and photographed under an optical microscope.

### Gene expression analysis

The total RNA II Extraction Kit (omega, USA) was used to extract RNA from WAT. A DNA/RNA concentration meter was used to determine total RNA purity and concentration. In accordance with the instructions of the FAST Quant Rt Kit (with gDNase), a reaction system of 20 μL was established for 50 ng – 2 g total RNA. The mixture was prepared according to the removal system of genomic DNA in Table 1 and thoroughly mixed. It was then centrifuged briefly and incubated at 42 °C for 3 min, then placed on ice. The mixture was prepared according to the reverse transcription reaction system in Table 2. The final cDNA was stored at low temperature, and the primers in Table 3 were used for qPCR detection. The obtained data were homogenized by internal reference genes. qPCR was used to assay related genes involved in WAT browning under cold exposure including PRDM16, BMP7, PPAR$\alpha$, COX-2 and PGC-1$\alpha$. Species-specific primer sets for PRDM16, BMP7, PPAR$\alpha$, COX-2 and PGC-1$\alpha$, and beta-actin in tree shrews were designed according to the gene sequences of reference (Mei et al. 2019). Each gene in each sample was repeated three times by qPCR. The relative quantity of gene expression was calculated by $2^{-\Delta\Delta Ct}$.

### Statistical analysis

Data were analysed using the software package SPSS 20.0. Prior to all statistical analyses, data were examined for assumptions of normality and homogeneity of variance using Kolmogorov-Smirnov and Levene tests, respectively. For experiment 1, differences in body mass, food intake and RMR for each group were analysed with a repeated measures ANOVA. For experiment 2, differences between groups on a single experimental day were examined using independent t-tests, differences in WAT mass and gene expression for each group were analysed with a one-way ANCOVA with body mass as a covariate, followed by Tukey's post hoc test. Results are presented as means ± SE, and $p < 0.05$ was considered statistically significant.

## Results

### Experiment 1

*Body mass, RMR and food intake*

Prior to the experiment there were no significant differences in body mass (t = –0.628, $p$ = 0.712), food intake (t = 1.257, $p$ = 0.635) or RMR (t = –0.573, $p$ = 0.832) between the two groups. Body mass changed significantly in the experimental group (F = 6.837, $p$ = 0.006), but there was no significant change in the control group (F = 1.647, $p$ = 0.545, Fig. 1A) throughout the acclimation. Food intake in the experimental group increased 45% over the 28 d (F = 4.767, $p$ = 0.008) but there was no significant change in the control group (F = 1.393, $p$ = 0.593;

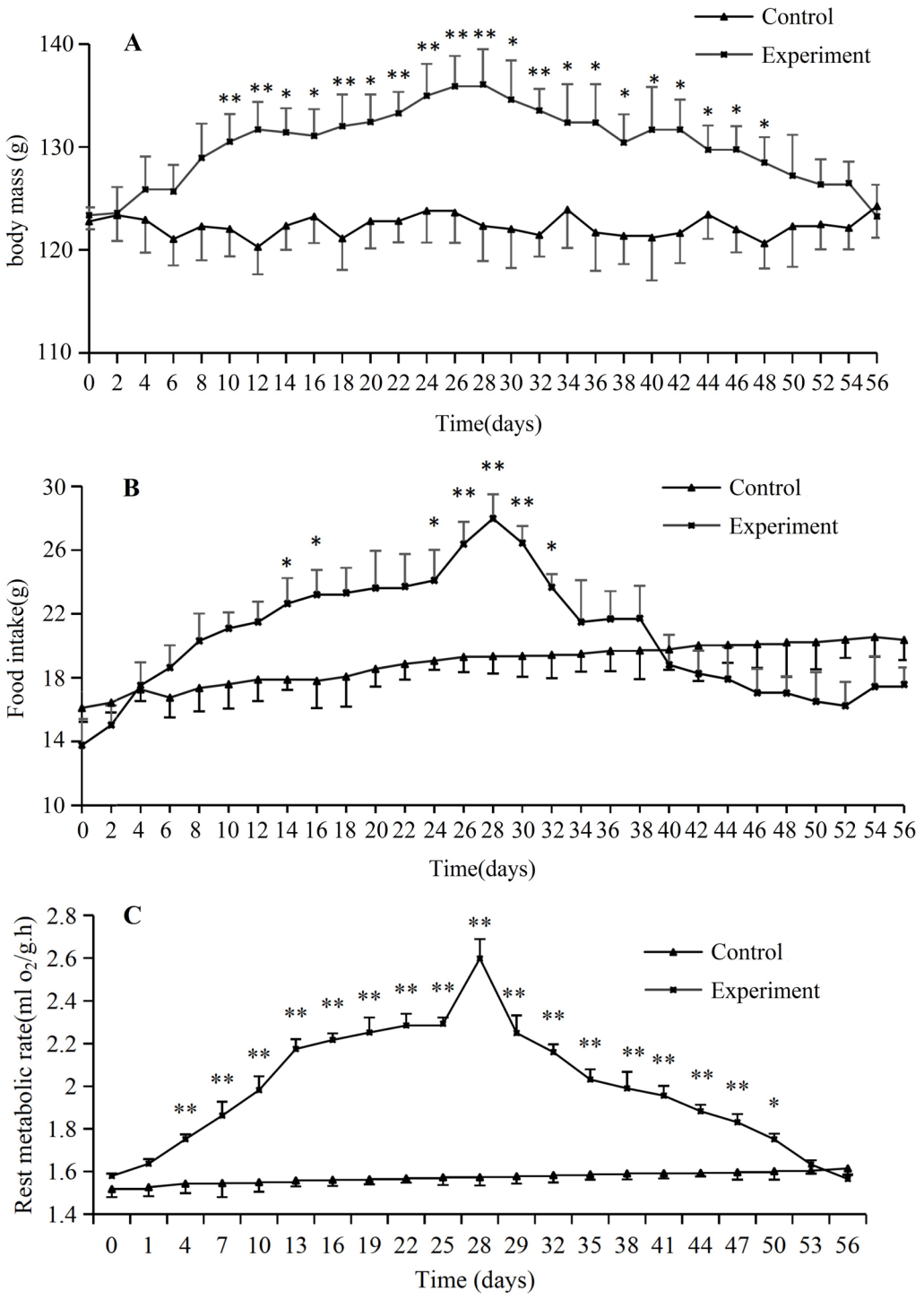


**Fig. 1.** Effects of cold acclimation and rewarming on A) body mass, B) food intake and C) RMR of *Tupaia belangeri*; *$p$ > 0.05, **$p$ > 0.01 (compared with control group).

Fig. 1B). RMR increased significantly under cold acclimation, reaching its highest value after 28 d (F = 34.609, $p$ = 0.001). There was no difference between the control group and the experimental group after 28 d rewarming (t = 1.174, $p$ = 0.656; Fig. 1C).

## Experiment 2

*WAT mass and morphology*

A significant difference was found in WAT mass in the experimental group (F = 13.587, $p$ = 0.003), but there was no significant difference in control group (F = 0.220, $p$ = 0.951; Fig. 2). WAT mass in experimental group recovered to the level of the control group after rewarming. WAT showed more multicellular fat cells, which had a smaller diameter after 28 d (Fig. 3A, B, D, E). The multicellular fat cells gradually disappeared and the diameter of more white fat cells increased during rewarming, showing no significant difference from the control group after 28 d (Fig. 3C, F).

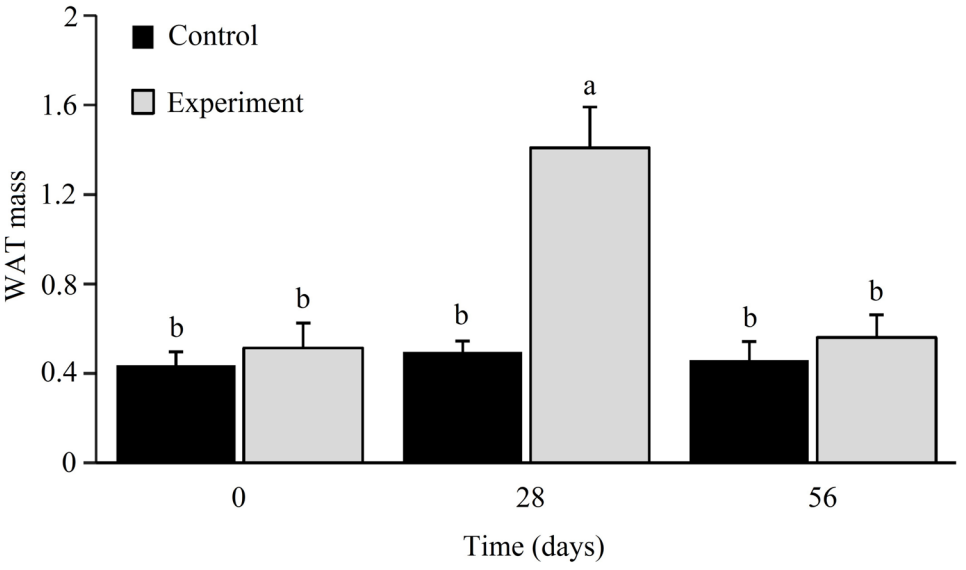


**Fig. 2.** Effects of cold acclimation and rewarming on WAT mass of *Tupaia belangeri*. Different letters indicate significant differences among the groups ($p < 0.05$).

### Gene expression

In the experimental group, PPAR$\alpha$ increased significantly after 28 d at low temperature (F = 19.515, $p = 0.001$), but recovered to the same level as the control group after rewarming. There was no significant change in the control group (F = 1.006, $p = 0.671$; Fig. 4A). Temperature had a significant effect on PGC-1$\alpha$ expression in the experimental group (F = 10.694, $p = 0.003$; Fig. 4B, which was significantly higher after 28 d cold acclimation and recovered to the control level after 28 d rewarming. BMP7, COXII and PRDM16 expression also increased after 28 d cold acclimation (BMP7: F = 4.325, $p = 0.033$; COXII: F = 3.638, $p = 0.046$; PRDM16: F = 8.253, $p = 0.005$), and returned to the control level after rewarming 28 d (Fig. 4C, D, E).

## Discussion

Environmental temperature plays an important role in body mass regulation in animals (Zhang & Wang 2006). Seasonal adjustments in body mass and thermogenesis in Mongolian gerbils, *Meriones unguiculatus*, showed that body mass was highest in winter (Li & Wang 2005), which is similar to tree shrews. In the present study, body mass increased during cold acclimation in tree shrews, reaching its peak value at 28 d and decreasing during

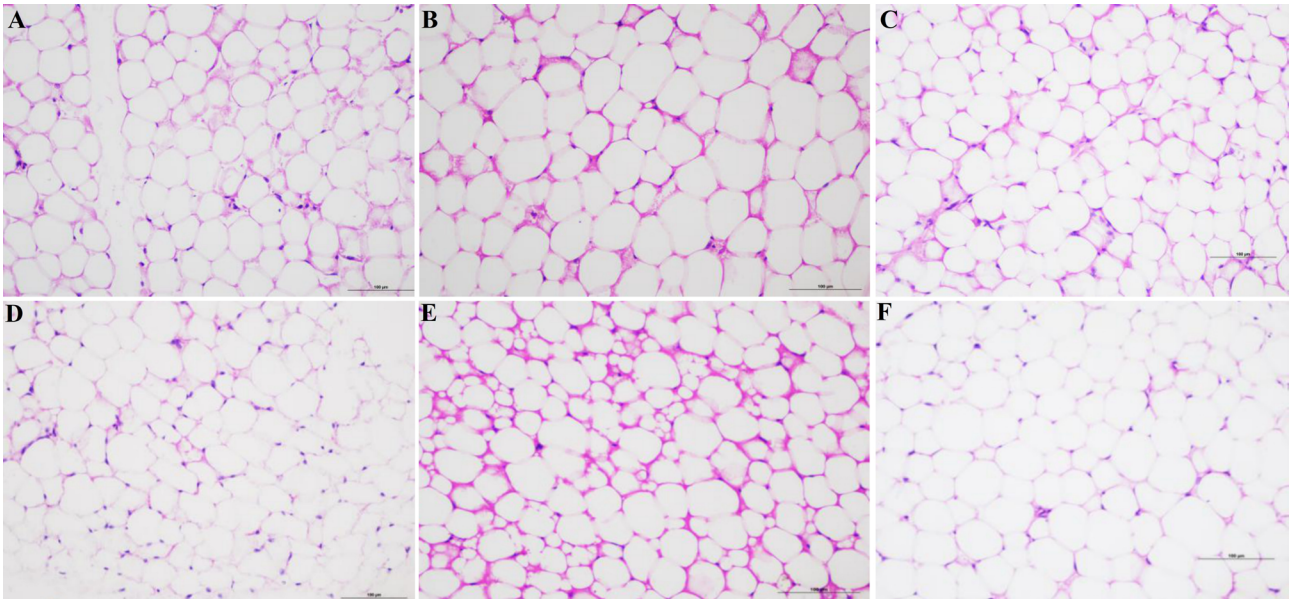


**Fig. 3.** Morphological study on WAT of *Tupaia belangeri* during cold acclimation and rewarming: A) 0 d control; B) 28 d control; C) 56 d control; D) 0 d experimental; E) 28 d experimental; F) 56 d experiential.

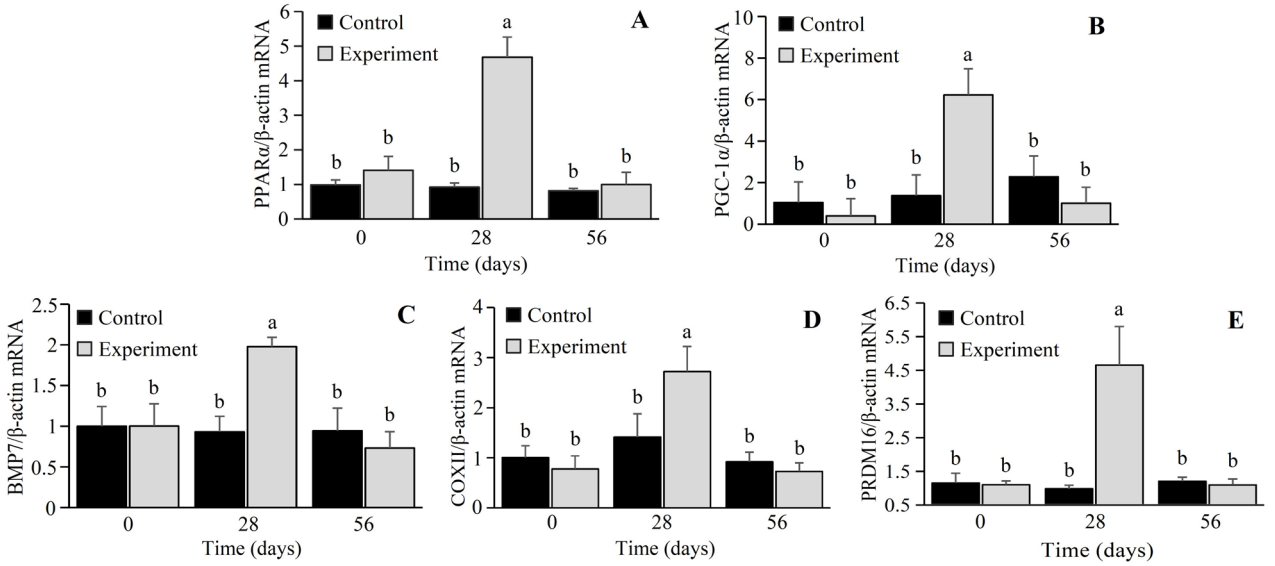


**Fig. 4.** Effect of cold acclimation and rewarming on the gene expression of WAT in *Tupaia belangeri*. Different letters indicate significant differences among the groups ($p < 0.05$): A) PPARα; B) PGC-1α; C) BMP7; D) COXII; E) PRDM16.

the rewarming process. These changes in body mass were associated with change in RMR and food intake which also increased in during cold acclimation and reduced after rewarming. These data indicate that ambient temperature drives changes in body mass and energy metabolism. Seasonal variation in body mass is considered to be an important adaptation strategy for small mammals (Concannon et al. 2001). An increase in RMR during cold acclimation may indicate the production of beige cells by WAT browning.

Morphological analysis showed cold acclimation induced browning of WAT, while WAT mass increased in response to increased energy consumption. After rewarming, the morphology and WAT mass returned to control levels, suggesting that thermogenesis of tree shrews decreased in the warm environment. Moreover, browning cells appear to return to white adipocytes, indicating plasticity of browning.

The increase in PRDM16 expression in WAT, likely induces the production of brown fat-like cells in WAT (Fisher et al. 2012). Brown adipocyte related genes in WAT, such as PGC-1α, show increased expression of browning in WAT (Wang et al. 2014). Due to the regulating role of COXII in beige cells, COXII excess activity in vivo was sufficient to protect genetically modified mice from weight gain on a high fat diet (Paschos et al. 2018). In our study, cold acclimation induced a significant increase in expression of PPARα, PGC-1α, BMP7, COXII and PRDM16 and the results showed that cold acclimation induced browning of the WAT. After rewarming, gene expression returned to the control level, showing browning plasticity.

In conclusion, cold acclimation induced WAT browning of tree shrews, and the restoration of relevant indices to the control level after rewarming, reflected strong browning plasticity of WAT in *T. belangeri*. In addition to the plasticity of body mass, RMR and food intake, the tree shrews also rely on the browning plasticity of WAT to adapt to changes in environmental temperature when facing cold temperatures in the wild.

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