

Thermogenic Property and its Hormonal Regulation in a South China Field Mouse, *Apodemus draco*, Under Seasonal Acclimatization

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Abstract.- Environmental factors, such as temperature, play an important role in the adjusting of body mass and thermogenesis in small mammals. In the present study, we examined thermogenic properties of a field mouse, *Apodemus draco*, under seasonal acclimatization. Body mass as well as several physiological, hormonal markers, and uncoupling-protein 1 (UCP1) contents were measured. The results showed that body mass decreased, while rest metabolic rate (RMR), nonshivering thermogenesis (NST), energy intake and UCP1 contents increased in winter. Serum leptin level, tri-iodothyronine (T₃), thyroxine (T₄), T₃/T₄, testosterone and melatonin concentration showed seasonal variation, while thyroid-stimulating hormone (TSH) showed no difference between seasons. There were significant negative correlation between serum leptin level and RMR, NST, energy intake, UCP 1 contents and serum T₃ ($r=-0.496$, $P<0.05$, $r=-0.700$, $P<0.01$, $r=-0.641$, $P<0.01$, $r=-0.552$, $P<0.05$, $r=-0.536$, $P<0.05$, respectively). Serum leptin level was positive correlated with body fat mass and T₄ ($r=0.764$; $P<0.01$, $r=0.484$, $P<0.05$). However, no correlation was found between serum leptin level and serum TSH concentrations ($r=0.361$, $P>0.05$). The present results suggested that *A. draco* adapted to winter cold stress by increasing energy intake, thermogenesis and decreasing body mass and body fat mass. Leptin may act as a starvation signal to permit the increase in energy intake for energy expenditure mainly as thermogenesis for winter adaptation. Furthermore, testosterone and melatonin seem to be involved in the regulation of body mass and energy balance in *A. draco*.

Key words: *Apodemus draco*, serum leptin level, uncoupling protein 1, testosterone; melatonin.

INTRODUCTION

Seasonal phenotypic flexibility of metabolism plays an important role in thermoregulation in endothermic animals (Dawson, 2003; Lovegrove, 2005; Bush *et al.*, 2008). Many rodent species require proper strategy and efficient thermoregulatory mechanisms to survive in seasonal environment, such as *Phodopus sungorus* (Klingenspor *et al.*, 2000), *Lasiopodomys brandtii* (Li and Wang, 2005a), *Meriones unguiculatus* (Li and Wang, 2005b), *Microtus oeconomus* (Wang *et al.*, 2006a), *Apodemus chevrieri* (Zhu *et al.*, 2012a), *Tupaia belangeri* (Zhu *et al.*, 2012b). The most important physiological strategy for small mammals to cope with winter or cold acclimation is to decrease body mass and increase the thermogenic capacity, *e.g.* *M. unguiculatus* (Li and Wang, 2005b), *Cricetulus barabensis* (Zhao *et al.*, 2010), *M. maximowiczii* (Chen *et al.*, 2012), *A. chevrieri* (Zhu *et al.*, 2011), *A. draco* (Zhu *et al.*, 2012c). UCP1 is an uncoupling protein found in the mitochondria of

brown adipose tissue (BAT). It is used to generate heat by non-shivering thermogenesis (Krauss *et al.*, 2005). Triiodothyronine (T₃) is a thyroid hormone, which affects almost every physiological process in mammals, including growth, development and metabolism (Klein, 2006). T₃ and its prohormone thyroxine (T₄) were activated by thyroid-stimulating hormone (TSH), which is released from the pituitary gland. Elevated concentrations of T₃ and T₄ in the blood plasma can inhibit the production of TSH in the pituitary gland (Mustonen *et al.*, 2000). Melatonin is a naturally occurring indoleamine molecule found in animals, plants, and microbes. In mammals, circulating levels of the hormone melatonin vary in a daily cycle, thereby allowing the entrainment of the circadian rhythms of several biological functions (Altun and Ugur-Altun, 2007). Change in duration of melatonin secretion thus serves as a biological signal for the organization of day length-dependent (photoperiodic) seasonal functions such as reproduction and behaviors, as well as thermogenic characteristics in seasonal animals (Arendt and Skene, 2005; Steinlechner and Heldmaier, 1982). Testosterone is a steroid hormone from the androgen group and is found in vertebrates, which is the principal male sex hormone and an

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anabolic steroid (Reed *et al.*, 2006).

Leptin is a 16 kDa protein hormone that plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism (Friedman and Halaas, 1998). It had been indicated that leptin is a potential signal that mediates the seasonal variations of body mass and energy balance (Klingenspor *et al.*, 2000; Li and Wang, 2005a). Serum leptin level is negatively correlated with UCP1 content in some seasonal mammals (Abelenda *et al.*, 2003; Li and Wang, 2005a; Wang *et al.*, 2006a; Zhang *et al.*, 2011, 2012). However, no relationship was found between leptin and UCP1 contents in cold-acclimated *M. unguiculatus* (Li *et al.*, 2004) and *M. agrestis* (Król *et al.*, 2006). Energy metabolism was regulated by the interactions between leptin and TSH. Fasting can decrease leptin and TSH concentrations, but injection of leptin can prevent the decline of T₄ concentrations. Injection of T₃ into the adipose tissue of hypothyroidism rat decreased the expression of leptin mRNA (Escobar-Morreale *et al.*, 1997). Some studies have also found that melatonin plays a regulatory role of circadian secretion of leptin level (Giyasettin *et al.*, 2001).

The South China field mouse, *Apodemus draco*, is the endemic species of the Hengduan mountains region (Zheng, 1993). The Hengduan Mountains region is located the boundary between the Palearctic region and the Oriental region. Environmental factors, such as photoperiods and cold, are effective cues that influence body mass and thermogenesis in *A. draco*, separately (Zhu *et al.*, 2012c, 2013). In the present research, we measured seasonal changes in thermogenesis including BMR, NST, energy intake and UCP1 contents of *A. draco*, and explored the potential role of leptin, testosterone and melatonin in the regulation of body mass and thermogenesis. We hypothesize that, similar to other small mammals, *A. draco* would change their thermogenesis seasonally and serum leptin level would change with body mass or body fat mass. We predict that *A. draco* would show a decrease in body mass and increase in thermogenesis in the cold season and that serum leptin level would be lower in winter than summer. Furthermore, testosterone and melatonin should be involved in the regulation of body mass and energy

balance in *A. draco*.

MATERIALS AND METHODS

Samples

A. draco were captured in a farmland (26°15'~26°45'N; 99°40'~99°55'E; altitude 2,590 m) at Jianchuan County, Yunnan province. The mean annual temperature is 9.1°C, average monthly temperature ranges from -4.0°C in winter to 24.1°C in summer. *A. draco* were captured in June (n=9), and December (n=8) in 2011, housed in plastic box (260×160×150mm³) individually. The subjects were fed *ad libitum* with standard rabbit food and water. All animal procedures were licensed under the Animal Care and Use Committee of Institute of Zoology, the Chinese Academy of Sciences. All pregnant, lactating or young individuals were excluded in present study. After the RMR, NST and energy intake measurement, subjects were sacrificed by puncture of the posterior vena cava within 7 days after capture for all the two seasons, and blood and tissue samples were taken for measurement of endocrine hormones concentrations and body fat mass (Wang *et al.*, 2006b). Total body fat mass was extracted from the dried carcass by ether extraction in a Soxhlet apparatus (Zhang and Wang, 2007).

Measurement of metabolic rates

Metabolic rates were measured by using an AD ML870 open respirometer (AD Instruments, Australia) at 25°C within the TNZ (thermal neutral zone), gas analysis were using a ML206 gas analysis instrument, the temperature was controlled by SPX-300 artificial climatic engine (±0.5°C), the metabolic chamber volume is 500ml, airflow rate is 200 ml/min. *A. draco* were stabilized in the metabolic chamber for at least 60 min prior to the RMR measurement, oxygen consumption was recorded for more than 120 min at 1 min intervals. Ten stable consecutive lowest readings were taken to calculate RMR (Li and Wang, 2005b). The method used for calculating the metabolic rate is detailed in Hill (1972):

$$MR = \frac{FR \times (FiO_2 - FeO_2) - FR \times FeO_2 \times (FeCO_2 - FiCO_2)}{1 - FeO_2}$$

Where FR = flow rate (ml/min), $FiO_2 = O_2$ input fractional concentration, $FiCO_2 = CO_2$ input fractional concentration, $FeO_2 = O_2$ excurrent fractional concentration, $FeCO_2 = CO_2$ excurrent fractional concentration.

Non-shivering thermogenesis (NST) was induced by subcutaneous injection of norepinephrine (NE) (Shanghai Harvest Pharmaceutical Co. Ltd) and measured at 25 °C. Two consecutive highest recordings of oxygen consumption more than 60 min at each measurement were taken to calculate the NST (Zhu *et al.*, 2010). The doses of NE were approximately 0.8-1.0 mg/kg according to dose-dependent response curves that were carried out before the experiment and the equation described by Heldmaier (1971): norepinephrine dosage (mg/kg) = $6.6M^{-0.458}$ (g).

Energy intake

Energy intake for each season was measured for 3 days (Song and Wang, 2002), and food were provided *ad libitum*. Animals were fed with food of fixed quantify at same time, next day weighted animal body mass, collected residual food. Residual food was dried in at 65°C for at least 72 h. Energy contents of food were measured by YX-ZR/Q automatic calorimeters instrument (U-therm Industry Co. Ltd. Changsha, China). The calorie of the diet fed to these animals was 18.0 ± 0.8 kJ/g. The calculation of energy intake was according to corresponding reports (Drozdz, 1975): Energy intake (kJ/d) = food (g/d) * energy content (kJ/g).

Measurement of hormone concentration

Serum leptin levels were determined by radioimmunoassay (RIA) with the ^{125}I Multi-species Kit (Cat. No. XL-85K, Linco Research Inc.). The lowest level of leptin that can be detected by this assay was 1.0 ng/ml when using a 100- μ l sample size. And the inter- and intra-assay variability for leptin RIA were <3.6% and 8.7%, respectively.

The concentrations of T_3 and T_4 in serum were determined using RIA kits (China Institute of Atomic Energy). These kits were validated for all species tested by cross-activity. The intra- and inter-assay coefficients of variation were 2.4% and

8.8% for the T_3 , 4.3% and 7.6% for T_4 , respectively. Serum melatonin concentrations were measurement by ELISA method (Bioteke Co. Beijing, China). TSH and testosterone concentrations were determined by radioimmunoassay kit (Linco Co. USA) (Du and You, 1992).

Measurements of UCP1 content

UCP1 content was measured by Western blotting. Total BAT protein (15 μ g per lane) was separated in a discontinuous SDS-polyacrylamide gel (12.5% running gel and 3% stacking gel) and blotted to a nitrocellulose membrane (Hybond-C, Amersham). To check for the efficiency of protein transfer, gels and nitrocellulose membranes were stained after transferring with Coomassie brilliant blue and Ponceau red, respectively. Unspecific binding sites were saturated with 5% nonfat dry milk in PBS. UCP1 was detected using a polyclonal rabbit UCP1 (1:5000) as a primary antibody (Abcam, Shanghai) and peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Jackson Immuno. Inc., USA) as the second antibody. Enhanced chemoluminescence (ECL, Amersham Biosciences, England) was used for detection of UCP signal. UCP1 concentration was determined from area readings by using Scion Image Software (Scion Corporation) (Li and Wang, 2005a).

Statistical analysis

Data were analyzed using the software package SPSS 15.0. Prior to all statistical analyses, data were examined for assumptions of normality and homogeneity of variance using Kolmogorov-Smirnov and Levene tests, respectively. Body mass, body fat mass, serum leptin level, UCP1 contents, RMR, NST, energy intake and other hormone concentrations were analyzed by independent-samples t-test. To detect possible associations of serum leptin with body fat mass, RMR, NST, energy intake and hormone concentration, we used Pearson-correlation analysis. Since no gender effects were found on almost all measured parameters, data from females and males were combined except when specified. Results are presented as means \pm SEM and $P < 0.05$ was considered to be statistically significant.

RESULTS

Body mass, body fat mass and serum leptin level

Body mass of *A. draco* showed significant seasonal variations ($t=4.21$, $df=15$, $P<0.05$; Table 1). Body mass was higher in summer and lower in winter. Body fat mass between two seasons showed significant differences ($t=2.40$, $df=15$, $P<0.05$; Table I). Body fat mass in summer was 51.69% higher than that in winter. Further, serum leptin level showed significant seasonal variations ($t=2.20$, $df=15$, $P<0.05$; Table II). Correlation analysis indicated that serum leptin levels were positively correlated with changes in body fat mass ($r =0.764$; $P<0.01$; Fig. 1).

Table I.- Body mass and thermogenetic markers in summer and winter in *Apodermus draco* (Results are presented as means \pm SEM).

Parameters	Summer	Winter	P value
Body mass (g)	41.48 \pm 1.59	35.11 \pm 2.15	$P<0.05$
Body fat mass (g)	5.15 \pm 0.64	3.40 \pm 0.28	$P<0.05$
RMR (ml O ₂ /g.h)	1.95 \pm 0.17	2.53 \pm 0.17	$P<0.05$
NST (ml O ₂ /g.h)	4.36 \pm 0.14	5.70 \pm 0.10	$P<0.01$
Energy intake (kJ/day)	78.57 \pm 4.26	95.49 \pm 4.16	$P<0.05$
UCP1 (mg/g.BAT tissue)	134.18 \pm 3.27	162.85 \pm 4.57	$P<0.01$

Table II.- Hormone concentrations in summer and winter in *Apodermus draco* (Results are presented as means \pm SEM).

Parameters	Summer	Winter	P value
Leptin (ng/ml)	3.85 \pm 0.71	2.13 \pm 0.22	$P<0.05$
T ₃ (ng/ml)	1.48 \pm 0.15	2.75 \pm 0.38	$P<0.01$
T ₄ (ng/ml)	49.07 \pm 2.09	41.56 \pm 0.80	$P<0.01$
T ₃ /T ₄	0.0297 \pm 0.0022	0.0660 \pm 0.0088	$P<0.01$
TSH (ng/ml)	1.47 \pm 0.08	1.37 \pm 0.10	$P>0.05$
Testosterone (ng/ml)	0.92 \pm 0.21	0.41 \pm 0.01	$P<0.05$
Melatonin (pg/ml)	44.91 \pm 0.93	48.78 \pm 3.84	$P<0.05$

Thermogenetic capacity

Significant seasonal effect on RMR of *A. draco* was observed ($t=-2.38$, $df=15$, $P<0.05$; Table 1). RMR in winter increased by 29.56% compared with that in summer. Similar to RMR, NST showed marked difference among seasons ($t=-7.74$, $df=15$, $P<0.01$; Table I). Winter showed higher NST than that in summer in *A. draco*. *A. draco* showed

seasonal variations in their energy intake ($t=-2.83$, $df=15$, $P<0.05$; Table I). There was significant change in UCP1 contents between the seasons ($t=-5.19$, $df=15$, $P<0.01$; Table I) with higher UCP1 contents in winter than in summer individuals. Correlation analysis showed that serum leptin levels were negatively correlated with RMR ($r=-0.496$, $P<0.05$; Fig. 2A), NST ($r=-0.700$, $P<0.01$; Fig. 2B), energy intake ($r=-0.641$, $P<0.01$; Figure 2C) and UCP1 contents ($r=-0.552$, $P<0.05$; Fig. 2D).

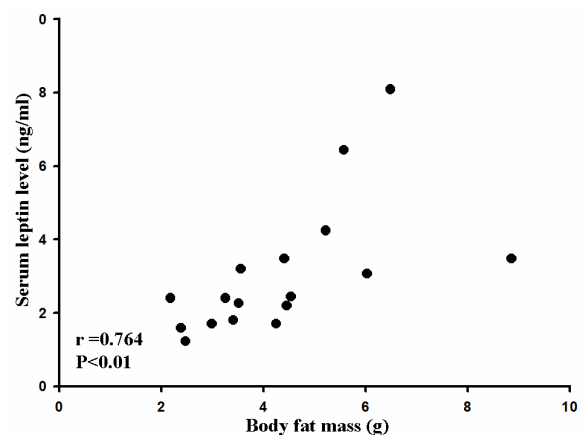


Fig. 1. Correlation of serum leptin level with body fat mass in *Apodemus draco* in different seasons.

Hormone concentrations

Serum T₃ concentration and T₃/T₄ in *A. draco* captured in winter was higher than that in summer ($t=-3.25$, $df=15$, $P<0.01$; $t=-3.98$, $df=15$, $P<0.01$, respectively; Table II). While serum T₄ concentration and testosterone concentrations were lower in winter and higher in summer ($t=3.19$, $df=15$, $P<0.01$; $t=2.17$, $df=15$, $P<0.05$, respectively; Table II). Seasonal alterations were also detected in melatonin concentrations ($t=-2.39$, $P<0.05$; Table II), which in winter was significantly higher than that in summer. However, there were no marked variations among seasons in TSH concentrations ($t=0.75$, $df=15$, $P>0.05$; Table II). Serum leptin level was negatively correlated with serum T₃ concentration ($r=-0.536$, $P<0.05$; Fig. 3A), and was positively correlated with serum T₄ concentration ($r=0.484$, $P<0.05$; Fig. 3B), but showed no correlation with TSH concentrations ($r=0.361$, $P>0.05$; Fig. 3C).

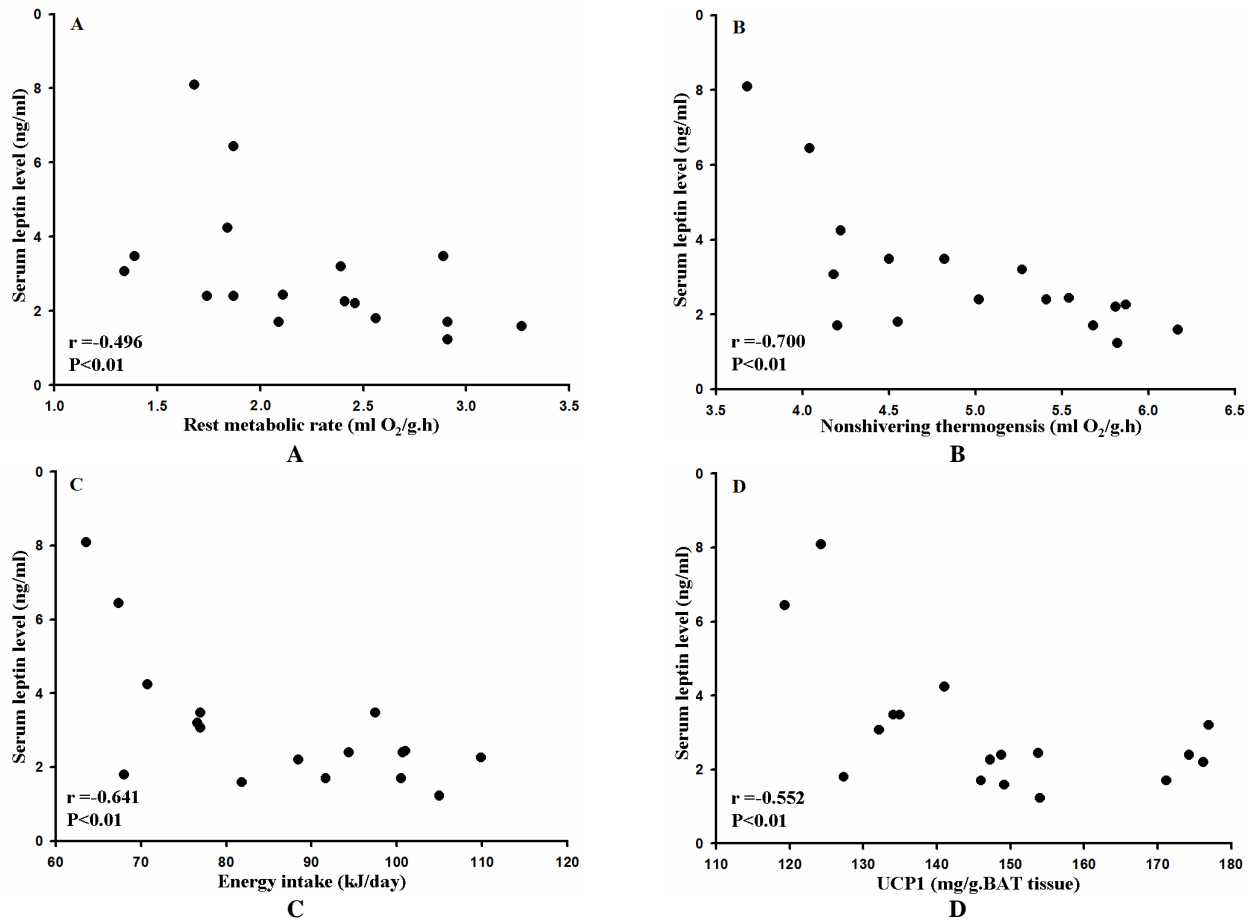


Fig. 2. Correlation of serum leptin level with RMR (A), NST (B), energy intake (C) and UCP 1 contents (D) in *Apodemus draco* in different seasons.

DISCUSSION

Body mass, body fat mass and serum leptin level

Many small mammals inhabiting the temperate regions have obvious seasonal variation in body mass and body fat mass (Bartness *et al.*, 2002). In the present study, it was clear that *A. draco* showed seasonal changes in body mass, which was lower in winter and higher in summer. To cope with winter stress and energy requirements, animals may reduce energy expenditure by decreasing their body weight (Li and Wang, 2005a). *A. draco* also displayed a significant decrease in body fat mass in winter compared to summer. Seasonal variation of body fat mass is closely related to the circulating levels of leptin

(Klingenspor *et al.*, 2000; Rousseau *et al.*, 2003). It suggested that leptin may be responsible for seasonal adjustments in body mass as a potential adiposity signal (Zhang and Wang, 2007), which showed a positive correlation with body fat mass (Considine *et al.*, 1996; Wolden-Hanson *et al.*, 1999; Woods *et al.*, 2000), such as in *Dicrostonyx groenlandicus* (Johnson *et al.*, 2004), *P. sungorus* (Klingenspor *et al.*, 2000), *M. domesticus* (Korhonen and Saarela, 2005), *M. unguiculatus* (Li and Wang, 2005b) and *Eothenomys miletus* (Zhu *et al.*, 2010). In the present study, it also showed a positive correlation between serum leptin concentration and body fat mass in *A. draco*, similar to findings in *A. draco* under cold exposure (Zhu *et al.*, 2012c). We suggest that serum leptin level in *A. draco* may act

as a starvation signal to increase energy intake in response to greater energy requirements in winter, and as a satiety signal to decrease energy intake to avoid obesity in summer.

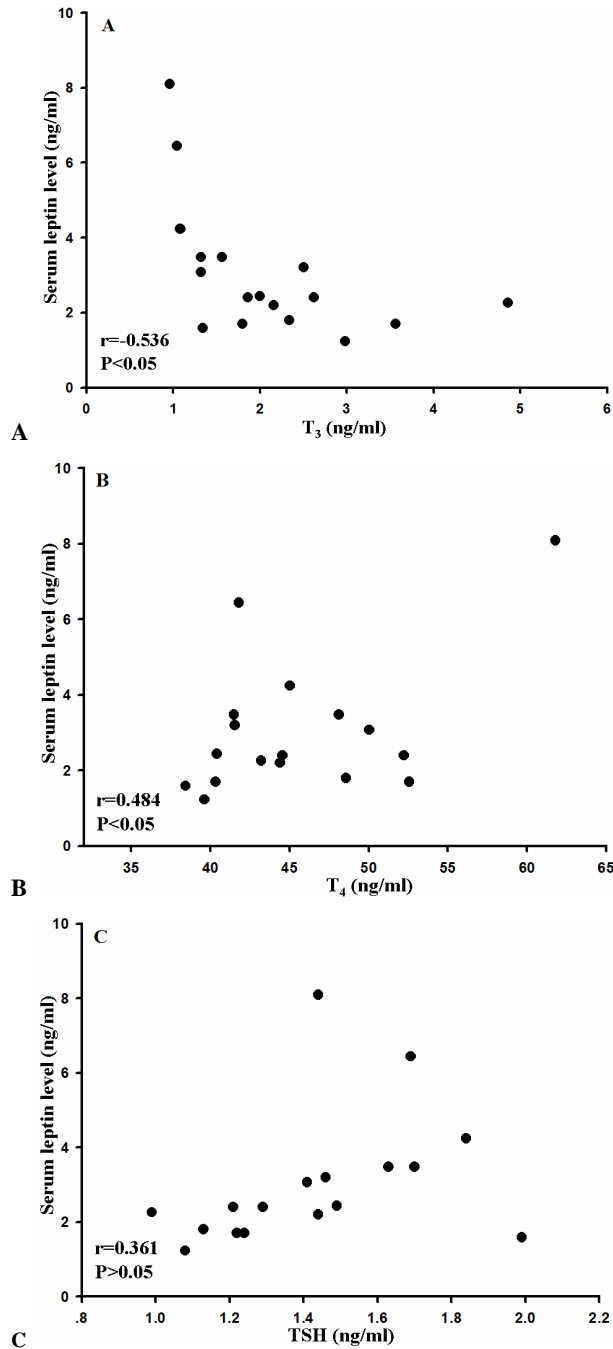


Fig. 3. Correlation of serum leptin level with T_3 (A), T_4 (B) and TSH concentration (C) in *Apodemus draco* in different seasons.

Thermogenic properties

It has been demonstrated that many small mammals showed a seasonal variation in thermogenesis capacity (Rousseau *et al.*, 2003). In present study, RMR and NST of wild *A. draco* showed seasonal plasticity with increased RMR and NST in cold seasons, which was also similar to other small mammals living in cold regions (Wang *et al.*, 2006a). In *A. draco*, energy intake increased during winter, partly as a response to increased RMR and NST. Decreased serum leptin level in winter or cold condition, which can be due to the increasing of norepinephrine secretion in vivo inhibits expression of leptin mRNA (Trayhurn *et al.*, 1995). Low concentrations of leptin under winter-like condition coupled with an increase in food intake were an adaptation to the large energy consumption in their natural environment (Bing *et al.*, 1998). Leptin mainly promotes thermogenesis through two ways: 1. inhibition secretion of hypothalamic neuropeptide Y (NPY) and 2. direct stimulation of the sympathetic nerve (Nieminen and Hyvarinen, 2000). During cold acclimation conditions, sympathetic nerve impulses increased heat production capacity in BAT, on the other hand, decomposed white adipose tissue resulting in reduction of body fat mass (Demas *et al.*, 2002). In the winter, *A. draco* decreased serum leptin level when faced to cold stress, thereby increased energy intake.

UCP1 contents may serve as an indicator of NST capacity. It has been demonstrated that cold- or short-photoperiod-induced elevation in NST was accompanied by increased UCP1 contents in several rodent species (Abelenda *et al.* 2003). In the present study, it shown that seasonal differences were found in UCP1 contents in the BAT of *A. draco*. Leptin-modulated changes of energy expenditure have been suggested to link to thermogenic activity of brown adipose tissue, but the conclusion is controversial (Chen *et al.*, 2012). Serum leptin level was found to be associated with increased BAT UCP1 gene expression in rats during cold acclimation (Bing *et al.*, 1998), however, no relationships was found between leptin and UCP1 contents in cold-acclimated *M. agrestis* (Król *et al.*, 2006), further, there was a negative correlation between serum leptin level and BAT UCP1 content

in *M. unguiculatus* (Zhang and Wang, 2007). In our study, it also shown a negative correlation between serum leptin level and BAT UCP1 content in *A. draco*, similar relationships have also been found in seasonally acclimatized captive Brandt's voles (Li and Wang, 2005a), wild plateau pikas and root voles (Wang *et al.*, 2006a,b). Our results also shown that serum leptin levels were negatively correlated with RMR and NST, similar results found in *Eptesicus fuscus* (Kunz *et al.*, 1999), suggesting that leptin might be involved in the regulation of thermogenesis in *A. draco*.

Roles of serum leptin level on hormones concentrations

Leptin secretion was negatively related with hypothalamic-pituitary-adrenal axis (HPA) function, namely leptin can directly inhibit adrenal secretion of glucocorticoids, and reduce the level of thyroxine releasing hormone (TRH) (Kakucaka *et al.*, 1995). In rodents, thyroid hormone had the interaction with serum leptin concentration (Escobar-Morreale *et al.*, 1997), leptin can influence the expression of TRH gene to regulate thyroid hormone, and thyroid hormone can influence body fat mass and TSH in the regulation of leptin (Pinkney *et al.*, 1998). In the present study, serum leptin level was negatively correlated with serum T₃ concentration, and was positively correlated with serum T₄ concentration, but showed no correlation with TSH concentrations in *A. draco*, suggesting that leptin might be involved in the regulation of thyroid hormones concentrations in seasonal variations.

Testosterone and melatonin concentrations

Testosterone concentration in summer was significantly higher than that in winter in *A. draco*, which may make them defend the territory (Gamboa *et al.*, 2005). The photoperiod in Hengduan Mountains region also showed seasonal change, resulting in seasonal plasticity of thermogenesis in *A. draco*. It indicated that changes of thermogenesis may be not only subject to endocrine regulation, but also depend on the changes of the external of photoperiodic signal of environment (Matthew *et al.*, 2008). This adjustment mechanism may consistent with "challenge hypothesis" (Wingfield *et al.*, 1990). Photoperiod inhibited synthesis of melatonin in the

most studied mammals, which is not only to inform the brain whether it is light or dark in the external environment, but also a coordinated propagation of immune, and other physiological processes, and then to increase survival in winter (Nelson and Demas, 1997). Injection of melatonin in some small mammals can stimulate enhancement of adaptive thermogenic capacity, including BAT proliferation, oxidative capacity enhancement, eventually leading to the enhancement of heat production to resist the low temperature in winter (Heldmaier and Lynch, 1986). Melatonin showed significant seasonal changes, which was significantly higher in summer than that in winter. It may be due to the long photoperiod in summer inhibited synthesis of melatonin, melatonin may act as an endogenous factor to play an important role on energy metabolism in *A. draco* of seasonal variation.

In conclusion, the results presented in this study shown that body mass, RMR, NST, energy intake, UCP1 contents, serum leptin level, T₃, T₄, T₃/T₄, testosterone and melatonin concentration showed significant variation, but TSH showed no difference between seasons in *A. draco*. There were significant negative correlation between serum leptin level and RMR, NST, energy intake, UCP 1 contents and T₃, and were positive correlation between serum leptin level and body fat mass and T₄. However, no correlation was found between serum leptin level and TSH. The present results indicated that *A. draco* adapt winter cold stress by increasing energy intake, thermogenesis and decreasing body mass and body fat mass. Leptin may act as a starvation signal to permit the increase in energy intake for energy exhaust mainly as thermogenesis for winter adaptation. Furthermore, testosterone and melatonin seem to be involved in the regulation of body mass and energy balance in *A. draco*.

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