Journal of Stress Physiology & Biochemistry, Vol. 10 No. 1 2014, pp. 26-36 ISSN 1997-0838

ORIGINAL ARTICLE

Effect of Cold Temperature and Food Restriction on Energy Metabolism and Thermogenesis in *Eothenomys miletus*

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Received August 8, 2013

The aim of the present study was to examine the energy strategy in response to the cold temperature and food shortage. The survival rate, body mass, body fat content, serum leptin levels, basal metabolic rate (BMR) and nonshivering thermogenesis (NST) as well as masses and the morphology of visceral organs and the digestive tract were measured in *Eothenomys miletus* that was subjected to the cold temperature (5°C) and food restriction (80% of ad libitum food intake). The results showed that body mass, body fat content, serum leptin levels, brown adipose tissue (BAT) mass and dry mass of digestive tract in cold and food restriction group were lower than those in control group. In contrasts, BMR and NST in cold and food restriction group was significantly higher relative to control group. The rate of survival was 18.18% in *E. miletus* during cold and food restriction after 4 weeks acclimation. In addition, serum leptin levels were positively correlated with body mass and body fat content, and negatively correlated with BMR and NST. These results suggested that *E. miletus* apply physiological adjustments to adapt cold and food lacking external environment by reducing body mass, body fat content, and increasing energy metabolism. However, energy intake is insufficient to compensate for the increase in energy requirement due to cold, led to body mass decreased and mortality rate increased. Moreover, serum leptin may acts as a fat signals, and may be involved in the regulation of energy balance and body mass in *E. miletus* under the cold temperature and food restriction.

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Energy metabolism plays an important role in distribution, richness and survival of species (Bozinovic, 1992). In the natural environment, the level of energy metabolism of wild animal is affected by many environmental factors, including temperature (Abelenda *et al*., 2003) and food (Bacigalupe & Bozinovic, 2002). Due to low temperature in winter, energy consumption increased and the available food resource relative reduced for mammals, which are important environmental factor affecting the metabolism and physiological action of energy of small mammals (Nagy *et al*., 1995). Leptin is a protein hormone secreted by fat cells, and plays an important role in the regulation of energy homeostasis and body mass of the animals, higher leptin concentration can inhibit the food intake, promote heat production and reduce body mass (Friedman and Halaas, 1998). Researches showed that the injection of exogenous leptin can inhibit energy intake, improve energy consumption (Abelenda *et al*., 2003). Abelenda (2003) studies showed that leptin is involved in the heat regulation and food intake under cold conditions. Many studies have showed that food restriction decreased the serum leptin concentration, and leptin was positively correlated with body mass, heat production ability (Rousseau *et al*., 2003), which indicated that serum leptin may be a hunger signals involved in adjustment of body mass and heat production under food deficient conditions (Zhao and Wang, 2007).

The Hengduan Mountains region is located the boundary between the Palaearctic region and the Oriental region. It is alpine with high mountains and gorges; the diversity and abundance of mammals is high and it is considered to be “the harbor in fourth ice age”. Therefore small mammals may differ from those from other regions. *Eothenomys* were the proper genus in China, were a special group in *Microtus*, and were the typical animals in Hengduan Mountains region. *E. miletus* is the inherent species in Hengduan mountains region (Zhu *et al*., 2012a), has special status in *Microtus*. In the present study, we examined the effect on body mass, thermogenesis and body composition in cold-acclimated *E. miletus* under food restriction. We predicted that *E. miletus* change their body mass, thermogenesis and body composition under simulated winter conditions, and leptin would be involved in the regulation of body mass and thermogenesis.

MATERIALS AND METHODS

Samples

E. miletus were captured in farmland (26°15´~26°45´N; 99°40´~99°55´E;altitude 2,590m) in Jianchuan County, Yunnan province, 2011. Average temperature is 9.1°C each year, minimum average temperature was -4.0°C in January, maximal average temperature was 24.1°C in July.

E. miletus were breed for two generations in School of life Science of Yunnan Normal University, park in plastic box(260mm×160mm×150mm), one in a box without any bedding material, and were maintained at the room temperature of 25±1°C, under a photoperiod of 12L:12D (with lights on at 08:00). After 1 month stabilization, animas were randomly divided into two groups(n=41). Food intake were measured by the food balance (Rosenmann and Morrison, 1974), a total of 5 days, quantitative timing (before day 11: 00 - 13: 00, food 9.5 G - 10.5 g) for animal feeding, the collecting, weighing surplus food (each collection time were in 11: 00 - 13: 00), calculation of the 5 day average food intake as part of their normal daily intake. Then all animal randomly divided into control group: free feeding at room temperature (temperature: 25 ± 1 ℃) (n=8); experimental group: 80% normal food intake and low temperature (temperature: 5 ± 1 ℃) in (n=33) last for 28 days. The survival rate was measured daily animal, every 7 days to measure body mass, BMR and NST. The voles were sacrificed between 09:00 and 11:00 by puncture of the posterior vena cava. Blood was centrifuged at 4000 rpm for 30 min, and serum was sampled and stored at −20°C for later measurement. All voles were dissected to evaluate organ morphology. All pregnant, lactating or young individuals were excluded.

Measurement of metabolic rates

Metabolic rates were measured by using AD ML870 open respirometer (AD Instruments, Australia) at 30°C within the TNZ (thermal neutral zone), gas analysis were using ML206 gas analysis instrument, the temperature was controlled by SPX-300 artificial climatic engine (±0.5°C), the metabolic chamber volume is 500 ml, flow is 200 ml/min. The tree shrew were stabilized in the metabolic chamber for at least 60 min prior to the RMR measurement, oxygen consumption was recorded for more than 60 min at 5 min intervals. Two stable consecutive lowest readings were taken to calculate RMR (Zhu *et al*., 2010). Calculate method of metabolic rate is detailed by Hills (1972) (Hill, 1972). All metabolic measurements were performed more than 400 hours.

Nonshivering 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).

Measurement of serum leptin level and thyroid hormones

Serum leptin levels were determined by radioimmunoassay (RIA) with the 125I 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-μl sample size. And the inter- and intra-assay variability for leptin RIA were <3.6% and 8.7%, respectively.

Body composition

Gastrointestinal tract (stomach, small intestine, cecum, large intestine) and the heart, lungs, pancreas, spleen, kidneys were extracted and weighed (±1 mg). The stomach and intestines were rinsed with saline to eliminate all the gut contents, before being dried and weighed. The remaining carcass and all the organs were dried in an oven at 60 °C to constant mass (at least 72 h), and then weighed again to obtain the dry mass. The remaining carcass and all the organs were dried in an oven at 60°C to constant mass (at least 72 h), and then weighed again to obtain the dry mass. The difference between the wet carcass mass and dry carcass mass was the water mass of carcass. Total body fat was extracted from the dried carcass by ether extraction in a Soxhlet apparatus (Zhao and Wang, 2007).

Statistical analysis

Data were analyzed using SPSS 15.0 software package. Prior to all statistical analyses, data were examined for assumptions of normality and homogeneity of variance, using Kolmogorov-Smirnov and Levene tests, respectively. Throughout the acclimation, animal survival rate, body mass, energy intake, body fat content, BMR, NST and body composition were analyzed by were analyzed by independent t-test. Pearson's correlation was performed to detect possible correlations among serum leptin and body mass, body fat mass, RMR and NST. Results were presented as mean ± SEM, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Changes of survival rate, body mass and body fat content

Before the experiment, no significant difference of body mass between low temperature restriction group and control group (t=-0.221, P>0.05, Table 1). It showed an significant difference between the two groups in 7 day (t=7.005, animal P<0.01), body mass in low temperature food restricted group were 13.44% lower than that in control group. It showed significant difference in 28 day (t=37.56, P<0.01), low temperature food restricted group were lower 33.55% than that in control group (fig 1). After the acclimation, food restricted group survival rate was 18.18% at low temperature, the control group had no death occurred, there also had an significant difference of body fat content between two groups (t=5.818, P<0.01) (Table 1). During acclimation, body mass in low temperature and food restricted group decreased significantly (F=60.51, P<0.01), but the control group remained stable (F=0.863, P>0.05) (fig 1).

RMR and NST

Before the experiment, no significant difference of RMR and NST were found between cold and food restriction group and the control group (RMR: t =-1.219, P>0.05; NST: t=1.191, P>0.05). During the acclimation, RMR and NST had no significant change in control group (BMR: F=0.247, P>0.05; NST: F=0.347, P>0.05), but RMR increased significantly in cold and food restriction group (F=7.139, P<0.01), RMR showed significant difference between two groups (RMR: T, =-6.159, P<0.01), and increased by 82.25% than that of the control group; NST in cold and food restriction group increased significantly in 28 day (F=11.228, P<0.01), NST significantly higher than the control group in 7 day and 14 day (7 days: NST: t =-5.918, P<0.01; 14 days: NST: t =-6.328, P<0.01, respectively), increased by 64.72% and 56.67% than that of the control group. It had no significant difference of NST in 21 days and 28 days (21 days: NST: t =-2.206, P>0.05; 28 days: NST: t =-1.19, P>0.05) (fig 2).

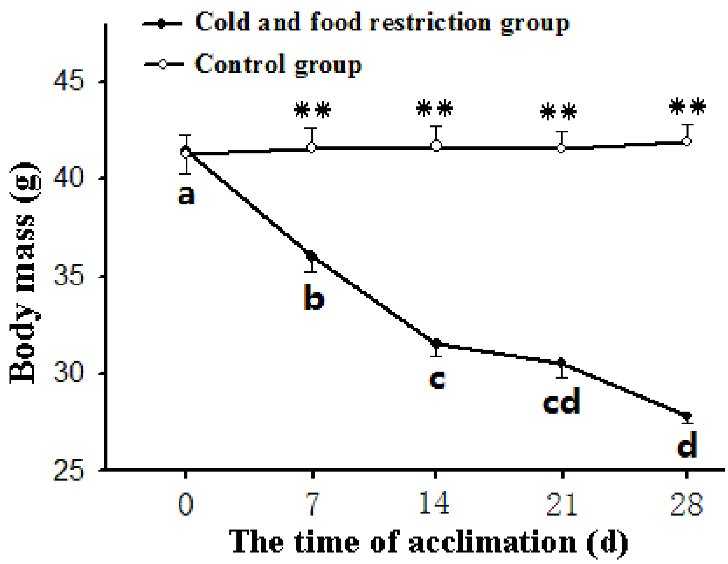

Figure 1. Body mass in *E. miletus* during cold and food restriction. Different letters indicate significant differences (P<0.05) (**P<0.01)

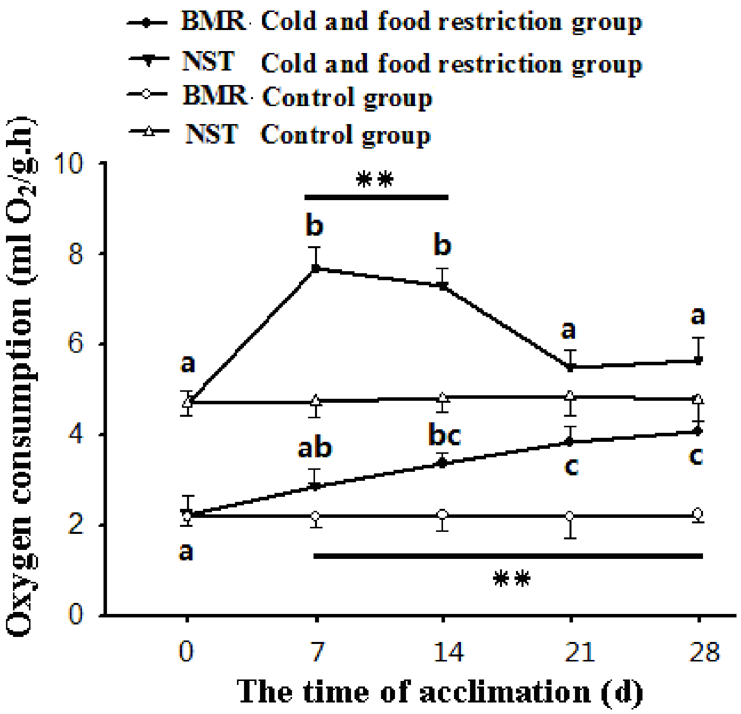

Figure 2. BMR and NST in *E. miletus* during cold and food restriction. Different letters indicate significant differences (P<0.05) (**P<0.01)

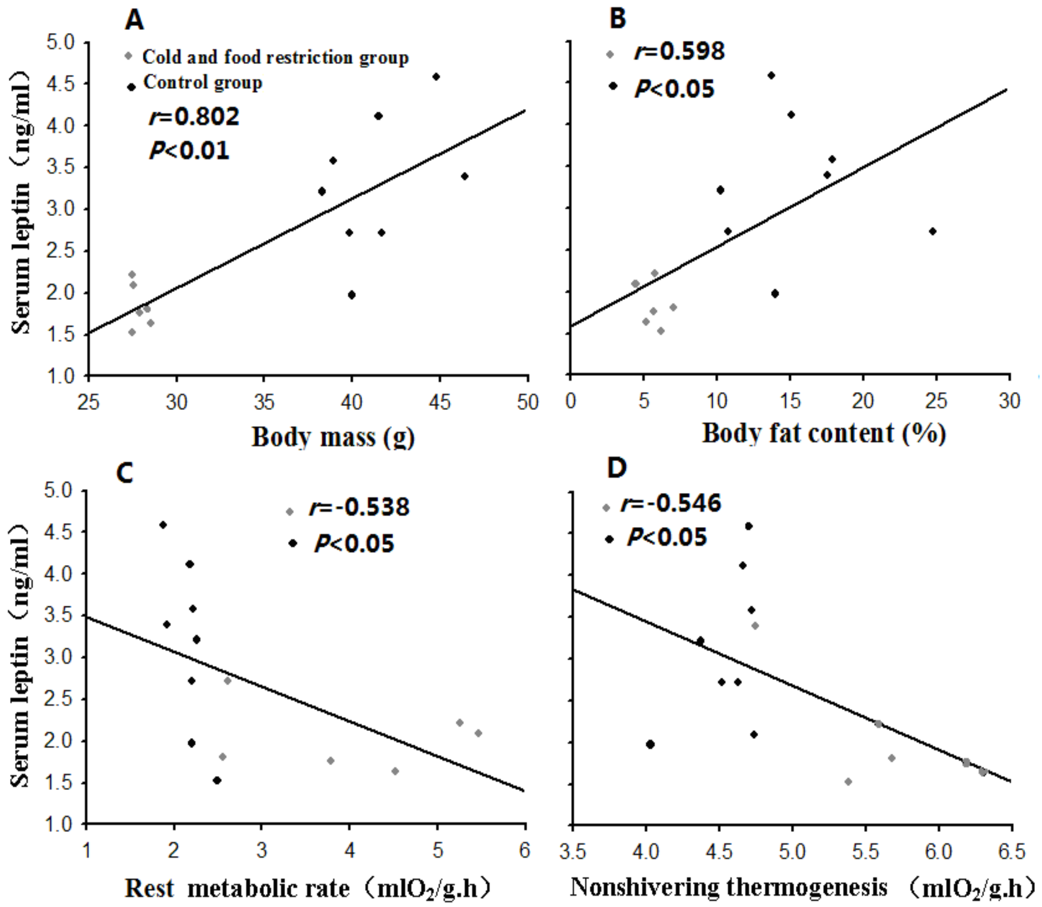

Figure 3. Correlation of serum leptin levels with body mass (A), body fat content (B), BMR (C) and NST (D) in *E. miletus*

Table 1 : Effect of cold and food restriction on rate of survival, body fat content, organ mass, and morphology of digestive organs in *E. miletus*.

	Cold and food restriction	Control group	*t*	*p*
Rate of survival (%)	18.18	100	*vs*	*vs*
Body mass (g)				
Initial	41.44±0.52	41.28±0. 98	-0.221	0.829
Final	27.83±0.35	41.88±0.95	37.56	0.000
Leptin (ng/ml)	1.84±0.27	3.29±0.83	4.071	0.002
Food intake (g/d)	4.47±0.24	5.58±0.30	10.00	0.015
Body fat mass (g)	0.58±0.07	1.67±0.58	6.023	0.000
Body fat content (%)	5.72±0.87	14.55±5.7	5.819	0.000
Carcass				
Wet mass (g)	21.33±0.44	34.06±1.11	9.698	0.000
Dry mass (g)	12.09±1.01	16.41±0.58	3.897	0.006
Water mass (g)	9.24±0.76	17.65±0.71	8.054	0.000
BAT mass (g)	0.24±0.038	0.64±0.08	4.84	0.001
Heart mass (g)	0.18±0.007	0.18±0.08	-0.377	0.715
Lungs mass (g)	0.23±0.02	0.19±0.05	-0.857	0.414
Liver mass (g)	1.70±0.16	1.73±0.38	0.086	0.933
Spleen mass (g)	0.047±0.008	0.068±0.005	2.125	0.63
Kidneys mass (g)	0.35±0.033	0.42±0.021	1.60	0.143
Digestive tract				
Length (cm)	74.06±1.64	72.41±1.94	-0.624	0.552
Mass with contents (g)	5.74±0.51	5.92±0.76	0.204	0.843
Wet mass (g)	2.51±0.23	2.9±0.26	1.114	0.294
Dry mass (g)	0.38±0.043	0.62±0.041	3.957	0.003
Stomch				
Length (cm)	2.66±0.09	2.81±0.04	1.64	0.176
Mass with contents (g)	1.32±0.15	1.78±0.29	1.487	0.171
Wet mass (g)	0.32±0.02	0.38±0.03	1.834	0.100
Dry mass (g)	0.07±0.005	0.12±0.009	4.459	0.002
Small intestine				
Length (cm)	32.96±3.13	37.5±1.39	1.428	0.196
Mass with contents (g)	1.56±0.21	1.69±0.20	0.466	0.652
Wet mass (g)	1.11±0.16	1.48±0.19	1.489	0.171
Dry mass (g)	0.15±0.02	0.30±0.03	4.529	0.001
Caecum				
Length (cm)	14.39±1.50	9.38±1.83	-2.142	0.061
Mass with contents (g)	2.08±0.30	1.63±0.36	-0.955	0.365
Wet mass (g)	0.60±0.13	0.51±0.09	-0.576	0.578
Dry mass (g)	0.09±0.027	0.10±0.019	0.266	0.796
Large intestine				
Length (cm)	23.22±3.10	22.72±1.05	-0.14	0.892
Mass with contents (g)	0.78±0.11	0.81±0.16	0.184	0.858
Wet mass (g)	0.48±0.06	0.52±0.09	0.415	0.688
Dry mass (g)	0.07±0.008	0.10±0.018	1.708	0.122

Serum leptin levels

After 4 weeks acclimation, serum leptin levels in cold and food restricted group was significantly lower than that of the control group (t=4.071, P<0.01) (Table 1). Significant positive correlation of serum leptin levels and body mass, body fat content, and RMR, NST were significantly negatively correlated (r=0.802, weight: $P<0.01$; body fat content: r=0.598, $P<0.05$; RMR: r=-0.538 $P<0.05$; NST:, r=-0.546, $P<0.05$) (fig 3).

Body composition

After 4 weeks acclimation, carcass mass (t =9.698, $P<0.01$), BAT (t =4.84, $P<0.01$), gastric dry weight (t =4.459, $P<0.01$) and intestine dry weight (t =4.529, $P<0.01$) in cold and food restricted group was significantly lower than the control group (Table 1).

DISCUSSION

In winter many small mammals reduced body mass or slow growth, which are considered to an energy strategy adapt to environment in the lack of food and under cold stress by reducing the total energy demand (Wang *et al*., 2006), such as *Lasiopodomys brandtii*, *Apodemus chevrieri*, and *Microtus pennsylvanicus* (Iverson and Turner, 1974; Li & Wang, 2005; Zhu *et al*., 2012b), decreased body mass in winter in order to reduce the total energy demand, so as to adapt to the low temperature and the lack of food resources in winter (Merritt *et al*., 2001). However, there are some mammals maintained body mass stability in winter or even increased (Klause *et al*., 1988), such as *Dicrostonyx groenlandicus* (Nagy, 1993), *Mesocricetus auratus* (Del Valle and Busch, 2003). In this study, similar in winter conditions (temperature and food restriction), *E. Miletus* reduced body mass in order to reduce the total energy demand and the use of body fat in response to low temperature and food shortage condition. However, decreased body mass will lead to unit body surface area (Surface-to-volume) increased, heat loss increased (Duarte *et al*., 2010), then the energy demand is high to supple the metabolic energy expenditure, and are vulnerable to food shortages stress (Gutman *et al*., 2007). *E. Miletus* in the cold and food restriction group cannot compensate energy demand increasing, leading to body mass decreased significantly, and increased mortality.

RMR and NST are the main energy expenditure of small mammals, play important roles in the regulation of energy balance (Hambly and Speakman, 2005). In winter many animals increased BMR and NST to adapt to the cold conditions in winter, such as Brandt's vole (Li and Wang, 2005), *Apodemus chevrieri* (Zhu *et al*., 2012b) and tree shrew (Zhu *et al*., 2012c). But there is also some animals decreased RMR in winter (Blaxter, 1989). It showed that small mammals have different energy strategy in the winter, choose the low BMR and NST, can reduce the total energy demand, and can therefore have a high survival rate in winter; or is to maintain a high BMR and NST, adapt to long cold condition in winter (Speakman, 1996). In the present study, a significant increase of RMR in cold and food restriction conditions in *E. miletus*, which indicated that increased RMR is one of adaptive strategy for *E. miletus* in response to low temperature and food shortages. NST is a effective and economic way of heat production for small mammals to adapt to the cold environment (Jansky, 1973). *E. miletus* of NST was significantly higher than that in the 7 day and 14 day group, but had no significant difference in 21 day and 28 day compared with control group, this is probably low caloric restriction caused great stress on survival in *E. miletus*, resulting insufficient heat production in cold and food restriction, and exhibit weak ability of adaptation to low temperature, so that the mortality rate high, reaching to 81.82%.

Leptin plays an important role in regulating the maintenance of homeostasis and body mass

(Wozniak *et al*., 2009). Feedback loop hypothesis showed that leptin concentrations reflect the body fat storage, and positively correlated with body fat content. Leptin signaling molecules form blood circulation through the stimulation of the hypothalamus, and then by the hypothalamus regulates food intake and energy expenditure, keeping the energy balance (Friedman and Halaas, 1998). Body mass and fat mass of many small mammals have annual change obviously, and the level of circulating Leptin was positively related, such as *Phodopus campbelli* (Heldmaier and Steinlechner, 1981). Recently studies showed that animals decreased the serum leptin concentration in winter, and leptin was positively correlated with body mass and fat mass, and significantly negative correlation on heat production ability, such as root vole (Wang *et al*., 2006). In our study, serum leptin levels decreased in cold and food restriction group. There had significantly positive correlation between serum leptin levels and body mass, body fat content, and showed significantly negative correlation between serum leptin levels and BMR and NST, suggesting that serum leptin may participate in the regulation of body mass and heat production in winter. In the cold and food shortage, serum leptin levels may mainly used to promote heat production, reduce body mass in order to adapt to the winter environment.

In conclusion, under low temperature and food restriction conditions, *E. miletus* mainly through reduced body mass and the use of stored body fat as a supplementary energy source to maintain energy balance. However, low caloric restriction caused larger stress to *E. miletus*, showing excessive mortality. Moreover, leptin may be involved in the regulation of body mass and thermogenesis under low temperature food restriction conditions in *E. miletus*.

ACKNOWLEDGMENTS

This research was financially supported by the National Key Technology Research and Development Program (No. 2014BAI01B00), International cooperation in science and technology project (SQS2014RR102), National Science Foundation of China (No. 31260097; 31360096), Basilic Project of Yunnan Province (No. 2013FA014; 2011FZ082).

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