Available online at www.sciencedirect.com
ScienceDirect
Zoology 112 (2009) 206–216
ZOOLOGY
www.elsevier.de/zool

Cold exposure does not decrease serum leptin concentration, but increases energy intake and thermogenic capacity in pregnant Brandt's voles (*Lasiopodomys brandtii*)

Xue-Ying Zhang[a], Bo-Bin Jing[a,b], De-Hua Wang[a,*]

[a]*State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Datun Lu, Chaoyang, Beijing 100101, China*
[b]*Graduate School of the Chinese Academy of Sciences, Beijing 100049, China*

Received 20 February 2008; received in revised form 21 August 2008; accepted 3 September 2008

Abstract

In most mammals, maternal body mass and fat mass increase during pregnancy due to hyperphagia. These physiological changes provide the fetus with energy and nutrients and prepare the mother for the high energetic demands of lactation. In the present study, metabolic changes in response to cold and pregnancy were examined in female Brandt's voles (*Lasiopodomys brandtii*). At $23\pm1\,^{\circ}C$, the voles increased body mass and deposited body fat during pregnancy. However, at $5\pm1\,^{\circ}C$ pregnant voles did not deposit body fat even though energy intake increased above the level in the warm. Serum leptin concentration increased during pregnancy and was not influenced by cold exposure. Thermogenic capacity, as indicated by uncoupling protein 1 (UCP1) content in brown adipose tissue (BAT), increased in cold-exposed pregnant voles. The number and mass of fetuses were not affected by cold exposure. Our data may indicate the importance of an increased serum leptin concentration for a successful outcome of the pregnancy and also the independence of leptin secretion from body fat in pregnant voles. It also implies the need to develop central leptin resistance with respect to control of energy balance for pregnant voles.

Keywords: Body mass; Energy balance; Leptin secretion; Brown adipose tissue; Pregnancy

Introduction

Hyperphagia during pregnancy is a natural state, during which body mass and fat mass can increase dramatically (Shirley, 1984), providing a useful model to investigate the underlying causes of food intake increase and fat deposition. The hyperphagia provides the growing fetus with energy and nutrients, and the mother with an energy reserve for lactation.

Long-term energy homeostasis requires a signal to the hypothalamus that varies in proportion to levels of body energy storage and energy status. Leptin is one such signal, and is secreted mainly from adipocytes (Zhang et al., 1994), but is also produced in some other tissues including the placenta, stomach and several fetal tissues (Masuzaki et al., 1997; Bado et al., 1998; Hoggard et al., 2000). Together with other metabolic feedback signals, for example insulin (Niswender et al., 2004), leptin acts in the hypothalamus to suppress food intake, and

*Corresponding author. Tel.: +86 10 64807073; fax: +86 10 64807099.
E-mail address: wangdh@ioz.ac.cn (D.-H. Wang).

0944-2006/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.zool.2008.09.003

coordinates thermogenesis and energy expenditure (Friedman and Halaas, 1998; Ahima and Flier, 2000). Exogenous leptin treatments can decrease food intake and increase uncoupling protein 1 (UCP1) mRNA expression (Pelleymounter et al., 1995; Scarpace and Metheny, 1998). UCP1, a 32 kDa protein uniquely expressed in the inner membrane of brown adipose tissue (BAT) mitochondria, uncouples oxidative metabolism from ATP synthesis (Cannon and Nedergaard, 2004). Cytochrome *c* oxidase (COX) is the terminal respiratory complex of the mitochondrial respiratory chain and is responsible for catalyzing the reduction of molecular oxygen. During pregnancy, COX activity and UCP1 mRNA expression in BAT decreased (Wade et al., 1986; Martin et al., 1989), while energy intake and serum leptin concentration increased significantly in numerous mammalian species (Butte et al., 1997; Amico et al., 1998; Zhang and Wang, 2008). Injection or infusion of leptin failed to decrease food intake in pregnant rats (Stocker et al., 2004), indicating that leptin resistance may occur during pregnancy. Whether leptin is related to the regulation of energy intake and/or thermogenic capacity during pregnancy in field rodents is unclear.

Brandt's voles (*Lasiopodomys brandtii*) mainly inhabit the grasslands of Inner Mongolia of China, Mongolia and the Baikal region of Russia, where the winter lasts for more than 5 months and average annual temperatures are 0–4 °C. They are non-hibernating herbivores, and store food in autumn for the long winter. The reproductive season of Brandt's voles is from March to August (Zhang and Wang, 1998). In Inner Mongolia of China, ambient temperatures in March and April are still mostly below zero (Wan et al., unpublished data). Therefore, in the early spring, Brandt's voles are faced with the simultaneous stressors of cold and breeding. Small mammals have increased thermoregulatory costs at low temperature. This energy demand for thermogenesis competes with reproduction (Bronson, 1985). Thus, Brandt's voles are a good model to study metabolic changes during reproduction in the cold.

The voles increased energy intake and thermogenic capacity in response to simultaneous lactation and cold exposure (Zhang and Wang, 2007). The objective of this study was to examine the effect of simultaneous pregnancy and cold exposure on energy intake, fat content, resting metabolic rate (RMR), and thermogenic capacity in female Brandt's voles, and whether this effect can be related to circulating leptin concentration. We hypothesized that cold-exposed Brandt's voles have the ability to maintain both maternal energy status and fetal development, with leptin mediating the regulation of maternal energy intake and thermogenesis. Thus maternal energy intake and thermogenic capacity would be expected to increase, while serum leptin concentration would decrease, and maternal body fat mass and fetal mass should not change during cold exposure.

Materials and methods

Animals

Adult virgin female Brandt's voles were offspring of the F6 generation of animals trapped in Inner Mongolian grasslands in May 1999 and raised at the Institute of Zoology, Chinese Academy of Sciences, Beijing. The voles were weaned at 21 days of age and housed as same-sex sibling pairs in plastic cages ($30 \times 15 \times 20$ cm) with sawdust as bedding in a temperature-controlled room (23 ± 1 °C) with a 12L:12D cycle (lights on at 08:00 h). Food (commercial rabbit pellet chow; KeAo Feed Co., Beijing, China) and water were provided *ad libitum*. The composition and energy content of the experimental diet is presented in Table 1.

All animal procedures were licensed by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

Experimental design

Females weighing 45–55 g (90–120 days old) were moved into individual cages for at least 2 weeks before the start of data collection. Then some ($n = 12$) were paired randomly with males and checked for vaginal emboli twice daily. Females were identified as pregnant as soon as a vaginal embolus was noticed and this day was defined as the mating day. Then the male was separated from the female (the male had been with the female for a maximum of 2 days). Four days after mating, half of the pregnant voles (pregnant voles in cold (PC), $n = 6$) were transferred to a room at 5 ± 1 °C for 2 weeks. The other half remained at 23 ± 1 °C (pregnant voles in warm (PW), $n = 6$). Also, non-reproductive voles (NR) remained at 23 ± 1 °C ($n = 6$). Body mass was measured before mating and on days 17 or 18 of pregnancy.

Table 1. Composition of macronutrients and energy content of the experimental diet based on dry mass.

	Standard rabbit pellet chow
Crude fat (%)	5.1 ± 1.0
Crude protein (%)	24.3 ± 2.2
NDF (%)	25.0 ± 2.8
ADF (%)	13.6 ± 2.2
Caloric value (kJ/g)	17.2 ± 4.5

NDF, neutral detergent fiber; ADF, acid detergent fiber.

Metabolic measurements

RMR (ml O_2/h) was measured between 09:00 and 17:00 h (to minimize the effect of circadian rhythms) on day 14 of pregnancy by using an established closed-circuit respirometer at 30 ± 0.5 °C (a temperature within the animals' thermal neutral zone), as described previously (Li and Wang, 2005). The metabolic chamber volume was 3.6 l and the temperature inside the chamber was controlled by a water bath. Potassium hydroxide (KOH) and silica gel were used to absorb carbon dioxide and water, respectively, in the metabolic chamber. The voles were weighed before the test, and were placed in the chamber for 60 min to acclimate and then oxygen consumption was recorded for another 60 min at 5 min intervals. Two stable consecutive lowest readings were taken to calculate RMR and corrected to standard temperature and pressure.

Energy intake

Energy intake was measured in metabolic cages for three consecutive days from day 15 to day 18 of pregnancy at the temperature the animals were living at (Liu et al., 2003). During the test, food was provided quantitatively and water was provided *ad libitum*. After the 3 day test, food residues and feces were collected, oven-dried at 60 °C to constant mass and separated manually. The caloric values of food and feces were determined by calorimetry (Parr 1281 oxygen bomb calorimeter, Parr Instrument Co., Moline, USA). Gross energy intake (GEI), digestible energy intake (DEI) and apparent digestibility (hereafter referred to as digestibility) were calculated according to Grodzinski and Wunder (1975) and Liu et al. (2003):

$$\mathrm{GEI(kJ/day)} = \text{dry matter intake(DMI, g/day)} \times \text{food caloric value(kJ/g)};$$

$$\mathrm{DEI(kJ/day)} = \mathrm{GEI(kJ/day)} - (\text{dry feces mass(g/day)} \times \text{feces caloric value(kJ/g)});$$

$$\text{Digestibility}(\%) = \mathrm{DEI/GEI} \times 100\%.$$

Measurements of mitochondrial protein content, cytochrome *c* oxidase activity and UCP1 content

All subjects were weighed and sacrificed by decapitation at 09:00–11:00 h on day 18 of pregnancy. Blood samples were collected by puncture of the posterior vena cava (Li and Wang, 2005; Zhang and Wang, 2007). The interscapular BAT and a piece of liver were carefully dissected, weighed, frozen in liquid nitrogen and stored at −80 °C. Mitochondrial protein (MP) content of BAT (mg/g BAT) was measured with the Folin phenol method, using bovine serum albumin as standards (Lowry et al., 1951). The COX activity of BAT (nmol O_2/min mg MP, nmol O_2/min g BAT, or nmol O_2/min in total BAT) was measured with the polarographic method using oxygen electrode units (Hansatech Instruments Ltd., King's Lynn, England) (Sundin et al., 1987; Li and Wang, 2005; Zhang and Wang, 2006). MP content and COX activity of liver were measured with the same methods as for BAT.

UCP1 content was measured by Western blotting. Total BAT protein (12 μg 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 Biosciences, England). To check for the efficiency of protein transfer, gels and nitrocellulose membranes were stained after transferring with Coomassie brilliant blue and Ponceau red, respectively. UCP1 was detected using a polyclonal rabbit anti-hamster UCP1 (1:5000) (supplied by Dr. M. Klingenspor, Department of Biology, Philipps University, Marburg, Germany) as a primary antibody, and peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Jackson ImmunoResearch Laboratories Inc., Indianapolis, USA) as the second antibody. Enhanced chemoluminescence (ECL, Amersham Biosciences, England) was used to detect specific binding sites. UCP1 content per MP was expressed as relative units (RU), quantified by using Scion Image Software (Scion Corporation, Maryland, USA) (Li and Wang, 2005; Zhang and Wang, 2006). UCP1 content per whole BAT can be calculated by multiplication: UCP1 per MP × MP as mg per g of BAT × BAT mass.

Serum leptin assay

Serum leptin concentration was measured by radioimmunoassay (RIA) using the Linco ^{125}I multi-species kit (Cat. No. XL-85 K, Linco Research Inc., St. Charles, USA). This method has been demonstrated to be valid for the measurement of leptin concentration in Brandt's voles (Li and Wang, 2005; Zhang and Wang, 2006). The lowest and highest concentrations of leptin that can be detected by this assay are 1.0 and 50 ng/ml, respectively, when using a 100 μl sample (instructions for multi-species leptin RIA Kit). Intra- and inter-assay coefficients of variation for leptin RIA reported by the manufacturer are $<3.6\%$ and 8.7%, respectively.

Body composition analysis

After interscapular BAT was removed, the visceral organs, including heart, lung, liver, kidneys, spleen, uterus and gastrointestinal tract (stomach, small intestine, cecum, proximal colon and distal colon) were extracted and weighed (± 1 mg). The fetuses and

placentas were taken out and weighed. The gastrointestinal tract was rinsed with saline to remove its contents and weighed. The body mass without visceral organs was defined as wet carcass mass. The carcass was dried in an oven at 60 °C for 10 days to constant mass, and then weighed again to obtain body water mass. Body fat (fat mass of individual organs was not measured) was extracted from the dried carcass by ether extraction in a Soxhlet apparatus (Gould, 1943).

Data analyses

Data analyses were performed using SPSS software (SPSS 1998). Distributions of all variables were tested for normality using the Kolmogorov-Smirnov test, and if not normal, were log-transformed. The percentage values were subjected to an arcsine square-root transformation. Analysis of variance (ANOVA) was performed on between-group differences in female body mass, COX activity, UCP1 content and serum leptin concentrations, followed by Tukey HSD tests. The differences in energy intake (DMI, GEI and DEI) and RMR were determined using ANCOVA with body mass as the covariate (RMR ANCOVA used body mass measured on day of RMR). Morphological parameters were analyzed by ANCOVA with wet carcass mass as a covariate. Differences in the number of fetuses (litter size), and in the overall mass of fetuses and placentas were determined by independent-samples *t*-tests. The mass of fetuses and placentas was further examined by ANCOVA, with litter size as a covariate. Pearson's correlation analysis was performed to determine the correlations between serum leptin concentrations and body fat mass, between residual serum leptin concentrations (corrected for body mass) and residual GEI (corrected for body mass), between serum leptin concentration and UCP1 content. Results are presented as arithmetic means ± SE and $P<0.05$ was considered to be statistically significant.

Results

Maternal body mass, body composition, organ mass and fetal number and mass

There was no difference in body mass among the three groups prior to the experiment (Table 2). At late pregnancy, maternal body mass increased both in the warm and cold, and there was no difference between pregnant voles in warm and cold conditions. The masses of carcass and body fat increased significantly in pregnant voles kept in the warm, as compared to the non-reproductive and cold-exposed pregnant voles. Body fat mass and fat content (fat mass relative to final body mass) in the cold-exposed pregnant voles were 43% and 32% lower, respectively, than in pregnant voles kept in the warm, and 13% and 21% lower than in non-reproductive voles in the warm. The fat-free carcass mass was higher in pregnant voles kept in the warm compared to that of non-reproductive voles. There was an almost significant difference in body water among the three experimental groups (Table 2).

Organ masses also increased during pregnancy. When the effect of carcass mass was removed, the masses of liver, spleen and uterus were higher in the warm- and cold-exposed pregnant voles than in the non-reproductive animals. The masses of stomach, small intestine, cecum and distal colon were higher in the cold-exposed pregnant voles than in the other two groups (Table 3).

There were no significant differences in the number and overall mass of fetuses, and in the overall mass of placentas between cold- and warm-exposed pregnant voles (Table 3). When the effect of litter size was removed, there were still no differences in the overall mass of fetuses ($F_{1,9} = 2.326$, $P = 0.162$) and placentas ($F_{1,9} = 0.066$, $P = 0.803$) between these two groups.

Table 2. Changes of body composition in non-reproductive and pregnant voles exposed to 23 °C and 5 °C.

	NR	PW	PC	$F_{2,15}$	P
Initial body mass (g)	49.9 ± 1.6	50.1 ± 1.8	49.9 ± 1.9	0.004	0.996
Final body mass (g)	50.6 ± 1.7	83.3 ± 2.0[a]	78.1 ± 3.6[a]	46.397	<0.001
Wet carcass mass (g)	36.331 ± 1.550	48.212 ± 1.859[a]	39.926 ± 2.330[b]	9.867	0.002
Body water mass (g)	19.578 ± 1.112	23.620 ± 1.560	23.213 ± 1.489	3.517	0.056
Fat-free wet carcass (g)	27.314 ± 1.186	34.387 ± 1.885[a]	32.068 ± 2.002	4.348	0.032
Body fat mass					
(g)	9.017 ± 0.647	13.826 ± 0.624[a]	7.858 ± 0.318[b]	20.700	<0.001
% (relative to final body mass)	24.8 ± 1.3	28.9 ± 1.6	19.7 ± 1.8[b]	8.325	0.004

Values are arithmetic means ± SE. a, significantly different at $P<0.05$ from NR; b, significantly different at $P<0.05$ from PW. NR, non-reproductive group; PW, pregnant females in the warm; PC, pregnant females in the cold.

Table 3. Mean wet organ and fetus masses in non-reproductive and pregnant voles exposed to 23 °C and 5 °C.

	NR	PW	PC	$F_{2,14}$	P
Heart (g)	0.238±0.013	0.294±0.013	0.321±0.016[ab]	10.800	0.001
Lungs (g)	0.368±0.045	0.399±0.041	0.489±0.053	1.731	0.213
Liver (g)	1.674±0.106	3.358±0.287[a]	3.462±0.222[a]	15.261	<0.001
Kidney	0.462±0.027	0.645±0.031[a]	0.766±0.019[ab]	26.669	<0.001
Spleen (g)	0.033±0.005	0.157±0.024	0.095±0.011[a]	4.907	0.024
Uterus (g)	0.105±0.015	1.153±0.065	1.213±0.108[a]	54.917	<0.001
Stomach (g)	0.327±0.020	0.448±0.013	0.550±0.042[ab]	23.246	<0.001
Small intestine (g)	0.528±0.015	0.678±0.038	0.909±0.080[ab]	11.168	0.001
Cecum (g)	0.417±0.008	0.601±0.038	0.823±0.068[ab]	16.496	<0.001
Proximal colon (g)	0.161±0.010	0.193±0.017	0.236±0.016[a]	6.137	0.012
Distal colon (g)	0.201±0.015	0.359±0.030	0.483±0.035[ab]	29.658	<0.001
Fetus numbers		7.7±0.6	9.2±1.0	$t = -1.265$, df = 10	0.234
Overall fetus mass (g)		7.480±1.506	7.231±1.456	$t = 0.119$, df = 10	0.908
Placental mass (g)		1.815±0.118	2.136±0.220	$t = -1.284$, df = 10	0.228

Values are arithmetic means±SE. Data were analyzed by ANCOVA with carcass mass as a covariate, or by independent-sample t-tests. a, significantly different at $P<0.05$ from NR; b, significantly different at $P<0.05$ from PW. NR, non-reproductive group; PW, pregnant females in the warm; PC, pregnant females in the cold.

Energy intake and RMR

When the effect of body mass on day 18 of pregnancy was removed, cold-exposed pregnant voles had higher dry matter (DMI) or gross energy intake ($F_{2,14} = 8.407$, $P = 0.004$) and digestible energy intake ($F_{2,14} = 6.482$, $P = 0.01$) than the other two groups (Fig. 1). There was no significant difference in digestibility among the three experimental groups ($F_{2,15} = 0.029$, $P = 0.972$; Fig. 1).

When the effect of body mass on RMR was removed, there was no significant difference in RMR between the non-reproductive and the pregnant voles in the warm, but the pregnant voles in the cold had a higher RMR than the pregnant voles in the warm ($F_{2,14} = 8.405$, $P = 0.004$; Fig. 2).

Mitochondrial protein content, COX activity in BAT and liver, and UCP1 content in BAT

There were no significant differences in BAT mass among the three experimental groups. The pregnant voles in the cold had lower relative BAT mass, but

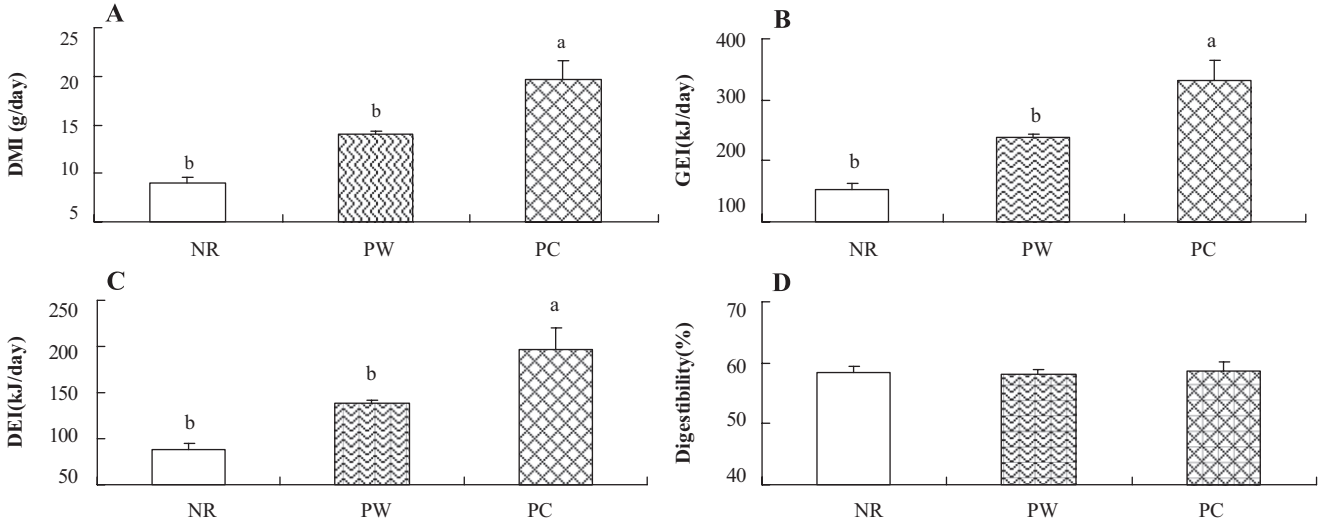

Fig. 1. Effect of cold exposure on energy intake in pregnant Brandt's voles. (A) Dry matter intake (DMI); (B) gross energy intake (GEI); (C) digestible energy intake (DIE); (D) digestibility. Values are arithmetic means±SE. DMI, GEI and DEI were analyzed by ANCOVA with body mass as the covariate. Different superscript letters indicate significant differences between groups. NR, non-reproductive group; PW, pregnant females in the warm; PC, pregnant females in the cold.

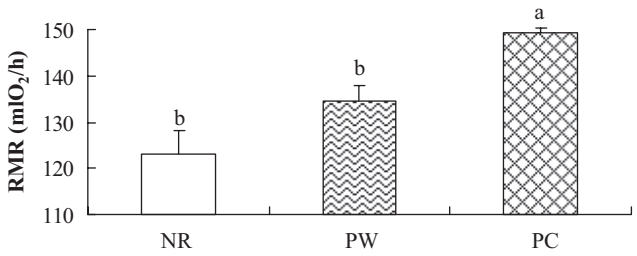

Fig. 2. Effect of cold exposure on resting metabolic rate (RMR) in pregnant Brandt's voles. Values are arithmetic means ± SE. RMR was analyzed by ANCOVA with body mass at the time of measurement as the covariate. Different superscript letters indicate significant differences between groups. NR, non-reproductive group; PW, pregnant females in the warm; PC, pregnant females in the cold.

higher COX activity per gram of BAT compared to pregnant voles in the warm. The cold-exposed pregnant voles also had higher MP content, but lower COX activity per mg MP compared to the other two groups (Table 4). The pregnant voles in the warm and cold had higher COX activity in whole liver compared to non-reproductive voles. The cold-exposed pregnant voles also had higher relative liver mass compared to non-reproductive voles, and higher liver COX activity per mg MP, but lower MP content in liver compared to non-reproductive voles and pregnant voles in the warm (Table 4).

UCP1 content per gram of MP in BAT in cold-exposed pregnant voles increased by 34% and 46% compared to non-reproductive and pregnant voles in the warm, respectively ($F_{2,15} = 5.424$, $P = 0.017$; Fig. 3A and B). UCP1 content per whole BAT in pregnant voles in the cold was 120% and 93% higher compared to non-reproductive and pregnant voles in the warm, respectively ($F_{2,15} = 6.334$, $P = 0.01$; Fig. 3C).

Serum leptin concentration

Pregnant voles had a higher serum leptin concentration than non-reproductive voles (PW vs. NR: 49%; PC vs. NR: 37%; $F_{2,15} = 21.698$, $P < 0.001$; Fig. 4A), and there was no difference in serum leptin concentration between pregnant voles in the warm and in the cold ($P = 0.144$). The correlation between serum leptin and body fat mass of all the voles was very weak ($r = 0.458$, $n = 18$, $P = 0.056$; Fig. 4B). When the data were analyzed separately, there was no correlation between serum leptin and body fat mass in any of the groups. When the effect of body fat on leptin was removed, pregnant voles both in the warm and in the cold still showed higher residual leptin concentrations than non-reproductive voles ($F_{2,15} = 15.197$, $P < 0.001$; Fig. 4C).

There was a weak correlation between serum leptin and gross energy intake ($r = 0.483$, $n = 18$, $P = 0.042$; Fig. 5A). However, when the effect of body mass was removed, residual serum leptin concentration was not correlated with residual gross energy intake ($r = 0.045$, $n = 18$, $P = 0.860$; Fig. 5B). There was no significant correlation between serum leptin concentration and UCP1 content ($r = 0.042$, $n = 18$, $P = 0.868$; Fig. 5C).

Table 4. MP content and COX activity in BAT and liver in non-reproductive and pregnant voles exposed to 23 °C and 5 °C.

	NR	PW	PC	*F*	*P*
Brown adipose tissue (BAT)					
(g)	0.266 ± 0.038	0.469 ± 0.076	0.241 ± 0.019	1.339	0.294
% (relative to body mass)	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.0^{b}	4.695	0.026
MP (mg/g BAT)	3.153 ± 0.367	2.254 ± 0.163	5.206 ± 0.656^{ab}	16.144	< 0.001
COX activity in BAT					
nmol O_2/min mg MP	2187.2 ± 195.6	2523.3 ± 150.1	1453.1 ± 142.3^{ab}	11.084	0.001
nmol O_2/min g BAT	6615.8 ± 388.7	5567.8 ± 95.74	7144.9 ± 310.3^{b}	7.534	0.005
nmol O_2/min in whole BAT	1729.9 ± 231.3	2594.2 ± 406.4	1715.7 ± 144.4	3.172	0.071
Liver					
% (relative to body mass)	3.3 ± 0.2	4.2 ± 0.3	4.4 ± 0.2^{a}	5.613	0.015
MP (mg/g liver)	11.383 ± 0.744	10.619 ± 0.249	8.920 ± 0.273^{ab}	7.246	0.006
COX activity in liver					
nmol O_2/min mg MP	253.96 ± 15.17	265.73 ± 6.27	360.44 ± 4.58^{ab}	35.198	< 0.001
nmol O_2/min g liver	2855.8 ± 157.5	2814.9 ± 32.1	3216.8 ± 114.4	3.769	0.047
nmol O_2/min in whole liver	4788.9 ± 429.8	9915.7 ± 924.2^{a}	10957.8 ± 718.3^{a}	25.577	< 0.001

Values are arithmetic means ± SE. The data for the mass of BAT (*g*) were analyzed by ANCOVA, with carcass mass as a covariate. Others were analyzed by ANOVA, followed by Tukey HSD tests. a, significantly different at $P < 0.05$ from NR; b, significantly different at $P < 0.05$ from PW. NR, non-reproductive group; PW, pregnant females in the warm; PC, pregnant females in the cold; MP, mitochondrial protein; COX, cytochrome *c* oxidase.

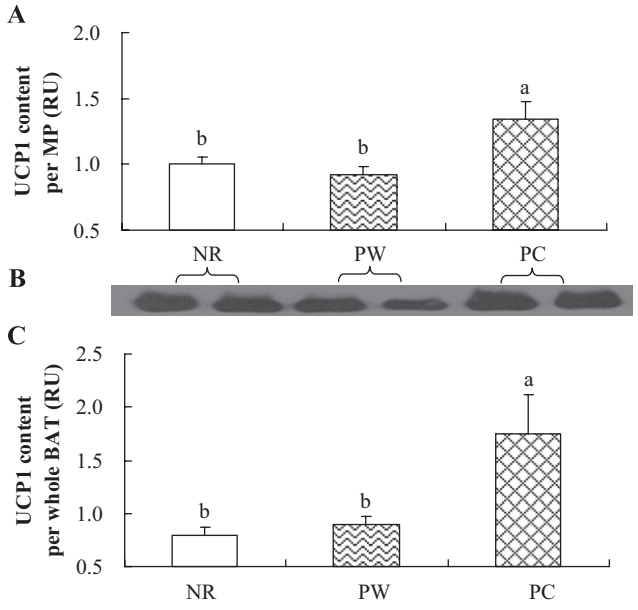

Fig. 3. Effect of cold exposure on uncoupling protein 1 (UCP1) content in brown adipose tissue (BAT) in pregnant Brandt's voles. (A) Compared with pregnant voles in the warm, the pregnant voles in the cold increased UCP1 content per mitochondrial protein by 46%. Values are arithmetic means ± SE. (B) Autoradiograms displaying UCP1 labeling from the non-reproductive and pregnant Brandt's voles in the warm and in the cold. Every two bars from left to right were consistent with those in (A). (C) UCP1 content per whole BAT in the cold-exposed pregnant voles was 93% higher than in pregnant voles in the warm. Different superscript letters indicate significant differences between groups. NR, non-reproductive group; PW, pregnant females in the warm; PC, pregnant females in the cold.

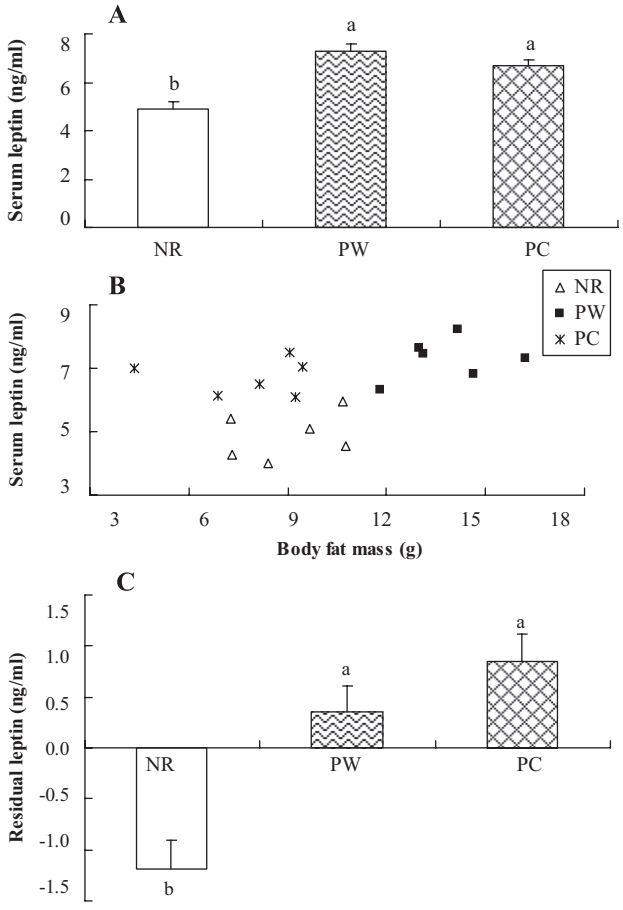

Fig. 4. Effect of cold exposure on serum leptin concentration in pregnant Brandt's voles. (A) Serum leptin in PW was 49% higher than that in NR. Serum leptin in PC was 37% higher than that in NR. (B) The correlation between serum leptin and body fat mass was weak ($r = 0.458$, $n = 18$, $P = 0.056$). (C) Serum leptin corrected for body fat mass. Residual leptin was higher in pregnant voles in the warm or in the cold than that in non-reproductive voles. Values are arithmetic means ± SE. Different superscript letters indicate significant differences between groups. NR, non-reproductive group; PW, pregnant females in the warm; PC, pregnant females in the cold.

Discussion

Small mammals generally increase BAT thermogenic capacity in response to cold, as indicated by increased COX activity and a higher UCP1 mRNA level (Cannon and Nedergaard, 2004). Our data support this. The cold-exposed pregnant voles had decreased body fat compared to reproduction in the warm, but the fetal development up to day 18 of gestation was not affected by cold exposure, which implies a trade-off in energy allocation to the mothers vs. their offspring. Further, hyperphagia and hyperleptinemia in pregnancy imply the need to develop a central leptin resistance with respect to control of energy balance.

Cold exposure enhanced thermogenic capacity and energy intake in pregnant voles

Most small mammals increase both RMR and non-shivering thermogenesis (NST) in response to cold. Breeding is highly energy demanding. During pregnancy, NST is inhibited, probably to save energy (Trayhurn et al., 1982). The pregnant voles exposed to cold had enhanced BAT thermogenic capacity. Another study showed that BAT thermogenic capacity in lactating Brandt's voles increased to the same level as in cold-exposed non-reproductive voles (Zhang and Wang, 2007). This reflects the well-known fact that increasing thermogenesis to maintain a stable body temperature is essential for survival in small mammals (Cannon and Nedergaard, 2004).

Small mammals are generally income breeders (i.e. increased energy intake is the main means to meet the extra energy demands of reproduction) (Stearns, 1989). Our present and previous data showed that the increased energy expenditure for thermogenesis in

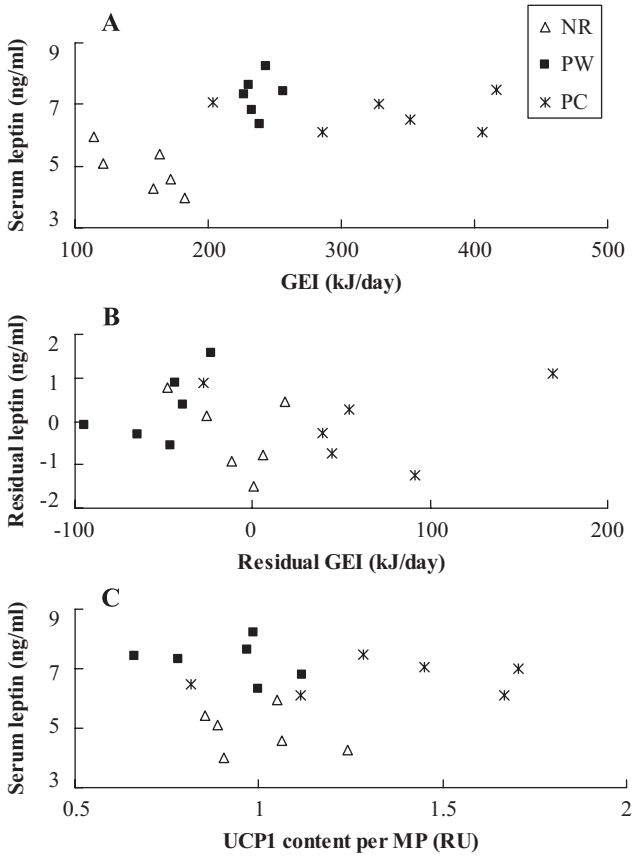

Fig. 5. Correlation between (A) serum leptin concentration and gross energy intake (GEI) ($r = 0.483$, $n = 18$, $P = 0.042$), (B) mass-specific leptin concentration and mass-specific energy intake ($r = 0.045$, $n = 18$, $P = 0.860$), (C) serum leptin concentration and UCP1 content per MP in BAT ($r = 0.042$, $n = 18$, $P = 0.868$). NR, non-reproductive group; PW, pregnant females in the warm; PC, pregnant females in the cold.

cold-exposed pregnant or lactating voles (Zhang and Wang, 2007) was mainly met by increasing energy intake. The assimilation energy was found to reach a maximum from 5 to 0 °C in Brandt's voles (Song and Wang, 2001). Pregnant rats (Luz and Griggio, 1992) or lactating mice and deer mice (*Peromyscus maniculatus*) (Hammond and Kristan, 2000; Johnson and Speakman, 2001) also increased energy intake in the cold. The increased masses of digestive organs (Hammond and Kristan, 2000; Johnson and Speakman, 2001; Zhang and Wang, 2007), and the increased intestinal digestive enzyme activity (Hammond and Diamond, 1994; Hammond, 1997; Liu, 2006) contributed to the increase in energy absorption during reproduction or cold exposure. But the voles did not rely on increased digestibility to compensate for high energy demands under these conditions. A previous study showed that in the early spring there was still enough food stored in the burrows of the voles (Shi et al., 1997). Therefore, food is not limited and this may explain why voles can commence reproduction in the early spring when ambient temperature is still very low.

Cold exposure did not affect fetal development but affected maternal body fat accumulation

Although energy demands during pregnancy increase because of fetal development, most small mammals such as rats, mice (Leshner et al., 1972; Richard and Trayhurn, 1985), Brandt's voles (Liu et al., 2003; Zhang and Wang, 2007) and Mongolian gerbils (*Meriones unguiculatus*) (Li, 2006) also increase body mass and body fat mass at the same time. The increased body mass and fat mass in our study indicate that females were in positive energy balance. The stored energy in fat can be mobilized during lactation. The pregnant voles neither decreased body mass to reduce energy expenditure nor reduced fetus number or mass, when they were exposed to the cold. Similar results were found for rats (Luz and Griggio, 1992). However, the fact that pregnant voles in the cold did not simply increase food intake for maintaining fat stores, similar to pregnant voles in the warm, suggests that their sustained energy intake may be centrally limited by aspects of the digestive process (Hammond and Diamond, 1992; Hammond et al., 1996; Johnson et al., 2001; Speakman and Król, 2005). Alternatively, the sustained energy intake may be peripherally limited at the sites of energy utilization (Hammond and Diamond, 1992; Hammond et al., 1996; Speakman and Król, 2005), or limited by the capacity to dissipate heat (Król and Speakman, 2003a, b). Although cold exposure during pregnancy increased the mother's energy burden, our data suggest that the voles did not sacrifice the fetuses to preserve themselves. In Brandt's voles, energy from body fat reserve catabolism is equivalent to about 13% of the total energy intake during lactation (Zhang and Wang, 2008). Therefore, mobilizing body fat is an important strategy to supply part of the energy required for milk production (Naismith et al., 1982). Since the pregnant voles in the cold could not accumulate body fat for the coming lactation, the effects of cold exposure during pregnancy and lactation on reproductive output and offspring development should be further studied.

The role of leptin in regulating maternal energy intake during pregnancy and cold exposure

Serum leptin, primarily secreted by adipose tissue, plays an important role in regulating energy balance and body mass (Friedman and Halaas, 1998). It has been shown that leptin is also secreted from the placenta and fetus in pregnant humans and rats (Masuzaki et al., 1997; Senaris et al., 1997) and plays a role in fetus

development (Harris, 2000; Henson and Castracane, 2006). Consistent with the results in humans, rats and mice (Butte et al., 1997; Tomimatsu et al., 1997; Amico et al., 1998), serum leptin concentration increased during pregnancy in Brandt's voles. The increased serum leptin concentration was not significantly affected by cold exposure. However, previous data from non-reproductive rodents such as mice (Korhonen and Saarela, 2005), rats (Bing et al., 1998), Siberian hamsters (*Phodopus sungorus*) (Larkin et al., 2001) and Brandt's voles (Zhang and Wang, 2006, 2007) showed that cold exposure decreased serum leptin concentration significantly. In the present study, there was no relation between serum leptin and body fat during pregnancy. Serum leptin was also independent from adiposity during fasting (Ahima et al., 1996; Mustonen et al., 2008), and during prehibernatory fattening (Kronfeld-Schor et al., 2000). These dissociations contribute to increasing energy intake and accumulating body fat. Hyperleptinemia during pregnancy plays a role in regulating fetus development and in modulating placental endocrine function (Henson and Castracane, 2006; Herrid et al., 2006). These data would appear to indicate the importance of an increased serum leptin level for a successful outcome of the pregnancy and also that leptin secretion is independent of body fat in pregnant voles.

Hyperleptinemia is accompanied by hyperphagia in pregnant Brandt's voles and many other mammals (Johnstone and Higuchi, 2001; Ladyman, 2008). The present and previous studies also showed that serum leptin was not correlated with UCP1 content during pregnancy (Zhang and Wang, 2008). However, injection of leptin in pregnant rats failed to reduce energy intake (Stocker et al., 2004), which suggests that leptin resistance may develop during pregnancy. Evidence showed that changes in hormonal environment due to gestation, for example through prolactin and progesterone, contribute to the development of pregnancy-induced hyperphagia and leptin resistance (Grattan, 2001; Grattan et al., 2007; Augustine and Grattan, 2008). The molecular mechanisms responsible for this resistance are unclear. Studies in rats showed that OB-Rb mRNA expression in the ventromedial nucleus of the hypothalamus and leptin-induced phosphorylation of signal transducers and activation of transcription-3 (STAT3) were reduced during pregnancy (Ladyman and Grattan, 2004, 2005). A state of leptin resistance would allow for an increase in energy intake and accumulation of maternal fat to support gestation (Ladyman, 2008), while keeping a high leptin level would be needed for proper fetal development. These findings emphasize the need to develop a central leptin resistance for the regulation of energy balance.

In summary, pregnant voles can increase energy intake to maintain the development of the fetus, but not to accumulate body fat, when exposed to cold. In the field, Brandt's voles store food for winter survival and commence breeding in early spring when the ambient temperature is still low. Whether cold exposure during pregnancy can influence the reproductive output and program the offspring's energy metabolism for their adulthood needs to be further investigated.

Acknowledgments

We are grateful to Dr. Martin Klingenspor at the Department of Biology, Philipps University Marburg, Germany, for supplying the hamster UCP1 antibody. Thanks to Ian D. Hume, Sydney University, Australia, for improving the English expression and helpful comments. This study was financially supported by the National Natural Science Foundation of China (No. 30625009), National Basic Research Program of China (2007BC109103), Chinese Academy of Sciences (KSCX2-YW-N-06) to DHW and the State Key Laboratory of Integrated Management of Pest Insects and Rodents (Grant No. IPM0801) to XYZ.

References

Ahima, R.S., Flier, J.S., 2000. Leptin. Annu. Rev. Physiol. 62, 413–437.

Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., Flier, J.S., 1996. Role of leptin in the neuroendocrine response of fasting. Nature 382, 250–252.

Amico, J.A., Thomas, A., Crowley, R.S., Burmeister, L.A., 1998. Concentrations of leptin in the serum of pregnant, lactating, and cycling rats and of leptin messenger ribonucleic acid in rat placental tissue. Life Sci. 63, 1387–1395.

Augustine, R.A., Grattan, D.R., 2008. Induction of central leptin resistance in hyperphagic pseudopregnant rats by chronic prolactin infusion. Endocrinology 149, 1049–1055.

Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J.P., Bortoluzzi, M.N., Moizo, L., Lehy, T., Guerre-Millo, M., Le Marchand-Brustel, Y., Lewin, M.J., 1998. The stomach is a source of leptin. Nature 394, 790–793.

Bing, C., Frankish, H.M., Pickavance, L., Wang, Q., Hopkins, D.F., Stock, M.J., Williams, G., 1998. Hyperphagia in cold-exposed rats is accompanied by decreased plasma leptin but unchanged hypothalamic NPY. Am. J. Physiol. 274, R62–R68.

Bronson, F.H., 1985. Mammalian reproduction: an ecological perspective. Biol. Reprod. 32, 1–26.

Butte, N.F., Hopkinson, J.M., Nicolson, M.A., 1997. Leptin in human reproduction: serum leptin levels in pregnant and lactating women. J. Clin. Endocrinol. Metab. 82, 585–589.

Cannon, B., Nedergaard, J., 2004. Brown adipose tissue: function and physiological significance. Physiol. Rev. 84, 277–359.

Friedman, J.M., Halaas, J.L., 1998. Leptin and the regulation of body weight in mammals. Nature 395, 763–770.

Gould, B.S., 1943. Simple adapters for continuous extraction of aqueous solutions in the Soxhlet extractor. Science 98, 546.

Grattan, D.R., 2001. The actions of prolactin in the brain during pregnancy and lactation. Prog. Brain Res. 133, 153–171.

Grattan, D.R., Ladyman, S.R., Augustine, R.A., 2007. Hormonal induction of leptin resistance during pregnancy. Physiol. Behav. 91, 366–374.

Grodzinski, W., Wunder, B.A., 1975. Ecological energetics of small mammals. In: Golley, F.B., Petrusewicz, K., Ryszkowski, L. (Eds.), Small Mammals: Their Productivity and Population Dynamics. Cambridge University Press, Cambridge, pp. 173–204.

Hammond, K.A., 1997. Adaptation of the maternal intestine during lactation. J. Mamm. Gland Biol. Neoplasia 2, 243–252.

Hammond, K.A., Diamond, J., 1992. An experimental test for a ceiling on sustained metabolic rate in lactating mice. Physiol. Zool. 65, 952–977.

Hammond, K.A., Diamond, J., 1994. Limits to dietary nutrient uptake and intestinal nutrient uptake in lactating mice. Physiol. Zool. 67, 282–303.

Hammond, K.A., Kristan, D.M., 2000. Responses to lactation and cold exposure by deer mice (*Peromyscus maniculatus*). Physiol. Biochem. Zool. 73, 547–556.

Hammond, K.A., Lloyd, K.C., Diamond, J., 1996. Is mammary output capacity limiting to lactational performance in mice? J. Exp. Biol. 199, 337–349.

Harris, R.B., 2000. Leptin – much more than a satiety signal. Annu. Rev. Nutr. 20, 45–75.

Henson, M.C., Castracane, V.D., 2006. Leptin in pregnancy: an update. Biol. Reprod. 74, 218–229.

Herrid, M., Nguyen, V.L., Hinch, G., McFarlane, J.R., 2006. Leptin has concentration and stage-dependent effects on embryonic development *in vitro*. Reproduction 132, 247–256.

Hoggard, N., Hunter, L., Lea, R.G., Trayhurn, P., Mercer, J.G., 2000. Ontogeny of the expression of leptin and its receptor in the murine fetus and placenta. Br. J. Nutr. 83, 317–326.

Johnson, M.S., Speakman, J.R., 2001. Limits to sustained energy intake. V. Effect of cold-exposure during lactation in *Mus musculus*. J. Exp. Biol. 204, 1967–1977.

Johnson, M.S., Thomson, S.C., Speakman, J.R., 2001. Limits to sustained energy intake. III. Effects of concurrent pregnancy and lactation in *Mus musculus*. J. Exp. Biol. 204, 1947–1956.

Johnstone, L.E., Higuchi, T., 2001. Food intake and leptin during pregnancy and lactation. Prog. Brain Res. 133, 215–227.

Korhonen, T., Saarela, S., 2005. Role of adiposity hormones in the mouse during fasting and winter-acclimatization. Comp. Biochem. Physiol. A 140, 217–223.

Król, E., Speakman, J.R., 2003a. Limits to sustained energy intake. VI. Energetics of lactation in laboratory mice at thermoneutrality. J. Exp. Biol. 206, 4255–4266.

Król, E., Speakman, J.R., 2003b. Limits to sustained energy intake. VII. Milk energy output in laboratory mice at thermoneutrality. J. Exp. Biol. 206, 4267–4281.

Kronfeld-Schor, N., Richardson, C., Silvia, B.A., Kunz, T.H., Widmaier, E.P., 2000. Dissociation of leptin secretion and adiposity during prehibernatory fattening in little brown bats. Am. J. Physiol. 279, R1277–R1281.

Ladyman, S.R., 2008. Leptin resistance during pregnancy in the rat. J. Neuroendocrinol. 20, 269–277.

Ladyman, S.R., Grattan, D.R., 2004. Region-specific reduction in leptin-induced phosphorylation of signal transducer and activator of transcription-3 (STAT3) in the rat hypothalamus is associated with leptin resistance during pregnancy. Endocrinology 145, 3704–3711.

Ladyman, S.R., Grattan, D.R., 2005. Suppression of leptin receptor messenger ribonucleic acid and leptin responsiveness in the ventromedial nucleus of the hypothalamus during pregnancy in the rat. Endocrinology 146, 3868–3874.

Larkin, J.E., Freeman, D.A., Zucker, I., 2001. Low ambient temperature accelerates short-day responses in Siberian hamsters by altering responsiveness to melatonin. J. Biol. Rhythms 16, 76–86.

Leshner, A.I., Siegel, H.I., Collier, G., 1972. Dietary self-selection by pregnant and lactating rats. Physiol. Behav. 8, 151–154.

Li, J.Y., 2006. Studies on reproductive energetics in Mongolian gerbils (*Meriones unguiculatus*). Ph.D. Thesis, Chinese Academy of Sciences, Beijing.

Li, X.S., Wang, D.H., 2005. Regulation of body weight and thermogenesis in seasonally acclimatized Brandt's voles (*Microtus brandtii*). Horm. Behav. 48, 321–328.

Liu, H., Wang, D.H., Wang, Z.W., 2003. Energy requirements during reproduction in female Brandt's voles (*Microtus brandtii*). J. Mammal. 84, 1410–1416.

Liu, Q.S., 2006. Digestive physiological adaptation under different energy demands in Mongolian gerbils and Brandt's voles. Ph.D. Thesis, Chinese Academy of Sciences, Beijing.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Luz, J., Griggio, M.A., 1992. Energy balance of pregnant rats in a cold environment. J. Therm. Biol. 17, 235–239.

Martin, I., Giralt, M., Vinas, O., Iglesias, R., Mampel, T., Villarroya, F., 1989. Adaptative decrease in expression of the mRNA for uncoupling protein and subunit II of cytochrome *c* oxidase in rat brown adipose tissue during pregnancy and lactation. Biochem. J. 263, 965–968.

Masuzaki, H., Ogawa, Y., Sagawa, N., Hosoda, K., Matsumoto, T., Mise, H., Nishimura, H., Yoshimasa, Y., Tanaka, I., Mori, T., Nakao, K., 1997. Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. Nat. Med. 3, 1029–1033.

Mustonen, A.M., Saarela, S., Nieminen, P., 2008. Food deprivation in the common vole (*Microtus arvalis*) and the tundra vole (*Microtus oeconomus*). J. Comp. Physiol. B 178, 199–208.

Naismith, D.J., Richardson, D.P., Pritchard, A.E., 1982. The utilization of protein and energy during lactation in the rat, with particular regard to the use of fat accumulated in pregnancy. Br. J. Nutr. 48, 433–441.

Niswender, K.D., Baskin, D.G., Schwartz, M.W., 2004. Insulin and its evolving partnership with leptin in the hypothalamic control of energy homeostasis. Trends Endocrinol. Metab. 15, 362–369.

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., Collins, F., 1995. Effects of the *obese* gene product on body weight regulation in ob/ob mice. Science 269, 540–543.

Richard, D., Trayhurn, P., 1985. Energetic efficiency during pregnancy in mice fed ad libitum or pair-fed to the normal energy intake of unmated animals. J. Nutr. 115, 593–600.

Scarpace, P.J., Metheny, M., 1998. Leptin induction of UCP1 gene expression is dependent on sympathetic innervation. Am. J. Physiol. 275, E259–E264.

Senaris, R., Garcia-Caballero, T., Casabiell, X., Gallego, R., Castro, R., Considine, R.V., Dieguez, C., Casanueva, F.F., 1997. Synthesis of leptin in human placenta. Endocrinology 138, 4501–4504.

Shi, D.Z., Hai, S.Z., Jin, X.M., Liu, X.L., 1997. Study on the forage behavior and selection for storage of forage plants of Brandt's voles (*Microtus brandti*) before overwintering. Acta Agrestia Sin. 5, 20–26 (in Chinese with English abstract).

Shirley, B., 1984. The food-intake of rats during pregnancy and lactation. Lab. Anim. Sci. 34, 169–172.

Song, Z.G., Wang, D.H., 2001. Maximum energy assimilation rate in Brandt's voles (*Microtus brandti*) from Inner Mongolia grassland. Acta Theriol. Sin. 21, 271–278 (in Chinese with English abstract).

Speakman, J.R., Król, E., 2005. Limits to sustained energy intake. IX. A review of hypotheses. J. Comp. Physiol. B 175, 375–394.

Stearns, S.C., 1989. Trade-offs in life-history evolution. Funct. Ecol. 3, 259–268.

Stocker, C., O'Dowd, J., Morton, N.M., Wargent, E., Sennitt, M.V., Hislop, D., Glund, S., Seckl, J.R., Arch, J.R., Cawthorne, M.A., 2004. Modulation of susceptibility to weight gain and insulin resistance in low birth weight rats by treatment of their mothers with leptin during pregnancy and lactation. Int. J. Obes. Relat. Metab. Disord. 28, 129–136.

Sundin, U., Moore, G., Nedergaard, J., Cannon, B., 1987. Thermogenin amount and activity in hamster brown fat mitochondria: effect of cold acclimation. Am. J. Physiol. 252, R822–R832.

Tomimatsu, T., Yamaguchi, M., Murakami, T., Ogura, K., Sakata, M., Mitsuda, N., Kanzaki, T., Kurachi, H., Irahara, M., Miyake, A., Shima, K., Aono, T., Murata, Y., 1997. Increase of mouse leptin production by adipose tissue after midpregnancy: gestational profile of serum leptin concentration. Biochem. Biophys. Res. Commun. 240, 213–215.

Trayhurn, P., Gouglas, J.B., McGuckin, M.M., 1982. Brown adipose tissue thermogenesis is suppressed during lactation in mice. Nature 298, 59–69.

Wade, G.N., Jennings, G., Trayhurn, P., 1986. Energy balance and brown adipose tissue thermogenesis during pregnancy in Syrian hamsters. Am. J. Physiol. 250, R845–R850.

Zhang, X.Y., Wang, D.H., 2006. Energy metabolism, thermogenesis and body mass regulation in Brandt's voles (*Lasiopodomys brandtii*) during cold acclimation and rewarming. Horm. Behav. 50, 61–69.

Zhang, X.Y., Wang, D.H., 2007. Thermogenesis, food intake and serum leptin in cold-exposed lactating Brandt's voles (*Lasiopodomys brandtii*). J. Exp. Biol. 210, 512–521.

Zhang, X.Y., Wang, D.H., 2008. Different physiological roles of serum leptin in the regulation of energy intake and thermogenesis between pregnancy and lactation in primiparous Brandt's voles (*Lasiopodomys brandtii*). Comp. Biochem. Physiol. C 148, 390–400.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425–432.

Zhang, Z.B., Wang, Z.W., 1998. Ecology and Management of Rodent Pests in Agriculture. Ocean Press, Beijing, China.