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ORIGINAL PAPER

Cold exposure inhibits hypothalamic *Kiss-1* gene expression, serum leptin concentration, and delays reproductive development in male Brandt's vole (*Lasiopodomys brandtii*)

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Abstract Cold commonly affects growth and reproductive development in small mammals. Here, we test the hypothesis that low ambient temperature will affect growth and puberty onset, associated with altered hypothalamic *Kiss-1* gene expression and serum leptin concentration in wild rodents. Male Brandt's voles (*Lasiopodomys brandtii*) were exposed to cold (4±1 °C) and warm (23±1 °C) conditions from the birth and sacrificed on different developmental stages (day 26, day 40, day 60, and day 90, respectively). Brandt's voles increased the thermogenic capacity of brown adipose tissue, mobilized body fat, decreased serum leptin levels, and delayed the reproductive development especially on day 40 in the cold condition. They increased food intake to compensate for the high energy demands in the cold. The hypothalamic *Kiss-1* gene expression on day 26 was decreased, associated with lower wet testis mass and testis testosterone concentration on day 40, in the cold-exposed voles compared to that in the warm. Serum leptin was positively correlated with body fat, testis mass, and testosterone concentration. These data suggested that cold exposure inhibited hypothalamic *Kiss-1* gene expression during the early stage of development, decreased serum leptin concentration, and delayed reproductive development in male Brandt's voles.

Keywords Brandt's voles (*Lasiopodomys brandtii*) · Cold exposure · Kiss-1 · Leptin · Reproductive development

List of abbreviations

COX	Cytochrome c oxidase
ECL	Enhanced chemiluminescence
EWAT	Epididymal white adipose tissue
iBAT	Inter-scapular brown adipose tissue
RIA	Radioimmunoassay
RU	Relative unit
UCP1	Uncoupling protein 1

Introduction

Inhibition of reproductive function in winter occurs commonly among small mammals living in the temperate zones (Bronson and Pryor 1983). Temperature is one of vital environmental factors impacting on metabolism and reproduction of small mammals (Bronson and Pryor 1983; Glądalski et al. 2014). In some rodents, it has been shown that cold exposure could suppress the growth and delay the onset of breeding (Bronson and Pryor 1983). Depression of fertility under harsh environmental conditions may avoid futile maternal investment.

For small mammals, increasing thermogenesis to maintain body temperature is the main strategy for survival in the cold environment. They usually increase food intake to compensate for the high energy expenditure, but they might still lose weight or grow slowly. They might also distribute less energy to the development of reproductive organs, resulting in delaying the onset of breeding (Bronson and Pryor 1983; Goldman 2001). The energy limitation may cause the delay

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of reproductive development as suggested by the trade-off theory (Garland 2014). For example, the very thin cows showed a delay in post-calving return to cyclicity (Kruip et al. 2001), and similar results were also found in rodents (Bronson and Manning 1991). In addition, bilateral removal of the epididymal white adipose tissue (EWAT) decreased spermatogenic activity and markedly atrophied the seminiferous tubules in rats (Srinivasan et al. 1986). These cases demonstrated that the reduction of body fat reserve might contribute to depression of reproductive function (Butler 2003).

Compelling lines of evidence point out that the adipocyte-derived hormone leptin, in addition to its main role in energy homeostasis, is also involved in the control of neuroendocrine systems for gonadal, adrenal, thyroid, and growth axis (Ahima et al. 2000). The absence of leptin led to infertility and lack of pubertal development, whereas leptin administration maintained reproductive cyclicity, rescued puberty onset, and prevented sterility in *ob*/*ob* mice (Chehab et al. 1996; Mounzih et al. 1997). Leptin administration also increased testicular mass and seminiferous tubule diameter and stimulated testicular function and testosterone synthesis in mice (Kus et al. 2005). Since its receptors (OB-Rb) exist in testis and a variety of other tissues or organs, leptin might act directly on the testicular tissue, or through a hypothalamic-pituitary-gonad axis to regulate reproductive function (Ahima et al. 2000). Some studies showed that leptin acted as a central neuroendocrine effect rather than a direct effect on testicular tissue (Barb et al. 2004; Soyupek et al. 2005). Leptin may in part be communicated to GnRH neurons via Kiss-1/GPR54 system (Luque et al. 2007; Kirilov et al. 2013; Martin et al. 2014), which are mainly expressed in hypothalamic nuclei and are potent stimulators of the GnRH/LH axis (Castellano et al. 2006; Kumar et al. 2014). The seasonal modulation of breeding in the temperate-zone mammals has been found to be influenced by the Kiss-1/GPR54 system (Revel et al. 2006; Janati et al. 2013; Boufermes et al. 2014).

Brandt's vole (*Lasiopodomys brandtii*), a typical nonhibernating herbivores, distributes mainly in the Inner Mongolian grassland of China, Mongolia, and the region of Baikal in Russia (Zhang and Wang 1998). The average annual temperature is 0–4 °C, and winter lasts for more than 5 months. The voles showed seasonal reproduction, and the offspring bred in the early spring would face with cold stress during their development. We have found that cold exposure decreased body fat mass and serum leptin concentrations but increased energy intake and thermogenic capacity in adult voles (Li and Wang 2005; Zhang and Wang 2006). Further study showed that hypoleptinemia in the cold condition contributed to hyperphagia and body fat mobilization in male adult voles (Tang et al. 2009). However, we still do not know the mechanism for how cold exposure affects growth and reproductive development under low ambient temperature. In this study, we hypothesized that the effects of low ambient temperature on growth and puberty onset would be associated with altered hypothalamic *Kiss-1* gene expression and serum leptin concentrations in wild rodents. We predicted that the growth, reproductive development, hypothalamic *Kiss-1* gene expression, and serum leptin concentrations would decrease under low temperature in male Brandt's voles.

Materials and methods

Animals and experimental design

All animal use procedures were permitted by the Institutional Animal Care and Committee of the Institute of Zoology, Chinese Academy of Science. Brandt's voles were from our laboratory breeding which were trapped in Inner Mongolian grasslands in 1999. The voles were housed with same gender sibling pairs in plastic cages (30×15×20 cm) and maintained at 23±1 °C under a 16 L: 8 D photoperiod with lights on at 04:00 h. Commercial rabbit pellets (Beijing Hua Fukang Feed Co.) and water were provided ad libitum.

The female voles were paired with males for 2 days to allow insemination, and then, the males were removed. On the day of parturition, the dams and their litters were transferred to a room at 4±1 °C (lactating in the cold, n=8) for 3 weeks. Other lactating females remained at 23±1 °C (lactating in the warm, n=8). All the voles were given the same amount of cotton (approximately 3–4 g) for their nest building. At weaning (21 days old), male offspring of the warm (23±1 °C) and cold (4±1 °C) groups were housed individually in plastic cages with sawdust bedding and maintained respectively at its primary temperature until sacrificed on the days of 26, 40, 60, and 90 (n=10–12), respectively, by carbon dioxide anesthesia. Body mass and food intake were monitored once a week.

Body compositions

After collecting trunk blood to get serum, the hypothalamus and inter-scapular brown adipose tissue (iBAT) were carefully dissected, frozen in liquid nitrogen, and then stored at −80 °C. The visceral organs, including the heart, lung, liver, kidneys, spleen, testis, epididymis, and gastrointestinal tract (stomach, small intestine, cecum, proximal colon, and distal colon), were extracted and weighed (±1 mg) for body composition analysis. The stomach and intestines were rinsed with 0.9 % saline to eliminate all the gut contents and weighed before being dried. At the same time, the WATs around the gonads, kidneys, and mesenterium were removed and weighed (±1 mg) and then kept together with the carcass to get the carcass mass. The remaining carcass and all the organs were dried in an oven at 60 °C to constant mass (at least 3 days), 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 with a Soxtec Fat Extraction System (Avanti 2050, FOSS, Hogänäs, Sweden) with petroleum ether following the manufacturer's directions.

Measurement of UCP1 and COX4 contents in iBAT

For measuring total protein content, iBAT was homogenized in the rate of 1:5 (*m*/*v*) with radio immunoprecipitation assay (RIPA) lysis buffer containing 10 mM Tris (pH 7.0), 158 mM NaCl, 1.0 % Triton X-100, 5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (PIC, Sigma, dilution 1:1000). The soluble fractions were obtained by centrifugation at 14,000*g* for 10 min at 4 °C. The protein concentration of iBAT was measured with Folin phenol method using bovine serum albumin as standards (Lowry et al. 1951).

The uncoupling protein 1 (UCP1) and cytochrome c oxidase IV (COX4) protein of iBAT was separated in a discontinuous SDS-polyacylamide gel (12.5 % running gel and 3 % stacking gel) according to the molecular weight with the same 90 μg protein per lane (Li and Wang 2005; Zhang et al. 2011a, b). After electrophoresis, the protein was transferred to PVDF membrane in Tris/glycine buffer (pH 9.2) containing 48 mM Tris, 1.3 mM SDS, 21.3 mM glycine (Amresco, Solon, OH), and 20 % methanol. For binding unspecific sites, membranes were soaked in a blocking buffer [5 % nonfat dried milk in phosphate-buffered saline (PBS, pH 7.6) containing 0.05 % Tween-20] for 1 h at room temperature. After the membrane was washed with TBS/T for three 10-min intervals, UCP1, COX4, and β-tubulin (inner reference) were detected respectively using rabbit anti-UCP1 (1:10000; Abcam), mouse anti-COX4 (1:1000; Santa Cruz Biotechnology), and mouse anti-β-tubulin (1:5000; DSHB) as a primary antibody overnight at 4 °C with gentle agitation. The membranes were washed with TBS/T for three 10-min intervals and then incubated with peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG as the secondary antibody (1:5000, Jackson ImmunoResearch Inc., Baltimore, PA, USA) for 3 h at room temperature. After three 10-min washes in TBS/T and two 10-min washes in TBS, the antigen-antibody peroxidase complex was detected by enhanced chemiluminescence (ECL; Amersham Biosciences) and was used for detection. Quantification of the immunoreactive bands was performed by using a Quantity One Ver.4.4.0 Software (Bio-Rad, USA). UCP1 and COX4 contents were expressed as relative unit (RU).

Serum assays

Serum leptin concentrations were measured by radioimmunoassay (RIA) with a [^{125}I] multispecies kit (Cat. No. XL-85 K, Linco Research Inc., USA), which had been validated previously in Brandt's voles (Li and Wang 2005; Zhang et al. 2011a, b). The measured range of the leptin concentrations that could be detected by this assay kit was from 1 to 50 ng/mL, and the intra- and inter-assay variations were <3.6 and <8.7 %, respectively. Serum leptin concentrations were determined in a single RIA and presented as nanogram per milliliter (see manufacturer's instructions for multispecies leptin RIA kit).

The concentrations of thyroid hormones (total T_3 and T_4) were measured in 50 μL of serum according to the manufacturer's instructions and determined by highly sensitive and specific 3,3′,5-triiodothyronine and thyroxine RIA kit (Human NO. S10930040, Atom-hitech Inc. CN). Intra- and inter-assay coefficients of variation were 2.4 and 8.8 % for T_3 and 4.3 and 7.6 % for T_4, respectively. The values are reported as nanogram per milliliter and in a single RIA for total T_3 and total T_4. All groups were assayed in the same run in order to avoid inter-assay variations (Zhang et al. 2011a, b).

Testosterone in testis tissue assays

The testes were quickly removed, weighed, frozen in dry ice, and kept at −80 °C. For the measurement of the testicular testosterone concentrations, one testis of each male was weighed and placed in tubes containing five times volume of the testis mass ice-cold Dulbecco's PBS buffer (NaCl 8.0 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15 g/L, KH_2PO_4 0.2 g/L, $MgCl_2 \cdot 6H_2O$ 0.1 g/L, $CaCl_2 \cdot 2H_2O$ 0.1 g/L at pH 7.3–7.4) with 0.25 M sucrose and homogenized (Korhonen et al. 2008). The samples were centrifuged at 600*g* for 10 min at 4 °C to precipitate nuclei and cellular debris. The supernatant was collected and centrifuged at 10,000*g* for 20 min at 4 °C to separate mitochondrial pellet. Finally, the supernatant was collected and kept at −80 °C until used for measurement. The concentrations of testosterone in testis homogenates were assessed by [^{125}I] testosterone RIA kit, and the intra- and inter-assay coefficient of variance are <10.0 and <15.0 %, respectively (Human NO. S10940093, BNIBT Inc. CN). The process follows the manufacturer's instructions of the testosterone RIA kit. The lowest limit of testosterone concentrations that could be detected by this assay was 0.1 ng/mL using 50 μL of serum. All groups were assayed in the same run in order to avoid inter-assay variations.

Hypothalamic *Kiss-1* gene expression

Total RNA isolation and cDNA synthesis

Hypothalamus total RNA was extracted using TRIzol reagent (Cat.No.15596- 026, Invitrogen, USA) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Cat. No. M6101, Promega, USA) for 30 min at 37 °C in order to denature any contaminating DNA.

Complementary DNA was generated from equal volumes (4 μg) of total RNA for each sample using reverse transcription kit (Cat. No. 1622, Fermentas, Lithuania) following the manufacturer's instructions, as described previously (Tang et al. 2009).

RT-qPCR

Hypothalamic *Kiss-1* messenger RNA (mRNA) expression was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), optimized for semiquantitative detection, used previously defined primer pairs and conditions (Castellano et al. 2005). As internal control for reverse transcription and reaction efficiency, amplification of primers set for β-actin protein mRNA was carried out in parallel in each sample. To verify changes in *Kiss-1* gene expression, RT-qPCR was performed using the SYBR Green I qPCR kit (Cat. NO. DRR041D, TaKaRa, Japan) in the Mx3000P quantitative PCR system (Stratagene, USA). The sample complementary DNA (cDNA) prepared as above was used as a template for the PCR. General procedures for RT-qPCR of *Kiss-1* mRNA were as previously described (Castellano et al. 2005). Real-time PCR was carried out in 12.5-μL reaction agent composed of 6.25 μL 2× SYBR® Premix EX Taq™ master mix, 1-μL cDNA templates, and 0.2 μmol/L primers. Each sample was analyzed in triplicate. The primers used were as follows: Kiss-1: (sense) 5′-ATGGGGAGGTCCTACGGG-3′, (anti-sense) 5′-CGTTAATGCCTGGGAAAAGG-3′; β-actin: (sense) 5′-TTGTGCGTGACATCAAAGAG-3′, (anti-sense) 5′-ATGCCAGAAGATTCCATACC-3′ (Tang et al. 2009). The Kiss-1 reaction conditions were as follows: 30 s at 95 °C for 1 cycle, then 40 cycles of 95 °C for 5 s and 60 °C for 20 s. At the end of the experiments, melting curve analysis showed that there were no nonspecific amplifications. PCR products were confirmed by melting curve information, 2 % agarose gel electrophoresis, and further confirmed by DNA sequencing. Standard curves were constructed for each gene via serial dilutions of cDNA (fivefold dilution). Analysis of standard curves between target genes and β-actin showed that they had similar amplification efficiency, which confirmed the validity of comparative quantity method. The data derived from Mx3000P quantitative software were expressed as relative amounts, calculated by normalizing the amount of target gene mRNA levels to the amount of β-actin mRNA levels. No amplification was detected in absence of template or in the no RT control. All RT-qPCR analysis standards and samples were run in triplicates, and the results were subjected to statistical analysis.

Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Prior to all statistical analyses, data were examined for assumptions of normality and homogeneity of variance, using Kolmogorov-Smirnov and Levene's tests, respectively. If not, data were log- or arcsine-square-root-transformed. The differences in body mass and food intake during development were analyzed by repeated measures analysis of variance (ANOVA) and analysis of covariance (ANCOVA) (body mass as a covariate) respectively, followed by least-significant difference (LSD) *post hoc* tests. Group differences at different time points between warm and cold were analyzed by independent *t* test in body mass and one-way ANCOVA in food intake with body mass as a covariate. All morphological parameters and molecular markers were analyzed by two-way ANCOVA (wet carcass mass as a covariate) and two-way ANOVA, respectively, followed by LSD *post hoc* tests. Pearson correlation analysis was performed to determine the correlation of serum leptin concentrations with body fat mass, hypothalamic *Kiss-1* gene expression, and testis testosterone concentrations and the correlation between hypothalamic *Kiss-1* gene expression and testosterone concentrations in testis tissue. Data were reported as mean± SE, and $P<0.05$ was considered to be statistically significant.

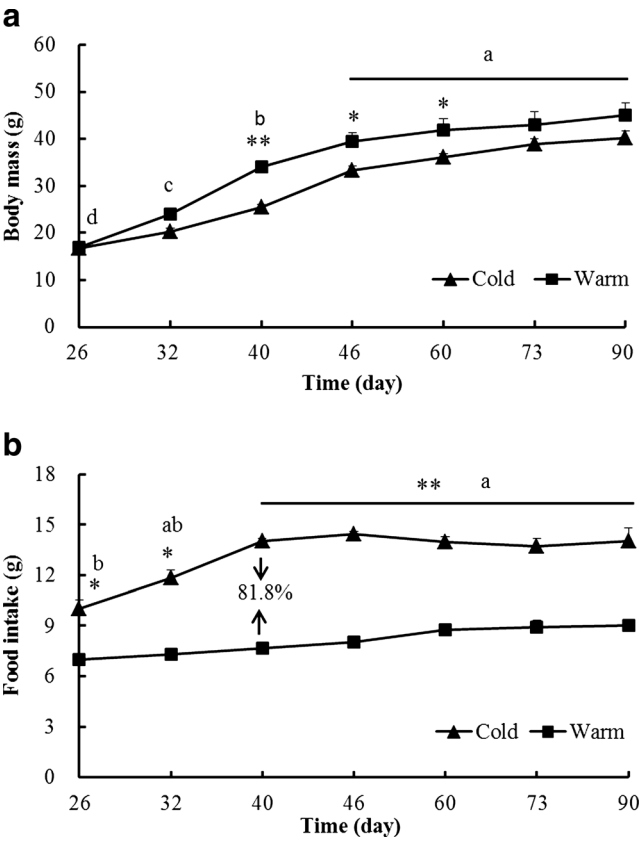

Fig. 1 Changes of body mass (**a**) and food intake (**b**) in male Brandt's voles during development under cold and warm conditions. Values are means±SE. *$P<0.05$; **$P<0.01$. *Bars with different letters* differed significantly from each other among different ages ($P<0.05$)

Results

Body mass, food intake, and body composition

Body mass on day 26 was 16.9±0.7 and 16.7±0.4 g at 23 and 4 °C, respectively, and there was no difference between two groups (t=0.24, df=18, P>0.05). Body mass on day 40 (t=10.192, df=18, P<0.01) and day 60 (t=2.308, df=18, P<0.05) was lower significantly by 20.0 and 11.9 %, respectively, in cold groups than in the warm groups. Body mass increased during development and kept stable after day 60 (repeated measures ANOVA, P<0.01) (Fig. 1a).

Food intake increased significantly from weaning to days 40 and then kept stable (Repeated measure ANOVA, P<0.01). Food intake increased by 42.9 ($F_{1,20}$=43.841, P<0.001), 81.8 ($F_{1,20}$=380.474, P<0.001), 53.9 ($F_{1,20}$=72.775, P<0.001), and 55.6 % ($F_{1,20}$=45.959, P<0.001) during days 26, 40, 60, and 90 in the cold compared to the warm, respectively (Fig. 1b).

Voles exposed to cold on day 40 had lower total body fat mass (by 49.2 %, $F_{1,32}$=11.698, P<0.01, Fig. 2a), epididymal fat mass (by 63.0 %, $F_{1,32}$=17.253, P<0.001, Fig. 2b), wet carcass mass (t=6.965, df=32, P<0.001), and fat-free body mass (t=6.576, df=32, P<0.001) (Table 1). Cold exposure increased organ mass, such as liver (P<0.01), colon (P<0.01), and total gut mass (P<0.01) (Table 2). Wet testis mass on day 40 decreased by 36.4 % in the cold voles compared to the warm ($F_{1,32}$=15.343, P<0.01, Fig. 2c). However, there were

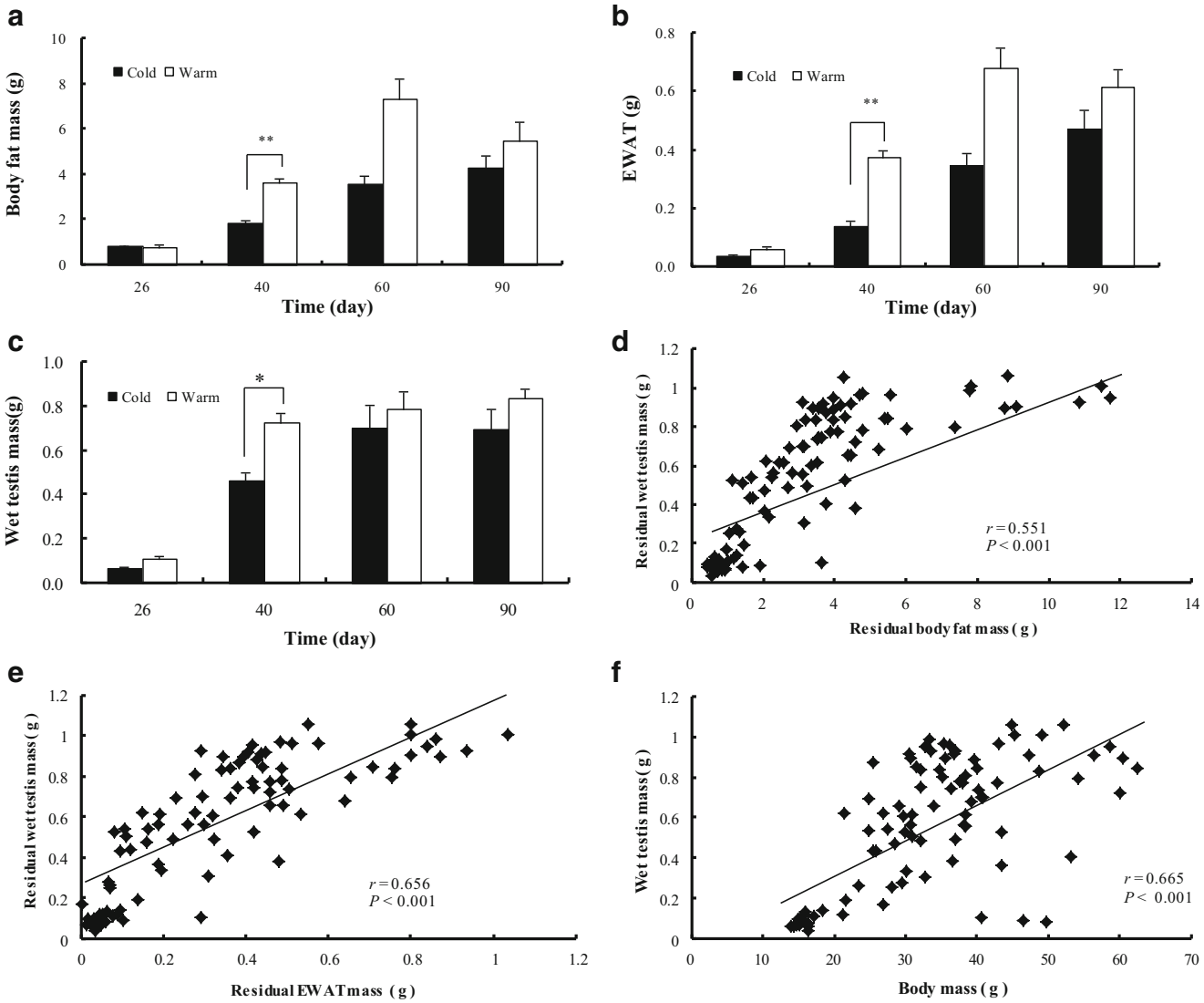

Fig. 2 Changes of body fat mass (**a**), epididymal white adipose tissue (EWAT) mass (**b**), and wet testis mass (**c**) in male Brandt's voles during development under the cold and warm conditions. Wet testis mass was positively correlated with body fat mass (**d**), EWAT (**e**), and body mass (**f**) in male Brandt's voles during development under cold and warm conditions. Values are means±SE. *P<0.05; **P<0.01

Table 1 Changes of body compositions during development in Brandt's voles between cold and warm (mean±SE)

Parameters	Day 26		Day 40		Day 60		Day 90		Statistical summary		
	23 °C	4 °C	23 °C	4 °C	23 °C	4 °C	23 °C	4 °C	T	A	T × A
Body mass (g)	16.85±0.685 c	16.66±0.397 c	36.230±1.473 b	25.480±1.473 b*	41.960±1.748 a	36.160±1.748 a*	44.700±1.834 a	40.260±1.834 a	<0.01	<0.01	<0.01
Wet carcass mass (g)	10.393±1.391 c	8.682±1.703 c*	23.900±1.287 b	16.483±1.135 b*	30.508±1.523 a	23.362±1.452 a*	31.675±1.523 a	25.506±1.523 a*	<0.01	<0.01	<0.01
Dry carcass mass (g)	4.012±0.549 c	3.651±0.672 c	9.227±0.508 b	6.931±0.448 b*	12.480±0.601 a	9.824±0.573 a*	12.228±0.601 a	10.724±0.601 a	<0.01	<0.01	<0.01
Body water mass (g)	6.381±0.842 c	5.031±1.032 c*	14.674±0.780 b	9.552±0.688 b*	18.028±0.923 a	13.538±0.880 a*	19.448±0.923 a	14.782±0.923 a*	<0.01	<0.01	<0.01
Fat-free wet carcass (g)	9.595±1.010 c	7.980±1.237 c*	20.244±0.935 b	14.356±0.825 b*	25.216±1.107 a	19.805±1.055 a*	26.196±1.107 a	21.195±1.107 a*	<0.01	<0.01	<0.01
Retroperitoneal fat(g)	0.019±0.043 bc	0.018±0.053 bc*	0.198±0.040 b	0.042±0.035 b*	0.345±0.047 a	0.133±0.045 a	0.330±0.047 a	0.167±0.047 a	<0.01	<0.01	<0.01
Mesenteric fat (g)	0.075±0.014 c	0.084±0.018 c*	0.183±0.013 b	0.175±0.012 b	0.274±0.016 a	0.242±0.015 a	0.216±0.016 a	0.250±0.016 a*	<0.01	ns	ns
Perirenal fat (g)	0.032±0.013 c	0.024±0.016 c	0.089±0.012 b	0.074±0.011 b	0.191±0.015 a	0.103±0.014 a	0.108±0.015 ab	0.108±0.015 ab	<0.01	<0.01	<0.01
iBAT (g)	0.123±0.018 b	0.143±0.022 b*	0.205±0.016 a	0.238±0.014 a*	0.219±0.018 a	0.242±0.019 a	0.228±0.011 a	0.248±0.019 a	<0.05	<0.01	ns

The different letters differed significantly from each other among different ages ($P<0.05$)

T temperature, *A* age; *T* × *A* the interaction between temperature and age, *ns* not significant, *iBAT* interscapular brown adipose tissue

*$P<0.05$ 4 vs 23 °C

no significant differences in testis mass between the two groups on days 26, 60, and 90 (Fig. 2c). Body fat mass, epididymal fat mass and wet testis mass increased significantly during development ($P<0.01$) and kept stable after day 60 (Fig. 2a–c). Residual wet testis mass was positively correlated with residual body fat mass ($r=0.551$, $P<0.001$, Fig. 2d) and residual epididymal fat mass ($r=0.656$, $P<0.001$, Fig. 2e). Wet testis mass was positively correlated with body mass ($r=0.665$, $P<0.001$, Fig. 2f)

Serum thyroid hormone concentrations and UCP1 and COX4 contents in iBAT

Serum T_3 concentrations increased significantly in the cold especially on day 26 (by 2.94 times, $P<0.001$) and day 60 (by 64.2 %, $P<0.01$, Fig. 3a), but T_4 concentrations decreased especially on day 60 (by 51.0 %, $P<0.05$, Fig. 3b) compared to those in the warm condition. The ratio of T_3/T_4 on days 26 ($P<0.001$), 40 ($P<0.05$), 60 ($P<0.05$), and 90 ($P<0.001$) increased significantly in the cold than in the warm (Fig. 3c). Serum T_3 and T_4 concentrations and the ratio of T_3/T_4 increased significantly from weaning to day 40 ($P<0.01$, Fig. 3a–c) and then kept stable both in cold and warm groups.

The absolute mass of iBAT increased by 16.3, 16.1, 10.1, and 8.8 % on day 26 ($P<0.05$), day 40 ($P<0.001$), day 60 ($P>0.05$), and day 90 ($P>0.05$) in the cold, respectively, compared to the warm (Table 1). Cold exposure significantly increased UCP1 (by 12.9 %, $P<0.05$, Fig. 4a) and COX4 (by 48.4 %, $P<0.01$, Fig. 4b) content of iBAT on day 40. However, there were no significant differences in UCP1 and COX4 content of iBAT on day 26, day 60, and day 90 between the two groups.

Hypothalamic *Kiss-1* gene expression

Hypothalamic *Kiss-1* mRNA levels on day 26 decreased significantly in the cold than in the warm (by 74.1 %, $P<0.05$). On day 40, *Kiss-1* mRNA levels in the cold decreased by 16.2 % compared with those in the warm, but the difference between warm and cold was not significant ($P>0.05$, Fig. 5). *Kiss-1* mRNA expression was affected significantly by the interaction of age and temperature ($F_{1,64}=4.677$, $P<0.05$, Fig. 5). *Kiss-1* mRNA level decreased significantly from weaning to day 40 ($P<0.01$, Fig. 5) and then kept stable in the warm, but the *Kiss-1* mRNA in the cold kept a stable level during the whole development ($P>0.05$, Fig. 5).

Serum leptin and testis testosterone assays

Voles in the cold on day 40 showed lower serum leptin (by 48.7 %, $P<0.01$, Fig. 6a) and testosterone concentrations in testis tissue (by 52.5 %, $P<0.01$, Fig. 6b) than those in the warm. There were no significant differences in serum leptin and testis testosterone concentrations on day 26, day 60, and

Table 2 Changes of wet organ mass during development in Brandt's voles between cold and warm (mean±SE)

Parameters	Day 26		Day 40		Day 60		Day 90		Statistical summary		
	23 °C	4 °C	23 °C	4 °C	23 °C	4 °C	23 °C	4 °C	T	A	T × A
Heart (g)	0.111±0.01 c	0.085±0.012 c	0.163±0.009 b	0.159±0.008 b*	0.230±0.011 a	0.216±0.01 a*	0.193±0.011 a	0.218±0.011 a*	<0.01	ns	ns
Lungs (g)	0.144±0.013 c	0.130±0.016 c	0.198±0.012 b	0.191±0.011 b*	0.327±0.014 a	0.261±0.014 a	0.277±0.014 a	0.238±0.014 a	<0.01	<0.01	ns
Liver (g)	0.688±0.081 c	0.591±0.099 c	1.334±0.075 b	1.241±0.066 b*	1.602±0.088 a	1.750±0.084 a*	1.424±0.088 a	1.749±0.088 a*	<0.01	ns	<0.05
Kidney	0.182±0.02 c	0.186±0.024 c*	0.367±0.018 b	0.382±0.016 b*	0.470±0.022 a	0.525±0.021 a*	0.440±0.022 a	0.539±0.022 a*	<0.01	<0.01	ns
Spleen (g)	0.024±0.002 a	0.026±0.003 a*	0.029±0.002 a	0.020±0.002 a	0.025±0.002 a	0.020±0.002 a	0.027±0.002 a	0.026±0.002 a	ns	<0.05	ns
Spermatheca (g)	0.006±0.034 c	0.005±0.042 c	0.205±0.032 b	0.048±0.028 b*	0.430±0.037 a	0.175±0.036 a	0.445±0.037 a	0.233±0.037 a	<0.01	<0.01	<0.01
Epididymis (g)	0.008±0.006 d	0.007±0.007 d	0.043±0.005 c	0.019±0.005 c	0.082±0.006 b	0.047±0.006 b	0.104±0.006 a	0.066±0.006 a*	<0.01	<0.01	<0.05
Stomach (g)	0.139±0.009 c	0.135±0.011 c	0.213±0.008 b	0.206±0.007 b*	0.250±0.010 a	0.267±0.009 a*	0.236±0.010 a	0.276±0.010 a*	<0.01	ns	ns
Small intestine (g)	0.159±0.034 c	0.207±0.042 c	0.349±0.032 b	0.343±0.028 b	0.315±0.038 ab	0.491±0.036 ab*	0.431±0.038 a	0.528±0.038 a	<0.01	<0.01	ns
Cecum (g)	0.227±0.022 c	0.221±0.027 c	0.355±0.020 b	0.357±0.018 b	0.370±0.024 a	0.471±0.023 a*	0.407±0.024 a	0.475±0.024 a	<0.01	ns	ns
Colon (g)	0.140±0.013 c	0.169±0.016 c*	0.270±0.012 b	0.316±0.011 b*	0.284±0.014 a	0.410±0.014 a*	0.300±0.014 a	0.399±0.014 a*	<0.01	<0.01	<0.01
Total gut mass (g)	0.665±0.068 c	0.733±0.083 c	1.186±0.063 b	1.223±0.056 b*	1.220±0.074 a	1.639±0.071 a*	1.374±0.074 a	1.679±0.074 a*	<0.01	<0.01	<0.05
Total content mass (g)	3.026±0.303 c	2.653±0.371 c*	3.487±0.281 b	5.481±0.247 b*	3.872±0.332 ab	6.418±0.317 ab*	3.910±0.332 a	7.376±0.332 a*	<0.01	<0.01	<0.01

The different letters differed significantly from each other among different ages ($P<0.05$)

T temperature, *A* age, *T* × *A* the interaction between temperature and age, *ns* not significant

*$P<0.05$ 4 vs 23 °C

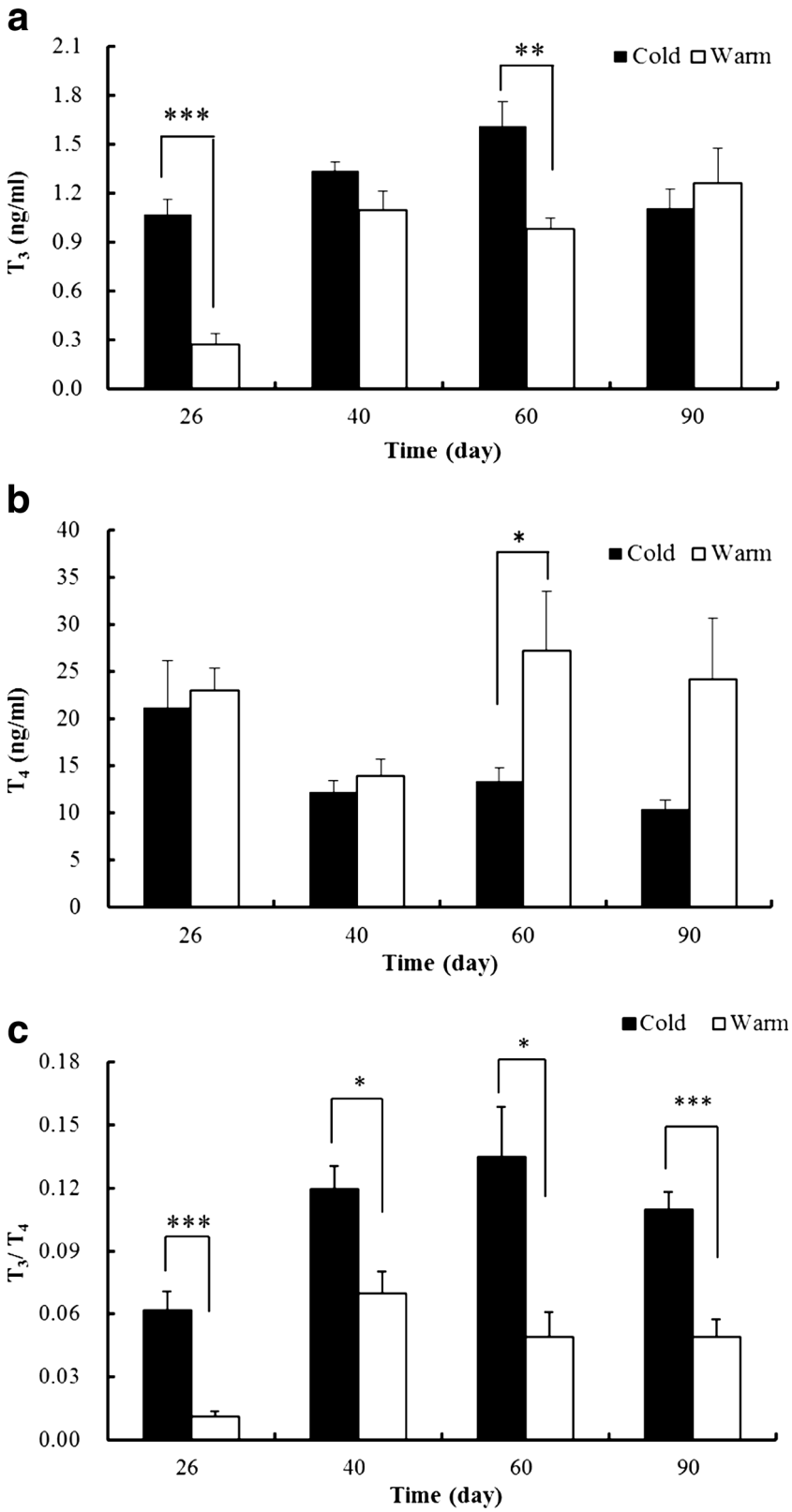

Fig. 3 Effect of cold exposure on serum T_3 (**a**), T_4 (**b**), and T_3 / T_4 (**c**) in male Brandt's voles during development. Values are means±SE. *P<0.05; **P<0.01; ***P<0.001

day 90 between the cold and warm groups. Serum leptin concentrations were not affected by the interaction of temperature × age ($F_{1,73}$=1.415, P>0.05), but testosterone concentrations in testis tissue were affected ($F_{1,73}$=3.851, P=0.013). Serum leptin concentrations increased significantly during development (P<0.01, Fig. 6a) and kept stable after

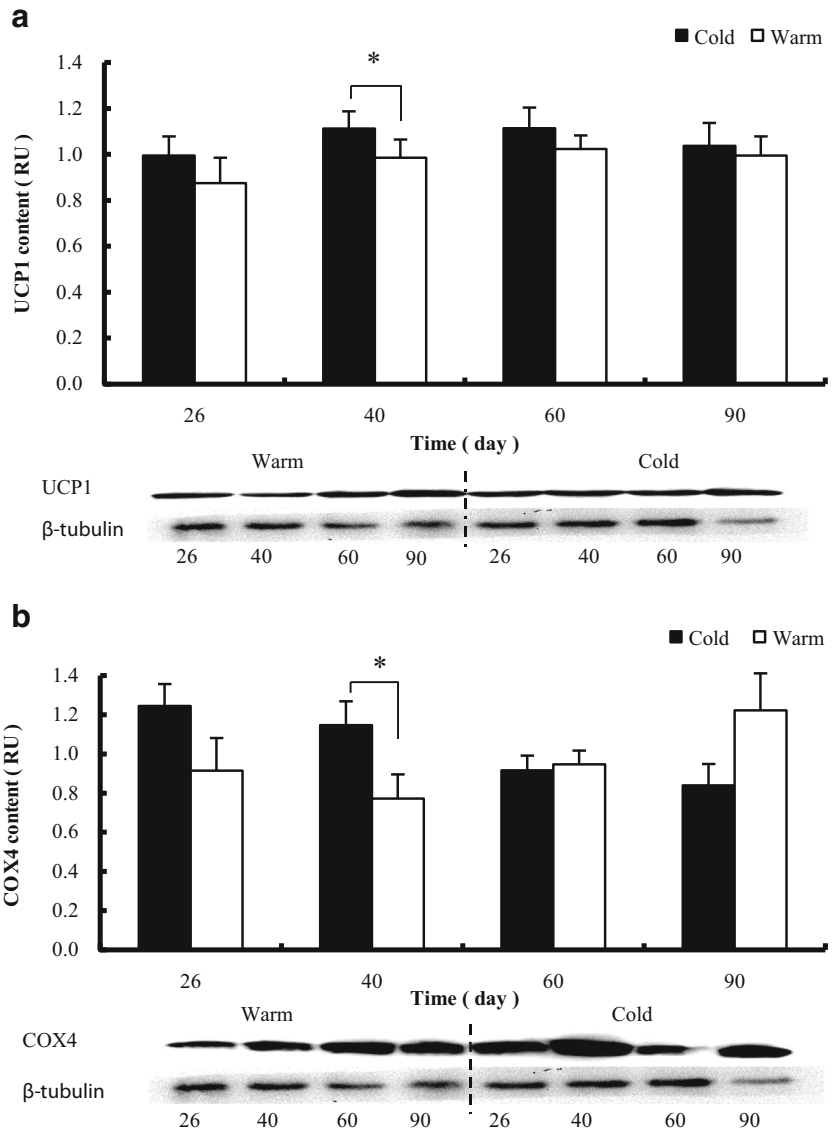

Fig. 4 Effect of cold exposure on uncoupling protein1 (UCP1) content (**a**) and cytochrome c oxidase IV (COX4) content (**b**) in brown adipose tissue (BAT) in male Brandt's voles during development. Values are means±SE. *$P<0.05$. *RU* relative unit

day 60 both in cold and warm groups. Testis testosterone concentrations increased significantly from weaning to day 40 ($P<0.01$, Fig. 6b) and then kept stable in warm, but the time when testis testosterone concentrations in the cold kept stable was delayed to day 60 ($P<0.01$, Fig. 6b).

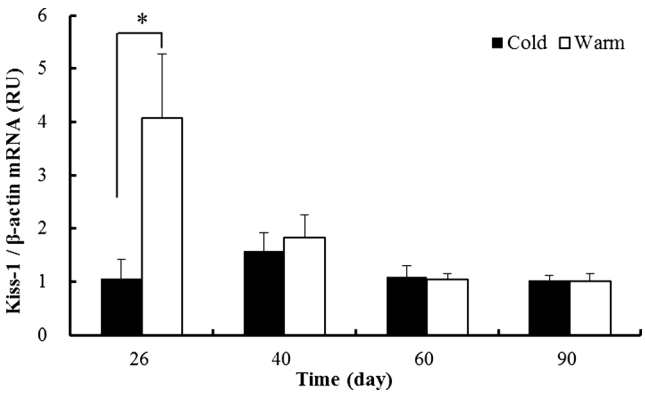

Fig. 5 Effect of cold exposure on Kiss-1/β-actin in hypothalamus in male Brandt's voles during development. Values are means±SE. *$P<0.05$. *RU* relative unit

Serum leptin concentrations were positively correlated with testosterone concentrations in testis tissue ($r=0.329$, $P<0.01$, Fig. 6c), wet testis mass ($r=0.330$, $P<0.05$, Fig. 6d), and epididymal fat mass ($r=0.451$, $P<0.001$, Fig. 6e). The testosterone concentration in testis tissue was positively related to wet testis mass ($r=0.496$, $P<0.001$, Fig. 6f).

Discussion

In this study, our data showed that cold exposure inhibited hypothalamic *Kiss-1* mRNA expression during the early stage

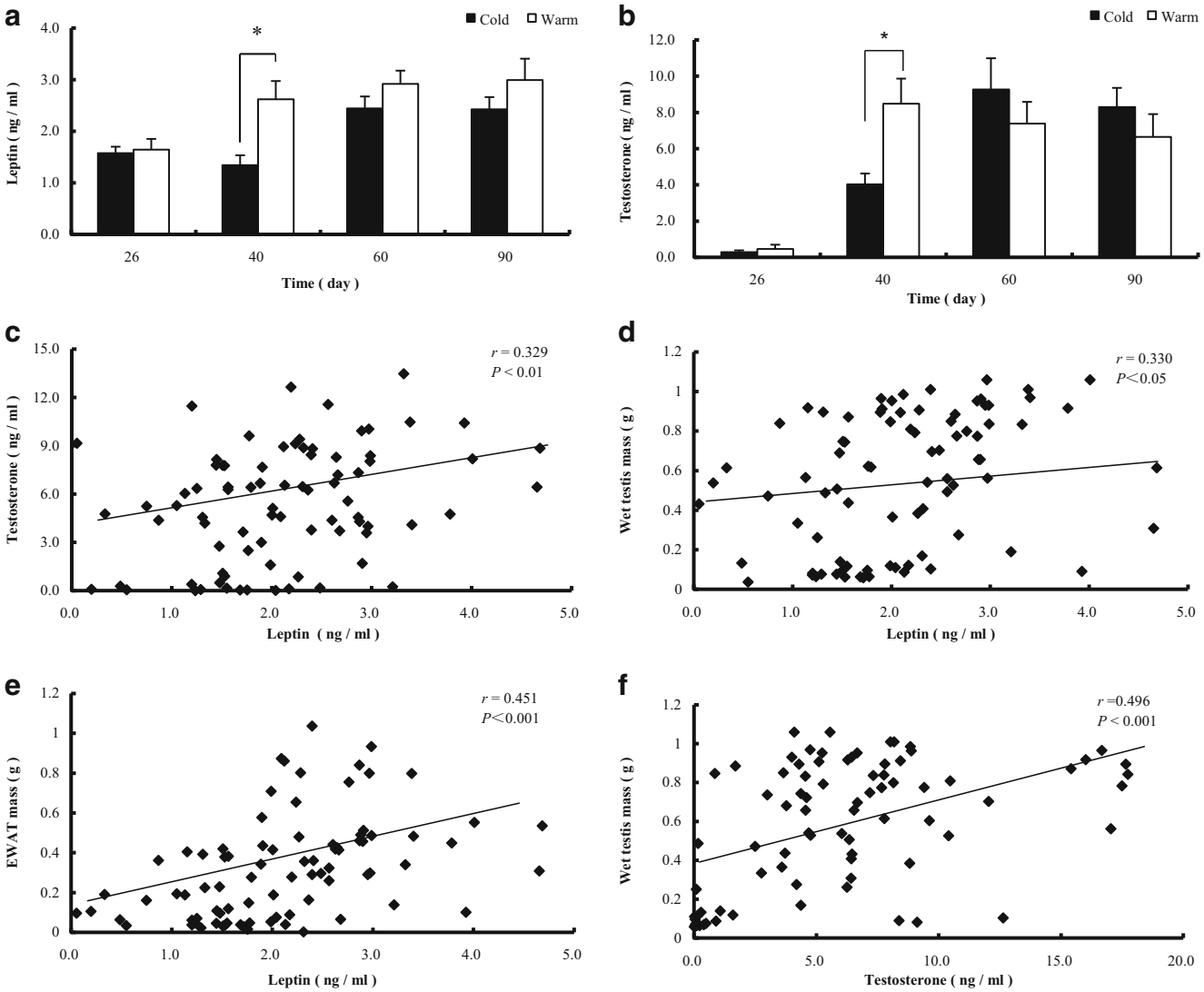

Fig. 6 Effect of cold exposure on serum leptin (**a**) and testis testosterone concentrations (**b**) in male Brandt's voles during development. Serum leptin concentrations were positively correlated with testis testosterone concentrations (**c**), wet testis mass (**d**), and epididymal fat mass (EWAT) (**e**) in male Brandt's voles during development under cold and warm conditions. Meanwhile, wet testis mass was positively correlated with testosterone concentrations in testis tissue (**f**). Values are means±SE. *$P<0.05$

of development, decreased serum leptin concentrations, and delayed the growth and reproductive development in male Brandt's voles.

Small mammals, because of their large surface to mass ratios, would experience a considerable thermoregulatory burden at low temperature (Hart 1971; Sugimoto et al. 2000). The increasing thermogenic capacity in BAT to maintain a stable body temperature is essential for survival in small mammals (Cannon and Nedergaard 2004; Cannon et al. 2000). UCP1 contents in BAT was enhanced in the cold in plateau pika (*Ochotona curzoniae*) (Wang et al. 2006a), root vole (*Microtus oeconomus*) (Wang et al. 2006b), Daurian ground squirrel (*Spermophilus dauricus*), Mongolian gerbil (*Meriones unguiculatus*), and Brandt's vole (Li et al. 2001; Li and Wang 2005). There were increases in the transcription levels of COX4 and other mitochondrial-encoded genes in hibernating ground squirrels (Fahlman et al. 2000). In this study, UCP1 and COX4 contents in iBAT were significantly higher in cold- than warm-reared young Brandt's voles. The rodents increased food intake and digestive function and mobilized body fat reserve to meet the high energy demands for thermoregulation in the cold (Bozinovic and Nespolo 1997; Zhang et al. 2011a). Thyroid hormones, critical in the central regulation of body temperature, can increase energy expenditure and stimulate basal thermogenesis by lowering metabolic efficiency (Tomasi 1991). Metabolic adjustment of thyroid hormones might correlate with BAT thermogenic capacity in tree shrews (*Tupaia belangeri*) and Mongolian gerbils exposed to cold (Li et al. 2001; Liu et al. 1997). Cold acclimation resulted in increases in serum T_3 concentration on

day 26 and day 60 and the ratio of serum T_3/T_4 at all stages in male Brandt's voles. These data suggested that the increase in the function of T3 was associated with the increase in cold-induced BAT thermogenesis for survival in the cold.

The trade-off theory of life history presumes that the increase in one function must be at the cost of the decrease in some others as a consequence of partitioning limited resources (Stearns 1992). The present study showed that the testis mass, testis testosterone concentrations, and EWAT mass decreased significantly in cold-reared male voles compared to their warm counterparts especially on day 40. These results were supported by the studies in mice, rats, Syrian hamster (*Mesocricetus auratus*), prairie voles (*Microtus ochrogaster*), and northern red-backed voles (*Clethrionomys rutilus*), which showed that the reproductive function was impaired at cold- or short-day exposure (Feist and Feist 1986; Kriegsfeld et al. 2000; Goldman 2001; Benderlioglu et al. 2006). These data suggested that cold-exposed animals shifted their metabolic priorities from development to basic survival, specifically thermogenesis.

Studies showed that both the *Kiss-1* and *GPR54* mRNA levels in the medial basal hypothalamus were the highest at the time of puberty in male rats and monkeys (Navarro et al. 2004; Shahab et al. 2005). *Kiss-1* mRNA in the hypothalamus decreased in short-day Syrian hamsters, and the chronic administration of kisspeptin-10, encoded by the *Kiss-1* gene, restored the testicular activity of these hamsters (Revel et al. 2006), indicating that the hypothalamic Kiss-1 system is critical for the regulation of reproduction and required for puberty onset (Kirilov et al. 2013; Martin et al. 2014). Our data showed that hypothalamic *Kiss-1* mRNA level on day 26 decreased significantly in the cold than that in the warm, prior to the decrease in reproductive organs. Moreover, *Kiss-1* mRNAs showed the highest level on day 26 in the warm but kept at low levels during the whole stage of development in the cold in male Brandt's voles. These findings suggested that the decreased expression of Kiss-1 in early stage of development might be a key gatekeeper for the delay of the initiation of puberty in the cold environment.

Some experiments in rodents have shown that the rise in leptin concentration may be the earlier signal of the initiation of puberty and may contribute to activation of the hypothalamic-pituitary-gonadal (HPG) axis, resulting in the testicular steroidogenesis (Gnessi et al. 1997). The administration of leptin stimulated *Kiss-1* expression in mouse hypothalamic cell line N6 and also in *ob*/*ob* mice (Luque et al. 2007). Our data showed that serum leptin concentrations decreased significantly in male Brandt's voles exposed to cold during the early stage of development, as indicated in the previous studies in adult rats (Trayhurn et al. 1995), Brandt's voles (Zhang and Wang 2006, 2007), and Siberian hamsters (*Phodopus sungorus*) (Larkin et al. 2001). The rising higher stage of serum leptin concentrations was from weaning to day 40 in warm, but from day 40 to 60 in cold in the present study. We also found that serum leptin concentrations were positively correlated with body fat mass, wet testis mass, and testis testosterone concentrations. All these data indicated that the lower serum leptin might link negative energy balance and delayed reproductive development in the cold.

In summary, we found that Brandt's voles increased thermogenesis and restrained the development of reproduction, supporting the trade-off between thermogenesis and reproduction in the cold environments. Serum leptin concentrations were correlated positively with body fat mass, testis mass, and testis testosterone concentrations, suggesting that lowered serum leptin in the cold might act as a signal to link the energy status to reproductive development. Importantly, the hypothalamic *Kiss-1* mRNA decreased only at the early stage before the reproductive onset. Together, the present data suggest the possible mechanism for leptin and Kiss-1 in mediating how cold-exposed wild rodents shift their metabolic priorities from development to basic survival, specifically thermogenesis, and the exact mechanisms should be further investigated.

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