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Pre- and post-weaning cold exposure does not lead to an obese phenotype in adult Brandt's voles (*Lasiopodomys brandtii*)

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ABSTRACT

Evidence has shown that postnatal undernutrition, overnutrition and cold stress are associated with imbalanced metabolic regulation as rodents achieve adulthood. In this study, we used a breeding colony of Brandt's voles (*Lasiopodomys brandtii*), a wild rodent species from the Inner Mongolia grasslands in China, to examine the effects of pre- and post-weaning cold exposure on the adult body (fat) mass, serum hormones and hypothalamic neuropeptides. Unlike laboratory rodents, vole offspring exposed to pre-weaning cold did not exhibit overweight or obese phenotypes in adulthood compared with unexposed controls. Moreover, adult male voles that remained in colder conditions had less body mass and lower serum leptin levels despite having higher food intake compared to other groups. To understand the mechanism of this unexpected regulation, hypothalamic gene expression was assessed for pre- and post-weaning cold exposure. Voles exposed to cold before weaning increased hypothalamic, orexigenic agouti-related protein (AgRP) and decreased anorexigenic proopiomelanocortin (POMC) mRNA expression at weaning. These expression changes were associated with hyperphagia and catch-up growth after weaning. Interestingly, these changes in hypothalamic neuropeptides were short lasting because in adult voles these differences were no longer apparent, which might explain why the pre-weaning, cold-exposed voles did not become obese in adulthood. These data suggest that some species do not develop an obese phenotype in response to early life cold stress.

Introduction

A multitude of perinatal, natural and social environmental factors can permanently alter the metabolic and neural systems that regulate energy homeostasis in offspring (Phillips and Young, 2000; Emack et al., 2008; Ryu et al., 2008). In general, most studies have focused on nutritional programming. For example, high-fat maternal diets during pregnancy and lactation (Howie et al., 2009), postnatal overnutrition induced by reduced litter size (Aubert et al., 1980; Faust et al., 1980), or post-weaning food restriction (Schroeder et al., 2010) resulted in hyperphagia, an overweight phenotype or an obese phenotype in adult rats and mice. These findings emphasize the important roles of food and nutrition during critical windows of development for programming metabolic phenotypes.

For temperate small mammals and even humans, environmental temperature is a crucial factor that can modulate thermoregulation, metabolism, reproduction and survival (Bronson, 2009). It is known that cold exposure in rodents during adulthood causes increased thermogenesis and food intake, but it also causes decreased body fat in rats (Fantino and Cabanac, 1984; Bing et al., 1998) and voles (Feist and Feist, 1986; Li and Wang, 2005; Zhang and Wang, 2006). It was also noted that cold exposure in neonatal rats increased sympathetic outflow to brown adipose tissue (BAT) (Morrison et al., 2000; Young et al., 2002) and resulted in more fat accumulation in adulthood (Young and Shimano, 1998). However, the long-term effects of perinatal cold exposure on feeding and thermogenesis were only studied in rats. It is still not clear whether the cold exposure response is universal in other wild rodents.

The hypothalamus is considered to be the main integrator of peripheral hormonal signals and controls homeostatic body regulation (Friedman and Halaas, 1998; Schwartz et al., 2000). One of the most studied hormonal signals is leptin, which is produced by adipose tissue, and serum leptin levels are directly proportional to fat mass in animals and humans (Zhang et al., 1994; Halaas et al., 1995). By activating the long form of the leptin receptor (OB-Rb), which is found predominantly in the hypothalamus, leptin regulates the expression of both orexigenic and anorexigenic peptides in the arcuate nucleus (ARC) (Friedman and Halaas, 1998; Schwartz et al., 2000). Neuropeptide Y (NPY) and agouti-related protein (AgRP) are the main orexigenic peptides that colocalize in the ARC, whereas proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) are the anorexigenic peptides in the ARC. Meanwhile, leptin activates suppressor of cytokine signaling 3 (SOCS3), a negative feedback regulator, to prevent overactivation of leptin signaling (Bjorbaek et al., 1998).

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Brandt's voles (*Lasiopodomys brandtii*) are nonhibernating herbivores that mainly inhabit the grasslands of Inner Mongolia in China, Mongolia, and the region of Beigaer in Russia. In these regions, the winters last for more than 5 months. These voles show seasonal reproduction from March to September (Zhang and Wang, 1998). They are still faced with cold temperatures during development if they are born in early spring or late summer; therefore, wild rodents are good models for examining cold exposure responses because such perturbations are ecologically relevant within their natural life cycles. These voles have been studied extensively regarding adult adaptive strategies to cold (Li and Wang, 2005; Zhang and Wang, 2006; Tang et al., 2009); however, it is still unclear whether cold challenges in early postnatal life impact the adult body (fat) mass, serum hormones and hypothalamic neuropeptides. Therefore, we used Brandt's voles as a model to examine the long-term effects of early postnatal (pre-weaning) cold exposure and the interaction with post-weaning cold exposure. We also aimed to elucidate the central mechanisms regulating phenotype formation. We predict that pre-weaning cold exposure would result in adult greater expression of orexigenic neuropeptides and obesity, while post-weaning cold exposure would decrease body mass in Brandt's voles.

Materials and methods

Animals

The Brandt's voles used in this study were the offspring of our laboratory breeding colony founded by field-captured animals from the Inner Mongolian Grasslands in May 1999. After weaning at 21 days of age, the voles were housed as same-gender sibling pairs in plastic cages (30×15×20 cm), maintained at 23±1 °C, and exposed to a photoperiod of 16:8 h (light:dark) with lights on beginning at 4:00 AM. All animals were provided standard rabbit pellet chow (KeAo Feed Co., Beijing) and water *ad libitum*.

At 3–4 months of age, seventy virgin female voles were housed individually, acclimated for 2 weeks and were then paired with males for 4 days to allow for insemination. On the day of parturition, a group of mothers ($n=20$) and their pups (the mothers with 6–8 pups/litter were chosen in this experiment) were transferred to a cold room (5± 1 °C) with the same photoperiod as their previous housing environment. They were provided with a small amount of cotton bedding (approximately 3 g) for nest material. The other group of mothers ($n=21$) and pups remained in a warm environment (23±1 °C). Nest temperatures were recorded with iButtons (DS1922L-F5, MAXIM). Of the twenty-one mothers in the warm environment, only one committed infanticide, which resulted in one pup death. In contrast, five out of twenty mothers in the cold environment committed infanticide, resulting in nine pup deaths. All the mothers in either the warm or cold environments had 5–8 pups and there was no difference in litter size between these two groups during the whole lactation. During weaning, one pup from each litter was sacrificed to collect tissues without regard to gender. One male and female from each litter were housed individually in a warm or cold environment until they reached 13 weeks old. In total, there were four groups for each gender based on environmental treatments: 1) pre-weaning warm and post-weaning warm (WW), 2) pre-weaning warm and post-weaning cold (WC), 3) pre-weaning cold and post-weaning cold (CC), and 4) pre-weaning cold and post-weaning warm (CW). During lactation, all the pups from a single nest were weighed every 3 days. After weaning, body mass was measured individually once every week. Diagram of experimental design was shown in Fig. 1.

All the voles were sacrificed by CO_2 overdose between 9:00 and 11:00 AM at weaning or week 13. Serum was collected and stored at −80 °C. The whole brain was rapidly removed from each body and placed on dry ice for slow freezing. The hypothalamus was dissected by sagittal cuts between the optic chiasm and the mammillary bodies, cuts through the edge of the septum and perihypothalamic sulcus, and by one horizontal cut immediately below the anterior commissure as previously described (Bing et al., 1998). The hypothalamus was frozen in liquid nitrogen immediately and stored at −80 °C until subsequent analysis. The interscapular brown adipose tissue (iBAT) was immediately and carefully dissected, weighed and stored at −80 °C until assayed. All experimental protocols were reviewed and approved by the Animal Care and Use Committee of the Institute of Zoology at the Chinese Academy of Sciences.

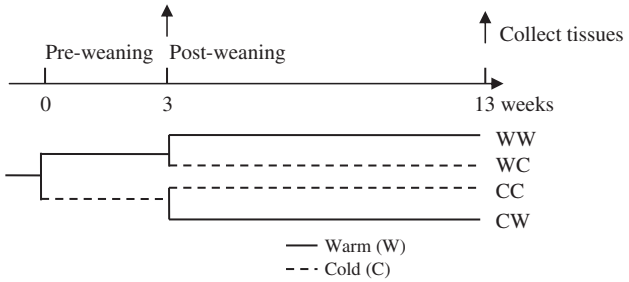

Fig. 1. Diagram of experimental design. During lactation (pre-weaning), the mothers and their pups were kept in a warm (23±1 °C, $n=21$) or cold (5±1 °C, $n=20$) environment. After weaning (post-weaning), one male and female from each litter were housed individually in a warm or cold environment until they reached 13 weeks old. Tissues were collected at 3 or 13 weeks of age. WW, pre-weaning warm and post-weaning warm; WC, pre-weaning warm and post-weaning cold; CC, pre-weaning cold and post-weaning cold; CW, pre-weaning cold and post-weaning warm.

Feeding measurement

Food intake was measured for 3 consecutive days once every two weeks after weaning. During the experiment, voles were housed individually in stainless steel mesh, metabolic cages (30×15×20 cm). Food and water were provided *ad libitum*. Uneaten food and feces were collected after each test, dried in an oven at 60 °C and then separated manually.

Serum assays

Serum leptin levels were determined by a radioimmunoassay (RIA) using the ^{125}I Multi-Species Leptin RIA Kit (Cat. No. XL-85K, Linco Research Inc.). This kit has been previously validated in Brandt's voles (Zhao and Wang, 2005; Zhang and Wang, 2006). The lower and upper limits of the assay when using a 100 μl sample were 1 and 50 ng/ml. The intra- and inter-assay coefficients of variation were 3.6% and 8.7%, respectively.

Serum 3,5,3′-triiodothyronine (T3) and thyroxine (T4) levels were quantified using the RIA kits (Institute of Chinese Atomic Energy, Beijing) according to the manufacturer's instructions. This kit was also previously validated for Brandt's voles (Zhao and Wang, 2005). The lower limit of the assay when using a 50 μl sample was 0.25 ng/ml for T3 and 3.96 ng/ml for T4. Intra- and inter-assay coefficients of variation were 2.4% and 8.8% for T3, and 4.3% and 7.6% for T4, respectively.

Measurement of UCP1, COX4 and SIRT1 contents in iBAT

The total protein concentration of iBAT was determined by the Folin phenol method using bovine serum albumin as a standard (Lowry et al., 1951). Uncoupling protein 1 (UCP1), cytochrome c oxidase 4 (COX4), and Sirtuin 1 (SIRT1) contents in iBAT were measured by western blotting as previously described (Li and Wang, 2005; Zhang and Wang, 2006). Total iBAT protein (90 μg/lane) was separated in a discontinuous SDS-polyacrylamide gel with the following formulations: 12.5% running gel and 3% stacking gel for

UCP1, COX4 and β-tubulin; and 8% running gel and 3% stacking gel for SIRT1 and β-tubulin. The protein was then transferred onto PVDF membranes (Hybond-P; Amersham, Buckinghamshire, UK), and the membrane was blocked in 5% milk in Tris-buffered saline-Tween for 1 h at room temperature. The membrane was incubated with rabbit anti-UCP1 (1:10,000; ab10983, Abcam, Cambridge, MA, USA), mouse anti-COX4 (1:1000; sc-58348, Santa Cruz Biotechnology, Inc., CA, USA), rabbit anti-SIRT1 (H-300) (1:1000; sc-15404, Santa Cruz Biotechnology, Inc.), and mouse anti-β-tubulin (1:5000; E7, DSHB, Iowa City, Iowa, USA) antibodies overnight at 4 °C. The immunoblot was visualized with horseradish peroxidase-conjugated secondary antibodies that were either goat anti-rabbit IgG or goat anti-mouse IgG (1:5000; ZSGB-BIO Co., Beijing, CHN). Protein bands were detected with ECL (Amersham Life Sciences, Little Chalfont, UK) and quantified using Quantity One software (version 4.4.0, BioRad, Hercules, CA). The protein content was expressed as arbitrary units (a.u.).

Real-time PCR for measurement of hypothalamic mRNA expression

Total RNA was isolated using Trizol (Cat. No. 15596-026, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After treating with DNase I (Cat. No. M6101, Promega, USA), 4 μg total RNA was reverse transcribed using a reverse transcription kit (Cat. No. 1622, Fermentas, Vilnius, the Republic of Lithuania).

Real-time PCR was performed using the SYBR Green I qPCR kit (Cat. No. DRR041D, TaKaRa, Shiga, Japan) and the Mx3005P quantitative PCR system (Stratagene, La Jolla, CA, USA). The thermal cycling conditions were as follows: 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 20 s. All samples were run in duplicate. All runs included the housekeeping gene β-actin as a control. Species-specific primers were designed and have been verified effectively in Brandt's voles (Tang et al., 2009; Zhang et al., 2011). The analysis of standard curves using serial dilutions of cDNA for the target genes and β-actin showed similar amplification efficiency, which ensures the validity of comparative quantifications. The data derived using the Mx3005P quantitative software were expressed as relative values and were calculated by normalizing the amount of target gene to the β-actin mRNA levels.

Body composition analysis

The organs and tissues, including heart, lungs, liver, kidneys, spleen, gonads, stomach, small intestine, cecum, colon, subcutaneous fat, epididymal fat, mesenteric fat, and epigonadal fat were extracted, weighed (±1 mg), and were then dried in an oven at 60 °C until a constant weight was achieved.

Statistical analysis

Results are expressed as the mean ± SEM. Data analysis was performed with SPSS software (SPSS, Chicago, IL, USA). Data on body mass over time were analyzed using an analysis of variance (ANOVA) with repeated measures followed by the Bonferroni adjustment. Data on food intake over time were analyzed using an analysis of covariance (ANCOVA) with repeated measures, using body mass as the covariate. Serum hormone levels, iBAT protein content, and hypothalamic mRNA expression were analyzed using a two-way ANOVA (treatment and gender), and fat and organ masses were analyzed using a two-way ANCOVA with body mass as the covariate when the data were normally distributed. When the data were not normally distributed, the data were log transformed or arcsine square root transformed to achieve normality of distribution before they were analyzed using two-way ANOVA or ANCOVA. Pearson correlation analyses were used to detect possible associations between serum leptin levels, body mass and food intake. A value of $P<0.05$ was considered significant.

Results

Nest temperature

We recorded the nest temperature with an iButton during lactation and found that there was a significant difference in nest temperatures

Table 1
The effects of pre- and post-weaning cold exposure on the dry organ masses but iBAT (wet mass) in adult male and female Brandt's voles.

Parameters (g)	WW	WC	CC	CW	F	P
Male	($n=10$)	($n=10$)	($n=10$)	($n=11$)		
iBAT (wet)	0.173 ± 0.016^{ab}	0.225 ± 0.011^{ab}	0.248 ± 0.017^{a}	0.221 ± 0.018^{b}	4.163	<0.05
Heart	0.047 ± 0.003^{bc}	0.062 ± 0.001^{a}	0.054 ± 0.004^{ab}	0.049 ± 0.003^{c}	14.991	<0.001
Lungs	0.071 ± 0.006	0.077 ± 0.009	0.056 ± 0.004	0.078 ± 0.016	0.475	>0.05
Liver	0.528 ± 0.061	0.618 ± 0.057	0.585 ± 0.071	0.631 ± 0.087	0.727	>0.05
Kidney	0.120 ± 0.010^{b}	0.144 ± 0.007^{a}	0.139 ± 0.007^{a}	0.110 ± 0.007^{b}	54.384	<0.001
Spleen	0.007 ± 0.001^{b}	0.007 ± 0.001^{ab}	0.007 ± 0.000^{a}	0.008 ± 0.001^{a}	4.613	<0.01
Testis	0.129 ± 0.007	0.105 ± 0.009	0.112 ± 0.013	0.114 ± 0.010	0.719	>0.05
Epididymis	0.034 ± 0.003	0.024 ± 0.004	0.021 ± 0.003	0.021 ± 0.002	0.815	>0.05
Seminal vesicle	0.110 ± 0.014^{a}	0.055 ± 0.009^{c}	0.060 ± 0.012^{bc}	0.104 ± 0.013^{ab}	6.719	<0.001
Stomach	0.057 ± 0.002^{bc}	0.069 ± 0.001^{a}	0.065 ± 0.003^{ab}	0.060 ± 0.003^{c}	7.707	<0.001
Small intestine	0.090 ± 0.010	0.125 ± 0.018	0.103 ± 0.011	0.095 ± 0.012	2.079	>0.05
Cecum	0.069 ± 0.004	0.084 ± 0.004	0.087 ± 0.014	0.076 ± 0.007	2.061	>0.05
Colon	0.059 ± 0.003^{ab}	0.075 ± 0.004^{a}	0.074 ± 0.003^{a}	0.058 ± 0.004^{b}	9.113	<0.001
Female	($n=13$)	($n=14$)	($n=15$)	($n=12$)		
iBAT (wet)	0.225 ± 0.034^{a}	0.121 ± 0.006^{b}	0.235 ± 0.019^{a}	0.244 ± 0.037^{a}	9.750	<0.001
Heart	0.042 ± 0.003^{b}	0.055 ± 0.001^{a}	0.056 ± 0.002^{a}	0.042 ± 0.003^{b}	30.664	<0.001
Lungs	0.061 ± 0.006^{ab}	0.074 ± 0.008^{a}	0.062 ± 0.004^{ab}	0.049 ± 0.004^{b}	3.051	<0.05
Liver	0.525 ± 0.040^{ab}	0.564 ± 0.054^{a}	0.510 ± 0.024^{ab}	0.396 ± 0.031^{b}	3.615	<0.05
Kidney	0.101 ± 0.005^{b}	0.123 ± 0.004^{a}	0.131 ± 0.004^{a}	0.090 ± 0.005^{b}	41.417	<0.001
Spleen	0.008 ± 0.001	0.007 ± 0.000	0.007 ± 0.001	0.007 ± 0.001	0.270	>0.05
Uterus	0.031 ± 0.009	0.030 ± 0.009	0.026 ± 0.002	0.024 ± 0.002	0.719	>0.05
Stomach	0.058 ± 0.003^{bc}	0.063 ± 0.002^{ab}	0.065 ± 0.003^{a}	0.055 ± 0.003^{c}	6.678	<0.001
Small intestine	0.098 ± 0.012^{ab}	0.123 ± 0.014^{a}	0.107 ± 0.010^{ab}	0.072 ± 0.008^{b}	3.132	<0.05
Cecum	0.082 ± 0.008	0.098 ± 0.015	0.079 ± 0.004	0.065 ± 0.005	2.148	>0.05
Colon	0.067 ± 0.003^{ab}	0.076 ± 0.004^{a}	0.073 ± 0.003^{ab}	0.062 ± 0.004^{b}	4.467	<0.01

Data are means ± SEM. Values for a specific parameter that share different superscripts (a, b or c) within rows are significantly different at $P<0.05$, determined by ANCOVA with body mass as a covariate and Bonferroni adjustment.

between the warm and cold environments (32.2 ± 0.6 °C vs. 28.1 ± 0.4 °C; $t = 5.451$, $df = 10$, $P < 0.001$).

Body mass

Although there was no difference in body mass between the warm and cold groups at birth ($t = 0.154$, $df = 39$, $P > 0.05$), offspring reared in the cold environment showed significantly slower growth rates than those in the warm environment during late lactation ($t = 3.337$, $df = 39$, $P < 0.01$; Fig. 2A).

From weaning until week 5, offspring exposed to the pre-weaning cold environment (CW, CC) had lower body masses when compared with animals exposed to the warm pre-weaning environment (WW, WC) (Week 3, $F_{3,\ 82} = 35.507$; $P < 0.001$; Week 4, $F_{3,\ 82} = 14.736$; $P < 0.001$; Week 5, $F_{3,\ 82} = 5.597$; $P < 0.01$; Figs. 2B, C). During weeks 4 to 13, male voles had higher body masses compared to female voles ($P < 0.001$). For the male voles, body mass values in CW were lower than those seen in other groups at weeks 3 ($F_{3,\ 30} = 36.264$; $P < 0.001$), 4 ($F_{3,\ 30} = 14.159$; $P < 0.001$), 5 ($F_{3,\ 30} = 6.043$; $P < 0.01$) and 6 ($F_{3,\ 30} = 3.720$; $P < 0.05$); but during weeks 9 to 13, body mass values in the CC group were lower than those seen in the CW group ($P < 0.05$; Fig. 2B). Interestingly, the case is not the same for the female voles. During weeks 5 to 13 for the female voles, there was no difference in body mass between treatments ($P > 0.05$; Fig. 2C). All voles had increasing body masses until 11 weeks of age ($P < 0.05$) and had stable body masses from 11 to 13 weeks of age (repeated measures ANOVA, $P > 0.05$).

Food intake

From weaning until week 13, voles exposed to post-weaning cold (WC and CC) had increased food intake, averaging 5.3 g/day in males and 4.7 g/day in females, when compared with voles exposed to the warm post-weaning environment (WW and CW) ($P < 0.001$; Figs. 2D, E). Food intake was not affected by gender at any age in this experiment ($P > 0.05$), but affected by the interaction between treatment and gender during weeks 8 ($F_{3,\ 87} = 5.878$, $P < 0.001$), and 10 ($F_{3,\ 87} = 4.955$, $P < 0.01$). During the course of the experiment, food intake first increased and then remained stable from weeks 8 to 13 (repeated measures ANCOVA, $P < 0.05$).

Biomarkers relative to thermogenesis in iBAT

At weaning (Figs. 3A–C), there were no differences in UCP1, COX4, and SIRT1 protein contents in iBAT between the warm- and cold-

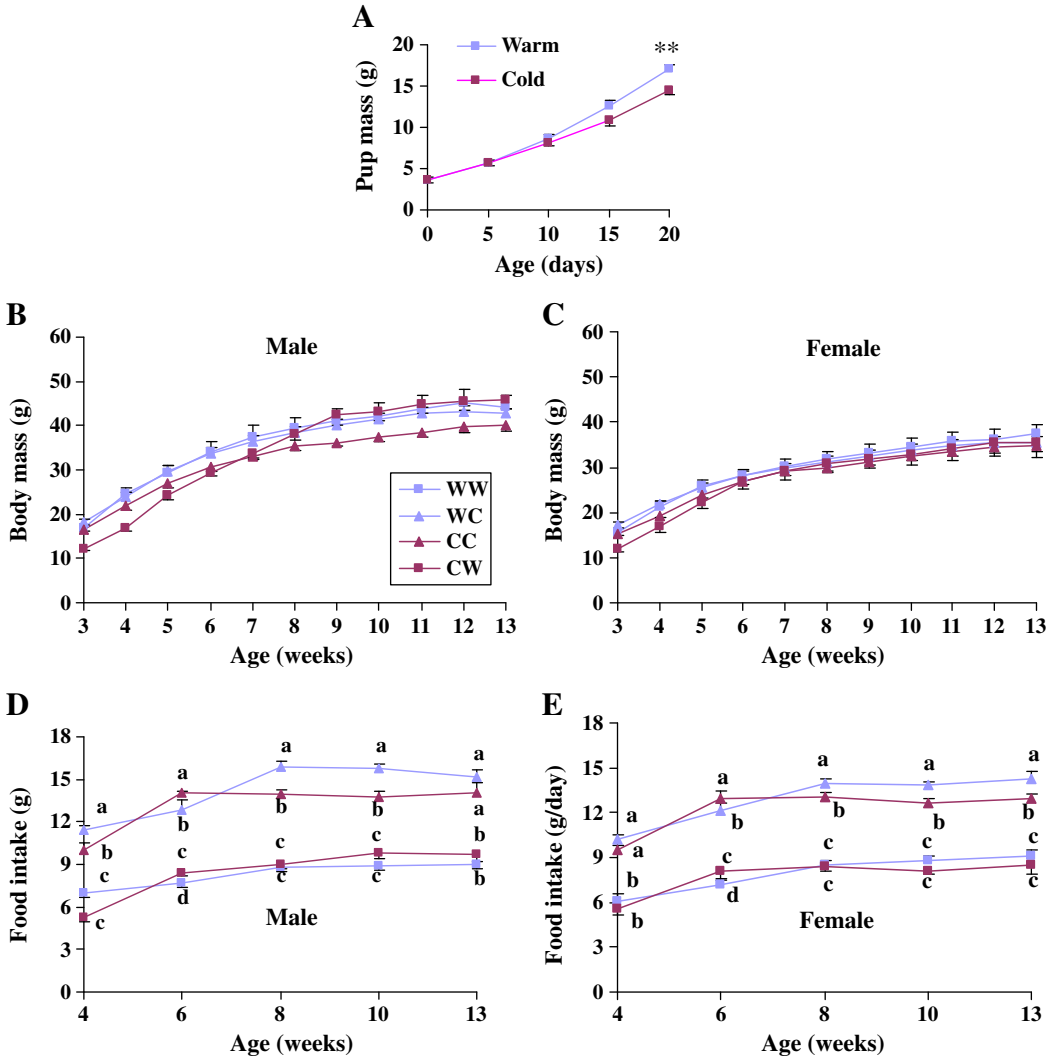

Fig. 2. Body mass (A–C)and food intake (D, E). Values are expressed as the mean ± SEM. **$P < 0.01$. Group differences are expressed as $P < 0.05$, and bars with different letters differ significantly from each other among groups.

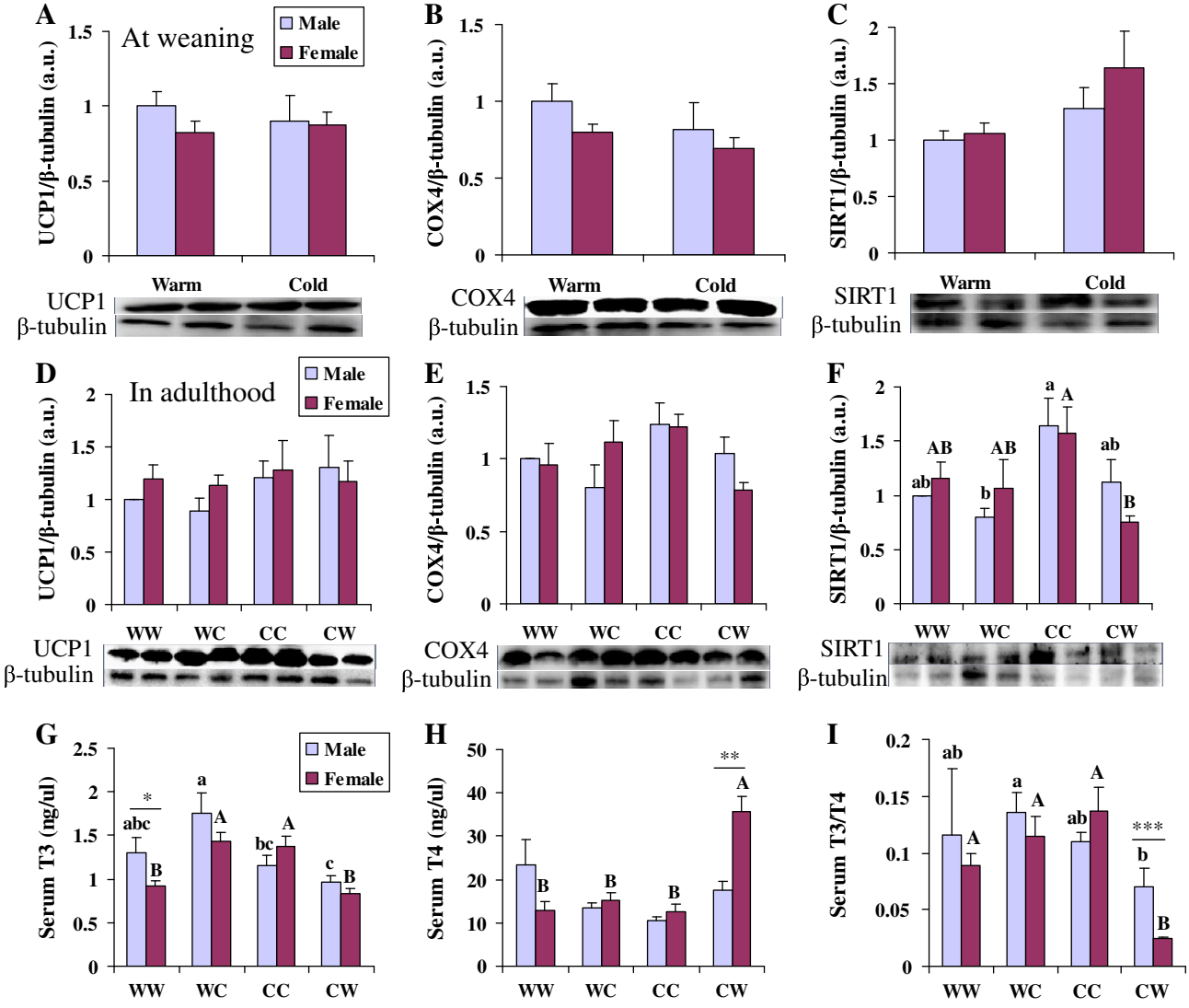

Fig. 3. UCP1, COX4 and SIRT1 contents in iBAT at weaning (A–C) and in adulthood (D–F), serum T3 (G), T4 (H), and T3/T4 (I) in male and female voles exposed to pre- and post-weaning warm or cold environments. Values are expressed as the mean ± SEM. Group differences are expressed as $P<0.05$, and bars with different letters differ significantly from each other among groups of the same gender. *$P<0.05$; **$P<0.01$; ***$P<0.001$. UCP1, uncoupling protein 1; COX4, cytochrome c oxidase 4; a.u., arbitrary units.

exposed offspring or between the male and female offspring ($P>0.05$).

In adulthood, UCP1 content was still not affected by treatment ($F_{3,\ 56}=0.743$, $P>0.05$; Fig. 3D); but COX4 ($F_{3,\ 56}=2.791$, $P<0.05$; Fig. 3E) and SIRT1 contents ($F_{3,\ 56}=2.791$, $P<0.05$; Fig. 3F) were affected by treatment. All these values were not affected by gender, or by the interaction between treatment and gender ($P>0.05$).

Serum T3, T4, and leptin levels

Serum T3 ($F_{3,\ 77}=9.876$, $P<0.001$; Fig. 3G), T4 levels ($F_{3,\ 77}=9.285$, $P<0.001$; Fig. 3H) and the ratio of T3/T4 ($F_{3,\ 77}=15.939$, $P<0.001$; Fig. 3I) in adulthood were significantly affected by the treatment. There were no differences between male and female voles in regards to T3 levels ($F_{1,\ 77}=2.852$, $P>0.05$), T4 levels ($F_{1,\ 77}=1.025$, $P>0.05$), or T3/T4 ($F_{1,\ 77}=3.193$, $P>0.05$). T4 ($F_{3,\ 77}=5.897$, $P<0.001$) and the ratio of T3/T4 ($F_{3,\ 77}=3.924$, $P<0.05$) were affected by the interaction between treatment and gender.

Serum leptin values were not significantly different between pre-weaning, cold-exposed offspring (20% lower) and pre-weaning, warm-exposed offspring at weaning ($F_{1,\ 27}=1.614$, $P>0.05$; Fig. 4A). Leptin values were also not different between genders at weaning ($F_{1,\ 27}=0.420$, $P>0.05$; Fig. 4A).

Leptin levels in the adults were increased in females than in males ($F_{1,\ 88}=4.882$, $P<0.05$; Fig. 4B). But the adult leptin levels were not affected by treatment ($F_{3,\ 88}=2.359$, $P=0.077$) or by the interaction between treatment and gender ($F_{3,\ 88}=0.494$, $P>0.05$). Serum leptin was positively correlated with body mass ($r=0.318$, $P<0.01$; Fig. 4D). When the effect of body mass was removed, the leptin values were even higher in female voles than in males ($F_{1,\ 87}=22.747$, $P<0.001$). For males, the offspring in WC had lower leptin levels in compared with those in WW and CW ($F_{3,\ 50}=3.513$, $P<0.05$). In contrast, there was no difference among different treatments for females ($F_{3,\ 50}=0.410$, $P>0.05$). Low leptin levels in WC (Fig. 4B) were associated with higher food intake (Fig. 4C), although no significant correlation was found between leptin and food intake among all the groups ($r=0.035$, $P>0.05$; Fig. 4E).

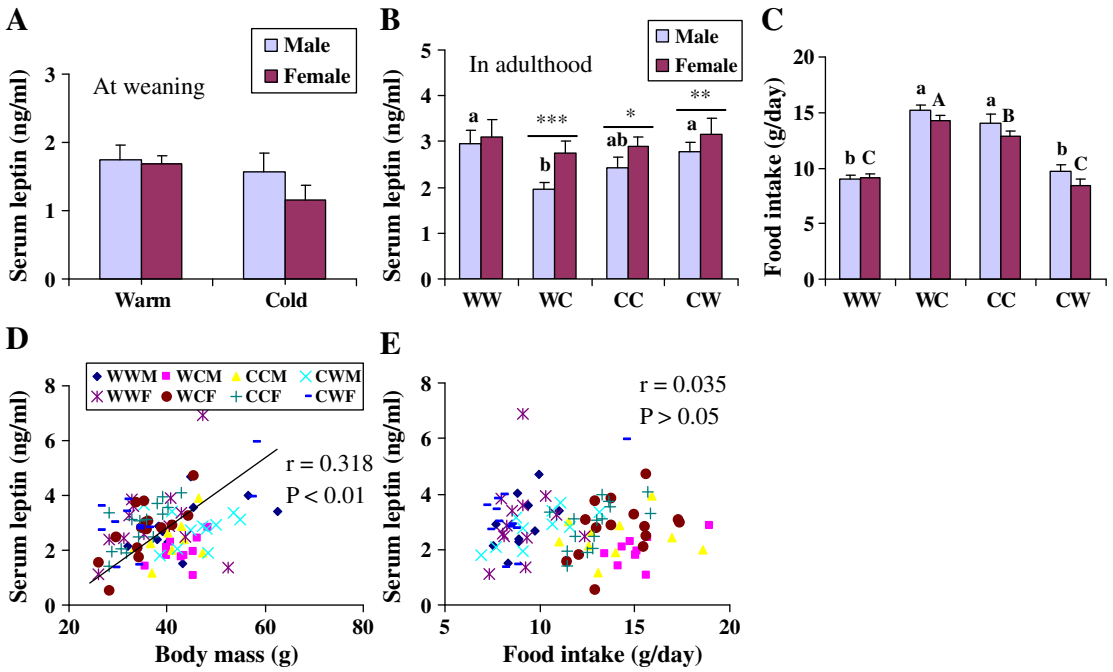

Fig. 4. Serum leptin levels at weaning (A) and in adulthood (B) in male and female voles exposed to pre- and post-weaning warm or cold environments. Low leptin levels in post-weaning, cold-exposed, adult voles (B) are associated with higher food intake (C). The adult serum leptin levels are positively correlated with body mass (D), but not with food intake (E). Values are expressed as the mean ± SEM. Group differences are expressed as $P<0.05$, and bars with different letters differ significantly from each other among groups of the same gender. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Hypothalamic neuropeptide mRNA expression

Pre-weaning cold exposure significantly increased AgRP ($F_{1,\ 20}=9.120$, $P<0.01$; Fig. 5d), but decreased POMC mRNA expression ($F_{1,\ 20}=4.467$, $P<0.05$; Fig. 5e) in the hypothalamus of weaning offspring. There were no differences in hypothalamic OB-Rb ($F_{1,\ 20}=0.023$, $P>0.05$), SOCS3 ($F_{1,\ 20}=0.279$, $P>0.05$), NPY ($F_{1,\ 20}=2.204$, $P>0.05$), or CART ($F_{1,\ 20}=1.691$, $P>0.05$) mRNA expression between pre-weaning warm or cold environment groups (Figs. 5a–c, f). There were also no differences in OB-Rb, SOCS3, NPY, AgRP, POMC or CART mRNA expression between male and female offspring at weaning ($P>0.05$; Figs. 5a–f).

Interestingly, the differences in neuropeptide expression induced by pre-weaning cold exposure were not found in adult voles (Figs. 5A–F). OB-Rb, SOCS3 and neuropeptide expression were not affected by treatment, gender, or the interaction between these two variables ($P>0.05$; Figs. 5A, C–F).

Body composition

There were only gender differences in the mass values for iBAT ($F_{1,\ 80}=3.081$, $P<0.01$), spleen ($F_{1,\ 80}=3.290$, $P<0.01$), kidney ($F_{1,\ 80}=2.695$, $P<0.05$) and colon ($F_{1,\ 80}=3.538$, $P<0.01$; Table 1). The mass values of iBAT ($F_{3,\ 80}=6.529$, $P<0.001$), heart ($F_{3,\ 80}=35.309$, $P<0.001$), kidney ($F_{3,\ 80}=68.135$, $P<0.001$), stomach ($F_{3,\ 80}=14.444$, $P<0.001$), small intestine ($F_{3,\ 80}=4.689$, $P<0.01$) and colon ($F_{3,\ 80}=14.046$, $P<0.001$) were affected by treatments. Only iBAT mass was affected by the interaction between treatment and gender ($F_{3,\ 80}=3.081$, $P<0.05$).

The female voles had more retroperitoneal ($F_{1,\ 80}=4.310$, $P<0.001$), mesenteric ($F_{1,\ 80}=7.059$, $P<0.001$) and perinephric ($F_{1,\ 74}=6.502$, $P<0.001$) fat pad mass, but they had less epigonadal fat ($F_{1,\ 80}=3.312$, $P<0.01$) compared with male voles (Table 2). There was no difference in carcass mass between the male and female voles ($F_{1,\ 80}=1.484$, $P>0.05$). Post-weaning cold exposure reduced the mesenteric fat ($F_{3,\ 80}=3.144$, $P<0.05$) and epigonadal fat mass ($F_{3,\ 80}=13.560$, $P<0.001$), and it also decreased the carcass mass ($F_{3,\ 80}=16.121$, $P<0.001$). Only epigonadal fat mass was affected by the interaction between treatment and gender ($F_{3,\ 80}=6.878$, $P<0.001$).

Discussion

This study demonstrates that pre-weaning cold exposure resulted in lower body mass during weaning in voles, and this effect was followed by an incomplete catch-up growth period, especially for the male offspring that were transferred back to a warm environment. In contrast to rats, the catch-up growth period in voles was short lasting (Table 3). Furthermore, this study indicates that the increased hypothalamic AgRP and decreased POMC mRNA expression during weaning induced by pre-weaning cold exposure may contribute to catch-up growth shortly after weaning.

Body mass, food intake and thermogenesis induced by pre- and post-weaning cold exposure

For endothermic small mammals, increasing thermogenesis to maintain stable body temperature is the primary strategy for survival in cold environments. Cold exposure can induce activation of the sympathetic nervous system innervating BAT (Morrison et al., 2000; Young et al., 2002) and peripheral conversion of T4 into T3 (Laurberg et al., 2005). Both factors stimulate the protein expression of several markers of increased thermogenesis (such as UCP1) and mitochondrial oxidative capacities, such as COX, in BAT in rats (Watanabe et al., 2008). In these studies, cold-reared rats (18 °C) showed elevated sympathetic input to BAT and increased gene expression for adrenergic receptors and UCP1 at 21 days. These effects persisted even 2 months after removal from the cold environment (Morrison et al., 2000; Young et al., 2002). We did not find any significant effects of pre-weaning cold exposure on UCP1 or COX4 content in iBAT during weaning in the study voles. It suggests that the vole pups during lactation may rely on huddling

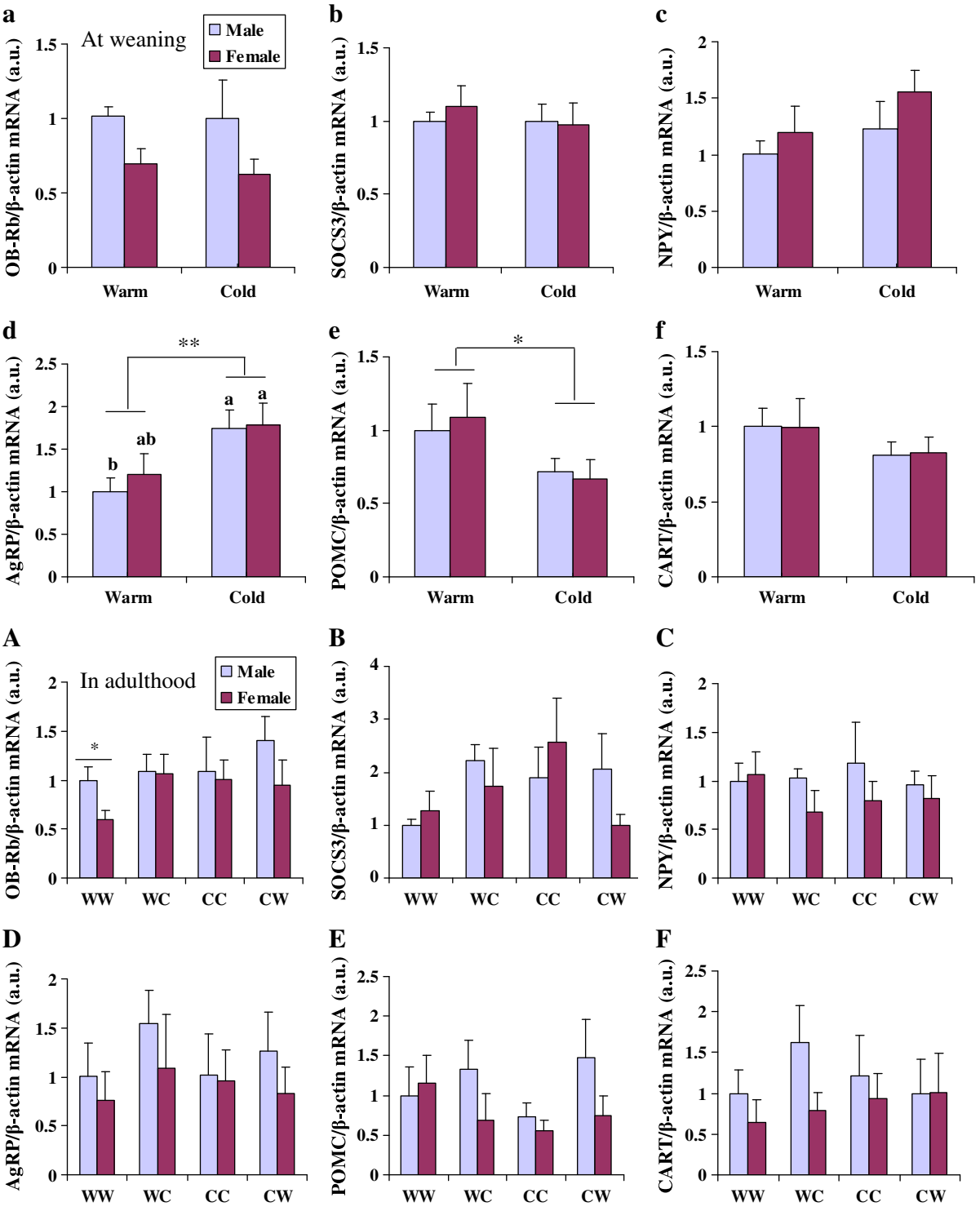

Fig. 5. Hypothalamic OB-Rb (a, A), SOCS3 (b, B), NPY (c, C), AgRP (d, D), POMC (e, E) and CART (f, F) mRNA expression at weaning (a–f) and in adulthood (A–F) in male and female voles exposed to pre- and post-weaning warm or cold environments. Values are expressed as the mean ± SEM. Group differences are expressed as $P<0.05$, and bars with different letters differ significantly from each other among groups of the same gender. *$P<0.05$. Ob-Rb, leptin receptor; SOCS3, suppressor of cytokine signaling 3; NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, proopiomelanocortin; CART, cocaine- and amphetamine-regulated transcript.

and the mother to keep stable body temperature. SIRT1 is an evolutionarily conserved NAD^+ dependent deacetylase that participates in the regulation of metabolism (Vaziri et al., 2001). We found that SIRT1 content in iBAT from adult voles exposed to pre- and post-weaning cold environment was increased. In our study, serum T3 levels and the ratio of T3 to T4 in adult voles were decreased in pre-weaning cold-exposed offspring, but these same values were increased by post-weaning cold exposure. This finding implies that pre-weaning cold exposure may delay thyroid development, whereas post-weaning cold exposure may stimulate thyroid function and increase thermogenesis.

Cold exposure during lactation decreased the body mass of offspring during weaning in rats (Gerrish et al., 1998), mice (Johnson and Speakman, 2001) and voles. The cold-reared voles demonstrated

Table 2
The effects of pre- and post-weaning cold exposure on the masses of fat pads and carcass in male and female Brandt's voles.

Parameters (g)	WW	WC	CC	CW	F	P
Male	($n=10$)	($n=10$)	($n=10$)	($n=11$)		
Retroperitoneal fat	0.330 ± 0.092	0.239 ± 0.064	0.167 ± 0.040	0.290 ± 0.043	0.593	>0.05
Mesenteric fat	0.216 ± 0.024^{b}	0.273 ± 0.019^{a}	0.250 ± 0.011^{b}	0.249 ± 0.018^{b}	4.100	<0.05
Perinephric fat	0.108 ± 0.029	0.096 ± 0.008	0.108 ± 0.008	0.133 ± 0.018	1.305	>0.05
Epigonadal fat	0.615 ± 0.063^{a}	0.362 ± 0.055^{c}	0.472 ± 0.064^{b}	0.559 ± 0.034^{b}	8.603	<0.001
Carcass mass	31.67 ± 2.49^{a}	27.01 ± 0.89^{b}	25.51 ± 1.14^{b}	31.01 ± 1.33^{a}	7.416	<0.001
Female	($n=13$)	($n=14$)	($n=15$)	($n=12$)		
Retroperitoneal fat	0.365 ± 0.095^{ab}	0.184 ± 0.051^{b}	0.220 ± 0.048^{ab}	0.373 ± 0.128^{a}	3.551	<0.05
Mesenteric fat	0.255 ± 0.035	0.256 ± 0.023	0.262 ± 0.022	0.233 ± 0.038	0.411	>0.05
Perinephric fat	0.175 ± 0.035	0.111 ± 0.017	0.125 ± 0.014	0.124 ± 0.036	1.697	>0.05
Epigonadal fat	0.206 ± 0.067^{ab}	0.145 ± 0.036^{bc}	0.142 ± 0.025^{c}	0.306 ± 0.102^{a}	8.871	<0.001
Carcass mass	25.55 ± 1.86^{a}	22.19 ± 1.17^{b}	21.74 ± 0.91^{b}	24.18 ± 2.52^{a}	13.221	<0.001

Data are means ± SEM. Values for a specific parameter that share different superscripts (a, b or c) within rows are significantly different at $P<0.05$, determined by ANCOVA with body mass as a covariate and Bonferroni adjustment.

hyperphagia and catch-up growth after weaning. In contrast to rats reared at 18 °C (Young and Shimano, 1998), the weight enhancing effect of cold exposure in voles was short lasting, and the voles did not develop an overweight phenotype in adulthood. Furthermore, pre-weaning, cold-reared offspring ate less during development than those reared in a warm environment, and the magnitude of this decrease was much larger in males than in females. As a result, the male voles exposed to a constantly cold environment after birth developed lower body masses than other male voles, but this trend did not occur in the females. Hormonal differences between the male and female may result in different adaptive response to cold. For example, a recent study indicated that estrogen may modulate central and peripheral thermogenesis to help the female to resist cold (Uchida et al., 2010). The diverse results in rats and voles may be derived from the differing extent of cold exposure. Fuller (1965) demonstrated that the effect of environmental temperature was biphasic, and the maximal weight gain seen in pigs occurred in those reared at 20 °C and 25 °C, whereas the lowest gain was seen at 10 °C and 30 °C. Alternatively, it is possible that Brandt's voles, like obesity-resistant S5B/Pl rats, differ from obesity-prone Sprague–Dawley or Osborne–Mendel rats (White et al., 2005) and do not gain extra weight when reared in cool environmental temperatures when compared to warm-reared animals.

Hypothalamic gene expression induced by pre- and post-weaning cold exposure

Adipose-derived leptin plays an important role in regulating food intake, energy expenditure and energy homeostasis (Friedman and Halaas, 1998; Schwartz et al., 2000). In this study, serum leptin levels in adult voles were not affected by pre-weaning cold exposure, and this result differs from rats that showed higher leptin levels with early cold exposure (White et al., 2005). However, postnatal cold exposure significantly decreased serum leptin in male voles, which is consistent with previous studies in cold-exposed adult rats (Bing et al., 1998; Puerta et al., 2002) and voles (Zhang and Wang, 2006, 2007; Tang et al., 2009). Decreased leptin levels act as a starvation signal to stimulate the appetite to compensate for high energy expenditure in cold environments.

In weaning and adult voles, we also analyzed the mRNA expression of hypothalamic leptin signaling and neuropeptides related to orexigenic and anorexigenic pathways induced by pre- and post-weaning cold exposure. Interestingly, pre-weaning cold-exposed voles showed higher AgRP and lower POMC expression at weaning, which suggests that the voles were in a state of negative energy balance. Increased AgRP expression was also noted in adult cold-exposed voles (Tang et al., 2009). This alteration in hypothalamic neuropeptide expression may contribute to hyperphagia and to catch-up growth after weaning. However, pre-weaning cold exposure did not have a long-term effect on adult hypothalamic neuropeptides, which may protect the voles from obesity. These data suggest that central alterations in neuropeptide expression may be involved in energy homeostasis regulation induced by interactions between pre- and post-weaning cold exposure.

In total, this study demonstrates that certain wild rodents have evolved special physiological and behavioral adaptation strategies in response to the complicated environments they encounter. These strategies can be different from the adaptive and developmental phenotypes of laboratory animal models such as rats. There are many factors impacting the survival and fitness of an animal, but these results may partially explain why there are rarely obese voles in wild populations regardless of when they are bred. Decreased serum leptin levels and altered expression of hypothalamic AgRP and POMC during weaning may contribute to increased energy intake in order to compensate for a low energy status. However, these alterations were short lasting in this study, which may make these voles resistant to developing obesity. Future studies investigating the mechanisms responsible for such adaptations in voles would be beneficial.

Table 3
Responses of body mass, food intake, fat pads, serum leptin, and hypothalamic neuropeptides to pre- and post-weaning cold exposure in rats and Brandt's voles.

	Rats		Brandt's voles	
	Pre-cold	Post-cold	Pre-cold	Post-cold
At weaning				
Body mass	↓		↓	
Neuropeptides			AgRP↑, POMC↓	
In adult				
Body mass	↑		–	–
Food intake	↑		First↑, then↓	↑
Fat pads	↑		–	↓
Leptin	↑		–	↓
Neuropeptides			–	–

The data for rats were adopted by literatures (Gerrish et al., 1998; Young and Shimano, 1998; Morrison et al., 2000; White et al., 2005). ↑ Increase; ↓ decrease; – no difference.

Abbreviations

AgRP	agouti-related protein
ARC	the arcuate nucleus
CART	cocaine- and amphetamine-regulated transcript
CC	pre-weaning cold and post-weaning cold
COX4	cytochrome c oxidase 4
CW	pre-weaning cold and post-weaning warm
iBAT	interscapular brown adipose tissue
NPY	Neuropeptide Y
OB-Rb	leptin receptor
POMC	proopiomelanocortin

SIRT1	Sirtuin 1
SOCS3	suppressor of cytokine signaling 3
T3	3,5,3′-triiodothyronine
T4	thyroxine
UCP1	uncoupling protein 1
WC	pre-weaning warm and post-weaning cold
WW	pre-weaning warm and post-weaning warm

Disclosure statement

No conflicts of interest, financial or otherwise, are declared by the authors.

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