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Limits to sustained energy intake. XI. A test of the heat dissipation limitation hypothesis in lactating Brandt's voles (*Lasiopodomys brandtii*)

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SUMMARY

The maximum rate of sustained energy intake (SusEI) may limit reproductive effort, thermoregulatory capability and other aspects of an animal's energy expenditure. Consequently, factors that limit SusEI are of interest. The 'heat dissipation limitation hypothesis' suggests that maximum SusEI during lactation is limited by the capacity to dissipate body heat generated as a by-product of processing food and producing milk. In the present study, we tested the heat dissipation limitation hypothesis in lactating Brandt's voles (*Lasiopodomys brandtii*). Female voles were mated and pregnant at 21(±1)°C. A random sample of animals was transferred into a hot room 30(±1)°C on the day of parturition. The energy intake of lactating voles at 30°C was always lower than that at 21°C. At peak lactation food intake was 3.3 g day^{-1} lower at 30°C than at 21°C. There was no significant difference in digestibility. With similar mean litter sizes (7.26±0.46 pups at 21°C and 7.78±0.39 pups at 30°C at the beginning of parturition, 6.83±0.51 pups at 21°C and 7.73±0.50 pups at 30°C at weaning), the milk energy output of mothers, evaluated from the difference between metabolizable energy intake and daily energy expenditure measured by doubly labelled water, at 30°C was 23.3 kJ day^{-1} lower than that at 21°C on days 14–16 of lactation. As for reproductive performance, there was a difference in the response to the higher temperature between mothers raising large and those raising small litters. For small litters (<7) there was no significant change in litter mass, but for large litters (≥7) there was a significant decrease at the higher temperature. On average, in larger litters the pups were 15.5 g heavier on day 12 of lactation when raised at 21°C. Our data from Brandt's voles support the suggestion that SusEI at peak lactation is limited by heat dissipation capacity, particularly for those voles raising large litters. In smaller litters the peripheral limitation hypothesis may be more relevant. The importance of heat dissipation limits in species raising exclusively small litters needs to be investigated.

Key words: Brandt's voles (*Lasiopodomys brandtii*), lactation, heat dissipation limitation hypothesis, food intake, digestive efficiency, metabolizable energy intake, daily energy expenditure, milk energy output, pup energy content, doubly labelled water.

INTRODUCTION

The sustainable maximum rate of energy intake (SusEI) is defined as the maximum rate of energy intake that animals can sustain over sufficiently long periods (days to weeks) to enable energy demands to be fuelled by food intake rather than depletion of energy reserves (Hammond and Diamond, 1997; Speakman and Król, 2005a). SusEI may impose limits on the reproductive effort of animals and their thermoregulatory capacities that together may define global distribution limits (Drent and Daan, 1980; Kirkwood, 1983; Peterson et al., 1990; Hammond and Diamond, 1992; Thompson, 1992; Hammond and Diamond, 1994; Root, 1988; Speakman, 2000; Humphries et al., 2002; Speakman and Król, 2005a). Late lactation is the most energetically demanding time for female mammals (Millar, 1977; Speakman and McQueenie, 1996). Consequently, limits on SusEI are particularly important during lactation, not only because these may determine the total investment that mammals can make in their offspring and may define maximum litter sizes (Johnson et al., 2001a) but also because the number and quality of offspring depends on milk production and quality (Rogowitz and McClure, 1995; Rogowitz, 1996; Rogowitz, 1998).

Following the pioneering study by Drent and Daan (Drent and Daan, 1980), several hypotheses were proposed concerning the proximate physiological causes of metabolic ceilings of small mammals. One hypothesis, called the 'central limitation hypothesis', was that the limitation is imposed by the capacity of the central 'energy-supplying' machinery (Kirkwood, 1983; Hammond and Diamond, 1992; Hammond and Diamond, 1994; Speakman, 2008). In this case, maximal sustained levels of energy expenditure for lactation, exercise and heat production in the same individual would all be equal and would reflect the ceiling imposed by intestinal absorption (Peterson et al., 1990). This idea is supported by some studies such as work in guinea pigs (*Cavia porcellus*) (Künkele, 2000; Laurien-Kehnen and Trillmich, 2003) and mice (Hammond and Diamond, 1992; Hammond and Diamond, 1994; Johnson et al., 2001a; Johnson et al., 2001b) that showed maternal food intake did not increase compared with that in unmanipulated mothers. However, other data are inconsistent with the hypothesis. Lactating mice (Hammond and Diamond, 1994; Johnson and Speakman, 2001), hispid cotton rats (*Sigmodon hispidus*) (Rogowitz, 1998) and Brandt's voles (*Lasiopodomys brandtii*) (Zhang and Wang, 2007) all increase food intake beyond the apparent limit in late lactation at 21°C when simultaneously exposed to low ambient temperature. In the light of these data, it was suggested that the limit must occur peripherally where energy is expended such as the mammary glands during lactation (Hammond, 1996; Rogowitz, 1998; Speakman, 2008). The 'peripheral limitation hypothesis' suggests that the mammary gland

at peak lactation should work at maximal capacity regardless of ambient temperature or other manipulations. To test this hypothesis, it is necessary to measure the milk energy output (MEO). Experiments including measures of MEO of mice lactating at different ambient temperatures, however, showed that MEO was not constant but rather increased as temperature declined (Johnson et al., 2001a; Johnson et al., 2001b; Johnson and Speakman, 2001; Król et al., 2003; Król and Speakman, 2003a; Król and Speakman, 2003b).

An alternative hypothesis to explain the proximate physiological causes of SusEI, called the 'heat dissipation limitation hypothesis', was subsequently proposed (Król and Speakman, 2003a; Król and Speakman, 2003b). The hypothesis is that the level of food intake at peak lactation is set by a central process independent of the capacity of the alimentary tract. This central limitation on food intake is the maximal capacity of the animal to dissipate body heat generated as a by-product of processing food and producing milk (Król and Speakman, 2003a; Król and Speakman, 2003b). Accordingly, lactating mice exposed to the cold can increase their SusEI because their capacity to dissipate heat is increased. This idea uniquely explains the simultaneous increase in milk production and the reproduction performance in the cold. Similarly, when animals were put into hot conditions, their capacity to dissipate heat was reduced, resulting in reduced food intake and milk production, which led to smaller pups being weaned (Król and Speakman, 2003a; Król and Speakman, 2003b; Speakman and Król, 2005a; Król et al., 2007; Speakman, 2008). Lactating MF1 mice at 21°C that were dorsally shaved to elevate their capacity to dissipate body heat also had increased food intake, increased MEO and a larger litter mass compared with unshaved mice, consistent with the heat dissipation limitation hypothesis (Król et al., 2007).

Different patterns of energy expenditure between species could be related to the life history strategy of each species (Koteja and Weiner, 1993; Koteja, 1995; Koteja, 1996a; Koteja, 1996b; Hammond and Diamond, 1997; Speakman, 2007). Accordingly, there is an implicit assumption that SusEI is adaptive (Bacigalupe and Bozinovic, 2002). To date, data supporting the heat dissipation limitation hypothesis have been generated predominantly in laboratory mice which raise rather large litters. The relevance of this hypothesis to wild mammals, for which litter sizes are generally much smaller, remains uncertain. Brandt's voles (*L. brandtii*) are a seasonal breeding small rodent that has been previously well studied (Li and Wang, 2005a; Zhao and Wang, 2006). They are non-hibernating herbivores that mainly inhabit the grasslands of Inner Mongolia, Mongolia and the Baikal region of Russia. Previous studies indicated that the limit of SusEI for Brandt's voles was not consistent with the central limitation hypothesis (Song and Wang, 2001; Liu et al., 2003; Zhang and Wang, 2007). However, the proximate physiological factors that impose limits on the energy budget of Brandt's voles during lactation remain unclear. To assess whether the limits to SusEI are imposed by the capacity to dissipate heat, we placed Brandt's voles at either 21°C or 30°C on the day of parturition. The lower critical temperature for this species is around 30°C (Song and Wang, 2001). In the wild, Brandt's voles live in burrows that can be up to 50 cm deep and therefore during the reproductive season they are probably normally well buffered from temperature extremes in either direction. In this manipulation we were not trying to mimic the free-living conditions encountered by wild voles but rather were attempting to experimentally manipulate them to establish the importance or not of heat dissipation constraints in a species raising small litters. It is well established that the capacity to dissipate heat depends on the conductivity of the insulating surface and the difference between body and ambient temperature (Król and Speakman, 2003a). The heat dissipation limitation hypothesis predicted that the food intake of lactating Brandt's voles would be reduced at 30°C compared with that at 21°C because of the reduced capacity to dissipate heat. Consequently, milk production and offspring growth are also predicted to be reduced. In contrast, the peripheral limitation hypothesis predicts reduced SusEI at 30°C due to lowered thermoregulation demands but milk production and offspring growth should be unchanged.

MATERIALS AND METHODS

Animals and experimental design

All animal procedures were licensed by the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences. Virgin female Brandt's voles (*Lasiopodomys brandtii* Radde 1861) were the offspring of our lab colony, which was trapped in the Inner Mongolian grasslands in 1999. Voles were weaned at 18–20 days of age and kept in single-sex groups of 3–4 animals in plastic cages (30 cm×15 cm×20 cm) that contained sawdust bedding, and exposed to a 16 h:8 h light/dark cycle (lights on 04:00 h) at an ambient temperature of 21±1°C. Food (commercial rabbit pellets, Beijing KeAo Feed Co., Beijing, China) and water were available *ad libitum*. Ninety virgin females, weighing 45–65 g and 100–150 days old, were individually housed for 1 month and acclimated to the experimental conditions for 1 week. After the acclimation period, half of the animals were randomly selected and were paired with males for 15 days. The males were then removed and used to mate the females in the other half of the group. Pregnant voles (N=46) were checked twice a day to determine the day of parturition (day 0 of lactation). On the day of parturition, half of the animals (N=23) were randomly selected and transferred into another room where the ambient temperature was 30±1°C. Following demonstration that the responses of the voles were significantly different between those raising large compared with small litters, we divided the animals into small or large litter size groups. If the litter size was less than seven [the mean litter size of Brandt's voles reported previously (Liu et al., 2003)], we regarded it as a small litter size. Voles raising ≥7 pups were considered to be raising large litters.

Body mass, food intake and reproductive performance

Body mass, food intake, litter mass (±0.1 g) and litter size were recorded between 15:00 and 17:00 h every day except the day of parturition. Food was given at the same time. The next day, food residues and faeces were collected together and oven-dried at 60°C to constant mass. Subsequently, food and faeces were separated by hand, and food intake was calculated from the difference between the food given and the food residue. Food samples were taken to determine the water content (8.6±0.4%, N=15). The energy density of food and faeces was determined by Parr1281 oxygen bomb calorimetry (Parr Instrument, Moline, IL, USA); gross energy intake (GEI, kJ day^{-1}), digestible energy intake (DEI, kJ day^{-1}) and apparent digestibility (%) were calculated as follows (Grodzinski and Wunder, 1975; Liu et al., 2003; Zhang and Wang, 2007):

$$\text{GEI} = \text{dry food intake} \times \text{food energy density},$$

$$\text{DEI} = \text{GEI} - \text{dry faeces mass} \times \text{faeces energy density},$$

$$\text{Digestibility} = \text{DEI} / \text{GEI} \times 100,$$

where dry food intake and dry faeces mass are in g day^{-1}, and energy density is in kJ g^{-1}. Measurements of metabolizable energy intake

(MEI) were estimated as the DEI assuming that urinary energy loss was 2% (Song and Wang, 2001; Liu et al., 2003; Zhang and Wang, 2007).

Daily energy expenditure

We measured daily energy expenditure (DEE) using the doubly labelled water (DLW) technique (Butler et al., 2004) on days 14–16 of lactation. This method has previously been validated by comparison with indirect calorimetry in a range of small mammals including rodents (Speakman and Król, 2005b) and provides an accurate measure of DEE over periods of several days. Day-to-day variability in estimated energy metabolism suggests that measurements spanning multiple 24 h periods may give a better estimate of average DEE (Speakman et al., 1994; Berteaux et al., 1996). Studies of lactating mammals suggest that the recycling of isotopes between a mother and her offspring is negligible (Scantlebury et al., 2000).

Lactating Brandt's voles were weighed (±0.1 g) and injected subcutaneously with approximately 0.31 g of water containing enriched ^{18}O (31.9 atom %) and ^{2}H (19.0 atom %). Syringes were weighed before and after administration (±0.0001 g) to calculate the mass of DLW injected. Blood samples were taken after 1 h of isotope equilibration to estimate initial isotope enrichments (Król and Speakman, 1999; Visser et al., 2000) and were also collected from unlabelled animals to estimate the background isotope enrichments [(Speakman and Racey, 1987) see their method C]. Blood samples were immediately heat sealed into 2×50 µl glass capillaries and stored at room temperature. A final blood sample was taken approximately 48 h later (Speakman and Racey, 1988) to estimate isotope elimination rates. Capillaries containing the blood samples were then vacuum distilled (Nagy, 1983) and water from the resulting distillate was used to produce CO_2 (Speakman et al., 1990) and H_2 (Speakman and Król, 2005b). The isotope ratios ^{18}O:^{16}O and ^{2}H:^{1}H were analysed using gas source isotope ratio mass spectrometry (ISOCHROMµGAS system and IsoPrime IRMS, Micromass, Manchester, UK). We ran three high-enrichment standards each day alongside the samples and corrected all the raw data to these standards (Meijer et al., 2000).

Initial isotope dilution spaces (moles) were calculated by the intercept method (Coward and Prentice, 1985), then converted to grams assuming a molecular mass of body water of 18.020 and expressed as a percentage of body mass before injection. Final dilution spaces were inferred from the final body mass, assuming the same percentage of body water as measured for the initial dilution spaces. The isotope elimination rate (k) was calculated following published methods (Lifson et al., 1955). Isotope enrichment was converted to DEE using a single pool model as recommended for animals under 10 kg (Speakman, 1993). We assumed a fixed evaporation of 25% of the water flux [see equation 7.17 of Speakman (Speakman, 1997)] which minimizes error in a range of conditions (Visser and Schekkerman, 1999; van Trigt et al., 2002; Speakman and Król, 2005b).

Energy equivalents for the rate of CO_2 production were calculated using a conversion factor of 24.026 J ml^{-1} CO_2, derived from the Weir equation (Weir, 1949) for a respiratory quotient of 0.85 (Speakman, 1997).

Milk energy output (MEO)

We used the DLW data to evaluate MEO, calculated from the difference between MEI and DEE (Król and Speakman, 2003b). MEI and DEE were measured simultaneously on days 14–16 of lactation. This timing was based on the fact that previous studies have indicated that Brandt's voles weaned their offspring completely at 18–21 days of lactation (Liu et al., 2003; Zhang and Wang, 2007; Zhang et al., 2008). In the present study, we found the pups began to eat food on day 12 of lactation. By partitioning total intake between the mother and her pups (see below), we discovered that maternal intake was already declining by days 14–16. However at this stage there was still a large difference in maternal energy intake between the two temperature treatment groups. We therefore assumed the difference in milk production we measured was reflective of a difference throughout the entire lactation period. To estimate the MEI of the mother it was necessary to separate the food eaten by the mother from that eaten by her offspring. This was done *via* the collection of faeces, which differ greatly in size between the mother and her offspring. We established a relationship between GEI and total faeces mass of the mother before the pups started to eat food, and used the measured maternal faeces production and this relationship to estimate the energy intake of the mother. The difference between this predicted intake and the total observed intake was inferred as the intake of the offspring. Animals appeared not to be affected by the DLW injections and bleeding, because their body mass, food intake and sucking behaviour did not change.

Pup energy content

On day 18 of lactation, two pups from each litter were selected randomly and killed to evaluate their energy density (Parr 1281 oxygen bomb calorimeter). They were oven-dried to a constant mass, then ground in a mill and well mixed. Pup body mass, pup dry mass and energy density of pups (dry mass), and gross energy content (kJ per pup) were calculated.

Body composition and organ mass

At day 18 of lactation, the mothers were killed by CO_2 overdose between 16:00 and 17:00 h. Voles were dissected into the following organs and tissues: heart, liver, spleen, lung, kidneys, stomach, small intestine, caecum, colon, mammary, ovary and uterus together as gonad, subcutaneous fat, epididymal fat, mesenteric fat, gonad fat. These were weighed to obtain the wet mass (±0.0001 g, digital balance; Sartorius, Göttingen, Germany). Individual organs and tissues were then oven-dried at 60°C to a constant mass, and weighed again to obtain the dry mass.

Statistics

Data are reported as means ± s.e.m. (N=sample size) and analysed using SPSS 13.0 software (SPSS 1988, Chicago, IL, USA). Maternal body mass during reproduction and food intake, litter mass and mean pup mass during lactation were analysed using two-way repeated measures ANOVA, with group (21°C *versus* 30°C) and day of lactation as factors. Bonferroni corrections were used to adjust the significance levels where appropriate. When the effect of experimental temperature group or the interaction 'group×day' was significant, one-way ANCOVA was used to determine differences between the groups within each day and P-values were corrected using the Bonferroni procedure. *A priori* the reason for this experiment was to establish the effects of heat dissipation in a species that raises smaller litters than the domesticated mice that have been the basis of previous tests. *A posteriori* we observed that the responses to temperature appeared to depend on litter size within the species. We examined the effect of litter size on peak lactation food intake of the mothers in the two experimental groups using ANCOVA with litter size as the covariate. This revealed a significant interaction effect between litter size and treatment group. To illustrate these effects we therefore divided the data into those raising

large (≥7) and small (<7) litters and performed analyses on these groups independently.

For single measurements such as pup dry mass and energy density of pups (dry mass), gross energy content and organ mass, we used one-way ANCOVA to determine differences (with Bonferroni correction). To remove the effect of body mass, the organs were adjusted by wet carcass mass. When the food intake and energy intake of mothers, and litter mass and pup mass were analysed, litter size was included as a factor. Correlations were described using Pearson correlation coefficients and *P*-values. Relationships between traits were calculated using least-squares linear regression analysis. $P<0.05$ was considered to be statistically significant. Bonferroni corrections were automatically included in the SPSS procedure.

RESULTS

Body mass and food intake

The initial body mass of female Brandt's voles before mating was 53.4±0.9 g and 52.9±1.0 g in the 21°C and 30°C groups, respectively. During the experiment, the body mass changed significantly from pregnancy to lactation (Fig. 1A, where day 0 is the day of parturition), but did not vary between the two temperature groups (repeated measures ANOVA; group, $F_{1,9}=0.48$, $P=0.506$; day, $F_{34,306}=141.5$, $P<0.001$; interaction group×day, $F_{34,06}=1.403$, $P=0.73$).

Maternal food intake of Brandt's voles increased significantly from day 1 to day 7 of lactation (repeated measures ANOVA, group, $F_{1,40}=21.441$, $P<0.001$; day, $F_{6,240}=42.652$, $P<0.001$; interaction group×day, $F_{6,240}=1.302$, $P=0.257$), from a mean of 11.16±0.69 g at 21°C and 7.87±0.61 g at 30°C on day 1 to 15.86±0.83 g and 11.03±0.58 g on day 7 (Fig. 1B). The next 5 days (that is, days 8–12 of lactation), maternal food intake at 30°C remained stable (repeated measures ANOVA, $F_{4,80}=2.002$, $P=0.102$); maternal food intake at 21°C remained stable on days 11–12 of lactation (repeated measures ANOVA, $F_{1,22}=0.069$, $P=0.795$). However, total food intake of mothers and pups of both groups increased significantly from day 13 to day 17 of lactation (repeated measures ANOVA, group, $F_{1,40}=4.085$, $P=0.050$; day, $F_{4,160}=118.72$, $P<0.001$; interaction group×day, $F_{4,160}=1.016$, $P=0.401$).

The relationship between faecal production and food intake of lactating voles was significant and positive (Fig. 1B; Pearson correlation, $R=0.982$, $P<0.001$). Between days 13 and 18, we separated the mothers' faeces from the pups' faeces based on their very different sizes (Fig. 1C). The maternal faecal production decreased significantly (repeated measures ANOVA, group, $F_{1,23}=29.251$, $P<0.001$; day, $F_{4,92}=24.733$, $P<0.001$; interaction group×day, $F_{4,92}=1.656$, $P=0.167$) after day 13 of lactation when the pups began to eat food. The faeces mass produced by the litter was 5.84±0.54 g and the maternal faecal production was 4.88±0.23 g on day 17 of lactation at 21°C. At 30°C the litter faeces production on day 17 was 5.91±0.52 g and maternal faeces production was 3.00±0.15 g. So, we conclude that the food intake of lactating Brandt's voles reached a maximum between days 8 and 12 of lactation. Heavier voles ate more food (Pearson correlation, $R=0.628$, $P=0.001$ at 21°C; $R=0.535$, $P=0.009$ at 30°C; Fig. 2A), and maternal food intake (mean values for days 8–12 of lactation) of lactating Brandt's voles was positively related to litter size (Pearson correlation, $R=0.670$, $P<0.001$ at 21°C; $R=0.550$, $P=0.007$ at 30°C. Fig. 2B). Maternal food intake at peak lactation (mean values for days 8–12 of lactation) using ANCOVA with litter size as covariate was significantly affected by litter size (Bonferroni correction, $F_{1,43}=26.125$, $P<0.001$), and the difference between temperature treatment groups was significant ($F_{1,43}=42.595$, $P<0.001$). The interaction effect between litter size and treatment group was also

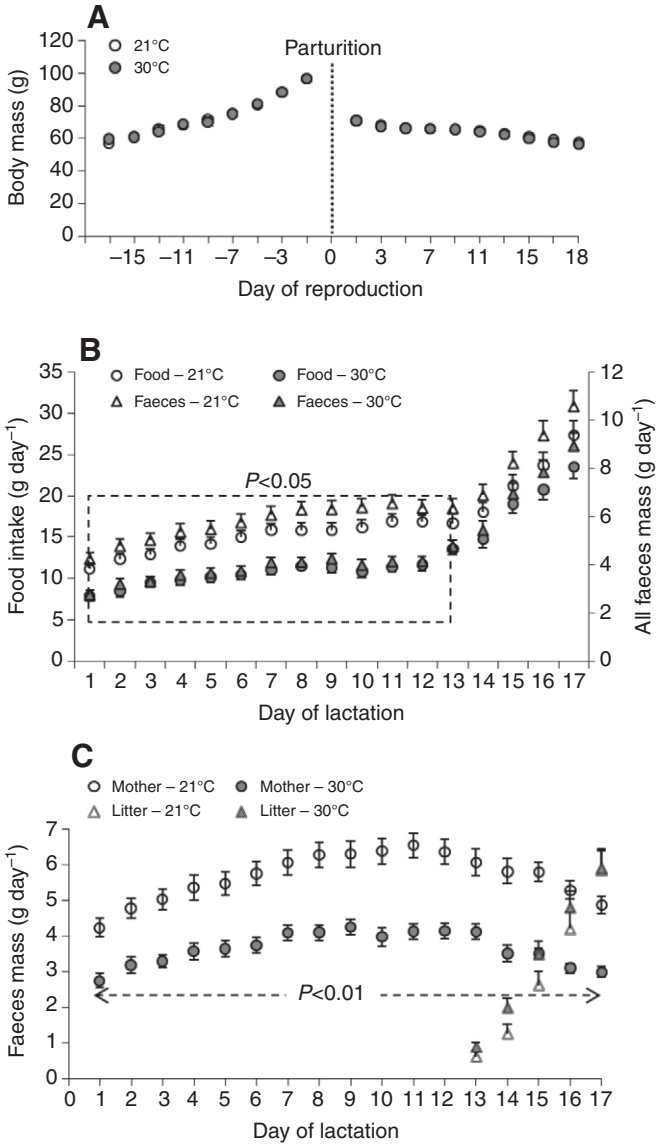

Fig. 1. Changes of body mass (A), food intake (B) and faeces mass (C) of Brandt's voles at 21°C (N=23) and 30°C (N=23) during lactation. Values are means ± s.e. High temperature did not induce compensatory effects in body mass. However, the dry matter intake was affected by high temperature; it was always lower in 30°C groups than in 21°C groups. The steep increase of dry matter intake after day 13 of lactation was because the pups began to eat food. Maternal dry matter intake reached a plateau on days 8–12 of lactation.

significant (litter size×group, $F_{2,43}=33.576$, $P<0.001$). Considering litter size as a factor, mothers raising large litters ($N\geq7$) ate more food than those raising small litters ($N<7$) at both temperatures (independent samples *t*-test, $P<0.05$). The changes in food intake of the mothers of each litter size group were similar to the trends in total food intake across the pooled groups on days 1–12 of lactation. However, maternal food intake was decreased on days 13–17 of lactation. The asymptotic food intake of the mother was stable between days 8 and 12 of lactation (repeated measured ANCOVA, day: $P>0.05$).

Metabolizable energy intake and digestive efficiency

The digestive efficiency of Brandt's voles was not significantly different between the treatment groups during lactation (Fig. 3A).

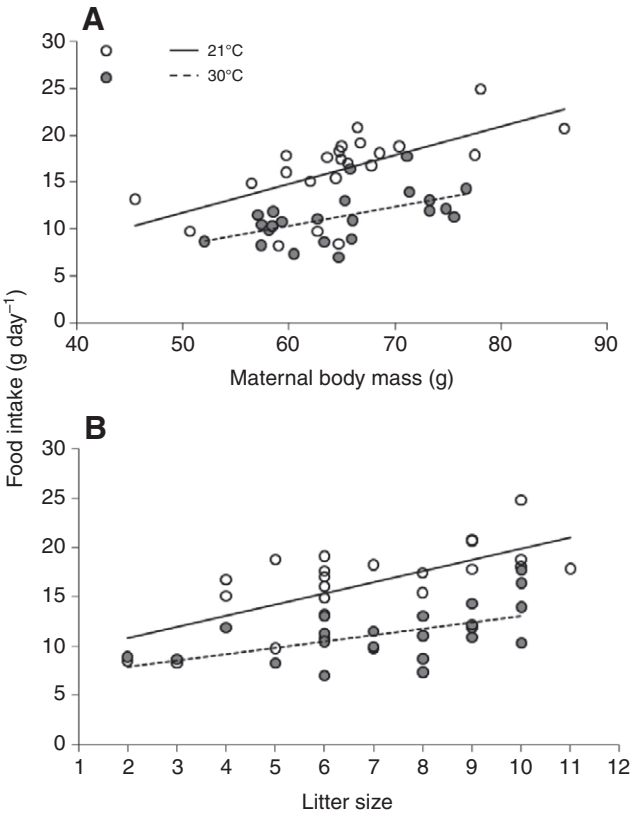

Fig. 2. The relationship between food intake and maternal body mass (A, R=0.628, P=0.001 at 21°C; R=0.535, P=0.009 at 30°C) or litter size (B, R=0.670, P<0.001 at 21°C; R=0.550, P=0.007 at 30°C). Food intake and female body mass are means for days 8–12 of lactation. Heavier voles ate more food, and food intake increased with litter size.

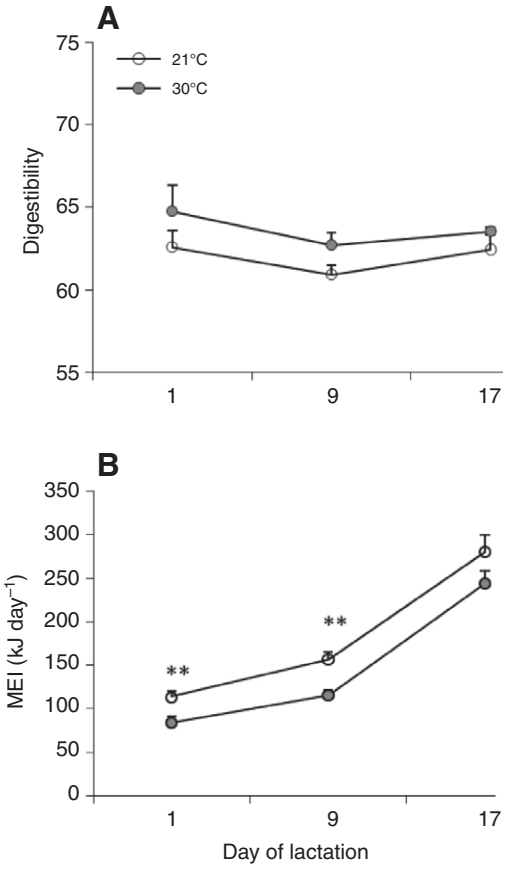

Fig. 3. Changes in digestive efficiency (A) and metabolizable energy intake (MEI, B) of Brandt's voles during lactation. **Significant difference between groups (P<0.01). Note, the data for day 17 come from intake of the mother and pups together.

The MEI on day 1 and day 9 of lactation showed a significant difference between groups. On day 17 of lactation (Fig. 3B) when intake reflected that of both mothers and their offspring there was no significant difference in MEI. The energy density of maternal faeces on days 14–16 of lactation did not vary significantly between temperature treatments, and was 16.01±0.11 kJ g^{-1} at 21°C and 15.87±0.11 kJ g^{-1} at 30°C (independent samples t-test, t_{43}=0.878, P=0.385). On days 14–16 of lactation, the total maternal mean MEI was 152.61±8.20 kJ day^{-1} at 21°C and 105.53±5.17 kJ day^{-1} at 30°C. When split into large and small litters, the MEI was 167.30±9.58 kJ day^{-1} and 139.25±12.00 kJ day^{-1} at 21°C, and 110.99±6.28 kJ day^{-1} and 97.06±8.60 kJ day^{-1} at 30°C for large and small litters, respectively. The MEI was significantly affected by litter size and temperature (two-way ANOVA, temperature, $F_{1,40}$=28.373, P<0.001; litter size, $F_{1,40}$=5.155, P=0.029; interaction temperature×litter size, $F_{1,40}$=0.584, P=0.449).

Daily energy expenditure

DEE estimated by doubly labelled water is presented in Table 1. Between days 14 and 16 of lactation, DEE of the 21°C group and 30°C group averaged 105.7±3.8 kJ day^{-1} (range 75.9–132.8 kJ day^{-1}) and 75.7±2.5 kJ day^{-1} (range 58.7–103.3 kJ day^{-1}), respectively. The difference between temperatures was significant (independent samples t-test, $t_{35.431}$=6.69, P<0.001). DEE and MEI were highly correlated (Pearson correlation, R=0.874, P<0.001, N=44, Fig. 4).

Milk energy output and reproductive performance

The MEO on days 14–16 of lactation was 53.1±8.2 kJ day^{-1} and 29.9±3.8 kJ day^{-1} at 21°C and 30°C, respectively. There was a significant difference between the two temperature groups (independent samples t-test, t_{42}=2.523, P=0.016.). The data were

Table 1. Results of doubly labelled water measurements of daily energy expenditure performed on lactating Brandt's voles

Trait	21°C	30°C
Body mass (g, initial)[a]	61.73±1.82	61.50±1.30
k_d (h^{-1})[b]	0.03±0.00	0.03±0.00
k_o (h^{-1})[c]	0.04±0.00	0.04±0.00
k_o/k_d	1.26±0.01	1.21±0.01
N_d (% of body mass)[d]	66.71±0.78	63.61±1.04
N_o (% of body mass)[d]	65.24±0.65	62.29±1.03
N_d/N_o	1.02±0.00	1.02±0.00
DEE (kJ day^{-1})[e]	105.67±3.79	75.67±2.53**
MEO	53.14±8.24	29.86±3.83**

Values are means ± s.e. DEE, daily energy expenditure (between days 14 and 16 of lactation in lactating Brandt's voles; see text for details). [a]Body mass before injection; [b]elimination rate of ^{2}H; [c]elimination rate of ^{18}O; [d]deuterium (N_d) and oxygen (N_o) dilution spaces (moles) were converted to grams assuming a molecular mass of body water of 18.02 and are expressed as % of body mass before injection; [e]daily energy expenditure was measured between days 14 and 16 of lactation.
**Significant difference between the two groups (P<0.01).

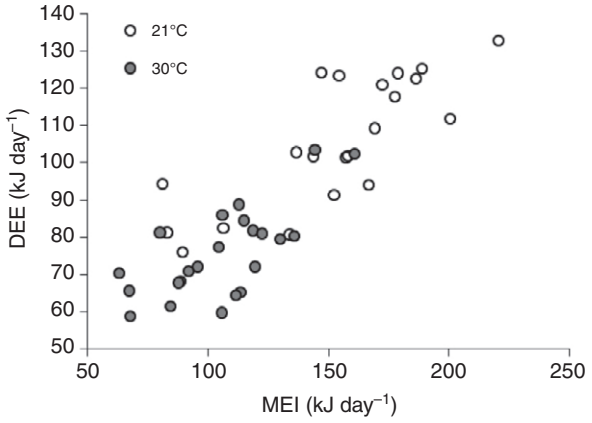

Fig. 4. Relationship between daily energy expenditure (DEE) and MEI in the two temperature groups (R=0.857, P<0.001, N=44).

divided into large and small litters. MEO was not significantly different in small litters between temperatures (independent samples t-test, t_{18}=1.286, P=0.215), but it was significantly different between the two temperatures in large litters (independent samples t-test, t_{22}=3.008, P=0.006). On average, the voles in the 21°C group exported 23.3 kJ day^{-1} more energy as milk than those in the 30°C group on days 14–16 of lactation. Milk production was correlated with maternal body mass (Fig. 5A; R=0.302, P=0.046). Moreover, there was significant positive correlation between MEO and litter size at 21°C but not at 30°C (Fig. 5B; R=0.537, P=0.012 at 21°C; R=0.238, P=0.274 at 30°C). A similar pattern of temperature effects was found for litter growth (Fig. 5C; R=0.589, P=0.005 at 21°C; R=0.318, P=0.139 at 30°C). Litter size also had a large, significant effect on litter growth (two-way ANOVA, $F_{1,42}$=29.854, P<0.001).

The number of dead pups in the two groups was 10 pups from 5 mothers at 21°C and 13 pups from 6 mothers at 30°C. This was 6.0% (10/167) and 6.9% (13/189) of total offspring at 21°C and 30°C, respectively. At the beginning of parturition, the mean litter size of the two groups was 7.26±0.46 at 21°C and 7.78±0.39 at 30°C, respectively (independent samples t-test, t_{44}=–0.860, P=0.395). At weaning, the mean litter size of the two groups was 6.83±0.51 at 21°C and 7.73±0.50 at 30°C (independent samples t-test, t_{45}=–0.737, P=0.465). Litter mass was positively related to litter size (Fig. 6A; Pearson correlation, R=0.967, P<0.001 at 21°C; R=0.843, P<0.001 at 30°C) on day 12 of lactation.

There was a negative correlation between mean pup mass and litter size (Fig. 6B; Pearson correlation, R=–0.301, P=0.042). The effect of temperature on litter mass was not significant. The effect of day of lactation on litter mass was significant, and the interaction between temperature and day was also significant (repeated measures ANOVA, group, $F_{1,41}$=0.605, P=0.441; day, $F_{17,697}$=304.043, P<0.001; interaction group×day, $F_{17,697}$=4.212, P<0.001). For pup body mass (litter mass divided by litter size) there was a borderline significant effect of the temperature treatment (repeated measures ANOVA, group, $F_{1,41}$=3.307, P=0.076) and highly significant effects of day ($F_{17,680}$=900.119, P<0.001) and the day×treatment interaction ($F_{17,680}$=4.259, P<0.001). Pooling all the data there was no significant difference in litter mass between the two temperature treatments, but a highly significant litter size effect (two-way ANOVA, group, P>0.05; litter size, P<0.001). In large litters there was a highly significant difference in litter mass between the two temperature treatments from day 4 to day 12 of lactation. On average the pups in large litters raised at 21°C were

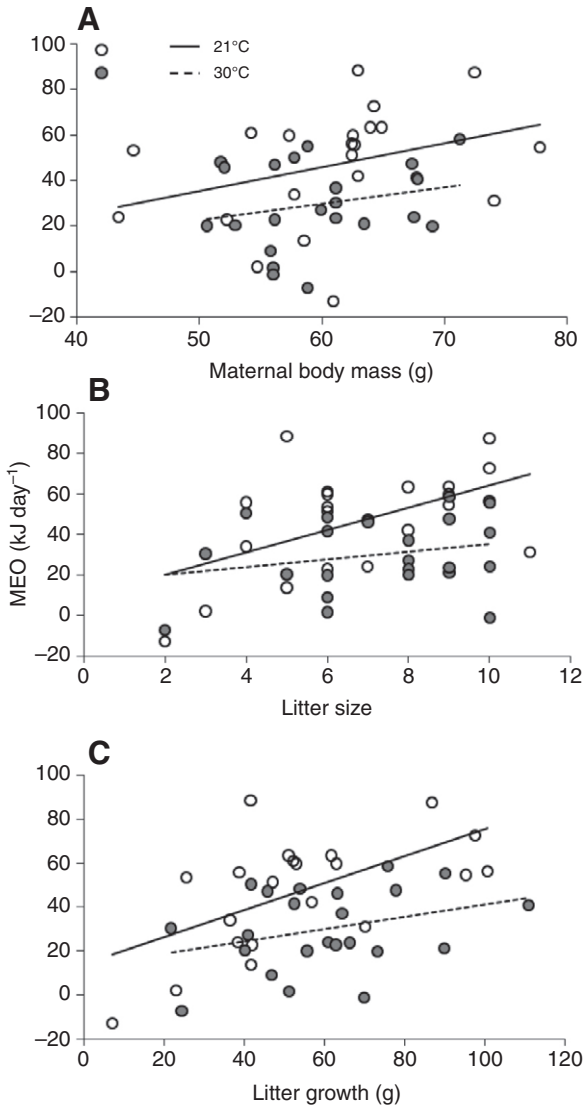

Fig. 5. The correlation between milk energy output (MEO) and maternal body mass (A, R=0.302, P=0.046), litter size (B, R=0.537, P=0.012 at 21°C; R=0.238, P=0.274 at 30°C) and litter growth (C, R=0.589, P=0.005 at 21°C; R=0.318, P=0.139 at 30°C). Maternal body mass refers to day 15 of lactation. Litter growth is defined as the difference between litter mass on day 16 and day 1 of lactation.

15.5 g heavier than pups in large litters raised at 30°C. In the small litters, however, there was no significant difference in litter mass between the two temperatures (Fig. 7A; independent samples t-test, P<0.05).

Individual pup mass showed the similar results: for days 4–12 of lactation, the body mass of the pups at 21°C was significantly greater than that for pups at 30°C in large litters and did not differ between temperatures in small litters (Fig. 7B; independent samples t-test, P<0.05). When pups began to eat food, after day 13 of lactation, the offspring of the 30°C group grew more quickly than those at 21°C. The mass gain at the two temperatures was similar from day 1 to day 12 of lactation in small litters. However, the litter mass gain was significantly greater at 21°C than at 30°C for large litters (Fig. 7C).

GEI varied in the same manner as total food intake during lactation (repeated-measures ANOVA, group, $F_{1,35}$=9.437, P=0.004;

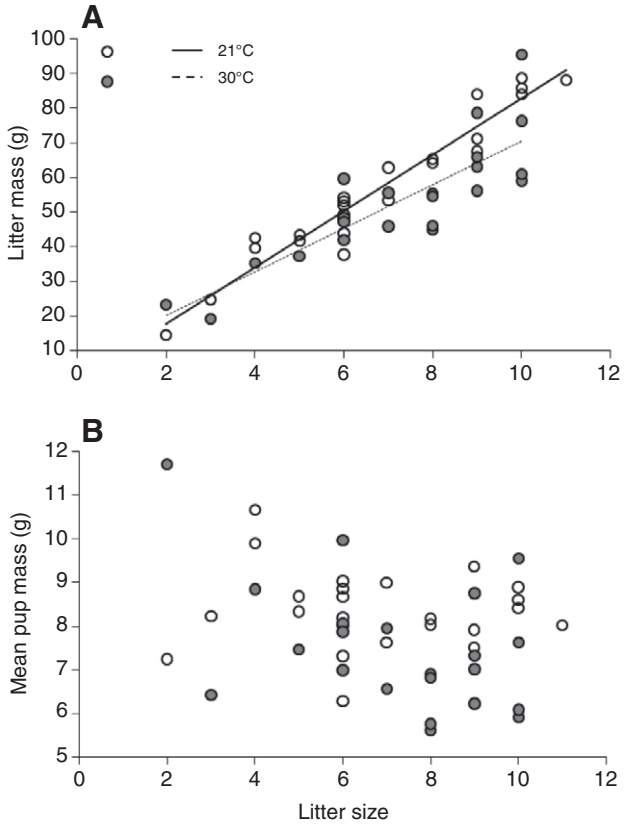

Fig. 6. The relationship between litter mass (A) or mean pup mass (B) and litter size. All parameters are those on day 12 of lactation. There was a positive correlation between litter mass and litter size (Pearson correlation, R=0.967, P<0.001 at 21°C; R=0.843, P<0.001 at 30°C); the correlation between mean pup mass and litter size was negative (R=–0.301, P=0.042).

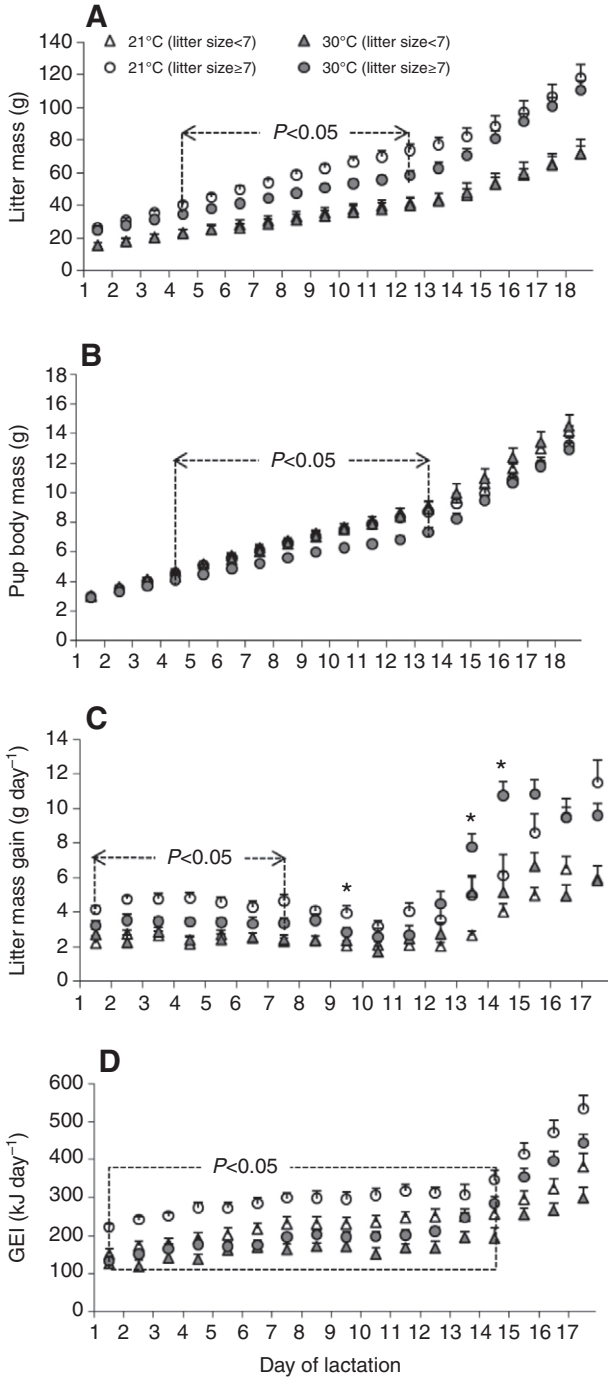

Fig. 7. Brandt's vole litter mass (A), pup mass (B), litter mass gain (C) and gross energy intake (GEI) of mothers and pups (D) at 21°C and 30°C during lactation. Values are means ± s.e. Litter mass and mean pup mass were significantly different between litter size groups from days 4 to 12 of lactation; energy intake was significantly different on days 1–14 of lactation (P<0.05, maternal body mass as a covariate). The data for energy intake after day 12 are the combined intake of the mother and her pups. When energy intake did not increase anymore on days 8–12 of lactation, the litter mass gain of the two groups decreased slowly. Litters at 30°C grew faster than those at 21°C after day 13 of lactation. Moreover, the litter mass gain was significantly greater at 21°C than that at 30°C on most days in larger litters.

day, $F_{16,560}$=114.595, P<0.001; interaction group×day, $F_{16,560}$=1.692, P=0.044), and was significantly different between temperature treatments from day 1 to day 14 of lactation (Fig. 7D; one-way ANCOVA maternal body mass as covariate, P<0.01). When the data for small and large litters obtained during the whole of lactation were separated, the results showed the maternal and offspring energy intake of large litters was always higher than that of small litters; moreover, it showed that in large litters there was a temperature effect (repeated-measures ANOVA, $F_{1,19}$=18.928, P<0.001) but in small litters there was not (repeated-measures ANOVA, $F_{1,14}$=2.344, P=0.148).

Greater pup body mass at weaning was associated with a higher dry mass content (Fig. 8A; Pearson correlation, R=0.391, P=0.010 at 21°C; R=0.609, P<0.001 at 30°C) and a higher energy density of pup dry mass (Fig. 8B, Pearson correlation, R=0.685, P<0.001 at 21°C; R=0.715, P<0.001 at 30°C). The relationship between gross energy content and pup body mass was positive (Fig. 8C, Pearson correlation, R=0.932, P<0.001 at 21°C; R=0.934, P<0.001 at 30°C). At weaning pups raised by the two groups did not differ in wet mass (ANCOVA, pup mass, $F_{1,84}$=0.139, P=0.710; group, $F_{1,84}$=0.002, P=0.963; interaction pup mass×group, $F_{1,84}$=0.028, P=0.868) or dry mass (ANCOVA, pup mass, $F_{1,84}$=0.151, P=0.699; group, $F_{1,84}$=1.451, P=0.232; interaction pup mass×group, $F_{1,84}$=0.145, P=0.704). However, there was a significant difference in proportional dry mass content (dry pup mass divided by wet pup mass) between groups (ANCOVA, dry mass content, $F_{1,84}$=0.043, P=0.836; group, $F_{1,84}$=18.885, P<0.001; interaction dry mass content×group, $F_{1,84}$=0.765, P=0.384). At weaning the energy density of pup dry mass did not differ between the two groups

Table 2. Organ mass (g) of lactating Brandt's voles exposed to 21°C and 30°C

	Wet mass			Dry mass		
Parameters	21°C	30°C	*P*	21°C	30°C	*P*
Heart	0.242±0.012	0.214±0.006	**0.001**	0.066±0.006	0.053±0.002	**0.007**
Liver	2.801±0.108	2.730±0.108	0.257	0.894±0.049	0.804±0.035	0.138
Spleen	0.054±0.004	0.059±0.007	0.751	0.017±0.002	0.015±0.002	0.319
Lung	0.356±0.021	0.322±0.018	0.158	0.087±0.008	0.083±0.003	0.742
Kidneys	0.529±0.015	0.445±0.011	**<0.001**	0.143±0.005	0.113±0.004	**<0.001**
Stomach	0.406±0.015	0.354±0.012	**0.002**	0.100±0.004	0.088±0.004	**0.046**
Small intestine	1.065±0.075	0.755±0.062	**0.001**	0.144±0.013	0.125±0.018	0.265
Caecum	0.860±0.045	0.689±0.040	**0.008**	0.116±0.007	0.083±0.006	**<0.001**
Colon	0.873±0.058	0.703±0.035	**0.009**	0.099±0.012	0.107±0.006	0.546
Alimentary tract	3.204±0.166	2.501±0.135	**0.001**	0.498±0.033	0.403±0.028	**0.016**
Gonads	0.107±0.009	0.155±0.047	0.389	0.027±0.003	0.027±0.003	0.945
Mammary gland	3.016±0.201	2.144±0.122	**<0.001**	0.886±0.056	0.691±0.037	**0.007**
Subcutaneous fat	1.644±0.211	2.337±0.286	0.081			
Epididymal fat	0.305±0.054	0.529±0.069	**0.020**			
Gonadal fat	0.499±0.053	0.615±0.067	0.240			
Mesenteric fat	0.347±0.027	0.315±0.021	0.249			
Total fat	2.801±0.304	3.796±0.410	0.080			
Carcass	30.226±0.751	31.124±0.754	0.405	11.488±0.357	12.708±0.407	**0.031**

Values are means ± s.e. Carcass, the body in the absence of any organs and fats. Carcass was analysed by independent samples *t*-test and *P*-values of other organs were determined by one-way ANCOVA, with carcass as covariate. *P*-values in bold indicate a significant difference.

(ANCOVA, pup energy density, $F_{1,84}$=0.057, P=0.811; group, $F_{1,84}$=1.053, P=0.308; interaction pup energy density×group, $F_{1,84}$=0.214, P=0.645). The body water content of pups did not vary between temperatures (ANCOVA, body water, $F_{1,84}$=0.127, P=0.723; group, $F_{1,84}$=0.367, P=0.546; interaction body water×temperature, $F_{1,84}$=0.004, P=0.948; data not shown).

Organ morphology

Wet and dry masses of nearly all organs of lactating voles at 30°C were lower than those at 21°C, and most were significantly different (Table 2). The main exceptions were the fat depots, which were generally not significantly different between temperature treatments, apart from epididymal fat which was significantly heavier in the

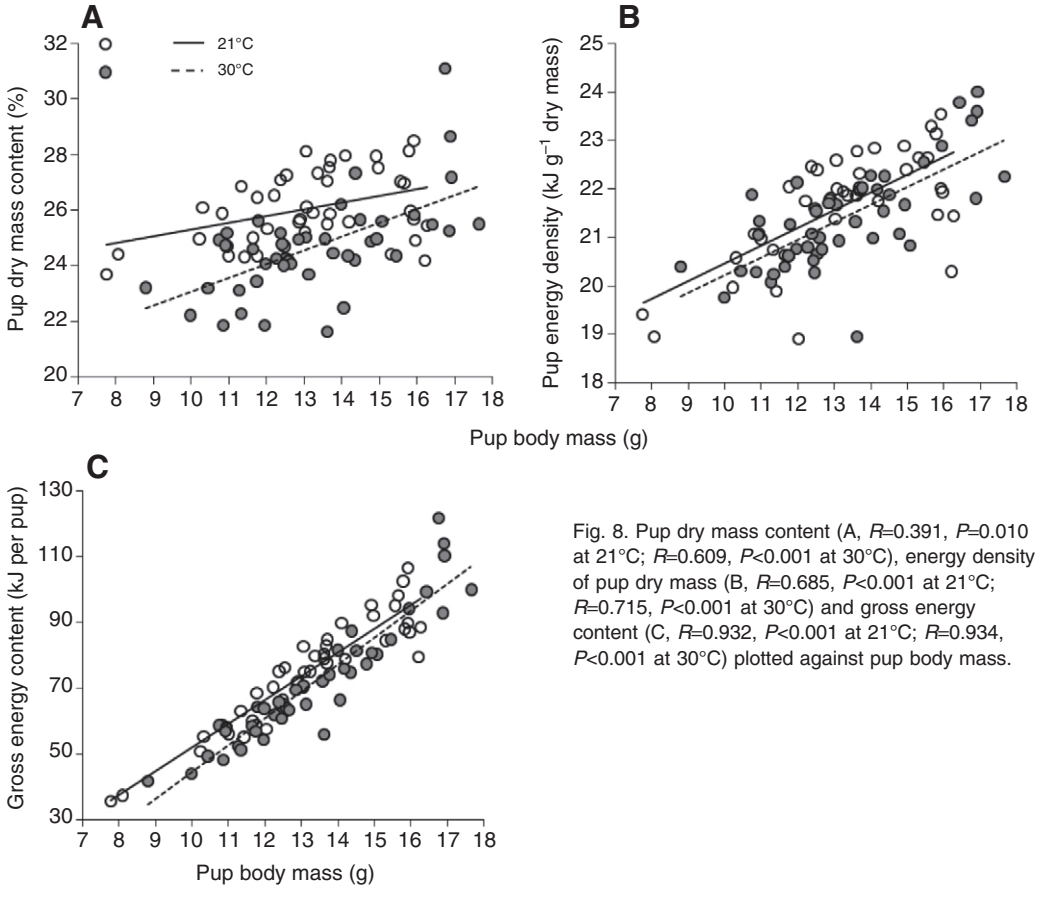

Fig. 8. Pup dry mass content (A, R=0.391, P=0.010 at 21°C; R=0.609, P<0.001 at 30°C), energy density of pup dry mass (B, R=0.685, P<0.001 at 21°C; R=0.715, P<0.001 at 30°C) and gross energy content (C, R=0.932, P<0.001 at 21°C; R=0.934, P<0.001 at 30°C) plotted against pup body mass.

voles kept at 30°C. In contrast the masses of the heart, kidneys, liver and alimentary tract were all significantly higher at 21°C than at 30°C (one-way ANCOVA, wet carcass as covariate, $P<0.05$). The mammary glands, as anticipated from the lower milk production at 30°C, were significantly lighter in voles exposed to 30°C (one-way ANCOVA, wet carcass as covariate, $F_{1,41}=16.240$, $P<0.001$).

DISCUSSION

We used a small wild rodent to test the heat dissipation limitation hypothesis concerning the limits to sustained energy intake at peak lactation. Previous studies attempting to test this hypothesis have been exclusively based on domesticated rodents selected for large litter sizes and high reproductive output. The current study used voles, which raise smaller litters, to assess the generality of the hypothesis. In present study, there were two simultaneous effects of exposing lactating Brandt's voles to 30°C. First there was the higher temperature, and second the rapid change in temperature. Our experiments do not allow us to separate these effects. It seems likely, however, that any effects of a change in temperature would be transient and during the short period following the change we did not record any overt differences between the experimental and control animals which might indicate they were stressed by the procedure – such as elevated mortality in the experimentally manipulated animals. Maternal body mass during lactation was not affected by the high temperature. Furthermore, the body water pool size (N_o) was nearly the same in the two temperature treatment groups, so compensatory changes in body fat content were also unlikely since the two groups had similar isotope-derived body fat content. Direct estimates of maternal body fat at weaning showed significant differences only in epididymal fat content. Lactating Brandt's voles at 30°C had less dry matter intake during the whole of lactation. This result is incompatible with the central limitation hypothesis. When we took into account individual variation in food wasting and individual estimates of apparent digestive efficiency, the GEI and MEI during lactation were significantly greater at 21°C than at 30°C.

Greater food intake at 21°C could simply reflect a combination of demands for lactation and thermoregulation: the peripheral limitation hypothesis. However, as predicted exclusively by the heat dissipation limitation hypothesis, the greater energy intake was used to generate more milk. Milk production was calculated from the difference between MEI and DEE (Król and Speakman, 2003b), and we used the DLW technique to obtain the DEE of the animals. With no significant differences in mean litter size (6.83±0.51 at 21°C and 7.73±0.50 at 30°C), mothers at 21°C exported on average 77.9% (23.3/29.9 kJ day^{-1}) more energy as milk than those at 30°C during days 14–16 of lactation. If the greater MEO at 21°C (23.3 kJ day^{-1}) was fuelled by the extra energy that was assimilated (53.14 kJ day^{-1}), the efficiency of conversion of MEI to MEO would be 43.8% (23.28/53.14 kJ day^{-1}). This value is lower than the net milk production efficiencies reported for other small mammals, e.g. 71.1% in MF1 mice (Król et al., 2007). This suggests that the extra capacity to dissipate heat during exposure to 21°C was not fully taken up by extra milk production.

Taken alone the lower milk production at 30°C does not necessarily reflect a heat dissipation-mediated limit on sustained maternal energy intake. An alternative explanation is that the milk production and hence intake are driven by pup energy demands, which would be lower at 30°C, because their thermoregulatory demands would be reduced. An important novel finding of this study is that the response of the voles to the temperature manipulation differed between small and large litters. For small litters ($N<7$) there was no apparent increase in milk production or litter mass at 21°C, despite the greater food intake of the mothers. For large litters ($N\geq7$), however, the greater food intake was translated into greater milk production and increased pup growth. This difference suggests that there may be different limits acting on different litter sizes. For example, in smaller litters pup growth may already be proceeding at a maximal rate unlimited by competition for maternal milk supply. Consequently, releasing the heat dissipation constraint on their mothers may not have resulted in greater growth because any increase in milk production could not be translated into greater pup growth. However, in larger litters the main constraint on growth was probably maternal milk supply – leading to an inverse relationship between litter size and pup mass because of competition effects between offspring for the limited milk supply (for review, see Speakman, 2008). Removal of the heat dissipation constraint on female voles raising larger litters could therefore be translated into greater milk production and, critically, greater offspring growth. This variability in the response in relation to litter size explains why the calculated efficiency of translating elevated food intake into milk production was much lower than previously reported.

The heat dissipation limitation hypothesis was generated from the study of domesticated mice which have large litters. Mean litter size in the MF1 mouse used in many studies is around 12 pups (Johnson et al., 2001a). Our data suggest that the relevance of the hypothesis may depend on litter size. It would be instructive therefore to know the impact of heat dissipation limits on species that raise even smaller litters than the Brandt's vole, which in our study raised an average of around seven pups. Overall the present data provide some qualified support for the heat dissipation limitation hypothesis, particularly when large litters are being raised.

In the present study, food intake and MEO of Brandt's voles was reduced when the voles were placed in high temperatures, and in larger litters this translated to lower pup growth. These data are consistent with other studies showing a negative effect of increased ambient temperature on lactation performance. This effect is even clearer in large domesticated animals such as dairy cattle (*Bos taurus*) (Cobble and Herman, 1951; Rensis and Scaramuzzi, 2003; Ahmed and El Amin, 1997), sheep (*Ovis aeries*) (Abdalla et al., 1993) and pigs (*Sus scrofa*) (Renaudeau et al., 2003). These larger animals often show chronic hyperthermia during lactation, e.g. sows (Ulmershakibaei and Plonait, 1992), and presumably their unfavourable surface to volume ratio for heat dissipation exacerbates the impact of ambient temperature on their lactation performance. Direct measurements of the maternal body temperature of some rodents confirm that lactating females also have a continuously elevated body temperature compared with non-reproducing individuals: mice (Speakman, 2008), rats (Croskerry et al., 1978; Leon et al., 1978) and Siberian hamsters (*Phodopus sungorus*) (Scribner and Wynne-Edwards, 1994a). Several studies have suggested that there are direct effects of the litter on maternal hyperthermia resulting from mother–pup contact (Leon et al., 1978; Adels and Leon, 1986; Scribner and Wynne-Edwards, 1994b). This has been inferred from studies of many rodent species (Stern and Azzara, 2002) and some livestock (El-Masry and Marai, 1991; Ulmershakibaei and Plonait, 1992; Silanikove, 2000).

The heat dissipation limitation hypothesis suggests that during lactation animals should decrease processes that generate heat but do not contribute to milk production. It has been known for a long time that thermogenic capacity in small mammals is suppressed during lactation (Trayhurn et al., 1982). This effect was previously interpreted as adaptive because it spared energy that could be

diverted into milk. However, it seems likely that this downregulation serves not to contribute energy to lactation but rather to minimize heat production. Consistent with the findings in mice (Johnson et al., 2001b) and Syrian hamsters (*Mesocricetus auratus*) (Wade et al., 1986), we have previously reported that the thermogenic capacity of brown adipose tissue decreased in lactating Brandt's voles, including a decrease in mitochondrial biogenesis and reduced gene expression for uncoupling protein 1 (UCP1) (Li and Wang, 2005b; Zhang and Wang, 2007). In rats, the extent of the decrease in thermogenic capacity is related to litter size (Isler et al., 1984), but this did not appear to be the case in Brandt's voles (Zhang et al., 2008).

In the present study we found large changes in the morphology of voles raised at 21 and 30°C. The alimentary tract and associated organs were remodelled to meet the changed energy demands. The wet or dry organs were mostly significantly smaller at 30°C than those at 21°C, especially the liver and kidneys. It has been known for at least half a century that during lactation there is an increase in the size of the liver (Kennedy et al., 1958) and pancreas (Jolicoeur et al., 1980). The most dramatic changes, however, are in the alimentary tract itself, involving major morphological increases in the absorptive surface of the intestinal mucosa and also increases in the length of the tract. Some data have shown that the size and capacity of the small intestine are highly responsive to changes in energy input or assimilation (e.g. Hammond et al., 1994). In addition, other organs such as the kidney also increase in mass with elevated energy demands in white mice (Toloza et al., 1991; Hammond and Diamond, 1992; Hammond et al., 1994; Hammond and Diamond, 1997) and Brandt's voles (Zhang and Wang, 2007). Our results suggest that alimentary tract and whole organ functional capacity for nutrient uptake under high temperature conditions also change with energy intake. The mammary glands of lactating Brandt's voles at 30°C were significantly smaller than at 21°C. This smaller size was consistent with the reduced MEO at 30°C.

In summary, the present study provides some support for the heat dissipation limitation hypothesis in a wild small mammal, particularly at large litter sizes, supporting previous work which has indicated the relevance of the idea in small domesticated rodents (Król and Speakman, 2003b; Król et al., 2007), which also raise large litters. A priority should now be to test the hypothesis in species raising exclusively small litters. This hypothesis is important because it indicates that changes in ambient temperature will have direct effects on reproductive performance, as well as indirect effects *via* impacts on food supply. Our data indicating the impact of litter size on the role of different factors that limit sustained energy intake may shed light on factors driving different maternal investment strategies, in relation to litter size, under conditions of varying ambient temperature.

LIST OF ABBREVIATIONS

DEE	daily energy expenditure
DEI	digestible energy intake
DLW	doubly labelled water
GEI	gross energy intake
MEI	metabolizable energy intake
MEO	milk energy output
SusEI	maximum rate of sustained energy intake

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