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RESEARCH ARTICLE

Limits to sustained energy intake. XIX. A test of the heat dissipation limitation hypothesis in Mongolian gerbils (*Meriones unguiculatus*)

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SUMMARY

We evaluated factors limiting lactating Mongolian gerbils (*Meriones unguiculatus*) at three temperatures (10, 21 and 30°C). Energy intake and daily energy expenditure (DEE) increased with decreased ambient temperature. At peak lactation (day 14 of lactation), energy intake increased from 148.7±5.7 kJ day^{-1} at 30°C to 213.1±8.2 kJ day^{-1} at 21°C and 248.7±12.3 kJ day^{-1} at 10°C. DEE increased from 105.1±4.0 kJ day^{-1} at 30°C to 134.7±5.6 kJ day^{-1} at 21°C and 179.5±8.4 kJ day^{-1} at 10°C on days 14–16 of lactation. With nearly identical mean litter sizes, lactating gerbils at 30°C exported 32.0 kJ day^{-1} less energy as milk at peak lactation than those allocated to 10 or 21°C, with no difference between the latter groups. On day 14 of lactation, the litter masses at 10 and 30°C were 12.2 and 9.3 g lower than those at 21°C, respectively. Lactating gerbils had higher thermal conductance of the fur and lower UCP-1 levels in brown adipose tissue than non-reproductive gerbils, independent of ambient temperature, suggesting that they were attempting to avoid heat stress. Thermal conductance of the fur was positively related to circulating prolactin levels. We implanted non-reproductive gerbils with mini-osmotic pumps that delivered either prolactin or saline. Prolactin did not influence thermal conductance of the fur, but did reduce physical activity and UCP-1 levels in brown adipose tissue. Transferring lactating gerbils from warm to hot conditions resulted in reduced milk production, consistent with the heat dissipation limit theory, but transferring them from warm to cold conditions did not elevate milk production, consistent with the peripheral limitation hypothesis, and placed constraints on pup growth.

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INTRODUCTION

The sustained maximum rate of energy intake (SusEI) is an important trait because it may provide an upper bound that constrains many aspects of animal performance, including reproductive output and thermoregulatory capabilities (Anderson and Jetz, 2005; Drent and Daan, 1980; Hammond and Diamond, 1997; Peterson et al., 1990; Speakman and Król, 2005a; Speakman, 2000; Weiner, 1992). Previous studies seeking to elucidate the nature of the limits to SusEI have focused on lactation, which is energetically the most demanding period for female mammals (Speakman and Król, 2005a; Speakman and Król, 2011).

The peripheral limitation hypothesis suggests that performance of lactating animals is limited by the capacities of their peripheral tissues, most likely in lactation by the capacity of mammary glands to produce milk (Hammond et al., 1994). This idea predicts that the mammary glands at peak lactation should work at maximal capacity regardless of ambient temperature. However, experiments including measures of milk energy output (MEO) of mice lactating at different ambient temperatures showed that MEO was not constant but rather increased as temperature declined (Johnson and Speakman, 2001; Johnson et al., 2001a; Johnson et al., 2001b; Król and Speakman, 2003a; Król and Speakman, 2003b).

The heat dissipation limitation (HDL) theory suggests that the limits to SusEI at peak lactation might be imposed by the capacity of the animal to dissipate body heat generated as a by-product of processing food and producing milk (Król et al., 2003; Król and Speakman, 2003a; Król and Speakman, 2003b). This idea uniquely explains the simultaneous increase in milk production and the reproduction performance in the cold. Lactating MF1 mice at 21°C that were dorsally shaved to elevate their capacity to dissipate body heat had increased food intake, milk production and a larger litter mass compared with unshaved mice (Krol et al., 2007). This study also excluded the possibility that the trends in maternal food intake with ambient temperature were a reflection of temperature on the pups rather than on the mother (but see Zhao et al., 2013b). Abundant evidence in domesticated livestock supports the HDL theory (Speakman and Król, 2011), which was generated from the study of domesticated mice with large litters. Some studies in wild animals working at much lower rates of productivity also support this hypothesis (Simons et al., 2011; Wu et al., 2009).

However, several studies in Swiss-Webster mice do not support this idea. Surgically removing half the mammary glands of Swiss-Webster mice did not result in an elevation of milk production in the remaining glands, suggesting that mammary gland performance

may impose the limit (Hammond et al., 1996). Moreover, shaving this strain of mouse at peak lactation resulted in elevated food intake but not a significantly greater milk production (Zhao and Cao, 2009; Zhao et al., 2010). Similar negative results were also observed by shaving Siberian hamsters (*Phodopus sungorus*) (Paul et al., 2010), and Valencak et al. (Valencak et al., 2010) found that European hares (*Lepus europaeus*) raising pups that were kept at different ambient temperatures from the mother responded in some ways that were consistent with the HDL theory but in other ways that matched more closely the peripheral limitation idea. Speakman and Król (Speakman and Król, 2011) developed a novel framework showing how the HDL and peripheral limitations are likely to be important in all animals, but to different extents, and they suggested that different limits are likely to come into play under different conditions in different species, and hence acceptance or rejection of the two theories (peripheral limitation and heat dissipation limitation) will depend on whether the species in question has a high, medium or low maximal milk production capacity, compared with a high, medium or low maximal heat dissipation capacity, and exactly what experimental protocol is used to test between them.

Mongolian gerbils [*Meriones unguiculatus* (Milne-Edwards, 1867)] are small, seasonally breeding, non-hibernating, granivorous rodents that are distributed in the desert and semi-arid regions of Mongolia and northern China (Walker, 1968). They experience marked seasonal fluctuations in environmental temperatures. The average shade temperature in the short summer is 18.8°C, and the average temperature in the winter, which lasts approximately 6–7 months, is −22.3°C (Chen, 1988). The thermal neutral zone of Mongolian gerbils is 26 to 38°C (Wang et al., 2000). In the wild, Mongolian gerbils live in burrows that can be on average 69.6 cm deep, approximately 35 cm in summer and autumn, and can reach to 135 cm in winter and spring, and therefore during any particular reproductive event they are probably normally well buffered from temperature variability from day to day in either direction. Mongolian gerbils have litters of between three and eight offspring, which is substantially fewer than laboratory mice [the average weaned litter size for an MF1 mouse is around 12 (Johnson et al., 2001b; Vaanholt et al., 2013) and for Swiss Webster mouse 10.6 (Zhao et al., 2013b)]. A previous study showed that litter mass in lactating gerbils exposed to 5°C was lower than in those exposed to 23°C, and also indicated that the limit of SusEI for Mongolian gerbils was not consistent with the central limitation hypothesis (Li, 2006). However, these results are insufficient to understand the proximate physiological factors that impose limits on the energy budget of Mongolian gerbils during lactation, as suggested by the framework mentioned above.

To assess whether the limits to SusEI are imposed by the capacity to dissipate heat or by the milk production capacity, we placed lactating Mongolian gerbils with their litters at 10, 21 or 30°C on day 1 after parturition. Meanwhile, non-reproductive Mongolian gerbils acclimated to the three ambient temperatures were also studied. We anticipated three possible scenarios for the experimental outcomes following the model presented in Speakman and Król (Speakman and Król, 2011). First, if the HDL theory applied across the whole range of ambient temperatures, then there would be a progressive increase in both food intake and milk production as temperature was reduced from 30 to 10°C. Second, if the animals' lactation performance was limited only by the capacity of the mammary glands, we would predict equivalent milk production at all ambient temperatures but that food intake would increase at lower ambient temperatures because of elevated thermoregulatory demands. Finally, if the HDL theory was applicable at higher temperatures but at lower ambient temperatures peripheral limits became more important, we would anticipate that the transfer from 21 to 30°C would result in reduced food intake and reduced milk production, but the transfer from 21 to 10°C would result in elevated food intake but a constant milk production.

MATERIALS AND METHODS

Animals

Female Mongolian gerbils (range 6–7 months old and weighing 59–88 g) from our breeding colony were maintained at the Institute of Zoology, Chinese Academy of Sciences. Gerbils were individually housed in plastic cages (30×15×20 cm) with sawdust as bedding for 2 weeks prior to the start of the experiment, and maintained at the room temperature of 21±1°C under a 16 h:8 h light:dark cycle (lights on at 04:00 h). Commercial standard rat pellet chow (Beijing KeAo Feed Co., Beijing, China) and water were provided *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

Experiment 1: The effect of ambient temperature on body mass, food intake, daily energy expenditure, resting metabolic rate and reproductive performance

Experimental design

Sixty-six virgin female Mongolian gerbils were paired with males for 15 days at 21±1°C. The males were then removed. Pregnant gerbils (N=36) were checked twice each day to determine the day of parturition (day 0 of lactation). On day 1 after parturition, all the lactating females were randomly allocated to three temperatures: 10±1°C (cold temperature and lactation, CL, N=13), 21±1°C (warm temperature and lactation, WL, N=11) and 30±1°C (high temperature and lactation, HL, N=12). These three groups were balanced for female body mass and litter size. Natal litter sizes were maintained. The mean litter sizes at CL, WL and HL were 6.0±0.4, 6.5±0.3 and 6.1±0.4, respectively. Animals that did not become pregnant were used as non-reproductive controls and were allocated to the three temperatures for 22 days acclimation (cold temperature and no reproduction, CN, N=10; warm temperature and no reproduction, WN, N=10; and high temperature and no reproduction, HN, N=10), and these three groups were also balanced for body mass.

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. Measurements of metabolizable energy intake (MEI) were made on days 14–16 of lactation or on days 19–21 of the temperature treatment for non-reproductive gerbils. Faeces during this period were collected. The energy density of food and faeces was determined by Parr 1281 oxygen bomb calorimetry (Parr Instruments, Moline, IL, USA); gross energy intake (GEI; kJ day^{-1}) and digestible energy intake (DEI; kJ day^{-1}) were calculated as follows (Grodzinski and Wunder, 1975; Liu et al., 2002):

$$\text{GEI} = \text{dry food intake} \times \text{food energy density}, \quad (1)$$

$$\text{DEI} = \text{GEI} - \text{dry faeces mass} \times \text{faeces energy density}, \quad (2)$$

where dry food intake and dry faeces mass are in g day^{-1}, and energy density is in kJ g^{-1}. Measurements of MEI were estimated as the DEI assuming that urinary energy loss was 2% (Liu et al., 2002).

Daily energy expenditure

We measured daily energy expenditure (DEE) using the doubly labelled water (DLW) technique (Butler et al., 2004; Speakman,

1998) on days 14–16 of lactation or days 19–21 of temperature treatment for non-reproductive gerbils. This method has previously been validated by comparison to indirect calorimetry in a range of small mammals (Speakman and Racey, 1988) and provides an accurate measure of DEE over periods of several days (Speakman and Król, 2005b). Day-to-day variability in estimated energy metabolism suggests that measurements spanning multiple 24 h periods may give a superior representation of energy metabolism (Berteaux et al., 1996; Speakman et al., 1994). Studies of lactating mammals suggest that recycling of isotopes between a mother and her offspring is negligible (Scantlebury et al., 2000).

Gerbils were weighed (±0.1 g) and injected intraperitoneally with ~0.56 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 [method C in Speakman and Racey (Speakman and Racey, 1987)]. Blood samples were immediately heat sealed into 2×50 µl glass capillaries and stored at room temperature. A final blood sample was taken ~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), and 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 eqn 7.17 of Speakman (Speakman, 1997)], which minimizes error in a range of conditions (Speakman, 1997; Speakman and Król, 2005b; Van Trigt et al., 2002).

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

We used the DLW data to evaluate MEO, calculated from the difference between MEI and DEE (Król and Speakman, 2003a). MEI and DEE were measured simultaneously on days 14–16 of lactation. At this stage there was still a large difference in maternal energy intake among the three temperature treatment groups. We therefore assumed the difference in milk production we measured was reflective of a difference throughout the entire peak lactation period.

Metabolic trials

Resting metabolic rate (RMR) was quantified as the rate of oxygen consumption, using an open-flow respirometry system (TSE LabMaster, TSE Systems, Bad Homburg, Germany) at 30°C. All the gerbils were kept at 25°C for 2 h before each metabolic measurement to reduce the effect of the large temperature change between the housing and measurement conditions. Body mass was weighed before each metabolic measurement. In brief, each animal was placed in a transparent plastic chamber (19.5×9.2×14.0 cm) with small pieces of tissue paper just enough to absorb animal wastes. An incubator (MIR-553, SANYO, Japan) was used to maintain the chamber at a constant ambient temperature of 30±0.5 [within the thermal neutral zone of Mongolian gerbils (Wang et al., 2000)]. Air from outside the building was pumped through the chamber at a mass flow rate of 0.8 l min^{-1}. The measurement of RMR for an animal lasted for 3 h, recorded at 5 min intervals. The lowest two consecutive recordings (10 min) were taken to calculate RMR.

Thermal conductance of pelage

At the end of the experiment, all the gerbils were killed by CO_2 overdose between 16:00 and 17:00 h. The pelage was removed by making a longitudinal ventral incision from the throat to the anus and then separating the pelage from the body cavity. Pelages were stored at 4°C until assayed (1–3 days). We wrapped the pelage around a small bottle filled with water (20 ml) that contained a temperature transmitter (15.5×6.5 mm, 1.1 g; Mini Mitter ModelG2 E-Mitter, Bend, OR, USA), and then attached it to the bottle with contact adhesive. An incubator (Yiheng Model LRH-250, Shanghai, China) was used to heat the bottle of water to 40°C. After reaching 40°C, the bottle was immediately put into another incubator at 20°C, and then the temperature, decreasing from 40 to 20°C, was monitored every 15 s. Each data point was transformed according to $\ln(x–20)$, where x is the temperature monitored during the temperature decreasing course. The slope calculated for these transformed data was defined as thermal conductance (after Zhao et al., 2013a).

Prolactin

Trunk blood was collected at the time of death, and the blood samples were allowed to clot for 30 min at 4°C. The serum was separated from each blood sample by centrifugation at 4°C for 30 min at 1500 ***g*** and stored at –80°C. Serum prolactin levels were quantified by radioimmunoassay using RIA kits (Beijing North Institute of Biological Technology, Beijing, China). Intra- and inter-assay coefficients of variation were 4.3 and 7.6%, respectively.

Body composition

Gerbils were dissected and the following organs and tissues were weighed wet: interscapular brown adipose tissue (iBAT), heart, liver, lung, kidneys, digestive tract (stomach, small intestine, caecum and colon) with and without contents, mammary gland, and ovary and uterus together as gonad. Tissues were weighed (±0.001 g) using a digital balance (Sartorius, Göttingen, Germany). Total body fat was extracted from the dried carcass by petroleum ether extraction in a Soxhlet apparatus.

Experiment 2: Effect of prolactin infusion on food intake, body mass, thermal conductance of the fur, UCP1 expression in iBAT, body temperature and physical activity levels

Experimental design

Fifteen female Mongolian gerbils were used in Experiment 2; these were randomly divided into two groups, the saline group (N=8) and the prolactin-treated group (N=7). Body mass and food intake were measured every day during the course of the experiment. After 7 days of baseline measurement, female gerbils were infused with ovine

prolactin (L6520, Sigma-Aldrich, St Louis, MO, USA) dissolved in saline or saline alone using miniosmotic pumps (Alzet model 2002, volume 200 µl, release rate 0.5 µl h^{-1}; Durect, Cupertino, CA, USA) for 2 weeks. The prolactin infusion rate was 90 IU kg^{-1} day^{-1}. Female gerbils were anaesthetized with pentobarbital sodium (*ca.* 30 mg kg^{-1}), and the pump was implanted subcutaneously on the dorsal side. At the end of the experiment all gerbils were killed, trunk blood was collected and the blood samples were allowed to clot for 30 min at 4°C, and serum was separated from each blood sample by centrifugation at 4°C for 30 min at 1500 ***g*** and stored at –80°C until assayed for prolactin (as described above for Experiment 1). The iBAT was immediately removed and dissected, weighed and stored at –80°C until assayed for uncoupling protein 1 (UCP1), and the pelage was removed and stored at 4°C until assayed for thermal conductance as described above (1–3 days). The following organs and tissues were dissected: heart, liver, lung, kidneys, digestive tract (stomach, small intestine, caecum and colon) with or without contents, and body fat (including subcutaneous, inguinal, retroperitoneal and mesenteric fat pads). These were weighed to obtain wet mass (±0.001 g, Sartorius digital balance).

Body temperature and physical activity

Two weeks before the experiment started, six gerbils (three in the PBS group and three in the prolactin group) were anaesthetized by pentobarbital sodium (*ca.* 30 mg kg^{-1}) and implanted intraperitoneally with a transmitter (15.5×6.5 mm, 1.1 g; Mini Mitter Model G2 E-Mitter). Transmitters and surgical apparatus were sterilized prior to surgery by immersion in a 75% by volume alcohol solution for 30 min. After 1 week recovery, core body temperature and physical activity were recorded telemetrically from the transmitter implanted in the abdomen. Individual cages were placed on receiver boards (Mini Mitter, Model ER-4000). All the receivers were connected to a computer with VitalView software (Mini Mitter). Records of core body temperature and physical activity were collected at 15 s intervals throughout the experiment.

Measurement of UCP1 content in iBAT

Frozen iBAT was homogenized on ice in RIPA buffer supplemented with anti-protease agents [1 mmol l^{-1} DTT, 1 mmol l^{-1} PMSF, 0.1 mmol l^{-1} EDTA, 1:1000 Protease Inhibitor Cocktails (Sigma-Aldrich)], and the homogenate was centrifuged at 4°C at 16,200 ***g*** for 10 min to obtain the whole protein of the tissue. Protein concentrations were determined using the Lowry assay.

Extracted protein (40 µg) was loaded and separated in a discontinuous SDS-polyacylamide gel, and then was transferred to PVDF membranes and incubated with bovine serum albumin solution overnight at 4°C. UCP1 and actin were detected using antibodies against UCP1 (Abcam, Cambridge, UK) and beta-actin (ZSGB-BIO, Beijing, China), respectively. Then the secondary antibody peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were added. Enhanced chemoluminescence (Amersham Biosciences, Pittsburgh, PA, USA) was used for detection. Film images were scanned (Bio-Rad Laboratories, Hercules, CA, USA) and results were quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistics

Data are reported as means ± s.e.m. and were analysed using SPSS 13.0 software (IBM, Armonk, NY, USA). Prior to all statistical analysis, data were examined for normality and homogeneity of variance using the Kolmogorov–Smirnov test and Levene's test, respectively.

In Experiment 1, maternal body mass, food intake, litter mass and pup body mass were analysed using two-way repeated-measures ANOVA, with temperature and day of lactation as factors. When the effect of ambient temperature or the temperature×day interaction was significant, one-way ANOVA was used to determine differences between the groups within each day followed by Tukey's *post hoc* comparisons. MEI, DEE, RMR, serum prolactin levels and thermal conductance were analysed using two-way ANOVA followed by Tukey's *post hoc* comparisons, with temperature and lactation as factors. Group differences in body compositions were analysed by a two-way ANCOVA with body mass as a covariate followed by Tukey's *post hoc* comparisons.

In Experiment 2, body mass and food intake, were analysed using two-way repeated-measures ANOVA, with prolactin treatment and time as factors. For the core body temperature and physical activity analysis we used the software package MINITAB (version 16, State College, PA, USA). We averaged body temperature and physical activity counts over hourly periods between days 9 and 15 of infusion and then analysed these hourly data using a general linear model with infusion day (9 to 15), time of day (0 to 23) and prolactin treatment as fixed factors and individual ID as a factor nested within group to account for repeated measurements. Because we have shown previously that physical activity causes an increase in body temperature (Gamo et al., 2013), we included physical activity as a covariate in the analysis of body temperature differences. Higher-level interactions were included. The model was simplified by removing non-significant interactions and factors, and comparisons were made using Tukey's *post hoc* comparisons. Serum prolactin levels, thermal conductance and UCP1 content were analysed using independent *t*-tests. Group differences in body compositions were analysed by a one-way ANCOVA with body mass as a covariate followed by Tukey's *post hoc* comparisons. Finally, Pearson correlation analysis was performed to determine the correlation between serum prolactin level and thermal conductance. $P<0.05$ was considered to be statistically significant.

RESULTS

Experiment 1

Body mass and food intake

The body masses of lactating gerbils allocated to cold, warm and hot temperature groups are shown in Table 1. Body mass did not vary significantly with temperature, but varied across days of lactation and did so in different ways at each temperature (two-way repeated-measures ANOVA; temperature, $F_{2,33}=0.4$, $P=0.663$; day, $F_{18,594}=21.5$, $P<0.001$; temperature × day, $F_{36,594}=5.4$, $P<0.001$; Fig. 1A). The mean food intake of lactating gerbils allocated to cold, warm and hot temperature groups is also shown in Table 1. Food intake was significantly increased by lower ambient temperature and varied with day of lactation (two-way repeated-measures ANOVA; temperature, $F_{2,33}=47.4$, $P<0.001$; day, $F_{17,561}=54.2$, $P<0.001$; temperature × day, $F_{34,594}=8.4$, $P<0.001$; Fig. 1B). The significant interaction indicated that the effect of day on food intake was significantly different at the different ambient temperatures. During lactation, food intake increased over the first 9 days but then reached a plateau between days 9 and 14. Thereafter the intake declined in the cold lactating group, although intakes remained elevated in the other two groups until weaning at day 18.

The body masses of non-reproductive gerbils allocated to cold, warm and hot temperature groups are shown in Table 1. Body mass was not affected by temperature treatment, but was affected by day of experiment (two-way repeated-measures ANOVA; temperature,

Table 1. Body mass, food intake, MEI and RMR in lactating and non-reproductive Mongolian gerbils acclimated to different environmental temperatures

	Lactation			No reproduction		
Parameter	10°C (CL)	21°C (WL)	30°C (HL)	10°C (CN)	21°C (WN)	30°C (HN)
Body mass (g)						
Day 0	75.1±2.2	75.1±1.7	76.9±2.2	64.9±1.4	64.9±1.4	64.3±1.8
Day 18 or 23	75.0±2.9	75.2±1.2	72.8±1.6	62.7±1.3	63.7±1.1	65.6±3.1
Food intake (g day^{-1})						
Day 1	7.7±0.5	8.5±0.5	8.5±0.7	5.9±0.3	5.5±0.2	5.9±0.5
Day 18 or 23	14.6±1.2	10.5±0.7	10.2±0.5	7.0±0.3	4.6±0.4	2.9±0.3
MEI (kJ day^{-1})	248.7±12.3	213.1±8.2	148.7±5.7	109.3±6.7	70.2±3.9	50.3±3.7
RMR (ml O_2 h^{-1} g^{-1})	1.5±0.1	1.7±0.1	1.3±0.1	1.3±0.1	1.3±0.1	1.1±0.1

MEI, metabolizable energy intake (between days 14 and 16 of lactation in lactating gerbils and between days 19 and 21 in non-reproductive gerbils; see Materials and methods); RMR, resting metabolic rate (on day 17 lactation for lactating gerbils or day 22 of temperature treatment for non-reproductive gerbils); CL, cold and lactation; WL, warm and lactation; HL, hot and lactation; CN, cold and no reproduction; WN, warm and no reproduction; HN, hot and no reproduction. Values are means ± s.e.m.

$F_{2,27}$=0.4, P=0.708; day, $F_{23,621}$=5.2, P<0.001; temperature × day, $F_{46,594}$=1.0, P=0.420; Fig. 1A). The mean food intake of non-reproductive gerbils allocated to cold, warm and hot temperature groups is also shown in Table 1. Food intake was significantly increased by lower ambient temperature and varied with day (two-way repeated-measures ANOVA; temperature, $F_{2,26}$=49.4, P<0.001; day, $F_{22,572}$=9.3, P<0.001; temperature × day, $F_{44,572}$=5.8, P<0.001; Fig. 1B). The significant interaction indicated that the response to ambient temperature over time differed between the three different temperature groups.

Metabolizable energy intake, daily energy expenditure and resting metabolic rate

For days 14–16 of lactation, the total maternal mean MEI values of lactating gerbils allocated to cold, warm and hot temperatures are shown in Table 1. MEI was significantly increased by both lactation and decreased ambient temperature (two-way ANOVA; lactation, $F_{1,60}$=414.1, P<0.001; temperature, $F_{2,60}$=53.3, P<0.001; lactation × temperature, $F_{2,60}$=5.3, P=0.008). The significant interaction indicated that the elevation due to lactation was not constant across the different ambient temperatures. Indeed at 10°C the mean increase was 149.4 kJ day^{-1} while at 30°C it was only 98.4 kJ day^{-1}. Results of the DLW measurements are presented in Table 2. DEE was significantly increased by both lactation and lower ambient temperature (two-way ANOVA; lactation, $F_{1,48}$=90.4, P<0.001; temperature, $F_{2,48}$=61.5, P<0.001; lactation × temperature, $F_{2,48}$=1.4, P=0.269). RMR (measured at 30°C) was significantly increased by lactation (two-way ANOVA; lactation, $F_{1,59}$=12.7, P=0.001; temperature, $F_{2,59}$=4.5, P=0.015; lactation × temperature, $F_{2,59}$=1.3, P=0.288; Table 1). The impact of ambient temperature was more complex because there was a reduction in RMR at 30°C relative to 21°C but no differences between 21 and 10°C.

Milk energy output and reproductive performance

On average, lactating gerbils allocated to the high temperature group exported significantly less energy as milk at peak lactation than those allocated to cold or warm temperatures (one-way ANOVA; temperature, $F_{2,31}$=3.7, P=0.038; Fig. 2A). At the end of lactation, mean litter size at CL, WL and HL was 5.6±0.4, 6.3±0.3 and 6.0±0.4, respectively, and it was not significantly affected by the temperature

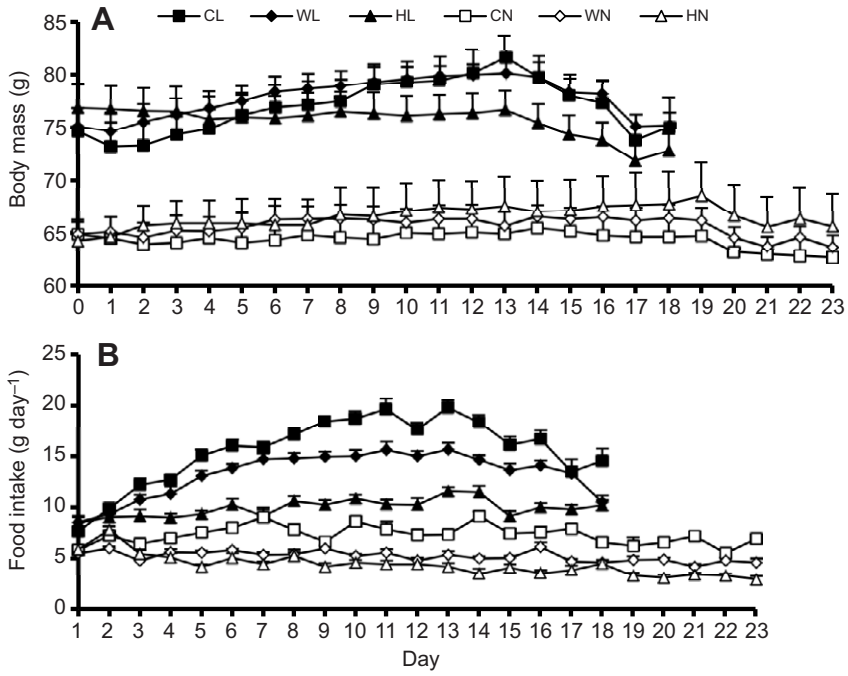

Fig. 1. Body mass (A) and food intake (B) of lactating and non-reproductive Mongolian gerbils exposed to cold (10±1°C), warm (21±1°C) and hot (30±1°C) temperatures. Values are means ± s.e.m. CL, cold and lactation; WL, warm and lactation; HL, hot and lactation; CN, cold and no reproduction; WN, warm and no reproduction; HN, hot and no reproduction.

Table 2. Results of doubly labelled water measurements of DEE performed on lactating and non-reproductive Mongolian gerbils acclimated to different environmental temperatures

	Lactation			No reproduction		
Parameter	10°C (CL)	21°C (WL)	30°C (HL)	10°C (CN)	21°C (WN)	30°C (HN)
Body mass (g)[a]	81.3±1.8	80.8±1.4	77.0±1.8	64.7±1.4	66.2±1.2	66.2±1.2
K_d (h^{-1})[b]	0.016±0.001	0.016±0.001	0.014±0.001	0.007±0.000	0.004±0.001	0.005±0.001
K_o (h^{-1})[c]	0.026±0.001	0.024±0.001	0.020±0.001	0.016±0.000	0.010±0.001	0.009±0.001
K_o/K_d	1.611±0.021	1.448±0.043	1.419±0.017	2.148±0.054	2.552±0.251	2.090±0.202
N_d (% of body mass)[d]	72.2±0.9	73.9±1.2	73.5±1.2	72.0±2.2	75.1±2.6	72.9±2.3
N_o (% of body mass)[d]	69.2±1.0	70.6±1.2	70.1±1.4	68.2±2.2	70.8±3.0	69.1±2.2
N_d/N_o	1.044±0.004	1.046±0.003	1.048±0.007	1.056±0.006	1.065±0.011	1.056±0.006
DEE (kJ day^{-1})[e]	179.5±8.4	134.7±5.6	105.1±4.0	124.7±2.9	89.0±3.8	69.4±4.5

DEE, daily energy expenditure (between days 14 and 16 of lactation in lactating gerbils and between days 19 and 21 in non-reproductive gerbils; see Materials and methods); CL, cold and lactation; WL, warm and lactation; HL, hot and lactation; CN, cold and no reproduction; WN, warm and no reproduction; HN, hot and no reproduction. Values are means ± s.e.m.

[a]Body mass before injection.

[b]Elimination rate of ^2H.

[c]Elimination rate of ^{18}O.

[d]Deuterium (N_d) and oxygen (N_o) dilution spaces expressed as % of body mass before injection.

[e]Daily energy expenditure was measured between days 14 and 16 of lactation (non-reproductive gerbils were measured between days 19 and 21 of temperature treatment).

treatment (one-way ANOVA; temperature, $F_{2,33}$=1.1, P=0.354). Litter mass was significantly affected by the day of experiment and temperature treatment (two-way repeated-measures ANOVA; temperature, $F_{2,33}$=7.5, P=0.002; day, $F_{18,594}$=1116.3, P<0.001; temperature × day, $F_{36,594}$=12.2, P<0.001; Fig. 2B). The significant interaction showed that the pattern of change in litter mass with day varied significantly between the different temperatures. These different patterns are evident from the data shown in Fig. 2B, where litter mass continued to increase between days 0 and 18 in both the 30 and 21°C groups, but in the 10°C group there was no further growth after day 14. Pup body mass was not affected by temperature treatment, but varied across days of lactation (two-way repeated-measures ANOVA; temperature, $F_{2,33}$=2.3, P=0.117; day, $F_{18,594}$=768.9, P<0.001; temperature × day, $F_{36,594}$=4.5, P<0.001; Fig. 2C). The significant interaction reflected the fact that, in the same way as for litter mass, individual pups in the cold temperature failed to grow after day 14, while those in the other two groups continued to grow until weaned on day 18.

Prolactin and thermal conductance

Lactating gerbils had higher serum prolactin levels (two-way ANOVA; lactation, $F_{1,57}$=481.9, P<0.001; temperature, $F_{2,57}$=0.3, P=0.782; lactation × temperature, $F_{2,57}$=2.2, P=0.120; Fig. 3A) and greater thermal conductance (two-way ANOVA; lactation, $F_{1,60}$=49.0, P<0.001; temperature, $F_{2,60}$=0.2, P=0.792; lactation × temperature, $F_{2,60}$=1.3, P=0.277; Fig. 3B) than non-reproductive gerbils regardless of temperature treatment. Across all individuals there was a positive correlation between prolactin and thermal conductance (r=0.548, P<0.001; Fig. 3C).

Body composition

iBAT, heart, liver, kidneys, gonads and digestive tract (stomach, small intestine, cecum and colon) with or without content and body fat (except lung and carcass) were significantly affected by lactation (Table 3). Only iBAT, heart, liver, kidneys, body fat and digestive tract with or without content (except stomach with content, colon without content and carcass) were significantly affected by ambient temperature. There was a significant interaction between lactation and ambient temperature on cecum and colon with content, and stomach, small intestine and caecum without content, showing that the effect of lactation was not constant at the different temperatures.

Experiment 2

Serum prolactin levels

Gerbils in prolactin group had higher serum prolactin levels than those in saline group (P<0.001; Fig. 4).

Thermal conductance and UCP1 content

Prolactin treatment had no effect on thermal conductance (P=0.6; supplementary material Fig. S1) and at the individual level there was no correlation between prolactin and thermal conductance (supplementary material Fig. S2). UCP1 content in iBAT in the prolactin group was significantly lower than that in the saline group (P=0.05; Fig. 5), although the effect was right on the borderline of significance.

Body mass, food intake and body composition

Body mass (two-way repeated-measures ANOVA; PRL, $F_{1,13}$=0.604, P=0.451; day, $F_{13,169}$=1.391, P=0.168; PRL×day, $F_{13,169}$=1.455, P<0.139; supplementary material Fig. S3) and food intake (two-way repeated-measures ANCOVA; PRL, $F_{1,9}$=0.005, P=0.947; day, $F_{11,99}$=0.836, P=0.604; PRL×day, $F_{11,99}$=0.645, P=0.786; supplementary material Fig. S4) were not affected by prolactin treatment. For body composition, none of the organs or tissues sampled was affected by prolactin treatment (supplementary material Table S1).

Body temperature and physical activity

Before prolactin treatment, no differences in body temperature and physical activity were found between the prolactin group and the saline group (P>0.05; supplementary material Fig. S5). Between days 9 and 15 of infusion there was no significant effect of day on either body temperature (P=0.293) or physical activity (P=0.271) and we therefore removed day from the model. For body temperature there was a significant effect of time of day ($F_{23,863}$=17.7, P<0.001; Fig. 6), treatment group ($F_{1,863}$=217.2, P<0.001), physical activity level ($F_{1,863}$=119.71, P<0.001) and individual nested within group ($F_{4,863}$=129.8, P<0.001), and a significant interaction between the treatment group and the time of day ($F_{23,863}$=1.92, P=0.006). For physical activity levels, there were significant effects of time

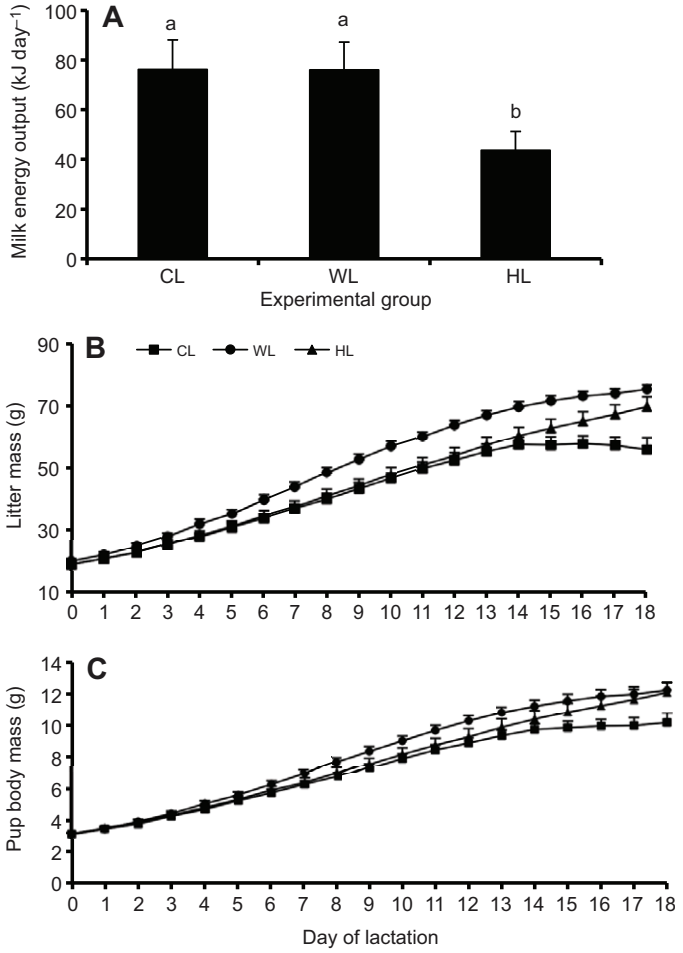

Fig. 2. Milk energy output (MEO; A), litter mass (B) and pup body mass (C) of lactating Mongolian gerbils exposed to cold (10±1°C), warm (21±1°C) and hot (30±1°C) temperatures. Significant differences are indicated by different letters if $P<0.05$. Values are means ± s.e.m. MEO was measured on days 14–16 of lactation. CL, cold and lactation; WL, warm and lactation; HL, hot and lactation.

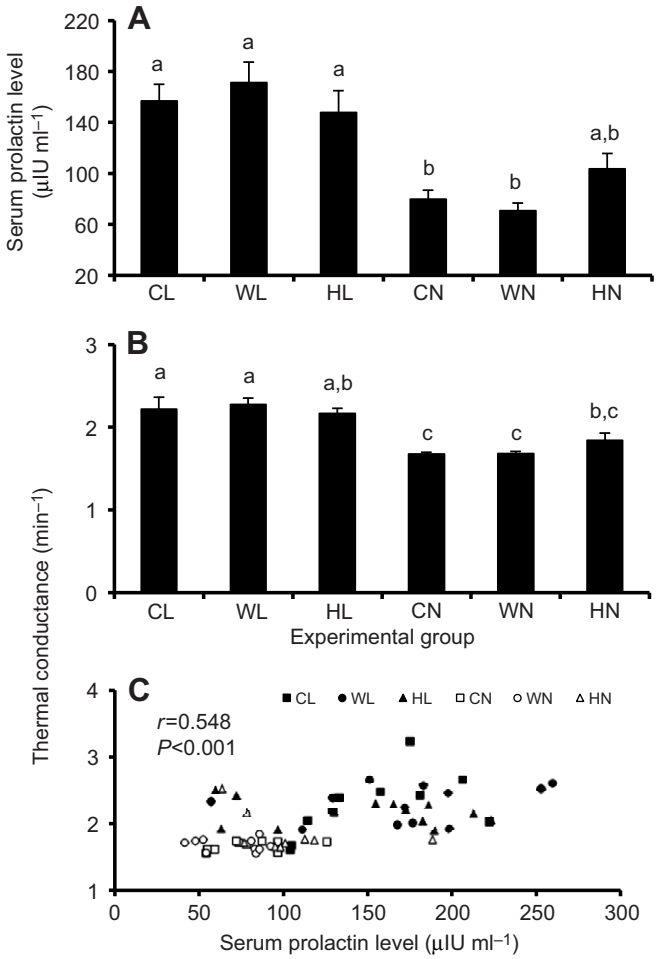

Fig. 3. Serum prolactin levels (A) and thermal conductance (B) of lactating Mongolian gerbils exposed to cold (10±1°C), warm (21±1°C) and hot (30±1°C) temperatures, and the correlation between prolactin and thermal conductance (C). Significant differences are indicated by different letters if $P<0.05$. Values are means ± s.e.m. CL, cold and lactation; WL, warm and lactation; HL, hot and lactation; CN, cold and no reproduction; WN, warm and no reproduction; HN, hot and no reproduction.

($F_{23,872}$=6.73, P<0.001), treatment group ($F_{1,872}$=132.0, P<0.001) and individual nested within group ($F_{4,872}$=10.39, P<0.001), and a significant treatment group × time of day interaction ($F_{23,872}$=2.89, P<0.001). A plot of body temperature against logged physical activity levels for all hourly measurements between days 9 and 15 across all individuals is shown in supplementary material Fig. S6. As anticipated, greater activity was linked to greater body temperature, but there was an offset between the relationships because of the impact of prolactin infusion. Mice infused with prolactin had on average 0.75°C lower body temperature than those infused with saline, which was reduced to a 0.5°C difference when the effects of physical activity differences between the groups were taken into account. These analyses show that the impact of prolactin on body temperature was not mediated by the effect on physical activity levels alone.

DISCUSSION

As demonstrated previously in several species of small mammal (Hammond and Diamond, 1992; Rogowitz, 1998; Johnson and Speakman, 2001; Hammond and Kristan, 2000; Zhang and Wang, 2007), lactating gerbils increased their food intake at reduced ambient temperature. Greater energy intake at lower temperature could simply reflect a combination of demands for lactation and thermoregulation, but the HDL theory uniquely predicts that the greater energy intake would be linked to the generation of more milk. Milk production was calculated from the difference between MEI and DEE (Król and Speakman, 2003b). With nearly identical mean litter sizes, lactating gerbils at 30°C exported 32.0 kJ day^{-1} less energy as milk at peak lactation than those allocated to 21°C. On day 14 of lactation, the pups raised at 30°C were smaller than those at 21°C. All these results were consistent with the predictions of the HDL theory. However, transferring lactating gerbils from 21 to 10°C did not increase milk production, and was associated with reduced total litter mass, possibly because at the lower ambient temperatures pup energy demands were greater than at 21°C (see also Zhao et al., 2013a). One interpretation of these data is that lactating gerbils below 21°C were limited in milk production peripherally by the capacity of the mammary glands. Similar data comparing food intake and milk production in animals transferred from 21°C to cold temperatures (5 to 11°C) were observed in cotton rats (Rogowitz, 1998). Also in Swiss mice, Brandt's voles and deer mice, exposure to cold conditions during lactation resulted in elevated food intake, but a reduction in litter growth compared

Table 3. Body compositions of lactating and non-reproductive Mongolian gerbils acclimated to different environmental temperatures

	Lactation			No reproduction			*P*		
Parameter	10°C (CL)	21°C (WL)	30°C (HL)	10°C (CN)	21°C (WN)	30°C (HN)	Lactation	T_a	Lactation×T_a
iBAT (mg)	161±9^{a,b}	132±8^{a}	135±11^{a,b}	181±13^{b}	156±8^{a,b}	151±17^{a,b}	0.037	0.019	0.936
Heart	358±14^{a,b}	340±21^{a}	283±14^{c}	305±7^{a,c}	247±6^{c,d}	229±11^{d}	<0.001	<0.001	0.373
Liver	3826±217^{a}	3495±94^{a,b}	3212±90^{b}	2381±97^{c}	2059±112^{c}	2195±134^{c}	<0.001	<0.001	0.202
Lung	416±26	383±20	421±33	410±38	383±21	388±17	0.702	0.563	0.707
Kidneys	839±22^{a}	704±18^{b}	691±19^{b}	706±20^{b}	586±18^{c}	526±19^{c}	<0.001	<0.001	0.446
Gonads	130±12^{a}	129±9^{a}	120±13^{a}					0.768	
Mammary gland	2114±185	2516±200	2339±180	70±8^{b}	90±7^{a,b}	92±14^{a,b}	<0.001	0.364	0.336
Mass with content									
Stomach	2887±438^{a}	2180±269^{a,b}	1980±216^{a,b,c}	1322±168^{b,c}	897±92^{c}	1019±81^{c}	<0.001	0.062	0.5
Small intestine	3675±174^{a,b}	3365±208^{a}	2794±102^{c}	2337±58^{c}	1595±99^{d}	1368±68^{d}	<0.001	<0.001	0.27
Cecum	2693±208^{a}	2791±147^{a}	1998±119^{b}	1644±87^{c}	1147±69^{c,d}	885±84^{d}	<0.001	0.001	0.086
Colon	1245±68^{b}	985±64^{a}	974±47^{a}	625±42^{c}	671±30^{c}	572±42^{c}	<0.001	0.002	0.013
Mass without content									
Stomach	524±22^{a,b}	553±33^{a}	438±31^{b,c}	408±12^{c}	341±17^{c}	369±19^{c}	<0.001	0.046	0.035
Small intestine	1112±129^{a,b}	1541±221^{a}	718±81^{b,c}	463±3^{c}	313±27^{c}	393±49^{c}	<0.001	0.03	0.005
Cecum	365±39^{b}	475±42^{a}	283±31b^{c,d}	315±29^{b,c}	187±15^{c,d}	176±13^{d}	<0.001	0.001	0.001
Colon	451±34^{a}	438±49^{a}	336±28^{a,b}	244±18^{b}	222±17^{b}	249±12^{b}	<0.001	0.226	0.096
Carcass (g)	50.1±2	50.2±0.8	50.8±0.9	47.1±1.1	49.9±1.0	52±2.6	0.638	0.134	0.299
Body fat (g)	3.9±0.5^{c}	4.1±0.3^{c}	4.4±0.4^{c}	5.2±0.8^{b,c}	7.3±0.7^{b}	10.2±2.1^{a}	<0.001	0.016	0.053

iBAT, interscapular brown adipose tissue; T_a, ambient temperature; CL, cold and lactation; WL, warm and lactation; HL, hot and lactation; CN, cold and no reproduction; WN, warm and no reproduction; HN, hot and no reproduction. Groups with different letters indicate statistically significant differences among group means (P<0.05). Values are means ± s.e.m.

with warm conditions (Hammond and Kristan, 2000; Zhang and Wang, 2007; Zhao et al., 2013b), perhaps indicating a failure to upregulate milk production, which was not measured directly in these latter studies.

However, another potential explanation of these data is that in the cold conditions the females were capable of upregulating their milk production but did not do so because of temperature-related limitations on pup growth capacity at low temperature (Simons et al., 2011; Zhao et al., 2013b). Some data from mice raising small litters at low temperatures support the idea that pup growth capacity at low ambient temperatures may be a limiting factor in female milk production (Zhao et al., 2013b). Further experimentation beyond the scope of the present study would be necessary to separate these ideas. Whatever the cause of the pattern, it is clear that different factors probably limit milk production in different situations and no single theory can explain the entirety of the available data (Speakman and Król, 2011).

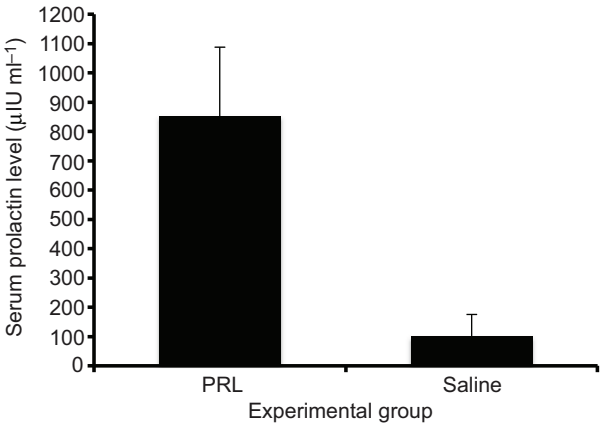

Fig. 4. Serum prolactin levels of female Mongolian gerbils infused with ovine prolactin (PRL) dissolved in saline or saline alone using miniosmotic pumps for 2 weeks. The prolactin infusion rate was 90 IU kg^{-1} day^{-1}. Values are means ± s.e.m.

Pelage insulation is a major constraint on heat loss. We have suggested that during lactation females should thin their pelage to facilitate heat dissipation (Speakman and Król, 2010). Consistent

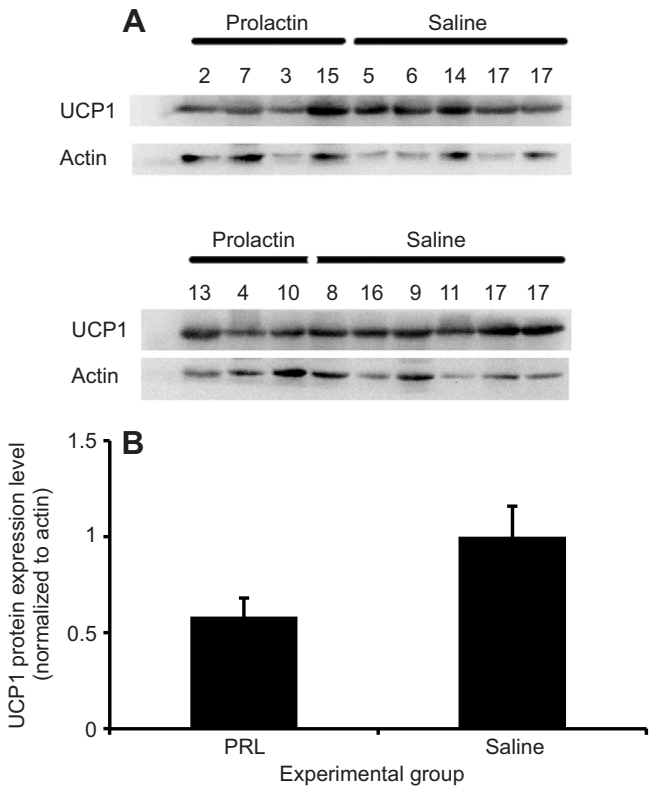

Fig. 5. Uncoupling protein 1 (UCP1) content in brown adipose tissue of female Mongolian gerbils infused with ovine prolactin (PRL) dissolved in saline or saline alone using miniosmotic pumps for 2 weeks. UCP1 expression for each gerbil (A) and means for PRL and saline groups (B) are shown. The prolactin concentration was 90 IU kg^{-1} day^{-1}. Values are means ± s.e.m.

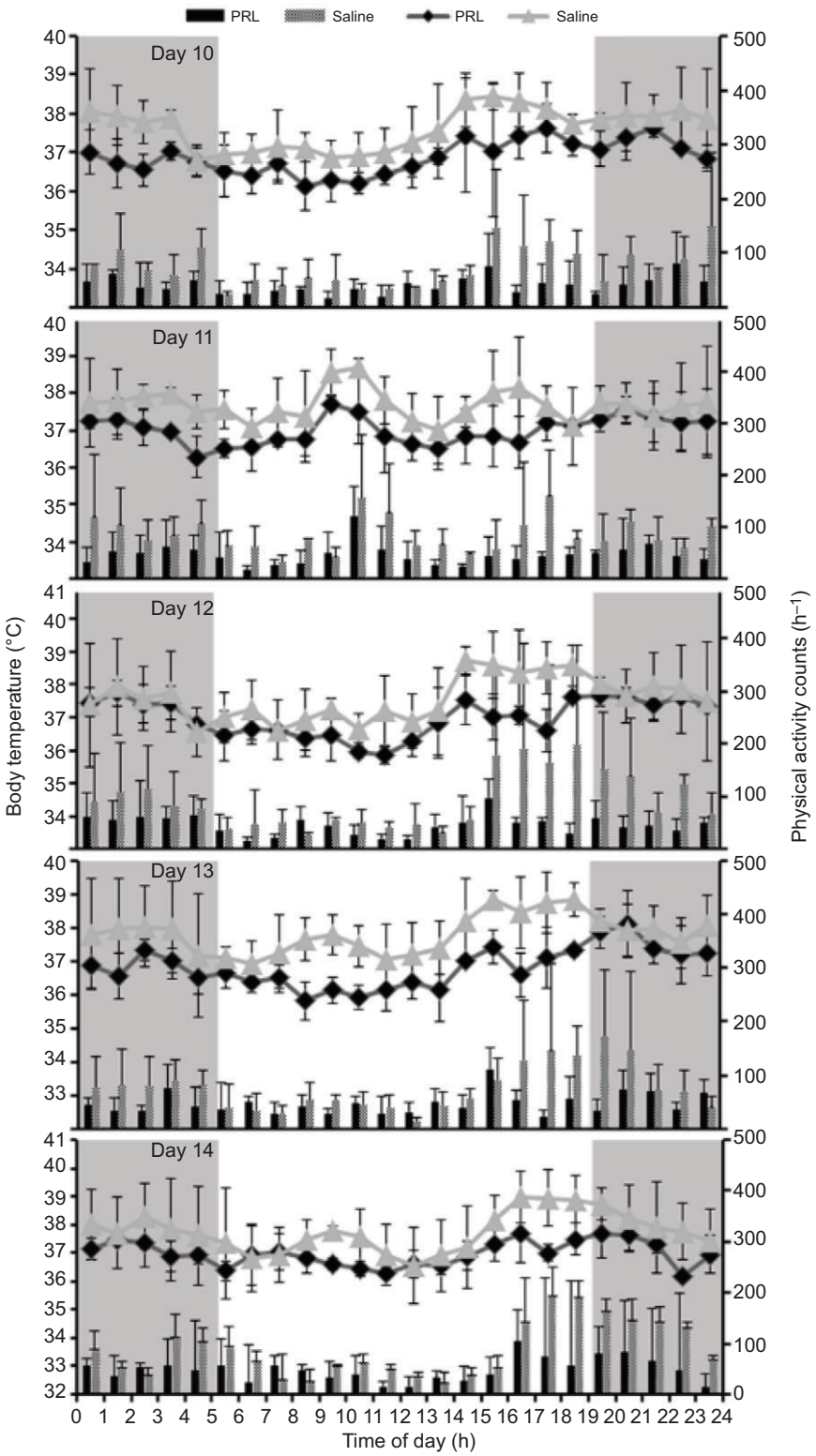

Fig. 6. Effects of prolactin (PRL) infusion (90 IU kg^{-1} day^{-1}) on body temperature and physical activity in Mongolian gerbils (*N*=3 per group). Grey shaded areas in the background represent the dark phase of the diurnal cycle. Values are means ± s.e.m.

with this suggestion, the thermal conductance of pelage was significantly increased during lactation independent of ambient temperature. Although a positive correlation between pelage thermal conductance and circulating levels of prolactin in Experiment 1 suggested that prolactin might be involved in the regulation of pelage thermal conductance, non-lactating gerbils given exogenous prolactin did not increase pelage thermal conductance (see supplementary material Fig. S1). This suggests that prolactin does not mediate increased pelage conductance during lactation. The correlation observed between thermal conductance and prolactin titres when data were pooled across the lactating and experimental groups therefore likely comes about only because prolactin levels are elevated in lactation to facilitate milk production at the same time that the pelage is thinned to facilitate heat loss at peak lactation.

Alternatively, it may be that prolactin receptor populations in the epidermis are elevated in lactation, making it more sensitive to circulating prolactin levels. At present we cannot separate these alternatives.

Body mass and food intake were also not affected by the exogenous prolactin. This latter result was inconsistent with previous work in rats that has indicated that prolactin may be part of the hormonal system stimulating the lactation hyperphagia (Noel and Woodside, 1993; Speakman and Król, 2005a; Woodside and Leon, 1980; Woodside et al., 1980). However, body temperature was lower in the prolactin-treated animals and they also had lower physical activity levels, showing that the infused prolactin was physiologically active. Lowered physical activity is characteristic of lactation (Slonaker, 1924; Wang, 1923; Zhao et al., 2013b). However, lactating females generally have greatly elevated body temperatures (Melanie et al., 1988; Scribner and Wynne-Edwards, 1994; Ulmershakibaei and Plonait, 1992). This inconsistent effect is explained by the observed impact of the prolactin treatment on UCP1 content of the iBAT. In the prolactin group this was significantly lower than that in the saline group. This reduction is consistent with the model suggested by Król et al. (Król et al., 2011), where gene expression in BAT during lactation appeared to be responsive to both prolactin and leptin levels. Normally in lactation the reduced heat production by BAT is more than compensated for by the heat production associated with milk synthesis. However, in non-lactating animals, as studied in Experiment 2, there is no compensatory heat production from milk synthesis for the reduced iBAT activity, and hence body temperature fell. The mechanism promoting greater thermal conductance of the pelage during lactation and mediating the elevated food intake in these animals remains unclear.

Our results showed that transferring lactating gerbils from warm to hot conditions resulted in reduced milk production, consistent with the HDL theory, but transferring them from warm to cold conditions did not elevate milk production, consistent with peripheral limitation hypothesis (or a limit on pup growth). As indicated by Speakman and Król (Speakman and Król, 2011), acceptance or rejection of the two theories (peripheral limitation and heat dissipation) depends on the milk production capacity relative to the heat dissipation capacity, and exactly what experimental protocol is used to test between them (Speakman and Król, 2011).

Thus, the key question is not whether the peripheral limitation or the HDL theory is correct, but rather at which ambient temperature peripheral limits (or pup growth) become more significant than heat dissipation limits. If the transitional temperatures between these controls are generally lower than the ambient temperatures experienced by the animals in the wild during their breeding seasons, then heat dissipation will be a more important phenomenon constraining lactation. However, if the transitional temperatures are normally higher than the temperatures experienced in the wild, then peripheral limits (or growth capacity) will be the more important constraint. For Mongolian gerbils we do not know the temperatures (or other factors driving heat balance such as solar radiation) that are routinely experienced by lactating females in the wild. However, ambient temperature and radiation conditions during summer, when they breed, probably oscillate each day between conditions where heat would impose a limit and conditions where peripheral limits would be more significant – making it difficult without hard data to judge which factor is most significant. Collecting data in the laboratory that define this transition temperature in more species, and comparing the derived transition temperature to field data on conditions experienced by lactating animals, should be a future priority.

LIST OF ABBREVIATIONS

DEE	daily energy expenditure
DEI	digestible energy intake
DLW	doubly labelled water
GEI	gross energy intake
HDL	heat dissipation limitation
iBAT	interscapular brown adipose tissue
MEI	metabolizable energy intake
MEO	milk energy output
RMR	resting metabolic rate
SusEI	maximum rate of sustained energy intake
UCP1	uncoupling protein 1

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AUTHOR CONTRIBUTIONS

D.-B.Y. and L.L. were involved in the design, execution and interpretation of the work, and in writing and revising the article. L.-P.W, Q.-S.C. and C.H. were involved in the execution of the work. D.-H.W. and J.R.S. were involved in the design and interpretation of the work and in writing and revising the article.

COMPETING INTERESTS

No competing interests declared.

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