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RESEARCH ARTICLE

Limits to sustained energy intake. XXI. Effect of exposing the mother, but not her pups, to a cold environment during lactation in mice

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SUMMARY

The capacity of females to dissipate heat may constrain sustained energy intake during lactation. However, some previous experiments supporting this concept have confounded the impact of temperature on the mothers with the impact on the pups. We aimed to separate these effects in lactating laboratory mice (MF1 strain) by giving the mothers access to cages at two ambient temperatures (10 and 21°C) joined by a tube. Food was available only in the cold cage, but females could also choose go to this cage to cool down while their pups were housed in the warmer cage. Control animals had access to the same configuration of cages but with both maintained at 21°C. We hypothesised that if females were limited by heat dissipation, alleviating the heat load by providing a cool environment would allow them to dissipate more heat, take in more food, generate more milk and hence wean heavier litters. We measured maternal energy budgets and monitored time courses of core body temperature and physical activity. To minimise the variance in energy budgets, all litters were adjusted to 12 (±1) pups. Females in the experimental group had higher energy intake ($F_{1,14}$=15.8, P=0.0014) and higher assimilated energy ($F_{1,13}$=10.7, P=0.006), and provided their pups with more milk ($F_{1,13}$=6.65, P=0.03), consistent with the heat dissipation limit theory. Yet, despite keeping demand constant, mean pup growth rates were similar ($F_{1,13}$=0.06, P=0.8); thus, our data emphasise the difficulties of inferring milk production indirectly from pup growth.

Key words: physiological limitation, choice, thermal conditions, heat dissipation limitation, MF1, body temperature, activity.

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INTRODUCTION

Sustained energy intake, the maximal amount of energy that can be ingested over a long period of high demand such as lactation, but also during physical exercise or cold exposure, sets the physiological boundaries in which an animal must reproduce, perform or exist (reviewed in Speakman and Król, 2011; Piersma and van Gils, 2011). During lactation in mammals, probably the most energy-demanding phase for females, peak sustained metabolism is reached and milk is synthesised at maximal rates (Millar, 1977; Glazier, 1985; Loudon and Racey, 1987; Speakman and Król, 2011). Several contrasting theories have been advanced to explain the limitations on maximal lactation performance in mammals. One idea is the 'heat dissipation limitation' (HDL) theory (Król et al., 2003; Król et al., 2007; Speakman and Król, 2010; Speakman and Król, 2011). Mechanistically, limits on heat dissipation could impact on females in several ways (Speakman, 2008). Milk synthesis might be lowered as a result of long-term elevation of body temperature and peak sustained energy intake. In addition, milk production could be further decreased as a consequence of the fact that blood flow is diverted away from the mammary gland to maximise thermal conductance. Alternatively, females might be obliged to terminate suckling bouts when faced with elevated hyperthermia due to being surrounded by large numbers of pups that impede heat loss (Leon et al., 1978; Scribner and Wynne-Edwards, 1994). All these ideas together point to a major underlying physiological constraint that arises from limitations in the capacity to dissipate heat during lactation. Yet, other limits on performance may also be important.

Based on a large body of work, it was essentially concluded that sustained energy intake in mammals is unlikely to be limited by the capacity of the energy-processing machinery (i.e. the central limitation hypothesis) (Hammond and Diamond, 1992; Rogowitz, 1998) (but see Sadowska et al., 2013). The peripheral limitation hypothesis suggests that all energy-expending organs such as the mammary glands would be limiting and some experiments actually provided support for the concept (Hammond et al., 1996; Zhao and Cao, 2009; Paul et al., 2010) (reviewed in Speakman and Król, 2011). In recent years, it it has also been suggested that pup demand could drive the females' peak sustained metabolism (Zhao et al., 2010; Simons et al., 2011; Duah et al., 2013) but experimental evidence for this concept has again been equivocal. Although most experimental work has so far been based on various strains of laboratory mice, evaluations of the limits on sustained lactation performance have also been performed in two species of vole (Wu et al., 2009; Simons et al., 2011), deer mice (Hammond and Kristan, 2000), cotton rats (Rogowitz, 1998) gerbils (Yang et al., 2013), hares (Hackländer et al., 2002; Valencak et al., 2009; Valencak et al., 2010), hamsters (Paul et al., 2010) and laboratory rats (Leon et al., 1983; Leon et al., 1985). Although the roles of peripheral limitation,

pup demand and heat dissipation in small mammals remain unclear, studies of the energy budgets of livestock animals such as cattle, goats, sheep, and rabbits strongly support the importance of heat loss during lactation as the major factor influencing milk yield (Forbes, 2007; Hale et al., 2003; Marai et al., 2001).

The primary data supporting the heat dissipation limit are the observations that as temperature is reduced, lactating females elevate their performance. Hence, performance (food intake and milk production) is always better at 21°C than it is at 30°C (Johnson et al., 2001; Król et al., 2003; Wu et al., 2009; Simons et al., 2011; Zhao and Cao, 2009; Yang et al., 2013), and is sometimes better at 10°C than it is at 21° (Johnson and Speakman, 2001), although this further increase at 10°C has only been partially replicated in several studies (i.e. food intake is increased but milk production is not, or has not been quantified) (e.g. Hammond and Diamond, 1992; Rogowitz, 1998; Yang et al., 2013). Although these data are consistent with heat dissipation limiting performance, particularly at higher temperatures, they are potentially confounded because both offspring and mother are exposed to the manipulated temperature. Thus, the effects could equally reflect the impact of temperature on pup demand (see also Zhao et al., 2013; Yang et al., 2013), potentially explaining the variable responses to the change from 21°C to 10°C. To avoid this potential problem, Król and colleagues (Król et al., 2007) shaved lactating female MF1 mice at 21°C, which elevated their capacity to dissipate heat, without influencing pup demand, and found an increase in their performance (significantly greater food intake, milk production and pup growth). Attempts to repeat this experiment have had mixed outcomes. Simons and co-workers (Simons et al., 2011) shaved voles and found a significant increase in pup growth, but the measured increase in milk production did not reach significance. Paul and colleagues (Paul et al., 2010) shaved hamsters and also found no impact, but there are some complexities in this experiment that make it less than ideal (see Paul et al., 2010; Speakman and Król, 2011). Zhao and colleagues (Zhao and Cao, 2009; Zhao et al., 2010) shaved Swiss mice and again found significantly increased food intake, but although trends in milk production and pup growth were in the anticipated direction, they were not statistically significant.

In a previous study, we (Valencak et al., 2010) utilised the intermittent suckling behaviour of lagomorphs to provide a different test of the HDL idea. Because hares only suckle their pups for a few minutes each day, it was feasible in this system to expose the mothers and offspring to different ambient temperatures. The consequences of these manipulations were complex and revealed an important role for pup demand early in lactation, and a potential role later on for peripheral limits or even HDL. Unlike lagomorphs, female mice are demand sucklers and feed their offspring continuously throughout the day. Because their food intake is elevated at peak lactation, and they also have significant water demands, they need to spend a considerable time away from their litter, feeding and drinking. By giving lactating females access to two cages, one at 21°C in which their pups were maintained and a second at 10°C where food and water were available, we could manipulate the capacity of females to dissipate heat, independent of the thermal environment experienced by their litters and hence pup demand. Note that females had to visit the cooler chamber to feed and drink, but could also go there voluntarily to potentially increase their heat dissipation capacity and elevate their performance, if they so wished.

We predicted that if the females were constrained by HDL, females feeding and drinking in the cooler environment would be capable of taking up more food, producing more milk for their pups and hence weaning larger pups, relative to control animals where the feeding cage was not cooled down. However, if the system were driven largely by pup demand then we would anticipate that the females with access to the cold would elevate their food intake, but would not elevate their milk production, and instead might have a lower extent of lactational hyperthermia (Gamo et al., 2013a).

MATERIALS AND METHODS

Animals and maintenance

MF1 mice (*Mus musculus* L.; 16 females and 16 males) aged 12 weeks were obtained from Charles River Laboratories (Margate, UK) for use in this study. After arrival at the Institute of Biological and Environmental Sciences, University of Aberdeen, female mice were housed in a custom-made cage setting that consisted of two plastic rodent cages connected *via* a V-shaped perforated plastic tube, facilitating locomotion between the cages (Fig. 1). The larger cage measured 48×15×13 cm while the smaller one had dimensions of 23×13×23 cm (Fig. 1). All 16 females were assigned at random to

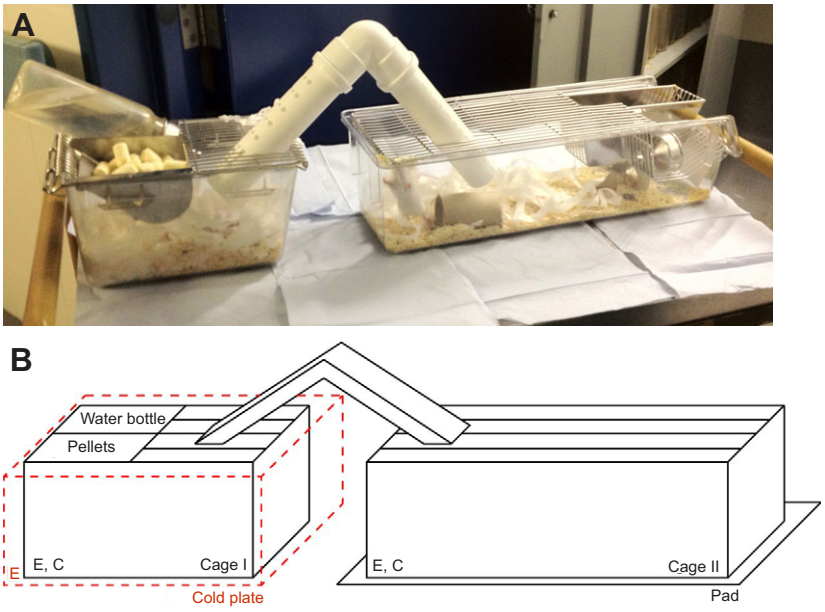

Fig. 1. Picture (A) and graphic depiction (B) of the cage set-up for each mouse in the experiment. Cage I (the smaller cage) contained food and water with a connecting pipe to allow free movement of the mice to the larger cage (cage II). The smaller cage was placed on a refrigerated counter to maintain the temperature at 10°C for the experimental (E) group. For the control (C) group, the temperature was maintained at 21°C. (The dashed line indicates that whereas the small cage was at 21°C in the control group, it was refrigerated in the experimental group.) Note that the hole for the drinking bottle in the larger cage had to be filled with a bottle lid to prevent young mice from leaving the cage.

one of two groups, an experimental group (E) and a control group (C). The two groups were treated alike, had the same parameters measured, but for females in the experimental group the small cage was cooled down to 10°C throughout lactation (Fig. 1B) and they were therefore forced to feed and drink at a lower ambient temperature. These mice could also choose to rest away from their pups at 10°C. To produce the lowered temperature in the small cage, it was placed on a refrigerated counter (Polar Refrigeration, Prestwick, Ayrshire, UK) (Fig. 1B). To familiarise mice in the experimental group with the lower ambient temperature in the food chamber, as well as to desensitise the females to the noise of the refrigeration device, we switched the refrigerated counter on for 2–3 h every day after the males had been removed during pregnancy and before it was switched on permanently for the remaining time of the study (2 days after parturition).

Females were paired with males for 11 days (Król and Speakman, 2003) to ensure all the females became pregnant. All animals were kept in a temperature-controlled room (21±1°C) on a 12 h:12 h light:dark cycle, with lights on at 05:00 h and with 20 min of dimmed light at either end of the light period. The room where the experiment took place was located within a closed specific pathogen-free (SPF) facility at the University of Aberdeen, with strict hygiene requirements and restricted access.

Two weeks before the pairing took place, female mice were surgically implanted with passive transponders in the peritoneum that monitored core body temperature and physical activity levels *via* a pad located underneath the larger of the two cages (Vital View System, Mini Mitter Inc., Bend, OR, USA) (see Gamo et al., 2013a; Gamo et al., 2013b) (Fig. 1B). As can be seen from the graphic depiction in Fig. 1B, no data on body temperature and activity could be obtained while the females were transferring from one cage to the other, and while they were in the small chamber (as there was no connection with the pad). From the periods when data were missing, it was possible to calculate the time spent in each of the cages, and the schedule of movements between the two cages. Although females put on weight substantially during pregnancy (Fig. 2) they were able at all times to easily transfer between the two cages.

Females had *ad libitum* access to food and water in the small cage throughout the experiment, and daily food intake was continuously monitored except during the mating period. Females were given two different diets during the experiment: (i) commercial rodent chow [CRM (P), Special Diets Services, BP Nutrition Witham, UK] with an energy content of 15.6 kJ g^{-1} during the acclimation and pregnancy phase and (ii) a diet containing 10% kcal from fat (12450B OpenSource Diet, Research Diets, Inc., New Brunswick, NJ, USA) with an energy content of 18.36 kJ g^{-1} from parturition onwards, with an acclimation phase of 2 days. This latter diet is harder and prevents grinding of food (Cameron and Speakman, 2010) and thus allows for more accurate food intake measurements while enabling the female to raise large litters. However, we previously observed a slightly elevated litter loss rate when mice fed on this diet and hence did not use it in pregnancy. Daily food intake data from periods when grinding occurred, as identified by the presence of pulverised pellet constituents during pregnancy primarily, were removed from the dataset. Equally, we removed data from one individual from the experimental group that reduced litter size from 12 to 9 pups by eating them on day 9 of lactation.

All 16 females gave birth ~21–23 days after introduction of the males and the average litter size observed was 12.5 pups (200 pups from 16 females). To reduce potential stress, measurements were suspended for 2 days around parturition. To reduce variation we adjusted litter size to 12±1 pups per female from day 4 of lactation. If natural litter size was 11, 12 or 13 we did not alter it, to keep the level of manipulation as low as possible. We did not observe any adverse effects on female food and pup milk intake or growth due to the adjustment of litter size. We have shown previously that peak energy demands in lactation depend only on litter size during lactation and are independent of pregnancy litter size (Duah et al., 2013).

All procedures concerning animal care and treatment were approved by the ethical committee for the use of experimental

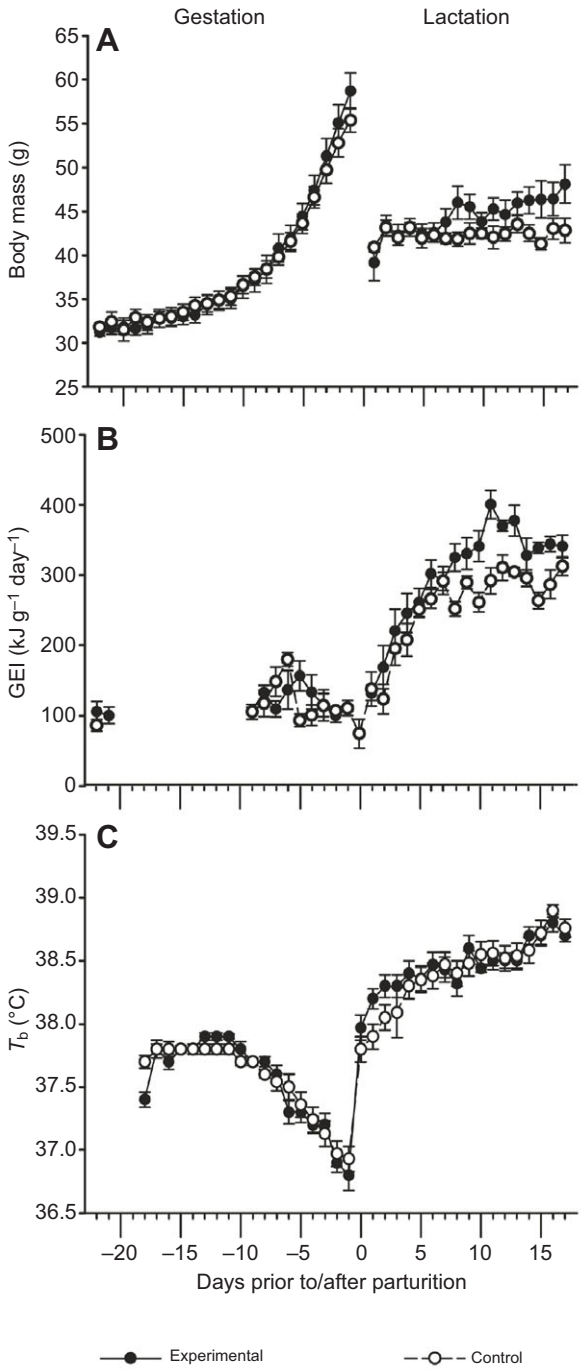

Fig. 2. Time courses of body mass (A), gross energy intake (GEI) in kJ day^{-1} (B) and core body temperature (T_b; C) over the duration of the experiment throughout pregnancy and lactation relative to parturition day. Means ± s.e.m.

animals of the University of Aberdeen, and licensed by the UK Home Office and performed under permit PPL 60/3705.

Data collection

From day 3 of lactation onwards we measured female body mass, food intake (FI), pup number and pup mass on a daily basis until weaning (day 18). In addition, we quantified daily energy expenditure in females between days 14 and 16 using the doubly labelled water (DLW) method (Butler et al., 2004). Briefly, mice were injected intraperitoneally with ~0.2 ml of DLW of known mass and characterised isotopic enrichment (*ca*. 329,000 p.p.m. ^{18}O, *ca*. 186,000 p.p.m. ^{2}H) on day 12 of lactation. The exact dose was quantified by weighing the syringe to the nearest 0.0001 g before and after administration. An initial blood sample of 100 µl was collected 1 h after the injection *via* tail tipping and stored in glass capillaries that were immediately flame-sealed with a blowtorch. The female was immediately returned to her cage and litter. A second and final blood sample was collected 49 h after the injection, timed to minimise the effects of diurnal variation in activity (Speakman and Racey, 1988). One blood sample from an additional mouse (that had no litter) and had not been injected with DLW was collected to assess the natural background abundance of ^{2}H and ^{18}O in the body water pools of the animals [Method C of Speakman and Racey (Speakman and Racey, 1987)]. Capillaries that contained the blood samples were vacuum distilled while water from the resulting distillate was used to produce CO_2 and H_2 (Vaanholt et al., 2013). The isotope ratios ^{18}O:^{16}O and ^{2}H:^{1}H were analysed using gas source isotope ratio mass spectrometry (Optima, Micromass IRMS and Isochrom µG, Manchester, UK). Samples were run alongside three laboratory standards for each isotope (calibrated to international standards) to correct delta values to p.p.m. (Vaanholt et al., 2013). Isotope enrichments were converted to values of daily energy expenditure using a single pool model as recommended for this size of animal (see Speakman, 1993). For the treatment of evaporative water loss in the calculation, we chose the assumption of a fixed evaporation of 25% of the water flux [eqn 7.17 from Speakman (Speakman, 1997)] as already successfully applied in lactation (Król et al., 2003). We monitored body temperature and physical activity of the mice continuously with records taken every minute for 23 h per day from the onset of pregnancy to the end of lactation. As given above, we used the Vital View transmitters (MiniMitter) (see Gamo et al., 2013b). The total raw dataset comprises thousands of measurements each of physical activity and body temperature. It is available to anyone wishing to use it on a collaborative basis.

Finally we collected faeces of the females over a 2 day period, dried the faeces to constant mass and determined the energy content with a bomb calorimeter (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL, USA) to assess metabolisable energy intake (MEI). MEI was computed as daily energy intake, i.e. gross energy intake (GEI, indicated as kJ g^{-1} day^{-1}), as follows:

$$\text{MEI} = \text{Dry food consumption} \times \text{Food energy content}, \quad (1)$$

where dry food consumption is in g day^{-1} and food energy content is in kJ g^{-1} dry $mass^{-1}$.

MEI was calculated as the difference between energy consumed and defecated, corrected for urinary protein losses. Urinary energy loss was assumed to be 3% of the digestible energy intake and thus digestive efficiency was determined as a percentage of gross energy intake digested (Drozdz, 1975). All litters were weaned on day 18 of lactation (see above). All procedures concerning animal care and treatment were approved by the ethical committee for the use of experimental animals of the University of Aberdeen, and licensed by the UK Home Office and performed under permit PPL 60/3705. Milk energy output (MEO) calculation was performed after Król and Speakman (Król and Speakman, 2003).

Data and statistical analysis

Data on food intake, GEI, average daily pup mass, daily energy expenditure (DEE) and body temperature were analysed with a repeated measures design as data were sampled from the same individuals. These models, so-called linear mixed effect models (lme models) included body mass of the female, experimental group and day of lactation as fixed factors and the ID of the female entered as a 'random' factor to fit separate intercepts for each animal. Assimilation efficiency (AE) data along with data for DEE were analysed in a separate data sheet with simple linear regression models (lm models) with each female going into the dataset once as we obtained faecal samples only once from each female as well as milk production. In the same way, we analysed data from peak lactation in the females, which was determined by the day when mean food intake was at a maximum. The observational data were compiled into a datasheet and a mean taken for each day for the experimental group mothers and the control group mothers, for each for the five activities. This was then taken as a percentage of the total minutes of observation that day. To analyse the observational data we again used linear mixed effects models in R with the exponential family set as 'binomial' because of the nature of the data (either in one cage or in the other, lmeR models included in the R package lme4). With the help of chi square tests that were run as test statistics, we analysed the data on the localisation of the females (time spent in the small cage or large cage). Again, these lmeR models were used to identify potential differences between the groups in the behaviour recordings. Before we ran statistical tests on the activity data that were assessed with the VitalView system, we compiled the data first to give the amount of time each mouse spent in the large cage in minutes. From that we calculated the amount of time spent in the small cage. Recall the small cage was where the ambient temperature was kept at 10°C in the experimental group. The day was split into four time periods, from 00:00 h to 05:59 h; 06:00 h to 11:59 h; 12:00 h to 17:59 h; and 18:00 h to 23:59 h.

Graphs were prepared in SigmaPlot 11.0 (Systat Software, Chicago, IL, USA) with all values used in the graphs presented as means with standard errors. We used linear mixed effects models to assess differences between the experimental and the control group for body mass, food intake and GEI during pregnancy and baseline period. All statistical analyses were conducted in R v2.13.1 (R Development Core Team, 2012).

RESULTS

Body mass and energy metabolism

At baseline, before the gestation period, body mass in the experimental and the control groups was 31.2±0.4 and 31.8±0.5 g, respectively, with no significant difference between them ($F_{1,14}$=0.21, P=0.6, n.s.). GEI at baseline was 99 kJ day^{-1} on average and, again, there was no difference between the two groups ($F_{1,14}$=0.43, P=0.53, n.s.). During pregnancy, body mass increased as expected in all females (partial for day of pregnancy: $F_{1,258}$=963.7, P<0.0001; Fig. 2A), independent of experimental group (partial for group: $F_{2,13}$=2.09, P=0.164). GEI during pregnancy was not affected by body mass of the individual, so as mice got heavier, they did not ingest more energy ($F_{1,104}$=0.02, P=0.9) and there was no difference in GEI between the two groups ($F_{1,13}$=0.0009, P=0.9; Fig. 2B). With respect to body mass over the entire experiment, we

Table 1. Body mass and energy metabolism for experimental and control groups

	Experimental	Control
Body mass (g)	44.54±0.4	42.38±0.2
Mean GEI (kJ day^{-1})	311.03±8.1	260.96±6.2
Asymptotic GEI (kJ day^{-1})	416.5±22.3	340.1±21.9
Asymptotic MEI (kJ day^{-1})	364.1±23.1	281.1±20.1
AE (%)	87.2±1.9	82.4±0.9
DEE (kJ day^{-1})	135.7±4.3	125.1±6.1
MEO (kJ day^{-1})	228.4±20.4	171.6±23.4
T_b (°C) at peak lactation	38.6±0.05	38.5±0.1
Litter mass at days 10–14 of lactation	85.3±1.7	83.3±5.7

Data are means ± s.e.m. over lactation and at asymptotic energy intake (days 10–14 of lactation).
GEI, gross energy intake; MEI, metabolisable energy intake; AE, assimilation efficiency; DEE, daily energy expenditure; MEO, milk energy output; T_b, body temperature.

observed a weak interaction between reproductive state of the females (baseline, pregnancy, lactation) and experimental group, with the lactating females in the experimental group being slightly heavier (reproductive state × experimental group: $F_{1,490}$=3.96, P=0.046; Fig. 2A). During lactation, GEI was dependent on body mass of the female ($F_{1,219}$=47.3, P<0.0001) and on the day of lactation ($F_{1,219}$=89.2, P<0.0001), and the mean was 16.1% higher in the experimental group than in the control group for whole of lactation ($F_{1,14}$=15.8, P=0.0014; Fig. 2B). Asymptotic GEI (days 10–14) was 18% higher in the experimental group than in the control group ($F_{1,13}$=5.96, P=0.029; Table 1) whereas MEI was 22% higher ($F_{1,13}$=7.2, P=0.02; Table 1). The efficiency of energy assimilation (AE) was 87.2% and 82.4% in the experimental and the control group and thus was significantly higher in the experimental females ($F_{1,13}$=10.7, P=0.006; Table 1).

Body temperature

Over the course of gestation, we observed similar body temperatures between the experimental and control group (partial for group: $F_{1,13}$=0.4, P=0.5; Fig. 2C). In addition, body temperature during pregnancy was affected by body mass (partial for body mass: $F_{1,196}$=283.1, P<0.0001) with the heavier females (towards parturition) having lower body temperatures (partial for body temperature: $F_{1,196}$=4.8, P<0.03).

During lactation, females significantly increased their body temperature by almost 1°C (partial for day of lactation: $F_{1,215}$=121.1, P<0.0001; Fig. 2C). However, we did not find any difference in body temperature between the experimental and control group (partial for experimental group: $F_{1,13}$=0.0, P=0.89). Equally, there was no difference in body temperature between the experimental and control group if the data were restricted to the period of peak lactation ($F_{1,13}$=0.03, P=0.87; Table 1).

Behavioural observations

Both the experimental and control animals spent about 60% of their time in the large cage and about 40% of their time in the small cage (data not shown). Between the four time periods, the mothers' activity was significantly different, i.e. the mothers spent more time in the small cage towards the end of the day and more time in the large cage during the start of the day (χ^2=178.34, P<0.0001, d.f.=3, chi square test). Interestingly, we observed that the control females shifted all their litters into the small cage where the food and drinking bottle were located, but none of the experimental group did this.

Over the course of lactation, we observed that the most frequent activity exhibited was suckling, which accounted for 55.6% of the experimental group's time and 52.4% of the control group's. This difference was not significant (Z=1.514, P=0.1300; Fig. 3). Resting was also found to be similar in the two groups, with the experimental group spending 14.5% of their time and the control group 14.7%, which was also not significantly different (Z=0.917, P=0.359). General activity, in contrast, showed a slightly bigger difference, taking up 11.3% of the experimental group's time and 13.9% of the control group's time, but this marginally failed to reach significance (Z=1.945, P=0.0517). The amount of time spent feeding (Z=0.562, P=0.574) and grooming (Z=0.636, P=0.525) also did not differ between experimental and control mice and amounted to 14.3% and 15.2% in the experimental and control group, respectively (Fig. 3). Grooming was the activity that the mothers spent the least time doing, with 4.3% for the experimental group and 3.8% for control group.

Over the course of lactation, females increased the time spent in the small cage (day of lactation: $F_{1,243}$=16.9, P=0.0001) with no difference between the groups (experimental group: $F_{1,13}$=0.7, P=0.4). While experimental females spent about 600 min per day at 10°C in the first week of lactation, they were found to stay 764 min per day there in the last week of lactation. Note that, as described above, all control females shifted their litters into the small cage and thus had the nest in the same cage as the food and the drinking water.

Milk energy transfer and pup growth

MEO, as assessed from the difference between MEI and DEE (measured with the DLW technique) (Król and Speakman, 2003), around the peak of lactation was 25% higher in the experimental than in the control females ($F_{1,13}$=6.65, P=0.03; Table 1). Further, as we allowed litter size to vary between 12±1 pups (see Materials and methods), we identified an interaction between experimental group and actual pup number (interaction: $F_{1,13}$=6.3, P=0.036), with the experimental females having more milk production for a given number of pups. As can be seen in Fig. 4, pups in both groups rapidly increased their body mass, with pups from experimental females weighing 6.2±0.2 g on day 10 and pups from controls weighing 5.8±0.2 g, respectively. This difference was, however, not significant ($F_{1,12}$=2.29, P=0.16). Mean total or average pup mass was influenced by individual female body mass ($F_{1,229}$=21.7, P<0.0001) but not by experimental group ($F_{1,13}$=0.06, P=0.8). Weaning mass was 8.88±1.3 and 8.77±1.9 g, respectively, in the experimental group and the control group and was again not significantly different ($F_{1,13}$=0.1, P=0.8).

DISCUSSION

By forcing females to be exposed to low ambient temperature while feeding and drinking, we clearly increased their sustained energy

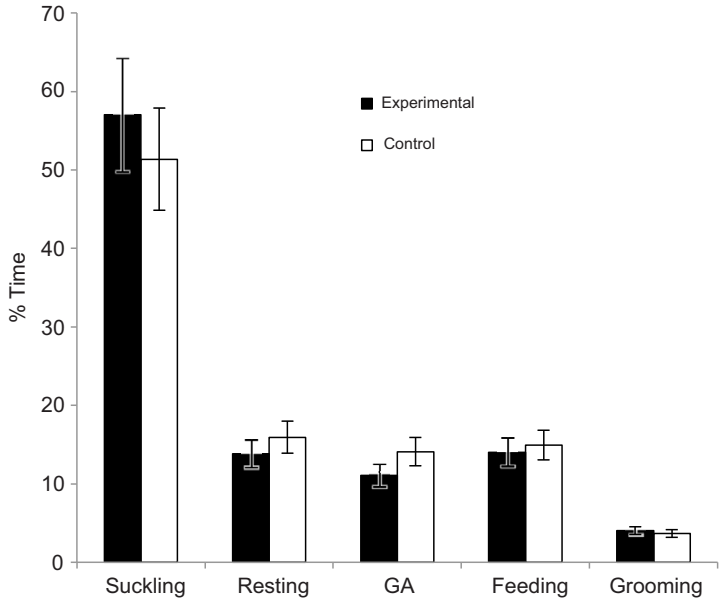

Fig. 3. Mean percentage of time spent on different behavioural activities [suckling, resting, general activity (GA), feeding, grooming] by experimental and control females. Means ± s.e.m. over the course of lactation.

intake and their milk production (Fig. 2B, Table 1). Thus, we conclude that the chilled environment of the small food chamber alleviated the physiological constraint on the females and allowed them to perform better by reaching higher energy intake and higher milk production. This experiment clearly indicates that females were not limited by the capacity of their gastrointestinal tract, as they could eat ~3 g or 50 kJ more than their counterparts from the control group when they had access to the cooler cage for feeding and drinking. Although it may not sound a large increase, 3 g food is approximately equal to the total daily food intake in a non-reproductive laboratory mouse (Johnson et al., 2001). Mice from the MF1 strain thus were not centrally limited as suggested by the central limitation hypothesis (Weiner, 1992; Koteja, 1996). These data support several previous studies that have also concluded that the capacity of the gastrointestinal tract to absorb energy is probably not a primary limiting factor on lactation performance (Johnson et al., 2001; Rogowitz, 1998; Hammond et al., 1996; Zhao and Cao, 2009; Wu et al., 2009).

Although female mice therefore appeared capable of increasing their food intake, providing females with more pups led to infanticide rather than to higher observed energy budgets (Hammond and Diamond, 1992; Johnson et al., 2001; Duah et al., 2013). In our chosen experimental setup, females could have reacted to the lower ambient temperature in the food chamber in the same way, i.e. by abandoning their litter early in lactation or reducing the number of pups to reduce demand. Instead, as the behavioural data showed, females in our experiment chose to rest away from their pups and simultaneously improved their lactation performance by producing more milk. Also, their energy assimilation was significantly improved, so they could extract nutrients from the ingested food more efficiently than control animals. These data on elevated milk production are also incompatible with the peripheral limitation hypothesis (Hammond et al., 1996; Zhao and Cao, 2009), which posits that the limiting factor in the system is the capacity of the mammary glands to synthesise milk.

One interpretation of our data is that the colder ambient temperature in the smaller cage enabled the females to more efficiently dissipate heat while feeding and drinking. The level of limitation in the experimental females thus may have been lower than in animals from the control group but with the same cage setting (Table 1). Interestingly, the females in the experimental group spent only 15% of their time feeding (Fig. 3), yet spent 40% of their time in the small cage. They consequently spent substantially more time in the cooler cage than they needed to meet their daily energy requirements. Comparisons with the control group in this respect, however, are not straightforward because all of the control animals moved their pups and nests into the smaller cage and they spent much of their time suckling in the small cage (P.W., A.W. and T.G.V., personal observation). The reason why the control animals moved their litters to the small cages is unclear, but given they all did, it suggests that the more confined space felt safer. Interestingly, we observed that the time spent in the small chilled cage increased over the course of lactation in both groups. We suggest that early in lactation, when pup demand might drive maternal milk production, females might have chosen to stay with their pups, but later in lactation it was more important to rest away from the pups in the cold cage to dissipate more heat and enhance their milk production. The finding that the control group showed the same increase in time spent in the small cage over the course of lactation was probably related to the fact that control females shifted their litters to the small cage in the first week of lactation and did not move them again later. All eight of the experimental animals chose to have the nest with their pups in the large cage that was at 21°C. This supports the idea that the optimal temperature during lactation may differ for the mothers and the pups (Simons et al., 2011). Generally, mothers may benefit from it being colder and hence alleviate heat dissipation constraints, leading to greater milk production, while pups may prefer it warmer where they grow more efficiently.

Consistent with the animals attempting to maximise milk production, temporary exposure to the lower temperature (10°C) did not lower the high body temperatures observed in the control lactating mice (Fig. 2C). These patterns in body temperature were also consistent with the patterns observed previously in MF1 mice at 21°C (Gamo et al., 2013a; Gamo et al., 2013b). Thus, females allowed body temperature to be high over lactation relatively independent of their thermal environment and fur thickness. This again suggests that lactating MF1 females face an intrinsic physiological limit imposed by their capacity to dissipate heat, instead of the alternative explanation that the capacity of their mammary glands (peripheral limitation) might limit the system.

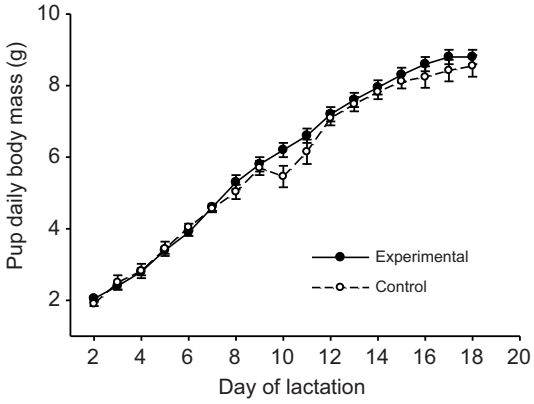

Fig. 4. Pup mean (±s.e.m.) daily body mass (g) in the course of lactation in both experimental and control females.

One confusing aspect of our data was why there was no impact of the elevated milk production in the experimental group (Table 1) on the trajectories of pup growth (Fig. 4). This might suggest that the pups were considerably less efficient at converting the milk into growth when their mothers had access to the cold cage. The difference in milk export at peak lactation was 56.8 kJ day^{-1}. Assuming this was sustained over the 8 days of peak lactation, this would amount to a total of 454.4 kJ and at an average litter size of 12 pups that would amount to 37.87 kJ pup^{-1}. According to pup growth rates at 21°C (Król et al., 2003), the energy actually available for growth would be about 10 kJ. If this was delivered and stored as fat then the actual difference in pup body mass would be about 0.25–0.3 g. This is close to the actual difference in pup mass at weaning of 0.18 g. The failure to detect an impact on growth could conceivably be a power issue at the low sample size (at a sample size of eight, the power to detect a difference of 0.27 g pup^{-1} with the observed variation is only 34%). However, if the energy was mostly delivered and stored as protein, then the body size effect would be much greater, in the region of 2 g, and the power to detect this magnitude of effect on growth is >99.9%. As we do not know the form in which the excess milk was delivered or stored, we do not know for certain whether the absence of a significant effect on pup growth is anomalous or not.

One additional possibility is that because the experimental group pups occupied a much larger cage than those in the control group, they were more physically active and this elevated their energy requirements making them less efficient at translating the extra milk into growth. We did not monitor physical activity in the offspring, and consequently we cannot eliminate this as a possibility. An extra 37.6 kJ day^{-1} on the energy budgets of the pups would, however, require an enormous amount of physical activity to be burned off. Although we did not make systematic observations of the pups while observing the mothers, this level of extra activity in the experimental pups was not immediately apparent. Nevertheless, without systematic observations we cannot eliminate it as a possibility. If this was the case, then an alternative explanation for the observations is that in the experimental group the greater milk production was driven by the greater pup demands.

It seems most likely that some combination of effects was at play, i.e. we had low power to detect an effect on growth because some of the extra milk was wasted on increased pup activity as they were located in the bigger cage, making growth less efficient, combined with the transfer of milk as fat making the expected impact on the growth curves very small. Hence, despite our best efforts to design an experimental situation that would separate the impacts of heat dissipation and pup demand, we ultimately failed to completely do so – although these observations are nonetheless valuable in unambiguously rejecting both the central and peripheral limitation hypotheses, and, apart from the impact on pup growth, are completely consistent with the heat dissipation limit hypothesis. Our experience suggests that designing experiments that allow complete separation between the heat dissipation limit theory and the pup demand idea will be difficult, but such experiments are an important future step in this field of research.

The conflicting observations on milk production and pup growth highlight the importance of the assessment of milk production, either directly, as is possible in lagomorphs such as hares (Valencak et al., 2009; Valencak et al., 2010), or indirectly, by using the DLW technique (Johnson et al., 2001; Król et al., 2007) (reviewed in Speakman and Król, 2011), to allow conclusions on physiological limits to be drawn. Simple observations that growth is not significantly increased in animals that ostensibly appear to be in identical conditions (e.g. Zhao and Cao, 2009; Zhao et al., 2010) cannot be used to infer that milk production of the female is unchanged, and hence that limits have not been altered.

LIST OF ABBREVIATIONS

AE	assimilation efficiency
DEE	daily energy expenditure
DLW	doubly labelled water
FI	food intake
GEI	gross energy intake
HDL	heat dissipation limitation
MEI	metabolisable energy intake
MEO	milk energy output
T_b	body temperature

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AUTHOR CONTRIBUTIONS

T.G.V. and J.R.S. conceived and designed the experiment; T.G.V., P.W., A.W., S.E.M., L.M.V., C.H., E.K. and J.R.S. carried out data collection, behavioural observations, implantation of transmitters, DLW analyses and interpretation of findings; T.G.V. and J.R.S. drafted and revised the manuscript.

COMPETING INTERESTS

No competing interests declared.

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