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 2 ambient temperatures (10 and 21°C) joined by a tube. Food was available only in the cold cage, but females could also choose to go to this cage to cool down while 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, intake 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 minimize the variance in energy budgets all litters were adjusted to 12 (+/- 1) pups. Females in the experimental group had higher energy intake (F1,14 = 15.8; p=0.0014), higher assimilated energy (F1,13 = 10.7; p=0.006) and provided their pups with more milk (F1,13 = 6.65; p=0.03), consistent with the heat dissipation limit theory. Yet, despite keeping demand constant, mean pup growth rates were similar (F1,13 = 0.06; p=0.8) thus our data emphasise the difficulties of inferring milk production indirectly from pup growth.
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 Krol, 2011 and Piersma and van Gils, 2012). 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 Krol, 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)” (Krol et al., 2003; Krol et al., 2007; Speakman and Krol, 2010; 2011). Mechanistically, limits on heat dissipation could impact on the females in several ways (Speakman, 2008). Milk synthesis might be lowered due to 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, the females might be obliged to terminate suckling bouts when faced by 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 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 largely concluded that sustained energy intake in mammals is unlikely 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 Krol, 2011). In recent years, it was also 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 been so far 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, in press), hares
(Hackländer et al., 2002; Valencak et al. 2009, 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; Krol et al., 2003; Wu et al., 2009; Simons et al., 2011; Zhao and Cao, 2009; Yang et al., 2013), and sometimes better at 10° 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 like in 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 form 21° to 10° C. To avoid this potential problem, Krol 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 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 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 discussion in Paul et al., 2010; Speakman and Krol, 2011). Zhao and Cao (2009) and Zhao et al., (2010) shaved Swiss mice and again found significantly increased food intake but trends in milk production and pup growth were in the anticipated direction but not statistically significant.

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 of which where their pups were maintained at 21°C, and a second where food and
water was available kept at 10°C, 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 would be constrained by HDL, females feeding and drinking
in the cooler environment would be capable of taking up more food, producing more milk for
the 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).

METHODS

Animals and maintenance

16 female and 16 male MF1 mice (Mus musculus L.) aged 12 weeks were obtained from
Charles River Laboratories, UK and used for 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. 1A, B). The
larger cage measured 48x15x13cm while the smaller one had dimensions of 23x13x23cm
(Fig.1). All 16 females were assigned at random to one of two groups, an experimental group
(E) and a control group (C). Both groups were treated alike, had the same parameters
measured, but females in the experimental group had the small cage cooled down to 10°C
throughout lactation (Fig. 1B) and 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 located in a refrigerated counter (Polar Refrigeration, 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 refrigerating device, we switched the refrigerated counter on for 2-3 hours every day after the males had been removed during pregnancy and before it was switched on permanently for the remaining time of the study (two days after parturition).

Females were paired with males for 11 days (Krol and Speakman, 2003) to ensure all the females became pregnant. All animals were kept in a temperature-controlled room (21°C ± 1°C) at a 12:12h light-dark cycle with lights on at 0500h with 20 minutes of dimmed light at either end of the light period. The room where the experiment took place was located within a closed 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 (Fig. 1B, Vital View System: Mini Mitter Inc., OR, US, see Gamo et al., 2013a; 2013b). 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 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 throughout the experiment in the small cage and daily food intake was continuously monitored except for 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) at an energy content of 15.6 kJ g⁻¹ during the acclimation and pregnancy phase and ii) a diet containing 10% kcal from fat (Open source diet, product code: 12450B, Research Diet Inc., NJ, US) at an energy content of 18.36 kJ g⁻¹ given to the animals 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 most accurate food intake measurements while enabling the female to raise large litters. However, we previously observed a slightly elevated litter loss rate when feeding on this diet and hence did not use it in pregnancy. Daily food intake data when grinding was observed by
identifying 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 sixteen females gave birth approximately 21-23 days after introduction of the males and the average litter size observed was 12.5 (200 pups from 16 females). To reduce potential stress, measurements were suspended for 2 days around parturition. To reduce variation we adjusted litter size at 12 ± 1 pups per female from day 4 of lactation. If natural litter size was 11, 12 or 13 we did not do any manipulation to keep the level of manipulation as low as possible. We did not observe any adverse effects on both female food and pup milk intake and 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).

Data collection

From day 3 of lactation onwards we measured female body weights, food intake (FI), pup number and pup mass on a daily basis until weaning (day 18). In addition, we quantified daily energy expenditure in the 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 doubly labelled water of known mass and characterized isotopic enrichment (c. 329000 ppm 18O, c. 186000 ppm 2H) on day 12 of lactation. The exact dose was quantified by weighing the syringe to the nearest 0.0001g before and after administration. An initial blood sample of 100ul was collected 1 hour 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. Forty-nine hours after the injection a second and final blood sample was collected timed to minimize the effects of diurnal variation in activity (Speakman and Racey, 1988). One blood sample of an additional mouse (that had no litter) and had not been injected with DLW was collected to assess the natural background abundances of 2H and 18O in the body water pools of the animals (Speakman and Racey, 1987 Method C). Capillaries that contained the blood samples were vacuum distilled while water from the resulting distillate was used to produce CO2 and H2 (Vaanholt et al., 2013). The isotope ratios 18O:16O and 2H:1H 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 ppm (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 by 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 (equation 7.17 from Speakman, 1997) as already successfully applied in lactation (Krol et al., 2003).

We monitored body temperature and physical activity of the mice continuously with records taken every minute for 23 hours per day from the onset of pregnancy to the end of lactation. As given above, we used the Vital View™ transmitters (MiniMitter OR, USA, 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 mass constancy and determined the energy content with a bomb calorimeter (Parr 6200 Calorimeter, Parr Instrument Company, Illinois, USA) to assess metabolisable energy intake (MEI). Metabolisable energy intake was computed as daily energy intake i.e. gross energy intake (indicated as kJ per gram per day) was calculated as follows:

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\text{Dry food consumption (g. day}^{-1}\times \text{Food energy content (kJ. g}^{-1}\text{ dry mass})
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Metabolisable Energy Intake (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 PPL 60/3705. Milk energy output (MEO) calculation was done after Krol and Speakman, (2003).

Data and Statistical analysis

Data on food intake, gross energy 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 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 5 activities. This
was then taken as a percentage of the total minutes of observation taken that day. To analyse
the observational data we again used linear mixed effects models in R with the exponential
family set as “binomial” due to 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
small cage or large cage). Again, these “lmeR” models were used to identify potential
differences between the groups in the behaviour recordings. Before we run 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 4
time periods, from 00:00 to 05:59; 06:00 to 11:59; 12:00 to 17:59; 18:00 to 23:59.

Graphs were prepared in SigmaPlot 11.0 (Systat Software, Chicago, IL, USA) with all values
used in the graphs means with standard errors. We used linear mixed effects models to assess
differences between the experimental and the control group of body weights, food intake,
gross energy intake (GEI) during pregnancy and baseline period. All statistical analyses were
conducted in R (2.13.1; R Development Core Team 2011).

RESULTS

Body weights and energy metabolism

At baseline, before the gestation period, body weights in the experimental and the control
groups were 31.2g ± 0.4 and 31.8g ± 0.5 respectively and were not significantly different
from each other (F1,14= 0.21; p=0.6 n.s.). Gross energy intake (GEI) at baseline was 99 kJ d⁻¹
on average and again, there was no difference between the two groups (F1,14 0.43; p=0.53
n.s.). During pregnancy, body weights increased as expected in all females (partial for day of
pregnancy: F1,258=963.7; p< 0.0001; Fig.2A), independent of experimental group (partial for
group: F2,13= 2.09; p=0.164). Gross energy intake (GEI) during pregnancy was not affected by
body mass of the individual, so as mice got heavier mice they did not ingest more energy
(F₁,₁₀₄ = 0.02; p=0.9) and there was no difference in GEI between the two groups (F₁,₁₃ = 0.0009; p=0.9, Fig. 2B). With respect to body weights over the entire experiment, we observed a weak interaction between reproductive state of the females (baseline, pregnancy, lactation) and experimental group, with the lactating females in the experimental groups being slightly heavier (reproductive state * experimental group: F₁,₄₀₀ = 3.96; p=0.046; Fig. 2A).

During lactation, gross energy intake was dependent on body mass of the female (F₁,₁₂₁₀ = 47.3; p<0.0001), on the day of lactation (F₁,₁₂₁₀ = 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 (F₁,₁₄ = 15.8; p=0.0014; Fig.2B). Asymptotic GEI (days 10 to 14) was 18% higher in the experimental group than in the control group (F₁,₁₃ = 5.96; p=0.029; Table 1) whereas MEI was 22% higher (F₁,₁₃ = 7.2; p=0.02; Table 1). The efficiency of energy assimilation was 87.2 and 82.4% in the experimental and the control group and thus was significantly higher in the experimental females (F₁,₁₃ = 10.7; p=0.006; Table 1).

**Milk energy transfer and pup growth**

Milk energy output (MEO) as assessed from the difference between MEI and DEE (measured with the DLW technique: Krol and Speakman, 2003), around the peak of lactation was 25% higher in the experimental than in control females (F₁,₁₃ = 6.65; p=0.03; Table 1). Further, as we allowed litter size to vary between 12 ± 1 (see Methods), we identified an interaction between experimental group and actual pup number (interaction: F₁,₁₃ = 6.3; p=0.036) with the experimental females having more milk production at a given number of pups. As can be seen in Fig. 6, pups in both groups rapidly increased their body mass, with pups from experimental females weighing 6.2g ± 0.2 on day 10 and pups from controls weighing 5.8g ± 0.2 respectively. This difference was however not significant (F₁,₁₂ = 2.29; p=0.16). Mean total or average pup mass was influenced by individual female body mass (F₁,₂₂₀ = 21.7; p<0.0001) but not by experimental group (F₁,₁₃ = 0.06; p=0.8). Weaning weights were 8.88g ± 1.3 and 8.77 ± 1.9 respectively in the controls and were again not significantly different (F₁,₁₃ = 0.1; p=0.8).

**Body temperature**

Over the course of gestation, we observed similar body temperatures between the experimental groups (partial for group: F₁,₁₃ = 0.4; p=0.5, Fig. 2C). In addition, body temperature during pregnancy was affected by body mass (partial for body mass:
F1,196=283.1; p<0.0001) with the heavier females (towards parturition) having lower body temperatures (partial for body temperature: F1,196=4.8; p<0.03).

During lactation, females significantly increased their body temperature by almost 1°C (partial for day of lactation: F1, 215= 121.1; p<0.0001, Fig. 2C). However, we did not find any difference in body temperatures between the two experimental groups (partial for experimental group: F1, 13=0.0; p=0.89). Equally, there was no difference in body temperature between the two experimental groups if the data was restricted to the period of peak lactation (F1, 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 and about 40% in the small cages (data not shown). Between the 4 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 (X² = 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 was 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 groups’ time and 52.4% of the control group. This difference was not significant (Z = 1.514; P = 0.1300; Fig. 3). Resting was also found to be similar in both 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, on the other hand, showed a slightly bigger difference with 11.3% of the experimental group and 13.9% of the controls group time spent doing general activities 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 least of their time doing, 4.3% of time the experimental mothers spent and 3.8% of the control group.

Over the course of lactation, females increased the time spent in the small cage (day of lactation: F1, 243=16.9, p=0.0001) with no difference between the groups (experimental group: F1, 13=0.7, p=0.4). While experimental females spent about 600 minutes per day at 10 °C in
the first week of lactation, they were found to stay 764 minutes per day there in the last week of lactation. Note that as described above, control females shifted all their litters into the small cage and thus had the nest in the same cage as the food and the drinking water.

**DISCUSSION**

By forcing females to be exposed to low ambient temperature while feeding and drinking, we clearly increased their sustained energy 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 of the female and allowed them to perform better by reaching higher energy intakes and higher milk production. This experiment clearly indicates that females were not limited by the capacity of their gastrointestinal tract, as they could eat approximately 3g, 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, 3g 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 et al., 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 lead 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 amount of 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 feeding and drinking 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 (Wright, Weir and Valencak, pers. observation). The reason why the control animals moved their litters to the small cages is unclear, but given they all did it suggested 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 the 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 fact that the control group showed the same increase in the time spent in the small cage over the course of lactation most likely was related to the fact that control females, shifted their litters to the small cage in the first week of lactation and later onwards did not move them again. All 8 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 temperatures 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 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; b). Thus, females allowed body temperature to be high over lactation relatively independent from 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.

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. 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. According to pup growth rates at 21°C (Krol 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 weight would be about 0.25 to 0.3g. This is close to the actual difference in pup mass at weaning of 0.18g. The failure to detect an impact on growth could conceivably be a power issue at the low sample size (at a sample size of 8 the power to detect a difference of 0.27g/pup with the observed variation was only 34%). However if the energy was mostly delivered and stored as protein then the body size effect would be much greater, and in the region of 2g, and the power to detect this magnitude of effect on growth was >99.9%. Since we do not know the form in which the excess milk was delivered or stored we do not know for certain if 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/d 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 was 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, which 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 are an important future step in this field of research.

The conflicting observations on milk production and pup growth highlight the importance of
assessments of milk production, either performed directly, like it can be done in lagomorphs
such as hares (Valencak et al., 2009; Valencak et al., 2010) or indirectly, by using the DLW
technique (Johnson et al., 2001; Krol et al., 2007; reviewed in Speakman and Krol, 2011), to
allow conclusions on physiological limits. 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.

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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
SPF Specific Pathogen Free
Tb Body Temperature

REFERENCES


Johnson, M. S. and Speakman, J. R. (2001). Limits to sustained energy intake. V. Effect of


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FIGURE LEGENDS

Fig. 1: Picture (panel A) and graphic depiction (panel B) of the cage set-up for each mouse in experiment. The cage on the left hand side allowed the mice access to food and water with a connecting pipe to allow free movement of the mice to the larger cage. Note that the hole for the drinking bottle in the big cage had to be filled with a bottle lid to prevent young mice from leaving the cage.

Fig. 2: Time courses of body weight (panel A), gross energy intake (GEI) in kJ day⁻¹ (panel B) and core body temperature (Tb) (panel C) over the duration of the experiment throughout pregnancy and lactation relative to parturition day. Means ± SEM

Fig. 3: Mean percentage of time spent on different behavioural activity classes (suckling, resting, general activity (GA), feeding, grooming) of experimental and control females. Means over course of lactation ± SEM.

Fig. 4: Pup average daily body weight (g) in the course of lactation in both experimental (filled circles) and control females (open circles). Means ± SEM
Fig. 2

A. Body weight (g) during gestation and lactation.

B. GEI (kJ g\(^{-1}\) d\(^{-1}\)) during gestation and lactation.

C. \(T_b\) (°C) during gestation and lactation.


- - - experimental
- - - control

days prior / after parturition
Fig. 3

The graph shows the mean percentage of time spent on different activities by the experimental and control groups. The activities include Suckling, Resting, GA, Feeding, and Grooming. The experimental group shows significantly higher time spent on Suckling compared to the control group. There is little difference in the other activities.
Fig. 4

Pup average daily body weight (g) vs. day of lactation for experimental and control groups.
Table 1: Energy budgets in experimental and control females over mean lactation and at asymptotic energy intake (days 10-14 of lactation) ± standard error of the mean

<table>
<thead>
<tr>
<th></th>
<th>Experimental</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>44.54 ± 0.4</td>
<td>42.38 ± 0.2</td>
</tr>
<tr>
<td>Mean GEI (kJ day⁻¹)</td>
<td>311.03 ± 8.1</td>
<td>260.96 ± 6.2</td>
</tr>
<tr>
<td>Asymptotic GEI (kJ day⁻¹)</td>
<td>416.5 ± 22.3</td>
<td>340.1 ± 21.9</td>
</tr>
<tr>
<td>Asymptotic MEI (kJ day⁻¹)</td>
<td>364.1 ± 23.1</td>
<td>281.1 ± 20.1</td>
</tr>
<tr>
<td>AE (%)</td>
<td>87.2 ± 1.9</td>
<td>82.4 ± 0.9</td>
</tr>
<tr>
<td>DEE (kJ day⁻¹)</td>
<td>135.7 ± 4.3</td>
<td>125.1 ± 6.1</td>
</tr>
<tr>
<td>MEO (kJ day⁻¹)</td>
<td>228.4 ± 20.4</td>
<td>171.6 ± 23.4</td>
</tr>
<tr>
<td>Tb (°C) at peak lactation</td>
<td>38.6 ± 0.05</td>
<td>38.5 ± 0.1</td>
</tr>
<tr>
<td>Litter weight at days 10-14 of lactation</td>
<td>85.3 ± 1.7</td>
<td>83.3 ± 5.7</td>
</tr>
</tbody>
</table>