

## RESEARCH ARTICLE

# Limits to sustained energy intake. XVIII. Energy intake and reproductive output during lactation in Swiss mice raising small litters

Zhi-Jun Zhao<sup>1,2,\*</sup>, De-Guang Song<sup>2</sup>, Zhen-Cheng Su<sup>2</sup>, Wen-Bo Wei<sup>2</sup>, Xian-Bin Liu<sup>2</sup> and John R. Speakman<sup>3,4,\*</sup>

<sup>1</sup>School of Life and Environmental Sciences, Wenzhou University, Wenzhou 325027, China, <sup>2</sup>School of Agricultural Science, Liaocheng University, Liaocheng, Shandong 252059, China, <sup>3</sup>Key State Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100100, China and <sup>4</sup>Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, AB24 2TZ, UK

\*Authors for correspondence (zhao73@foxmail.com; J.Speakman@abdn.ac.uk)

### SUMMARY

Limits to sustained energy intake (SusEI) during lactation in Swiss mice have been suggested to reflect the secretory capacity of the mammary glands. However, an alternative explanation is that milk production and food intake are regulated to match the limited growth capacity of the offspring. In the present study, female Swiss mice were experimentally manipulated in two ways – litter sizes were adjusted to be between 1 and 9 pups and mice were exposed to either warm (21°C) or cold (5°C) conditions from day 10 of lactation. Energy intake, number of pups and litter mass, milk energy output (MEO), thermogenesis, mass of the mammary glands and brown adipose tissue cytochrome *c* oxidase activity of the mothers were measured. At 21 and 5°C, pup mass at weaning was almost independent of litter size. Positive correlations were observed between the number of pups, litter mass, asymptotic food intake and MEO. These data were consistent with the suggestion that in small litters, pup requirements may be the major factor limiting milk production. Pups raised at 5°C had significantly lower body masses than those raised at 21°C. This was despite the fact that milk production and energy intake at the same litter sizes were both substantially higher in females raising pups at 5°C. This suggests that pup growth capacity is lower in the cold, perhaps due to pups allocating ingested energy to fuel thermogenesis. Differences in observed levels of milk production under different conditions may then reflect a complex interplay between factors limiting maternal performance (peripheral limitation and heat dissipation: generally better when it is cooler) and factors influencing maximum pup growth (litter size and temperature: generally better when it is hotter), and may together result in an optimal temperature favouring reproduction.

Key words: temperature, heat dissipation limit, reproductive performance, milk energy output, peripheral limit, pup growth, thermogenesis.

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### INTRODUCTION

The sustained maximum rate of energy intake (SusEI) is the maximal rate of energy intake that animals can maintain over sufficiently long periods so that energy demands are fuelled only by food intake, rather than by depletion of energy reserves (Hammond and Diamond, 1997; Piersma and van Gils, 2011). Limits on SusEI are important because they establish the upper energetic constraint on the ability of animals to survive and reproduce (Kirkwood, 1983; Peterson et al., 1990; Hammond and Diamond, 1992; Weiner, 1992; Hammond and Diamond, 1997; Piersma and van Gils, 2011). Lactation is the most energetically demanding period for female mammals, and has previously been used extensively to attempt to elucidate the nature of the limitations on SusEI (Hammond and Diamond, 1997; Johnson et al., 2001a; Speakman, 2008; Speakman and Król, 2005; Valencak et al., 2010; Piersma and van Gils, 2011). Earlier studies suggested that SusEI might be imposed by the capacities of the energy-consuming organs, such as the mammary glands during lactation – the ‘peripheral limitation’ hypothesis (Peterson et al., 1990; Hammond and Diamond, 1992; Weiner, 1992; Hammond et al., 1994; Hammond and Kristan, 2000; Johnson et al., 2001a; Bacigalupe and Bozinovic, 2002; Speakman and Król, 2005).

Consistent with the peripheral limitation hypothesis, Hammond and colleagues (Hammond et al., 1996) manipulated Swiss mice by surgically removing half of the mammary tissue. They found that productivity in the halved glands did not increase, indicating that the mammary tissue was already working at maximal capacity (Hammond et al., 1996). However, milk production was increased in MF1 mice after exposure to the cold, and was also decreased in hot conditions (Johnson and Speakman, 2001; Król and Speakman, 2003a; Król and Speakman, 2003b; Speakman and Król, 2005). These last observations were contrary to the predictions of the peripheral limitation hypothesis. To explain these data, it has been suggested that SusEI may be limited by the capacity of the female to dissipate body heat – the ‘heat dissipation limit (HDL)’ theory (Speakman and Król, 2005; Speakman and Król, 2010). Under the HDL theory, cold exposure resulted in a relaxation of the limitation on the heat dissipation capacity, allowing the lactating mice to increase both food intake and milk production. In contrast, when exposed to the hot conditions, the females had a reduced ability to dissipate heat, resulting in a lower food intake and less milk production (Speakman and Król, 2011). Consistent with this idea, dorsally shaved MF1 mice had increased thermal conductivity, and were thus able to produce more milk and wean heavier litters (Król

et al., 2007). Studies on Brandt's voles (Wu et al., 2009) and Mongolian gerbils (Yang et al., 2013) both revealed that high temperatures (30°C) induced a reduction in milk production. Although these data supported the HDL theory, the effect in voles was greatest in females raising the largest litters, and in small litters there was no significant effect of high temperature (Wu et al., 2009). This could mean that another limit was reached reflecting the capacity of the pups to grow. It is therefore uncertain whether small mammals raising small litters would normally be affected by limits on the capacity to dissipate heat, perhaps also calling into question the wider validity of the HDL theory.

We have previously repeated the shaving experiment of Król and colleagues (Król et al., 2007) on Swiss mice according to the same protocol with that performed on MF1 mice, and found that fur removal had an effect on food intake but although the effect on pup growth was in the expected direction it was not statistically significant (Zhao and Cao, 2009; Zhao et al., 2010a). These data were different from those observed in MF1 mice, and provided support for the peripheral limitation hypothesis. Speakman and Król provided a potential explanation for the observed strain differences, i.e. MF1 mice and Swiss mice might have similar maximum capacities to dissipate heat in relation to ambient temperature, but different maximum capacities to produce milk (Speakman and Król, 2011). For MF1 mice, the ability to dissipate heat might be below the maximum milk production capacity, and hence when the ability to dissipate heat was elevated by exposure to cold, milk production was increased (Speakman and Król, 2011). In contrast, when heat dissipation capacity was increased by cold exposure in Swiss mice, they failed to upregulate milk production because their maximal milk production capacity was lower, relative to their capacity to dissipate heat (Zhao and Cao, 2009; Zhao et al., 2010a; Speakman and Król, 2011). An alternative hypothesis, however, is that milk production in Swiss mice is regulated by pup growth capacity. Hence, the females may not have upregulated their milk production when placed in the cold, not because they were unable to do so but because their pups would have been unable to convert this extra milk production into growth (Simons et al., 2011). To test this idea, in the present study we manipulated litter sizes raised by Swiss mice to 1, 3, 5, 7 and 9 pups (compared with the natural average litter size of 12) and also exposed half of the animals to cold temperatures (5°C) from day 10 of lactation. In these manipulated females it was hypothesised that the maximal capacity of the mammary glands to produce milk would always exceed the growth capacity of the pups. Hence, milk production and maternal energy budgets would be expected to vary in relation to the pup growth capacity and energy requirements – being greater at higher litter sizes and in the cold. Moreover, growth of the pups would be expected to be independent of litter sizes and temperature over the range studied, because mammary gland capacity would be able to respond to individual pup needs. In addition, we predicted that below the maximal capacity of the mammary glands to produce milk (the peripheral limitation hypothesis), energy intake of females would be affected by the capacity of mothers to dissipate heat (the HDL theory) as well as the growth capacity of the pups.

## MATERIALS AND METHODS

### Animals and experimental protocol

Virgin female Swiss mice, 10–11 weeks old, were obtained from a laboratory colony from the Experimental Animal Centre of Shandong University, and were housed individually in plastic cages (29×18×16 cm) with fresh sawdust bedding. Standard rodent chow (total energy content 17.6 kJ g<sup>-1</sup>; Beijing KeAo Feed Co., Beijing,

China) and water were available *ad libitum*, and environmental temperature was kept constant at 21±1°C with a 12 h:12 h light:dark cycle (lights on at 08:00 h).

Females ( $N=155$ ) were paired with males for 11 days, after which the males were removed. Of these females, 141 became pregnant and gave birth. On day 10, females were randomly assigned into one of five litter size (LS) groups: LS 1, 3, 5, 7 and 9 pups. We allowed the females to raise 1 pup only in the LS 1 group, 3 pups in the LS 3 group, etc.; the rest of the pups were removed. Half of the females with their offspring were transferred to a cold room (5±1°C,  $N=14, 15, 15, 13, 13$  in LS 1, 3, 5, 7 and 9 groups, respectively), and the rest were maintained at room temperature (21±1°C,  $N=14, 17, 14, 13, 13$  in LS 1, 3, 5, 7 and 9 groups, respectively). All pups were weaned on day 17 of lactation. We observed that some pups were killed at 5°C between days 11 and 17. This only happened in some females. We counted the offspring on a daily basis to control for the effects that this altered litter size might have on the experiment.

All experimental protocols were approved by the Animal Care and Use Committee of Liaocheng University.

### Body mass and gross energy intake

Female body mass ( $M_b$ ) and gross energy intake ( $FI_{GE}$ ), as well as number of pups and litter mass were measured on a daily basis. Food intake was calculated as the mass of food missing from the hopper every day, subtracting orts mixed in the bedding (Zhao, 2011).  $FI_{GE}$  (kJ day<sup>-1</sup>) was calculated as food intake (g day<sup>-1</sup>)×dry matter content of the diet (90.06%)×energy content of the diet (17.6 kJ g<sup>-1</sup>). Asymptotic energy (food) intake ( $FI_{AS}$ ) during peak lactation was calculated as the mean daily  $FI_{GE}$  between days 13 and 17 of lactation because there was no significant difference in daily energy intake over this period using ANOVA for repeated measurements ( $P>0.05$ ).

### Basal metabolic rate and non-shivering thermogenesis

Basal metabolic rate (BMR) of females was quantified at weaning (day 18 of lactation) as the rate of oxygen consumption, using an open-flow respirometry system (Sable Systems, Las Vegas, NV, USA). Air was pumped at a rate of 600–850 ml min<sup>-1</sup> through a cylindrical sealed Perspex chamber. Temperature was controlled to within ±0.5°C in an incubator. Gases leaving the chamber were directed through the oxygen analyser at a flow rate of 150–175 ml min<sup>-1</sup>. Incurrent air to the chamber and excurrent gases were dried using anhydrous silica gel. The data were collected every 10 s by an analog-to-digital converter (STD-UI2, Sable Systems). Females were fasted for 4 h and transferred into chambers for 1 h for adaptation to the chamber. BMR was measured for 3 h at 30±0.5°C (within the thermoneutral zone of this species) (Song and Wang, 2003) and calculated from the lowest consecutive readings over 5 min, using the following equation:  $\dot{V}_{O_2} = \dot{V}(F_{IO_2} - F_{EO_2})/[1 - F_{IO_2} \times (1 - RQ)]$ , where  $\dot{V}$  is the flow rate,  $F_{IO_2}$  is the input fractional concentration of O<sub>2</sub> to the chamber,  $F_{EO_2}$  is the excurrent fractional concentration of O<sub>2</sub> from the chamber, and RQ is the respiratory quotient (Arch et al., 2006; Zhao, 2012). Here, RQ was assumed to be 0.85 (Withers, 1977; Chi and Wang, 2011).

Non-shivering thermogenesis ( $NST_{max}$ ) was determined as maximal oxygen consumption induced by subcutaneous injection of noradrenaline (norepinephrine; NA) (Shanghai Harvest Pharmaceutical Co. Ltd, Shanghai, China) at 25±1°C. A mass-dependent dosage of NA (in mg kg<sup>-1</sup>) was calculated according to the equation:  $NA = 6.6 M_b^{-0.458}$  (Heldmaier, 1971) (where  $M_b$  is in g).  $NST_{max}$  was determined for another hour after BMR

measurements.  $NST_{max}$  was calculated from the same equation as for BMR calculation but instead used the highest consecutive readings over 5 min. BMR ( $ml\ O_2\ h^{-1}$ ) and  $NST_{max}$  ( $ml\ O_2\ h^{-1}$ ) were corrected to standard temperature and air pressure conditions and were converted to energy equivalents using the equation of Weir ( $1\ ml\ O_2\ h^{-1}=20.9\ kJ\ h^{-1}$ ) (Weir, 1949; Speakman, 1999; Johnson et al., 2001a). All measurements were made between 10:00 h and 17:00 h.

### Milk energy output

Milk energy output (MEO) of females during peak lactation was estimated from the energy budget of the litters between day 14 and 15 of lactation according to a modified method previously described (Król and Speakman, 2003b). Briefly, the pups depend entirely on milk, and their total energy requirements are the sum of energy allocated to respiration (daily energy expenditure, DEE, of the pups) and energy accumulated as new tissue. DEE was determined in pups from both 21 and 5°C conditions on day 15 of lactation using an open-flow respirometry system. Pups were separated from their mothers and entire litters were placed in the chamber for 1 h during which they were able to be active but had access to neither food nor water. DEE was estimated from the accumulated oxygen consumption over the 1 h measurement, and this was expanded to cover a 24 h period. DEE ( $ml\ O_2\ h^{-1}$ ) was converted to energy expenditure, using the equation of Weir (Weir, 1949; Speakman, 1999; Johnson et al., 2001a).

The modified equation used to estimate MEO ( $kJ\ day^{-1}$ ) was:

$$MEO = [DEE + (LMI \times GE_{pups})] \times 100 / d_{milk} \quad (1)$$

where LMI ( $g\ day^{-1}$ ) is the litter mass increase between day 14 and 15 of lactation,  $GE_{pups}$  ( $kJ\ g^{-1}$  wet mass) is the gross energy content of pups and  $d_{milk}$  is the apparent digestibility of milk ( $d_{milk}=96\%$ ) (Ofteidal and Iverson, 1987; Król and Speakman, 2003b). The mean values of  $GE_{pups}$  used in this formula were determined using a Parr 1281 oxygen bomb calorimeter using 10 pups from the 21°C groups and 10 pups from the 5°C groups (21°C,  $6.06 \pm 0.15\ kJ\ g^{-1}$ ; 5°C,  $6.20 \pm 0.12\ kJ\ g^{-1}$ ). Measuring the energy demands of the pups for 1 h during which they do not have access to food, and then expanding this 1 h up to a full day to reflect the total litter DEE will produce a slight underestimate of the real 24 h energy demands because the heat increment of feeding (HIF) will have been slightly underestimated. We have measured the HIF in adult mice fed diets with varying fat, carbohydrate and protein content and found that the HIF is between 2% and 5% of the total energy intake (Kajya-Agyemang, 2008) for diets where protein was around 20% of the diet. It is unclear to what extent the procedure we used will lead to an underestimate of this HIF effect because the HIF continues to contribute to the metabolic rate for up to 2.5 h after feeding has stopped (Z.-J.Z., personal observation). In the protocol used here, the mice were separated from their mothers and measured for 1 h; therefore, some of the HIF relating to milk intake immediately prior to the separation will be accounted for in the respiration measurement of the mice during the following hour. We can therefore expect an error somewhat lower than the 2–5% estimate of HIF in adults. This small theoretical error with the approach we used is probably why the estimated MEO using this 'litter energy budget' approach did not differ significantly from that using the gold standard method based on the difference between maternal metabolisable energy intake and maternal energy expenditure using the doubly labelled water (DLW) technique, in a previous validation of different methods for determining MEO of mice (Król and Speakman, 2003b). In fact, the mean MEO using the metabolisable

energy intake (MEI)–DLW method across 24 mice was  $79.5\ kJ\ day^{-1}$  and for the estimate based on the litter energy budget method, used here, it was  $80.3\ kJ\ day^{-1}$  (mean 1% higher using the technique employed in the current paper). Moreover, the individual estimates across the two techniques were strongly correlated across the 24 individuals ( $r=0.94$ ,  $P<0.001$ ) [see fig. 7C of Król and Speakman (Król and Speakman, 2003b)]. We are confident therefore that our estimates of the MEO of the females are a true reflection of the females' lactation performance.

### Brown adipose tissue cytochrome c oxidase activity

Females were killed by decapitation at 09:00 h–11:00 h after BMR and NST measurements. Interscapular brown adipose tissue (iBAT) was removed quickly, weighed and homogenised. Mitochondrial protein was prepared as previously described (Wiesinger et al., 1989; Zhao and Wang, 2005). Mitochondrial protein concentration was determined by the Folin phenol method with BSA as standard (Lowry et al., 1951). Cytochrome c oxidase (COX) activity in the whole BAT tissue was measured polarographically with oxygen electrode units (Hansatech Instruments Ltd, King's Lynn, Norfolk, UK) (Sundin et al., 1987; Zhao and Wang, 2005; Zhao et al., 2010b; Zhao et al., 2010c).

### Mammary glands and body fat content

After BAT was removed, the mammary glands was separated carefully, pooled and weighed (to 1 mg). Gastrointestinal tract, heart, liver, lung, spleen and kidney were then removed and the remaining carcass (including head and tail) were weighed to obtain the wet mass, dried in an oven at 60°C for at least a week and then reweighed to obtain the dry mass (to 1 mg). Total body fat mass was extracted from the dried carcass by ether extraction in a Soxhlet apparatus (Zhao and Wang, 2006; Zhao et al., 2010b).

### Statistics

Data were analysed using SPSS 13.0 statistical software. Changes over time in maternal energy intake, number of pups and litter mass throughout lactation were examined using ANOVA for repeated measurements. Two-way ANOVA (temperature  $\times$  number of pups) was used to examine the effects of temperature and litter size on  $FI_{AS}$ , litter mass, MEO, fat content and BAT COX activity. The effects of temperature and litter size on BMR,  $NST_{max}$  and mass of mammary glands were determined using two-way ANCOVA with mass of the whole body or carcass as covariates. Differences between LS 1, 3, 5, 7 and 9 groups in either 21 or 5°C were examined using Tukey's HSD *post hoc* tests where appropriate. Relationships between the number of pups, litter mass,  $FI_{AS}$ , energy intake, MEO, BMR,  $NST_{max}$ , BAT COX activity, mass of the mammary glands and fat content were examined using Pearson's correlation analysis. Data are reported as means  $\pm$  s.e.m. Statistical significance was determined at  $P<0.05$ .

## RESULTS

### $FI_{GE}$

$FI_{GE}$  significantly increased in all groups over the first 9 days of lactation (repeated measures, days 3–9; 21°C,  $F_{6,420}=71.36$ ,  $P<0.001$ ; 5°C,  $F_{6,414}=74.90$ ,  $P<0.001$ ; Fig. 1A). After the litters were manipulated on day 10,  $FI_{GE}$  declined in females at 21°C. By the day of weaning it was decreased by 55.5%, 38.8%, 22.4% and 9.8% compared with the intake on day 9 in LS 1, 3, 5 and 7 groups, respectively (*post hoc* test,  $P<0.05$ ). Females raising litters of 9 pups at 21°C consumed a similar amount of energy at weaning to that on day 9 (*post hoc* test,  $P>0.05$ ). Cold-exposed females also showed a

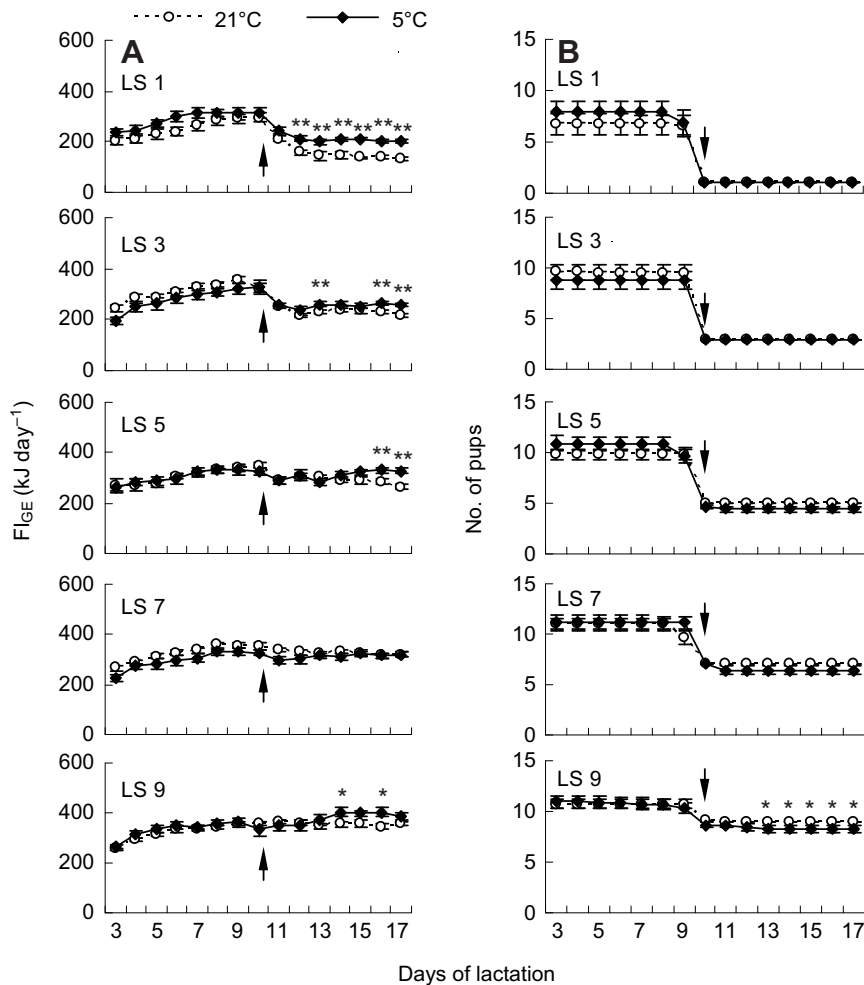


Fig. 1. Effect of cold exposure on gross energy intake ( $FI_{GE}$ , A) and litter size (B) during lactation in Swiss mice. Arrows indicate that females with offspring were exposed to 5°C from day 10 of lactation. On day 10, litters were experimentally reduced to 1, 3, 5, 7 and 9 pups (LS, litter size). Data are presented as means  $\pm$  s.e.m. Asterisks indicate a significant difference between data for 21 and 5°C: \* $P$ <0.05, \*\* $P$ <0.01.

reduction of  $FI_{GE}$  in LS 1 and 3 groups between day 10 and 17, such that energy intake at weaning was lower by 35.5% and 19.4% than that on day 9 (*post hoc* test,  $P$ <0.05).  $FI_{GE}$  was significantly affected by cold exposure on day 13 until weaning (day 13,  $F_{1,131}=4.94$ ,  $P$ <0.05; day 17,  $F_{1,131}=42.35$ ,  $P$ <0.001). Litter size had a significant effect on energy intake on day 11 until 17 (day 11,  $F_{1,131}=35.87$ ,  $P$ <0.001; day 17,  $F_{1,131}=127.67$ ,  $P$ <0.001; Fig. 1A).

Cold-exposed females showed significantly higher  $FI_{AS}$  than females lactating at 21°C (higher by 47%, 12%, 10% and 11% for the mothers raising 1, 3, 5 and 9 pups, respectively,  $F_{1,131}=28.56$ ,  $P$ <0.01; Fig. 2A).  $FI_{AS}$  was also significantly affected by experimental group ( $F_{4,131}=132.59$ ,  $P$ <0.01). Females increased  $FI_{AS}$  in parallel with the increase in the number of pups they raised at either 21 or 5°C, such that females had 153% higher  $FI_{AS}$  in LS 9 than in LS 1 groups at 21°C and 90% higher  $FI_{AS}$  at 5°C (*post hoc* test, 21°C,  $P$ <0.05; 5°C,  $P$ <0.05; Fig. 2A).

#### $M_b$

Female  $M_b$  did not differ between 21 and 5°C, or between LS 1, 3, 5, 7 and 9 groups on day 3 until day 9 (day 3, 5°C,  $F_{1,131}=0.45$ ,  $P$ >0.05; LS,  $F_{4,131}=2.18$ ,  $P$ >0.05; day 9, 5°C,  $F_{1,131}=2.07$ ,  $P$ >0.05; LS,  $F_{4,131}=2.23$ ,  $P$ >0.05). On day 10 and thereafter, the effect of cold exposure on body mass was also not statistically different (day 17,  $F_{1,131}=3.08$ ,  $P=0.08$ ; Fig. 2B). Experimental group, however, had a significant effect on body mass, in that females raising 3, 5, 7 and 9 pups were heavier than females supporting 1 pup (day 17,  $F_{4,131}=10.09$ ,  $P$ <0.05; *post hoc* test,  $P$ <0.05; Fig. 2B).

#### Number of pups and litter mass

In the cold, females raising 7 and 9 pups lost some of their pups. Cold exposure had a significant effect on the number of pups on day 11 till day 17 of lactation (day 11,  $F_{1,131}=6.50$ ,  $P$ <0.05; day 17,  $F_{1,131}=11.68$ ,  $P$ <0.01; Fig. 1B). No difference was observed in the number of pups between the 21 and 5°C groups in LS 1, 3 and 5 groups throughout the lactation period (day 17, LS 1,  $t_{26}=1.05$ ,  $P$ >0.05; LS 3,  $t_{30}=1.07$ ,  $P$ >0.05; LS 7,  $t_{24}=1.34$ ,  $P$ >0.05). However, the number of pups was significantly decreased to  $6.5\pm 0.4$  in LS 7 and to  $8.3\pm 0.3$  in LS 9 groups after exposure to the cold (day 17, LS 5,  $t_{27}=2.28$ ,  $P$ <0.05; LS 9,  $t_{24}=2.11$ ,  $P$ <0.05; Fig. 1B).

There was no effect of cold and number of pups on litter mass between days 3 and 9 prior to manipulation (day 9,  $F_{1,130}=1.36$ ,  $P$ >0.05; LS,  $F_{4,130}=1.56$ ,  $P$ >0.05; Fig. 2C). Cold exposure had a significant effect on litter mass, so that in LS 1, 3, 5, 7 and 9 groups litter mass was lower by 14%, 13%, 31%, 30% and 19% at 5°C than at 21°C (day 17,  $F_{1,130}=64.92$ ,  $P$ <0.01; Fig. 2C). Mean pup mass at weaning was affected by cold exposure, with females raising lighter pups at 5°C than at 21°C ( $F_{1,131}=34.37$ ,  $P$ <0.01; Fig. 2D). There was a significant effect of the number of pups on mean pup mass; the more pups the females raised, the lighter the pups weaned (day 17,  $F_{1,131}=8.93$ ,  $P$ <0.01). Litter mass was positively correlated with the number of pups (21°C; Fig. 3A) and  $FI_{AS}$  (Fig. 3B).

#### BMR and $NST_{max}$

Cold exposure had significant effects on BMR and  $NST_{max}$ , and both BMR and  $NST_{max}$  were significantly higher in females in the



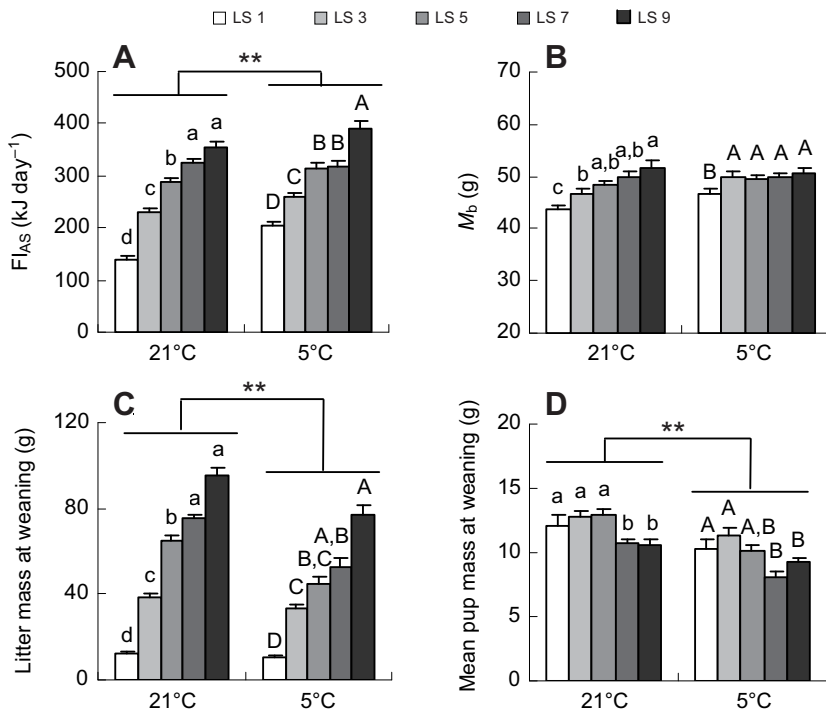


Fig. 2. Effect of cold exposure on asymptotic energy intake ( $FI_{AS}$ , A), body mass ( $M_b$ , B), litter mass (C) and mean pup mass (D) in Swiss mice. Females with offspring were exposed to 5°C on day 10 of lactation. On day 10, litters were experimentally reduced to 1, 3, 5, 7 and 9 pups (LS, litter size). Data are presented as means  $\pm$  s.e.m. \*\*Significant effect of cold exposure ( $P < 0.01$ ). Different lowercase/uppercase letters above columns indicate a significant difference within the 21 or 5°C groups ( $P < 0.05$ ).

5°C group than in those in the 21°C group (BMR,  $F_{1,49}=177.12$ ,  $P < 0.01$ ;  $NST_{max}$ ,  $F_{1,49}=69.14$ ,  $P < 0.01$ ; Fig. 4A,B). BMR was significantly affected by the number of pups ( $F_{3,49}=3.93$ ,  $P < 0.01$ ). Females raising larger litters showed higher BMR than mothers supporting small litters at 21°C (*post hoc* test,  $P < 0.05$ ), while at 5°C the difference between LS 1, 3, 5, 7 and 9 groups was not statistically significant (*post hoc* test,  $P > 0.05$ ; Fig. 4A). Experimental group had a significant effect on  $NST_{max}$ , and females raising larger litters (LS 5, 7 and 9) appeared to have lower  $NST_{max}$  than females supporting small litters (LS 1 and 3) (*post hoc* test, 21°C,  $P = 0.08$ ; 5°C,  $P < 0.05$ ; Fig. 4B).

### MEO

There was a significant effect of temperature on MEO, and females lactating at 5°C showed 63%, 83%, 58%, 49% and 25% higher MEO in LS 1, 3, 5, 7 and 9 groups than their counterparts lactating at 21°C ( $F_{1,50}=39.06$ ,  $P < 0.01$ , *post hoc* test,  $P < 0.05$ ; Fig. 4C). Experimental group also had a significant effect on MEO ( $F_{4,50}=47.01$ ,  $P < 0.01$ ). Females raising 3, 5, 7 and 9 litters produced 1.1, 2.5, 2.9 and 4.5 times more milk than females supporting 1 pup at 21°C (*post hoc* test,  $P < 0.05$ ), and 1.3, 2.4, 2.6 and 3.2 times more milk at 5°C (*post hoc* test,  $P < 0.05$ ; Fig. 4C). MEO was positively correlated with energy intake (Fig. 5A) and litter mass (Fig. 5B). There was a positive correlation between MEO and BMR at 21°C, but a negative correlation between MEO and BMR at 5°C (Fig. 5C). A negative correlation was observed between MEO and  $NST_{max}$  in females at both 21 and 5°C (Fig. 5D).

### Body fat content

Body fat content was significantly affected by cold exposure ( $F_{1,71}=6.51$ ,  $P < 0.05$ ; Fig. 6A). For example, in the LS 1 group, body fat content was 25.0% higher at 5°C than at 21°C ( $t_{14}=2.00$ ,  $P = 0.07$ ). We also observed a significant inverse effect of litter size on body fat content ( $F_{4,71}=30.40$ ,  $P < 0.01$ ). Females had 56.3% lower body fat content in the LS 9 group than in the LS 1 group at 21°C (*post*

*hoc* test,  $P < 0.05$ ), and 65.8% lower body fat content at 5°C (*post hoc* test,  $P < 0.05$ ; Fig. 6A). Body fat content was negatively correlated with  $FI_{AS}$  (21°C,  $R^2 = -0.58$ ,  $P < 0.01$ ; 5°C,  $R^2 = -0.42$ ,  $P < 0.01$ ).

### Mammary glands

The mass of the mammary glands was significantly different between the groups of females raising different numbers of pups ( $F_{1,71}=9.96$ ,  $P < 0.01$ ; Fig. 6B). In females at 21°C, mammary gland mass averaged 1.50 $\pm$ 0.20 g in the LS 1 group, and increased to 3.54 $\pm$ 0.38 g in the LS 9 group (increase of 135.3%, *post hoc* test,  $P < 0.05$ ). However, the mass of the mammary glands was not different between mothers raising different numbers of pups at 5°C (*post hoc* test,  $P > 0.05$ ), and the effect of cold exposure was not significant ( $F_{1,71}=2.02$ ,  $P = 0.16$ ). We observed heavier mammary glands in the LS 1, 3 and 5 groups at 21°C than at 5°C (heavier by 66.1% in LS 1,  $t_{14}=3.16$ ,  $P < 0.01$ ; 25.9% in LS 3,  $t_{18}=1.87$ ,  $P = 0.08$ ; 22.3% in LS 5,  $t_{15}=2.34$ ,  $P < 0.05$ ), whereas no difference was found between 21 and 5°C in the LS 7 and 9 groups (LS 7,  $t_{12}=1.12$ ,  $P > 0.05$ ; LS 9,  $t_{12}=1.46$ ,  $P > 0.05$ ; Fig. 6B). The mass of the mammary glands was positively correlated with  $FI_{AS}$  at either 21 or 5°C (21°C,  $R^2 = 0.79$ ,  $P < 0.01$ ; 5°C,  $R^2 = 0.21$ ,  $P < 0.01$ ). A positive relationship was observed between the mass of the mammary glands and litter mass at 21°C, but not at 5°C (21°C,  $R^2 = 0.72$ ,  $P < 0.01$ ; 5°C,  $R^2 = 0.08$ ,  $P > 0.05$ ).

### BAT COX

There was a significant effect of experimental group on BAT COX activity. BAT COX activity was higher in the LS 1 group than in the LS 7 and 9 groups ( $F_{4,71}=10.27$ ,  $P < 0.01$ ; Fig. 7). Females had 60.9% lower BAT COX activity in the LS 9 group than in the LS 1 group at 21°C and 73.8% lower BAT COX activity at 5°C (*post hoc* test, 21°C,  $P < 0.05$ ; 5°C,  $P < 0.05$ ). The effect of cold exposure on BAT COX activity was not significant ( $F_{1,71}=2.68$ ,  $P = 0.11$ ; Fig. 7). Negative relationships were observed between BAT COX activity and  $FI_{AS}$  at both 21 and 5°C (21°C,  $R^2 = -0.28$ ,  $P < 0.01$ ; 5°C,

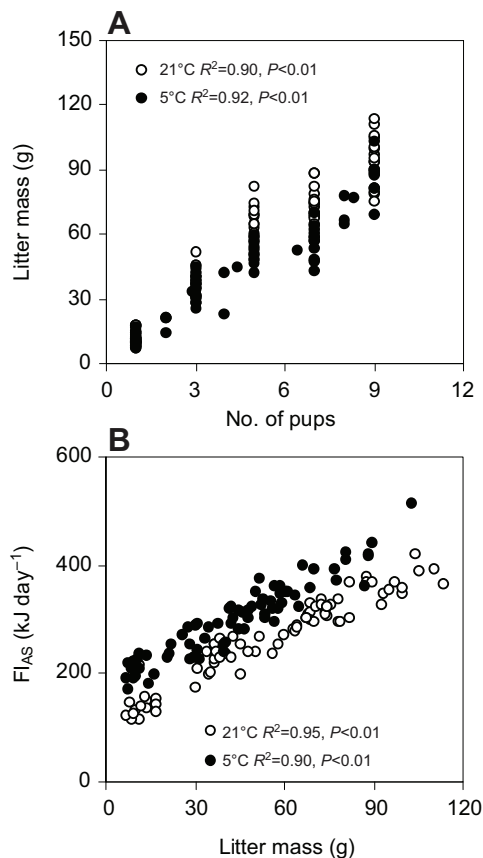


Fig. 3. Relationship between litter mass and (A) litter size and (B)  $FI_{AS}$  in Swiss mice. Females with offspring were exposed to 5°C on day 10 of lactation.

$R^2=-0.41$ ,  $P<0.01$ ). Additionally, BAT COX activity was negatively correlated with litter mass (21°C,  $R^2=-0.22$ ,  $P<0.05$ ; 5°C,  $R^2=-0.46$ ,  $P<0.01$ ).

### DISCUSSION

During lactation there is normally a large increase in food intake relative to that of non-reproductive animals, particularly during peak lactation (Koteja, 1996b; Hammond and Diamond, 1997; Speakman and Król, 2005). The factors that limit the intake of food at peak lactation, and hence overall reproductive performance, have been the subject of repeated experimentation (Peterson et al., 1990; Hammond and Diamond, 1992; Weiner, 1992; Hammond et al., 1994; Koteja, 1996a; Rogowitz, 1998; Hammond and Kristan, 2000; Johnson et al., 2001a; Bacigalupe and Bozinovic, 2002; Król and Speakman, 2003a; Król and Speakman, 2003b; Zhang and Wang, 2007; Zhang and Wang, 2008; Wu et al., 2009; Zhao and Cao, 2009; Speakman and Król, 2011; Zhao, 2011; Zhao, 2012). Among several others, two factors that have emerged from this work appear to be significant: (i) the capacity of the mammary glands to produce milk and (ii) the capacity of the female to dissipate body heat (Peterson et al., 1990; Hammond and Diamond, 1992; Weiner, 1992; Hammond et al., 1994; Koteja, 1996a; Rogowitz, 1998; Hammond and Kristan, 2000; Johnson et al., 2001a; Bacigalupe and Bozinovic, 2002; Król and Speakman, 2003a; Król and Speakman, 2003b; Speakman and Król, 2005; Speakman and Król, 2011). An additional factor that may also influence milk production and hence the  $FI_{AS}$  of females at peak lactation is the growth capacity of the offspring.

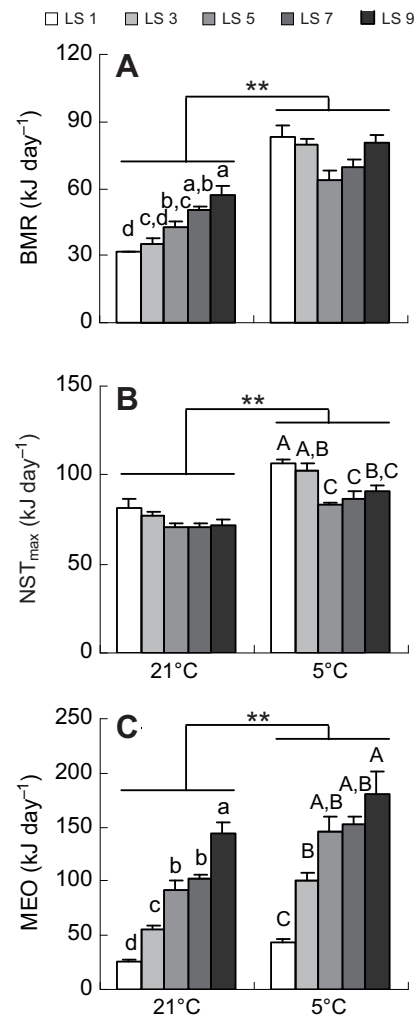


Fig. 4. Effect of cold exposure on basal metabolic rate (BMR, A), maximum non-shivering thermogenesis ( $NST_{max}$ , B) and milk energy output (MEO, C) in Swiss mice. Groups were the same as those shown in Fig. 2. \*\*Significant effect of cold exposure ( $P<0.01$ ). Different lowercase/uppercase letters above columns indicate a significant difference within 21 or 5°C groups ( $P<0.05$ ).

In the present study, we reduced the litter sizes raised by Swiss mice, at two ambient temperatures, to explore whether limitations in pup growth capacity might affect maternal intake and milk production. We predicted that if maternal food intake and milk output were governed only by mammary gland capability and heat dissipation capacity, litter size manipulations would have a negligible effect on these parameters, but pups in smaller litters would grow substantially larger than those in larger litters because of access to much greater resources for fuelling growth. Moreover, if heat dissipation was more important than limits on mammary gland function, then pups raised at 5°C would grow larger than those raised at 21°C. In contrast, if pup growth capacity was a limiting factor, then we would anticipate weaned pup size to be independent of the manipulations, and instead maternal milk production and food intake would be strongly dependent on the manipulated litter size and temperature. Consistent with the hypothesis that pup demands might play a role in defining maternal performance, we found a strong correlation between  $FI_{AS}$  and the manipulated litter size. The fewer pups a female was given to raise, the lower the level of food she

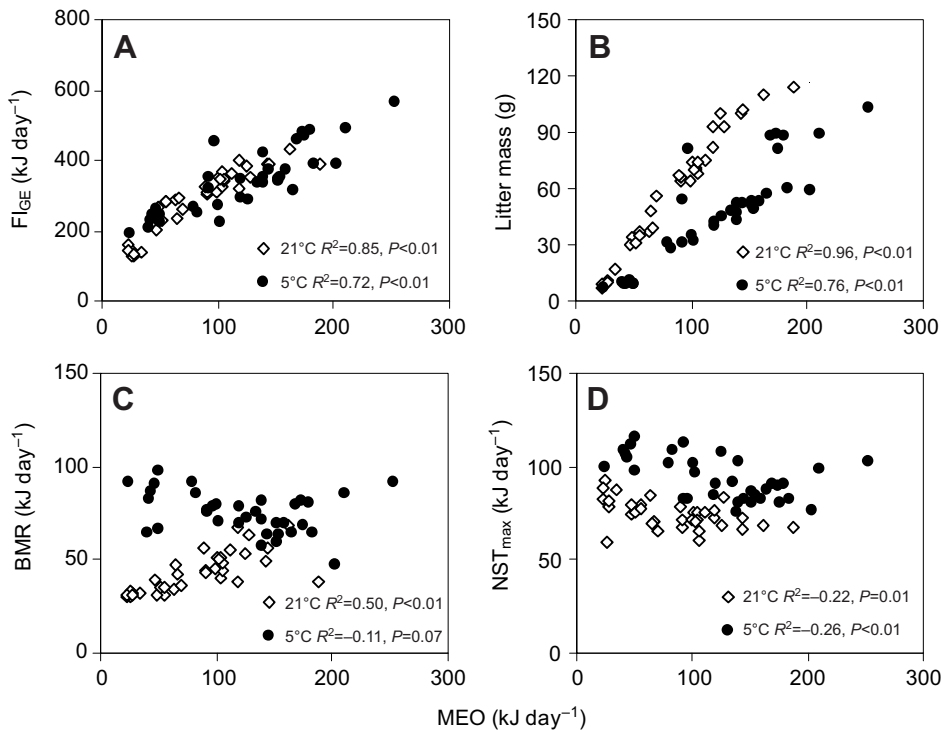


Fig. 5. Relationship between MEO and  $FI_{GE}$  (A), litter mass (B), BMR (C) and  $NST_{max}$  (D) in Swiss mice.

consumed and the lower the amount of milk she produced. These adjustments by the female in  $FI_{AS}$  and milk production were such that at 21°C the size of the weaned pups was almost independent of litter size. Pups in litters of 1, 3 and 5 weaned at the same average mass, while those in litters of 7 and 9 were slightly (but significantly) smaller. At 5°C we observed a similar pattern to that at 21°C. Energy intake and milk production were strongly related to the litter size manipulation with the consequence that the weaned pup mass was largely (but not completely) independent of litter size. This means that mothers raising smaller litters were not limited by the capacity of their mammary glands. The body masses of female mice raising different sized litters at either room or cold temperatures were constant, indicating that they were probably in energy balance.

Comparisons between the mothers raising pups at 21 and 5°C revealed some interesting patterns consistent with the hypothesis that pup demand was a key factor driving maternal performance at these litter sizes. First, the energy intake of females when supporting the experimental litter sizes was clearly below the sustained food intake limit found previously in this strain of mouse. Moreover, at any given litter size the  $FI_{AS}$  and milk production were much greater at 5 than at 21°C, yet the resultant litter and pup masses at 5°C were lower. Indeed, for any particular litter mass the milk production at 5°C was approximately twice that at 21°C. This pattern would be consistent with pup energy demands at 5°C being substantially elevated as a result of their thermoregulatory requirements. Although the pups raised at 5°C were on average smaller than those raised at 21°C, this was not because the females had reached an asymptote in their milk production capabilities at the lower temperature. For example, pups raised in litters of 1 at 5°C were smaller than those raised in litters of 1, 3 and 5 at 21°C, yet milk production of the females raising singletons at 5°C was substantially lower than that of females raising larger litters at this temperature. These data suggest that in this strain pup growth capacity at 5°C is lower than that at 21°C.

The reasons for this difference in pup growth capacity at different ambient temperatures are currently unclear. However, there are two

obvious possibilities. Pups only develop independent thermoregulatory capacities during late lactation (Chi and Wang, 2005; MacArthur and Humphries, 1999). If this capacity is limited then pups at 5°C may have lower body temperatures, which may influence their capacity for growth. Growth capacity in ectotherms is strongly dependent on body temperature (Ligon et al., 2012; Zuo et al., 2012). Alternatively, pups may have limited capacity for energy ingestion and processing, and when a large fraction of this must be devoted to thermoregulation at 5°C then the amount remaining to fuel growth may be reduced. Whatever the physiological reason for this effect, it has some important potential implications. First, when we previously exposed female mice of this strain but raising large litters of 12 pups to cold temperatures, they did not upregulate their milk production (Zhao, 2012). This may not necessarily have been because their mammary glands were incapable of such an increase – as suggested by the peripheral limitation hypothesis. The females may not have increased their milk production because at the lower temperature their pups would have been unable to convert any extra milk into additional growth due to higher maintenance costs. Several other studies have also shown that at cold temperatures milk production was not increased by lactating females [Swiss Webster mice (Hammond and Diamond, 1992), cotton rats *Sigmodon hispidus* (Rogowitz, 1998), Brandt's voles (when raising small litters) (Wu et al., 2009) and Mongolian gerbils (D. B. Yang, L. Li, L. P. Wang, Q. S. Chi, C. Hambly, D. H. Wang and J.R.S., submitted)]. While these data are often interpreted as supporting the peripheral limitation hypothesis they are also consistent with a limitation on pup growth at lower temperatures dictating maternal milk production.

Distinguishing between the roles of peripheral limitation in the mother and the growth limits of the pups that are dependent on ambient temperature could be achieved by keeping the mother and pups at different ambient temperatures. As far as we are aware only one study has used this design. Valencak and colleagues (Valencak et al., 2010) took advantage of the discontinuous suckling behaviour of brown hares (*Lepus europaeus*) to raise pups at a different ambient

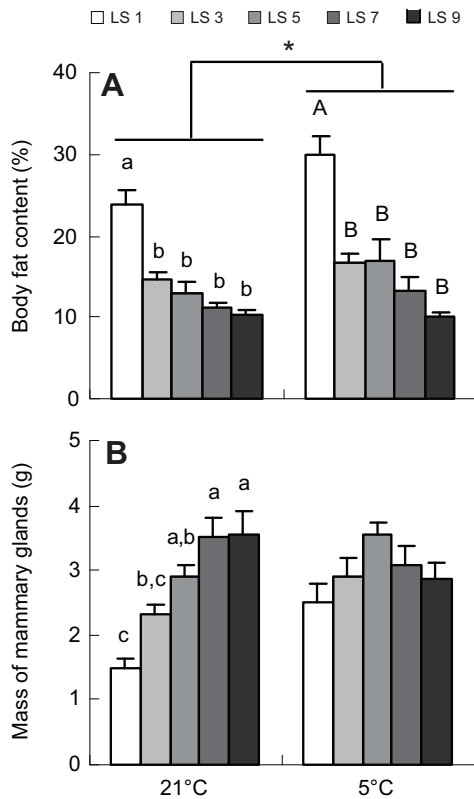


Fig. 6. Effect of cold exposure on (A) body fat content and (B) mass of the mammary glands in Swiss mice. Groups were same as those in Fig. 2. \*Significant effect of cold exposure ( $P < 0.05$ ). Different lowercase/uppercase letters above columns indicate a significant difference within 21 or 5°C groups ( $P < 0.05$ ).

temperature to that at which their mothers were kept. They showed that in early lactation, hare pups raised in the cold (5°C) stimulated greater milk production from their mothers than pups kept in warm conditions (21°C), independent of whether the mother herself was in the cold or warm temperature – suggesting that at this stage pup demand might be the most important factor driving maternal milk production. By the end of lactation, however, the milk production of the mothers did not differ between litters raised in the warm or cold, which is more consistent with a limit imposed on maternal milk production. The results were consequently inconclusive, and perhaps differ from the situation in mice because of the precocial nature of hare offspring, which rapidly develop their thermoregulatory capabilities after birth (Zörner, 1996). So far, studies in mice separating the temperature at which the mother and offspring are kept are lacking – probably because of the technical difficulties in designing such an experiment in an animal where the offspring suckle more continuously. Nevertheless, although pup growth limitations may explain why Swiss mice raising natural litters exposed to the cold do not significantly upregulate their milk production (Zhao, 2012) despite elevated food intake, this cannot explain why dorsally shaved Swiss mice at 21°C also do not significantly increase their milk production (Zhao and Cao, 2009; Zhao et al., 2010a) in contrast to MF1 mice (Król et al., 2007). This suggests that at least under some circumstances peripheral limitation at the mammary glands may be a limit on performance in this strain of mouse.

The second implication pertains to the potential disconnect between limitations imposed by the pup growth capacity and

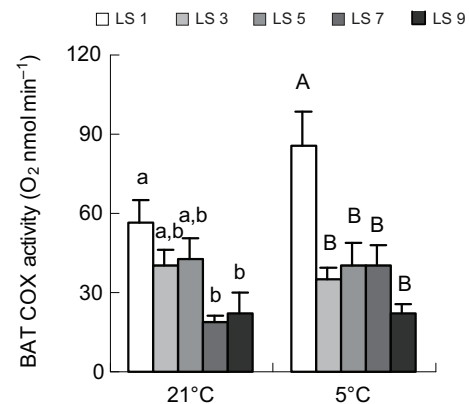


Fig. 7. Effect of cold exposure on the activity of brown adipose tissue cytochrome c oxidase (BAT COX) in Swiss mice. Groups were same as those in Fig. 2. Different lowercase/uppercase letters above columns indicate a significant difference within the 21 or 5°C groups ( $P < 0.05$ ).

limitations imposed by maternal milk production capacity. The HDL hypothesis suggests that the milk production capacity of females should increase as it gets colder. Yet the data presented here suggests that the pups' ability to turn such milk into growth may be greater at warmer temperatures. This may be because of the need for pups to utilise more energy for thermoregulation when it is colder, leaving less energy available for growth, or because of some intrinsic limit to growth performance related to temperature. Hence, there may be an optimal ambient temperature at which the capacities of mother and pups are best matched. This may provide an additional explanation (to changes in food availability and quantity) for why small mammals generally breed in the warmest period of the year despite the prediction of the HDL hypothesis that maternal performance would be maximised in colder conditions. Simons and colleagues similarly found that in common voles (*Microtus arvalis*) breeding under hot conditions (30°C), maternal milk production was lower than at cooler temperatures (21°C) but the offspring survived better, pointing to a discrepancy between the temperature conditions that are best for female milk production and those that are best for pup growth and development (Simons et al., 2011).

In addition to the dramatic increase in energy intake, a large reduction in the size of the adipose tissue stores occurred during lactation (Speakman and McQueenie, 1996). This has generally been interpreted as withdrawal of stored energy to support energy delivery, and thus decrease the energy intake demands in peak lactation (Speakman, 2008). In the present study, we found a negative correlation between body fat content and litter size. Females raising 9 pups had only half the body fat of females supporting 1 pup only. We also observed a negative correlation between body fat content and FI<sub>AS</sub>, suggesting that females not only elevated food intake but also increased mobilisation of fat reserves to meet the highest energy demands of themselves and of their offspring (=energetic costs of lactation). However, in laboratory mice the contribution of fat to the overall energy budget is relatively trivial (Johnson et al., 2001b; Speakman, 2008) (but see Duah et al., 2013). One suggestion is that the reduction in the size of fat tissue may reduce the production of leptin and other adipokines, which then serves to stimulate food intake (Halaas et al., 1997; Abelenda et al., 2003; Speakman, 2008; Cui et al., 2011).

Small mammals exposed to cold conditions such as 5°C need to elevate their capacity to produce heat to maintain body temperature



and increase their intake of energy to balance the increased heat loss (Hammond et al., 1994; Rogowitz, 1998; Hammond and Kristan, 2000; Johnson and Speakman, 2001; Zhang and Wang, 2007). We also observed a significant effect of cold exposure on  $FI_{AS}$  in Swiss mice raising experimentally reduced litters. In addition to elevated milk production, which is an exothermic process supplying heat that can be used for thermoregulation and elevated food intake, which also generates heat as a byproduct of processing the food (diet-induced thermogenesis), the mice exposed to the cold also had elevated BMR,  $NST_{max}$  and BAT COX activity. We observed negative relationships between  $FI_{AS}$ , milk production,  $NST_{max}$  and BAT COX activity. These data suggest that when heat (as a byproduct of food intake) and when milk production were highest, the mice had less need for heat produced directly from the BAT and this was downregulated accordingly. We have similarly shown recently that MF1 mice at 21°C downregulate gene expression linked to thermogenesis in BAT in direct relation to milk production (Król et al., 2011).

The growth of mammary tissue generally occurs during pregnancy for most female mammals, which facilitates milk production during the subsequent lactation period (Speakman, 2008). Here, we found that the mass of the mammary glands at the end of lactation was positively correlated to litter size and litter mass, and was directly related to milk production. Females raising 3, 5, 7 and 9 pups had 53%, 92%, 135% and 135% heavier mammary glands and produced 110%, 250%, 290% and 450% more milk than mothers supporting 1 pup, respectively. This demonstrates that there is considerable postpartum potential to remodel the mammary tissue in response to pup demands or environmental conditions favouring high or low levels of milk production (see also Duah et al., 2013). Furthermore, given the much greater expansion in milk production compared with the differences in mass, it also shows that in addition to changes in mass there must be other modifications of the mammary tissue to facilitate greater milk production in mothers supporting larger litters. Moreover, cold-exposed females had significantly heavier mammary glands and produced more milk than females in warm conditions.

In conclusion, the results of the present study show that when female Swiss mice were given small litters (between 1 and 9 pups) to raise, the primary factor that determined the milk production and  $FI_{AS}$  levels of the mothers was the growth capacity of the pups. This resulted in milk output and energy intake levels in the mothers that were closely linked to litter size and litter mass, and pup sizes that were virtually independent of litter size. Pups at low ambient temperatures such as 5°C demanded more milk from their mothers, resulting in greater milk production and greater energy intake at lower temperatures. Yet, despite this the pups grew to smaller sizes by the end of lactation than pups at 21°C. This result suggests that pup growth capacity was temperature dependent, as is widely observed in ectotherms. This temperature dependency of pup growth may explain why raising some small mammals at cold temperatures does not result in the anticipated elevation in milk production predicted by the HDL hypothesis. Separating this effect from peripheral limitations imposed at the mammary glands may prove difficult. The discrepancy in the temperatures at which mothers perform best (generally colder is better) and at which pups perform best (generally warmer is better) may lead to optimal ambient temperatures for reproduction.

#### LIST OF ABBREVIATIONS

BAT	brown adipose tissue
BMR	basal metabolic rate
COX	cytochrome <i>c</i> oxidase

DEE	daily energy expenditure
$FI_{AS}$	asymptotic energy intake
$FI_{GE}$	gross energy intake
GE	gross energy content
GLM	general linear model
HDL	heat dissipation limit
LS	litter size
$M_b$	body mass
MEO	milk energy output
NST	non-shivering thermogenesis
SusEI	sustainable energy intake

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#### AUTHOR CONTRIBUTIONS

Z.-J.Z. and J.R.S. conceived and designed the experiments. D.-G.S., Z.-C.S., W.-B.W. and X.-B.L. performed the experiments. Z.-J.Z. analyzed the data. Z.-J.Z. and J.R.S. wrote the paper.

#### COMPETING INTERESTS

No competing interests declared.

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