Effect of cold exposure on energy budget and thermogenesis during lactation in Swiss mice raising large litters

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Summary

In Swiss mice sustained energy intake (SusEI) during peak lactation has been previously suggested to be constrained by the capacity of the mammary glands to produce milk, supporting the "peripheral limitation" hypothesis. Here we experimentally examined if SusEI in these mice was not only limited peripherally but also constrained by the ability to dissipate heat. Female Swiss mice were provided with additional offspring above their natural litter sizes and were maintained during lactation either in warm (23℃) or cold (5℃) conditions. Food intake, thermogenesis, litter size and mass, and the weight of the mammary glands were measured. No differences were observed in asymptotic food intake at peak lactation, litter mass and thermogenesis between females raising litters of different size. Cold-exposed females increased food intake and thermogenic capacity, but weaned significantly smaller and lighter litters with smaller pup sizes compared with females in warm conditions. The weight of the mammary glands did not differ between warm and cold-exposed females, but within temperatures was positively related to litter mass. These data suggested that cold exposure increased food intake, but had no effect on the capacity of the mammary glands to secret milk because they were already working maximally in the females raising larger litters. The factors causing this limit in the mammary capacity remain elusive.

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Key words: Cytochrome c oxydase (COX), Food intake, Lactation, Nonshivering thermogenesis (NST), Resting metabolic rate (RMR), Swiss mice

Introduction

Lactation is probably the most energy-demanding period in the lives of most female mammals, and energy intake during this phase appears to be limited (Koteja, 1996; Hammond and Diamond, 1997; Rogowitz, 1998; Speakman and Król, 2005, 2011). Understanding the nature of the limits during lactation has wide ecological relevance (Speakman and Król, 2011). Many studies have been performed in several different species (and strains of mice) to determine the factors that limit the sustained energy intake (SusEI) during peak lactation (Drent and Daan, 1980; Peterson et al., 1990; Hammond and Diamond, 1992; Weiner, 1992; Hammond et al., 1994; Koteja, 1996; Rogowitz, 1998; Hammond and Kristan, 2000; Johnson et al., 2001; Bacigalupe and Bozinovic, 2002; Król and Speakman, 2003a,b; Speakman and Król, 2005, 2011; Wu et al, 2009; Naguib et al., 2010; Valencak et al., 2010). It seems that SusEI is not constrained physiologically by the capacity of the alimentary tract to absorb energy (the central limitation hypothesis), but may rather be limited by a combination of demands arising from peripheral tissues – the 'peripheral limitation' hypothesis (Hammond et al., 1994) or by the capacity of animals to dissipate heat – the 'heat dissipation limitation (HDL)' theory (Król and Speakman, 2003a,b; Speakman and Król, 2005, 2011).

The HDL theory suggests that SusEI at peak lactation is imposed by the capacity of an animal to dissipate body heat generated as a by-product of processing food and producing milk (Król and Speakman, 2003a,b). Under this theory, an animal has limited capacity to get rid of the heat by physical constraints, combined with an inability to store the heat internally because of the risks of hyperthermia (Speakman and Król, 2005, 2011). When the limits to heat dissipation are removed, such as during cold exposure, females consume more food and export significantly more milk and consequently wean larger litters (Król and Speakman, 2003a,b; Speakman and Król, 2005). The data from studies on MF1 mice strongly support the HDL theory. These data include observations that milk energy output during peak lactation is increased in females exposed to the cold, but decreased in females exposed to hot temperatures (Johnson and Speakman, 2001; Król and Speakman, 2003a,b; Speakman and Król, 2005, 2011). Removal of the fur also allowed MF1 females to elevate energy intake and to produce more milk compared with unshaved controls (Król et al., 2007).

We have previously performed studies on Swiss mice to determine factors limiting SusEI during peak lactation, and observed different responses to those observed in MF1 mice (Zhao and Cao, 2009; Zhao et al., 2010a). For example, when Swiss mice were dorsally shaved during peak lactation, fur-removal had no effect on milk production and litter growth, consistent with the prediction of 'peripheral limitation' hypothesis. These findings were also consistent with the previous studies on the same strain performed by Hammond and colleagues (Hammond and Diamond, 1992, 1997; Hammond et al., 1994; Hammond and Kristan, 2000). These results for Swiss mice differ from the same studies in MF1 mice, but do not necessarily refute the HDL theory. Speakman and Król (Speakman and Król, 2011) proposed a potential interpretation of the strain differences regarding to limits to SusEI during peak lactation, i.e. the two strains were constrained by the capacity to produce milk and also limited by the ability to dissipate body heat, but to different extents (Speakman and Król, 2011). In other words, the two strains may have similar maximum capacities to dissipate heat in relation to ambient temperature but may differ in their maximum capacity to synthesise milk (Speakman and Król, 2011). For MF1 mice the capacity to produce milk may be well above their capacity to dissipate heat while in Swiss mice the maximal milk production capacity may lie below their capacity to dissipate heat.

The purpose of the present experiment was to test this idea in Swiss mice. We experimentally added additional pups to female mice raising offspring at 23℃ and thus ensured females were working at their maximal capacity at this temperature. We then exposed half of these manipulated females to cold (5℃) ambient temperatures from day 8 of lactation onwards. Food intake, litter size and mass, thermogenesis and weight of the mammary glands were measured. If the maximum capacity of the mammary glands to process energy was higher than the limit imposed by the capacity to dissipate heat, then transferring these mice from 23 to 5℃ would release them from this constraint and their energy intake and milk production would increase, and potentially growth of the pups would increase as well. However, if the limit imposed by maximal milk production capability defined by the capacity of the mammary glands was lower than the limit imposed by the capacity to dissipate heat, energy intake would increase to meet the elevated thermoregulatory demands but this extra intake could not be translated into greater milk production. Because the pups might also have greater energy requirements in the cold this inability of the female to upregulate milk production might then lead to reduced growth of the pups when exposed to the cold. Because elevated milk production would not generate extra heat to offset thermoregulatory demands in this latter scenario we predicted there would also be an increase in non-shivering thermogenic capacity in the females, but if elevated milk production occurred in the cold then non-shivering thermogenic capacity would be unaltered.

Materials And Methods

Animals and experimental protocol

Virgin female Swiss mice, 9–10 weeks old, were obtained from a laboratory colony from the Experimental Animal Centre of Shandong University, and were housed individually in plastic cages (29 cm × 18 cm × 16 cm). Animals were given free access to food (Standard rodent chow, produced by Beijing KeAo Feed Co., Beijing, China) and water. Environmental temperature was kept constant at 23±1℃ with a 12h: 12 h light: dark cycle (lights on at 0800h). All experimental procedures were in compliance with the Animal Care and Use Committee of Liaocheng University.

One hundred and fifteen females were paired with males for 11 days, after which the males were removed. On the day of parturition, females had their litter sizes manipulated. We provided females with additional pups, and allowed them to raise 11, 13, 15, 17 or 19 offspring (hereafter refer to as LS=11, 13, 15, 17 and 19 groups, 16 females of each group). On day 8 of lactation, we transferred half of the females with their offspring in each group to the cold (5±1℃, Cold) with the rest still housed at room temperature (23±1℃, Warm). All pups were weaned on day 17 of lactation. Litter size and litter mass were measured daily between days 7 and 17 of lactation.

Asymptotic food intake

Food intake of the females was measured daily. Food intake was calculated as the mass of food missing from the hopper every day, subtracting orts mixed in the bedding (Cameron and Speakman, 2010). As there were no different differences in food intake between days 11 to 17 in females lactating at either cold or warm conditions, asymptotic food intake during peak lactation was calculated as the mean daily food intake between these days (days 11–17).

Resting metabolic rate (RMR) and nonshivering thermogenesis (NST)

RMR of the females was quantified as the rate of oxygen consumption, using an open-flow respirometry system (Sable system, USA). Air was pumped at a rate of 600–850 ml/min through a cylindrical sealed Perspex chamber, which was immerged in water bath (±0.5℃). Gases leaving the chamber were dried (silica gel) and directed through the oxygen analyzer at a flow rate of 150–175 ml/min. The data were averaged and collected every 10 s by a computer connected analogue-to-digital converter (STD-UI2, Sable system), and analyzed using a standard software (Sable system). On day 17 of lactation, RMR was measured for 2 hours at 30±0.5℃ (within the thermal neutral zone of this species) (Speakman and Rossi, 1999). RMR was calculated from the lowest rate of oxygen consumption over 5 min. Oxygen consumption was calculated using the equation: VO_2= FR (FiO_2-FeO_2)/(1-FiO_2 × (1-RQ)), where FR is the flow rate, FiO_2 is input fractional concentration of O_2 to the chamber, FeO_2 is excurrent fractional concentration of O_2 from the chamber, and RQ is respiratory quotient (Arch et al., 2006). Here, RQ was assumed to be 0.85 (Withers, 1977; Chi and Wang, 2011).

On day 17 of lactation, NST was measured next to RMR measurements. NST was quantified as the maximal rate of oxygen consumption induced by subcutaneous injection of norepinephrine (NE) (Shanghai Harvest Pharmaceutical Co. Ltd.) at 25±1℃. A mass-dependent dosage of NE was calculated according to the equation: NE (mg/kg)=$6.6 \times M_b^{-0.458}$(g) (Heldmaier, 1971). NST measurement was carried out for 1 hour, and the highest rate of oxygen consumption over 5 min was averaged to estimate NST_{max}. NST_{max} was calculated according to the same conversion equation as that used in RMR calculation. NST was calculated as NST_{max} minus RMR. RMR, NST_{max} and NST were corrected to standard temperature and air pressure conditions and expressed as ml O_2/h (Zhao et al., 2010b,c). All measurements were made between 0900 and 1700h.

Sample collection and COX activity

After NST measurements on day 17 of lactation, females were sacrificed by decapitation between 0900 and 1100 h. Interscapular BAT was quickly removed and weighed and, mitochondria were prepared as described previously (Wiesinger et al., 1989; Zhao and Wang, 2005). Mitochondrial concentrations were measured using the Folin phenol method with bovine serum albumin as standard (Lowry et al., 1951). COX activity in the whole BAT tissue was determined polarographically with oxygen electrode units (Hansatech Instruments Ltd., England) (Zhao and Wang, 2005; Sundin et al., 1978). After BAT was removed, the mammary gland was separated carefully, pooled and weighed (to 1 mg).

Statistics

Data were analyzed using SPSS 13.0 statistic software. Two-way ANOVA (temperature × litter size) was used to examine effect of temperature and litter size on asymptotic food intake, litter size, litter mass, mean pup mass and BAT COX activity. Effects of temperature and litter size on RMR, NST and the weight of the mammary glands were determined using two-way ANCOVA with mass of whole body or carcass as covariates. Further, differences between LS=11, 13, 15, 17 and 19 females in either the cold or warm conditions were examined using Tukey's HSD post-hoc tests where appropriate. Pearson's correlation was performed to examine correlations between litter mass and litter size, asymptotic food intake, BAT COX activity and the weight of the mammary glands. Data were reported as means ± s.e.m. Statistical significance was determined at $P< 0.05$.

Results

Asymptotic food intake

Asymptotic food intake was significantly affected by cold exposure; cold-exposed females consumed more food than females in warm conditions ($F_{1,62}=12.19$, $P<0.01$) (Fig. 1). No significant differences were observed in asymptotic food intake between females raising 11, 13, 15, 17 and 19 offspring ($F_{4,62}=0.59$, $P>0.05$) (Fig. 1).

Litter losses

Initial litter sizes are not different between the cold and warm conditions (day 0, $F_{1,62}=1.31$, $P>0.05$). The sizes of litters

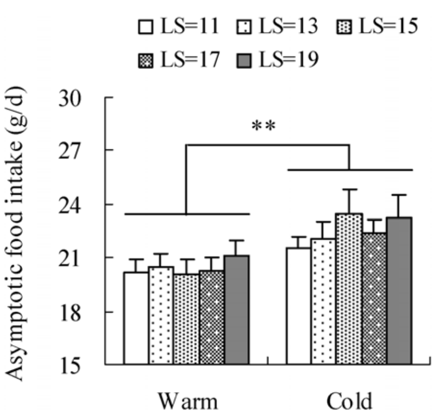

Fig. 1. Effect of cold exposure on asymptotic food intake during lactation in Swiss mice raising large litters. Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring (litter size, LS=11, 13, 15, 17 or 19). On day 8 of lactation, half of females with their offspring in each group were transferred to the cold (5±1℃, Cold), the rest were still maintained in warm conditions (23±1℃, Warm). Data are presented as means ± s.e.m. **, significant effect of cold exposure ($P<0.01$).

differed significantly between LS=11, 13, 15, 17 and 19 groups on day 7 of lactation; LS=17 and 19 females had larger litters than LS=11 and 13 females (Table 1). A difference was observed in the sizes of litters throughout the lactation period in females exposed to warm conditions. Litters losses occurred throughout the lactation period in the mothers lactating in the cold, in particular in the mothers raising the larger litters. Consequently, no difference in the sizes of litters was observed between LS=11, 13, 15, 17 and 19 groups on days 11–17 in the cold-exposed females. Litter size in the cold on the day of weaning was independent of the starting litter size when first exposed to the cold. As expected cold exposure did not have any effect on the sizes of litters on day 7 – the day before they were exposed to cold conditions. On day 9 and thereafter, cold exposure had a significant effect on litter mortality, and cold-exposed females lost more offspring and consequently raised smaller litters than females exposed to warm conditions (Table 1).

Litter mass and mean pup mass

We observed a significant difference in the litter mass between LS=11, 13, 15, 17 and 19 groups on day 7 and 8 of lactation, but did not observe differences on days 9–17 for either warm or cold-exposed females (Table 2). Litter mass was not different between warm and cold-exposed females prior to cold exposure. Cold exposure had a significant effect on litter mass on days 8–17. Consequently, litters were weaned with a significantly lower mass in the cold-exposed females than females lactating in warm conditions (Table 2). Litter mass was positively correlated with litter size in the cold ($r=0.90$, $P<0.01$) and also in warm conditions ($r=0.64$, $P<0.01$) (Fig. 2A). A positive correlation between litter mass and asymptotic food intake was also observed in the cold ($r=0.73$, $P<0.01$) and warm conditions ($r=0.68$, $P<0.01$) (Fig. 2B). Mean pup mass was significantly affected by litter size throughout lactation, by which the larger litters the mothers supported, the lighter pups they raised and weaned (Table 3). Cold exposure had significant effect on mean pup mass on day 8 to 15 of lactation, and the pups raised in warm conditions were heavier than those in the cold (Table 3).

RMR and NST

Cold exposure had significant effect on RMR; cold-exposed females had a higher RMR at weaning than females lactating in warm conditions ($F_{1,39}=5.63$, $P<0.05$) (Fig. 3A). $\mathrm{NST_{max}}$ and NST were also significantly higher in females in the cold than warm conditions ($\mathrm{NST_{max}}$, $F_{1,39}=52.78$, $P<0.01$) (Fig. 3B); (NST, $F_{1,39}=31.01$, $P<0.01$) (Fig. 3C).

BAT COX activity

We observed a significant effect of cold exposure on BAT COX activity ($F_{1,55}=15.68$, $P<0.01$) (Fig. 3D). Consistent with the changes in RMR, $\mathrm{NST_{max}}$ and NST, BAT COX activity was increased in the cold-exposed females compared with that of warm-exposed females, by which COX activity was higher by 32%, 89%, 108%, 55% and 58% in females in the cold than warm conditions in LS=11, 13, 15, 17 and 19 groups, respectively. No differences were observed in RMR, $\mathrm{NST_{max}}$, NST or BAT COX activity between LS=11, 13, 15, 17 and 19 females (RMR, $F_{4,39}=0.48$, $P>0.05$) (Fig. 3A); ($\mathrm{NST_{max}}$, $F_{4,39}=0.28$, $P>0.05$) (Fig. 3B); (NST, $F_{4,39}=0.39$, $P>0.05$) (Fig. 3C); (COX,

Table 1. Litter size throughout the lactation period in cold-exposed Swiss mice raising large litters.

	Warm					Cold					
	LS=11	LS=13	LS=15	LS=17	LS=19	LS=11	LS=13	LS=15	LS=17	LS=19	
Day of lactation	$n=7$	$n=7$	$n=7$	$n=7$	$n=7$	$n=8$	$n=8$	$n=7$	$n=7$	$n=8$	P
7	10.7±0.2	12.1±0.3	13.9±0.5	15.7±0.3	17.7±0.6	10.9±0.2	12.6±0.3	15.0±0.1	16.3±0.3	16.9±0.7	LS,**
8	10.7±0.2	11.9±0.3	13.6±0.8	15.7±0.3	17.9±0.5	10.6±0.2	12.3±0.4	14.7±0.3	14.3±0.6	15.6±0.6	LS,**
9	10.6±0.2	11.9±0.3	13.4±0.8	15.6±0.4	17.6±0.6	9.4±0.9	11.8±0.5	12.9±0.6	13.6±0.8	13.3±1.5	T,**; LS,**
10	10.6±0.2	11.9±0.3	13.1±0.9	15.6±0.4	17.4±0.8	9.0±0.9	10.9±0.6	12.6±0.7	13.1±0.8	12.5±1.5	T,**; LS,**
11	10.3±0.3	11.9±0.3	12.9±0.9	15.4±0.5	17.1±0.7	8.9±0.9	10.5±0.5	10.9±1.3	12.6±1.0	11.9±1.8	T,**; LS,**
12	10.3±0.3	11.9±0.3	13.0±0.9	15.4±0.5	16.9±0.7	8.5±0.9	10.6±0.5	10.7±1.3	11.6±0.9	10.9±1.5	T,**; LS,**
13	10.3±0.3	11.9±0.3	12.7±0.9	15.4±0.5	16.9±0.7	8.1±0.9	10.5±0.5	10.4±1.4	10.4±0.8	9.6±1.5	T,**; LS,**
14	10.3±0.3	11.9±0.3	12.9±0.9	15.3±0.4	16.9±0.7	8.1±0.9	9.6±0.8	10.4±1.4	9.0±0.9	9.1±1.3	T,**; LS,**
15	10.3±0.3	11.9±0.3	12.9±0.9	15.1±0.4	16.7±0.7	7.9±0.9	8.5±0.8	10.1±1.3	8.6±0.9	8.6±1.3	T,**; LS,**
16	10.3±0.3	11.9±0.3	12.9±0.9	15.1±0.4	16.6±0.6	7.8±0.8	7.6±1.1	9.9±1.2	7.7±1.1	8.4±1.3	T,**;LS,**
17	10.3±0.3	11.9±0.3	12.9±0.9	14.9±0.5	16.3±0.7	7.5±0.8	7.3±1.0	9.0±1.1	7.3±1.2	8.0±1.3	T,**;LS,**

Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring. On day 8 of lactation, half of females with their offspring in each group were transferred to the cold (5±1°C, Cold), the rest were still maintained at the warm (23±1°C, Warm). Values are presented as the means ±s.e.m. T, temperature; LS, litter size;*, $P<0.05$, **, $P<0.01$.

Table 2. Litter mass (g) throughout the lactation period in cold-exposed Swiss mice raising large litters.

	Warm					Cold					
	LS=11	LS=13	LS=15	LS=17	LS=19	LS=11	LS=13	LS=15	LS=17	LS=19	
Day of lactation	*n*=7	*n*=7	*n*=7	*n*=7	*n*=7	*n*=8	*n*=8	*n*=7	*n*=7	*n*=8	P
7	57.0±3.3	60.7±1.8	59.8±3.9	66.0±1.4	70.9±3.7	54.6±1.9	60.7±2.7	67.0±2.8	72.7±1.6	73.0±1.9	LS,**
8	63.2±3.4	66.7±1.8	65.2±4.8	72.8±1.6	77.6±3.9	55.6±2.4	62.0±3.0	67.5±3.8	65.9±2.7	70.9±2.7	T,**; LS,**
9	67.8±3.6	72.7±2.1	69.9±5.4	78.5±1.8	82.2±4.7	53.8±5.7	63.3±2.5	63.3±6.6	65.3±2.7	62.3±7.7	T,**
10	72.0±3.5	77.5±2.0	73.7±5.7	83.0±1.5	84.7±5.2	52.4±5.8	61.1±2.7	64.4±7.3	64.1±3.6	62.2±8.5	T,**
11	72.9±3.8	82.0±2.0	75.6±5.9	86.2±1.7	87.2±5.4	53.9±5.5	60.8±2.1	59.1±10.5	63.0±4.2	62.2±10.1	T,**
12	76.3±3.7	86.2±2.5	78.8±6.2	89.8±1.7	90.8±5.5	53.6±5.5	62.8±2.4	62.1±11.0	59.4±3.4	58.9±8.9	T,**
13	79.5±3.6	90.0±2.5	82.4±6.0	93.2±2.1	94.4±5.3	54.9±5.6	63.4±2.2	61.9±10.4	56.2±3.5	55.4±8.5	T,**
14	81.2±3.4	94.1±4.0	84.6±6.1	94.2±3.0	96.0±5.3	56.7±5.4	60.5±4.3	63.7±10.4	51.2±5.0	56.8±7.5	T,**
15	81.8±3.5	92.0±3.6	86.5±6.1	95.0±3.6	98.4±5.4	54.0±4.7	53.5±4.6	62.6±9.2	46.8±6.4	54.4±7.2	T,**
16	82.5±3.7	93.9±3.8	87.2±6.1	97.3±3.8	98.8±5.7	53.9±4.2	48.7±6.8	61.4±8.1	46.7±6.0	55.0±6.9	T,**
17	82.6±4.2	94.6±3.5	88.3±5.9	95.3±4.3	98.1±5.9	51.8±3.3	46.0±6.9	56.1±6.0	44.8±6.3	53.1±6.8	T,**

Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring. On day 8 of lactation, half of females with their offspring in each group were transferred to the cold (5±1°C, Cold), the rest were still maintained at the warm (23±1°C, Warm). Values are presented as the means ±s.e.m. T, temperature; LS, litter size; *, $P<0.05$, **, $P<0.01$.

$F_{4,55}=0.45$, $P>0.05$) (Fig. 3D). No correlation was observed between COX activity and asymptotic food intake in females lactating in the cold or warm conditions (Warm, $r=-0.35$, $P>0.05$; Cold, $r=-0.32$, $P>0.05$) (Fig. 4A). COX activity was negatively correlated with litter mass in females lactating in warm conditions ($r=-0.38$, $P<0.05$), but such a correlation was not significant in the females lactating in the cold ($r=-0.28$, $P>0.05$) (Fig. 4B).

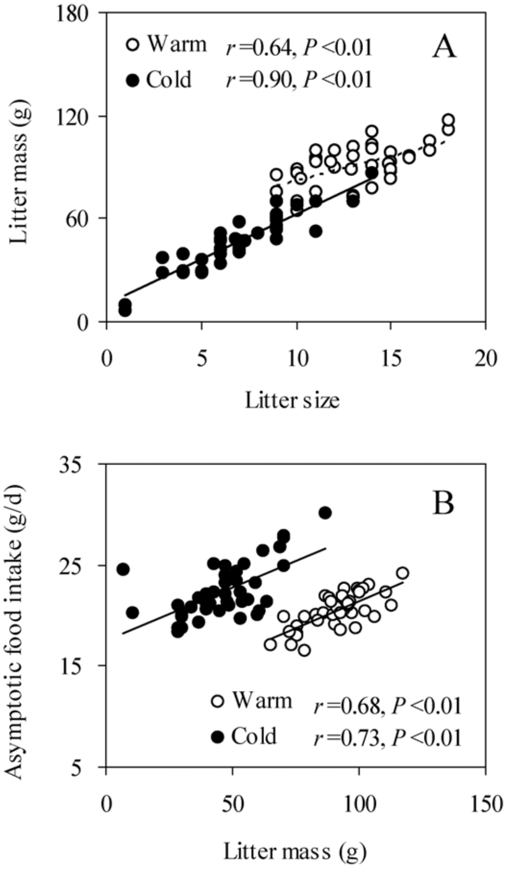

Fig. 2. Relationships between litter mass and litter size (A) and asymptotic food intake (B) during peak lactation in cold-exposed Swiss mice raising large litters. Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring. On day 8 of lactation, half of females with their offspring were transferred to the cold (5±1℃, Cold), the rest were still maintained in warm conditions (23±1℃, Warm).

Mammary glands

There was no significant effect of cold exposure on the weight of the mammary glands ($F_{1,50}=1.36$, $P>0.05$) (Fig. 5). For mice lactating in warm conditions, the weight of the mammary glands was heavier by 75% and 68% in LS=17 and 19 females respectively than in LS=13 females ($F_{4,26}=3.19$, $P<0.05$). There was no difference in the weight of the mammary glands between LS=11, 13, 15, 17 and 19 females in the cold conditions (Warm, 3.39±0.35, 3.79±0.32, 3.50±0.73, 5.92±0.97 and 5.67±0.78 g; Cold, 3.84±0.25, 4.02±0.54, 4.22±0.55, 3.76±0.42 and 4.50±1.24 g in LS=11, 13, 15, 17 and 19 groups respectively, $F_{4,25}=0.22$, $P>0.05$). The weight of the mammary glands was positively correlated with litter mass at weaning (Warm, $r=0.46$, $P<0.01$, Cold, $r=0.53$, $P<0.01$) (Fig. 6).

Discussion

The peripheral limitation hypothesis suggests that the central 'delivery' organs have more processing and delivery capacity than the peripheral organs can use. During lactation, the capacity of the mammary glands to produce milk is suggested to be the factors limiting SusEI and therefore reproductive output. (Hammond and Diamond, 1997; Speakman and Król, 2005). In contrast the heat dissipation limit hypothesis suggests that SusEI and reproductive output are primarily constrained by the ability to dissipate heat. In the present study, cold exposure had a significant effect on asymptotic food intake during peak lactation. Cold-exposed females (5℃) consumed 2.1 g/d on average more food compared with females lactating in warm conditions (23℃). This increase is consistent with both the peripheral limitation and HDL ideas. Under the peripheral limitation idea the increase is interpreted to reflect the elevated demands for thermogenesis to maintain body temperature. Under the HDL theory the increase reflects a relaxation of the heat dissipation limit. The main

Table 3. Mean pup mass (g) throughout the lactation period in cold-exposed Swiss mice raising large litters.

	Warm					Cold					
	LS=11	LS=13	LS=15	LS=17	LS=19	LS=11	LS=13	LS=15	LS=17	LS=19	
Days of lactation	*n*=7	*n*=7	*n*=7	*n*=7	*n*=7	*n*=8	*n*=8	*n*=7	*n*=7	*n*=8	P
7	5.3±0.2	5.0±0.1	4.3±0.2	4.2±0.1	4.0±0.1	5.0±0.2	4.8±0.2	4.5±0.2	4.5±0.1	4.4±0.1	LS,**
8	5.9±0.2	5.6±0.1	4.8±0.2	4.6±0.1	4.3±0.1	5.2±0.2	5.1±0.2	4.6±0.2	4.6±0.1	4.6±0.2	T,*; LS,**
9	6.4±0.2	6.1±0.1	5.2±0.3	5.0±0.1	4.7±0.1	5.7±0.2	5.4±0.2	4.9±0.4	4.9±0.2	4.7±0.2	T,**; LS,**
10	6.8±0.2	6.5±0.1	5.6±0.2	5.4±0.1	4.8±0.1	5.8±0.2	5.7±0.2	5.0±0.3	4.9±0.2	4.9±0.3	T,**; LS,**
11	7.1±0.2	6.9±0.1	5.9±0.2	5.6±0.1	5.1±0.1	6.1±0.2	5.9±0.3	5.2±0.4	5.1±0.3	5.1±0.3	T,**; LS,**
12	7.4±0.3	7.2±0.1	6.1±0.3	5.8±0.2	5.4±0.1	6.4±0.3	6.0±0.2	5.6±0.5	5.3±0.4	5.4±0.3	T,**; LS,**
13	7.7±0.3	7.6±0.2	6.5±0.3	6.0±0.2	5.6±0.1	6.9±0.3	6.1±0.3	5.8±0.4	5.6±0.4	5.8±0.3	T,**; LS,**
14	7.9±0.3	7.9±0.4	6.7±0.4	6.2±0.2	5.7±0.1	7.2±0.4	6.4±0.3	6.1±0.4	5.7±0.4	6.4±0.3	T,**; LS,**
15	8.0±0.3	7.8±0.2	6.8±0.4	6.3±0.2	5.9±0.1	7.2±0.5	6.4±0.3	6.2±0.4	5.5±0.6	6.6±0.4	T,*; LS,**
16	8.0±0.3	7.9±0.3	6.9±0.4	6.4±0.2	5.9±0.2	7.3±0.6	6.6±0.4	6.4±0.4	6.2±0.4	6.8±0.4	LS,**
17	8.0±0.3	8.0±0.3	7.0±0.5	6.4±0.2	6.0±0.1	7.3±0.6	6.7±0.5	6.5±0.5	6.3±0.4	7.0±0.5	LS,**

Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring. On day 8 of lactation, half of females with their offspring in each group were transferred to the cold (5±1°C, Cold), the rest were still maintained at the warm (23±1°C, Warm). Values are presented as the means ±s.e.m. T, temperature; LS, litter size; *, $P<0.05$, **, $P<0.01$.

difference between the two ideas is that the HDL theory predicts the animals can exploit their greater capacity to dissipate heat, when it is cold, by elevating their milk production, while the peripheral limitation hypothesis suggests that the mammary glands are assumed to already be working at their maximum capacity and therefore milk production will not changed after females are exposed to the cold.

Females generally increase food intake to meet the high energy demanding periods like lactation or cold exposure (Hammond and Diamond, 1992, 1997; Hammond et al., 1994, 1996; Koteja, 1996; Rogowitz, 1998; Hammond and Kristan, 2000; Król and Speakman, 2003a,b; Speakman and Król, 2005, 2011; Wu et al., 2009). We found positive correlations between asymptotic food intake and litter mass, indicating that increased food intake was largely allocated to milk production and hence pup growth in both warm and cold mice. Females that were manipulated to support 19 pups at parturition (LS=19) weaned on average 16.3 pups on day 17 of lactation at 23℃, higher by 58% than the 10.3 pups at weaning in LS=11 mothers ($P<0.01$). However, the weaned litter mass was not different between LS=11, 13, 15, 17 and 19 females ($P>0.05$). These data suggest that the mammary glands of the females were working at maximal capacity and the

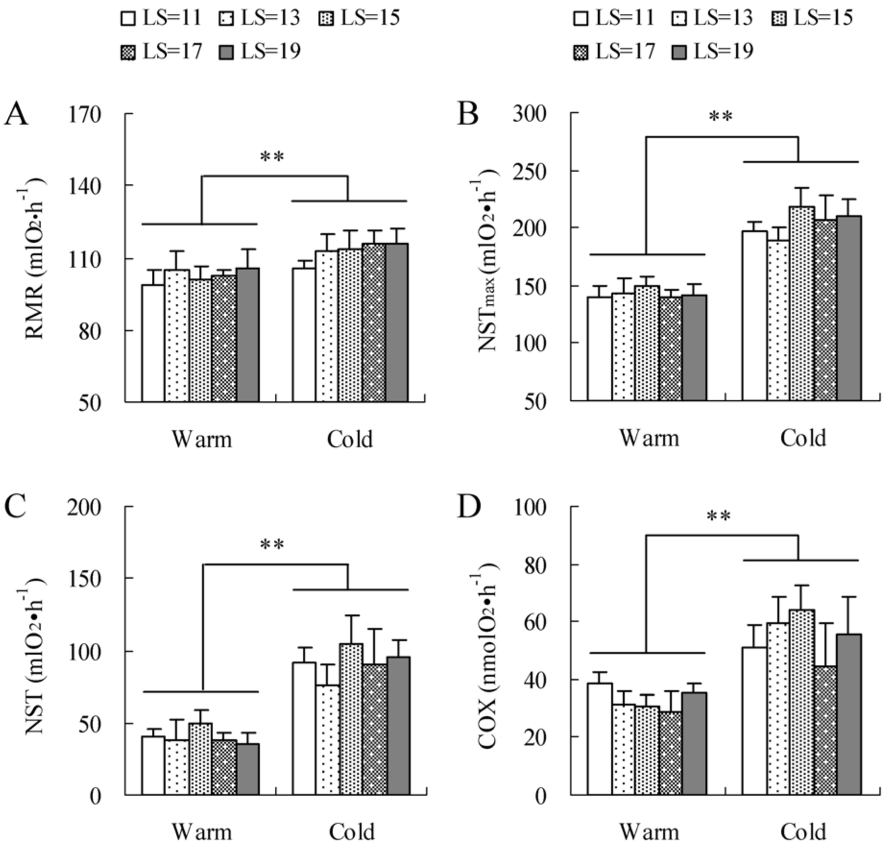

Fig. 3. Cold exposure on resting metabolic rate (RMR) (A), maximum nonshivering thermogenesis (NST_{max}) (B), NST (C) and activity of cytochrome c oxydase of brown adipose tissue (COX) (D) at weaning in cold-exposed Swiss mice raising large litters. Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring (litter size, LS=11, 13, 15, 17 or 19). On day 8 of lactation, half of females with their offspring in each group were transferred to the cold (5±1℃, Cold), the rest were still maintained in warm conditions (23±1℃, Warm). Data are presented as means ± s.e.m. **, significant effect of cold exposure ($P<0.01$).

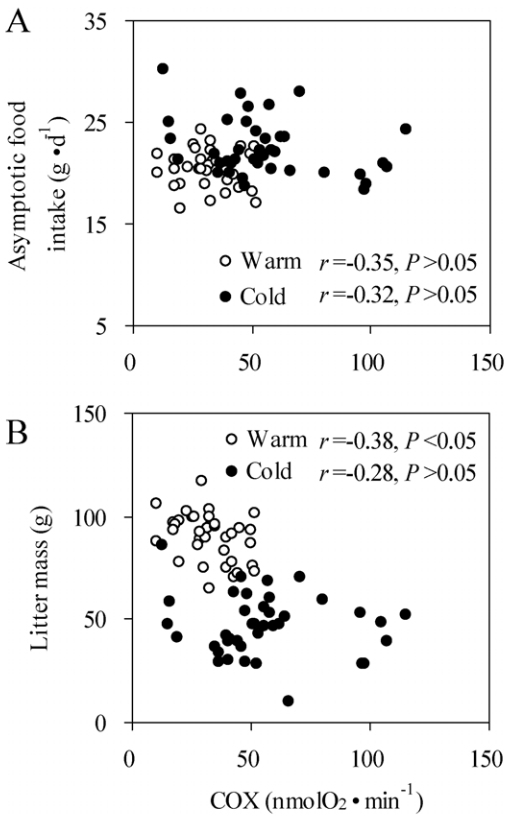

Fig. 4. Relationships between activity of cytochrome c oxydase of brown adipose tissue (COX) and asymptotic food intake (A) and litter mass (B) during peak lactation in cold-exposed Swiss mice raising large litters. Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring. On day 8 of lactation, half females with their offspring were transferred to a cold temperature (5±1℃, Cold), the rest were still maintained at the room temperature (23±1℃, Warm).

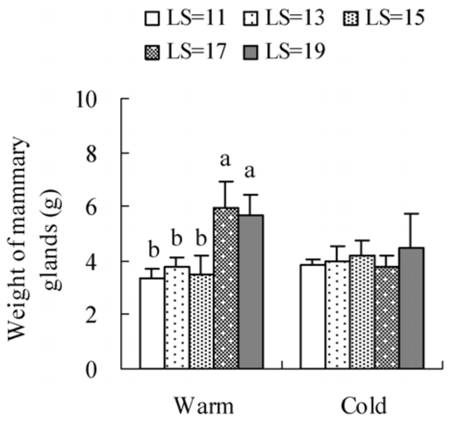

Fig. 5. Effects of cold exposure on the weight of the mammary glands in Swiss mice raising large litters. Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring (litter size, LS=11, 13, 15, 17 or 19, six groups in total). On day 8 of lactation, half females with their offspring in each group were transferred to a cold temperature (5±1℃, Cold), the rest were still maintained at the room temperature (23±1℃, Warm). Data are presented as means ± s.e.m. Different letters above the columns indicate significant difference between LS=11, 13, 15, 17 and 19 groups ($P<0.05$).

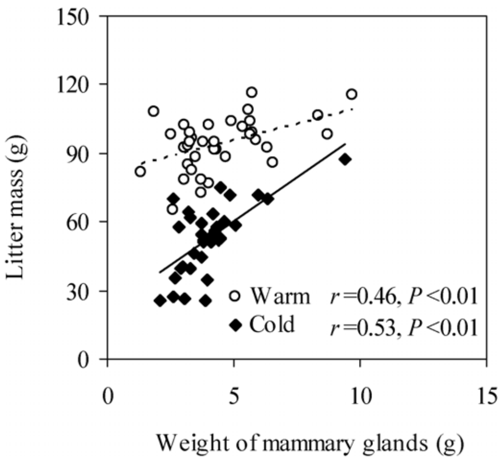

Fig. 6. Relationship between the weights of the mammary glands and litter mass at weaning in cold-exposed Swiss mice raising large litters. Females were provided with additional pups on the day of parturition and then allowed to raise 11, 13, 15, 17 or 19 offspring. On day 8 of lactation, half females with their offspring were transferred to a cold temperature (5±1℃, Cold), the rest were still maintained at the room temperature (23±1℃, Warm).

resultant milk was divided between more offspring as litter size was increased. This result is consistent with the previous studies in the same mouse strain (Zhao and Cao, 2009; Zhao et al., 2010a). For example, Hammond et al. (Hammond et al., 1996) manipulated female Swiss mice by surgically removing their mammary glands during late lactation but did not reduce litters they raised. They found that productivity in the halved glands did not change (Hammond et al., 1996; Speakman and Król, 2005).

The key question is whether the milk secretion activity of the mammary glands was defined by intrinsic aspects of the gland itself, or whether the activity was dependent on the entire females capacity to dissipate heat. In other words, was activity of the mammary glands elevated in the cold (consistent with the HDL) or were the glands operating at the same level at 5℃ as they do at 23℃ (consistent with the peripheral limitation).

In our study females in the cold weaned smaller and lighter litters than females lactating in warm conditions. There are two interpretations for these data. First the cold-exposed mothers may have failed to upregulate their milk production (consistent with the peripheral limitation hypothesis). Combined with elevated pup demands in the cold this may have meant they were unable to maintain the large litter size, and consequently dams culled litter size to a level they could sustain. This would be consistent with previous studies indicating that this strain of mouse is peripherally limited at 23℃ (Zhao and Cao, 2009; Zhao et al., 2010a). However, another interpretation is that the female did upregulate her milk production in the cold (consistent with the HDL) but this increase was insufficient to offset these elevated pup demands, leading her to cull some of the pups. Unfortunately we do not have direct measures of milk production to separate between these alternative interpretations of the data.

If the females exposed to the cold were using their elevated capacity to dissipate heat to synthesise more milk they would not need to increase their thermogenic capacity using the more familiar routes of increased thermogenesis involving brown adipose tissue (Heldmaier et al., 1982; Klaus et al., 1988; Li and Wang, 2005; Wang et al., 2006; Cannon and Nedergaard, 2011). Yet cold-exposed females elevated RMR, NST and BAT COX activity compared with females lactating in warm conditions, indicating that females had increased the capacity of their BAT to produce heat to meet the heat loss caused by cold exposure. It suggests that mammary gland may set a peripheral limitation, leading us towards the former interpretation.

The growth of the mammary tissue is generally initiated during pregnancy which facilitates milk production during the subsequent lactation (Speakman, 2007, 2008). However, post-partum mammogenesis continues and is responsive to changes in demands during lactation. In the present study, consistent with this previous work, weight of the mammary glands was related to litter size. Females raising 17–19 pups in warm conditions had heavier mammary glands than mothers supporting 11–15 pups. This finding suggests that the mammary glands grew to enable greater milk production to support the larger litters. Yet, as discussed above the litter mass remained unchanged across the different litter sizes, suggesting that milk production was not elevated despite having larger glands. These data are consistent with prior work in this strain which indicate the mammary glands impose limits on reproductive performance (Hammond and Diamond, 1992, 1997; Hammond et al., 1994, 1996; Hammond and Kristan, 2000; Zhao and Cao, 2009; Zhao et al., 2010a). The new data however raise important questions about what imposes such limits on the mammary gland performance in these mice. Clearly the limit is not on the growth of the tissue. However, if the heavier glands in mice that raised larger litters did not generate more milk, the implication is that this enlarged gland actually generated less milk per gram of tissue, suggesting some constraint on milk secretion activity external to the gland itself. The nature of this constraint remains elusive. The manifest ability of the mice to grow their glands in response to litter size also begs the question of why the glands did not also grow when the mothers were exposed to the cold. At present there is no answer to this question.

Contrary to the apparent situation in Swiss mice, in MF1 mice milk production was increased as temperature got colder (Johnson and Speakman, 2001) and pups raised in the cold were consequently larger than those raised at 21℃, and those raised under hot conditions (30℃) were smaller (Johnson and Speakman, 2001; Król and Speakman, 2003a; Speakman and Król, 2005, 2011). The different results between MF1 mice and Swiss mice may be due to different levels of the capacity of the mammary glands to produce milk, relative to the limits imposed by the capacity to dissipate heat (Speakman and Król, 2011). For MF1 mice, the capacity to dissipate heat appears to be a constraint on maximum milk production. Cold-exposure or fur-removal elevates heat dissipation, allowing females to secrete more energy in milk and wean larger litters (Król and Speakman, 2003a; Speakman and Król, 2011). In contrast, Swiss mice seemed to have lower maximal milk production capacity relative to their capacity to dissipate heat. We have highlighted here that the nature of the limit on the mammary gland performance may be extrinsic to the mammary tissue itself, but the nature of such a limit remains unclear.

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Competing Interests

The author declares no competing interests.

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