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RESEARCH ARTICLE

Sustained energy intake in lactating Swiss mice: a dual modulation process

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ABSTRACT

Limits to sustained energy intake (SusEI) during lactation are important because they provide an upper boundary below which females must trade off competing physiological activities. To date, SusEI is thought to be limited either by the capacity of the mammary glands to produce milk (the peripheral limitation hypothesis) or by a female's ability to dissipate body heat (the heat dissipation hypothesis). In the present study, we examined the effects of litter size and ambient temperature on a set of physiological, behavioral and morphological indicators of SusEI and reproductive performance in lactating Swiss mice. Our results indicate that energy input, energy output and mammary gland mass increased with litter size, whereas pup body mass and survival rate decreased. The body temperature increased significantly, while food intake (18 g day^{-1} at 21°C versus 10 g day^{-1} at 30°C), thermal conductance (lower by 20–27% at 30°C than 21°C), litter mass and milk energy output decreased significantly in the females raising a large litter size at 30°C compared with those at 21°C. Furthermore, an interaction between ambient temperature and litter size affected females' energy budget, imposing strong constraints on SusEI. Together, our data suggest that the limitation may be caused by both mammary glands and heat dissipation, i.e. peripheral limitation is dominant at room temperature, but heat dissipation is more significant at warm temperatures. Further, the level of the heat dissipation limits may be temperature dependent, shifting down with increasing temperature.

KEY WORDS: Heat dissipation limits, Lactation, Litter size, Sustained energy intake, Mammary gland, Temperature

INTRODUCTION

Sustained energy intake (SusEI) is the maximal rate of energy intake that animals can sustain while allowing their energy demands to be met by food consumption rather than by the depletion of previously accumulated energy reserves (Hammond and Diamond, 1997; Piersma, 2011; Speakman and Król, 2005; Zhao et al., 2013a). Lactation, in which females increase rates of food intake and energy assimilation several fold to meet the demands of suckling offspring, is the most energy-demanding aspect of mammalian reproduction (Hammond and Diamond, 1997; Speakman and Król, 2005; Valencak and Ruf, 2009). However, the fact that SusEI and milk energy output (MEO) reach a ceiling during peak lactation indicates that there is an upper limit to SusEI (Speakman and Król, 2005). This limit is important because it represents an upper boundary under which females must trade off the competing energy demands of different physiological processes (Hammond and Diamond, 1997; Johnson et al., 2001; Koteja, 1996a,b; Piersma, 2011).

A variety of intrinsic and extrinsic factors have been found to affect SusEI, and several hypotheses have been proposed to explain its upper limit (Speakman and Król, 2005). For example, the peripheral limitation (PL) hypothesis proposes that SusEI is constrained by the capacity of energy-expending organs, such as the mammary glands, during lactation (Hammond and Diamond, 1997; Speakman and Król, 2005). This hypothesis has been supported by experimental data showing that female Swiss mice were unable to increase their food intake following substantial increases in litter size (e.g. up to 23 pups) (Diamond and Hammond, 1992). In addition, when half the mammary glands were surgically removed, milk production from the remaining half did not increase, suggesting that the glands were already working at maximal capacity (Hammond et al., 1996). In constrast, the heat dissipation limitation (HDL) hypothesis proposes that SusEI is constrained by the maximal capacity to dissipate body heat (Król and Speakman, 2003a,b; Speakman and Król, 2010). This hypothesis is supported by data showing that milk production in MF1 mice was less in lactating females kept at a warmer ambient temperature (Johnson et al., 2001; Król and Speakman, 2003a,b; Speakman and Król, 2005). Experiments have been conducted on a variety of animal species to examine the roles of intrinsic and extrinsic factors on SusEI (Diamond and Hammond, 1992; Hammond et al., 1994, 1996; Hammond and Kristan, 2000; Johnson et al., 2001; Król et al., 2007; Król and Speakman, 2003a,b; Leon et al., 1983; Paul et al., 2010; Rogowitz, 1998; Simons et al., 2011; Valencak et al., 2010; Wu et al., 2009; Yang et al., 2013). It is interesting to note that although there is evidence to support both hypotheses, the data are not always consistent. In addition, it seems that the two hypotheses are not mutually exclusive, and that the results of experiments are sometimes species specific (Simons et al., 2011; Speakman and Król, 2005, 2011; Zhao et al., 2013a). Although differences in the maximum capacities to synthesize milk and to dissipate heat have been argued to attribute to the contrasting results between different animal species (Speakman and Król, 2011), the interpretation for divergent data is still lacking. Because maximum capacities of both milk synthesis and heat dissipation are not only associated with the energy demands of raising different litter size, but also significantly affected by the embient temperature, it is reasonable to predict that both limitations may act simultaneously on the same individuals, but to a different extent in a stimulus-specific manner. To the best of our knowledge, no study has yet systematically examined the possibility that SusEI and reproductive output are constrained by the capacity to both produce milk and dissipate heat.

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List of symbols and abbreviations

AgRp	agouti-related protein
CART	cocaine- and amphetamine-regulated transcript
C_{wet}	wet thermal conductance
DEI	digestive energy intake
GE	gross energy
GEI	gross energy intake
HDL	heat dissipation limit
LS	litter size
MEO	milk energy output
NPY	neuropeptide Y
Ore	orexin
POMC	pro-opiomelanocortin
Prl-R	prolactin receptor
RMR	resting metabolic rate
RT	room temperature, 21±1°C
RT-qPCR	real-time quantitative polymerase chain reaction
SusEI	sustained energy intake
T_b	body temperature
WT	warm temperature, 30±1°C

Lactating Swiss mice have been previously used as an animal model in which to study the effects of intrinsic and environmental factors on SusEI (Hammond et al., 1996; Zhao and Cao, 2009; Zhao, 2012; Zhao et al., 2013a, 2016). For example, it has been shown that the effect of litter size on SusEI and reproductive performance in this species is limited because neither asymptotic food intake nor reproductive output continued to increase with substantial increases in litter size (Zhao, 2012). Further support for the PL hypothesis is provided by the finding that female Swiss mice that had their dorsal fur shaved increased their rate of thermal conductance, but not their milk production, relative to non-shaved controls (Zhao et al., 2010b). In contrast, the fact that lactating females kept at a warmer ambient temperature significantly decreased their food intake and produced less milk compared with counterparts kept in cooler conditions suggests that SusEI can also be constrained by the capacity of animals to dissipate heat (Zhao et al., 2016). It has been suggested that SusEI during peak lactation in Swiss mice can be constrained by a combination of peripheral demands and heat dissipation capacity, although the specific contribution of each of these variables, their interactions and the underlying mechanisms remain unknown (Zhao and Cao, 2009; Zhao et al., 2010b).

A possible mechanism linking heat dissipation capacity to lactation performance is suggested to be suckling schedules and prolactin secretion (Speakman and Król, 2005). Continual maternal hyperthermia in relation to the suckling unit of mother and pups at peak lactation forces the female to discontinue suckling, and therefore directly inhibits prolactin secretion, thereby reducing milk production (Croskerry et al., 1978; Speakman and Król, 2005). Prolactin, released from the pituitary in response to suckling stimulation, has been implicated in a wide range of physiological systems, including reproduction, development and metabolism (Woodside, 2007; Carré and Binart, 2014). Its effects are produced by binding to a membrane receptor [prolactin receptor (Prl-R)], which is expressed in brain areas associated with the regulation of neuropeptides in the hypothalamus, including orexigenic peptides, such as neuropeptide Y (NPY), agouti-related peptide (AgRp) and orexin (Ore; hypocretin), and the anorexigenic neuropeptides, e.g. pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Woods et al., 1998; Mercer and Speakman, 2001; Kokay and Grattan, 2005). This raises the possibility that prolactin is involved in energy balance (Ben-Jonathan et al., 2006). Evidence suggests that prolactin acts centrally to stimulate food intake during lactation, indicating that prolactin has orexigenic effects (Gerardo-Gettens et al., 1989; Noel and Woodside, 1993; Woodside, 2007). Additionally, prolactin secretion may reach a maximum during peak lactation, when the mammary glands have been working at a maximum rate. This may suggest that it is involved in the food regulation of the females that have been peripherally limited (Zhao et al., 2010a). Therefore, prolactin is a candidate for involvement in the combination of the PL and HDL hypotheses.

In this study, we used lactating Swiss mice to test both the PL and HDL hypotheses. We tested whether the SusEI and reproductive performance of lactating females were constrained by expenditure to peripheral organs (peripheral limitations) by experimentally altering their litter size. In addition, we tested the role of heat dissipation as a limiting factor for SusEI by comparing the SusEI and reproductive performance of females at two different ambient temperatures. We took a comprehensive approach to systematically examine variation in body temperature, food intake, body mass, and the resting metabolic rate (RMR) of females and litters, as well as serum prolactin levels during lactation, and to test for an interaction between litter size and ambient temperature on these variables. In addition, we examined the expression of several neuropeptide genes, including NPY, AgRp, Ore, CART and POMC in the hypothalamus. We hypothesized that the two limiting factors (i.e. litter size and ambient temperature) can interact in constraining animals' SusEI and reproductive performance by acting on overlapping regulating mechanisms.

MATERIALS AND METHODS

Experimental subjects

Experimental subjects were the female offspring of a breeding colony of Swiss mice maintained at Wenzhou University, Wenzhou, China. Animals were housed individually in plastic cages (29×18×16 cm) with sawdust bedding, and kept under a 12 h:12 h light:dark cycle (lights on at 08:00 h) at a constant temperature of 21±1°C. Food (D12450B, Research Diets, Inc., New Brunswick, NJ, USA) and water were provided *ad libitum*. All experimental procedures complied with the Wenzhou University Animal Care and Use Committee's (WU-ACUC) guidelines, and this experiment was approved by the WU-ACUC.

Virgin female mice (9–10 weeks of age) were paired with males for 11 days, after which the males were removed. The 144 females that subsequently became pregnant and gave birth were randomly assigned to one of four experimentally adjusted litter size (LS) treatment groups on the day of parturition (day 0). The litter sizes of these groups were 2 (LS-2), 6 (LS-6), 12 (LS-12) and 18 (LS-18). Half the animals in each group were kept at an ambient temperature of 21±1°C [hereafter the room temperature (RT) group] and the other half at 30±1°C [hereafter the warm temperature (WT)] group. Therefore, there were a total of eight treatment groups: LS-2 (n=11), LS-6 (n=18), LS-12 (n=18) and LS-18 (n=23) under RT conditions, as well as LS-2 (n=15), LS-6 (n=18), LS-12 (n=21) and LS-18 (n=20) under WT conditions. The experiment continued from day 0 (parturition) until offspring were weaned (day 18).

Physical and behavioral measurements

The body mass of individual mice was measured daily at 16:00 h on days 1–16 of lactation, and food intake was also measured and calculated as the mass of food missing from the hopper, minus orts mixed in the bedding (Cameron and Speakman, 2010). Asymptotic food intake during peak lactation was calculated as the mean food

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intake between days 11 and 15 of lactation. Pup survival and body mass were also measured daily on days 0–16 of their mother's lactation. The survival of pups for each litter was calculated as: (number of pups alive/total number of pups)×100%.

Subcutaneous body temperature

The subcutaneous body temperature (subcutaneous T_b, referred to as T_b hereafter), instead of core body temperature, was measured in the females. Specifically, an encapsulated thermo-sensitive passive transponder (diameter 2 mm and length 14 mm; Destron Fearing, South St Paul, MN, USA) was implanted subcutaneously in the dorsolateral hip region of each subject on day 3 of lactation. A Pocket Reader was then used to receive and collect T_b data without disturbance to the mother and pups. T_b was measured at 15:30 h daily on days 5–16 of lactation.

Energy intake and apparent energy absorption efficiency

Gross energy intake (GEI), digestive energy intake (DEI) and apparent energy absorption efficiency (AEAE) were measured between days 13 and 15 of lactation. Specifically, a known quantity of food was provided at 16:00 h on day 13, and any uneaten food and orts mixed with the bedding material were collected, along with feces from each animal, at 16:00 h on day 15. Food and feces were separated manually after drying at 60°C to constant mass. Gross energy content of food and feces were determined using an IKA C2000 oxygen bomb calorimeter (IKA, Germany). GEI, DEI, AEAE and gross energy (GE) of feces were calculated as described previously (Zhao et al., 2014; Zhang et al., 2015): GEI (kJ day^{-1})=[food provided (g day^{-1})×dry matter content of food (%)−dry spillage of food and uneaten food]×gross energy content of food (kJ g^{-1}); DEI (kJ d^{-1})=GEI (kJ d^{-1})–GE of feces; AEAE (%)=DEI/GEI×100%; and GE of feces=dry feces mass (g day^{-1})×gross energy content of feces (kJ g^{-1}).

Resting metabolic rate

The resting metabolic rate (RMR) of each female was quantified on day 17 by measuring the rate of oxygen consumption with an O_2 measuring module high-speed sensor unit (994620-CS-HSP-01) for calorimetric measurements in an open-flow respirometry system (TSE, Germany). Air was pumped through a cylindrical sealed Perspex chamber at a rate of 1 l min^{-1} at a temperature of 30±0.5°C, which was within the thermal neutral zone of this species. Gases leaving the chamber were dried and sampled using an oxygen analyzer at a flow rate of 0.38 l min^{-1}. Data were collected every 10 s by a computer connected via an analogue-to-digital converter, and analyzed using standard software (TSE). The consecutive minimum rate of oxygen consumption over 10 min was used to calculate RMR, which was corrected to standard temperature and air pressure conditions and expressed as ml O_2 h^{-1}. RMR was measured for 2.5 h, and all measurements were made between 09:00 and 18:00 h. The RMR of each litter was quantified in the same way on day 17 of their mother's lactation.

Thermal conductance

Wet thermal conductance (C_{wet}; ml O_2 g^{-1} h^{-1} $°C^{-1}$), which is the rate of heat loss relative to the thermal gradient, uncorrected for evaporative heat loss, was calculated using the following equation:

$$C_{wet} = \text{metabolic rate}/(T_b - T), \quad (1)$$

where metabolic rate (ml O_2 g^{-1} h^{-1}) is the rate of metabolism at each temperature, T_b is the subject's body temperature and T is the ambient temperature (Gordon, 1993; Gordon, 2012; McNab, 1974, 1980; Schleucher and Withers, 2001; Zhao et al., 2010b).

Milk energy output

MEO during peak lactation (days 13–14) was assessed from the energy budget of litters, as described previously (Król and Speakman, 2003b). Energy available to the pups is obtained from their mother's milk, so total energy was calculated as the sum of energy allocated daily to energy expenditure [daily energy expenditure (DEE)] of the pups and for the growth of new tissue (Zhao et al., 2013a,b). DEE was predicted from pup body mass on the basis of the relationship between RMR and body mass under the assumption that DEE=1.4×RMR to take into account the energetic costs of the pups' activity. The equation used was (Król and Speakman, 2003b):

$$\text{MEO} = [(7.28 + 0.17 \times M_L) \times \text{CF} + M_{L,inc} \times \text{GE}_{pups}] \times 100/d_{milk}, \quad (2)$$

where MEO (kJ day^{-1}) is the milk energy output, M_L (g) is the litter mass on day 14 of lactation, CF is the correction factor (CF=1.4, the mean ratio of daily energy expenditure to RMR) and GE_{pups} (kJ g^{-1} wet mass) is the gross energy content of the pups. The mean GE_{pups} values used in this formula for the eight treatment groups were determined using an IKA C2000 oxygen bomb calorimeter. $M_{L,inc}$ (g day^{-1}) was the increase in litter mass between days 13 and 14 of lactation, and d_{milk} was the apparent digestibility of milk (d_{milk}=96%) (Król and Speakman, 2003b).

Body composition and body fat content

Females were killed by decapitation after they had suckled their pups for at least 2 h between 08:00 and 12:00 h on day 18 of lactation. Trunk blood was collected from each subject and serum was separated from each blood sample by centrifugation (3000 ***g*** min^{-1}×15 min) and stored at –20°C. The hypothalamus and two pads of mammary glands were quickly removed and flash frozen in liquid nitrogen. Then the gastrointestinal tract, heart, liver, lungs, spleen and kidneys, as well as the remainder of the mammary glands, were also removed, and the remaining carcass (including the head and tail) was weighed to the nearest 1 mg to obtain its wet mass before being dried in an oven at 60°C for at least 2 weeks to obtain its dry mass (to 1 mg). Total body fat mass was extracted from the dried carcasses by ether extraction in a Soxhlet apparatus (Zhao and Wang, 2006; Zhao et al., 2010a).

Serum prolactin concentrations

Serum prolactin levels were determined by radioimmunoassay using RIA kits (Beijing North Institute of Biological Technology, Beijing, China). The lowest and highest detection limits were 125 and 2000 μIU ml^{-1}, respectively. This RIA kit has been previously validated for Swiss mice (Zhao et al., 2010b).

Real-time RT-qPCR analysis

Total RNA was prepared from the hypothalamus and the mammary glands using TRIzol agent (TAKARA, Dalian, China). Real-time RT-qPCR analysis was carried out as described previously (Zhao et al., 2014). Briefly, 2 μl cDNA samples were used as a template for the subsequent PCR reaction using gene-specific primers (Table S1). The final reaction volume of 20 μl contained 10 μl of 2× SYBR Premix EX Tag TM (TAKARA), 2 μl cDNA template, and 0.4 μl of forward and reverse primer (final concentration 0.2 μmol l^{-1} per primer; Table S1). The qPCR was performed using

a Roche Light Cycler 480 real-time qPCR system (Forrentrasse CH-6343, Rotkreuz, Switzerland). All samples were quantified for relative gene expression using actin expression as an internal standard.

Data analyses

Data were analyzed using SPSS statistical software (version 20.0). Repeated-measures ANOVA was used to identify significant differences in body mass, T_b and food intake between the different treatment groups over the period of lactation. The effects of litter size and temperature on GEI, DEI, AEAE, RMR, mammary gland mass, serum prolactin levels and the expression of hypothalamic neuropeptide genes were examined using two-way ANOVA or ANCOVA, with body mass as a covariate where required. Significant differences between treatment groups were evaluated using the Student–Newman–Kuels (SNK) *post hoc* test. Pup survival rate, body mass and litter RMR were also analyzed by two-way ANOVA. Correlation coefficients between different variables were evaluated using Pearson's correlation coefficient. All data are presented as means ±s.e.m.; *P*-values <0.05 were considered statistically significant.

RESULTS

Subcutaneous T_b

T_b increased significantly with litter size ($F_{3,40}$=18.67, P<0.01): T_b of the LS-6 group was higher than that of the LS-2 group, but lower than that of the LS-12 and LS-18 groups (Table 1). Furthermore, WT mice had higher T_b than their RT counterparts ($F_{1,40}$=81.95, P<0.01; Table 1). A significant litter size–temperature interaction was also apparent ($F_{3,40}$=18.00, P<0.01): the increase in T_b with litter size was only significant in the WT group (Fig. 1A).

Body mass

Body mass was significantly affected by both litter size ($F_{3,135}$=19.88, P<0.01) and temperature ($F_{1,135}$=360.57, P<0.01). The body mass of the LS-18 group was significantly higher than that of the other three litter size treatment groups, which did not differ significantly from one another (Table 1). The WT group had lower body mass than the RT group (Table 1). A significant litter size–temperature interaction was found ($F_{3,135}$=22.75, P<0.01), and *post hoc* tests indicate that litter size significantly affected the body mass of the RT group (Fig. 1B). This litter size–temperature interaction had different temporal patterns among the different litter size groups ($F_{48,2160}$=6.29, P<0.01). Body mass increased with litter size in RT mice, but not in their WT counterparts (Fig. S1A).

Food intake

Food intake was significantly affected by litter size ($F_{3,135}$=225.95, P<0.01). Food intake was higher in the LS-6 group, and higher still in the LS-18 group relative to the LS-2 group (Table 1). Food intake was also affected by temperature ($F_{1,135}$=484.38, P<0.01): the RT group consumed more food than the WT group (Table 1). A significant litter size–temperature interaction was also found ($F_{3,135}$=22.46, P<0.01). In the RT group, food intake was higher in the LS-6 group, and higher still in the LS-12 and LS-18 groups, compared with the LS-2 group. However, in the WT group, there was no significant difference in food intake between the LS-6, LS-12 and LS-18 groups, all of which were significantly higher than that in the LS-2 group (Fig. 1C). Despite some fluctuations, there were significant temporal differences in food intake between the RT and WT groups over the course of lactation ($F_{45,1770}$=3.73, P<0.01; Fig. S1B).

Pup survival and mean body mass

Pups in the LS-18 group had significantly lower survival compared with those in the other three groups ($F_{3,136}$=21.32, P<0.01; Table 2). Pups in the WT group also had lower survival than those in the RT group ($F_{1,136}$=4.67, P<0.05; Table 2) but there was no significant litter size–temperature interaction. Mean pup body mass was significantly affected by litter size ($F_{3,135}$=229.02, P<0.01): mean body mass in the LS-12 group was lower than that in the LS-2 and LS-6 groups, but higher than that in the LS-18 group (Table 2). The mean body mass of pups in the WT group was also lower than that of those in the RT group ($F_{1,135}$=10.50, P<0.01; Table 2). There was a significant litter size–temperature interaction ($F_{3,135}$=3.19, P<0.05): LS-12 pups in the WT group had a lower mean body mass than their counterparts in the RT group (Fig. 2A). Significant negative correlations between mean pup mass and litter size were found in both the RT (R^2=0.83, P<0.01) and WT groups (R^2=0.79, P<0.01; slope, $F_{1,140}$=2.84, P>0.05; intercept, $F_{1,141}$=15.43, P<0.01; Fig. 2B). Temporal patterns of litter size–temperature interactions affecting pup body mass differed among different litter size groups ($F_{45,2040}$=5.24, P<0.01). For example, in the LS-12 and LS-18 groups, RT pups were significantly heavier than their WT counterparts from days 4–5 to the end of lactation. This was not the case in the LS-2 and LS-6 groups (Fig. S1C).

Energy intake and apparent energy absorption efficiency

Litter size had significant effects on GEI ($F_{3,73}$=54.32, P<0.01): the LS-2 group had the lowest GEI, which was not significantly different among the other treatment groups (Table 1). GEI was also affected

Table 1. Main effects of litter size and ambient temperature on selected morphological, behavioral, and metabolic variables of lactating Swiss mice

	Litter size					Ambient temperature (°C)		
	LS-2	LS-6	LS-12	LS-18	*P*	21	30	*P*
Subcutaneous T_b (°C)	36.80±0.13^a	37.51±0.27^b	38.43±0.31^c	38.34±0.22^c	<0.01	37.28±0.13	38.25±0.23	<0.01
Body mass change (%)	−0.07±0.01^a	−0.05±0.02^a	−0.03±0.02^a	0.04±0.03^b	<0.01	0.09±0.01	−0.13±0.01	<0.01
Asymptotic food intake (g day^{-1})	7.13±0.26^a	12.82±0.50^b	13.81±0.66^{b,c}	14.93±0.65^c	<0.01	16.02±0.46	9.54±0.24	<0.01
GEI (kJ day^{-1})	135.39±6.90^a	233.70±13.51^b	241.32±18.29^b	262.11±16.60^b	<0.01	293.09±10.32	164.05±5.44	<0.01
DEI (kJ day^{-1})	123.86±6.63^a	215.65±12.40^b	223.73±17.01^b	241.93±15.33^b	<0.01	270.04±9.66	152.05±5.37	<0.01
AEAE (%)	91.38±0.44	92.30±0.33	92.66±0.40	92.29±0.25	n.s.	92.07±0.18	92.42±0.30	n.s.
GE of feces (kJ day^{-1})	11.50±0.58^a	18.07±1.34^b	17.59±1.56^b	20.18±1.42^b	<0.01	23.05±0.83	12.00±0.39	<0.01
RMR of females (ml O_2 h^{-1})	114.48±9.42	110.58±6.98	112.07±6.98	122.36±5.92	n.s.	129.37±4.24	101.32±4.63	<0.01
Thermal conductance (ml O_2 g^{-1} h^{-1} °C^{-1})	0.37±0.03	0.33±0.03	0.30±0.02	0.31±0.02	n.s.	0.36±0.01	0.30±0.02	<0.05
MEO (kJ d^{-1})	42.14±1.38^a	102.16±2.79^b	119.60±4.26^c	126.17±5.04^c	<0.01	114.49±5.94	95.38±4.49	<0.01

T_b, body temperature; GEI, gross energy intake; DEI, digestive energy intake; AEAE, apparent energy absorption efficiency; GE, gross energy; RMR, resting metabolic rate; MEO, milk energy output. Data are presented as means±s.e.m. Different superscript letters indicate significant between-group differences identified by Student–Newman–Keuls (SNK) *post hoc* tests. n.s., not significant.

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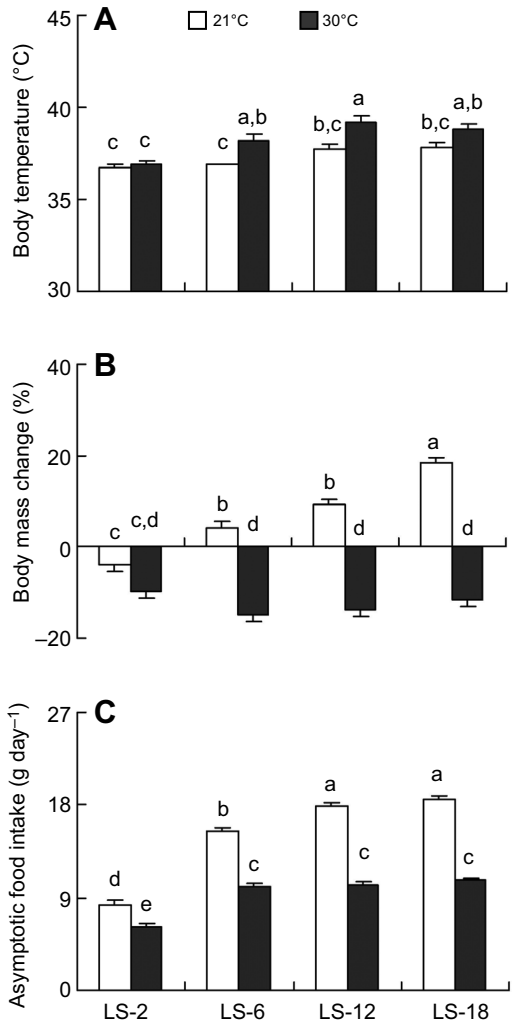

Fig. 1. Effect of temperature on body temperature, body mass and food intake during peak lactation in Swiss mice. Bars show (A) body temperature, (B) body mass and (C) asymptotic food intake in Swiss mice lactating at 21 and 30°C. Females were randomly assigned to one of four litter size (LS) treatment groups that raised 2, 6, 12 or 18 pups (LS-2, LS-6, LS-12 or LS-18). Data are presented as means±s.e.m. Different letters indicate significant between-group differences as determined by a Student–Newman–Keuls (SNK) *post hoc* test (P<0.05).

by temperature ($F_{1,73}$=309.31, P<0.01): mice in the WT group had lower GEI than their counterparts in the RT group (Table 1). A significant litter size–temperature interaction was also apparent ($F_{3,73}$=15.01, P<0.01). *Post hoc* test results indicate that the GEI of each litter size group was higher in RT mice than in their WT counterparts (Fig. S2A). Furthermore, the GEI of the RT group increased with litter size whereas that of the WT group only significantly increased from LS-2 to LS-6 (Fig. S2A). DEI was similarly affected by litter size ($F_{3,73}$=48.38, P<0.01; Table 1), temperature ($F_{1,73}$=263.36, P<0.01, Table 1) and their interaction ($F_{3,73}$=12.70, P<0.01; Fig. S2B). AEAE was not affected by litter size and temperature (Table 1, Fig. S2C) but fecal GE was affected by litter size ($F_{3,73}$=25.65, P<0.01; Table 1), temperature ($F_{3,73}$=246.94, P<0.01; Table 1) and their interaction ($F_{3,73}$=13.85, P<0.01; Fig. S2D). *Post hoc* test results indicate that fecal GE only increased with litter size in the RT group (Fig. S2D).

Maternal RMR

Nursing females in the WT group had lower RMR than their WT counterparts ($F_{1,73}$=13.11, P<0.01; Table 1). Although litter size had no significant effect on RMR (Table 1), there was a significant litter size–temperature interaction ($F_{3,73}$=3.15, P<0.05); RMR in the LS-12 group was only significantly lower that of the other litter size groups in WT mice (Fig. S3A). There was a significant positive correlation between RMR and asymptotic food intake in the RT group (R^2=0.16, P<0.05), but not in the WT group (R^2=0.02, P>0.05; Fig. S4A).

RMR of litters

Litter size had a significant effect on the RMR of litters ($F_{3,73}$=75.87, P<0.01; Table 2). *Post hoc* test results indicate that litters in the LS-2 group had the lowest RMR, and that RMR increased steadily with litter size (Table 2). Temperature did not significantly affect litter RMR, nor was there a significant litter size–temperature interaction (Table 2, Fig. S3B).

Thermal conductance

The thermal conductance of the WT group was lower than that of the RT group ($F_{1,40}$=6.26, P<0.05, Table 1). Litter size had no significant effect on thermal conductance, nor was there any significant litter size–temperature interaction (Table 1, Fig. S3C).

MEO

MEO was affected significantly by litter size ($F_{3,73}$=76.91, P<0.01): the MEO of the LS-6 group was higher than that of the LS-2 group, but lower than that of the LS-12 and LS-18 groups (Table 1, Fig. S3D). Furthermore, the MEO of the WT group was lower than that of the RT group ($F_{1,73}$=13.17, P<0.01; Table 1). MEO was positively correlated with asymptotic food intake in both the RT (R^2=0.86, P<0.01) and WT groups (R^2=0.78, P<0.01; slope, $F_{1,77}$=11.46, P<0.01; intercept, $F_{1,78}$=289.13, P<0.01; Fig. S4B).

Mass of the mammary glands and serum prolactin level

The mass of the mammary glands was significantly affected by litter size ($F_{3,73}$=57.12, P<0.01). The LS-18 group had the heaviest mammary glands, and those of the LS-12 group were heavier than those of the LS-2 and LS-6 groups (Table 3). Mammary glands were significantly lighter in the WT group than in the RT group ($F_{1,73}$=76.06, P<0.01; Table 3). There was also a significant litter size–temperature interaction ($F_{3,73}$=17.68, P<0.01); only the LS-12

Table 2. Main effects of litter size and ambient temperature on the survival, body mass and resting metabolic rate (RMR) of Swiss mouse litters

	Litter size					Ambient temperature		
	LS-2	LS-6	LS-12	LS-18	P	21°C	30°C	P
Survival rate (%)	98.08±1.92^b	100.00±0.00^b	99.57±1.86^b	90.64±8.47^a	<0.05	97.69±0.63	95.85±1.04	<0.01
Mean pup mass (g)	12.42±0.34^c	12.26±0.21^c	7.91±0.13^b	6.19±0.10^a	<0.01	9.50±0.33	9.11±0.36	<0.01
RMR of litters (ml O_2 h^{-1})	54.15±2.11^a	169.96±5.95^b	182.37±7.17^{b,c}	197.36±6.69^c	<0.01	172.09±8.49	155.15±9.40	n.s.

Data are presented as means±s.e.m. Different superscript letters indicate significant between-group differences identified by SNK *post hoc* tests. n.s., not significant.

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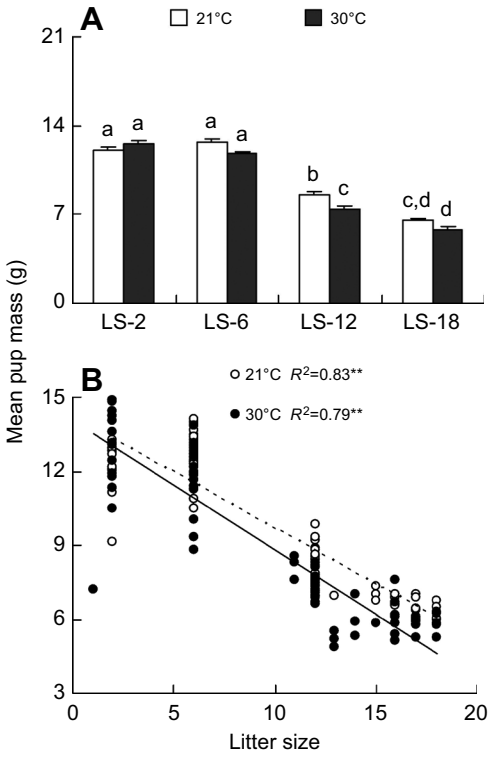

Fig. 2. Effect of temperature on mean pup mass and the correlation between litter size and mean pup mass during peak lactation in Swiss mice. Mean pup mass (A) and the correlation between litter size and mean pup mass (B) in Swiss mice lactating at 21 and 30°C. Females were randomly assigned to one of four litter size (LS) treatment groups that raised 2, 6, 12 or 18 pups (LS-2, LS-6, LS-12 or LS-18). Data are presented as means±s.e.m. Different letters indicate significant between-group differences as determined by an SNK *post hoc* test (P<0.05). **P<0.01.

and LS-18 groups of WT mice had lighter mammary glands than their RT counterparts (Fig. 3A). In addition, the mass of the mammary glands was positively correlated with asymptotic food intake in both the RT (R^2=0.59, P<0.01) and WT groups (R^2=0.30, P<0.01; Fig. 3B). There was a positive correlation between the mass of the mammary glands and T_b in the RT group (R^2=0.61, P<0.01), but not the WT group (R^2=0.02, P>0.05) (slope, $F_{1,44}$=34.60, P<0.01; intercept, $F_{1,45}$=26.56, P<0.01; Fig. 3C), and between mammary gland mass and MEO in both the RT (R^2=0.55, P<0.01) and WT groups (R^2=0.26, P<0.01) (slope, $F_{1,77}$=9.67, P<0.01; intercept, $F_{1,78}$=27.87, P<0.01; Fig. 3D). Serum levels of prolactin were not significantly affected by litter size, temperature or their interaction (Table 3).

Gene expression

In general, neurochemical gene expression was not affected by litter size, temperature or the interaction between these factors (Table 4). There were, however, a few exceptions. POMC gene expression in the hypothalamus was significantly lower in the WT group than in the RT group ($F_{3,73}$=4.01, P<0.05; Table 4). Furthermore, there was a significant litter size–temperature interaction on NPY levels in the hypothalamus ($F_{3,72}$=2.93, P<0.05; Table 4). *Post hoc* test results indicate that the WT LS-18 group had a higher level of NYP gene expression in the hypothalamus than the RT LS-12 group (Table 4). There were positively significant correlations between the gene expressions of several hypothalamus neuropeptides, whereas no correlations were observed between GEI and any of the tested neuropeptides (Table S2).

DISCUSSION

Lactation is the most energy demanding period in the life cycle of many female mammals and thus is an ideal period in which to study energy intake and expenditure, as well as the effects of environmental and intrinsic factors on maternal energy balance (Speakman and Król, 2005; Thompson and Nicoll, 1986; Thompson, 1992). We examined the effects of differences in litter size and ambient temperature on body temperature, body mass, food intake and RMR of lactating Swiss mice. We also examined maternal serum prolactin levels and the expression of some neuropeptide genes thought to play a role in development, metabolism and reproduction. Collectively, our results show that litter size and ambient temperature both independently and interactively affected energy intake and reproductive output, indicated by SusEI and MEO at peak lactation and litter mass at weaning. These results not only provide support for both the PL and HDL hypotheses, but also suggest that the limitation of SusEI is a dynamic process influenced by multiple factors acting on overlapping physiological mechanisms.

The effects of litter size on energy balance

Mammals usually increase their food intake several fold to meet the energy requirements of lactation, which vary with litter size (Hammond and Diamond, 1997; Speakman and Król, 2005; Valencak and Ruf, 2009). In the present study, female mice raising larger litters had significant higher food and energy intake (including asymptotic food intake, GEI and DEI) during lactation than those raising smaller litters. The former also had higher energy output, as measured by MEO, compared with the latter. However, it is notable that neither the food intake nor energy output of the LS-18 group was significantly higher than that of the LS-12 group. These data are consistent with the results of previous research on mice (Hammond et al., 1996; Zhao et al., 2010a, 2013a; Zhao, 2012) and other rodent species (Fiorotto et al., 1991; Kenagy et al., 1990; Rogowitz, 1996; Zhao et al., 2010a), suggesting that there are limits to SusEI during lactation (Hammond and Diamond, 1997; Koteja, 1996a,b; Speakman and Król, 2005). SusEI in lactating Swiss mice is probably already at maximum capacity and females are consequently unable to increase milk production to support more than 12 pups (Zhao et al., 2010b). Consequently, the LS-18 group had a significantly lower pup survival rate compared with the other three LS treatment groups. These data support the PL hypothesis.

Table 3. Main effects of litter size and ambient temperature on the mammary gland mass and serum prolactin levels of lactating Swiss mice

	Litter size					Ambient temperature (°C)		
	LS-2	LS-6	LS-12	LS-18	P	21	30	P
Mammary gland mass (g)	2.00±0.12^a	3.10±0.25^a	5.34±0.56^b	7.42±0.63^c	<0.01	6.63±0.53	3.22±0.22	<0.01
Serum prolactin (μIU ml^{-1})	287.56±20.40	256.70±12.30	239.11±10.04	259.42±10.80	n.s.	270.69±8.39	245.00±9.37	n.s.

Data are presented as means±s.e.m. Different superscript letters indicate significant between-group differences identified by SNK *post hoc* tests. n.s., not significant.

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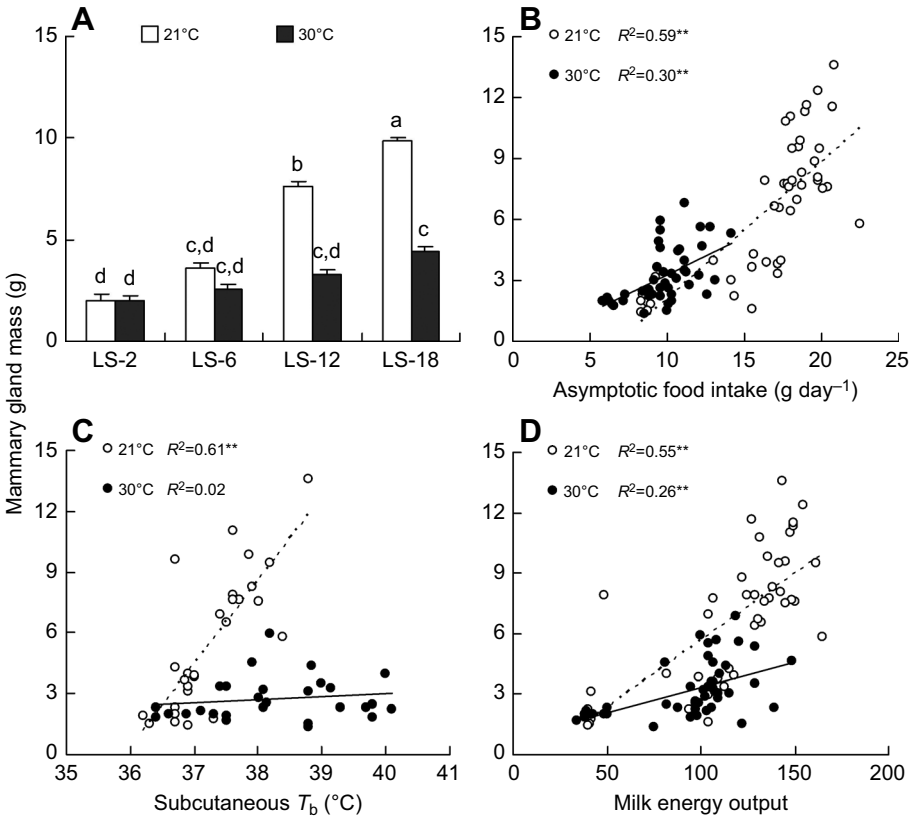

Fig. 3. Effect of temperature on mammary gland mass and the correlations between mammary gland mass and asymptotic food intake, body temperature and milk energy output during peak lactation in Swiss mice. Mammary gland mass (A) and correlations between mammary gland mass and asymptotic food intake (B), body temperature (C) and milk energy output (D) in Swiss mice lactating at 21 and 30°C. Females were randomly assigned to one of four litter size (LS) treatment groups that raised 2, 6, 12 or 18 pups (LS-2, LS-6, LS-12 or LS-18). Data are presented as means±s.e.m. Different letters indicate significant between-group differences as determined by an SNK *post hoc* test (P<0.05). **P<0.01.

However, these findings do not provide strong evidence of the HDL hypothesis at all, although it is somewhat consistent with the prediction of the HDL idea. Based on the HDL hypothesis, the females raising a large litter size may have more heat risk because they are nursing more pups, and thus have higher body temperatures than those supporting a smaller litter size. In this study, however, the T_b of females did not change significantly in the LS-18 group compared with that of females in the LS-12 group. Consistently, T_b during nursing in MF1 mice was not higher than that recorded during other behaviors, and there was no indication that mothers discontinued suckling from the offspring because of a progressive rise in their T_b while suckling (Gamo et al., 2016). This suggests that HDL may have little effect on SusEI and reproductive performance for the females lactating at room temperature.

The effects of ambient temperature on energy balance

Our data also indicate that ambient temperature had marked effects on the indicators of food intake and energy expenditure that we monitored. The WT group had significantly lower food and energy intake (including asymptotic food intake, GEI and DEI) and energy expenditure and output (as measured by RMR, MEO and fecal GE) than the RT group. For example, the females supporting 2, 6, 12 and 18 pups showed 25.4, 34.6, 42.7 and 42.6% less asymptotic food intake at the warm temperature than at room temperature. The females raising two pups did not differ in MEO between the two temperatures, whereas the females supporting 6, 12 and 18 pups produced 8.5, 20.4 and 16.3% less milk, respectively, at the warm temperature than at room temperature. The lower MEO of the WT group might also contribute to this group's lower mean pup mass at weaning and lower pup survival compared with the RT group. Warmer temperatures have been found to reduce both energy expenditure and milk production in lactating mice (Hammond and Kristan, 2000; Król and Speakman, 2003a,b), rats (Leon et al., 1983; Rogowitz, 1998), voles (Simons et al., 2011; Wu et al., 2009), gerbils (Yang et al., 2013) and hamsters (Paul et al., 2010). It should be noted that the WT group had lower thermal conductance, but higher body temperature, than the RT group. The increased metabolic cost of dissipating body heat at warmer temperatures

Table 4. Effects of temperature and litter size on the expression of selected neuropeptide genes in the hypothalamus and the prolactin receptor gene in the mammary glands of lactating Swiss mice

	21°C–LS-2	21°C–LS-6	21°C–LS-12	21°C–LS-18	30°C–LS-2	30°C–LS-6	30°C–LS-12	30°C–LS-18	P_T	P_{LS}	$P_{LS\times T}$
NPY	1.51±0.36^{a,b}	1.51±0.15^{a,b}	1.00±0.15^{a}	1.06±0.11^{a,b}	1.23±0.17^{a,b}	1.29±0.15^{a,b}	1.30±0.14^{a,b}	1.62±0.19^{b}	n.s.	n.s.	<0.05
AgRp	1.01±0.34	1.37±0.36	1.00±0.26	1.01±0.19	0.62±0.14	1.12±0.29	1.37±0.28	1.96±0.47	n.s.	n.s.	n.s.
Ore	1.54±0.40	1.67±0.33	1.00±0.20	0.81±0.13	1.09±0.23	0.94±0.20	0.68±0.12	1.05±0.21	n.s.	n.s.	n.s.
CART	1.41±0.24	1.40±0.16	1.00±0.21	0.98±0.08	0.94±0.14	1.10±0.16	0.98±0.15	1.88±0.59	n.s.	n.s.	n.s.
POMC	1.46±0.61	1.32±0.29	0.99±0.38	0.69±0.17	0.99±0.50	0.64±0.24	0.40±0.14	0.76±0.25	<0.05	n.s.	n.s.
Prl-R	1.76±0.48	1.38±0.18	1.00±0.22	0.64±0.12	0.94±0.25	1.20±0.10	1.26±0.29	1.25±0.35	n.s.	n.s.	n.s.

NPY, neuropeptide Y; AgRp, agouti-related protein; Ore, orexin; CART, cocaine- and amphetamine-regulated transcript; POMC, pro-opiomelanocortin; Prl-R, prolactin receptor. Data are presented as means±s.e.m. P_T, P_{LS}, $P_{LS\times T}$: P-values of differences between temperature treatment groups, litter size treatment groups and their interaction. Different superscript letters indicate significant between-group differences identified by SNK *post hoc* tests. n.s., not significant.

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may be an important factor constraining SusEI (Król and Speakman, 2003a,b; Speakman and Król, 2005, 2011). A previous comparison of two mouse strains selected for different levels of thermal conductance found that the strain with lower thermal conductance assimilated less energy, produced less milk and had lighter litters at weaning than the strain with higher thermal conductance (Al Jothery et al., 2014). The results of our temperature experiment support the HDL hypothesis, which suggests that animals subject to warmer ambient temperatures have to restrict the production of body heat associated with food input and energy expenditure (Speakman and Król, 2005, 2011).

Interaction between litter size and ambient temperature

An interesting finding in the present study is the interaction between litter size and ambient temperature on several indicators of food intake and energy expenditure. For example, asymptotic food intake increased with litter size up to LS-12 in the RT group but only up to LS-6 in the WT group. Similarly, GEI and DEI increased with litter size in the RT group, but not in the WT group. Specifically, the females raising smaller litter sizes showed similar energy intake and milk production, and raised pups of similar body mass between the two temperatures, while the females supporting larger litter sizes consumed significantly less food, produced less milk and weaned significantly lighter pups at the warm temperature than at room temperature, indicating a strong interaction of litter size and ambient temperature. Furthermore, increased litter size also resulted in a moderate, but significant, increase in RMR and fecal GE in the RT group but not in the WT group. These results suggest that energy intake and expenditure of females at peak lactation reach an asymptote with increasing litter size, while the asymptote is considerably attenuated under the higher temperature compared with that at lower temperature. It is possible that an animal's SusEI under normal ambient temperature (i.e. 21°C) is mainly constrained by its capacity to meet increased demands, such as those caused by an increase in litter size, as suggested by the PL hypothesis. However, at warmer temperatures, the ability to dissipate heat, as suggested by the HDL hypothesis, becomes a major factor that interacts with such peripheral factors to constrain SusEI. Our results indicate that SusEI was limited by increased litter size, and that this limitation intensified at a higher ambient temperature (30±1°C). As mentioned above, females lactating at warm temperature showed significantly lower thermal conductance (lower by 16.7% at 30°C than at 21°C) and higher body temperatures than those at room temperature. Thus, the females may stop suckling from their pups to decrease the risk of the progressive rise in their T_b. It has been observed that Swiss mice lactating at 30°C spent 25.0% less time suckling their pups than females lactating at 21°C (Zhao et al., 2016). Consequently, reproductive performance has been notably impaired at higher ambient temperature, indicating a role for HDL.

It should be noted that in our experiment, increases in litter size elevated body temperature but had no effects on RMR or thermal conductance. The increases in food intake and energy expenditure associated with increased litter size during lactation can generate additional heat as a by-product (Speakman and Król, 2005), whereas milk production and the suckling of young also affect maternal heat retention (Gamo et al., 2016). Therefore, the main effects of litter size on SusEI during lactation may be modified by an interaction between an animal's maximum reproductive capacity (Diamond and Hammond, 1992; Speakman and Król, 2005) and its ability to dissipate heat (Speakman and Król, 2005).

This notion is further supported by our data on mammary gland mass. Mammary gland mass significantly increased with litter size, and this increase was much more pronounced in the RT group than in the WT group. In addition, mammary gland mass was strongly correlated with food intake, body temperature and MEO in the RT group, but only weakly correlated with these variables in the WT group. These data again suggest that the limitation of SusEI imposed by the maximum capacity of the mammary glands is intensified at higher ambient temperatures, possibly by the requirement to dissipate excess heat.

Prolactin and neuropeptide gene expression in the hypothalamus

Prolactin is known to stimulate lactation and mammary gland development, as well as facilitate milk production and secretion (Kennett and McKee, 2012; Patil et al., 2014). It has been proposed that the relationship between the capacity to dissipate heat and lactation is mediated by prolactin (Speakman and Król, 2005). Thus, in addition to its effects on reproduction, prolactin may be directly involved in regulating SusEI (Speakman and Król, 2005). However, although both litter size and ambient temperature affected mammary gland mass, the development and growth of which is regulated by prolactin, we found no significant differences in prolactin levels among treatment groups. One possible explanation for this apparently anomalous result is that mice with large litters were at risk of hyperthermia owing to increased milk production. These animals may consequently have attempted to reduce milk production, especially during peak lactation, leading to a reduction in prolactin production and release (Croskerry et al., 1978; Speakman and Król, 2005) and the consequent absence of between-group differences in prolactin levels. Regulation of the mammary glands by prolactin in the WT group may have be attenuated, which suggests that SusEI may be regulated by downstream physiological, morphological and behavioral factors associated with mammary gland function.

We also examined the effects of litter size and ambient temperature on the expression of several neuropeptides genes in the hypothalamus that have key roles in development, metabolism and reproduction. To our surprise, there were, with few exceptions, no significant differences in the expression of most of these neuropeptide genes among treatment groups. There are several possible explanations for this. One is that the effects of the experimental treatments on gene expression were transient and, consequently, not detected. This hypothesis could be tested by a time-course experiment, and experiments measuring neuropeptide protein expression and release will also be necessary. Another possible explanation is that litter size and ambient temperature may only affect neuropeptide expression in a brain-region-specific manner. If so, our approach of processing tissue punches from the entire hypothalamus may lead to a 'floor effect', reducing the quantitative sensitivity and preventing us from detecting treatment effects on the neuropeptide expression in a particular hypothalamic nucleus. Additional experiments with better quantitative sensitivity and anatomical resolution on measuring neuropeptide expression and release as well as their receptor activity will be required.

In summary, our results show that changes in litter size and ambient temperature can affect a comprehensive set of behavioral, physiological and morphological indicators of SusEI in lactating Swiss mice. This supports both the PL and HDL hypotheses. In addition, the fact that the same indicators were affected by both litter size and ambient temperature indicates a significant interaction between the two factors. The limitation of SusEI induced by increased litter size may be regulated by the capacity of the mammary glands under normal temperature conditions. However, increased ambient temperature appears to interact with litter size to constrain SusEI, possibly causing animals to restrict the production of excess body

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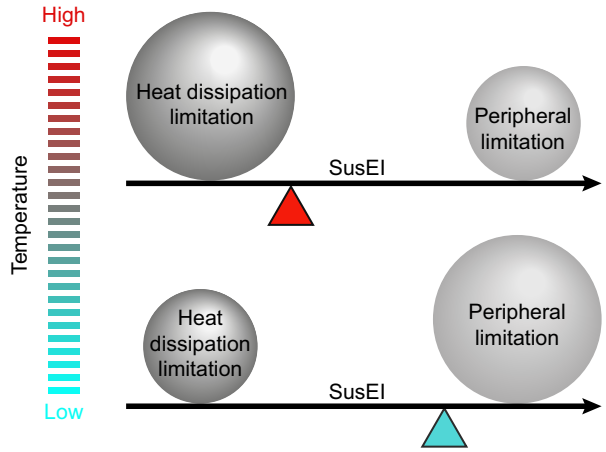

Fig. 4. Schematic diagram illustrating the constraints on sustained energy intake (SusEI) during lactation imposed by the dynamic interaction between reproductive energy requirements and heat dissipation. Animals increase their SusEI to meet increased energy demands associated with reproduction (e.g. increased litter size), which, under normal temperature conditions (bottom), is constrained mainly by peripheral limitation. However, under warmer temperatures (top), heat dissipation limitation may further constrain SusEI, shifting maximum SusEI to the left (indicated by the triangle). Therefore, SusEI during lactation is constrained by multiple factors and their interactions operating within overlapping physiological mechanisms.

heat. As shown in Fig. 4, we propose that SusEI during lactation is a dynamic process regulated and constrained simultaneously by both limitations in an ambient-temperature-dependent manner. Under normal temperature conditions (Fig. 4, bottom), SusEI is constrained mainly by peripheral limitation, such as the capacity of the mammary glands to produce milk. When the ambient temperature is rising from low to high (Fig. 4, top), heat dissipation limitation may generate a synergistic effect, further constraining SusEI, making the constraining point shift to the left. This model is supported by the data showing that both SusEI and reproductive performance are constrained in cold-exposed female mice (Hammond and Kristan, 2000; Zhao, 2012), whereas SusEI is notably constrained in the lactating mice simultaneously forced to run to obtain food (Perrigo, 1987; Zhao et al., 2013b). Therefore, SusEI is constrained during lactation by multiple factors, acting both independently and interactively in a dynamic pattern on overlapping physiological mechanisms (Fig. 4). Although it is beyond the scope of the present study, such dynamic interactions between intrinsic and extrinsic factors on energy intake/expenditure should be examined in other energy-demanding phases, such as during excessive exercise or under extreme low environmental temperature conditions (Speakman and Król, 2005, 2011).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Methodology: J.W., S.T., Q.-Q.Q., Y.H., J.-S.L., Z.-J.Z.; Software: J.C.; Formal analysis: J.C.; Data curation: J.W., S.T., W.-J.F., Z.-J.Z.; Writing - original draft: J.W., Z.-X.W., Z.-J.Z.; Writing - review & editing: Z.-X.W., Z.-J.Z.; Supervision: J.-S.L., Z.-J.Z.

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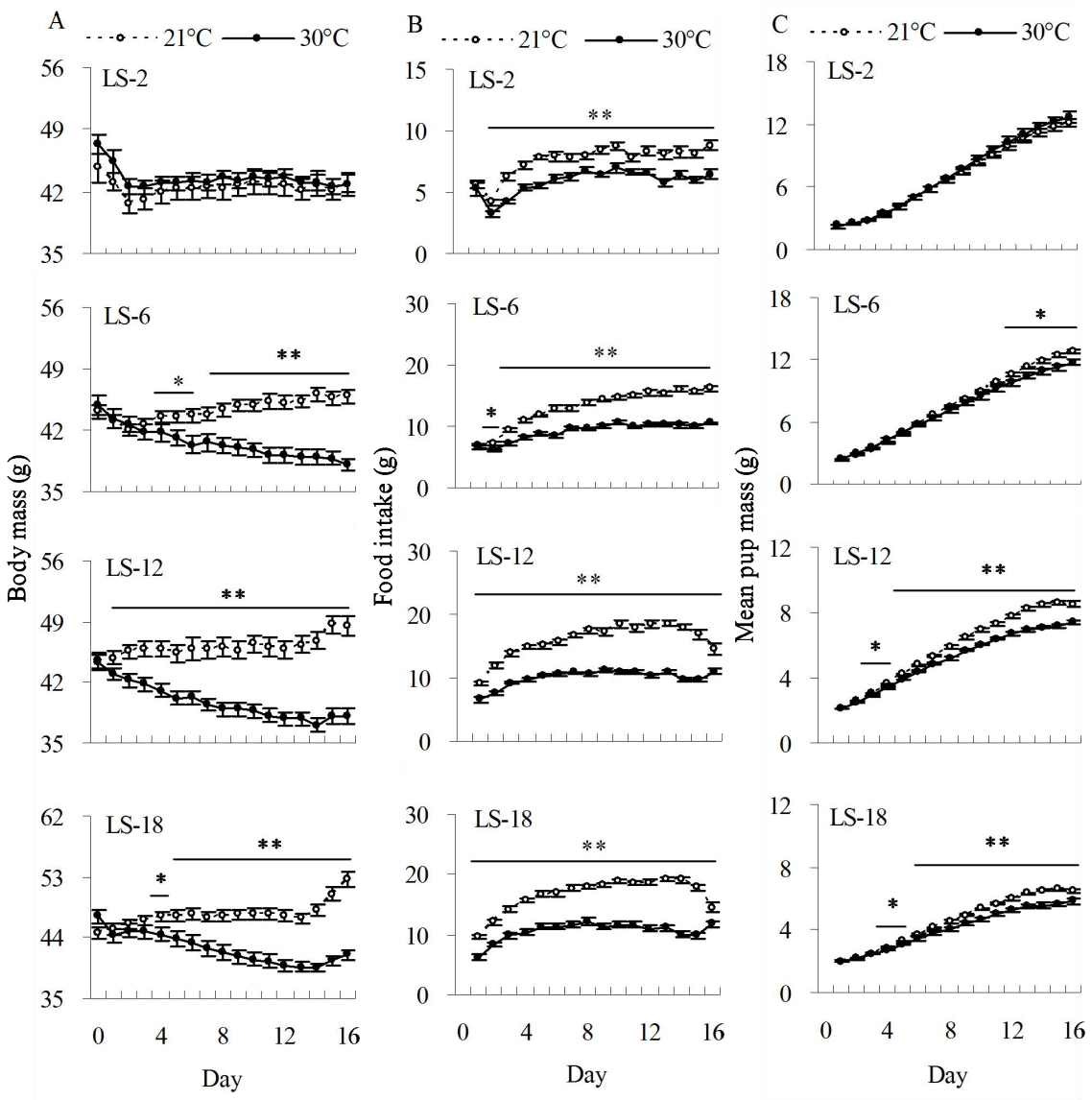

Fig. S1. Body mass (A), food intake (B) and mean pup mass (C) Swiss mice lactating at 21°C and 30°C, respectively. Females were randomly assigned to one of four litter size (LS) treatment groups that raised 2, 6, 12 or 18 pups (LS-2, LS-6, LS-12 or LS-18). Data are presented as means ± SEM, *p<0.05, **p<0.01.

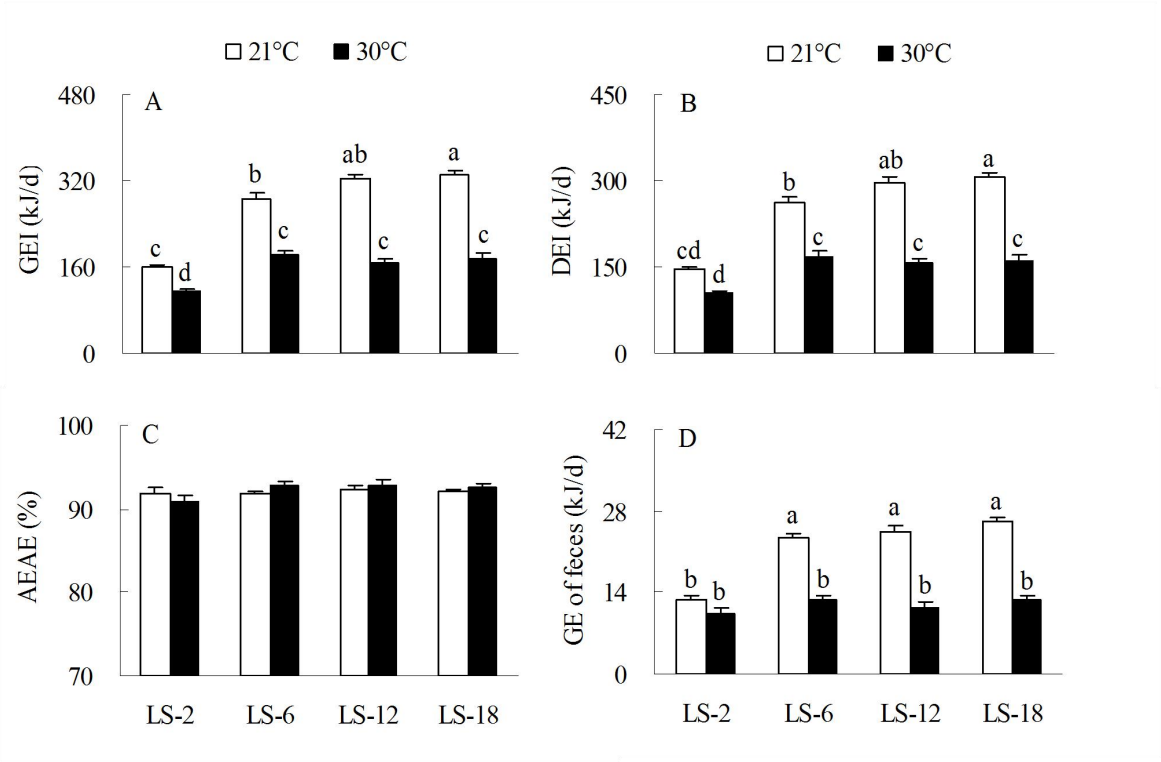

Fig. S2. Gross energy intake (GEI, A), digestive energy intake (DEI, B), apparent energy absorption efficiency (AEAE, C) and gross fecal energy (GE, D) in Swiss mice lactating at 21°C and 30°C, respectively. Females were randomly assigned to one of four litter size (LS) treatment groups that raised 2, 6, 12 or 18 pups (LS-2, LS-6, LS-12 or LS-18). Data are presented as means ± SEM. Different letters indicate significant between-group differences as determined by an SNK post-hoc test (p<0.05).

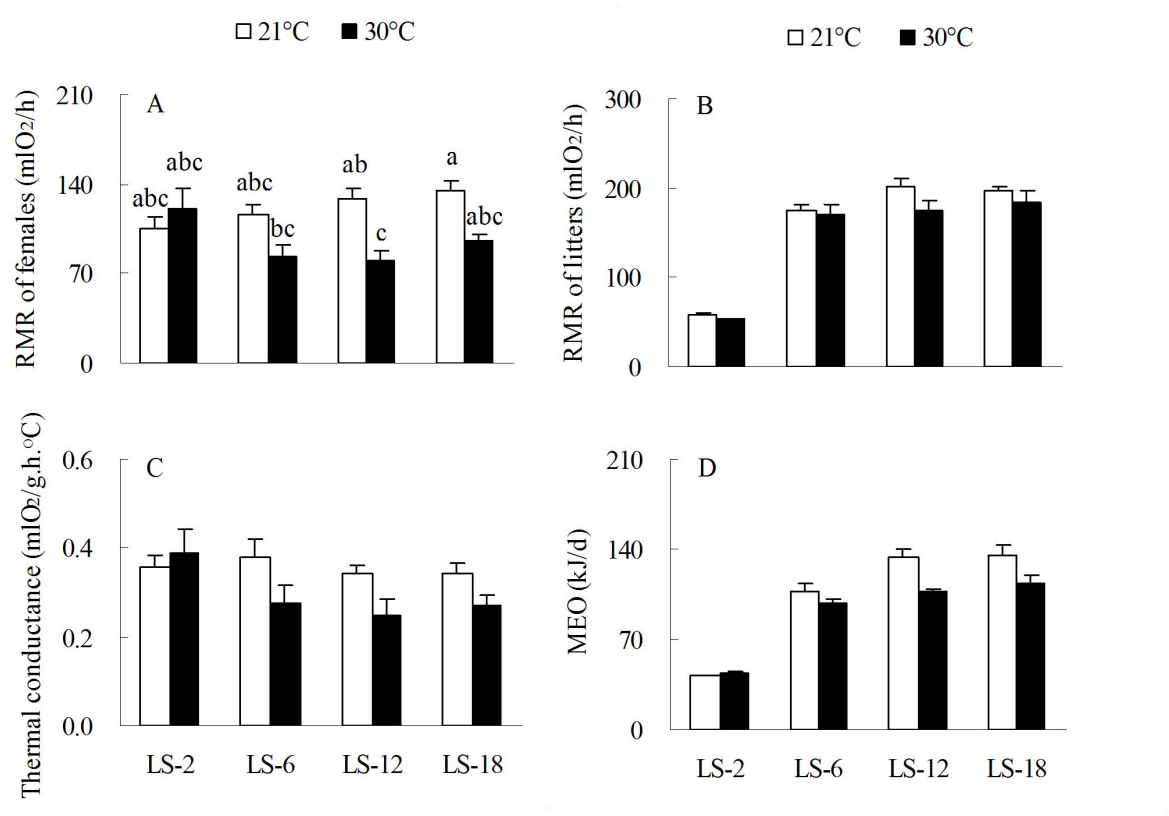

Fig. S3. Resting metabolic rate (RMR) of females (A) and litters (B), and thermal conductance (C), and milk energy output (MEO, D), in Swiss mice lactating at 21°C and 30°C, respectively. Females were randomly assigned to one of four litter size (LS) treatment groups that raised 2, 6, 12 or 18 pups (LS-2, LS-6, LS-12 or LS-18). Data are presented as means ± SEM. Different letters indicate significant between-group differences as determined by an SNK post-hoc test (p<0.05).

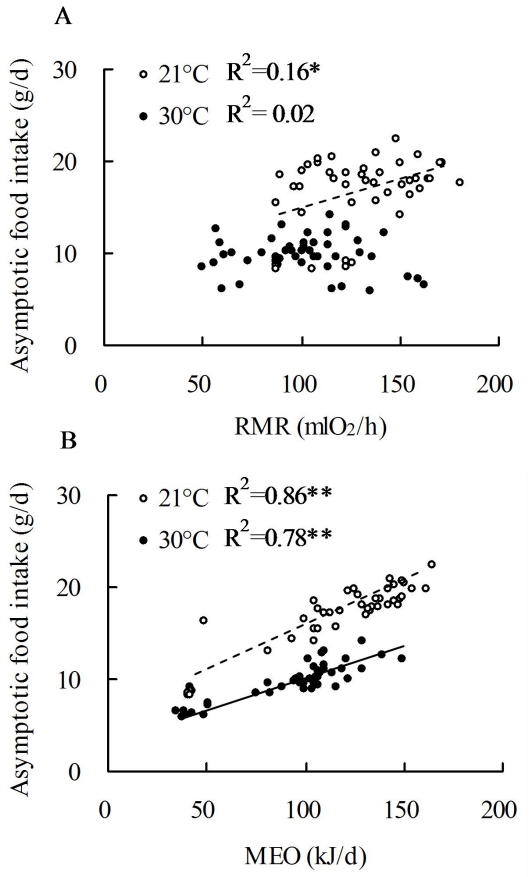

Fig. S4. Correlations between asymptotic food intake and resting metabolic rate (RMR; A) and milk energy output (MEO; B) in lactating Swiss mice at either 21ºC or 30ºC ambient temperature. *, the coefficient of correlation is significant (p<0.05), **, p<0.01.

Table S1. Gene-specific primer sequences used for real-time RT-QPCR analysis of the expression of neuropeptide genes and the prolactin receptor gene in lactating Swiss mice.

Gene	Primers	(5'to3')
NPY	forward	5’-TCGTGTGTTTGGGCATTCTG-3’
	reverse	5’-TCTGGTGATGAGATTGATGTAGTG-3’
AgRp	forward	5’-ACCTTAGGGAGGCACCTCAT-3’
	reverse	5’- AGCAACATTGCAGTCAGCAT-3’
Orx	forward	5’-AACTTTCCTTCTACAAAGGTTCC-3’
	reverse	5’-CGCTTTCCCAGAGTCAGGAT-3’
CART	forward	5’-ACGAGAAGGAGCTGCCAAG -3’
	reverse	5’-GCTCTCCAGCGTCACACAT-3’
POMC	forward	5’-GAAGATGCCGAGATTCTGCT-3’
	reverse	5’- CTCCAGCGAGAGGTCGAGTT-3’
Prl-R	forward	5’-ATAAAAGGATTTGATACTCATCTGCTAGAG-3’
	reverse	5’-TGTCATCCACTTCCAAGAACTCC-3’
Actin	forward	5’-CGTAAAGACCTCTATGCCAA-3’
	reverse	5’-GCGCAAGTTAGGTTTTGTC-3’

Table S2. The coefficient of correlations between neuropeptides and energy intake in Swiss mice lactating at 21°C and 30°C.

		GEI	NPY	AgRp	Ore	CART	POMC
21°C	GEI	1					
	NPY	-0.30	1				
	AgRp	0.04	0.32*	1			
	Orx	-0.19	0.41**	0.48**	1		
	CART	-0.14	0.72**	0.49**	0.58**	1	
	POMC	-0.20	0.18	0.59**	0.55**	0.47**	1
30°C	GEI	1					
	NPY	-0.04	1				
	AgRp	-0.05	0.27	1			
	Ore	-0.22	0.37*	0.65**	1		
	CART	0.08	0.57**	0.66**	0.67**	1	
	POMC	-0.31	0.17	0.51**	0.67**	0.36*	1

Data are coefficient of correlations. *, significant correlation (P<0.05), **, P<0.01. GEI, gross energy intake during peak lactation; NPY, neuropeptide Y; AgRp, agouti-related protein; Ore, Orexin; CART, cocaine and amphetamine regulated transcript; POMC, pro-opiomelanocortin. There were positively significant correlations between the gene expressions of several hypothalamus neuropeptides, whereas, no correlations were observed between GEI and neuropeptides.