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RESEARCH ARTICLE

Limits to sustained energy intake. XXII. Reproductive performance of two selected mouse lines with different thermal conductance

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ABSTRACT

Maximal sustained energy intake (SusEI) appears limited, but the factors imposing the limit are disputed. We studied reproductive performance in two lines of mice selected for high and low food intake (MH and ML, respectively), and known to have large differences in thermal conductance (29% higher in the MH line at 21°C). When these mice raised their natural litters, their metabolisable energy intake significantly increased over the first 13 days of lactation and then reached a plateau. At peak lactation, MH mice assimilated on average 45.3% more energy than ML mice (222.9±7.1 and 153.4±12.5 kJ day^{-1}, *N*=49 and 24, respectively). Moreover, MH mice exported on average 62.3 kJ day^{-1} more energy as milk than ML mice (118.9±5.3 and 56.6±5.4 kJ day^{-1}, *N*=subset of 32 and 21, respectively). The elevated milk production of MH mice enabled them to wean litters (65.2±2.1 g) that were on average 50.2% heavier than litters produced by ML mothers (43.4±3.0 g), and pups that were on average 27.2% heavier (9.9±0.2 and 7.8±0.2 g, respectively). Lactating mice in both lines had significantly longer and heavier guts compared with non-reproductive mice. However, inconsistent with the 'central limit hypothesis', the ML mice had significantly longer and heavier intestines than MH mice. An experiment where the mice raised litters of the opposing line demonstrated that lactation performance was not limited by the growth capacity of offspring. Our findings are consistent with the idea that the SusEI at peak lactation is constrained by the capacity of the mothers to dissipate body heat.

KEY WORDS: Artificial selection, Cross-fostering, Daily energy expenditure, Heat dissipation limit, Milk production, Lactation

INTRODUCTION

Factors limiting maximal rates of sustained energy intake (SusEI) and sustained energy expenditure (SusMR) have been of interest for at least 30 years, since the suggestion that both are constrained at some multiple of basal metabolism (Drent and Daan, 1980; Kirkwood, 1983). Four different ideas have emerged to explain why intake and expenditure might be limited (reviewed in Speakman and Król, 2005a; Piersma and van Gils, 2010; Speakman and Król, 2011). The 'central limitation hypothesis' (Weiner, 1989; Weiner, 1992; Peterson et al., 1990; Sadowska et al., 2013) suggests that limits are imposed by the uptake capacity of the alimentary tract. The 'peripheral limitation hypothesis' (Hammond et al., 1996) posits the limit resides in the capacities of the tissues where the energy is expended. The 'heat dissipation limit (HDL) theory' (Speakman and Król, 2010) suggests that intake is constrained by the capacity to dissipate the heat generated as a by-product of food utilisation and milk production. Finally, a trade-off idea suggests that working beyond a certain limit generates negative physiological consequences that impact survival (Drent and Daan, 1980; Daan et al., 1996; Piersma, 2011; Piersma and van der Velde, 2012). The HDL theory could be considered a special case of this latter idea, because the implication is that processing food and elevating metabolic rate beyond the heat dissipation capacity leads to hyperthermia, with direct or indirect negative consequences for survival.

One of the the most popular models for exploring the question of where the limit resides is lactation (Hammond and Diamond, 1992; Speakman and McQueenie, 1996). During lactation food intake increases enormously (Johnson et al., 2001a) and conspicuously reaches a plateau in late lactation that is resistant to attempts to breach it by imposing additional workloads on the female, for example, by manipulating litter size or pup demands (Hammond and Diamond, 1992; Johnson et al., 2001a; Laurien-Kehnen and Trillmich, 2003; Duah et al., 2013), by making females simultaneously pregnant (Johnson et al., 2001c), or by forcing them to run to obtain their food (Perrigo, 1987; Zhao et al., 2013a). However, when lactating animals are placed in the cold, they are able to eat significantly more than at room temperature (Hammond et al., 1994; Hammond and Kristan, 2000; Johnson and Speakman, 2001; Rogowitz, 1998; Zhang and Wang, 2007), and conversely when kept in hot conditions their maximal intake declines (Król and Speakman, 2003a; Wu et al., 2009; Yang et al., 2013). This effect could be explained either by the HDL theory, the summed peripheral demands idea or temperature-dependent variations in pup energy demands. In MF1 mice, observations of milk production and pup growth at the different temperatures [enhanced in the cold and reduced in the heat (Johnson and Speakman, 2001; Król and Speakman, 2003b)] strongly supported only the HDL idea. Yet in other studies, cold exposure did not have an impact on pup growth (Zhang and Wang, 2007; Zhao and Cao, 2009; Zhao et al., 2010; Zhao et al., 2013b; Yang et al., 2013), supporting the other two ideas.

Attempts to differentiate between the ideas that the intake is limited by the heat dissipation capacity of the mother, the peripheral capacities of the mammary glands or the demand of the pups have produced a confusion of results. Shaving MF1 mice to increase their

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List of abbreviations

ADE	apparent digestive efficiency
BAT	brown adipose tissue
BMR	basal metabolic rate
DEE	daily energy expenditure
DLW	doubly labelled water
HDL	heat dissipation limit
H-L	MH mothers with cross-fostered ML pups
L-H	ML mothers with cross-fostered MH pups
MEI	metabolisable energy intake
MEO	milk energy output
MH	high maintenance line
ML	low maintenance line
RMRt	resting metabolic rate at thermoneutrality
SusEI	sustained energy intake
SusMR	sustained energy expenditure

heat dissipation capacity showed that the females ate more food, produced more milk and weaned larger pups (Król et al., 2007) – consistent only with the HDL idea. However, shaving Swiss mice resulted in significantly elevated food intake, but the inferred changes in milk production and pup growth, although in the predicted direction, were not statistically significant (Zhao and Cao, 2009; Zhao et al., 2010). In a later study, the interpretation of pup growth capacity was rejected, by raising small litters in the cold and showing that they could grow faster than larger litters (Zhao et al., 2013b), thereby implicating the milk-production capacity, as the factor limiting sustained intake in Swiss mice. Conversely, shaving lactating field voles increased offspring growth, but milk production was again in the expected direction but not significantly different (Simons et al., 2011).

A novel approach was used to address the issue in brown hares, by keeping mother and pups at different temperatures (Valencak et al., 2010). The results suggested that in the early phase of lactation pup demand might drive intake, but that later in lactation it is less clear what factors imposed the limit (Valencak et al., 2009). This approach was later expanded to mice (Valencak et al., 2013). However, again the results were not clear cut, because although the mothers with access to the cold elevated their intake and milk production (consistent with the HDL theory), their pups did not grow more, potentially pointing to increased pup demand driving the intake and milk production effects.

Overall, the current data are extremely confusing and suggest that different species and strains operate under different constraints. Moreover, multiple constraints may apply in the same individuals under different conditions, for example, at different ambient temperatures (Yang et al., 2013) or at different litter sizes (Wu et al., 2009). More experimental data across a range of different animal models are needed to enhance our understanding of the factors that are of potential importance in limiting SusEI. Here, we propose a direct test of the HDL theory using two related mouse lines (MH and ML). The lines had been divergently selected on their maintenance requirements (Hastings et al., 1997; Bünger et al., 1998). We have previously shown that a correlated trait for such selection has been thermal conductance, whereby MH mice have higher thermal conductance than the ML mice by 23–55%, depending on the ambient temperature (29% at 21°C) (Selman et al., 2001b). We predicted *a priori* from the HDL theory that if heat dissipation constrains both food intake at peak lactation and peak lactation performance, the MH line with greater capacity to dissipate heat, would have greater peak lactation energy intake, greater milk production and elevated pup growth. Moreover, these traits would be conserved if the mothers were given pups of the opposing line to raise, reflecting the heat-dissipation capacity of the mother, rather than the growth capacity of the offspring.

RESULTS

Experiment with natural litters

Maternal body mass

Body mass during baseline was 25.7±0.4 g (N=33) in MH and 25.2±0.5 g (N=16) in ML mice (ANOVA, line, $F_{1,47}$=0.5, P=0.5). Female body mass increased significantly over the last 10 days of pregnancy. Although there was no significant line effect on pregnant body mass, the interaction between day and line was highly significant (ANOVA, line, $F_{1,51}$=2.4, P=0.13; day, $F_{9,459}$=555.5, P<0.001; interaction line×day, $F_{9,459}$=25.5, P<0.001). Maternal body mass of MH mice increased significantly more than that of the ML mice in the last few days of pregnancy (Fig. 1A). Maternal body mass did not differ between lines across days of lactation, but the interaction between line and day was significant (ANOVA, line, $F_{1,71}$=2.6, P=0.11; day, $F_{12,852}$=2.7, P=0.002; interaction line×day, $F_{12,852}$=2.6, P=0.002; Table 1). Body mass of ML mice remained unchanged throughout lactation but body mass of MH mice exhibited a significant drop over the last 3 days (15–18). The average reduction in body mass was 0.8±0.3 g (Fig. 1A).

Metabolisable energy intake (MEI) and apparent digestive efficiency (ADE)

On days 12–14 of lactation, MH mice produced on average 2.3±0.1 g (N=32) dry mass of faeces daily compared with 1.6±0.1 g (N=21) dry mass in ML mice. Faecal production was highly correlated with food intake (r=0.78, P<0.001) and this relationship did not differ significantly between the lines (line, $F_{1,50}$=1.8, P=0.18; food intake, $F_{1,50}$=20.2, P<0.001, Fig. 1B). Despite this, there was a significant difference in the ADE between lines (MH mice 83.1±0.3%, ML mice 80.7±0.9%; t_{51}=2.7, P=0.01). We applied these estimates of ADE from the feeding trial to convert estimated food intake into MEI during baseline and throughout reproduction (see Materials and methods for more details).

MEI during baseline was 51.1±1.8 kJ day^{-1} (N=33) and 37.1±2.4 kJ day^{-1} (N=16) in the MH and ML mice, respectively (ANOVA, line, $F_{1,47}$=35.7, P<0.001, Fig. 1C). During pregnancy, MEI increased significantly over the last 5 days of pregnancy, when line and day of pregnancy both had significant effects (ANOVA, line, $F_{1,51}$=28.9, P<0.001; day, $F_{4,204}$=3.2, P=0.015; interaction line×day, $F_{4,204}$=1.1, P=0.36). During lactation, MH mice had higher MEI than ML mice, and MEI also varied significantly with day of lactation (ANOVA, line, $F_{1,71}$=26.7, P<0.001; day, $F_{12,852}$=36.7, P<0.001; interaction line×day, $F_{12,852}$=2.6, P=0.003). A significant line×day interaction indicated that MH and ML mice responded differently over time during lactation. In both lines, MEI increased over the first 13 days and reached a plateau at day 13. MEI of ML mice remained at this plateau until day 18. However, MH mice remained at this asymptotic level for only 3 days before MEI dropped significantly: coincident with the period over which the same mice were losing mass (see above). The average fall in MEI over days 15–18 of lactation was 58.3 kJ. This reduction in MEI was correlated with the reduction of body mass over the same period (r=0.55, P<0.001, Fig. 1D). Mice that reduced their intake more over the last 3 days of lactation also lost more weight over the same interval.

At peak lactation (days 13–15), MH mice had a higher MEI of 222.9±7.1 kJ day^{-1} (N=49) compared with 153.4±12.5 kJ day^{-1} (N=24) in ML mice. Asymptotic MEI (days 13–15) was positively correlated with body mass at peak lactation (r=0.58, P<0.001), litter

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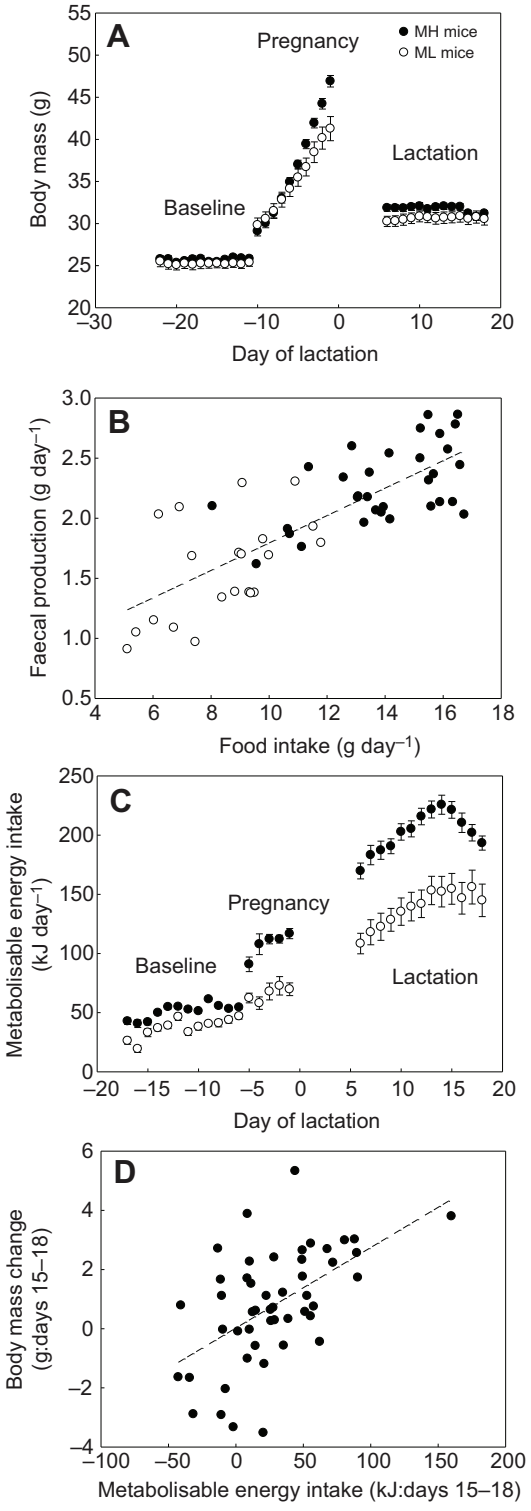

Fig. 1. Parameters of energy budget in MH mice (filled circles) and ML mice (open circles) raising natural litters. (A) Mean body mass (±s.e.m.) during baseline, pregnancy and lactation (for sample size details, see Results). (B) Relationship between faecal production and daily food intake in lactating MH (N=32) and lactating ML mice (N=21). The fitted line represents a linear regression (y=0.65+0.11x, r^2=0.61) for the pooled data (N=53). (C) Mean metabolisable energy intake (±s.e.m.) during baseline, pregnancy and lactation (for sample size details, see Results). (D) Relationship between body mass changes (days 15–18) and metabolisable energy intake changes (days 15–18) for lactating MH (N=49). The fitted line represents a linear regression (y=0.03+0.03x, r^2=0.30).

Table 1. Reproductive performance of lactating mice with high (MH) and low (ML) thermal conductance, raising natural litters

Trait	MH mice	ML mice
Body mass (g) on day 15	31.9±0.4	30.9±0.7
MEI (kJ day^{-1}) over days 12–14	209.6±6.3	128.1±6.3
DEE (kJ day^{-1}) over days 15–17	90.7±2.3	71.5±1.9
MEO (kJ day^{-1})	118.9±5.3	56.6±5.4
Litter size at weaning	6.7±0.3	5.7±0.6
Litter mass (g) at weaning	65.2±2.1	43.4±3.0
Pup mass (g) at weaning	9.9±0.2	7.8±0.2

MEI, metabolisable energy intake; DEE, daily energy expenditure; MEO, milk energy output. Values are means ± s.e.m.; N=49 and N=24 for MH and ML, respectively (body mass, litter size, litter mass, and pup mass); N=32 and N=21 for MH and ML, respectively (MEI, DEE and MEO).

size (r=0.68, P<0.001), and litter mass (r=0.73, P<0.001, Fig. 2). Using GLM with mean body mass at peak lactation, litter size, and pup mass at weaning (day 18) as covariates indicated that the effect of line on MEI remained significant when these additional factors were added to the model.

Daily energy expenditure (DEE)

DEE of MH and ML mice (Table 2) averaged 90.7±2.3 kJ day^{-1} (N=32) and 71.5±1.9 kJ day^{-1} (N=21), respectively (t_{51}=5.9, P<0.001; Table 1). DEE was highly correlated with MEI (r=0.78, P<0.001). The relationship between DEE and MEI was independent of the line (GLM, line, $F_{1,50}$=0.3, P=0.57; MEI, $F_{1,50}$=27.4, P<0.001, Fig. 3).

MEO and reproductive performance

Over days 12–14 of lactation, MH mice had significantly higher MEI than the ML mice. The average MEI in the subset of MH mice for which DEE had been measured was 209.6±6.3 kJ day^{-1} (N=32) compared with the average of 128.1±6.3 kJ day^{-1} (N=21) in the ML mice (line effect, t_{51}=8.7, P<0.001; Table 1). MH mice also had significantly higher MEO compared with the ML mice (t_{51}=7.92, P<0.001), averaging 118.9±5.3 and 56.6±5.4 kJ day^{-1} in the MH and ML mice, respectively (Table 1). MEO was highly correlated with body mass at peak lactation (r=0.48, P<0.001), litter size (r=0.59, P<0.001), litter mass (r=0.79, P<0.001) and pup mass (r=0.59, P<0.001, Fig. 4). Using GLM with mean body mass at peak lactation, litter size, litter mass and pup mass at weaning as covariates indicated that the effect of line on MEO remained significant when these additional factors were added to the model.

Litter size did not differ significantly between lines at birth and at weaning (at birth, t_{71}=1.5, P=0.136; at weaning, t_{71}=1.9, P=0.067). At birth, the average litter size was 7.0±0.3 (N=49) and 6.1±0.6 (N=24) in the MH and ML mice, respectively. At weaning, the litter size of MH mice (6.7±0.3, N=49) was also not significantly different compared with 5.7±0.6 (N=24) in ML mice (Table 1). Litter mass in both MH and ML mice increased significantly throughout lactation (ANOVA, line, $F_{1,71}$=32.2, P<0.001; day, $F_{12,852}$=962.7, P<0.001; interaction line×day, $F_{12,852}$=28.5, P<0.001, Fig. 5A). At weaning, the litter mass of MH mice (65.2±2.1 g, N=49) was significantly (P<0.001) heavier than the litter mass of ML mice (43.4±3.0 g, N=24; Table 1). Because litter sizes were not significantly different between the lines, pup mass in both lines also increased significantly throughout lactation (ANOVA, line, $F_{1,71}$=78.2, P<0.001; day, $F_{12,851}$=1818.1, P<0.001; interaction line×day, $F_{12,851}$=19.1, P<0.001, Fig. 5B) with the pups from the MH line being significantly (P<0.001) heavier than those of the ML line. The pup mass at weaning for the MH mice was 9.9±0.2 g compared with

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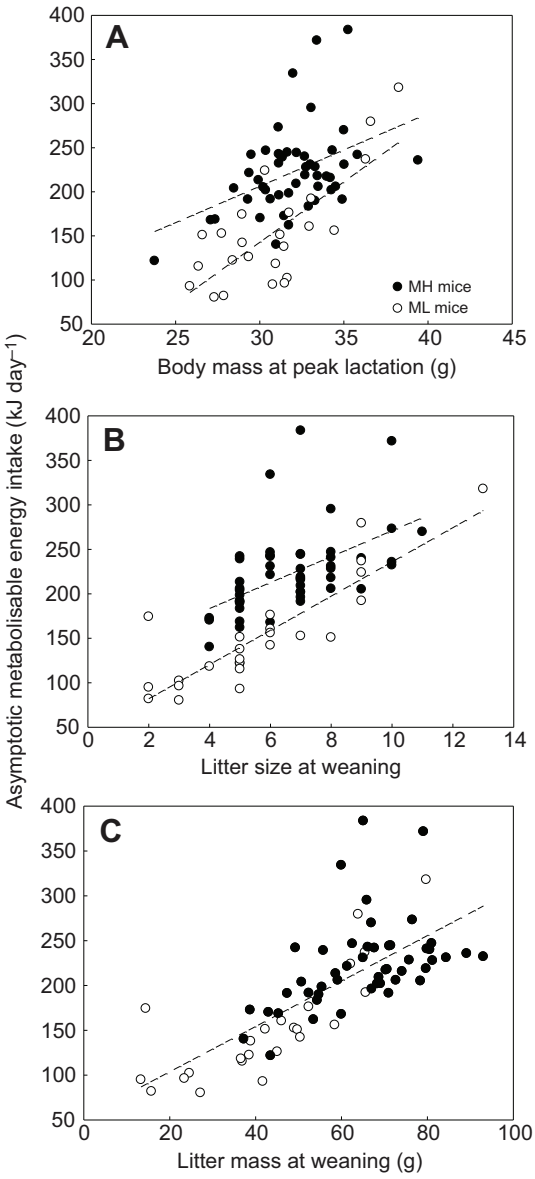

Fig. 2. Estimated values of asymptotic metabolisable energy intake (days 13–15 of lactation) in MH mice (*N*=49, filled circles) and ML mice (*N*=24, open circles) raising natural litters. MEI plotted against (A) body mass at peak lactation (MH: $y=-41.03+8.25x$, $r^2=0.20$; ML: $y=-269.32+13.74x$, $r^2=0.61$), (B) litter size at weaning (MH: $y=125.4+14.57x$, $r^2=0.27$; ML: $y=43.50+19.25x$, $r^2=0.75$), (C) litter mass at weaning (MH and ML pooled: $y=53.67+2.53x$, $r^2=0.53$).

7.8±0.2 g in ML mice (Table 1). The growth rate was higher over the first days of lactation compared with later in lactation. The average litter growth rate on day 7 was 4.3±0.2 g and 2.7±0.2 g compared with 1.1±0.1 g and 0.7±0.2 g on day 18 in MH and ML mice, respectively, Fig. 5C). Pup mass at weaning was negatively correlated with litter size ($r=0.28$, $P=0.01$), but including litter size in the model did not change the significant difference in pup mass between lines (GLM, line, $F_{1,70}=104.7$, $P<0.001$, litter size, $F_{1,58}=36.4$, $P<0.001$, Fig. 6B).

Organ morphology

The average wet masses of several internal organs in lactating and non-reproductive mice are presented in Table 3. Using GLM with reproductive status and line as fixed factors showed that mean maternal body mass of lactating mice on the day of dissection was significantly different compared with the non-reproductive mice, and the interaction line×reproductive status was also significant (GLM, line, $F_{1,53}=0.5$, $P=0.83$; reproductive status, $F_{1,53}=6.2$, $P=0.01$; interaction line×reproductive status, $F_{1,53}=4.6$, $P=0.03$). Differences between the lines may then only be a reflection of the overall size differences. Analyses of the data for organ morphology in lactating and non-reproductive lactating mice with body mass as covariate are therefore also presented in Table 3.

Reproductive status and line had significant effects on the length of small intestine, caecum and whole gut. Lactating mice had significantly longer intestines than non-reproductive individuals and the ML line had longer intestines than the MH line. Lactating mice had significantly heavier full and empty guts than non-reproductive individuals and the ML line had heavier full and empty guts than the MH line. In addition to a significant line effect, among the lactating mice there was a significant positive relationship between the MEI on day 18 and the mass of the full gut (GLM, $F_{1,33}=25.5$, $P<0.001$), mass of the empty gut (GLM, $F_{1,33}=25.5$, $P<0.001$, Fig. 7A) and length of the small intestine (GLM, $F_{1,33}=25.5$, $P<0.001$, Fig. 7B). For the mass of the empty gut, the effect of the interaction between body mass with line was significant, but the interactions with line were not significant for the full gut and the length of the small intestine. There were no significant relationships between MEI on day 18 and the lengths of the large intestine and caecum ($P>0.05$ in both cases).There were no significant differences in mean wet mass of BAT or mammary glands between lines. Mean wet masses of mammary glands were positively but weakly correlated with MEI ($r=0.36$, $P=0.03$), DEE ($r=0.34$, $P=0.037$) and MEO ($r=0.32$, $P=0.05$). The relationship between mass of the mammary gland and MEI, DEE and MEO was not different between the two lines (Fig. 8).

Experiment with cross-fostered litters

Maternal body mass

Mean body mass changed significantly across the days of pregnancy and differed between the two lines (ANOVA, line, $F_{1,14}=25.3$, $P<0.001$; day, $F_{13,182}=429.4$, $P<0.001$; interaction line×day, $F_{13,182}=36.5$, $P<0.001$). During lactation, the body mass of H-L mice (MH mothers with cross-fostered ML pups) was higher than that of L-H mice (ML mothers with cross-fostered MH pups) (ANOVA, line, $F_{1,19}=10.6$, $P=0.004$; day, $F_{11,209}=12.8$, $P<0.001$; interaction line×day, $F_{11,209}=6.6$, $P<0.001$; H-L ($N=10$) and L-H ($N=11$); Table 4). The day×line interaction was significant, indicating that the body mass changed differently during lactation in the two lines. Similar to the MH mice raising MH pups, the body mass of H-L mice fell over the last 3 days of lactation by an average of 2.9±0.6 g (Fig. 9A).

MEI and ADE

Faecal production of lactating mice monitored over days 13–15 was significantly correlated with food intake ($r=0.49$, $P=0.05$). There was no significant line effect when food intake was included as a covariate (GLM, line, $F_{1,12}=0.7$, $P=0.42$; food intake, $F_{1,12}=0.1$, $P=0.7$, Fig. 9B). On days 13–15 of lactation, there was no significant difference ($t_{13}=0.9$, $P=0.41$) in the average ADE between the lines, which averaged 86.8±1.5% ($N=6$) and 85.4±0.9% ($N=9$) in H-L and L-H mothers, respectively. Using these estimates of ADE we converted food intake estimates throughout reproduction into MEI. MEI increased significantly over the last 5 days of pregnancy and was different between the lines (ANOVA, line, $F_{1,14}=46.9$, $P<0.001$; day, $F_{4,56}=4.1$, $P=0.006$; interaction line×day, $F_{4,56}=4.1$, $P=0.006$, Fig. 9C).

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Table 2. Results of doubly labelled water measurements of daily energy expenditure performed on lactating mice with high (MH) and low (ML) thermal conductance, raising natural or cross-fostered ML and MH pups

	Experiment with natural litters		Experiment with cross-fostered litters	
Trait	MH mice	ML mice	MH mothers with ML pups (H-L)	ML mothers with MH pups (L-H)
Body mass (g)[a]	31.8±0.5	29.7±0.5	35.4±0.9	30.4±1.6
k_d (h^{-1})[b]	0.052±0.003	0.054±0.005	0.032±0.001	0.03±0.002
k_o (h^{-1})[c]	0.073±0.004	0.073±0.006	0.043±0.002	0.040±0.003
k_o/k_d	1.410±0.009	1.380±0.019	1.372±0.032	1.38±0.030
N_d (% of body mass)[d]	73.8±0.6	70.2±0.8	80.5±2.1	82.6±2.9
N_o (% of body mass)[d]	69.5±0.6	65.9±0.8	70.1±0.4	71.7±1.2
N_d/N_o	1.062±0.006	1.073±0.005	1.153±0.030	1.15±0.032
DEE (kJ day^{-1})[e]	90.7±2.3	71.5±1.9	98.5±8.3	84.5±8.4

Values are means ± s.e.m.; N=32 for MH lactating mice; N=21 for ML lactating mice (experiment with natural litters) and N=6 for H-L lactating mice; N=8 for L-H lactating mice (experiment with cross-fostered litters). [a]Body mass before injection; [b]elimination rate of ^2H; [c]elimination rate of ^{18}O; [d]deuterium (N_d) and oxygen (N_o) dilution spaces expressed as % of body mass before injection; [e]daily energy expenditure measured over days 15–17 of lactation.

During lactation, H-L mice had a significantly higher MEI than L-H mice, and MEI also varied significantly with the day of lactation (ANOVA, line, $F_{1,19}$=27.2, P<0.001; day, $F_{11,209}$=18.4, P<0.001; interaction line×day, $F_{11,209}$=3.2, P=0.001). The pattern observed in the L-H mice was very similar to that observed for ML mice in the experiment with natural litters. MEI increased over the first 13 days of lactation and reached a plateau over days 13–18. In contrast, the MEI of the H-L mice mirrored that of the MH mice raising natural litters. MEI increased to a plateau which only lasted from day 13 to day 15 and thereafter there was a decline (Fig. 9C). The average drop over days 15–18 of lactation was 107.4 kJ. This reduction in MEI was correlated with the reduction of body mass over the same period (r=0.79, P=0.007, Fig. 9D). Between days 13–15 of lactation, H-L mothers (N=10) assimilated on average 242.5±8.8 kJ day^{-1} compared with 165.3±9.8 kJ day^{-1} in L-H mice (N=11). Use of GLM with mean body mass at peak lactation, litter size and litter mass at weaning as covariates, indicated that the effect of line on MEI remained significant when these other factors were added to the model. Asymptotic MEI (days 13–15) was positively correlated with body mass at peak lactation (r=0.85, P<0.001) and litter mass at weaning (r=0.68, P=0.001), but it was not significantly correlated with litter size (r=0.32, P=0.15) (Fig. 10).

DEE

DEE of H-L and L-H mice averaged 98.5±8.3 kJ day^{-1} (N=6) and 84.5±8.4 kJ day^{-1} (N=8), respectively (t_{12}=1.2, P=0.27: Tables 2, 4).

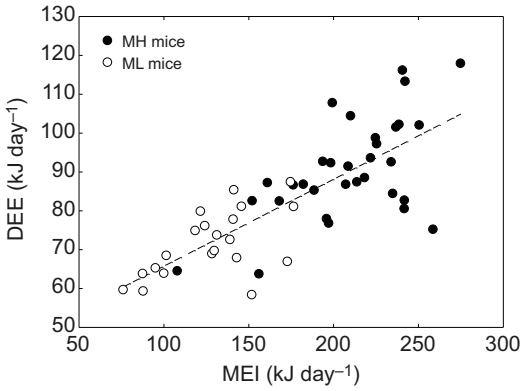

Fig. 3. Daily energy expenditure (DEE) and metabolisable energy intake (MEI) in MH mice (*N*=32, filled circles) and ML mice (*N*=21, open circles) raising natural litters. Both parameters were measured at peak lactation. The fitted line represents a linear regression (y=43.39+0.22x, r^2=0.61) for the pooled data (N=53).

MEO and reproductive performance

Over days 13–15 of lactation, H-L mice had significantly higher MEI than L-H mice (line effect, $t_{12=}$5.9, P<0.001; Table 4). This led to them having significantly higher MEO compared with L-H mice (t_{12}=4.1, P=0.001). MEO was significantly higher in H-L than L-H mice (Table 4). Using GLM with mean body mass at peak lactation, litter size, litter mass and pup mass at weaning as covariates, indicated that the effect of line on MEO remained significant when these factors were added to the model. MEO was not significantly correlated with litter size (r=0.21, P=0.47) or pup mass (r=0.32, P=0.27) (Fig. 11). There was, however, a positive correlation between MEO and body mass at peak lactation (days13–15) (r=0.85, P<0.001) and litter mass (r=0.64, P=0.01) (Fig. 11).

Litter size did not differ significantly between lines when the litters were swapped (t_{19}=0.4, P=0.7). The average litter size after swapping was 7.2±0.5 and 7.5±0.8 in H-L and L-H mice, respectively. No pups were lost. Litter masses of both H-L and L-H mothers increased significantly throughout lactation (ANOVA, line, $F_{1,19}$=10.5, P=0.003; day, $F_{11,208}$=211.8., P<0.001; interaction line×day, $F_{11,209}$=3.7, P<0.001). Although litter mass did not differ significantly between lines from day 7 to day 11 of lactation, ML pups supported by MH mothers were significantly heavier than MH pups supported by ML mothers from day 12 until weaning (pairwise comparison, day 12, P=0.036; day 13, P=0.015 and days 14–18, P<0.01). At weaning, the average litter mass of ML pups supported by MH mothers was greater than that for MH pups supported by ML mothers (Fig. 12A, Table 4). At weaning, pup mass of MH pups supported by ML mice was significantly greater than that of the MH pups raised by ML mice (Table 4). Growth rate of litters in both lines varied significantly throughout lactation but marginally failed to reach significance between lines (ANOVA, line, $F_{1,19}$=3.1, P=0.08; day, $F_{10,189}$=3.3, P<0.001; interaction line×day, $F_{10,189}$=0.6, P=0.8, Fig. 12C). Greater litter mass at weaning was highly correlated with litter size (r=0.86, P<0.001) and litter mass at weaning of H-L mothers was significantly greater than L-H mothers when litter size was added to the model (GLM, line, $F_{1,18}$=20.2, P<0.001, litter size, $F_{1,18}$=114.9, P<0.001, Fig. 13A). Pup mass at weaning was negatively correlated to litter size (r=0.74, P<0.001) and the average mass of ML pups supported by MH mice was significantly heavier than MH pups supported by ML mice, when litter size was added to the model (GLM, line, $F_{1,18}$=13.3, P=0.002, litter size, $F_{1,18}$=35.6, P<0.001, Fig. 13B).

Comparison of cross-fostered and natural litters

We pooled the data collected with respect to the natural and cross-fostered litters and examined the effects of group (mother–offspring

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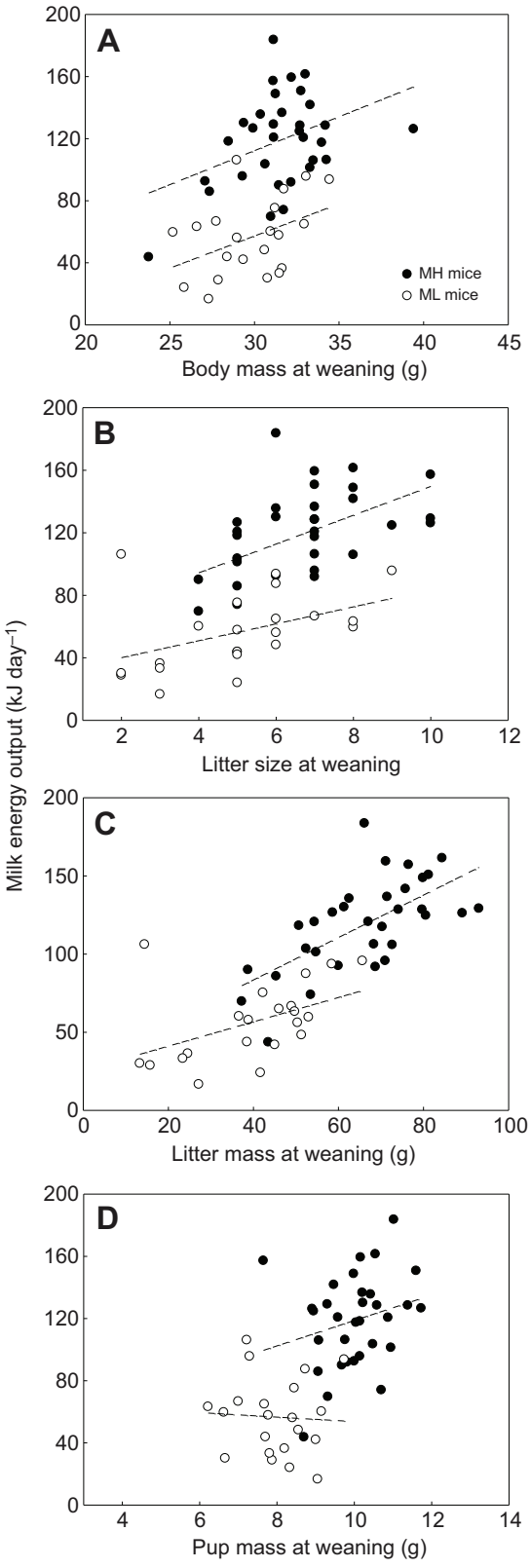

Fig. 4. Milk energy output (MEO) in MH mice (*N*=32, filled circles) and ML mice (*N*=21, open circles) raising natural litters. MEO plotted against (A) body mass at peak lactation (MH: y=45.85+5.36x, r^2=0.22; ML: y=71.48+4.31x, r^2=0.20), (B) litter size at weaning (MH: y=57.62+9.21x, r^2=0.26; ML: y=29.19+5.44x, r^2=0.20), (C) litter mass at weaning (MH: y=29.24+1.36x, r^2=0.42; ML: y=25.58+0.78x, r^2=0.22), (D) pup mass at weaning (MH: y=37.10+8.17x, r^2=0.06; ML: y=81.72−3.10x, r^2=0.01).

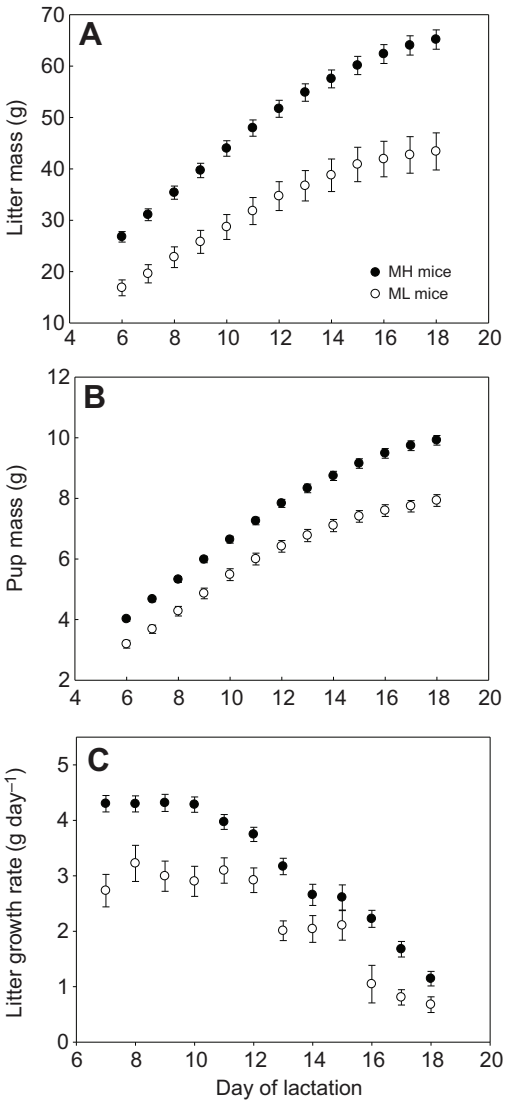

Fig. 5. Parameters of reproductive performance in MH mice (*N*=49, filled circles) and ML mice (*N*=24, open circles) raising natural litters. Litter mass (A), mean pup mass (B), and mean litter growth rate (C) throughout lactation. The data are expressed as means ± s.e.m.

source: H-H, H-L, L-L and L-H) on the peak metabolisable energy intake, milk energy output and litter mass at day 18, with litter size as a covariate. For MEI, there was a significant effect of litter size ($F_{1,89}$=68.2, P<0.001) and a significant group effect ($F_{3,89}$=20.1, P<0.001). For MEO, there was a significant effect of litter size ($F_{1,61}$=21.8, P<0.001) and a significant group effect ($F_{3,61}$=20.8, P<0.001). For the litter mass at weaning, there was a significant effect of litter size ($F_{1,82}$=335.8, P<0.001) and a significant group effect ($F_{3,82}$=35.3, P<0.001). For all three variables, *post hoc* Tukey test comparisons revealed that the high mothers differed from the low mothers (P<0.05) but there was no difference between the high mothers raising high or low pups (P>0.05), and no difference between the low mothers raising either high or low pups (P>0.05).

DISCUSSION

The goal for this study was to test the HDL theory by comparing the reproductive performance of two lines of mice previously shown to have high and low thermal conductance. The HDL theory suggests that at peak lactation mammals are constrained by their capacity to

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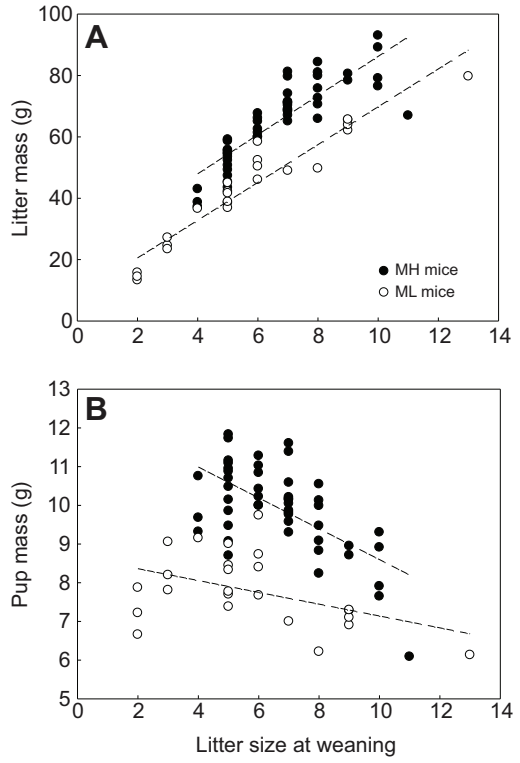

Fig. 6. Litter and pup masses at weaning for MH mice (*N*=49, filled circles) and ML mice (*N*=24, open circles) raising natural litters. Litter mass (A) (MH: $y=22.47+6.38x$, $r^2=0.73$; ML, $y=8.22+6.16x$, $r^2=0.92$) and pup mass (B) (MH: $y=12.58-0.4x$, $r^2=0.40$; ML, $y=9.09-0.2x$, $r^2=0.34$) are plotted against litter size at weaning.

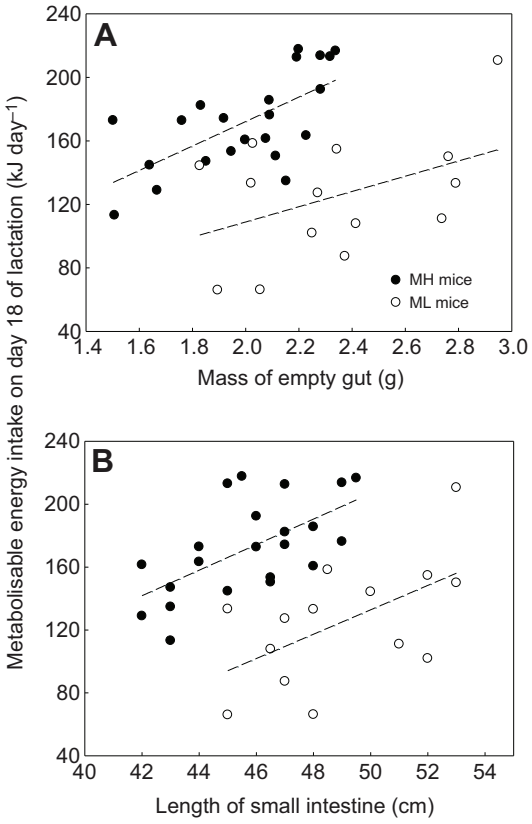

Fig. 7. Metabolisable energy intake (MEI) on day 18 of lactation MH mice (*N*=22, filled circles) and ML mice (*N*=14, open circles) raising natural litters. MEI plotted against (A) wet masses of empty gut (MH: $y=18.22+77.01x$, $r^2=0.44$; ML: $y=12.94+48.02x$, $r^2=0.20$), (B) length of small intestine (MH: $y=199.36+8.13x$, $r^2=0.38$; ML: $y=255.26+7.77x$, $r^2=0.32$).

dissipate body heat, and hence predicts that the MH mice, with greater thermal conductance, should have greater peak energy intake, permitting them to invest more energy in milk production and hence produce heavier litters and pups.

During lactation, mice in both lines increased MEI significantly over the first 13 days and then reached a plateau (asymptotic food intake). These findings are consistent with previous research on food intake during lactation in different animal models. Lactating MF1 mice reach a plateau around day 11 of lactation (Johnson et al., 2001a; Król et al., 2003; Vaanholt et al., 2013; Gamo et al., 2013; Duah et al., 2013), lactating common voles on day 14 (*Microtus arvalis*) (Simons et al., 2011), lactating Brandt's voles

Table 3. Wet masses of tissues and organs of lactating MH (*N*=22) and ML (*N*=15) mice and non-reproductive MH and ML (*N*=10 for both lines) mice

	Organ wet mass (g)				Organ length (cm)			
	BAT	Mammary gland	Full gut	Empty gut	Small intestine	Large intestine	Caecum	Whole gut
Means								
Lactating MH mice	0.083±0.002	3.03±0.2	4.28±0.2	1.99±0.1	45.7±0.5	8.2±0.3	3.3±0.16	57.2±0.6
Lactating ML mice	0.076±0.004	2.89±0.2	4.93±0.3	2.32±0.1	49.1±0.7	7.8±0.4	3.3±0.21	60.2±1.0
Non-reproductive MH mice	0.077±0.003		2.50±0.1	1.69±0.1	41.2±0.6	7.4±0.3	2.0±0.15	50.6±0.5
Non-reproductive ML mice	0.086±0.005		2.83±0.2	1.94±0.1	45.5±1.0	7.3±0.3	2.2±0.13	54.9±1.3
Statistics								
Line	$P=0.005^a$	n.s.	$P=0.003^b$	$P<0.001^b$	$P<0.001^b$	n.s.	n.s.	$P<0.001^b$
RS	n.s.		$P<0.001^c$	$P=0.002^c$	$P<0.001^c$	n.s.	$P<0.001^b$	$P<0.001^c$
BM	$P=0.007$	$P=0.009$	$P=0.005$	$P<0.001$	$P=0.001$	n.s.	n.s.	$P<0.001$
L x RS	$P=0.031$		n.s.	$P=0.007$	n.s.	n.s.	n.s.	n.s.
L x BM	$P=0.005$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
RS x BM	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L x RS x BM	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

RS, reproductive status; BM, body mass minus respective organ mass; n.s., not significant ($P>0.05$); [a]MH>ML; [b]ML>MH; [c]Lactating mice>non-reproductive mice. Values are presented as means ± s.e.m. *P*-values indicate statistical significance of effects. Line, reproductive status, interaction between line and reproductive status, interaction between line and body mass, interaction between reproductive status and body mass, and interaction among three traits were used in a GLM model. Body mass at dissecting day minus the organ mass being considered as the dependent variable was used as a covariate for organ mass parameters. Body mass at day of dissection was used as a covariate for organ length parameters. Non-significant interactions were removed from the models.

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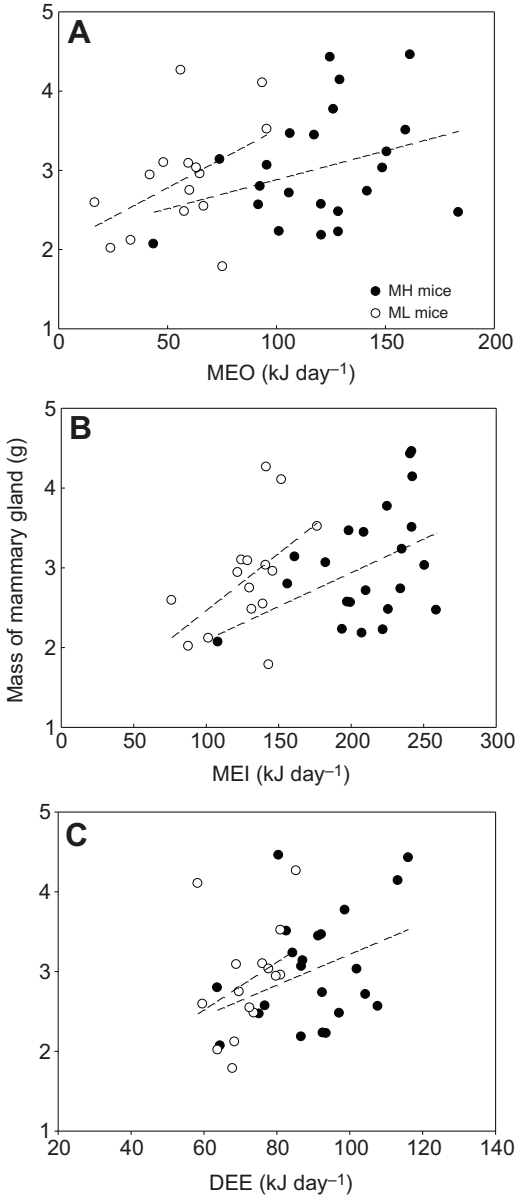

Fig. 8. Wet masses of mammary gland at the end of lactation in MH mice (*N*=22, filled circles) and ML mice (*N*=15, open circles) raising natural litters. Mean mass of wet mammary gland plotted against (A) milk energy output (MH: $y=2.15+0.07x$, $r^2=0.10$; ML: $y=2.03+0.02x$, $r^2=0.22$), (B) metabolisable energy intake (MH: $y=2.15+0.01x$, $r^2=0.11$; ML: $y=1.03+0.01x$, $r^2=0.28$), (C) daily energy expenditure (MH: $y=1.30+0.02x$, $r^2=0.14$; ML: $y=0.73+0.03x$, $r^2=0.12$).

(*Lasiopodomys brandtii*) on day 8 (Wu et al., 2009), lactating European hares (*Lepus europaeus*) during weeks 3–4 (Valencak and Ruf, 2009) and lactating Mongolian gerbils (*Meriones unguiculatus*) on day 9 (Yang et al., 2013). Consistent with the prediction of the HDL theory, the peak metabolisable energy intake in lactation (days 13–15) was significantly higher in the MH line compared with the ML line. This was, in turn, translated into a greater milk production, which led to a greater growth of the litters in the MH line mice and ultimately led to them weaning heavier pups. The litter mass of MH mice at weaning was 50.2% heavier compared with the average litter mass weaned by ML mice. Similarly, the mass of individual pups raised by MH mice was 27.2% greater than those raised by the ML mice. The MH females exported on average 62.3 kJ day^{-1} more

Table 4. Reproductive performance of lactating mice with high (MH) and low (ML) thermal conductance, raising cross-fostered ML and MH pups

Trait	MH mothers with ML pups (H-L)	ML mothers with MH pups (L-H)
Body mass (g) on day 15	35.2±0.6	30.8±1.2
MEI (kJ day^{-1}) over days 13–15	248.9±8.1	160.2±12
DEE (kJ day^{-1}) over days 15–17	98.5±8.3	84.5±8.4
MEO (kJ day^{-1})	150.4±13.6	75.8±10.1
Litter size at weaning	7.2±0.5	7.5±0.8
Litter mass (g) at weaning	69.3±3.4	60.3±5.0
Pup mass (g) at weaning	9.8±0.3	8.5±0.4

MEI, metabolisable energy intake; DEE, daily energy expenditure; MEO, milk energy output. Values are means ± s.e.m.; *N*=10 and *N*=11 for H-L and L-H, respectively (body mass, litter size, litter mass, and pup mass); *N*=6 and *N*=8 for H-L and L-H, respectively (MEI, DEE and MEO).

energy as milk than ML females. Because the increase in MEO in MH mice was fuelled by extra MEI (81.5 kJ day^{-1}), the efficiency for converting the MEI to MEO was 76.4%. This is consistent with previous efficiency estimates (Romero et al., 1976; Baldwin et al., 1980; Freetly et al., 2006; Król et al., 2007). Our findings are corroborated by a previous study that was conducted on laboratory mice that had been selected for high and low heat loss (Nielsen et al., 1997a; Nielsen et al., 1997b). It was demonstrated by using a weigh–suckle–weigh method that high heat loss mice synthesised on average 20.6% more milk than low heat loss mice. As a consequence, they weaned litters on average 10.1 g heavier (McDonald and Nielsen, 2006).

The asymptotic MEI in the ML line remained stable over days 13–18, consistent with studies in other mouse strains and other small rodents and lagomorphs (Johnson et al., 2001a; Król and Speakman, 2003a; Król and Speakman, 2003b; Król et al., 2003; Król et al., 2007; Wu et al., 2009; Simons et al., 2011; Zhao and Cao, 2009; Valencak and Ruf, 2009; Zhao et al., 2010; Vaanholt et al., 2013; Gamo et al., 2013; Yang et al., 2013; Duah et al., 2013). In contrast, the pattern observed in the MH mice was different. There was a significant drop in MEI across days 15–18 amounting to a total deficit of 58.3 kJ. At the same time, the MH females lost 0.8 g of body mass. This reduction in body mass could be just reduced gut fill reflecting the lower food intake. However, if this loss of weight was caused by withdrawal of fat reserves, it would represent ~31 kJ of energy (39 kJ g^{-1}×0.8 g) (Johnson et al., 2001c; Speakman, 2008) that could supplement the reduced intake. Because fat is the most energy-dense tissue, this is the maximal level of energy that could be supplied by the lost body mass. Hence, making these limiting assumptions, the lactating MH mice had between 58.3 and 27.3 kJ (14.6 to 6.7 kJ day^{-1}) lower energy intake over the last few days of lactation than they would have had if they had sustained their energy intake at the peak level. Since the milk energy output at peak lactation was 118.9±5.3 kJ day^{-1}, the reduction in daily milk production would have been at least 5.6% and up to 12.5%, assuming that all the deficit was paid for by reduced milk production. Over this period, the growth of the MH litters declined steeply (Fig. 5C), yet they still retained greater growth than the ML litters, consistent with the fact the ML litters were receiving on average only 56.6 kJ day^{-1} of milk. Exactly why the MH mice used a strategy of fuelling late lactation by a reduction in energy intake possibly supplemented by a withdrawal of reserves is unclear. Because it occurred in both experiments with natural litters and cross-fostered litters, it was a strategy adopted by the mothers, independent of the pups they were suckling.

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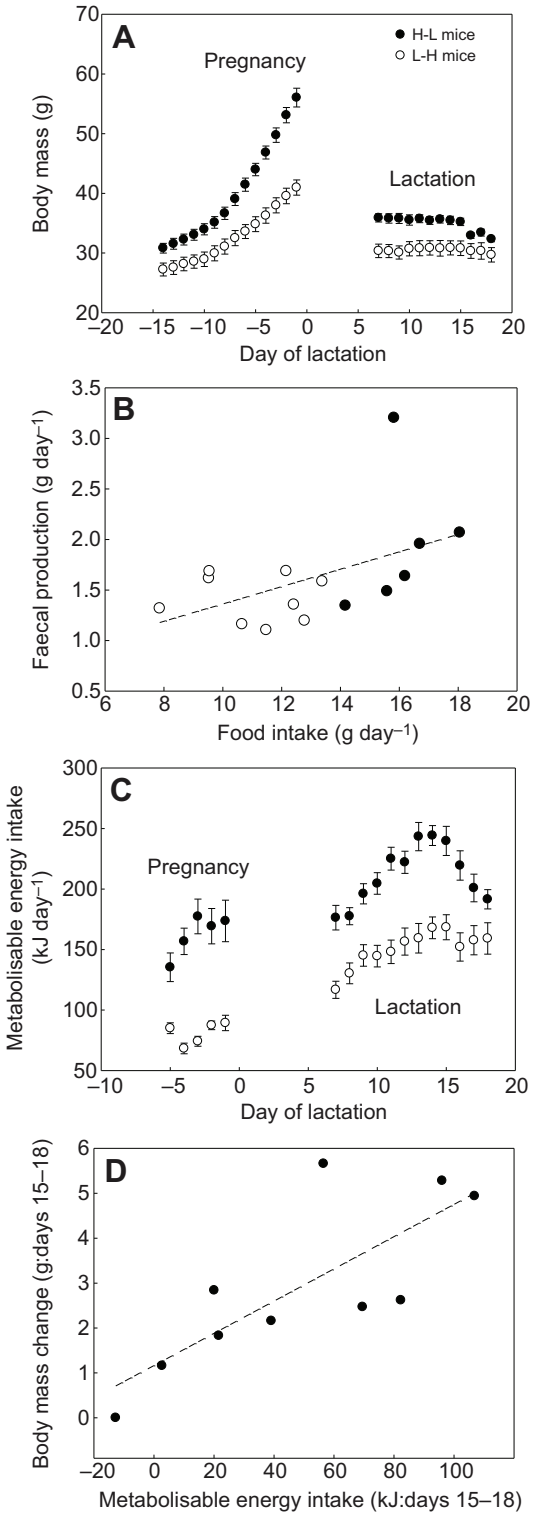

Fig. 9. Parameters of energy budget in H-L mice (filled circles) and L-H mice (open circles) raising cross-fostered pups. (A) Mean body mass (±s.e.m.) during pregnancy and lactation (for sample size details, see Results). (B) Relationship between faecal production and daily food intake in lactating H-L (*N*=6) and lactating L-H mice (*N*=9). The fitted line represents a linear regression (y=0.5+0.09x, r^2=0.24) for the pooled data (*N*=15). (C) Mean metabolisable energy intake (±s.e.m.) during pregnancy and lactation (for sample size details, see Results). (D) Relationship between body mass changes (days 15–18) and metabolisable energy intake changes (days 15–18) for lactating H-L (*N*=10). The fitted line represents a linear regression (y=1.17+0.04x, r^2=0.62).

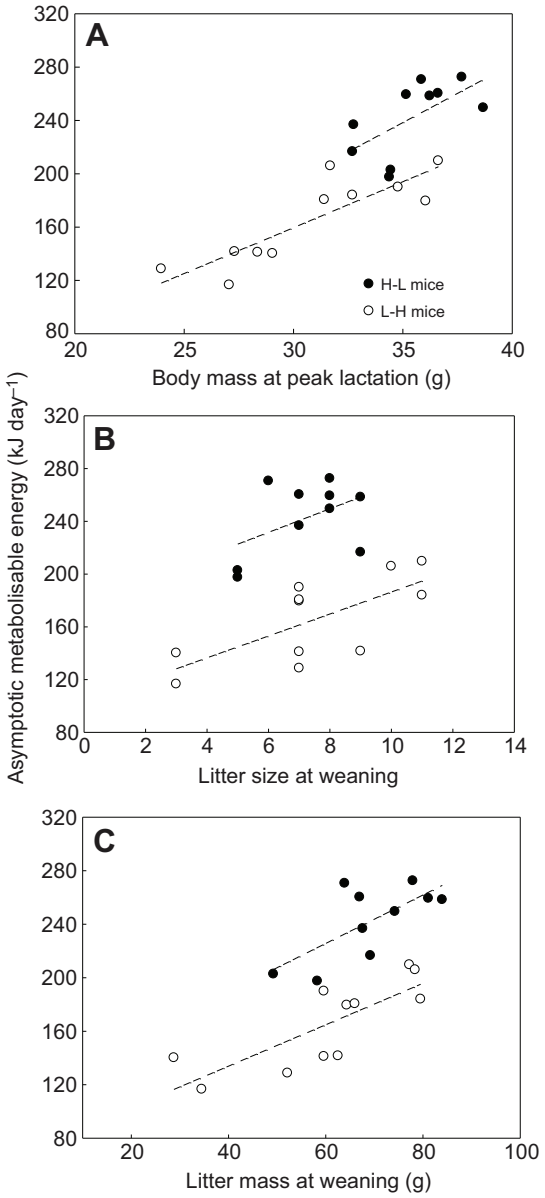

Fig. 10. Estimated values of asymptotic metabolisable energy intake (days 13–15 of lactation) in H-L mice (*N*=10, filled circles) and L-H mice (*N*=11, open circles) raising cross-fostered pups. MEI plotted against (A) body mass at peak lactation (H-L: y=−68.24+8.77x, r^2=0.39; L-H: y=−47.22+6.90x, r^2=0.74), (B) litter size at weaning (H-L: y=178.07+8.94x, r^2=0.23; L-H: y=103.15+8.3x, r^2=0.50), (C) litter mass at weaning (H-L: y=117.03+1.8x, r^2=0.48; L-H: y=71.85+1.55x, r^2=0.63.

Examination of the internal organs of lactating mice revealed a significant increase in the size and mass of several organs compared with non-reproductive mice. These changes included the whole gut length, small intestine length, caecum length, empty and full gut masses. However, no significant differences were found in the mass of BAT and length of the large intestine between lactating and non-reproductive mice. Our findings were similar to the patterns that were found in previous work in a diversity of rodent species. This previous work has shown substantial increases in lactation of the alimentary tract and associated organs such as liver and pancreas (Kennedy et al., 1958; Jolicoeur et al., 1980; Wu et al., 2009; Speakman and McQueenie, 1996). The increase in the sizes of the components of the alimentary tract during lactation are consistent with the central limit

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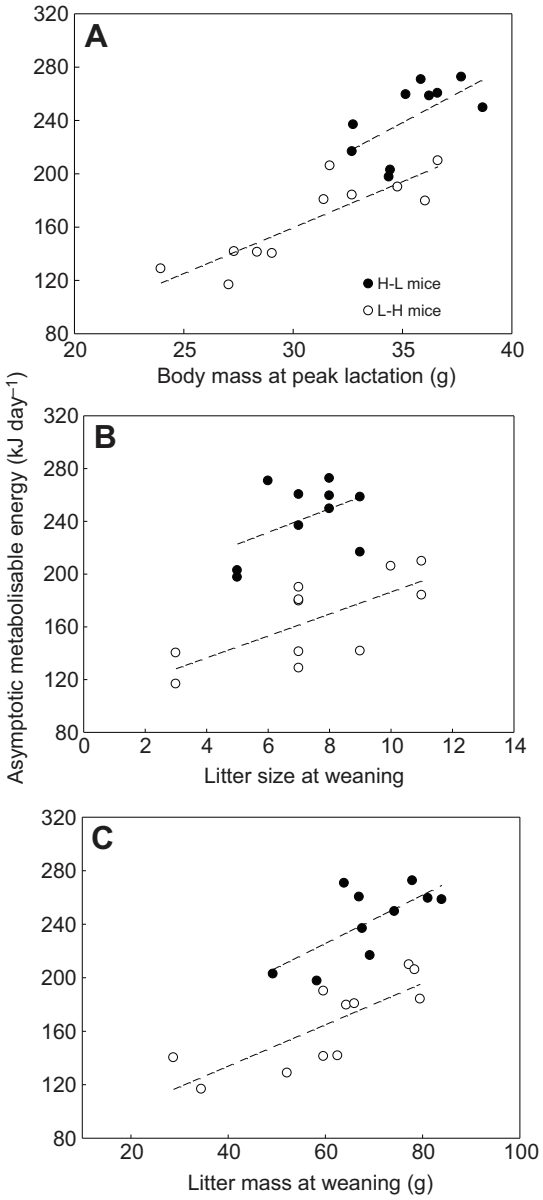

Fig. 11. Milk energy output (MEO) in H-L mice (*N*=6, filled circles) and L-H mice (*N*=8, open circles) raising cross-fostered pups. MEO plotted against (A) body mass at peak lactation (H-L: $y=2.92+4.15x$, $r^2=0.18$; L-H: $y=-147.22+7.33x$, $r^2=0.74$), (B) litter size at weaning (C) litter mass at weaning (H-L: $y=141.50+0.12x$, $r^2=0.001$; L-H, $y=8.71+0.1.36x$, $r^2=0.25$), (D) pup mass at weaning (H-L: $y=-70.78+23.77x$, $r^2=0.68$; L-H: $y=92.87-2.03x$, $r^2=0.007$).

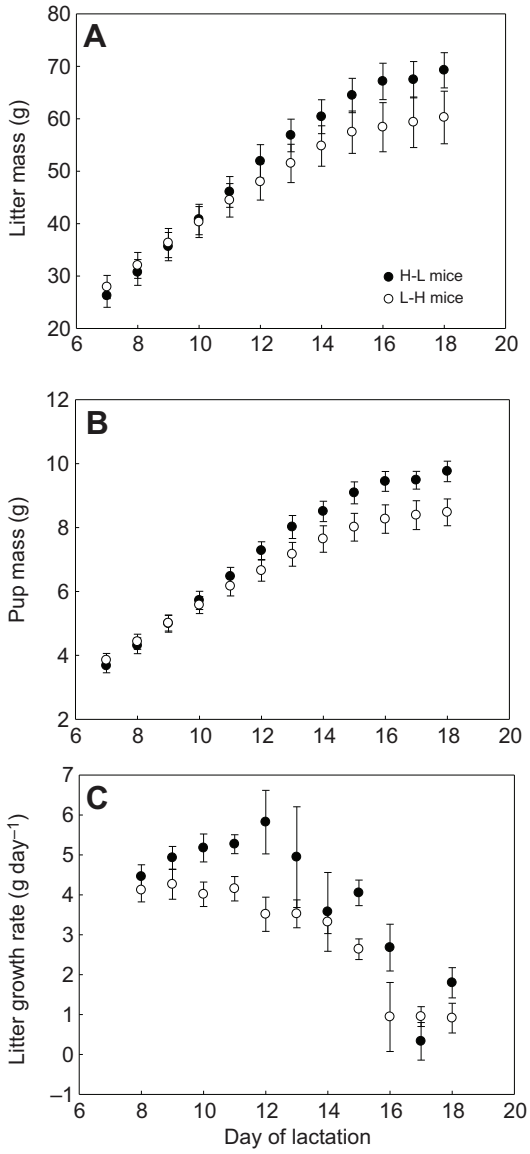

Fig. 12. Parameters of reproductive performance in H-L mice (*N*=10, filled circles) and L-H mice (*N*=11, open circles) raising cross-fostered pups. Litter mass (A), mean pup mass (B), and mean litter growth rate (C) throughout lactation. The data are expressed as means ± s.e.m.

theory wherein intake is constrained by the uptake capacity of the alimentary tract (Kirkwood, 1983; Perrigo, 1987; Hammond and Diamond, 1992; Hammond and Diamond, 1994; Koteja, 1996; Künkele, 2000; Johnson et al., 2001a; Johnson et al., 2001b; Laurien-Kehnen and Trillmich, 2003; Speakman, 2008). However, it seems highly unlikely that such changes underpin the difference in intake between the MH and ML lines at peak lactation, because the differences between lines were in the opposite direction. The ML mice had longer whole guts, mostly attributed to their significantly longer small intestines. Moreover, ML mice had significantly greater wet masses of empty and full guts. These data are consistent with previous work on these lines (Selman et al., 2001a) where the ML mice had significantly greater dry mass of the stomach and large intestine. However, whole gut length was not measured in that study. Surprisingly, at the end of lactation (day 18), there was a significant positive relationship between the MEI and the masses of both the full and empty gut, and the length of the small intestine, within each of the lines, in complete contrast to the difference between the lines (MH mice with higher food intake had shorter and lighter guts than ML mice). Consequently, although it seems unlikely that a central limit imposed by gut capacity was responsible for the line difference, it remains feasible that the individual differences within lines could be attributed to such an effect.

Sadowska et al. (Sadowska et al., 2013) studied the reproductive performance of mice that had been selected for high and low basal metabolic rate (BMR) and found that those with high BMR had greater reproductive performance. They attributed this difference to differences in the assimilation capacity of the alimentary tract, and hence concluded their data were consistent with the central limitation hypothesis. The mice we studied also differ in their resting

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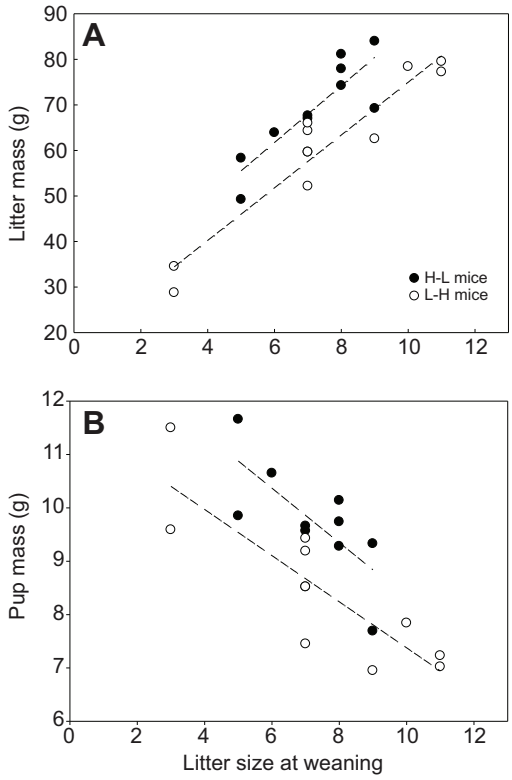

Fig. 13. Mean litter size at weaning in MH mice (*N*=49, filled circles) and ML mice (*N*=24, open circles) raising natural litters. Litter size plotted against (A) litter mass at weaning (H-L: $y=24.39+6.23x$, $r^2=0.75$; L-H, $y=17.06+5.79x$, $r^2=0.97$), (B) pup mass at weaning (H-L: $y=13.43-0.51x$, $r^2=0.55$; L-H: $y=11.7-0.43x$, $r^2=0.73$).

metabolic rate in the thermoneutral zone [RMRt: which is similar to BMR (Speakman et al., 2004)] with non-reproductive MH mice having higher RMRt than the ML mice (Selman et al., 2001a). However, we have shown previously that these differences in RMRt between the M lines are not linked to morphological differences in the alimentary tract (Selman et al., 2001a). Moreover, given the strain differences discussed above, this seems an unlikely explanation for the data presented here. However, the positive correlation between RMRt and thermal conductance observed in the M strain mice (Selman et al., 2001b) suggests a potential alternative explanation for the observations on mice selected for high and low BMR (Sadowska et al., 2013). It is potentially the case that the mice with higher BMR also had higher thermal conductance, and were hence able to dissipate more heat, and this was the primary factor regulating the level of their reproductive performance, with the observed changes in the alimentary tract in that study a secondary response. Unfortunately, thermal conductance differences between the strains were not measured in this previous study (Sadowska et al., 2013). The thermal conductance of these mice was measured by Gębczyński (Gębczyński, 2005) at generation 19, and no significant differences were noted. The relevance of these measurements to the mice studied by Sadowska et al. (Sadowska et al., 2013) is however uncertain because they are separated by 8 years (and 13 generations) of continued selection, hence there has been ample time for a difference in thermal conductance as a correlated trait to develop between these two studies. Note that although a similar time elapsed between the characterisation of thermal conductance of the lines studied here and the present study of their reproductive performance a reduction in the difference in thermal conductance between the lines over this interval seems unlikely. For the trait under selection (food intake), at generations 8–9, the difference was 20.5%, at generations 14–15 it was 28.7% and at generations 21–23 the difference was 45.2% (Hastings et al., 1997); at generation 38 when selection stopped, it was 58.7% (Bünger et al., 1998). After repeated rounds of inbreeding, at generation 47 we characterised the thermal conductance. We did not measure the food intake of the lines in this generation, but did so in generation 50 when the difference averaged 45.5%. In the current study (generations 68–83), the difference in baseline food intake between the lines persisted at the similar level of 37.7%. Hence, there has been only a slight reduction in the difference, between generation 50 and 83. At generation 47, we performed a pilot study to explore the lactation performance of the two lines, and found that at peak lactation the intake of the high line was 55% greater than that of the low line. This compares with 31% at generation 68, 64% at generation 75, 56% at generation 80 and 47% at generation 83. Clearly, these values vary a lot from generation to generation, but the overall average for the data presented here (generations 68–83) is 49.5%, a slight reduction on the value of 55% at generation 47, consistent with the slight reduction in the difference between the baseline food intakes over the same period. These data clearly indicate that the metabolic phenotype of the MH and ML mice has remained virtually unchanged over the last 35–40 generations since the period of inbreeding designed to fix the genetics, hence we are confident the difference in thermal conductance probably also persisted through this period.

Although DEE and MEO were uncorrelated with features of the alimentary tract, these traits and MEI were positively correlated with the mass of the mammary glands. Despite this weak correlation there was no significant difference in the mass of the mammary glands between the two lines. Similar results were found in lactating MF1 mice that were exposed to warm and hot conditions at peak lactation, which also had a highly significant difference in their MEO but not in the masses of their mammary glands (Król et al., 2003). Indeed, it has been recently shown in MF1 mice that mice rearing experimentally manipulated small litters actually had heavier mammary glands compared with mice rearing experimentally manipulated large litters. This was partially attributable to the differences in the fat contents of mammary glands between the two groups, with the heaviest mammary glands in those raising the smallest litters containing more fat (Duah et al., 2013). These data further emphasise that mass of the mammary gland is a poor index of lactation performance in mice. Greater attention should be paid in future to the use of more informative techniques such as measuring the activity and the number of secretory cells [e.g. the bromodeoxyuridine-labelling index (Capuco et al., 2002) or the explants method (Wilde et al., 1999)].

An alternative explanation to the HDL theory for the results observed in the experiment with natural litters is that female milk production is driven by pup demand (Speakman and Król, 2005a; Zhao et al., 2013b); thus the greater food intake and milk production of the MH line results from the MH pups demanding more milk from their mothers. To test whether the system was regulated by the performance of the mother or the demands of the pups, we cross-fostered litters in the second experiment. In this experiment the performance of the MH mothers raising ML pups matched exactly their performance when raising MH pups, and the same was also true of ML mothers raising MH pups, compared with their performance when raising ML pups. Although the comparison between the mothers raising natural litters and mothers raising cross-fostered litters is not ideal because those raising natural litters

received a different level of disturbance, this comparison also shows that the difference between the lines resides in the mothers and not in the offspring. These data very clearly show that the overall energy flux of the mother–pup system is controlled by factors that affect the performance of the female, rather than the growth capacity of the pups. This is consistent with the data generated elsewhere (Zhao et al., 2013b).

In summary, in the experiment with natural litters, we showed that the mice selected for high and low food intake were limited in their maximum energy intake and reached a plateau at day 13 of lactation. Reproductive performance in the MH mice was significantly higher than that of the ML mice. MH mice ate more food and produced more milk and weaned heavier pups. Our morphological findings suggest that mice at peak lactation were unlikely to be constrained centrally by the capacity of the alimentary tract (central limit hypothesis). Furthermore, it was demonstrated that the reproductive performance was driven by factors affecting the mothers rather than growth capacity of the pups. Our results support the hypothesis that the capacity to dissipate heat is the physiological mechanism shaping the maximum energy intake and the reproductive performance in mice selected for high and low food intake, as a result of the correlated effects of selection on thermal conductance.

MATERIALS AND METHODS

Source of mouse lines

We used mice from the maintenance (M) lines (Hastings et al., 1997; Bünger et al., 1998), which originated from a common background generated by a three strain cross, between two inbred strains (JU and CBA) and one outbred CFLP strain (Sharp et al., 1984). The mice were divergently selected over 38 generations for high and low food intake (MH and ML, respectively) at the University of Edinburgh, UK. Because food intake is related to body mass, the selection was based on food intake corrected for average body mass. Three independent replicate lines were selected in each direction. At generation 20, inter-crossing was made in each of three replicates, and only one resultant line in each direction was maintained till generation 38, after which the selective breeding was terminated. At the beginning of generation 43, partially inbred lines were produced by sib–sib mating for four generations to facilitate mapping work. Mice were subsequently random bred within each line, avoiding sib–sib mating. The current studies were performed over a period of 5 years spanning the approximate generations 68 to 83. The MH and ML lines were shown to have different thermal conductance (Selman et al., 2001b).

Breeding protocol

Virgin female mice aged 9–12 weeks were individually housed in shoebox cages (48 cm×15 cm×13 cm) under a 12 h:12 h light:dark photoperiod at 21±2°C and a relative humidity of 59±5%. All cages were provided with sawdust, paper bedding and a cardboard tube. Animals had *ad libitum* access to water and food (details below). After 12 days of baseline females were mated with non-sibling males for 11–15 days. Pregnant mice were monitored daily to establish the day of parturition (day 0), and the timing for pregnancy was back calculated from the day of birth as day −1 (last day of pregnancy) to day −18 (beginning of pregnancy). Adult females and their pups were subjected to various measurements (details below) until day 18 of lactation.

Experiment with natural litters

Data were collected for 4 years (2007–2010), resulting in a total sample size of 49 lactating MH and 24 lactating ML mice. In 2007, mice were fed CRM diet (Pelleted Rat and Mouse Breeder and Grower Diet, Special Diets Services, BP Nutrition, Witham, UK) and in the other years, they were fed D12450B diet (Research Diets, New Brunswick, NJ, USA). Because not all lactating females were monitored for body mass and food intake during their baseline period and/or during pregnancy, the pre-lactation sample sizes are smaller than those during lactation and varied depending on the parameter. Specifically, the body mass and food-intake measurements during the baseline period were performed on 33 MH and 16 ML mice, and during pregnancy on 40 MH and 13 ML mice, respectively. Mice were allowed to raise their natural litters to weaning. Ten age-matched females from each line were not mated to provide non-reproductive controls. On day 18 of lactation, all lactating (N=73) and non-reproductive (N=20) mice were sacrificed and a subsample dissected to evaluate organ morphology (details below).

Experiment with cross-fostered litters

Data on reproductive performance of MH mothers (N=10) rearing cross-fostered ML pups (H-L) and ML mothers (N=11) rearing cross-fostered MH pups (L-H) were collected in 2011. Mice had *ad libitum* access to water and food (D12450B, Research Diet, New Brunswick, NJ, USA). Mothers and their naturally born pups were left undisturbed for a period of 1–2 days after birth. Cross-fostering of pups was performed on days 2–4 of lactation (the exact day of swap varied between mothers because of the asynchronous nature of the births).

To allow mothers and their cross-fostered pups to settle, they were left undisturbed for another 2 days before monitoring of body mass and energy balance resumed. Because not all females were monitored during pregnancy, the pre-lactation sample sizes are smaller than those during lactation. Specifically, the body mass and food intake measurements during pregnancy were performed on eight individuals from each line, with no data collected during the baseline.

Body mass, food intake and reproductive performance

Female body mass and food intake were measured (±0.01 g) daily between 12:00 and 14:30 h. No food intake measurements were taken when females were housed with males. Litter size and mass (±0.01 g) were recorded daily on days 5–18 of lactation, and the average pup mass was calculated as the litter mass divided by litter size. The growth rate of litter (g day^{-1}) was calculated as the difference in litter mass between two consecutive days of lactation.

MEI

Measurements of MEI were performed either on days 12–14 of lactation (experiment with natural litters) or on days 13–15 of lactation (experiment with cross-fostered litters). Females and their litters were placed in cages with fresh sawdust, and a weighed portion of D12450B food was added to the hopper at the beginning of the 48 h feeding trial. Samples of food were taken to determine dry mass content (93.8±0.2%, N=8), and the food remaining in the hopper was reweighed at the end of feeding trial. Any uneaten, fragmented food and faeces were removed from the cage, dried to a constant mass at 60°C and weighed. The gross energy content of D12450B food (17.8±0.17 kJ g^{-1} dry mass, N=3) and faeces (MH mothers, 15.4±0.04 kJ g^{-1} dry mass, N=32; ML mothers, 15.7±0.1 kJ g^{-1} dry mass, N=21; H-L mothers, 15.4±0.08 kJ g^{-1} dry mass, N=6; L-H mothers, 15.8±0.1 kJ g^{-1} dry mass, N=9) were measured by bomb calorimetry (Parr 6200 calorimeter with semi-micro oxygen bomb 1109A, Scientific and Medical Products Ltd, Cheadle, UK). Dry food consumption (g day^{-1}) was calculated by multiplying the food intake (g day^{-1}) by the food dry mass content (%). Gross energy intake (GEI, kJ day^{-1}) was then calculated by multiplying the dry food consumption (g day^{-1}) by the food energy content (kJ g^{-1} dry mass). Energy lost through faeces (kJ day^{-1}) was calculated by multiplying dry faecal production (g day^{-1}) by the faecal energy content (kJ g^{-1} dry mass). MEI (kJ day^{-1}) was calculated as the difference between GEI and energy lost through faeces, assuming that the energy loss via urine was 3% of the energy digested (Drożdż, 1975). The ADE was calculated as the percentage of GEI that was digested.

Because CRM and D12450B diets had different apparent digestibility, all energy-intake data were presented as MEI rather than GEI. Evaluation of MEI during baseline, pregnancy and lactation was based on the measured values of GEI, assuming that both ADE and the 3% loss of energy via urine remained stable through the whole experiment (Król and Speakman, 2003a; Król et al., 2007). For mice fed with CRM diet, MEI was calculated using the measured value of dry mass content of food (94.4±0.3%, N=10) and previously published values of the diet energy content (17.97 kJ g^{-1} dry mass) and the diet-specific ADE (79.8%) (Król et al., 2007). MEI in mice

fed with D12450B diet was calculated using the parameters measured in the current study, including the energy content of D12450B diet (17.8 kJ g^{-1} dry mass) and the diet-specific ADE (MH mothers, 83.1%; ML mothers, 80.7%; H-L mothers 86.8% and L-H mothers, 85.4%).

DEE

The doubly labelled water (DLW) technique (Butler et al., 2004) was used to measure DEE over days 15–17 of lactation (MH, *N*=32 and ML, *N*=21 for experiment with natural litters; H-L, *N*=6 and L-H, *N*=8 for experiment with cross-fostered litters). Previous work has indicated the accuracy of this method to measure DEE in small mammals (Speakman and Król, 2005b). Measurements were made across 2 days to minimise the potential day-to-day variability in DEE (Speakman et al., 1994; Berteaux et al., 1996). Recycling of isotopes between the mother and her pups was considered negligible (Scantlebury et al., 2000). On day 15 of lactation, mice were weighed (±0.01 g) and injected intraperitoneally with ~0.25 g of water enriched with ^{18}O (27.8 atom%) and ^{2}H (15.9 atom%). Syringes were weighed before and after injection (±0.0001 g) to calculate the exact dose of DLW injected. Blood samples were collected after 1 h to evaluate initial isotope enrichments (Król and Speakman, 1999; Visser et al., 2000a) and were also taken from unlabelled mice to evaluate the background isotope enrichments (method D in Speakman and Racey, 1987). Blood samples were immediately heat sealed into two 50 µl glass capillaries. Two days after dosing, a final blood sample was collected as close as possible to 48 h after the initial sample to minimise circadian effects (Speakman and Racey, 1988b). Capillaries containing the blood samples were then distilled using a vacuum (Nagy, 1983) and the produced water was used to generate CO_2 (Speakman et al., 1990) or H_2 (Speakman and Król, 2005b). The isotope ratios ^{18}O:^{16}O in CO_2 and ^{2}H:^{1}H in H_2 were analysed using gas source isotope ratio mass spectrometry (ISOCHROMµGAS system and IsoPrime IRMS, Micromass, Manchester, UK). Three high-enrichment standards bracketing the experimental samples were run each day (Meijer et al., 2000). Initial isotope dilution spaces (mol) were evaluated by the intercept method (Coward and Prentice, 1985), and converted to grams considering a molecular mass of body water of 18.020 and expressed as a percentage of the body mass prior to injection. The intercept method was used instead of a plateau method because the actual body water pool estimated by desiccation was more accurately predicted by the intercept approach (Speakman and Król, 2005b). The isotope elimination rate (*k*) was evaluated following published methods (Lifson et al., 1955). The single-pool model equation 7.19 was used (Speakman, 1997) to determine the rate of CO_2 production, which has been shown to be most appropriate for this size of animal (Visser and Schekkerman, 1999; Visser et al., 2000b; Speakman and Król, 2005b). Energy equivalents of the rate of CO_2 production were evaluated using a conversion factor of 24.026 J ml^{-1} CO_2 (Weir, 1949).

MEO

We subtracted the estimated DEE from MEI to calculate MEO (Król and Speakman, 2003b).

Organ morphology

Reproductive females on day 18 of lactation (*N*=22 MH and *N*=15 ML) along with non-reproductive females (*N*=10 for both MH and ML) were sacrificed by CO_2 overdose. The brain and liver were collected for other studies not reported here. Brown adipose tissue (BAT), mammary glands and the alimentary tract were removed and then weighed (±0.0001 g; Ohaus Analytical plus Balance, Nänikon, Switzerland). The alimentary tract was separated into small intestine, large intestine and caecum, and the lengths of these components were measured with a ruler (±1 mm). The total length of the three components was reported as the whole gut length. The sections were weighed first with the gut content (full) and then empty.

Statistical analysis

Data were tested for normality using the Shapiro–Wilks test and natural logarithms were used to normalise them where required. We determined the changes in body mass and MEI throughout two stages of the experiment (baseline and pregnancy) using ANOVA, accounting for repeated measures by including individual as a nested random factor within line. During lactation, changes in body mass, MEI, litter mass, pup mass and growth rate of litters were determined also using ANOVA, accounting for repeated measures by including individual as a nested random factor within line and litter size as a time-varying covariate to correct for litter losses. When the effect of line or the interaction between line×day was significant, a *post hoc* test (Tukey pairwise comparisons) was used to determine the differences between lines. Significant differences between days were also determined using a *post hoc* comparisons test (Tukey pairwise comparisons). General linear modelling (GLM) was performed to explore the relationships between asymptotic MEI and litter size, body mass, litter mass and pup mass with line as a fixed factor and other factors as covariates when appropriate. Relationships between body mass, asymptotic MEI, litter size, litter mass and pup mass were determined using Pearson correlation and the lines were fitted using a linear regression analysis. Arcsine transformations were performed prior to analysis for percentage data (ADE), but untransformed data are quoted in the summary statistics. Independent *t*-tests were performed to determine the differences in MEO, DEE, ADE and litter size between lines. Differences in organ masses between two lines were tested using GLM with line, reproductive status and interaction between line and reproductive status as fixed factors and body mass (minus organ mass) on day 18 of lactation as a covariate. The relationships between organ masses and MEI, MEO and DEE were established by using GLM with organ mass as the independent variable and line and reproductive status as fixed factors and the body mass minus the mass of the respective organ mass as a covariate. A full factor model with all two-way and three-way interactions was fitted and then non-significant interaction terms were removed. Comparisons between the natural and cross-fostered litters for MEI, MEO and litter mass on day 18 were made using GLM with the group (mother-offspring: H-H, H-L, L-L and L-H) as a fixed factor and litter size on day 18 as a covariate. All data are presented as means ± s.e.m. Minitab (Version 16; Minitab Inc., State College, PA, USA) was used to perform all statistical analyses.

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.R.S. and E.K. designed the experiments; A.H.A.J., E.K., J.H., A.C., A.S.L. and Y.G. collected the data in the experiment with natural litters; S.C.S. and T.V. collected the data in the experiment with cross-fostered litters. W.G.H. and L.B. performed the original selection and provided the mice, C.H. analysed the DLW samples; A.H.A.J., L.V., T.V., E.K. and J.R.S. analysed the data, A.H.A.J. and J.R.S. wrote the paper and it was further edited by E.K. and L.V., and commented on and approved by the other authors.

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