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Published in:

Journal of Experimental Biology

DOI:

[10.1242/jeb.103705](https://doi.org/10.1242/jeb.103705)

Print publication: 01/01/2014

Document Version

Peer reviewed version

[Link to publication](#)

Citation for published version (APA):

Al Jothery, AH., Krol, E., Hawkins, J., Chetoui, A., Saint-Lambert, A., Gamo, Y., Shaw, SC., Valencak, T., Bunger, L., Hill, WG., Vaanholt, LM., Hambly, C., & Speakman, JR. (2014). Limits to sustained energy intake. XXII. Reproductive performance of two selected mouse lines with different thermal conductance. *Journal of Experimental Biology*, 217, 3718 - 3732. <https://doi.org/10.1242/jeb.103705>

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

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Running title: Reproductive performance and thermal conductance

1 ABSTRACT

2 Maximal sustained energy intake (SusEI) appears limited, but the factors imposing the limit
3 are disputed. We studied reproductive performance in two lines of mice selected for high and
4 low food intake (MH and ML, respectively), and known to have large differences in thermal
5 conductance (29% higher in the MH line at 21 °C). When these mice raised their natural
6 litters, their metabolisable energy intake significantly increased over the first 13 days of
7 lactation and then reached a plateau. At peak lactation, MH mice assimilated on average 45.3 %
8 more energy than ML mice (222.9 ± 7.1 and 153.4 ± 12.5 kJ day⁻¹, $N=49$ and 24 , respectively).
9 Moreover, MH mice exported on average 62.3 kJ day⁻¹ more energy as milk than ML mice
10 (118.9 ± 5.3 and 56.6 ± 5.4 kJ day⁻¹, N =subset of 32 and 21 , respectively). The elevated milk
11 production of MH mice enabled them to wean litters (65.2 ± 2.1 g) that were on average 50.2%
12 heavier than litters produced by ML mothers (43.4 ± 3.0 g), and pups that were on average
13 27.2% heavier (9.9 ± 0.2 and 7.8 ± 0.2 g, respectively). Lactating mice in both lines had
14 significantly longer and heavier guts compared to non-reproductive mice. However,
15 inconsistent with the central limit hypothesis, the ML mice had significantly longer and
16 heavier intestines than MH mice. An experiment where the mice raised litters of the opposing
17 line demonstrated that lactation performance was not limited by offspring growth capacity.
18 Our findings are consistent with the idea that the SusEI at peak lactation is constrained by the
19 capacity of the mothers to dissipate body heat.

20
21 **KEY WORDS: Artificial selection, Cross-fostering, Daily energy expenditure, Heat**
22 **dissipation limit, Milk production, Lactation**

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1 INTRODUCTION

2 Factors limiting maximal rates of sustained energy intake (SusEI) and sustained energy
3 expenditure (SusMR) have been of interest for at least 30 years, since the suggestion that both
4 are constrained at some multiple of basal metabolism (Drent and Daan, 1980; Kirkwood,
5 1983). Four different ideas have emerged to explain why intake and expenditure might be
6 limited (reviewed in Speakman and Król, 2005a; Piersma and van Gils, 2010; Speakman and
7 Król, 2011). The central limitation hypothesis (Weiner, 1989; Weiner, 1992; Peterson et al.,
8 1990; Sadowska et al. 2013) suggests that limits are imposed by the uptake capacity of the
9 alimentary tract. The peripheral limitation hypothesis (Hammond et al., 1996) posits the limit
10 resides in the capacities of the tissues where the energy is expended. The heat dissipation
11 limit (HDL) theory (Speakman and Król, 2010) suggests that intake is constrained by the
12 capacity to dissipate the heat generated as a by-product of food utilisation and milk
13 production. Finally, a trade-off idea suggests that working beyond a certain limit generates
14 negative physiological consequences that impact survival (Drent and Daan, 1980; Daan et al.,
15 1996; Piersma, 2011; Piersma and van der Velde, 2012). The HDL theory could be
16 considered a special case of this latter idea, since the implication is that processing food and
17 elevating metabolic rate beyond the heat dissipation capacity, leads to hyperthermia, with
18 direct or indirect negative consequences for survival.

19 Among the most popular models for exploring the question of where the limit resides
20 is lactation (Hammond and Diamond, 1992; Speakman and McQueenie, 1996). During
21 lactation food intake increases enormously (Johnson et al., 2001a) and conspicuously reaches
22 a plateau in late lactation that is resistant to attempts to breach it by imposing additional
23 workloads on the female, by manipulating litter size or pup demands (Hammond and
24 Diamond, 1992; Johnson et al., 2001a; Laurien-Kehnen and Trillmich, 2003; Duah et al.,
25 2013), making females simultaneously pregnant (Johnson et al., 2001c), or forcing them to
26 run to obtain their food (Perrigo, 1987; Zhao et al., 2013a). Yet, when lactating animals are
27 placed into the cold, they are able to eat significantly more than at room temperature
28 (Hammond et al., 1994; Hammond and Kristan, 2000; Johnson and Speakman, 2001;
29 Rogowitz, 1998; Zhang and Wang, 2007), and conversely when kept in hot conditions their
30 maximal intake declines (Król and Speakman, 2003a; Wu et al., 2009; Yang et al., 2013).
31 This effect could be explained either by the HDL theory, the summed peripheral demands
32 idea, or temperature dependent variations in pup energy demands. In MF1 mice, observations
33 of milk production and pup growth at the different temperatures (enhanced in the cold and
34 reduced in the heat: Johnson and Speakman, 2001; Król and Speakman, 2003b) strongly

1 supported only the HDL idea. Yet in other studies cold exposure did not have impacts on pup
2 growth (Zhang and Wang, 2007; Zhao and Cao, 2009; Zhao et al., 2010; Zhao et al., 2013b;
3 Yang et al., 2013) supporting the other two ideas.

4 Attempts to separate between the ideas that the intake is limited by the heat
5 dissipation capacity of the mother, the peripheral capacities of the mammary glands, or the
6 demand of the pups, have produced a confusion of results. Shaving MF1 mice to increase
7 their heat dissipation capacity showed that the females ate more food, produced more milk
8 and weaned larger pups (Kříd et al., 2007) – consistent only with the HDL idea. However,
9 shaving Swiss mice resulted in significantly elevated food intake, but the inferred milk
10 production and pup growth changes, while in the predicted direction, were not statistically
11 significant (Zhao and Cao, 2009; Zhao et al., 2010). In a later study, the pup growth capacity
12 interpretation was rejected, by raising small litters in the cold, and showing they could grow
13 faster than larger litters (Zhao et al., 2013b) thereby implicating the milk production capacity,
14 as the factor limiting sustained intake in Swiss mice. Conversely, shaving lactating field voles
15 increased offspring growth, but milk production was again in the expected direction but not
16 significantly different (Simons et al., 2011).

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

25 Overall, the current data are extremely confusing and suggest different species and
26 strains operate under different constraints. Moreover, multiple constraints may apply in the
27 same individuals under different conditions, for example, at different ambient temperatures
28 (Yang et al., 2013) or at different litter sizes (Wu et al., 2009). More experimental data across
29 a range of different animal models are needed to enhance our understanding of the factors
30 that are of potential importance in limiting SusEI. In the current paper, we proposed a direct
31 test of the HDL theory using two related mouse lines (MH and ML). The lines had been
32 divergently selected on their maintenance requirements (Hastings et al., 1997; B ünger et al.,
33 1998). We have previously shown that a correlated trait for such selection has been thermal
34 conductance, whereby MH mice have higher thermal conductance than the ML mice by 23 to

1 55% depending on the ambient temperature (29% at 21 °C) (Selman et al., 2001b). We
2 predicted *a priori* from the HDL theory that if heat dissipation constrains both food intake at
3 peak lactation and peak lactation performance, the MH line with greater capacity to dissipate
4 heat, would have greater peak lactation energy intake, greater milk production and elevated
5 pup growth. Moreover, these traits would be conserved if the mothers were given pups of the
6 opposing line to raise, reflecting heat dissipation capacity of the mother, rather than growth
7 capacity of the offspring.

8

9 RESULTS

10 Experiment with natural litters

11 *Maternal body mass*

12 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
13 (ANOVA, line, $F_{1,47}=0.5$, $P=0.5$). Female body mass increased significantly over the last 10
14 days of pregnancy. Although there was no significant line effect on pregnant body mass, the
15 interaction between day and line was highly significant (ANOVA, line, $F_{1,51}=2.4$, $P=0.13$;
16 day, $F_{9,459}=555.5$, $P<0.001$; interaction line \times day, $F_{9,459}=25.5$, $P<0.001$). Maternal body
17 mass of MH mice increased significantly more than that of the ML mice in the last few days
18 of pregnancy (Fig.1A). Maternal body mass did not differ between lines across days of
19 lactation, but the interaction between line and day was significant (ANOVA, line, $F_{1,71}=2.6$,
20 $P=0.11$; day, $F_{12,852}=2.7$, $P=0.002$; interaction line \times day, $F_{12,852}=2.6$, $P=0.002$; Table 1).
21 Body mass of ML mice remained unchanged throughout lactation but body mass of MH mice
22 exhibited a significant drop over the last 3 days (15 to 18). The average reduction in body
23 mass was 0.8 ± 0.3 g (Fig. 1A).

24

25 *MEI and ADE*

26 On day 12-14 of lactation, MH mice produced on average 2.3 ± 0.1 g ($N=32$) dry mass of
27 faeces daily compared to 1.6 ± 0.1 g ($N=21$) dry mass in ML mice. Faecal production was
28 highly correlated with food intake ($r=0.78$, $P<0.001$) and this relationship did not differ
29 significantly between the lines (line, $F_{1,50}=1.8$, $P=0.18$; food intake, $F_{1,50}=20.2$, $P<0.001$, Fig.
30 1B). Despite this, there was a significant difference in the ADE between lines (MH mice
31 $83.1 \pm 0.3\%$, ML mice $80.7 \pm 0.9\%$: $t_{51}=2.7$, $P=0.01$). We applied these estimates of ADE from
32 the feeding trial to convert estimated food intake into MEI during baseline and throughout
33 reproduction (see Methods for more details).

MEI during baseline was $51.1 \pm 1.8 \text{ kJ day}^{-1}$ ($N=33$) and $37.1 \pm 2.4 \text{ kJ day}^{-1}$ ($N=16$) 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 \times 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 \times day, $F_{12,852}=2.6$, $P=0.003$). A significant line \times 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 $222.9 \pm 7.1 \text{ kJ day}^{-1}$ ($N=49$) compared to $153.4 \pm 12.5 \text{ kJ day}^{-1}$ ($N=24$) in ML mice. Asymptotic MEI (days 13-15) was positively correlated with body mass at peak lactation ($r=0.58$, $P<0.001$), litter 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 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 3) averaged $90.7 \pm 2.3 \text{ kJ day}^{-1}$ ($N=32$) and $71.5 \pm 1.9 \text{ kJ day}^{-1}$ ($N=21$), 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 $209.6 \pm 6.3 \text{ kJ day}^{-1}$ ($N=32$) compared to the average of $128.1 \pm 6.3 \text{ kJ day}^{-1}$ ($N=21$) in the ML mice (line

1 effect, $t_{51}=8.7$, $P<0.001$; Table 1). MH mice also had significantly higher MEO compared to
 2 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
 3 ML mice, respectively (Table 1). MEO was highly correlated with body mass at peak
 4 lactation ($r=0.48$, $P<0.001$), litter size ($r=0.59$, $P<0.001$), litter mass ($r=0.79$, $P<0.001$), and
 5 pup mass ($r=0.59$, $P<0.001$, Fig. 4). Using GLM with mean body mass at peak lactation,
 6 litter size, litter mass, and pup mass at weaning as covariates indicated that effect of line on
 7 MEO remained significant when these additional factors were added to the model.

8 Litter size did not differ significantly between lines at birth and at weaning (at birth,
 9 $t_{71}=1.5$, $P=0.136$; at weaning, $t_{71}=1.9$, $P=0.067$). At birth, the average litter size was 7.0 ± 0.3
 10 ($N=49$) and 6.1 ± 0.6 ($N=24$) in the MH and ML mice, respectively. At weaning, the litter size
 11 of MH mice (6.7 ± 0.3 , $N=49$) was also not significantly different compared to 5.7 ± 0.6 ($N=24$)
 12 in ML mice (Table 1). Litter mass in both MH and ML mice increased significantly
 13 throughout lactation (ANOVA, line, $F_{1,71}=32.2$, $P<0.001$; day, $F_{12,852}=962.7$, $P<0.001$;
 14 interaction line \times day, $F_{12,852}=28.5$, $P<0.001$, Fig. 5A). At weaning, the litter mass of MH
 15 mice (65.2 ± 2.1 g, $N=49$) was significantly ($P<0.001$) heavier than the litter mass of ML mice
 16 (43.4 ± 3.0 g, $N=24$; Table 1). Because litter sizes were not significantly different between the
 17 lines, pup mass in both lines also increased significantly throughout lactation (ANOVA, line,
 18 $F_{1,71}=78.2$, $P<0.001$; day, $F_{12,851}=1818.1$, $P<0.001$; interaction line \times day, $F_{12,851}=19.1$,
 19 $P<0.001$, Fig. 5B) with the pups from the MH line being significantly ($P<0.001$) heavier than
 20 those of the ML line. The pup mass at weaning for the MH mice was 9.9 ± 0.2 g compared to
 21 7.8 ± 0.2 g in ML mice (Table 1). The growth rate was higher over the first days of lactation
 22 compared later. The average litter growth rate on day 7 was 4.3 ± 0.2 g and 2.7 ± 0.2 g
 23 compared to 1.1 ± 0.1 g and 0.7 ± 0.2 g on day 18 in MH and ML mice, respectively, Fig. 5C).
 24 Pup mass at weaning was negatively correlated with litter size ($r=0.28$, $P=0.01$), but
 25 including litter size into the model did not change the significant difference in pup mass
 26 between lines (GLM, line, $F_{1,70}=104.7$, $P<0.001$, litter size, $F_{1,58}=36.4$, $P<0.001$, Fig. 6B).

27

28 *Organ morphology*

29 The average wet masses of several internal organs in lactating and non-reproductive mice are
 30 presented in Table 4. Using GLM with reproductive status and line as fixed factors showed
 31 that mean maternal body mass of lactating mice on the day of dissection was significantly
 32 different compared to the non-reproductive mice, and the interaction line \times reproductive
 33 status was also significant (GLM, line, $F_{1,53}=0.5$, $P=0.83$; reproductive status, $F_{1,53}=6.2$,
 34 $P=0.01$; interaction line \times reproductive status, $F_{1,53}=4.6$, $P=0.03$). Differences between the

1 lines may then only be a reflection of the overall size differences. Analyses of the data for
 2 organ morphology in lactating and non-reproductive lactating mice with body mass as
 3 covariate are therefore also presented in Table 4.

4 Reproductive status and line had significant effects on the length of small intestine,
 5 caecum, and whole gut. Lactating mice had significantly longer intestines than non-
 6 reproductive individuals and the ML line had longer intestines than the MH line. Lactating
 7 mice had significantly heavier full and empty guts than non-reproductive individuals and the
 8 ML line had heavier full and empty guts than the MH line. In addition to a significant line
 9 effect, among the lactating mice there was a significant positive relationship between the MEI
 10 on day 18 and the mass of the full gut (GLM, $F_{1,33}=25.5$, $P<0.001$), mass of the empty gut
 11 (GLM, $F_{1,33}=25.5$, $P<0.001$, Fig. 7A) and length of the small intestine (GLM, $F_{1,33}=25.5$,
 12 $P<0.001$, Fig. 7B). For the mass of the empty gut, the effect of the interaction between body
 13 mass with line was significant, but the interactions with line were not significant for the full
 14 gut and the length of the small intestine. There were no significant relationships between MEI
 15 on day 18 and the lengths of the large intestine and caecum ($P>0.05$ in both cases). There
 16 were no significant differences in mean wet mass of BAT or mammary glands between lines.
 17 Mean wet masses of mammary glands were positively but weakly correlated with MEI
 18 ($r=0.36$, $P=0.03$), DEE ($r=0.34$, $P=0.037$) and MEO ($r=0.32$, $P=0.05$). The relationship
 19 between mass of the mammary gland and MEI, DEE, and MEO was not different between
 20 the two lines (Fig. 8).

21

22 **Experiment with cross-fostered litters**

23 *Maternal body mass*

24 Mean body mass changed significantly across the days of pregnancy and differed between the
 25 two lines (ANOVA, line, $F_{1,14}=25.3$, $P<0.001$; day, $F_{13,182}=429.4$, $P<0.001$; interaction line \times
 26 day, $F_{13,182}=36.5$, $P<0.001$). During lactation, the body mass of H-L mice (MH mothers with
 27 cross-fostered ML pups) was higher than that of L-H mice (ML mothers with cross-fostered
 28 MH pups) (ANOVA, line, $F_{1,19}=10.6$, $P=0.004$; day, $F_{11,209}=12.8$, $P<0.001$; interaction line \times
 29 day, $F_{11,209}=6.6$, $P<0.001$; H-L ($N=10$) and L-H ($N=11$); Table 2). The day \times line interaction
 30 was significant, indicating that the body mass changed differently during lactation in the two
 31 lines. Similar to the MH mice raising MH pups, the body mass of H-L mice fell over the last
 32 3 days of lactation by on average 2.9 ± 0.6 g (Fig. 9A).

33

34 *MEI and ADE*

1 Faecal production of lactating mice monitored over days 13-15 was significantly correlated
 2 with food intake ($r=0.49$, $P=0.05$). There was no significant line effect when food intake was
 3 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).
 4 On days 13-15 of lactation, there was no significant difference ($t_{13}=0.9$, $P=0.41$) in the
 5 average ADE between the lines which averaged $86.8 \pm 1.5\%$ ($N=6$) and $85.4 \pm 0.9\%$ ($N=9$) in
 6 H-L and L-H mothers, respectively. Using these estimates of ADE we converted food intake
 7 estimates throughout reproduction into MEI. MEI increased significantly over the last 5 days
 8 of pregnancy and was different between the lines (ANOVA, line, $F_{1,14}=46.9$, $P<0.001$; day,
 9 $F_{4,56}=4.1$, $P=0.006$; interaction line \times day, $F_{4,56}=4.1$, $P=0.006$, Fig. 9C).

10 During lactation, H-L mice had a significantly higher MEI than L-H mice, and MEI
 11 also varied significantly with the day of lactation (ANOVA, line, $F_{1,19}=27.2$, $P<0.001$; day,
 12 $F_{11,209}=18.4$, $P<0.001$; interaction line \times day, $F_{11,209}=3.2$, $P=0.001$). The pattern observed in
 13 the L-H mice was very similar to that observed for ML mice in the experiment with natural
 14 litters. MEI increased over the first 13 days of lactation and reached a plateau over days 13-
 15 18. In contrast, the MEI of the H-L mice mirrored that of the MH mice raising natural litters.
 16 MEI increased to a plateau which only lasted from day 13 to 15 and thereafter there was a
 17 decline (Fig. 9C). The average drop over days 15-18 of lactation was 107.4 kJ. This reduction
 18 in MEI was correlated with the reduction of body mass over the same period ($r=0.79$,
 19 $P=0.007$, Fig. 9D). Between days 13 to 15 of lactation, H-L mothers ($N=10$) assimilated on
 20 average 242.5 ± 8.8 kJ day⁻¹ compared to 165.3 ± 9.8 kJ day⁻¹ in L-H mice ($N=11$). Using GLM
 21 with mean body mass at peak lactation, litter size, and litter mass at weaning as covariates,
 22 indicated that effect of line on MEI remained significant when these other factors were added
 23 to the model. Asymptotic MEI (days 13-15) was positively correlated with body mass at peak
 24 lactation ($r=0.85$, $P<0.001$) and litter mass at weaning ($r=0.68$, $P=0.001$), but it was not
 25 significantly correlated with litter size ($r=0.32$, $P=0.15$) (Fig. 10).

26 *DEE*

27 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 ($N=8$),
 28 respectively ($t_{12}=1.2$, $P=0.27$: Table 2, 3).

29 *MEO and reproductive performance*

30 Over days 13-15 of lactation, H-L mice had significantly higher MEI than L-H mice (line
 31 effect, $t_{12}=5.9$, $P<0.001$; Table 2). This led to them having significantly higher MEO
 32 compared to L-H mice ($t_{12}=4.1$, $P=0.001$). MEO was significantly higher in H-L than L-H
 33
 34

1 mice (Table 2). Using GLM with mean body mass at peak lactation, litter size, litter mass,
 2 and pup mass at weaning as covariates, indicated that effect of line on MEO remained
 3 significant when these factors were added to the model. MEO was not significantly correlated
 4 with litter size ($r=0.21$, $P=0.47$) or pup mass ($r=0.32$, $P=0.27$) (Fig. 11). There was, however,
 5 a positive correlation between MEO and body mass at peak lactation (days 13-15) ($r=0.85$,
 6 $P<0.001$) and litter mass ($r=0.64$, $P=0.01$) (Fig. 11).

7 Litter size did not differ significantly between lines when the litters were swapped
 8 ($t_{19}=0.4$, $P=0.7$). The average litter size after swapping was 7.2 ± 0.5 and 7.5 ± 0.8 in H-L and
 9 L-H mice, respectively. No pups were lost. Litter masses of both H-L and L-H mothers
 10 increased significantly throughout lactation (ANOVA, line, $F_{1,19}=10.5$, $P=0.003$; day,
 11 $F_{11,208}=211.8$, $P<0.001$; interaction line \times day, $F_{11,209}=3.7$, $P<0.001$). Although litter mass
 12 did not differ significantly between lines from day 7-11 of lactation, ML pups supported by
 13 MH mothers were significantly heavier than MH pups supported by ML mothers from day 12
 14 until weaning (pairwise comparison, day 12, $P=0.036$; day 13, $P=0.015$ and days 14-18,
 15 $P<0.01$). At weaning, the average litter mass of ML pups supported by MH mothers was
 16 greater than that for MH pups supported by ML mothers (Fig. 12A, Table 2). At weaning,
 17 pup mass of MH pups supported by ML mice was significantly greater than that of the MH
 18 pups raised by ML mice (Table 2). Growth rate of litters in both lines varied significantly
 19 throughout lactation but marginally failed to reach significance between lines (ANOVA, line,
 20 $F_{1,19}=3.1$, $P=0.08$; day, $F_{10,189}=3.3$, $P<0.001$; interaction line \times day, $F_{10,189}=0.6$, $P=0.8$, Fig.
 21 12C). Greater litter mass at weaning was highly correlated with litter size ($r=0.86$, $P<0.001$)
 22 and litter mass at weaning of H-L mothers was significantly greater than L-H mothers when
 23 litter size was added to the model (GLM, line, $F_{1,18}=20.2$, $P<0.001$, litter size, $F_{1,18}=114.9$,
 24 $P<0.001$, Fig. 13A). Pup mass at weaning was negatively correlated to litter size ($r=0.74$,
 25 $P<0.001$) and the average mass of ML pups supported by MH mice was significantly heavier
 26 than MH pups supported by ML mice, when litter size was added to the model (GLM, line,
 27 $F_{1,18}=13.3$, $P=0.002$, litter size, $F_{1,18}=35.6$, $P<0.001$, Fig. 13B).

28

29 **Comparison of cross-fostered and natural litters**

30 We pooled the data collected with respect to the natural and cross fostered litters and
 31 examined the effects of group (mother-offspring source: H-H, H-L, L-L and L-H) on the peak
 32 metabolisable energy intake, milk energy output and litter mass at day 18, with litter size as a
 33 covariate. For MEI, there was a significant effect of litter size ($F_{1,89}=68.2$, $P<0.001$) and a
 34 significant group effect ($F_{3,89}=20.1$, $P<0.001$). For MEO, there was a significant effect of

1 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
2 litter mass at weaning, there was a significant effect of litter size ($F_{1,82}=335.8$, $P<0.001$) and a
3 significant group effect ($F_{3,82}=35.3$, $P<0.001$). For all three variables, *post hoc* Tukey test
4 comparisons revealed that the high mothers differed from the low mothers ($P<0.05$) but there
5 was no difference between the high mothers raising high or low pups ($P>0.05$), and no
6 difference between the low mothers raising either high or low pups ($P>0.05$).

7

8 **DISCUSSION**

9 The goal for this study was to test the HDL theory by comparing the reproductive
10 performance of two lines of mice previously shown to have high and low thermal
11 conductance. The HDL theory suggests that at peak lactation mammals are constrained by
12 their capacity to dissipate body heat, and hence predicts that the MH mice, with greater
13 thermal conductance, should have greater peak energy intake, permitting them to invest more
14 energy in milk production and hence produce heavier litters and pups.

15 During lactation, mice in both lines increased MEI significantly over the first 13 days
16 and then reached a plateau (asymptotic food intake). These findings are consistent with
17 previous research on food intake during lactation in different animal models. Lactating MF1
18 mice reach a plateau around day 11 of lactation (Johnson et al., 2001a; Krđ et al., 2003;
19 Vaanholt et al., 2013; Gamo et al., 2013; Duah et al., 2013), lactating common voles on day
20 14 (*Microtus arvalis*) (Simons et al., 2011), lactating Brandt's voles (*Lasiopodomys brandtii*)
21 on day 8 (Wu et al., 2009), lactating European hares (*Lepus europaeus*) during weeks 3-4
22 (Valencak and Ruf, 2009) and lactating Mongolian gerbils (*Meriones unguiculatus*) on day 9
23 (Yang et al., 2013). Consistent with the prediction of the HDL theory, the peak metabolisable
24 energy intake in lactation (days 13-15) was significantly higher in the MH line compared
25 with the ML line. This was in turn translated into a greater milk production, which led to a
26 greater growth of the litters in the MH line mice and ultimately led to them weaning heavier
27 pups. The litter mass of MH mice at weaning was 50.2% heavier compared the average litter
28 mass weaned by ML mice. Similarly, the mass of individual pups raised by MH mice was
29 27.2% greater than those raised by the ML mice. The MH females exported on average 62.3
30 kJ day⁻¹ more energy as milk than ML females. Since the increase in MEO in MH mice was
31 fuelled by extra MEI (81.5 kJ day⁻¹), the efficiency for converting the MEI to MEO was
32 76.4%. This is consistent with previous efficiency estimates (Romero et al., 1976; Baldwin et
33 al., 1980; Freetly et al., 2006; Krđ et al., 2007). Our findings are corroborated by a previous
34 study that was conducted on laboratory mice which had been selected for high and low heat

1 loss (Nielsen et al., 1997a; Nielsen et al., 1997b). It was demonstrated by using a weigh-
2 suckle-weigh method that high heat loss mice synthesized on average 20.6% more milk than
3 low heat loss mice. As a consequence, they weaned litters on average 10.1 g heavier
4 (McDonald and Nielsen, 2006).

5 The asymptotic MEI in the ML line remained stable over days 13 to 18, consistent
6 with studies in other mouse strains and other small rodents and lagomorphs (Johnson et al.,
7 2001a; Krđ and Speakman, 2003a; Krđ and Speakman, 2003b; Krđ et al., 2003; Krđ et al.,
8 2007; Wu et al., 2009; Simons et al., 2011; Zhao and Cao, 2009; Valencak and Ruf, 2009;
9 Zhao et al., 2010; Vaanholt et al., 2013; Gamo et al., 2013; Yang et al., 2013; Duah et al.,
10 2013). In contrast, the pattern observed in the MH mice was different. There was a significant
11 drop in MEI across days 15-18 amounting to a total deficit of 58.3 kJ. At the same time, the
12 MH females lost 0.8 g of body mass. This reduction in body mass could be just reduced gut
13 fill reflecting the lower food intake. However, if this loss of weight represented withdrawal of
14 fat reserves, it would represent approximately 31 kJ of energy ($39 \text{ kJ g}^{-1} \times 0.8 \text{ g}$) (Johnson et
15 al., 2001c; Speakman, 2008) that could supplement the reduced intake. Since fat is the most
16 energy dense tissue, this is the maximal level of energy that could be supplied by the lost
17 body mass. Hence, making these limiting assumptions, the lactating MH mice had between
18 58.3 and 27.3 kJ (14.6 to 6.7 kJ day^{-1}) lower energy intake over the last few days of lactation
19 than they would have had if they had sustained their energy intake at the peak level. Since the
20 milk energy output at peak lactation was $118.9 \pm 5.3 \text{ kJ day}^{-1}$, the reduction in daily milk
21 production would have been at least 5.6% and up to 12.5%, assuming that all the deficit was
22 paid for by reduced milk production. Over this period, the growth of the MH litters declined
23 steeply (Fig. 5C), yet they still retained greater growth than the ML litters, consistent with the
24 fact the ML litters were receiving on average only 56.6 kJ day^{-1} of milk. Exactly why the MH
25 mice used a strategy of fuelling late lactation by a reduction in energy intake possibly
26 supplemented by a withdrawal of reserves is unclear. Since it occurred in both experiments
27 with natural litters and cross-fostered litters, it was a strategy adopted by the mothers,
28 independent of the pups they were suckling.

29 Examination of the internal organs of lactating mice revealed a significant increase in
30 the size and mass of several organs compared to non-reproductive mice. These changes
31 included the whole gut length, small intestine length, caecum length, empty and full gut
32 masses. However, no significant differences were found in the mass of BAT and length of the
33 large intestine between lactating and non-reproductive mice. Our findings were similar to the
34 patterns that were found in previous work in a diversity of rodent species. This previous work

1 has shown substantial increases in lactation of the alimentary tract and associated organs such
2 as liver and pancreas (Kennedy et al., 1958; Jolicoeur et al., 1980; Wu et al., 2009; Speakman
3 and McQueenie, 1996). The increase in the sizes of the components of the alimentary tract
4 during lactation are consistent with the central limit theory that intake is constrained by the
5 uptake capacity of the alimentary tract (Kirkwood, 1983; Perrigo, 1987; Hammond and
6 Diamond, 1992; Hammond and Diamond, 1994; Koteja, 1996; Künkele, 2000; Johnson et al.,
7 2001a; Johnson et al., 2001b; Laurien-Kehnen and Trillmich, 2003; Speakman, 2008).
8 However, it seems highly unlikely that such changes underpin the difference in intake
9 between the MH and ML lines at peak lactation, because the differences between lines were
10 in the opposite direction. The ML mice had longer whole guts, mostly attributed to their
11 significantly longer small intestines. Moreover, ML mice had significantly greater wet
12 masses of empty and full guts. These data are consistent with previous work on these lines
13 (Selman et al., 2001a) where the ML mice had significantly greater dry mass of the stomach
14 and large intestine. However, whole gut length was not measured in that study. Surprisingly,
15 at the end of lactation (day 18), there was a significant positive relationship between the MEI
16 and the masses of both the full and empty gut, and the length of the small intestine, within
17 each of the lines, which completely contrasted the difference between the lines. Consequently,
18 while it seems unlikely that a central limit imposed by gut capacity was responsible for the
19 line difference, it remains feasible that the individual differences within lines could be
20 attributed to such an effect.

21 Sadowska et al. (2013) studied the reproductive performance of mice that had been selected
22 for high and low basal metabolic rate (BMR) and found that those with high BMR had
23 greater reproductive performance. They attributed this difference to differences in the
24 assimilation capacity of the alimentary tract, and hence concluded their data were consistent
25 with the central limitation hypothesis. The mice we studied also differ in their resting
26 metabolic rate in the thermoneutral zone (RMRt: which is similar to BMR Speakman et al.
27 2004) with non-reproductive MH mice having higher RMRt than the ML mice (Selman et al.
28 2001a). However, we have shown previously that these differences in RMRt between the M
29 lines are not linked to morphological differences in the alimentary tract (Selman et al. 2001a).
30 Moreover, given the strain differences discussed above, this seems an unlikely explanation
31 for the data presented here. However, the positive correlation between RMRt and thermal
32 conductance observed in the M strain mice (Selman et al. 2001b) suggests a potential
33 alternative explanation for the observations on mice selected for high and low BMR
34 (Sadowska et al. 2013). It is potentially the case that the mice with higher BMR also had

1 higher thermal conductance, and were hence able to dissipate more heat, and this was the
2 primary factor regulating the level of their reproductive performance, with the observed
3 changes in the alimentary tract in that study a secondary response. Unfortunately, thermal
4 conductance differences between the strains were not measured in this previous study
5 (Sadowska et al. 2013). The thermal conductance of these mice was measured by Gębczyński
6 (2005) at generation 19, and no significant differences were noted. The relevance of these
7 measurements to the mice studied by Sadowska et al. (2013) is however uncertain because
8 they are separated by 8 years (and 13 generations) of continued selection, hence there has
9 been ample time for a difference in thermal conductance as a correlated trait to develop
10 between these two studies. Note that although a similar time elapsed between the
11 characterisation of thermal conductance of the lines studied here and the present study of
12 their reproductive performance a reduction in the difference in thermal conductance between
13 the lines over this interval seems unlikely. For the trait under selection (food intake), at
14 generation 8-9, the difference was 20.5%, at generation 14-15 it was 28.7%, and at
15 generations 21-23 the difference was 45.2% (Hastings et al. 1997). At generation 38 when
16 selection stopped it was 58.7% (Bunger et. al 1998). After repeated rounds of inbreeding, at
17 generation 47 we characterised the thermal conductance. We did not measure the food intake
18 of the lines in this generation, but did so in generation 50 when the difference averaged
19 45.5%. In the current study (generations 68-83), the difference in baseline food intake
20 between the lines persisted at the similar level of 37.7%. Hence, there has been only a slight
21 reduction in the difference, between generation 50 and 83. At generation 47, we performed a
22 pilot study to explore the lactation performance of the two lines, and found that at peak
23 lactation the intake of the high line was 55% greater than that of the low line. This compares
24 with 31% at generation 68, 64% at generation 75, 56% at generation 80 and 47% at
25 generation 83. Clearly, these values vary a lot from generation to generation, but the overall
26 average for the data presented here (generations 68 to 83) is 49.5%, a slight reduction on the
27 value of 55% at generation 47, consistent with the slight reduction in the difference between
28 the baseline food intakes over the same period. These data clearly indicate that the metabolic
29 phenotype of the MH and ML mice has remained virtually unchanged over the last 35-40
30 generations since the period of inbreeding designed to fix the genetics, hence we are
31 confident the difference in thermal conductance probably also persisted through this period.

32

33 Although DEE and MEO were uncorrelated with features of the alimentary tract,
34 these traits and MEI were positively correlated with the mass of the mammary glands.

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

14 An alternative explanation to the HDL theory for the results observed in the
15 experiment with natural litters is that female milk production was driven by pup demand
16 (Speakman and Krd 2005a; Zhao et al., 2013b). By this idea, the greater food intake and
17 milk production of the high line was because the MH line pups demanded greater milk from
18 their mothers. To test whether the system was regulated by the performance of the mother or
19 the demands of the pups, we cross-fostered litters in the second experiment. In this
20 experiment the performance of the MH mothers raising ML pups matched exactly their
21 performance when raising MH pups, and the same was also true of ML mothers raising MH
22 pups, compared to their performance when raising ML pups. Although the comparison
23 between the mothers raising natural litters and mothers raising cross-fostered litters is not
24 ideal because those raising natural litters received a different level of disturbance, this
25 comparison also shows that the difference between the lines resides in the mothers and not in
26 the offspring. These data very clearly show that the overall energy flux of the mother-pup
27 system is controlled by factors that affect the performance of the female, rather than the
28 growth capacity of the pups. This is consistent with the data generated elsewhere (Zhao et al.,
29 2013b).

31 **Conclusions**

32 In the experiment with natural litters, we showed that the mice selected for high and low food
33 intake were limited in their maximum energy intake and reached a plateau at day 13 of
34 lactation. Reproductive performance in the MH mice was significantly higher than that of the

1 ML mice. MH mice ate more food and produced more milk and weaned heavier pups. Our
2 morphological findings suggest that mice at peak lactation were unlikely to be constrained
3 centrally by the capacity of alimentary tract (central limit hypothesis). Furthermore, it was
4 demonstrated that the reproductive performance was driven by factors affecting the mothers
5 rather than growth capacity of the pups. Our results support the hypothesis that capacity to
6 dissipate heat is the physiological mechanism shaping the maximum energy intake and the
7 reproductive performance in mice selected for high and low food intake, due to the correlated
8 effects of selection on thermal conductance

9

10 **MATERIALS AND METHODS**

11 **Source of mouse lines**

12 We used mice from the maintenance (M) lines (Hastings et al., 1997; Bünger et al., 1998),
13 which originated from a common background generated by a three strain cross, between two
14 inbred strains (JU and CBA) and one outbred CFLP strain (Sharp et al., 1984). The mice
15 were divergently selected over 38 generations for high and low food intake (MH and ML,
16 respectively) at the University of Edinburgh, UK. Because food intake is related to body mass,
17 the selection was based on food intake corrected for average body mass. Three independent
18 replicate lines were selected in each direction. At generation 20, inter-crossing was made in
19 each three replicates, and only one resultant line in each direction was maintained till
20 generation 38, after which the selective breeding was terminated. At the beginning of
21 generation 43, partially inbred lines were produced by sib-sib mating for 4 generations to
22 facilitate mapping work. Mice were subsequently random bred within each line, avoiding sib-
23 sib mating. The current studies were performed over a period of five years spanning the
24 approximate generations 68 to 83. The MH and ML lines were shown to have different
25 thermal conductance (Selman et al., 2001b).

26

27 **Breeding protocol**

28 Virgin female mice aged 9-12 weeks were individually housed in shoebox cages (48 cm x 15
29 cm x 13 cm) under a 12 h L: 12 h D photoperiod at 21 ± 2 °C and a relative humidity of $59 \pm 5\%$.
30 All cages were provided with sawdust, paper bedding and a cardboard tube. Animals had *ad*
31 *libitum* access to water and food (details below). After 12 days of baseline females were
32 mated with non-sibling males for 11-15 days. Pregnant mice were monitored daily to
33 establish the day of parturition (day 0), and the timing for pregnancy was back calculated

1 from the day of birth as day -1 (last day of pregnancy) to day -18 (beginning of pregnancy).
2 Adult females and their pups were subjected to various measurements (details below) until
3 day 18 of lactation.

4 5 **Experiment with natural litters**

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

19 20 **Experiment with cross-fostered litters**

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

27 To allow mothers and their cross-fostered pups to settle down together, they were left
28 undisturbed for another two days, before monitoring of body mass and energy balance
29 resumed. Because not all females were monitored during pregnancy, the pre-lactation sample
30 sizes are smaller than those during lactation. Specifically, the body mass and food intake
31 measurements during pregnancy were performed on 8 individuals from each line, with no
32 data collected during the baseline.

33 34 **Body mass, food intake and reproductive performance**

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

7 **Metabolisable energy intake (MEI)**

8 Measurements of MEI were performed either on days 12-14 of lactation (experiment with
9 natural litters) or on days 13-15 of lactation (experiment with cross-fostered litters). Females
10 and their litters were placed in cages with fresh sawdust, and a weighed portion of D12450B
11 food was added to the hopper at the beginning of the 48 h feeding trial. Samples of food were
12 taken to determine dry mass content ($93.8 \pm 0.2\%$, $N=8$), and the food remaining in the hopper
13 was reweighed at the end of feeding trial. Any uneaten, fragmented food and faeces were
14 removed from the cage, dried to a constant mass at $60\text{ }^{\circ}\text{C}$ and weighed. The gross energy
15 content of D12450B food ($17.8 \pm 0.17\text{ kJ g}^{-1}$ dry mass, $N=3$) and faeces (MH mothers,
16 $15.4 \pm 0.04\text{ kJ g}^{-1}$ dry mass, $N=32$; ML mothers, $15.7 \pm 0.1\text{ kJ g}^{-1}$ dry mass, $N=21$; H-L
17 mothers, $15.4 \pm 0.08\text{ kJ g}^{-1}$ dry mass, $N=6$; L-H mothers, $15.8 \pm 0.1\text{ kJ g}^{-1}$ dry mass, $N=9$) were
18 measured by bomb calorimetry (Parr 6200 calorimeter with semi-micro oxygen bomb 1109A,
19 Scientific and Medical Products Ltd, Cheadle, UK). Dry food consumption (g day^{-1}) was
20 calculated by multiplying the food intake (g day^{-1}) by the food dry mass content (%). Gross
21 energy intake (GEI, kJ day^{-1}) was then calculated by multiplying the dry food consumption
22 (g day^{-1}) by the food energy content (kJ g^{-1} dry mass). Energy lost through faeces (kJ day^{-1})
23 was calculated by multiplying dry faecal production (g day^{-1}) by the faecal energy content
24 (kJ g^{-1} dry mass). MEI (kJ day^{-1}) was calculated as the difference between GEI and energy
25 lost through faeces, assuming that the energy loss via urine was 3% of the energy digested
26 (Drożdż, 1975). The apparent digestive efficiency (ADE) was calculated as the percentage of
27 GEI that was digested.

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

1 (Král et al., 2007). MEI in mice fed with D12450B diet was calculated using the parameters
2 measured in the current study, including the energy content of D12450B diet (17.8 kJ g^{-1} dry
3 mass) and the diet-specific ADE (MH mothers, 83.1%; ML mothers, 80.7%; H-L mothers
4 86.8% and L-H mothers, 85.4%).

6 **Daily energy expenditure (DEE)**

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

1 (Speakman, 1997) to determine the rate of CO₂ production, which has been shown to be most
2 appropriate for this size of animal (Visser and Schekkerman, 1999; Visser et al., 2000b;
3 Speakman and Król, 2005b). Energy equivalents of the rate of CO₂ production were
4 evaluated using a conversion factor of 24.026 J ml⁻¹ CO₂ (Weir, 1949).

6 **Milk energy output (MEO)**

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

9 **Organ morphology**

10 Reproductive females on day 18 of lactation ($N=22$ MH and $N=15$ ML) along with non-
11 reproductive females ($N=10$ for both MH and ML) were sacrificed by CO₂ overdose. The
12 brain and liver were collected for other studies not reported here. Brown adipose tissue
13 (BAT), mammary glands, and the alimentary tract were removed and then weighed (± 0.0001
14 g; Ohaus Analytical plus Balance, Nänikon, Switzerland). The alimentary tract was separated
15 into small intestine, large intestine and caecum, and the lengths of these components were
16 measured with a ruler (± 1 mm). The total length of the three components was reported as the
17 whole gut length. The sections were weighed first with the gut content (full) and then empty.

19 **Statistical analysis**

20 Data were tested for normality using the Shapiro-Wilks test and natural logarithms were used
21 to normalize them where required. We determined the changes in body mass and MEI
22 throughout two stages of the experiment (baseline and pregnancy) using ANOVA, accounting
23 for repeated measures by including individual as a nested random factor within line. During
24 lactation, changes in body mass, MEI, litter mass, pup mass, and growth rate of litters were
25 determined also using ANOVA, accounting for repeated measures by including individual as
26 a nested random factor within line and litter size as a time varying covariate to correct for
27 litter losses. When the effect of line or the interaction between line \times day was significant, a
28 *post hoc* test (Tukey pairwise comparisons) were used to determine the differences between
29 lines. Significant differences between days were also determined using a *post hoc*
30 comparisons test (Tukey pairwise comparisons). General linear modelling (GLM) was
31 performed to explore the relationships between asymptotic MEI and litter size, body mass,
32 litter mass, and pup mass with line as a fixed factor and other factors as covariates when
33 appropriate. Relationships between body mass, asymptotic MEI, litter size, litter mass, and
34 pup mass were determined using Pearson correlation and the lines were fitted using a linear

1 regression analysis. Arcsine transformations were performed prior to analysis for percentage
 2 data (ADE), but untransformed data are quoted in the summary statistics. Independent *t*-tests
 3 were performed to determine the differences in MEO, DEE, ADE, and litter size between
 4 lines. Differences in organ masses between two lines were tested using GLM with line,
 5 reproductive status and interaction between line and reproductive status as fixed factors, and
 6 body mass (minus organ mass) on day 18 of lactation as a covariate. The relationships
 7 between organ masses and MEI, MEO and DEE were established by using GLM with organ
 8 mass as the independent variable and line and reproductive status as fixed factors and the
 9 body mass minus the mass of the respective organ mass as a covariate. A full factor model
 10 with all 2-way and 3-way interactions was fitted and then non-significant interaction terms
 11 were removed. Comparisons between the natural and cross-fostered litters for MEI, MEO and
 12 litter mass on day 18 were made using GLM with the group (mother-offspring: H-H, H-L, L-
 13 L and L-H) as a fixed factor and litter size on day 18 as a covariate. All data are presented as
 14 mean \pm s.e.m. Minitab (Version 16; Minitab Inc., State College, PA, USA) was used to
 15 perform all statistical analyses.

16
 17
 18
 19

20 **List of abbreviations**

| | | |
|----|-----|--|
| 21 | ADE | apparent digestive efficiency |
| 22 | BAT | brown adipose tissue |
| 23 | BMR | basal metabolic rate |
| 24 | DEE | daily energy expenditure |
| 25 | DLW | doubly labelled water |
| 26 | HDL | heat dissipation limit |
| 27 | H-L | MH mothers with cross-fostered ML pups |
| 28 | L-H | ML mothers with cross-fostered MH pups |
| 29 | MEI | metabolisable energy intake |
| 30 | MEO | milk energy output |
| 31 | MH | high maintenance line |
| 32 | ML | low maintenance line |

1 RMRt resting metabolic rate at thermoneutrality

2 SusMR sustained energy expenditure

3 SusEI sustained energy intake

4

5 **Acknowledgments**

6 We are grateful to the animal house staff that looked after the mice. Peter Thomson provided
7 invaluable technical support for the isotope analysis.

8

9 **Author contribution**

10 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.
11 collected the data in the experiment with natural litters; S.C.S. and T.V. collected the data in
12 the experiment with cross-fostered litters. W.G.H. and L.B. performed the original selection
13 and provided the mice, C.H. analysed the DLW samples; A.H.A.J., L.V., T.V., E.K. and
14 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.
15 and L.V., and commented on and approved by the other authors.

16

17 **Competing interests**

18 No competing interests declared.

19

20 **Funding**

21 The mice were selected under grants from BBSRC to W.G.H. and L.B. who was additionally
22 supported by Cotswold International. A.H.A.J. was supported by a scholarship from the Iraqi
23 government. A.C. and A.S.L. were supported by the Erasmus program and Y.G. was
24 supported by a scholarship from the international rotary foundation. The work was also partly
25 funded by research grants from NERC to J.R.S. and C.H. and from BBSRC to J.R.S. and E.K.
26 J.R.S. was also supported by a 1000 talents professorship during the final phase of the work.

27

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17 **Figure legends**

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

27
28 **Fig. 2. Estimated values of asymptotic metabolisable energy intake (days 13-15 of**
 29 **lactation) in MH mice ($N=49$, filled circles) and ML mice ($N=24$, open circles) raising**
 30 **natural litters.** MEI plotted against (A) body mass at peak lactation (MH: $y=-41.03+8.25x$,
 31 $r^2=0.20$; ML: $y=-269.32+13.74x$, $r^2=0.61$), (B) litter size at weaning (MH: $y=125.4+14.57x$,
 32 $r^2=0.27$; ML: $y=43.50+19.25x$, $r^2=0.75$), (C) litter mass at weaning (MH and ML pooled:
 33 $y=53.67+2.53x$, $r^2=0.53$).

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

6 **Fig. 4. Milk energy output (MEO) in MH mice ($N=32$, filled circles) and ML mice ($N=21$,**
 7 **open circles) raising natural litters.** MEO plotted against (A) body mass at peak lactation
 8 (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:
 9 $y=57.62+9.21x$, $r^2=0.26$; ML: $y=29.19+5.44x$, $r^2=0.20$), (C) litter mass at weaning (MH:
 10 $y=29.24+1.36x$, $r^2=0.42$; ML: $y=25.58+0.78x$, $r^2=0.22$), (D) pup mass at weaning (MH:
 11 $y=37.10+8.17x$, $r^2=0.06$; ML: $y=81.72-3.10x$, $r^2=0.01$).

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

16 **Fig. 6. Litter and pup masses at weaning for MH mice ($N=49$, filled circles) and ML**
 17 **mice ($N=24$, open circles) raising natural litters.** Litter mass (A) (MH: $y=22.47+6.38x$,
 18 $r^2=0.73$; 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,
 19 $y=9.09-0.2x$, $r^2=0.34$) are plotted against litter size at weaning.

21 **Fig. 7. Metabolisable energy intake (MEI) on day 18 of lactation MH mice ($N=22$, filled**
 22 **circles) and ML mice ($N=14$, open circles) raising natural litters.** MEI plotted against (A)
 23 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)
 24 length of small intestine (MH: $y=199.36+8.13x$, $r^2=0.38$; ML: $y=255.26+7.77x$, $r^2=0.32$).

26 **Fig. 8. Wet masses of mammary gland at the end of lactation in MH mice ($N=22$, filled**
 27 **circles) and ML mice ($N=15$, open circles) raising natural litters.** Mean mass of wet
 28 mammary gland plotted against (A) milk energy output (MH: $y=2.15+0.07x$, $r^2=0.10$; ML:
 29 $y=2.03+0.02x$, $r^2=0.22$), (B) metabolisable energy intake (MH: $y=2.15+0.01x$, $r^2=0.11$; ML:
 30 $y=1.03+0.01x$, $r^2=0.28$), (C) daily energy expenditure (MH: $y=1.30+0.02x$, $r^2=0.14$; ML:
 31 $y=0.73+0.03x$, $r^2=0.12$).

32

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

10

11 **Fig. 10. Estimated values of asymptotic metabolisable energy intake (days 13-15 of**
 12 **lactation) in H-L mice ($N=10$, filled circles) and L-H mice ($N=11$, open circles) raising**
 13 **cross-fostered pups.** MEI plotted against (A) body mass at peak lactation (H-L: $y=-68.24+$
 14 $8.77x$, $r^2=0.39$; L-H: $y=-47.22+6.90x$, $r^2=0.74$), (B) litter size at weaning (H-L:
 15 $y=178.07+8.94x$, $r^2=0.23$; L-H: $y=103.15+8.3x$, $r^2=0.50$), (C) litter mass at weaning (H-L:
 16 $y=117.03+1.8x$, $r^2=0.48$; L-H: $y=71.85+1.55x$, $r^2=0.63$).

17

18 **Fig. 11. Milk energy output (MEO) in H-L mice ($N=6$, filled circles) and L-H mice ($N=8$,**
 19 **open circles) raising cross-fostered pups.** MEO plotted against (A) body mass at peak
 20 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
 21 weaning (C) litter mass at weaning (H-L: $y=141.50+0.12x$, $r^2=0.001$; L-H, $y=8.71+0.136x$,
 22 $r^2=0.25$), (D) pup mass at weaning (H-L: $y=-70.78+23.77x$, $r^2=0.68$; L-H: $y=92.87-2.03x$,
 23 $r^2=0.007$).

24

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

29

30 **Fig. 13. Mean of litter size at weaning in MH mice ($N=49$, filled circles) and ML mice**
 31 **($N=24$, open circles) raising natural litters.** Litter size plotted against (A) litter mass at
 32 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
 33 weaning (H-L: $y=13.43-0.51x$, $r^2=0.55$; L-H: $y=11.7-0.43x$, $r^2=0.73$).

34

1 Table 1. Reproductive performance of lactating mice with high (MH) and low (ML) thermal
 2 conductance, raising natural litters. Values are means \pm s.e.m.; $N=49$ and $N=24$ for MH and
 3 ML, respectively (body mass, litter size, litter mass, and pup mass); $N=32$ and $N=21$ for MH
 4 and ML, respectively (MEI, DEE, and MEO).

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

7
 8 MEI, metabolisable energy intake; DEE, daily energy expenditure; MEO, milk energy output.
 9

1 Table 2. Reproductive performance of lactating mice with high (MH) and low (ML), raising
 2 cross-fostered ML and MH pups. Values are means \pm s.e.m.; $N=10$ and $N=11$ for H-L and L-
 3 H, respectively (body mass, litter size, litter mass, and pup mass); $N=6$ and $N=8$ for H-L and
 4 L-H, respectively (MEI, DEE, and MEO).

5

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

6

7 MEI, metabolisable energy intake; DEE, daily energy expenditure; MEO, milk energy output.

1 Table 3. Results of doubly labelled water measurements of daily energy expenditure
 2 performed on lactating mice with high (MH) and low (ML) thermal conductance, raising
 3 natural or cross- fostered ML and MH pups.

4

| Trait | Experiment with natural litters | | Experiment with cross-fostered litters | |
|--|---------------------------------|-------------|--|-------------------------------|
| | 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 |

5

6 Values are means ± s.e.m.; $N=32$ for MH lactating mice; $N=21$ for ML lactating mice
 7 (experiment with natural litters) and $N=6$ for H-L lactating mice; $N=8$ for L-H lactating mice
 8 (experiment with cross-fostered litters).

9 ^aBody mass before injection; ^belimination rate of ²H; ^celimination rate of ¹⁸O; ^ddeuterium (N_d)
 10 and oxygen (N_o) dilution spaces expressed as % of body mass before injection; ^edaily energy
 11 expenditure measured over days 15-17 of lactation.

12

13

1 Table 4. Wet masses of tissues and organs of lactating MH ($N=22$) and ML ($N=15$) mice and
 2 non-reproductive MH and ML ($N=10$ for both lines) mice. Values are presented as mean \pm
 3 sem. P values indicate statistical significance of effects. Line, reproductive status, interaction
 4 between line and reproductive status, interaction between line and body mass, interaction
 5 between reproductive status and body mass, and interaction among three traits were used in a
 6 GLM model. Body mass at dissecting day minus the organ mass being considered as the
 7 dependent variable was used as a covariate for organ mass parameters. Body mass at day of
 8 dissection was used as a covariate for organ length parameters. Non-significant interactions
 9 were removed from the models.

| | 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 \pm 0.002 | 3.03 \pm 0.2 | 4.28 \pm 0.2 | 1.99 \pm 0.1 | 45.7 \pm 0.5 | 8.2 \pm 0.3 | 3.3 \pm 0.16 | 57.2 \pm 0.6 |
| Lactating ML mice | 0.076 \pm 0.004 | 2.89 \pm 0.2 | 4.93 \pm 0.3 | 2.32 \pm 0.1 | 49.1 \pm 0.7 | 7.8 \pm 0.4 | 3.3 \pm 0.21 | 60.2 \pm 1.0 |
| Non-reproductive MH mice | 0.077 \pm 0.003 | | 2.50 \pm 0.1 | 1.69 \pm 0.1 | 41.2 \pm 0.6 | 7.4 \pm 0.3 | 2.0 \pm 0.15 | 50.6 \pm 0.5 |
| Non-reproductive ML mice | 0.086 \pm 0.005 | | 2.83 \pm 0.2 | 1.94 \pm 0.1 | 45.5 \pm 1.0 | 7.3 \pm 0.3 | 2.2 \pm 0.13 | 54.9 \pm 1.3 |
| Statistics | | | | | | | | |
| Line | $P=0.005^a$ | ns | $P=0.003^b$ | $P<0.001^b$ | $P<0.001^b$ | ns | ns | $P<0.001^b$ |
| RS | ns | | $P<0.001^c$ | $P=0.002^c$ | $P<0.001^c$ | ns | $P<0.001^b$ | $P<0.001^c$ |
| BM | $P=0.007$ | $P=0.009$ | $P=0.005$ | $P<0.001$ | $P=0.001$ | ns | ns | $P<0.001$ |
| Line x RS | $P=0.031$ | | ns | $P=0.007$ | Ns | ns | ns | ns |
| Line x BM | $P=0.005$ | ns | ns | Ns | Ns | ns | ns | ns |
| RS x BM | ns | | ns | Ns | Ns | ns | ns | ns |
| Line x RS x BM | ns | | ns | Ns | Ns | ns | ns | ns |

10

11

12 RS, reproductive status; BM, body mass minus respective organ mass; ns, not significant
 13 ($P>0.05$); ^aMH > ML; ^bML > MH; ^cLactating mice > non-reproductive mice.

14

























