

RESEARCH ARTICLE

Limits to sustained energy intake. XXVII. Trade-offs between first and second litters in lactating mice support the ecological context hypothesis

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ABSTRACT

Increased reproductive effort may lead to trade-offs with future performance and impact offspring, thereby influencing optimal current effort level. We experimentally enlarged or reduced litter size in mice during their first lactation, and then followed them through a successive unmanipulated lactation. Measurements of food intake, body mass, milk energy output (MEO), litter size and litter mass were taken. Offspring from the first lactation were also bred to investigate their reproductive success. In their first lactation, mothers with enlarged litters ($n=9$, 16 pups) weaned significantly smaller pups, culled more pups, and increased MEO and food intake compared with mothers with reduced litters ($n=9$, 5 pups). In the second lactation, no significant differences in pup mass or litter size were observed between groups, but mothers that had previously reared enlarged litters significantly decreased pup mass, MEO and food intake compared with those that had reared reduced litters. Female offspring from enlarged litters weaned slightly smaller pups than those from reduced litters, but displayed no significant differences in any of the other variables measured. These results suggest that females with enlarged litters suffered from a greater energetic burden during their first lactation, and this was associated with lowered performance in a successive reproductive event and impacted on their offspring's reproductive performance. Female 'choice' about how much to invest in the first lactation may thus be driven by trade-offs with future reproductive success. Hence, the 'limit' on performance may not be a hard physiological limit. These data support the ecological context hypothesis.

KEY WORDS: Milk energy output, Fitness, Reproductive success, Lactation

INTRODUCTION

The maximum rate at which animals can process energy over protracted periods of time (i.e. sustained energy intake, SusEI) is a trait of key significance because it imposes an upper boundary on animal performance, including reproductive output and thermoregulatory capabilities (Drent and Daan, 1980; Hammond and Diamond, 1997; Peterson et al., 1990; Speakman and Król,

2005; Weiner, 1992). Considerable interest has been focused on the factors that impose intrinsic physiological limits on this maximum. Several physiological limitations have been proposed, i.e. limits imposed by the energy-supplying machinery (such as the alimentary tract and associated organs) (Hammond and Diamond, 1992; Hammond et al., 1994; Koteja, 1996b; Perrigo, 1987), limits imposed by the capacities of the energy-utilising machinery (such as milk production by mammary tissue) (Hammond et al., 1996; Koteja, 1996a; Rogowitz, 1998) and limits to the ability to dissipate excess heat (Krol et al., 2007; Speakman and Król, 2010).

A model system that has been extensively used to test ideas concerning limitations on maximal SusEI is the period of late lactation in small rodents (Hammond and Diamond, 1992; Hammond et al., 1994; Perrigo, 1987; Rogowitz, 1998; Speakman and McQueenie, 1996; Speakman and Król, 2005, 2011; Valencak et al., 2013; Wu et al., 2009). Late lactation is a particularly useful phase in which to test these ideas because daily food intake in this phase of the life cycle is generally higher than in any other phase, and the products of this process (offspring) provide a tangible link to fitness. Experimental manipulations of female mice during late lactation to increase their energy demands, for example, artificially enlarging litter size (Hammond and Diamond, 1992; Johnson et al., 2001), prolonging lactation (Hammond et al., 1994) and forcing animals to run to obtain their food (Perrigo, 1987; Zhao et al., 2013a), have suggested that there is a limit on SusEI that the mice are unable, or choose not, to breach. However, other manipulations, in particular exposing mice in late lactation to cold conditions, have shown that mice are sometimes capable of breaching these limits (Hammond and Diamond, 1992; Hammond and Kristan, 2000; Johnson and Speakman, 2001). It has been suggested that these data are consistent with a peripheral limitation imposed by the milk production capacity of the mammary glands (Hammond et al., 1994) and that mice regulate their food intake to match this limit. Hence, when manipulations which require the female to elevate milk production are performed, food intake does not increase because the extra food intake cannot be converted into additional milk. However, when lactating animals are faced with an additional demand which does not require elevated milk production, the animals demonstrate their capacity to process additional food (Hammond and Diamond, 1994; Hammond and Kristan, 2000; Johnson and Speakman, 2001; Kenagy et al., 1989; Rogowitz, 1998).

This interpretation suggests that the energy exported as milk should be fixed during late lactation independent of ambient temperature, a prediction confirmed in hispid cotton rats (*Sigmodon hispidus*) (Rogowitz, 1998). In contrast, MF1 mice (*Mus musculus*) show elevated milk production in parallel with elevated food intake during cold exposure, suggesting that the mammary glands do not work at maximal capacity at 21°C and therefore cannot impose a

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List of symbols and abbreviations

| | |
|---------------------|--|
| AsFI | asymptotic food intake |
| AssEI | assimilated energy intake |
| d_e | apparent digestibility |
| d_{milk} | apparent digestibility of milk |
| DEE | daily energy expenditure |
| DMC _{food} | dry mass energy content food |
| DOL | day of lactation |
| GE _{food} | gross energy content of food |
| GE _{pups} | gross energy content of pups |
| LM | litter mass |
| LMI | litter mass increase |
| MEI | metabolisable energy intake |
| MEO | milk energy output |
| RM ANOVA | repeated measures analysis of variance |
| UEL | urinary energy loss |

peripheral limit on maximal SusEI (Johnson and Speakman, 2001). Moreover, shaving MF1 mice in late lactation enables mice to increase food intake and raise heavier litters (Krol et al., 2007). Both manipulations suggest that mice may be limited by their ability to dissipate heat [heat dissipation limitation (HDL) theory] and, when this limitation is lifted (by reducing ambient temperature or removing fur), females are able to increase their performance (Johnson and Speakman, 2001; Krol et al., 2007). Similar effects of temperature or shaving on lactation performance have been observed in common voles (*Microtus arvalis*), Brandt's voles (*Lasiopodomys brandtii*), bank voles (*Myodes glareolus*) and Mongolian gerbils (*Meriones unguiculatus*) (Sadowska et al., 2016; Simons et al., 2011; Wu et al., 2009; Yang et al., 2013). However, contradictory results have been found in lactating European hares (*Lepus europaeus*) (Valencak et al., 2010), and manipulations in striped hamsters (*Cricetulus barabensis*), Siberian hamsters (*Phodopus sungorus*) and Swiss mice resulted in increased food intake, but unchanged milk production and/or pup growth (Paul et al., 2010; Zhao et al., 2010; Zhao and Cao, 2009; Zhao, 2011). The HDL theory thus appears to play an important role in limiting lactation performance in several, but not all, small mammals. It may be that the limit that is reached first differs between species (e.g. HDL or peripheral limits), which may explain some of the observed discrepancies (see Speakman and Król, 2011; Wen et al., 2017).

An assumption in all of these previous studies is that in any particular situation – when the ambient temperature is changed or other manipulations are performed – the female maximises the amount of energy that she allocates to the litter she is raising. An alternative viewpoint has been called the ‘ecological context’ hypothesis (Speakman and Król, 2005). This hypothesis recognises that mice are a product of an evolutionary process and have evolved to maximise their lifetime fitness. Therefore, mice may not maximise their performance under all conditions because, in some circumstances, doing so may have a detrimental effect on their future reproductive performance or survival. In other words, we may be unable to fully understand the limits on SusEI outside the context of an evolved life history strategy that maximises lifetime fitness.

An important concept in life history theory (Stearns, 1989) is that there are trade-offs between life history components – notably current and future reproductive effort. Perhaps MF1 mice at 21°C could invest more in their litters (as indicated by their performance at 8°C), but they choose not to because normally, at this temperature in the wild, in a temperate seasonal environment, they would be

expecting to raise additional litters. Greater investment in the current event might jeopardise future reproductive output or survival. In contrast, a mouse at 8°C, coming into winter, might have little prospect of future reproductive events or survival and therefore might make a much greater investment in the current reproductive attempt. Ambient temperature may then be regarded as an environmental cue, signalling the appropriate level at which investment should be made. The inferred ‘limits’ in performance are then not physical, or physiologically imposed, but the result of the mother’s strategic decisions that take into account fitness consequences across more than single litters. In other words, the limits observed for mice raising their first litters may not be ‘hard’, unbreachable physiological limits, but a ‘soft’ limit imposed by the choices of the female.

In our previous studies using manipulated litter size of MF1 mice (e.g. Johnson et al., 2001), we found that when mice were given small litters to raise they ate significantly less food at peak lactation than females given larger litters. If trade-offs exist between current and future reproductive performance in these mice, we anticipate that allocation to a second litter, immediately following the first, may be contingent on their SusEI during the first litter. To test these ideas, we experimentally manipulated the litters of lactating mice, shortly after birth, to be either artificially large (16 pups) or small (5 pups), and re-mated the females 14 days after their litters weaned. In addition, we bred the offspring from the first litters to examine whether there were any detrimental effects on their reproductive success. We examined the data for evidence of trade-offs between the investment during the first lactation and that during the second lactation, and trade-offs on the reproductive success of offspring.

MATERIALS AND METHODS**Animals and housing**

Virgin female mice ($n=18$, *Mus musculus* Linnaeus 1758; outbred strain MF1), aged 9–10 weeks and bred from in-house stock, were housed individually in shoebox cages with sawdust as bedding. They were provided with rodent chow [CRM (P), 17.35 kJ g⁻¹ dry mass, Special Diet Services, BP Nutrition, UK] and water *ad libitum*, and placed under a 12 h:12 h light:dark photoperiod at 21±1°C for 7 days to acclimate to their new environment.

The female mice were individually paired with males of the same age. Mating pairs had access to *ad libitum* rodent chow and water. All mating pairs were left undisturbed for 11 days (Król et al., 2007), after which the males were removed, and then female body mass and food intake were measured daily (to ±0.01 g; Top-pan balance, Sartorius, Göttingen, Germany) until parturition (day 0 of lactation). Newborn pups and mothers were left undisturbed on the day of parturition. From day 1 of lactation until weaning on day 18 of lactation, maternal body mass, food intake, litter mass and litter size were measured daily. On day 1, pups were removed from their mothers, and mixed with pups from other litters born on the same day (±1 day). Females were randomly allocated five or 16 pups (reduced versus enlarged litters, $n=9$ females in each group).

Following weaning of pups after the first lactation, female mice ($n=18$) were left undisturbed for 2 weeks except for daily measurement of body mass and food intake. After 14 days, the female mice were paired with males (10–12 weeks old) supplied by Harlan UK Ltd (second lactation). During the second lactation, litter size was not manipulated, and mothers suckled pups in their natural litters until they were weaned on day 18 of lactation. As in the first lactation, body mass, food intake, litter mass and litter size were measured daily from day 1 to 18 of lactation. All procedures concerning animal care and treatment were approved by the Life

Sciences and Medicine Ethics Review Board (CERB) ethics committee for the use of experimental animals of the University of Aberdeen, and licensed by the UK Home Office (project licence: PPL 60/3606).

Offspring of first reproductive event

Randomly selected female offspring from the first lactation event ($n=16$ from reduced litters and $n=16$ from enlarged litters) were housed individually (2–4 weeks of age) and maintained under the same conditions as described above for their mothers.

At 10–12 weeks of age, the females were randomly paired with male mice reared under the same conditions (i.e. in enlarged or reduced litters). Body mass, food intake, litter mass and litter size were measured throughout lactation as described above. Mice were injected with doubly labelled water (DLW) on day 14 of lactation to determine their daily energy expenditure (DEE), and faeces were collected and manually sorted over days 12–18 of lactation to enable an estimation of MEO (see full details below).

Milk energy output

MEO (kJ day^{-1}) was estimated for all lactation events from the energy budget of the litter between day 14 and 15 of lactation, using the method described in Krol and Speakman (2003b, appendix A) and Zhao et al. (2013b). In short, pups depend entirely on milk for their energy intake, and their total energy requirement is the sum of the energy allocated to respiration (DEE, kJ day^{-1}) and the energy accumulated as new tissue (growth). DEE was predicted from litter mass on day 14 (LM, g) using the relationship established previously in MF1 mice for 23 litters { $\text{DEE}=[(7.28 + 0.71 \times \text{LM}) \times 2.2]$; Krol and Speakman}. MEO was then calculated using the following formula:

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

where LMI (g day^{-1}) is the litter mass increase between day 14 and 15 of lactation, GE_{pups} (8.95 kJ g^{-1} wet mass; Krol and Speakman, 2003b) is the gross energy content of pups and d_{milk} is the apparent digestibility of milk ($d_{\text{milk}}=96\%$; Krol and Speakman, 2003b).

In offspring of the first lactation event, MEO was also estimated using the doubly labelled water technique (Lifson, 1966; Speakman, 1997). MEO was estimated from the difference between the estimated DEE by DLW and metabolisable energy intake (MEI; Krol and Speakman, 2003b). On day 14 of lactation, lactating females were weighed and immediately injected intraperitoneally with approximately 0.1 g of water containing enriched deuterium (^2H ; 18.79 atom%) and ^{18}O (30.63 atom%). Syringes were weighed (accurate to 0.0001 g, Analytical Plus Balance, Ohaus) immediately before and after injections to calculate the mass of DLW injected. Injections were administered within 10 min of each other, and 1 h was allowed for isotopes to reach equilibrium (Speakman, 1997) to estimate initial isotope enrichments. During this period, the mice were returned to the housing conditions in their respective cages and had free access to food and water. After the isotopes had reached equilibrium, initial blood samples were collected by tail tipping into glass capillaries (50 μl , Vitrex Camlab Ltd) and immediately flame sealed. The mice were returned to their cages and allowed free access to food and water for 48 h. Final blood samples (40–50 μl) were collected (by tail tipping) after the 48 h period, treated in the same manner as the initial samples and stored in room temperature pending analysis. Capillaries containing the blood samples were vacuum distilled and water from the resulting distillate was used to produce CO_2 and H_2 . The isotope ratios $^{18}\text{O}:^{16}\text{O}$ and $^2\text{H}:^1\text{H}$ were analysed

using gas-source isotope ratio mass spectrometry (ISOCHROM- μG , UK). High enrichment standards were run alongside the samples and the raw data were corrected to the standards to reduce the problem of inlet cross contamination. Dilution space (total body water) was estimated, and ^2H and ^{18}O turnover rates (k_d and k_o , respectively) were estimated from the gradients of their exponential declines. From these estimates, the flux rates of ^2H and ^{18}O were calculated. The isotope elimination rate (k) was calculated, following published methods (Lifson, 1966). The single-pool model equation was used to calculate the rate of CO_2 production, and the energy equivalent of the rate of CO_2 production was calculated using a conversion factor of $24.026 \text{ J ml}^{-1} \text{ CO}_2$, derived from the Weir equation (Weir, 1949) for a respiratory quotient of 0.85 (Speakman, 1997).

MEI (kJ day^{-1}) at peak lactation was estimated from individual measurements of asymptotic food intake (FI), i.e. mean food intake for days 12–18 of lactation (g day^{-1}), and mean values for dry mass content of food ($\text{DMC}_{\text{food}}=94.4\%$), gross energy content of food ($\text{GE}_{\text{food}}=17.35 \text{ kJ g}^{-1}$ dry mass) (as determined by bomb calorimetry) (Parr 6200, Scientific and Medical Products Ltd, UK), and apparent digestibility of energy on days 12–18 of lactation ($d_e=83.0\%$) (Krol and Speakman, 2003b). To determine apparent digestibility, faeces were collected and manually sorted over days 12–18 of lactation and the energy contained therein was determined using bomb calorimetry (see Krol for a full description of the procedure). We assumed that urinary energy loss (UEL) was 3% of the digestible energy intake. MEO (kJ day^{-1}) was calculated from the difference between MEI and DEE of each individual, according to:

$$\text{MEO} = [\text{FI} \times \text{DMC}_{\text{food}}/100 \times \text{GE}_{\text{food}} \times d_e/100 \times (100 - \text{UEL})/100] - \text{DEE}. \quad (2)$$

Statistics

Effects of day of lactation (DOL) and manipulation of body mass, food intake, pup mass and litter size for first and second litters separately were analysed using repeated measures general linear models (RM GLM) in SPSS version 24.0 (IBM, Armonk, NY, USA). In these models, DOL was entered as a repeated factor and litter size manipulation (reduced versus enlarged) was entered as a fixed factor. Where significant effects were found, *post hoc t*-tests were performed. Independent *t*-tests and paired *t*-tests were used to compare estimates of MEO between groups and first and second lactations. Pearson correlations were performed to determine associations between traits. All data were tested for normality using the Kolmogorov–Smirnov test in SPSS (version 18), and *post hoc* power analysis using Minitab 18 was performed to ascertain whether we had sufficient power (0.8) to observe significant differences with the effect sizes used. All tests were two-tailed and significance was set at $P<0.05$.

RESULTS

First lactation

The litter size at birth resulting from the first mating, prior to the litter size manipulation, was slightly lower in females in the enlarged group (10.1 \pm 3.8 pups) than that in females in the reduced group (13.6 \pm 1.6 pups) (*t*-test, $P=0.022$; Table 1). After manipulation, the reduced litter mice had an average of 5 pups per litter ($n=9$) whereas the enlarged litter group had 16 pups per litter ($n=9$). The females in the reduced group weaned 5.0 \pm 0.0 pups on average, but those in the enlarged group weaned on average 13.8 \pm 1.7 pups (first litter; Table 1, Fig. 1A). Pup mortality during lactation was significantly higher in

Table 1. Litter size, food intake, body mass, litter mass and pup mass in first and second litters of females raising reduced or enlarged litters

| | First lactation | | Second lactation | |
|---|-----------------|----------------|------------------|----------------|
| | Reduced (N=9) | Enlarged (N=9) | Reduced (N=7) | Enlarged (N=8) |
| Litter size at birth (pups) | 13.6±1.6 | 10.1±3.8* | 13.7±2.5 | 12.4±3.2 |
| Litter size on day 1 (pups) | 5±0 | 16.0±0.0* | 13.7±2.7 | 12.0±3.4 |
| Litter size on day 18 (pups) | 5±0 | 13.8±1.7* | 12.8±2.6 | 10.1±3.0 |
| Asymptotic food intake (g day ⁻¹) | 21.9±1.2 | 24.7±2.3* | 29.3±4.0 | 23.9±4.3 |
| Body mass on day 1 (g) | 40.3±2.8 | 38.4±3.5 | 45.7±3.7 | 41.2±2.6* |
| Body mass on day 18 (g) | 42.6±2.9 | 43.4±2.6 | 51.5±3.1 | 46.7±4.6 |
| Litter mass on day 1 (g) | 9.8±0.4 | 32.0±1.5* | 28.3±4.0 | 23.5±5.1 |
| Litter mass on day 18 (g) | 67.2±5.2 | 92.9±8.5* | 104.3±11.5 | 87.7±12.2* |
| Pup mass on day 1 (g) | 2.0±0.1 | 2.0±0.1 | 2.1±0.3 | 2.0±0.2 |
| Pup mass on day 18 (g) | 13.4±1.0 | 6.8±0.8* | 8.4±1.8 | 9.1±2.1 |

Means and s.d. of variables measured in female mice during a first lactation where litter size was experimentally reduced to 5 pups or enlarged to 16 pups, and during a successive (second) unmanipulated lactation. Day of lactation at which measurements were taken is indicated for each variable. Asymptotic food intake was calculated as the mean food intake over days 14–16 of lactation. Repeated measures general linear models (RM GLM) were performed for all variables for first and second lactation separately. Day of lactation, manipulation, and the interaction between day of lactation and manipulation were added to the models as fixed factors. Where significant effects were found, *post hoc* independent *t*-tests were performed. Asterisks indicate a significant difference between females with enlarged versus reduced litters (*post hoc t*-test).

the mice that were given large litters to raise (average loss 2.2±1.7 pups, range 0–4) than in those given reduced litters, which lost none. There was no significant relationship between the number of pups added to the litters and the number of pups that were lost ($R^2=0.01$, $P>0.87$). In the enlarged group, the losses were distributed throughout the whole period of lactation.

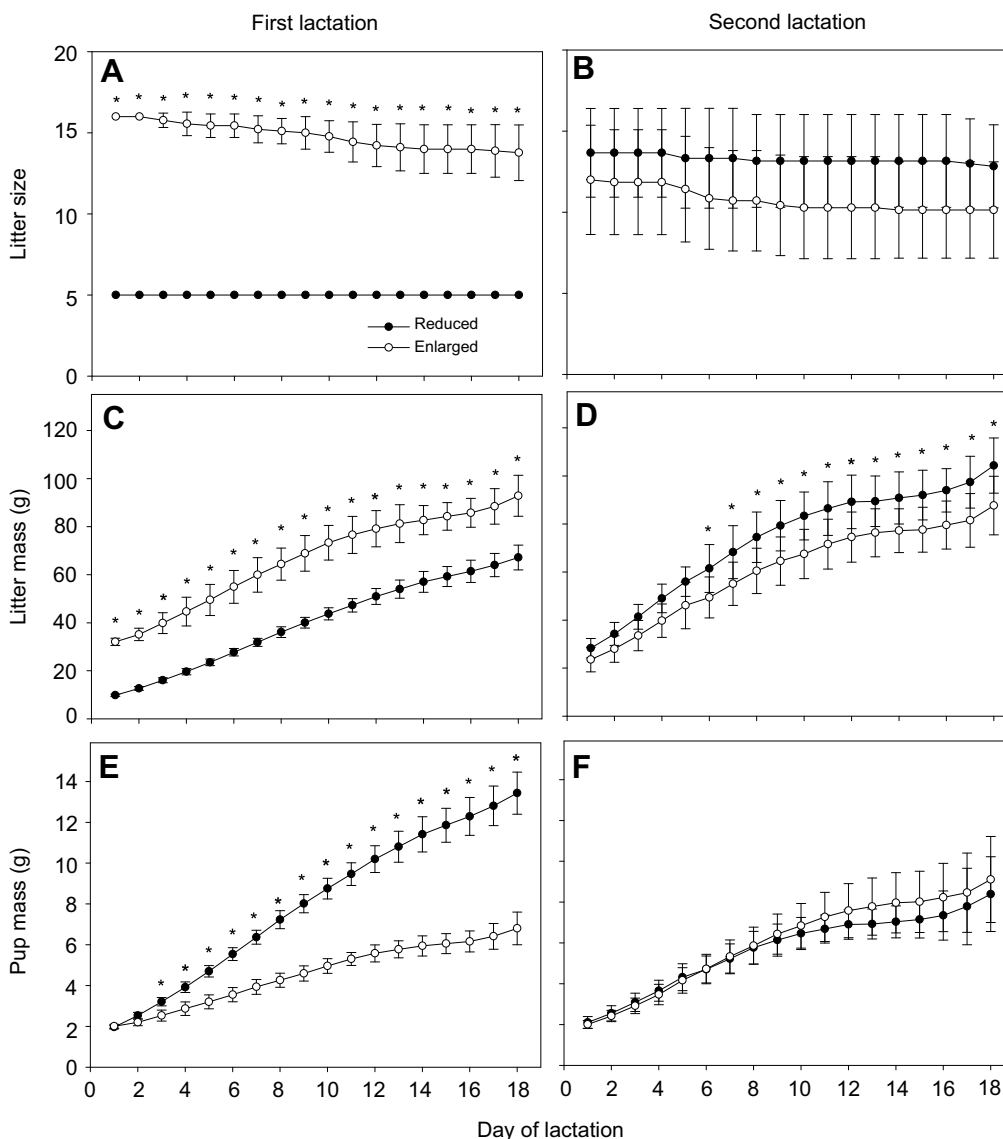


Fig. 1. Mean ± s.d. litter size, litter mass and pup mass during lactation. (A,C,E) First lactation: females raised artificially reduced (litter size=5, $n=9$) or enlarged litters (litter size=16, $n=9$). (B,D,F) Second lactation: females reared natural (unmanipulated) litter sizes (reduced $n=7$, enlarged $n=8$). Repeated measures general linear models (RM GLM) were performed for all variables to investigate the effect of day of lactation and manipulation (reduced versus enlarged). Where significant effects were found, *post hoc t*-tests were performed. Significant differences between groups are indicated with an asterisk (*post hoc t*-test, $P<0.05$).

Litter mass was significantly higher in enlarged litters than in reduced litters throughout lactation (RM GLM: day of lactation, $F_{17,272}=849.1$, $P<0.001$; manipulation: $F_{1,16}=169.4$, $P<0.001$; manipulation \times day: $F_{17,272}=2.8$, $P<0.001$; Table 1, Fig. 1C). On day 1, pup mass (pup mass=litter mass/litter size) was not significantly different for reduced and enlarged litters (t -test, $P>0.05$; Table 1) and, in both groups, pup mass increased throughout lactation (GLM: day of lactation, $F_{17,272}=1118.1$, $P<0.001$; Fig. 1E); however, pup mass increased faster and was significantly higher at the end of lactation in the reduced litters (GLM: manipulation, $F_{1,16}=320.5$, $P<0.001$; manipulation \times day: $F_{17,272}=193.4$, $P<0.001$; Table 1, Fig. 1E).

Before breeding, female mice had a mean body mass of 29.5 ± 2.1 g and there were no significant differences in body mass between mothers given reduced or enlarged litters (29.9 ± 2.1 and 29.0 ± 2.1 g, respectively). The average body mass of females was significantly affected by DOL (GLM: $F_{17,272}=35.4$, $P<0.001$; Table 1, Fig. 2A), but not by manipulation (GLM: $F_{1,16}=1.3$, $P=0.28$); however, there was a significant interaction between DOL and manipulation (GLM: $F_{17,272}=9.5$, $P<0.001$). Body mass increased with DOL, but more so in females raising enlarged litters than in females raising reduced litters.

Food intake during the first lactation increased linearly between day 1 and day 14 of lactation, when it reached an asymptote (Fig. 2B). The amount of food eaten was significantly affected by the DOL (GLM: $F_{16,224}=28.25$, $P<0.001$) and the manipulation (GLM: $F_{1,14}=49.59$, $P<0.001$), and there was a significant interaction between DOL and manipulation (GLM: $F_{16,224}=4.82$, $P<0.001$). From day 17 onwards, the pups started to nibble at the

food and, consequently, the FI recorded from the food missing from the hopper was slightly higher than that eaten by the female. Asymptotic food intake (AsFI) was calculated as the mean food intake during days 14–16 of lactation, when food intake reached an asymptote (Table 1, Fig. 2C). MEO was calculated using the energy budget of the litter (see Materials and methods, Fig. 3). Females raising enlarged litters had an increased AsFI (+13%; Table 1) and MEO (approximately +27%) compared with those of females raising reduced litters (first lactation; independent t -tests; $P=0.001$; Fig. 4A).

Second lactation

Mean litter size in the second litters did not differ significantly between females that had previously raised reduced litters and those that had raised enlarged litters (second litter, GLM: DOL, $F_{17,187}=4.8$, $P<0.001$; manipulation: $F_{1,13}=2.4$, $P=0.15$; interaction: $F_{17,187}=1.3$, $P=0.18$; Table 1, Fig. 1B). Loss of pups was 6.1% (0.8 ± 1.0 pups) on average in mothers that had previously raised reduced litters and 14.9% (1.9 ± 2.3 pups) in those that had raised enlarged litters (second litter, t -test: $P>0.05$; Table 1). In both the reduced and enlarged groups, most of the losses occurred during the first 10 days of lactation.

Litter mass showed a similar increase with DOL in both groups (GLM: DOL, $F_{17,187}=215.0$, $P<0.001$; interaction between DOL and manipulation: $F_{17,187}=1.4$, $P=0.14$; Table 1, Fig. 1D), but was significantly higher in females that had reared reduced litters in their first lactation than in those that had reared enlarged litters ($F_{1,11}=8.3$, $P=0.015$; Fig. 1D). Pup mass, however, did not differ significantly between the groups at any point during lactation

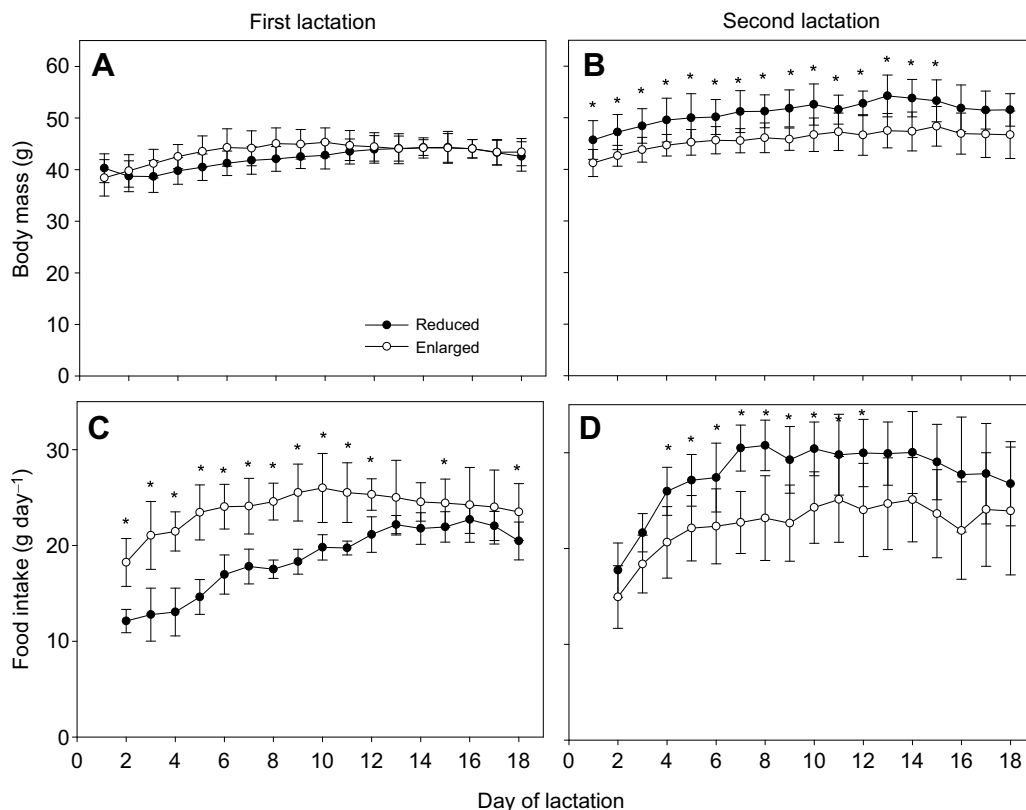


Fig. 2. Mean \pm s.d. body mass and food intake of female mice during lactation. (A,C) First lactation: females raised artificially reduced (litter size=5, $n=9$) or enlarged litters (litter size=16, $n=9$). (B,D) Second lactation: females reared natural litter sizes (reduced $n=7$, enlarged $n=8$). RM GLM were performed for all variables to investigate the effect of day of lactation and manipulation. Where significant effects were found, *post hoc t*-tests were performed. Significant differences between groups are indicated with an asterisk (*post hoc t*-test, $P<0.05$).

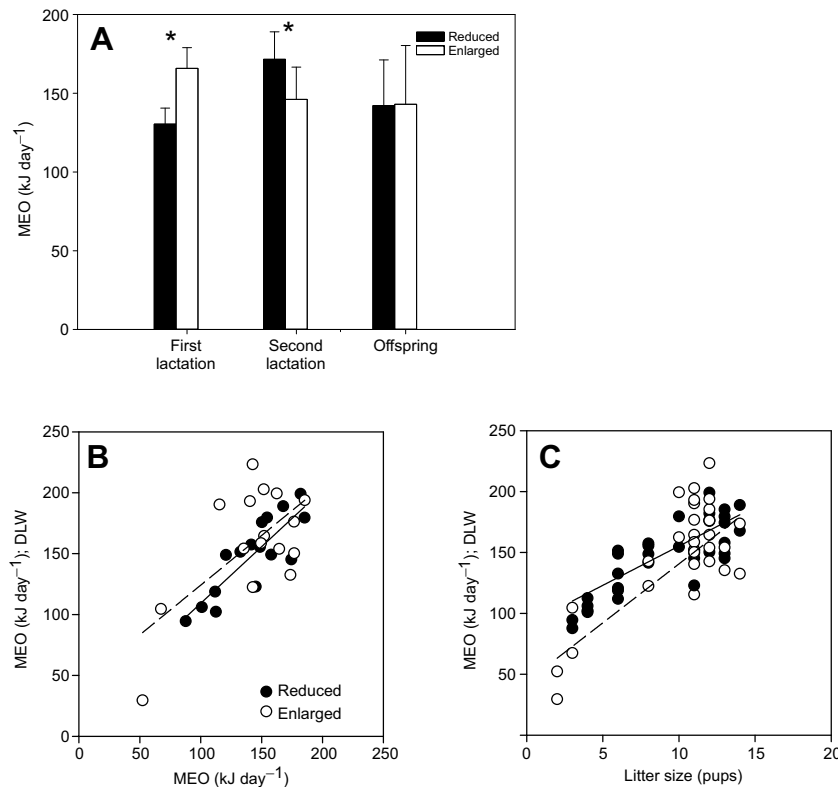


Fig. 3. Milk energy output (MEO) during first and second lactation and in offspring. (A) MEO of females raising reduced (litter size=5, $n=9$) and enlarged (litter size=16, $n=9$) litters (first lactation), MEO of the same females during a second lactation with no litter size manipulation ($n=7$ and $n=8$ for reduced and enlarged, respectively) and MEO of offspring from the first lactation rearing their own natural litters ($n=16$). Asterisks indicate a significant difference between enlarged and reduced groups (independent sample t -test, $P<0.05$). (B) Correlation between MEO measured using the energy budget method and that measured using the doubly labelled method. (C) Correlation between MEO and litter size in females (that previously raised enlarged or reduced litters) during the second lactation.

(GLM: DOL, $F_{17,187}=153.8$, $P<0.001$; manipulation: $F_{1,11}=0.5$, $P=0.50$; interaction: $F_{17,187}=1.6$, $P=0.065$; Fig. 1F).

As in the first lactation, an increase in body mass was observed during the second lactation (GLM: DOL, $F_{17,187}=16.5$, $P<0.001$), and body mass was approximately 10% lower in females that had previously raised enlarged litters throughout lactation (GLM: manipulation, $F_{1,11}=9.6$, $P=0.010$; second litter; Table 1, Fig. 2B).

There was a similar pattern of food intake as a function of the DOL as during the first lactation (GLM: DOL, $F_{16,176}=14.0$, $P<0.001$; Fig. 2D). However, food intake was significantly lower – by 17% on average between days 3 and 15 of lactation – in mice that had previously raised enlarged litters than in those that had raised reduced litters (manipulation, $F_{1,11}=9.5$, $P=0.011$, second litters and *post hoc t*-tests; Table 1, Fig. 2D).

During the second lactation, where no manipulation was done, both AsFI and MEO were significantly decreased, by about 17%, in females that had previously raised enlarged litters compared with those in females that had raised reduced litters (second lactation; independent t -test; $P=0.046$ for AsFI and $P=0.036$ for MEO; Table 1, Fig. 3A). When comparing changes in MEO and AsFI between the first and second lactation within groups, females with reduced litters had significantly increased MEO and AsFI during their second lactation than during their first lactation (paired t -test: $P=0.002$ for MEO and $P=0.010$ for AsFI), whereas females with enlarged litters had significantly reduced MEO in their second lactation compared with their first lactation (paired t -test, $P=0.029$) but AsFI did not change ($P=0.46$).

Combining data for both groups, there was a significant positive relationship between AsFI and MEO in the first ($r=0.54$, $P=0.021$) and second ($r=0.74$, $P=0.004$) lactation. However, MEO during the first lactation was not related to MEO during the second lactation ($r=-0.5$, $P=0.1$) and the same was true for AsFI during the first and second lactation ($r=0.02$, $P=0.94$). A significant correlation

between litter size and MEO was found during the second lactation ($r=0.69$, $P=0.01$).

Lactation in offspring from first lactation

Body mass in the offspring (male and female) of females raising reduced litters was significantly increased at weaning compared with that in the offspring of females raising enlarged litters (Fig. 2C, Table 1). This difference persisted into adulthood and throughout their first lactation (RM GLM: DOL, $F_{17,510}=43.0$, $P<0.001$; manipulation: $F_{1,30}=347.0$, $P=0.009$; Table 2, Fig. 4A). Offspring of reduced and enlarged litters gave birth to a similar number of pups (GLM: manipulation, $F_{1,30}=243.9$, $P=0.29$; Table 2) and experienced similar pup losses, at approximately 5%. Litter mass increased steadily during lactation and did not differ significantly between groups (GLM: DOL, $F_{17,510}=414.6$, $P<0.001$; manipulation: $F_{1,30}=0.31$, $P=0.53$; interaction: $F_{7,510}=0.2$, $P=0.99$; Fig. 1D). Pup mass was slightly increased in offspring from reduced litters from day 10 of lactation onwards (GLM: DOL, $F_{17,510}=361.0$, $P<0.001$; manipulation: $F_{1,30}=4.76$, $P=0.037$; interaction: $F_{17,510}=243.9$, $P<0.001$ with *post hoc t*-tests; Fig. 1E). Food intake did not differ significantly between the groups (GLM: manipulation, $F_{1,30}=1.2$, $P=0.29$; Fig. 4B).

AsFI and MEO (estimated using the energy budget method) were similar in offspring raised in reduced and enlarged litters during their first lactation event (offspring; independent t -test: $P=0.92$; Table 2, Fig. 3A). For the offspring, MEO was also calculated using the DLW technique. There was a significant correlation between the estimates derived using both methods ($r=0.71$, $P<0.001$; Fig. 3B) and no significant difference between females derived from enlarged and reduced groups (MEO= 148.4 ± 29.1 and 143.0 ± 37.3 kJ d⁻¹ in reduced and enlarged, respectively; independent t -test: $P=0.32$). A significant correlation between litter size and MEO was observed in both groups ($r=0.72$, $P<0.001$; Fig. 3C).

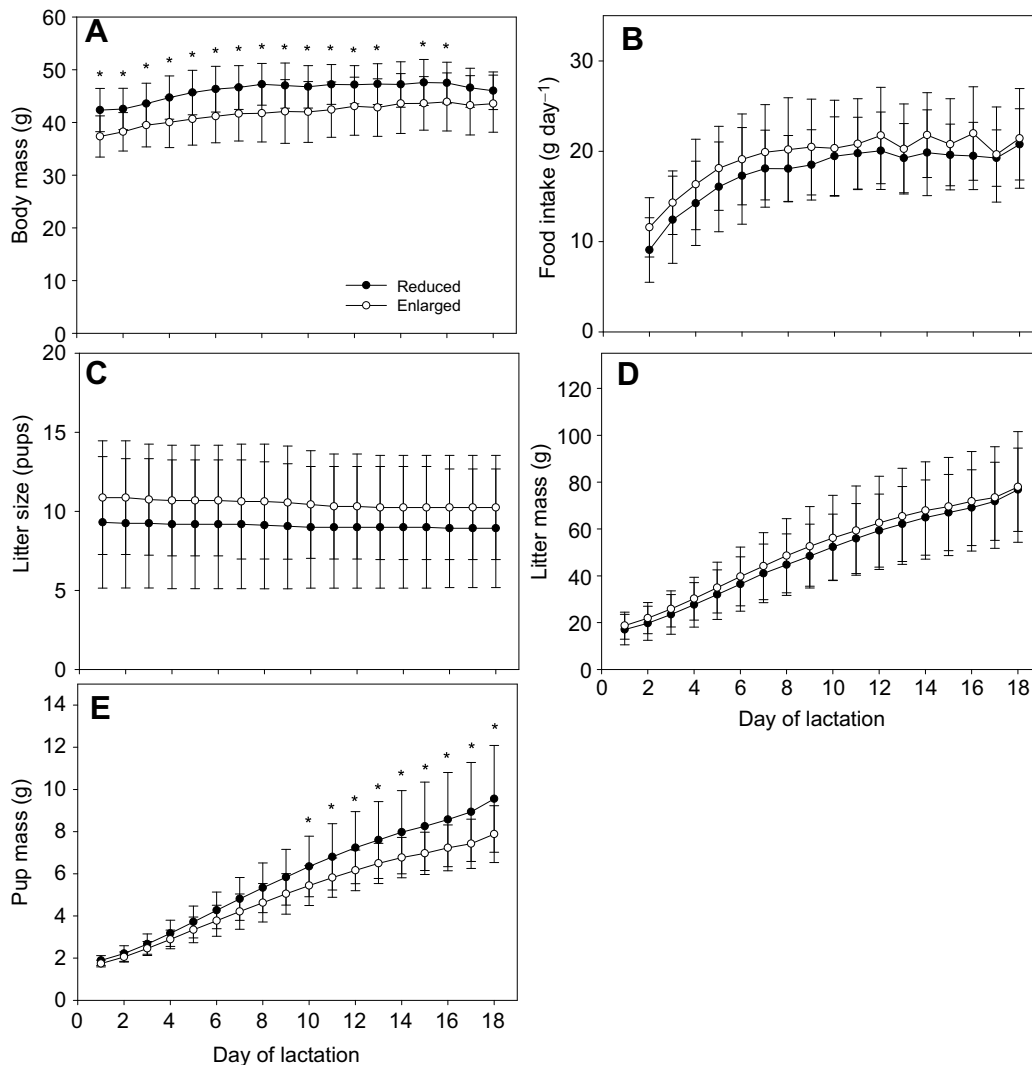


Fig. 4. Reproductive parameters measured during the first lactation of female offspring from reduced or enlarged litters. Values shown are mean \pm s.d for body mass (A), food intake (B), litter size (C), litter mass (D) and pup mass (E) on day 0–18 of lactation. RM GLM were performed for all variables to investigate the effect of day of lactation and manipulation (reduced versus enlarged, $n=16$ per group). Where significant effects were found, *post hoc t*-tests were performed. Significant differences between groups are indicated with an asterisk (*post hoc t*-test, $P<0.05$).

DISCUSSION

Females that received a greater energetic burden during their first reproductive event had lower AsFI and reduced milk production during their second reproductive event compared with those of females that experienced a smaller energetic burden during their first litter. The lower milk production led to a lower total litter mass weaned. These results indicate that females experience a trade-off between the first and second reproductive efforts. This contrasts with several previous studies which have suggested that increased nursing costs of larger litters only affect the success of current reproduction (Hare and Murie, 1992; Kenagy et al., 1990; Mappes et al., 1995; Neuhaus, 2000; Oksanen et al., 2001). Instead, the results of the present study are consistent with those of other studies showing a trade-off (Koivula et al., 2003; Lehto Hurlimann et al., 2014)

The immediate cost of experimentally elevated litter size was the small size of pups at the time of weaning. For *M. musculus*, the suggested minimal physiological weaning mass, at which pups reach a state of independence and are able to survive on their own, is around 9 g (König and Markl, 1987). The mean weaning mass

of the pups from the enlarged litters was lower than this (i.e. 6.8 ± 0.8 g), which may have corresponded to a future fitness cost if they were in the wild. During the first lactation, the pups in the enlarged litters were smaller because they had to share the milk produced by the mother with a larger number of siblings. Females with enlarged litters had higher food intake and produced more milk (MEO was increased by approximately 27% compared with that of females with reduced litters), but this was insufficient to match their pups' energy demands; i.e. litter size at weaning was 276% larger but total litter mass was only 38% greater, resulting in significantly smaller pups in enlarged litters than in reduced ones. Other studies in rodents have shown similar effects of enlarged litters (Lehto Hurlimann et al., 2014; Mappes et al., 1995; Oksanen et al., 2001; Rogowitz, 1998; Speakman et al., 2001). For instance, in common voles, *Microtus arvalis*, females with an enlarged litter showed increased reproductive effort (i.e. higher metabolic rate and alternative body mass dynamics), but weaned smaller pups (Lehto Hurlimann et al., 2014). In contrast, when bank voles, *Myodes (=Clethrionomys) glareolus*, were supplemented with extra food, enlarging litters had no effect on the weaning size of pups (Koskela

Table 2. Litter size, food intake, body mass, litter mass and pup mass in offspring previously raised in reduced or enlarged litters

| | Reduced (n=16) | Enlarged (n=16) |
|---|-------------------|--------------------|
| Litter size at birth (pups) | 9.3±4.2 | 10.9±3.6 |
| Litter size on day 18 (pups) | 8.9±3.8 | 10.3±3.3 |
| Asymptotic food intake (g day ⁻¹) | 19.6±4.0 | 21.5±5.0 |
| Body mass on day 1 (g) | 42.4±4.1 | 37.4±3.9 |
| Body mass on day 18 (g) | 46.0±3.6 | 43.6±5.4 |
| Litter mass on day 1 (g) | 17.0±6.5 | 18.7±5.8 |
| Litter mass on day 18 (g) | 76.8±17.8 | 78.0±23.6 |
| Pup mass on day 1 (g) | 1.9±0.2 | 1.7±0.2 |
| Pup mass on day 18 (g) | 9.6±2.5 | 7.9±1.4* |

Means and s.d. of variables measured during a first lactation of offspring raised in experimentally reduced or enlarged litters. Day of lactation at which measurements were taken is indicated for each variable. Asymptotic food intake was calculated as the mean food intake during days 14–16 of lactation. RM GLM were performed for all variables. Day of lactation, manipulation, and the interaction between day of lactation and manipulation were added to the models as fixed factors. Where significant effects were found, *post hoc* independent *t*-tests were performed. The asterisk indicates a significant difference between females with enlarged versus reduced litters (*post hoc t*-tests).

et al., 1998). Similarly, in grasshopper mice, *Onychomys leucogaster*, naturally larger litters (i.e. unmanipulated) did not lead to smaller pups at the time of weaning (Sikes, 1995).

Mothers can naturally adjust the size of their litters before and after birth, so as to maximise the number of high quality young at weaning. Because the adjustments are potentially matched to each individual mother's abilities and resources, the same negative relationships between litter size and pup weight found in experimentally altered litters are commonly not present in unmanipulated litters (Mendi, 1988). This may suggest that mothers tailor their litter size in pregnancy to match their capabilities in lactation. However, previous studies have indicated that energy allocated to litters during lactation is independent of pregnancy litter size, but depends only on the litter size during lactation (Duah et al., 2013). One way in which mothers can adjust the size of their litters after birth is through infanticide (Mendi, 1988; Sikes, 1995). Infanticide provides the mother with the chance to reverse any mistake she made by producing a litter that is too large for her to feed (Sikes, 1995). Often, mothers faced with extreme energetic demands – rather than working harder, and being adversely affected themselves – either give up on reproduction completely (Bronson, 1989; Murie and Dobson, 1987) or cull pups in order to reduce the energetic effort (Johnson and Speakman, 2001). In the present study, the greatest energetic burden fell on the mothers of enlarged litters, who responded with a higher rate of culling than the mothers of reduced litters (14% culling rate compared with 0% in reduced litters). It has also been suggested that the excessive infanticide by mothers with artificially augmented litters could have resulted from the extreme disturbance caused by the pups to the mother (Mendi, 1988). This could explain why, during their next reproductive attempt, the females with previously enlarged litters culled more pups than did those with previously reduced litters (6% versus 14% culling), even though both groups had a similar litter size. Also, females with previously enlarged litters had lower food intake and MEO than those of females with previously reduced litters. However, pup mass and litter size at weaning were not affected significantly and did not differ significantly between the females of either group.

In contrast with our study, observations by others seem to suggest that varying maternal workloads at peak lactation, attributable to differences in litter size, are unlikely to form the basis for a trade-off with future fecundity (Hare and Murie, 1992; Kenagy et al., 1990; Koskela et al., 1998; Mappes et al., 1995; Neuhaus, 2000; Oksanen et al., 2001). In Cascade golden-mantled ground squirrels, *Callospermophilus saturatus*, litter size is mainly determined by variations in the mother's behaviour and the quality of the habitat (Kenagy et al., 1990). Similarly, female bank voles that were supplemented with food, increasing the quality of the habitat, suffered no ill effects from raising large litters on subsequent reproductive success (Koskela et al., 1998). The number of young a mother has might be adjusted to body condition, which in itself reflects environmental conditions (Murie and Dobson, 1987).

Nevertheless, there are many previous studies that have suggested trade-offs between litters. For instance, a 3 year field experiment in bank voles, *C. glareolus*, revealed that litter enlargements reduced survival and fecundity of mothers (Koivula et al., 2003), and a laboratory experiment with wild-derived common voles, *Microtus arvalis*, showed that females with previously enlarged litters reared smaller pups in a second attempt (Lehto Hurlimann et al., 2014). In the present study, mothers that raised enlarged litters in their first lactation were not able to match the level of allocation to milk production in the second attempt, i.e. MEO and food intake were 17% lower than those of mothers that had previously raised reduced litters, and MEO was 14% lower than that of the same group during their first attempt. Despite this reduction in MEO, litter size and pup mass in the second lactation did not differ significantly between females that had raised reduced and enlarged litters. These data are confusing and one must wonder why there was no impact of the reduced milk production on the trajectories of pup growth (Fig. 1F). If anything, the pups from mothers that had previously reared enlarged litters (and that had reduced food intake and MEO) grew slightly faster than the pups from mothers that had reared reduced litters ($P=0.065$; Fig. 1F). A previous study in MF1 mice had found similar results, where an increase in MEO by 25% did not result in an effect on pup growth (Valencak et al., 2013). The lack of an effect of MEO on pup growth might suggest that there were differences in the efficiency of converting milk into growth between the pups. Pups could differ in the type of tissue that they laid down (e.g. fat versus protein) or in their physical activity (Valencak et al., 2013), but without data on body composition and activity levels of pups it is not possible to determine whether there were differences in pup growth efficiencies in the current study. In agreement with the MEO data, however, total litter mass at weaning was significantly decreased (by approximately 16%) in mothers previously rearing enlarged litters and, even though the difference was not significant, litter size was decreased by 21%. Over both reproductive events, mothers of enlarged litters reared 23.9 pups compared with the 17.8 pups that were reared by mothers of reduced litters, which – assuming similar pup survival – would give females raising enlarged litters a greater fitness.

The main detrimental effects of the litter size manipulation on the future reproductive effort of the females were in the form of increased infanticide and reduced MEO. An additional impact may be experienced by their offspring. We found that the offspring raised in reduced and enlarged litters differed significantly in their body mass, and poor early nutrition has been previously linked to impairment of performance and reduced survival in several species (Gibson et al., 2015; Plumel et al., 2014). In line with these previous suggestions, we found that pup mass at weaning was reduced in females from enlarged litters compared with females from reduced

litters, indicating that the similar litter sizes (9.3 ± 4.2 and 10.9 ± 3.6 pups in females from reduced and enlarged litters, respectively) typical of the MF1 strain (Johnson et al., 2001) were comparable in mass at birth, but not at weaning. Investment in their litters was similar, as indicated by their identical MEO, and mortality rates in the litter were comparable, resulting in females successfully weaning a similar number of pups (8.9 ± 3.9 versus 10.3 ± 3.3).

Females that had been reared and weaned from litters of only five pups, and therefore were well nourished, were significantly (approximately 19%) heavier than the females raised in litters of 16 pups, and remained heavier at the end of lactation by approximately 8%. From the perspective of energetics, the larger females may have had superior energy stores than those of their smaller counterparts, which could have benefited them during the entire reproductive bout or particularly during lactation, when energy availability and its intake are extremely important (Speakman et al., 2001; Speakman and Król, 2011). Drent and Daan (1980) emphasised the importance of energy stores or body condition on breeding success in birds, and hypothesised that energy stores at the onset of reproduction impact the rate of energy expenditure during reproduction. Also, in species in which body reserves or energy stores provide only a small portion of reproductive expenditure, reserves may be essential to supplement physiological limitations on the rate of intake at peak energy demands or to buffer short-term reductions in food availability (Heldstab et al., 2017). However, because reproduction in the MF1 mouse is primarily characterised by increases in food or energy intake (Krol and Speakman, 2003a,b; Speakman and McQueenie, 1996) and stable body condition or energy balance (Johnson et al., 2001; Krol and Speakman, 2003a), it does not appear that the mice drew on these larger energy stores to fuel reproduction. Within each group, body mass was constant during peak lactation, which suggests stable body condition or energy balance. Therefore, in the present investigation, it seems that energy stores did not play a role in the reproduction processes. The outcome of the present study contrasts with a study in prairie voles, *Microtus ochrogaster*, that showed no significant difference in the reproductive success or survival between large and small laboratory female prairie voles, and a study in Columbian ground squirrels, *Urocitellus columbianus*, where total litter loss, survival of adult females and the probability of weaning young the following year were not affected by the litter size manipulation (Neuhaus, 2000). A recent study using a phylogenetic comparative approach on a sample of 87 mammalian species has indicated that annual variation in body mass is reduced in species with allomaternal care (Heldstab et al., 2017).

In the current investigation, the persistence of a maternal effect on body mass into adulthood indicated that the small females did not show compensatory growth. Similar results have been found in *Peromyscus maniculatus*, but opposing results have also been found (Sikes, 1996, 1998) in grasshopper mice (*Onychomys leucogaster*) and eastern wood rats (*Neotoma floridana*). Body size at weaning has a strong influence on life expectancy (Skibieli et al., 2013) and, although no trade-off was found of the smaller size of females weaned from enlarged litters on their first reproductive event, longer-term fitness consequences of the litter size manipulations may exist (Lehto Hurlimann et al., 2014).

During studies of the limits on female performance, it is often assumed that, in any particular situation, the female maximises her investment in the litter that she is raising (Hammond and Diamond, 1992; Johnson et al., 2001). However, in the current study, females raising reduced litters showed a significant increase in both MEO and AsFI when rearing a successive litter, whereas females that had

previously reared enlarged litters were not capable of matching their MEO in the second lactation. In addition, despite a similar litter size at birth, females that had previously raised enlarged litters 'chose' to cull a greater number of pups (14% versus 6%). These results are in agreement with the 'ecological context' hypothesis (Speakman and Król, 2005), which recognises that mice are a product of an evolutionary process that aims to maximise lifetime fitness. Therefore, mice may not maximise their performance under all conditions because, in some circumstances, greater investment in the current event might jeopardise future reproductive output or survival. Females that had previously raised reduced litters may have chosen to invest more during the second attempt because they had only reared 5 pups in the first attempt, whereas females that had previously reared enlarged litters maximised their performance and chose to reduce their efforts during a second attempt (i.e. by reducing MEO and culling more pups). Thus, the limits experienced by the females in the first litter may not have been a 'hard', unbreachable physiological limit, but a 'soft' limit imposed by the choices of the female.

Conclusions

Greater investment in the first reproductive event negatively impacted performance in the second event in MF1 mice. In addition, offspring reared in enlarged litters weaned the same number of, but significantly smaller, pups, indicating that the manipulation also had a detrimental effect on the reproductive success of offspring. This suggests that the observed asymptote in food intake and investment in the first lactation may be a 'soft' limit dependent on female 'choice' about how much to invest, rather than a 'hard', unbreachable limit defined by aspects of maternal physiology, as is commonly assumed.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: O.A.D., J.R.S.; Methodology: L.M.V., O.A.D., S.B., S.E.M., C.H., J.R.S.; Formal analysis: L.M.V., O.A.D.; Investigation: J.R.S.; Resources: J.R.S.; Data curation: L.M.V., S.B.; Writing - original draft: L.M.V., O.A.D.; Writing - review & editing: L.M.V., J.R.S.; Visualization: L.M.V.; Supervision: J.R.S.; Project administration: J.R.S.; Funding acquisition: O.A.D., J.R.S.

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