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RESEARCH ARTICLE

Limits to sustained energy intake. XVII. Lactation performance in MF1 mice is not programmed by fetal number during pregnancy

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SUMMARY

Several studies have suggested that lactation performance may be programmed by the number of fetuses during pregnancy, whereas other studies indicate that processes during lactation are more important. As gestation litter size and litter size in lactation are usually strongly correlated, separating the roles of pregnancy and lactation in lactation performance is difficult. To break this link, we experimentally manipulated litter size of MF1 mice to five or 16 pups per litter by cross-fostering. Litter size and mass at birth were recorded on day 1 of lactation prior to litter size manipulation. Maternal body mass and food intake, litter size and litter mass were measured daily throughout. After weaning, the potential differential utilisation of body tissues of the mothers was investigated. Relationships between maternal mass and food intake, including asymptotic daily food intake at peak lactation, offspring traits and other maternal parameters suggested that the number of fetuses the females had carried during pregnancy had no effect on lactation performance. Litter mass increases depended only on maternal food intake, which was highly variable between individuals, but was independent of fetal litter size. The sizes of key organs and tissues like the liver and alimentary tract were not related to maximal food intake at peak lactation or to fetal litter size, but the masses of the pelage, mammary glands and retroperitoneal fat pad were. These data suggest that while growth of the mammary glands and associated structures may be initiated in gestation, and vary in relation to the number of placentas, the ultimate sizes and activities of the tissues depends primarily on factors during lactation.

Key words: reproductive performance, lactation, litter size, growth, fetal programming, laboratory mouse.

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INTRODUCTION

Although pregnancy involves a significant increase in energy expenditure (Garton et al., 1994; Gittleman and Thompson, 1988; Speakman and McQueenie, 1996), lactation is widely agreed to be the most energetically demanding component of mammalian reproduction (Glazier, 1985a; Glazier, 1985b; Millar, 1979; Millar, 1981; Randolph et al., 1977; Speakman, 2008). Over the past 20 or so years there has been considerable interest in the factors that limit maternal lactation performance, focusing in particular on the sustained rate of maximal energy intake (SusEI) (Hackländer et al., 2002; Hammond et al., 1994; Hammond et al., 1996; Laurien-Kehnen and Trillmich, 2003; Rogowitz, 1996; Valencak et al., 2010; Zhang et al., 2008; Zhao and Cao, 2009; Zhao et al., 2010) (reviewed in Speakman and Król, 2011; Piersma and van Gils, 2011). SusEI in lactation appears to be physiologically constrained. The nature of this constraint has been much debated, and over time insight into the important factors has developed and accumulated. Early work emphasised the importance of the capacity of the alimentary tract to absorb energy (Drent and Daan, 1980; Peterson et al., 1990; Weiner, 1989; Weiner, 1992; Valencak and Ruf, 2006); this was largely superceded by a focus on the ability of the mammary glands to synthesise milk (Hammond et al., 1996; Rogowitz, 1998; Zhao and Cao, 2009) and more recently the capacity of the lactating female to dissipate heat (Król and Speakman, 2003; Król et al., 2003; Król et al., 2007; Speakman and Król, 2010).

Whatever this limiting process (or processes) proves to be, it remains true that the SusEI at peak lactation varies widely between individuals. A body of work (reviewed below) suggests that this variability may be pre-programmed during pregnancy by the number of gestated fetuses, and hence the expected number of offspring that the female anticipates she will have to support during lactation. However, females show a remarkable capacity to modulate their maximal food intake during peak lactation, milk production and resultant pup growth when environmental conditions (notably ambient temperature) are altered during lactation (Hammond et al., 1994; Hammond and Kristan, 2000; Johnson and Speakman, 2001; Król and Speakman, 2003; Speakman and Król, 2005; Król et al., 2007), suggesting there is no rigid pre-programming of sustained food intake or milk production capacity during gestation. In the present study we aimed to experimentally explore the extent to which maximal food intake, pup growth and maternal lactation morphology are pre-set by maternal expectations of lactation burden established from fetal numbers during gestation or are responsive to conditions during lactation.

Data indicating that peak lactation performance may be set during pregnancy relate to the fact that in many species mammary

development commences during pregnancy, stimulated by placental lactogens and oestrogens. Crucially, both of these hormones are produced in direct proportion to the number of placentas. In this way, it is suggested, mammogenesis is adjusted to the number of neonates to be fed during lactation (Bateman, 1957; Feldman et al., 1993; Forsyth, 1994; Horseman, 1999). For example, Hayden and colleagues (Hayden et al., 1980) reported that in goats (*Capra hircus*) during late pregnancy, lactogenic activity increased with the number of fetuses, and the mass of the lobulo-alveolar component of the mammary glands correlated positively with placental mass and fetal number. Moreover, milk yield correlated with the weekly mean of placental lactogen titres between week 11 of gestation and term. This supports the view that placental lactogen has a significant role in the control of normal mammary development and function in goats. Other data in goats indicate that diet in pregnancy may significantly impact on mammogenesis and hence lactation performance. Sahlu and colleagues (Sahlu et al., 1995) reported that milk production, and milk fat and protein content of goats increased in response to prepartum protein and metabolizable energy concentrations.

These effects are not restricted to larger mammals. In mice, the mass of the mammary tissue has long been established to be related to litter size at birth (Bateman, 1957). Nagasawa and Yanai confirmed that this correlation in mice occurs because of the effect of the number of placentas on mammary growth by surgically removing some embryos and their placentas, and thereby reducing lactogenic activity (Nagasawa and Yanai, 1971). They found that both prepartum and postpartum mammogenesis were reduced in relation to the number of placentas removed. A strong impact of pregnancy is also implied by studies demonstrating that nutritional events during hormone-sensitive growth phases, especially during late gestation, can significantly affect mammary development and subsequent lactation performance (Kim et al., 1998; Moon and Park, 1999; Moon and Park, 2002) in dairy cattle, beef heifers and female rats. These improvements in performance have been linked to increased cell proliferation with concurrent elevations in gene expression of key genes involved in cell proliferation and differentiation.

Despite these studies, there are also equally compelling data suggesting that lactation performance can be immediately responsive to changes in demand that occur during lactation. We have already mentioned the changes that occur when mice are exposed to altered ambient temperatures, however, there are numerous other examples. When dairy cows were milked twice daily, there was an increase in milk yield from parturition to peak lactation, which was due to increased secretory activity per cell (Capuco et al., 2003). Increased milking frequency during the first weeks of lactation increased milk yield, even after a return to less frequent milking, with increases of ~8% over the entire lactation, involving a mammary cell proliferation response. Milking cows four times daily during early lactation not only increased milk yield during the treatment period but also elicited an increase in milk yield for the entire lactation (Hale et al., 2003; Hillerton et al., 1990), again linked to an increase in cell proliferation, mammary growth and hence a carryover effect on milk production for the majority of lactation. Increased milking frequency also increased milk production of goats as a result of a rapid increase in the activity of mammary secretory cells, often followed by proliferation of secretory tissues (Knight et al., 1990; Wilde et al., 1987).

As litter sizes in lactation are normally highly correlated with the number of fetuses during pregnancy, the separate roles played by gestational programming and factors unique to lactation are often obscured. To explore these relationships more closely, and specifically to address the extent to which lactation performance is set during gestation, we experimentally manipulated litter sizes of mice the day after parturition to break the correlation between lactation litter size and gestation fetal numbers. We then asked to what extent maternal lactation performance (SusEI, litter growth rate and maternal morphology) was dependent on maternal expectations set in gestation (reflected by litter size at birth).

MATERIALS AND METHODS

Virgin female laboratory mice (*Mus musculus*; outbred strain MF1), supplied by Harlan UK Ltd (Bicester, UK), aged 9–10 weeks were individually housed in shoebox cages (44×12×13 cm) containing sawdust and paper bedding. They were provided with rodent chow [17.35 kJ g^{-1} dry mass CRM(P), Special Diet Services, BP Nutrition, Witham, UK] and water *ad libitum*, and placed under a 12 h:12 h light:dark photoperiod at 21±1°C. Mice were randomised into two groups that did not differ significantly in their baseline body mass and food intake. Female mice were individually paired with males. Mating pairs had access to *ad libitum* rodent chow and water and were left undisturbed for 11 days (Król et al., 2007). At the end of the 11 days, the males were removed and from day 12 onwards body mass and food intake of the females were measured daily (to ±0.01 g; Sartorius top-pan balance, Epsom, Surrey, UK) until parturition (day 0). Days of pregnancy were retrospectively numbered backwards from the day of parturition. New-born pups and mothers were not weighed on the day of parturition to reduce disturbance.

All procedures concerning animal care and treatment were approved by the ethical committee for the use of experimental animals of the University of Aberdeen, and were licensed by the UK Home Office and performed under PPL 60/3705.

On day 1 of lactation, mothers and their litters were weighed (±0.01 g). Pups were separated from their mothers and the pups from litters born on the same day were pooled in a measuring pot with bits of the bedding material. Females were then allocated either a small (N=5 pups) or large (N=16 pups) litter depending on their original randomised group. For both large and small litters, the pups consisted of a mix of the mother's own offspring and offspring from other mothers. We started with 60 females but because litters were not always born on the same days we ended up with 50 randomised litters (25 small and 25 large). There was no difference in the average litter size at birth between the mice that were allocated to small or large litters. From day 2 of lactation onwards, maternal body mass, maternal food intake, litter mass and litter size were recorded daily until day 18 when the pups were weaned. Throughout the experiment maternal food intake was calculated daily (afternoon) from the mass of food missing from the hopper. The bedding was manually sorted for uneaten food items each day, which was also weighed and subtracted from the apparent intake. Previous studies suggested that undiscovered food loss in the bedding constituted less than 2% of the total food consumed (Johnson et al., 2001a) and therefore was considered negligible.

Following the weaning of pups on day 18, each mother was weighed (±0.1 g) then killed by CO_2 inhalation. Whole-body dissection was carried out in the afternoon and 19 body components removed and their wet masses immediately recorded (±0.001 g; Ohaus Analytic Plus Balance, Nänikon, Switzerland) (see Johnson et al., 2001b). These included the brain, brown adipose tissue (BAT), three white adipose tissue depots (WAT), mammary tissue (including subcutaneous WAT), gonads, liver, kidneys, heart, lungs, spleen, pancreas, stomach, and small and large intestines. The stomach was cut open longitudinally, its contents removed and the stomach then

weighed. The contents of the small and large intestines were also removed prior to the intestines being weighed. For each animal, the remaining body parts were divided into tail, pelage and carcass, including both skeletal muscle and bone. All tissues and organs were oven-dried at 60°C (Gallenkamp air fan oven) for 14 days (Król and Speakman, 1999) and reweighed (±0.001 g) to determine dry mass.

Data analysis

Data are reported as means ± s.d. We explored the changes in food intake and body mass during pregnancy using ANOVA, accounting for repeated measures by including individual as a nested factor within group. We used linear regression to explore relationships between parameters measured in pregnancy and the litter size and mass at birth. During lactation, we summarised the changes in body mass and food intake for the two treatment groups (large and small litters) and compared them using ANOVA. As for pregnancy, we accounted for repeated measures by including individual as a nested factor within group. Relationships were examined between maternal gestation and lactation body mass, mean and asymptotic food intake (FI_{AS}), and litter growth using least squares linear regression analysis. Changes in maternal gestation and lactation food intake over time and the difference in lactation food intake between the two groups was tested using ANOVA. General linear modelling (GLM) was used to explore the relationships between maternal FI_{AS} and pup body mass or litter mass with treatment group as a factor. Effects of gestation (litter size at birth) on lactation performance were explored using regression. Litter sizes were not normally distributed and were consequently normalised using the Box–Cox procedure. Relationships between female morphology and lactation performance and morphological differences between the groups were explored using GLM. The effect of the Box–Cox transformed litter size on female morphology was explored using GLM with litter size as a covariate and treatment group as a factor.

Proportional water content of the tissues were calculated as the difference between the wet and dry tissue masses divided by the wet mass. The relationship between proportional water content and tissue mass was obviously non-linear and so we log converted the tissue masses prior to regression analysis. We used stepwise regression with backward elimination to investigate the association between tissue mass and individual variability in food intake at peak lactation. The analysis was repeated using wet and dry masses.

All statistical analyses were conducted using Minitab for Windows (Version 16; Minitab Inc., State College, PA, USA). Statistical significance was accepted when $P<0.05$, and where multiple testing was employed (e.g. in comparisons of multiple tissues) a Bonferroni correction was applied to the significance criterion.

RESULTS

Pregnancy

During the last 10 days of pregnancy, females significantly increased in body mass (ANOVA, $F_{9,431}=50.89$, $P<0.001$) from an average of 40.06±3.12 g to a maximum of 55.86±7.56 g prior to parturition (day −1). The mean body mass of pregnant females throughout gestation was significantly positively related to litter size at birth ($R^2=0.241$, $F_{1,47}=14.60$, $P<0.001$; Fig. 1A) and litter mass at birth ($R^2=0.261$, $F_{1,47}=16.27$, $P<0.001$; Fig. 1B). Females significantly increased food intake during the last part of pregnancy (ANOVA, $F_{5,275}=4.13$, $P<0.001$) from an average of 6.54±1.87 g on day −10 to a maximum of 7.98±1.82 g on day −3, after which their mean daily food intake declined to 7.06±0.92 g on the day immediately prior to parturition (day −1). Over the period of pregnancy, the mean body mass of pregnant females was significantly ($R^2=0.113$, $F_{1,47}=5.33$, $P=0.026$) related to the mean daily food intake (Fig. 1C). However, maternal food intake throughout gestation was not related to the litter size or litter mass at birth (size: $F_{1,47}=1.35$, $P=0.251$; mass: $F_{1,47}=0.68$, $P=0.415$).

Lactation

Body mass of the females on day 1 of lactation prior to the manipulations was not significantly different between groups that were given large or small litters to raise (ANOVA: $F_{1,47}=0.45$, $P=0.83$). After manipulation, the body masses diverged over a period of about 10 days during which the mean mass of both groups increased, but the body mass of the females raising large litters increased more rapidly. Body mass then stabilised between days 12 and 16 (Fig. 2A). During this period of stable body mass the females rearing large litters weighed on average 48.18±4.60 g and were significantly heavier than females that reared small litters, which weighed on average 43.48±3.38 g (ANOVA, $F_{1,47}=16.94$, $P<0.001$).

Fig. 1. Mean body mass of pregnant females averaged over the last 10 days of gestation in relation to (A) litter size and (B) litter mass at birth. (C) Food intake averaged over the last 10 days of gestation in relation to mean body mass over the same period. Lines are fitted regressions.

Food intake of females in both litter size groups increased over time in lactation but then reached an asymptote (Fig. 2B). Food intake of the females that reared small litters increased slowly over the first 10 days of lactation and then reached a plateau between days 11 and 15, during which time the females ate on average 19.27±3.7 g day^{-1}. For the females raising larger litters, food intake increased more rapidly in early lactation reaching a plateau by day 6. Between days 11 and 15 the food intake of females that reared large litters averaged 20.46±3.48 g day^{-1}. This FI_{AS} was not significantly different between the females raising small and large litters (ANOVA, $F_{1,47}$=1.36, P=0.249). The litter size of the group raising small litters remained constant at five pups throughout lactation (Fig. 2C). However, all the females in the group raising large litters (with one exception) lost pups during lactation. The days this happened and the numbers involved varied between litters, resulting on average in a slow linear decline in the mean litter size for this group. On average, the weaned litter size was 11.2 pups (s.d.=2.1 pups), which despite the losses was still highly significantly greater than the litter size in the group raising small litters (ANOVA, $F_{1,47}$=209.7, P<0.001).

The mean mass of the large litters increased rapidly from on average 32.62±3.1 g on day 2 to 58.71±12.35 g on day 12 (Fig. 3A). Thereafter, the total litter mass did not increase significantly until the litters were weaned on day 18 when on average they weighed 60.77±23.18 g. In contrast, the small litters grew more slowly but increased in mass continuously throughout lactation, from 12.39±1.26 g on day 2 to a maximum of 59.92±9.78 g on day 18. From day 15 onwards the total litter mass supported was not significantly different between the two groups (Fig. 3A).

Pups increased in mean body mass during lactation (Fig. 3B). Pups reared in small litters increased significantly in mean body mass from 2.48±0.25 g on day 2 of lactation to a maximum of 12.28±1.78 g on day 18 when they were weaned. Pups reared in large litters weighed on average 2.04±0.18 g on day 2 of lactation and also increased significantly in mass over the period of lactation, and were weaned at an average mass of 5.75±1.79 g on day 18. The average pup mass over the last 3 days of lactation was highly significantly different between the two groups (ANOVA, $F_{1,47}$=222.64, P<0.001).

There was a significant positive relationship between the average pup mass at the end of lactation (last 3 days) and the FI_{AS} between

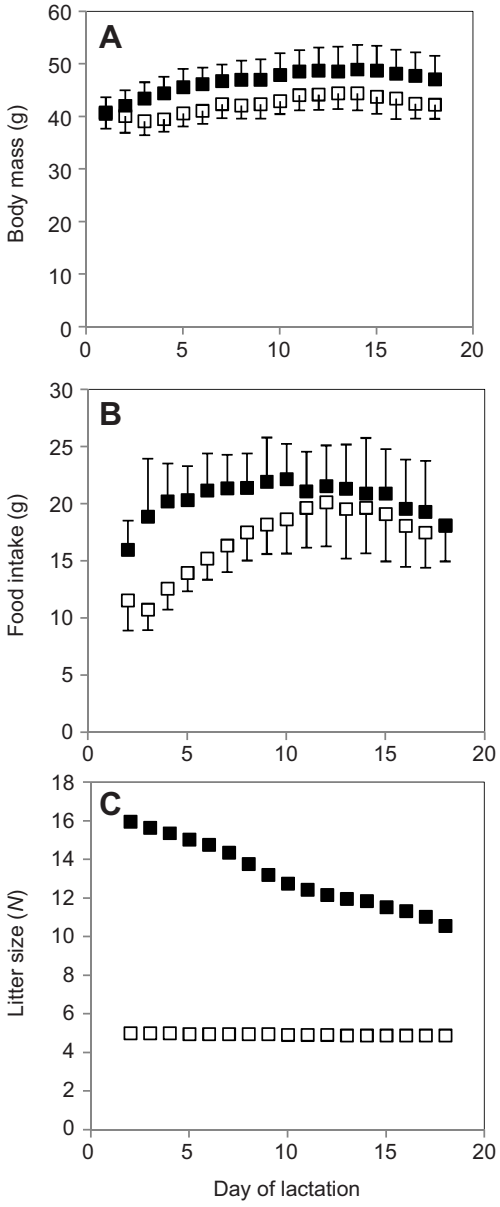

Fig. 2. (A) Maternal body mass, (B) maternal food intake and (C) litter size over the days of lactation. In all cases, the filled squares are for females raising large litters and the open squares are for females raising small litters. Data are means ± s.d.

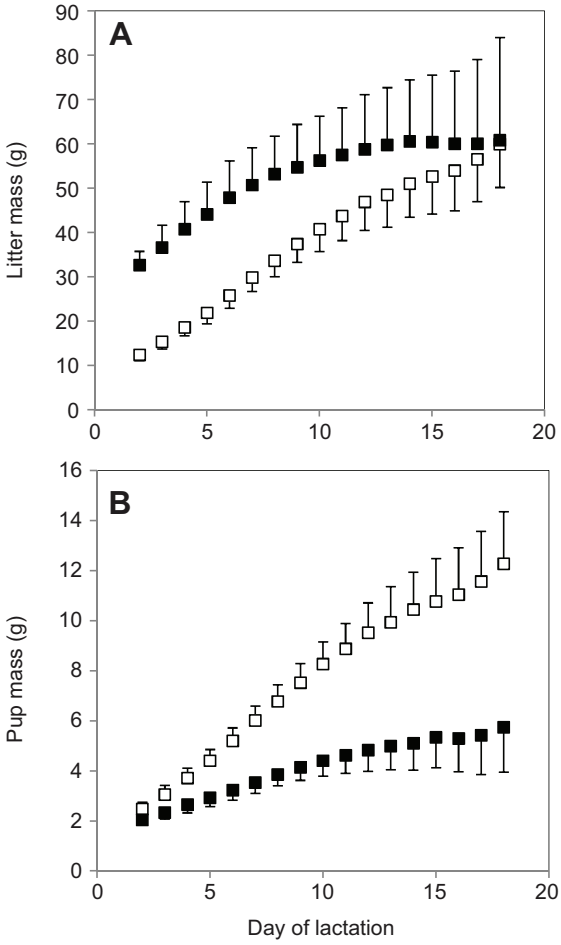

Fig. 3. (A) Total litter mass and (B) individual average pup mass over the duration of lactation for females raising large (filled squares) and small (open squares) litters. Data are means ± s.d.

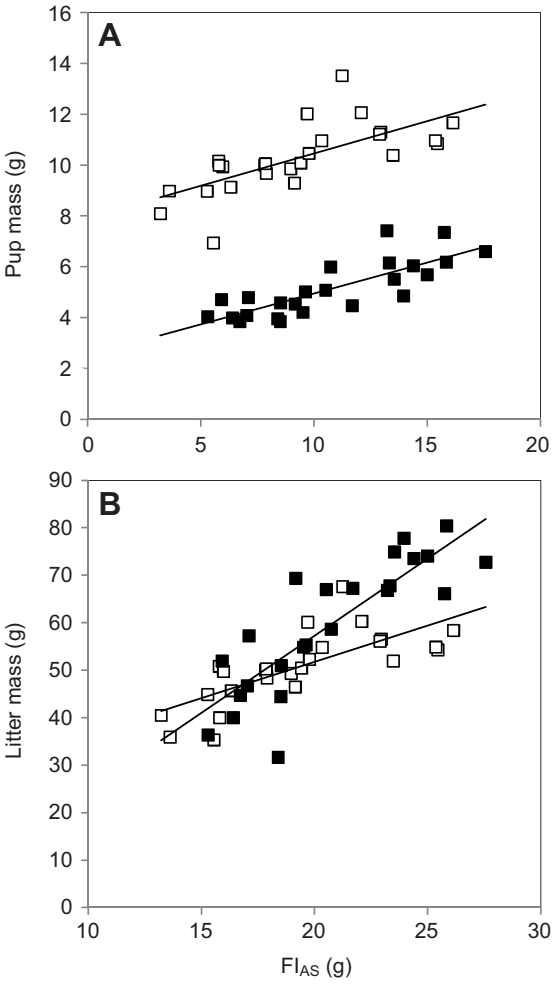

Fig. 4. (A) Individual mean pup mass and (B) total litter mass in relation to the asymptotic daily food intake (FI_{AS}) of females at peak lactation (days 11–15). Open symbols are small litters and filled symbols are large litters. Lines are fitted regressions.

days 11 and 15 in both groups, and there was a highly significant group effect (Fig. 4A; ANOVA, food intake effect: $F_{1,47}$=51.69, P<0.001; group effect: $F_{1,47}$=13.82, P=0.001). There was no significant interaction between group and FI_{AS} on pup mass ($F_{1,47}$=0.03, P=0.86). Similarly, there was a significant relationship between average litter mass at the end of lactation (last 3 days) and the FI_{AS} between days 11 and 15 in both groups (Fig. 4B; ANOVA, FI_{AS} effect, $F_{1,47}$=60.94, P<0.001), but in this case the effect of group was smaller and less significant ($F_{1,47}$=4.15, P=0.047) and there was a significant intake × group interaction effect ($F_{1,47}$=6.92, P=0.012).

Carryover effects of pregnancy on lactation

The mean litter size weaned in the group that were given 16 pups to raise was 11.3 pups. The mean natural litter size across all females was 11.8 pups (s.d.=2.1 pups) but restricting the analysis only to the females who were given large litters to raise, the natural litter size was also 11.3 pups (paired t-test, t=0.0, P=1.0). However, there was no significant relationship in this group between the natural litter size and the litter size that was ultimately weaned (Fig. 5; regression, F=1.18, P=0.289); in fact, the mother that gave birth to the smallest natural litter (N=6 pups) was the only female who successfully raised all 16 foster pups without loss.

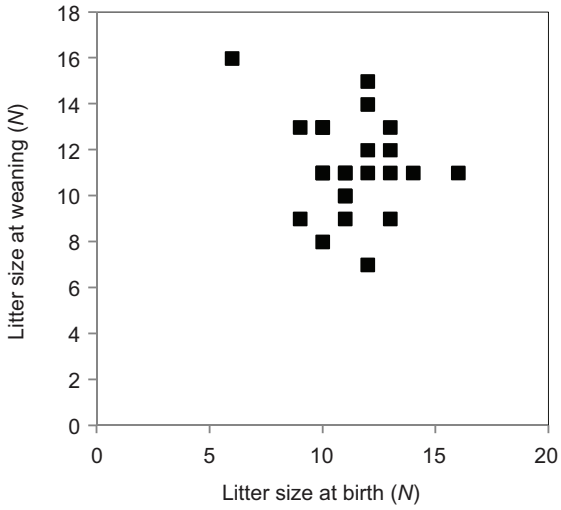

Fig. 5. Litter size at weaning in relation to litter size at birth for females that reared pups in large litters. Litter size at weaning was the number of live pups that remained with each female on the day of weaning (day 18 of lactation). There was no significant relationship (P>0.05).

Litter size at birth was not normally distributed; we therefore transformed litter size using a Box–Cox transformation (λ=2.2). We used the resulting Box–Cox converted values to establish the contributory roles played by the birth litter size and the manipulation group (small *versus* large litters in lactation) on the morphology of the female mice. We entered the mass of each organ as the dependent variable in a GLM analysis with the manipulation group as a factor and the birth litter size as a covariate; analyses were rerun excluding the interaction term if it was not significant. The results for wet mass of all tissues are in Table 1 and for dry mass are shown in Table 2. There was no significant effect (P<0.05) of the birth litter size on any of the organs when weighed either wet or dry. The closest relationships were for the dry masses of the large intestine and stomach (P=0.028 and 0.006, respectively, both not significant at P=0.05 when Bonferroni correction applied). There were, however, several significant effects of the experimental manipulation of litter size during lactation on the morphology of the females. In particular, there were significant effects on the wet and dry masses of all three WAT depots (gonadal, mesenteric and retroperitoneal) and the interscapular (i)BAT depot. In all four cases, the direction of the effect was that the adipose tissues were smaller in the animals raising large litters. These differences were quite large, with the depots in the females raising small litters being about five times larger than the depots for those raising large litters. In addition, there was a significant effect of group on dry pelage mass (effect on wet pelage mass marginally significant group effect, P=0.007 *versus* Bonferroni adjusted significance criterion of 0.003). The dry mass of the pelage was significantly lower in females raising large litters.

The percentage water content of the adipose tissues (both WAT and BAT) was strongly dependent on the mass of the tissue, with heavier tissues having much less water in them. The relationships, however, varied significantly between the different depots (Fig. 6A; GLM using arcsin converted proportion of water as the dependent variable and ln tissue mass as the covariate; tissue mass effect: $F_{1,192}$=164.6, P<0.001; fat store effect: $F_{3,192}$=2.65, P=0.05; interaction: $F_{3,192}$=16.17, P<0.001). Pairwise comparisons revealed that mesenteric fat and BAT did not differ from each other but both

Table 1. Wet mass (g) of tissues and organs of females that reared pups in large or small litters

Tissue	Small litters (mean ± s.d.)	Large litters (mean ± s.d.)	Birth litter size		Group		Interaction	
			F	*P*	*F*	*P*	*F*	*P*
Brain	0.333±0.1	0.315±0.067	0.05	0.83	0.51	0.477	0.08	0.774
Pelage	4.681±0.95	4.021±0.546	0.35	0.56	8.09	0.007	0.07	0.800
MG	3.654±1.785	3.817±0.967	0.00	1.00	0.01	0.922	0.05	0.832
gWAT	1.663±1.115	0.303±0.225	0.01	0.93	21.49	**<0.001**	0.05	0.832
mWAT	0.731±0.371	0.269±0.131	0.16	0.69	32.00	**<0.001**	0.54	0.466
aWAT	0.655±0.508	0.254±0.235	0.03	0.86	10.74	**0.002**	0.34	0.563
iBAT	0.394±0.153	0.291±0.055	0.42	0.52	14.01	**0.001**	0.00	0.969
Liver	3.353±0.602	3.483±0.449	0.04	0.84	1.22	0.276	0.86	0.359
Heart	0.315±0.072	0.32±0.064	0.71	0.40	0.00	0.984	0.00	0.982
Lungs	0.579±0.094	0.539±0.082	0.40	0.53	3.76	0.059	2.56	0.117
Kidneys	0.579±0.086	0.548±0.064	0.78	0.38	0.55	0.461	0.06	0.800
Spleen	0.121±0.027	0.118±0.025	3.54	0.07	1.56	0.218	1.52	0.224
Pancreas	0.516±0.141	0.526±0.154	0.01	0.93	3.88	0.055	4.60	0.037
Gonads	0.309±0.068	0.324±0.096	0.14	0.71	0.50	0.482	0.35	0.559
SI	0.872±0.232	0.918±0.166	0.57	0.46	0.56	0.457	0.37	0.546
LI	0.457±0.085	0.498±0.077	4.04	0.05	1.47	0.232	0.03	0.870
Stomach	0.271±0.036	0.271±0.038	9.82	**<0.001**	0.01	0.911	0.01	0.925
Tail	1.031±0.109	1.046±0.087	0.33	0.57	0.21	0.65	0.26	0.613
Carcass	17.39±1.53	16.98±1.161	0.07	0.79	0.00	0.97	0.16	0.694

Effects of group (large or small litters) and the birth litter size are shown. Significant effects (P<0.05) are shown in bold (applying Bonferroni correction actual critical significance level=0.003).

MG, mammary glands; gWAT, gondal white adipose tissue; mWAT, mesenteric white adipose tissue; aWAT, abdominal or retroperitoneal white adipose tissue; iBAT, interscapular brown adipose tissue; SI, small intestine; LI, large intestine.

differed from the other two depots, which also differed from each other. When controlled for tissue depot size, on average mesenteric (m)WAT and iBAT both had a higher water content (Tables 1, 2). The water content was significantly greater in the depots removed from the females who were raising large litters (Table 3). As water content is inversely proportional to fat content, this indicates that the tissues were smaller because the females raising large litters withdrew fat from their adipose tissue depots – including the iBAT depot. The relationship between the wet tissue and dry tissue mass of the mammary glands was different between the two groups (Fig. 6B; effect on dry mass GLM–wet mass: $F_{1,47}$=112.55, P<0.001; group: $F_{1,47}$=35.23, P<0.001; interaction: $F_{1,47}$=2.22, P=0.123).

We sought associations between the individual variations in tissue mass and the individual variability in the FI_{AS} at peak lactation. Using stepwise regression with backward elimination of variables in order of their P-values revealed five significant predictors based

Table 2. Dry mass (g) of tissues and organs of females that reared pups in large or small litters

Tissue	Small litters (mean ± s.d.)	Large litters (mean ± s.d.)	Birth litter size		Group		Interaction	
			F	*P*	*F*	*P*	*F*	*P*
Brain	0.075±0.021	0.072±0.015	0.10	0.759	0.01	0.931	0.11	0.739
Pelage	2.289±0.602	1.627±0.258	0.48	0.493	22.08	**<0.001**	0.22	0.641
MG	1.936±1.000	1.315±0.351	0.09	0.771	7.35	0.009	0.05	0.832
gWAT	1.469±1.045	0.219±0.197	0.00	0.991	30.48	**<0.001**	0.02	0.884
mWAT	0.547±0.327	0.122±0.101	0.12	0.795	35.27	**<0.001**	0.47	0.495
aWAT	0.491±0.423	0.143±0.165	0.01	0.919	12.55	**0.001**	0.21	0.645
iBAT	0.256±0.102	0.152±0.041	1.32	0.257	22.90	**<0.001**	0.01	0.904
Liver	0.927±0.183	0.949±0.101	0.00	0.985	0.22	0.639	0.19	0.663
Heart	0.079±0.016	0.077±0.016	1.09	0.302	0.03	0.87	0.01	0.926
Lungs	0.144±0.026	0.123±0.021	0.11	0.738	8.30	0.006	2.92	0.094
Kidneys	0.156±0.030	0.136±0.018	0.78	0.381	0.55	0.461	0.06	0.800
Spleen	0.027±0.007	0.025±0.006	2.51	0.120	1.39	0.244	2.07	0.157
Pancreas	0.141±0.034	0.137±0.038	0.01	0.931	3.88	0.055	4.60	0.037
Gonads	0.134±0.054	0.120±0.075	0.44	0.511	0.21	0.651	0.69	0.412
SI	0.220±0.059	0.216±0.040	0.43	0.517	0.05	0.827	0.14	0.705
LI	0.122±0.019	0.121±0.017	5.17	0.028	0.57	0.455	0.05	0.832
Stomach	0.071±0.010	0.071±0.011	8.14	0.006	0.14	0.714	0.31	0.583
Tail	0.473±0.057	0.442±0.035	0.35	0.557	7.09	0.011	0.10	0.757
Carcass	5.8610.714	5.1470.327	0.22	0.640	19.79	**<0.001**	0.03	0.845

Effects of group (large or small litters) and the birth litter size are shown. Significant effects (P<0.05) are shown in bold (applying Bonferroni correction actual critical significance level=0.003).

MG, mammary glands; gWAT, gondal white adipose tissue; mWAT, mesenteric white adipose tissue; aWAT, abdominal or retroperitoneal white adipose tissue; iBAT, interscapular brown adipose tissue; SI, small intestine; LI, large intestine.

on wet tissues mass. These were, with their associated coefficients, *t*- and *P*-values in the multiple regression model: abdominal or retroperitoneal (a)WAT (coefficient=–4.21, t=3.12, P=0.003), pelage (coefficient=2.01, t=2.79, P=0.008), gonads (coefficient=–15.65, t=–2.80, P=0.008), mammary glands (coefficient=–0.76, t=–2.77, P=0.022) and pancreas (coefficient=6.99, t=2.11, P=0.041). Together, these variables accounted for 33.2% of the variation in FI_{AS}. For dry mass, only one of these variables was significant. The dry mass of the mammary gland explained 13.0% of the variation in FI_{AS} (coefficient=–1.60, t=–2.68, P=0.01; Fig. 7). Individuals with heavier mammary glands had lower FI_{AS}. However, taking the two groups separately, the effect of mammary gland dry mass was not significant in the females raising large litters (r^2=0.024, t=0.76, P=0.45) but was highly significant in the females raising small litters (r^2=0.273, t=–2.94, P=0.003). This negative effect of the dry mass of the mammary gland on FI_{AS} was also reflected in a negative association between dry mammary gland mass and weaned litter mass (coefficient=–4.86, t=–2.41, P=0.02). Despite several wet tissue mass variables being significantly linked to food intake, using stepwise regression none of these variables was also linked to the weaned litter mass, which instead correlated only with wet mass of the stomach (coefficient=–105.5, t=–2.41, P=0.02).

There was no significant relationship between FI_{AS} and litter size at birth (linear regression using Box–Cox transformed litter size as the predictor variable: $F_{1,47}$=0.01, P=0.972). This remained non-significant if the group allocated to in lactation was included as a factor in the analysis.

DISCUSSION

The patterns of change in body mass and in food intake during pregnancy were consistent with the changes that we have reported previously in this strain of mice (Gamo et al., 2013; Johnson et al., 2001a; Speakman and McQueenie, 1996). Specifically, food intake increased slightly during pregnancy to a peak about 2–3 days prior to parturition, while body mass increased exponentially to a maximum on the day of parturition. Much of this mass increase (about 25 g on average) was the mass of the litter, as there was a close correspondence between maternal mass gain, litter size and litter mass. However, there was also significant accumulation of somatic tissue during pregnancy, as the mice on the first day of lactation were about 10 g heavier than they were prior to mating. This increase in mass would include not only the structures necessary to support fetal growth *in utero* but also increases in the size of the alimentary tract (Campbell and Fell, 1964; Fell et al., 1963; Jolicoeur et al., 1981a; Jolicoeur et al., 1981b) and liver (Kennedy et al., 1958; Speakman and McQueenie, 1996) to cope with the anticipated increase in food intake during lactation, as well as pre-partum mammogenesis in preparation for milk production in lactation (Bateman, 1957). These somatic modifications (or other changes such as fat distribution and pelage density, and hence heat dissipation capacity) initiated during pregnancy might theoretically play a role in determining the SusEI at peak lactation, and thus a match between actual energy requirements for offspring growth and those anticipated from the number of gestating fetuses.

The growth of the litters, in both litter size groups, was primarily affected by the food intake of the females during peak lactation. There was no significant difference in the FI_{AS} in response to the litter size manipulation. This shows that at peak lactation the system is primarily limited by the capacity of the mother to ingest food and convert it to milk. Two observations, however, suggest that pup demands do play an additional role. First, the females raising large litters reached their FI_{AS} much faster (by day 6) compared with the females raising small litters, who did not reach FI_{AS} until day 11. This suggests that the rate of increase in food intake towards the asymptotic level over the initial part of lactation was primarily driven by pup demand – higher in the larger litters – but this then reached an asymptote that was largely independent of pup demand and regulated by factors intrinsic to the female. Second, the relationship between FI_{AS} and weaned litter mass was slightly but significantly different between the two groups: notably, the gradient for the small litters was significantly lower than that for the large litters. This meant that at the highest FI_{AS}, the litter mass achieved in the small litters was lower than that in the large litters (see Fig. 4B). This would be consistent with a limit on growth as the pups get larger.

Nevertheless, the dominant factor influencing differences in lactation performance (weaned litter mass) in the current experiment was the FI_{AS} of the mothers. This varied widely between individuals (from 13.2 to 27.6 g day^{-1}) but was independent of the experimental manipulation. Mice with greater FI_{AS} raised much heavier litters, independent of the number of suckling offspring. Because litter sizes

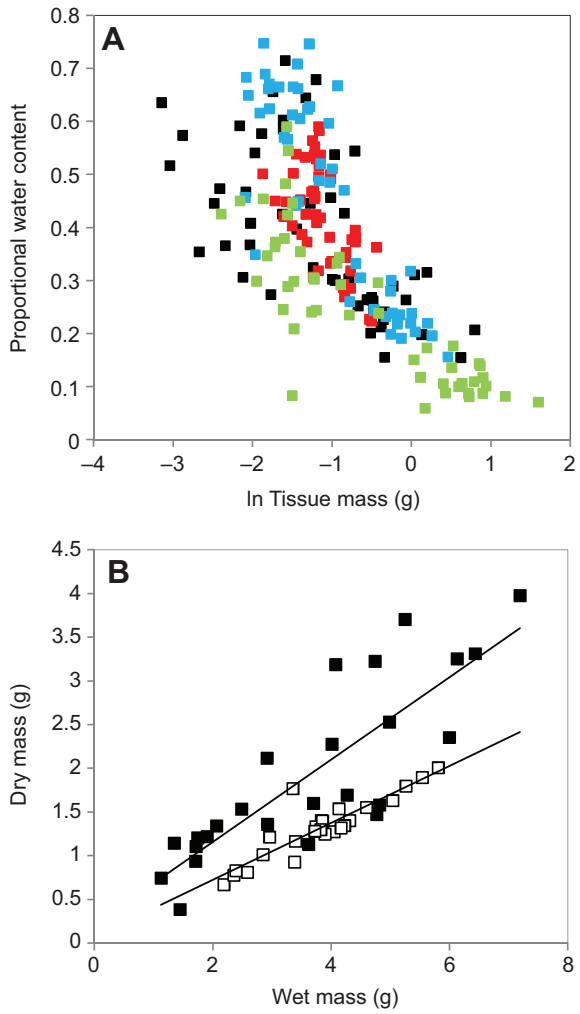

Fig. 6. (A) Proportional water content of all fat tissue samples (white adipose tissue, WAT; and brown adipose tissue, BAT) from all individuals plotted against tissue mass (ln converted). Different colours reflect different adipose tissue depots (WAT and BAT): black squares represent abdominal WAT, green squares gonadal WAT, blue squares mesenteric WAT and red squares intrascapular BAT. (B) Dry mass of the mammary glands plotted against wet mass of the same tissue for mice raising small (filled squares) and large (open squares) litters.

Table 3. Proportional water content of adipose tissues collected from females that had raised either large or small litters in lactation

Depot	Small (mean ± s.d.)	Large (mean ± s.d.)	*F*	*P*
gWAT	0.187±0.148	0.317±0.101	14.8	**<0.001**
iBAT	0.364±0.083	0.481±0.068	29.3	**<0.001**
aWAT	0.314±0.132	0.471±0.136	16.9	**<0.001**
mWAT	0.324±0.179	0.578±0.109	37.0	**<0.001**

Significant effects are shown in bold.
iBAT, interscapular brown adipose tissue; gWAT, gondal white adipose tissue; mWAT, mesenteric white adipose tissue; aWAT, abdominal or retroperitoneal white adipose tissue.

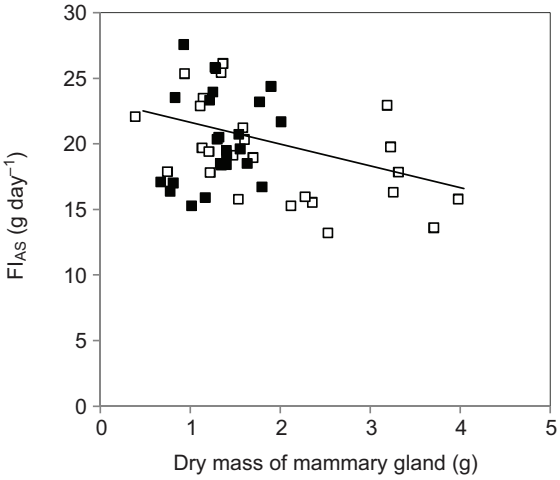

Fig. 7. FI_{AS} at peak lactation (days 11–15) in relation to the dry mass of the mammary glands. Large litters (filled squares) small litters (open squares). Line is the fitted regression.

were very different between the two groups, this led to profound differences in pup size between groups (with the pups weaned from the small litters weighing on average more than double those weaned from the large litters). The fact pups in the small litters were able to grow this large when competition for milk is dramatically reduced suggests that the females in the large litters (and in natural litters) were limited in their capacity to take in food and produce milk at peak lactation, rather than being responsive to pup demands or regulating their intake to optimize pup growth [as suggested by Valencak (Valencak et al., 2010)].

We found several significant differences in the sizes of various organs of the females at the end of lactation between the two groups. These differences mostly reflected differences in the size of adipose tissue depots (smaller in the animals raising larger litters). There are several alternative explanations for the differences in the sizes of the fat depots between the groups. First, females raising large litters, which had reached their FI_{AS} relatively early in lactation, may have supplemented their energy intake by withdrawing fat to support milk production. Consistent with this hypothesis, we found that as the fat depots got smaller they also increased in water content – reflecting a lowered fat content. The fact that the BAT depot also increased in water content as it got smaller was also consistent with the contraction in the size of the tissue being primarily through withdrawal of triglycerides. This suggests that the reduction in the heat production capacity of iBAT during lactation is probably predominantly driven by the reduction in the molecular machinery underpinning heat generation (Król et al., 2011; Trayhurn and Bing, 2006; Trayhurn and Richard, 1985) rather than reductions in the size of the tissue.

Quantitatively, the direct contribution of this withdrawn fat to milk production has previously been suggested to be unlikely to be an important factor influencing the overall lactation performance between the mothers raising large and small litters (Speakman, 2008). The difference in body fat at weaning between the groups across the gonadal, mesenteric and retroperitoneal WAT depots plus the iBAT depot averaged 2.3 g. This was equivalent to about 90.5 kJ of energy assuming an energy density of 39.0 kJ g^{-1} (Brisbin, 1966). In contrast, the accumulated food intake over the last 10 days of lactation, across all the animals was 198.7 g, equivalent to around 3200 kJ of net energy uptake, assuming a metabolisable energy content for the diet of 16.1 kJ g^{-1} (Gamo et al., 2013). In rough figures therefore, the fat withdrawal may have at most contributed an additional 2.8% to the energy available to support peak lactation. However, this calculation assumes that the withdrawal of the fat occurs during the final phase of the lactation event. In fact, from the available data we do not know exactly when the fat is withdrawn. If it was withdrawn during the first 5 days of lactation then the contribution of the fat to the available energy would be higher. During the first 5 days of lactation the intake of the females raising large litters was 75.3 g, equivalent to 1234 kJ, so 90.5 kJ from fat would increase the net available energy by 7.3%. This might be significant in allowing the females to meet pup demands at a time when they are rapidly increasing. Nevertheless, while this withdrawal might be significant at certain times, its overall impact is small. This probably explains why, despite the fact the fat stores of the mothers raising large litters were only about one-fifth the size of those in the group raising small litters, there were no significant differences in the eventual weaned litter masses on day 18 of lactation. Although fat stores may have contributed relatively little to overall lactation performance in the current study, we do not mean to imply this low contribution is the same in all small mammals. Valencak and colleagues, for example, found that female brown hares (*Lepus europaeus*) adjusted their investment in their young in relation to the size of their fat reserves (Valencak et al., 2009).

Reduced fat stores may have contributed to lactation performance in two further ways. First, lowering fat levels might reduce circulating leptin levels, which is a potent orexigenic signal (Mercer and Speakman, 2001). A reduction in circulating leptin may have facilitated the rapid elevation in intake in the large litter size group during the early phase of lactation. Manipulations of leptin in lactation, however, have yielded ambiguous results, with some studies showing reduced intake in response to repletion of leptin levels (Cui et al., 2010; Stocker et al., 2007) but others showing no effect (Xu et al., 2009), so the importance of this effect is unclear. Second, lowered fat, in combination with lowered pelage mass, might aid heat dissipation capacity and modulate the hypothesised heat dissipation constraint on intake (Speakman and Król, 2010; Speakman and Król, 2011). If this was correct, then we would anticipate that the mass of the pelage and the size of the fat stores would be significant factors related to the individual variability in FI_{AS}. This was indeed the case; both the wet mass of the pelage and the wet mass of the retroperitoneal fat pad were significant predictors of FI_{AS}. However, it seems unlikely that these relationships were due to the tissue masses modulating the heat loss capacity and hence the FI_{AS} as the relationship between FI_{AS} and pelage mass was positive. In addition, while the effect of retroperitoneal fat was in the correct direction to support the heat dissipation idea, it is unclear why this fat pad alone was significantly related to food intake. Both

of these variables had no effect on litter mass, suggesting their links with intake were not causal.

In addition to the effects of the pelage and the retroperitoneal fat pad, there was also a significant relationship between the mass of the mammary tissue and the FI_{AS} (Fig. 7). This relationship was also in the opposite direction to that anticipated, i.e. the mice with the heaviest mammary glands had the lowest intake and weaned the smallest litters. However, the difference in the water content of mammary tissue between the females raising small and large litters (Fig. 6B) suggests the main reason for the effect was that the mammary tissues varied in their fat content. Heavier mammary glands were heavier primarily because they contained more fat not because they contained additional mammary buds. Although we found several significant morphological features correlated with the FI_{AS}, these effects explained less than 35% of the variance, and perhaps more importantly appeared not to be functionally related to the intake but a consequence of additional complicating factors – such as the fat content of the mammary tissue. The organs that *a priori* might be most expected to be linked to individual variability in intake – the alimentary tract organs and the liver – were all not significant. This accords with previous failed attempts to link together intake and expenditure at peak lactation with organ sizes of the alimentary tract (Johnson et al., 2001b; Król and Speakman, 2003; Speakman et al., 2004). The repeated absence of gross morphological effects suggests that further studies are needed at the physiological and molecular levels to understand the individual variability in sustained FI_{AS} during lactation.

The large individual differences in FI_{AS} were directly linked to the large differences in litter growth and hence pup size within groups. Our original hypothesis was that these profound individual differences in female performance might be programmed during pregnancy in relation to variable *in utero* litter sizes. However, we found no evidence to support this hypothesis. FI_{AS}, litter mass and pup mass, as well as the female morphology at peak lactation, were all independent of the litter size at birth. Although the mothers given litters of 16 to raise generally failed to raise this number and ultimately on average weaned exactly the same number of offspring as the average natural litter size at birth (11.2 pups), there was no correlation between the number of pups that a female had given birth to and the number that she successfully weaned. Overall, the present data strongly indicate that SusEI at peak lactation in these mice was not programmed by events during pregnancy.

The primary source of the variability between individuals in FI_{AS} remains uncertain. However, the present data also permit us to discount the possibility that the differences are largely driven by variation in pup demand, as the FI_{AS} was unaffected by a major litter size manipulation (five pups compared with 16 pups), and within each group there was tremendous variation. Although we have shown that asymptotic lactation performance is not programmed during pregnancy, and is largely unresponsive to pup demand, the key physiological and morphological features that drive the major differences between individual mice in their FI_{AS} remain obscure. In the accompanying paper (Vaanholt et al., 2013), we have shown that the variability in FI_{AS} of this strain of mouse is heritable. Selective breeding of mice for both high and low FI_{AS} may thus be a viable strategy to shed more light on the morphological, physiological and molecular mechanisms that underpin this crucial trait.

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AUTHOR CONTRIBUTIONS

O.A.D. co-designed the experiment, performed the majority of the laboratory work, and did a preliminary analysis of the data. K.A.M. assisted with the data analysis. C.H. and E.K. assisted with the laboratory work. J.R.S. conceived and co-designed the experiments, analysed the data and wrote the first draft of the paper. K.A.M., C.H. and E.K. commented on and revised the paper.

COMPETING INTERESTS

No competing interests declared.

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