

RESEARCH ARTICLE

Limits to sustained energy intake. XXVIII. Beneficial effects of high dietary fat on lactation performance in mice

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ABSTRACT

Maximal animal performance may be limited by the ability of an animal to dissipate heat: the heat dissipation limitation (HDL) theory. Because the incidental heat produced during digestion [specific dynamic action (SDA)] varies among diets, the HDL theory predicts that lactating female mice consuming diets with lower SDA should have increased reproductive performance. Dietary fat has a lower SDA than dietary carbohydrate. Female mice were fed low (LF), medium (MF) or high (HF) fat diets (10, 45 and 60% energy from fat, respectively) from days 4–18 of lactation. HF- and MF-fed mice weaned significantly heavier litters than LF mice. This was because they not only consumed more energy [metabolisable energy intake (E_{mei}); HF: 306.5 ± 25.0 , MF: 340.5 ± 13.5 kJ day⁻¹] at peak lactation, but also delivered more milk energy to their pups [milk energy output (E_{milk}); HF: 203.2 ± 49.9 , MF 229.3 ± 42.2 kJ day⁻¹] than the LF-fed mice ($E_{mei} = 266.7 \pm 4.5$, $E_{milk} = 164.60 \pm 30.59$ kJ day⁻¹). A mathematical model based on the predictions from the HDL theory showed that this effect was greater than predicted from differences in SDA between the diets. Fatty acid profiles of the diets, milk and pups showed significant correlations between the profiles. Besides reduced SDA, HF- and MF-fed mice were probably able to directly transfer absorbed dietary fat into milk, reducing the heat production of lactogenesis and enabling them to perform better than expected from the HDL model. In summary, HF and MF diets had beneficial effects on reproductive performance compared with the LF diet because they enabled mice to generate milk more efficiently with less incidental heat production.

KEY WORDS: Central and peripheral limits, Heat dissipation limit, Dietary fat, Asymptotic food intake, Digestibility, Lactation performance, Laboratory mouse

INTRODUCTION

The sustained maximal rate of energy intake (E_{max} ; sometimes called SusEI) is the maximum rate of energy intake that animals can sustain over prolonged periods of time (Drent and Daan, 1980; Hammond and Diamond, 1992, 1997; Peterson et al., 1990; Speakman and Król, 2005a,b; Weiner, 1992). Energy is a key resource, and limits on its availability or expenditure may play an important role in shaping evolution of physiological, morphological and behavioural traits


(Johnson et al., 2001). In female mammals, limits to E_{max} are likely to be particularly important during the period of the highest energy demand, i.e. peak lactation (Hammond and Diamond, 1992; Hammond et al., 1994; Rogowitz, 1998; Speakman and Król, 2005a, 2011; Wu et al., 2009). Limits at peak lactation may determine the total investment that female mammals can make to their offspring and thus define maximum litter and offspring sizes, which are important life-history traits (Speakman, 2008).

The physiological basis of mechanisms imposing limits on E_{max} during reproduction has been the matter of much debate (Drent and Daan, 1980; Hammond and Diamond, 1992; Koteja, 1996; Krol et al., 2007; Rogowitz, 1998; Wu et al., 2009). Several theories have been advanced to explain the apparent limits on E_{max} (Hammond et al., 1996; Krol and Speakman, 2003a,b; Vaanholt et al., 2018; Wen et al., 2017). The two theories most supported by the current data are the ‘peripheral limitations’ theory (Hammond and Diamond, 1994; Hammond and Kristan, 2000; Koteja, 1996; Rogowitz, 1998) and the ‘heat dissipation limitation’ (HDL) theory (Sadowska et al., 2016; Simons et al., 2011; Wu et al., 2009; Yang et al., 2013), or a combination thereof (Speakman and Król, 2011; Wen et al., 2017). The ‘peripheral limitation hypothesis’ suggests that lactating animals are limited peripherally by the capacity of the mammary glands to produce milk (Hammond et al., 1996). Studies showing that female rodents with prolonged lactation (Hammond et al., 1994) or that had mammary tissue surgically removed (Hammond et al., 1996) could not respond to the increased demands with an increased translation of food intake into milk production show support for this idea. Also, studies of several species have suggested that, in the cold, despite the increased thermoregulatory demands of their offspring, energy exported as milk (E_{milk}) was not elevated, which is also consistent with the idea that milk production capacity of the mammary gland imposes the limit (Rogowitz, 1998; Yang et al., 2013; Zhao, 2011a,b, 2012).

However, experiments in which MF1 mice were exposed to varying ambient temperatures at peak lactation showed that lactating mice consumed more food and produced more milk to support the growth of heavier offspring in the cold compared with warm or hot conditions (Johnson et al., 2001; Krol and Speakman, 2003a,b). These results did not support the idea that limits to E_{max} during lactation are imposed peripherally at the mammary glands, but may be explained by the HDL theory (Krol and Speakman, 2003a,b). According to this theory, the limits to food intake at peak lactation are imposed centrally by processes of heat production during digestion and milk production that place the female mouse at risk of fatal hyperthermia. The HDL theory has found support, for instance, in experiments where animals were shaved to increase heat dissipation (Krol et al., 2007; Sadowska et al., 2016; Simons et al., 2011; Wu et al., 2009) or when animals were housed in hot conditions (Krol and Speakman, 2003a,b; Simons et al., 2011), although conflicting results have also been observed (Paul et al.,

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List of symbols and abbreviations

DLW	doubly labelled water
$E_{D EE}$	daily energy expenditure
E_{gei}	gross energy intake
E_{max}	maximum sustained energy intake
E_{mei}	metabolisable energy intake
E_{milk}	milk energy output
E_{ob}	obligatory energy expenditure
E_{SDA}	energy expenditure due to SDA
GE_{faeces}	gross energy content faeces
GE_{food}	gross energy content food
HDL	heat dissipation limitation
HF	high fat
l	efficiency of lactation
LF	low fat
MF	medium fat
M_{faeces}	dry mass faeces
M_{food}	dry mass food
M_{litter}	litter mass
M_{pup}	pup mass
s	proportion of net intake devoted to SDA
SDA	specific dynamic action

2010; Valencak et al., 2010; Zhao and Cao, 2009; Zhao et al., 2010; Zhao, 2011b). Discrepancies between studies may be explained by the idea that limits on E_{max} may be determined by an interplay of the peripheral limitation theory and HDL theory, with one becoming more dominant depending on the species/strain and/or the ambient temperatures at which animals are lactating (Speakman and Król, 2011; Wen et al., 2017).

Diets with different macronutrient contents have different specific dynamic action (SDA; also called heat increment of feeding) (Kagya-Agyemang et al., 2010; Secor, 2009). SDA refers to the increased metabolism (i.e. heat production) above resting rates after ingestion of food associated with digestion, assimilation and biosynthesis. It has been previously reported that diets with high carbohydrate content have higher SDA, i.e. cause a larger increase in metabolism, than those with high fat (HF) content at constant levels of dietary protein (Kagya-Agyemang et al., 2010). In MF1 mice, SDA was measured at 6.1, 4.5 and 3.9% (as a percentage of energy intake) for high-carbohydrate [low fat (LF)], medium-fat (MF) and HF diets, respectively (Kagya-Agyemang et al., 2010). Based on these differences in SDA, the HDL theory predicts that mice fed a MF/HF diet should be able to consume more food at peak lactation, with positive effects for milk production and growth of their offspring. To test this hypothesis, diets with constant protein content (20%) but variable contents of fat and carbohydrate were fed to MF1 mice between days 4 and 18 of lactation at 21°C.

Previous studies have focussed on energy requirements of pups without considering that the limits on growth of offspring might be related to their fat intake. Hence, limits on E_{max} of a MF or HF diet could be set by lower heat production from the females' perspective, but how this translates to growth of offspring may depend on the fat content in the milk. Therefore, samples of diets, milk produced by lactating females on LF, MF and HF diets, and offspring from females on LF, MF and HF diets were collected to determine their fatty acid profiles.

MATERIALS AND METHODS**Animal housing**

Sixty virgin female mice (*Mus musculus* Linnaeus 1758; outbred MF1, Harlan UK Limited, UK) aged 9- to 10-weeks old were

individually housed in cages (44×12×13 cm) with sawdust, paper wool bedding and cardboard tubes. Animals were maintained at 21°C (range 20–22°C) on a 12 h:12 h light:dark photoperiod, with lights on at 07:00 h. Food (standard rodent chow; 15.6 kJ g⁻¹ gross energy, 18.8% crude protein, 60.3% carbohydrate, 3.4% crude oil, 3.7% crude fibre and 3.8% ash; Special Diets Services, BP Nutrition, Witham, UK) and water were provided *ad libitum*. All procedures concerning animal care and treatment were approved by the Life Sciences and Medicine Ethics Review Board (CERB) of the University of Aberdeen and licensed by the UK Home Office (project licence: PPL 60/3606).

Experimental protocol

After acclimation to the housing conditions, mice were weighed and were allocated into three experimental groups ($N=20$ per group) ensuring an equal range of body masses in each group. Each female was paired with a male for 11 days, after which the males were removed. Mating was staggered so that each animal was likely to reach the end of gestation, or lactation, on a different day.

After the males had been removed, daily measurements of female body mass and food intake were taken 1 h after lights on using a top-pan balance (Mettler Toledo, Switzerland, ±0.01 g). On the day of parturition (day 0 of lactation), no measurements were made on the lactating mothers and their pups. From days 1 to 18 of lactation, maternal body mass, litter size, litter mass (M_{litter}) and pup mortality were monitored daily. On days 2–3 of lactation, mothers from each experimental group were presented with either a HF (60% energy from fat; D12492, Research Diets), MF (45% energy from fat; D12451) or LF (10% energy from fat; D12450B) diet while still supplied with standard rodent chow *ad libitum* (Research Diets, New Brunswick, NJ, USA). From day 4 onwards, the animals were switched from the mixed diets to HF, MF or LF diet exclusively. Maternal food intake was measured daily between days 5–18 of lactation. Four females did not get pregnant, reducing the final sample size to 19, 19 and 18 in HF, MF and LF groups. All pups were weaned on day 18 of lactation, culled by CO₂ overdose and frozen until further analysis (see Fatty acid analyses of diets, milk and pup tissues).

Metabolisable energy intake (E_{mei})

Total faeces produced by mice between days 11 and 15 of lactation were collected. The faeces were manually separated from the sawdust. Samples of each diet and faeces were weighed and dried (Gallenkamp oven at 60°C) for 14 days to obtain dry mass. These were subsequently analysed for gross energy content using bomb calorimetry (Gallenkamp Autobomb Adiabatic Bomb Calorimeter; Rowett Research Institute Analytical Services, Bucksburn, Aberdeen, UK; for a detailed description see Krol and Speakman, 2003b). The gross energy content of the diets (GE_{food} , kJ g⁻¹) and the gross energy content of the faeces (GE_{faeces} , kJ g⁻¹) were used to estimate gross energy intake (E_{gei} , kJ day⁻¹ = $M_{dfi} \times GE_{food}$, where M_{dfi} is the dry mass of the food eaten in g) and energy lost in faeces (E_f , kJ day⁻¹ = $M_f \times GE_{faeces}$, where M_f is dry mass of faeces produced in g). For each individual throughout lactation, metabolisable energy intake (E_{mei} ; previously also referred to as MEI) was obtained by subtracting E_f from E_{gei} , and assimilation efficiency (AE, %) of the diets was estimated using the following formula: $AE = [(E_{gei} - E_f) / E_{gei}] \times 100$.

Doubly labelled water measurements

The doubly labelled water (DLW) method (Butler et al., 2004) was used to measure daily energy expenditure (E_{DEE}) from the

elimination rates of ^2H (deuterium) and ^{18}O in lactating females during peak lactation. Measures of E_{DEE} were made to determine the milk energy output (E_{milk} ; previously MEO) from the difference between E_{mei} and E_{DEE} (Krol and Speakman, 2003b).

The DLW measurements were conducted on the 56 lactating females (HF, $N=19$; MF, $N=19$; and LF $N=18$). On day 16 of lactation (between 8:00 h and 11:00 h), individual mice were weighed to ± 0.01 g using a balance (Mettler Toledo, Switzerland) and labelled with an intra-peritoneal injection of approximately 0.2 g of water containing enriched ^2H (36.3 atoms%) and ^{18}O (59.9 atoms%). Syringes used to inject the DLW were weighed (± 0.0001 g; Ohaus Analytical Plus, Brooklyn, USA) immediately before and after the injection to provide an accurate measurement of the amount of the isotope injected. Mice were placed in their cages during the 1 h equilibration period. An initial 30–80 μl blood sample was collected by tail tipping 1 h after the injection (Krol and Speakman, 1999). Blood samples were immediately flame-sealed into pre-calibrated 50 μl pipettes and stored at 4°C until analysis. A final blood sample was collected approximately 48 h after the initial blood sample was collected to estimate isotope elimination rates.

Samples of blood in capillaries were vacuum-distilled (Nagy, 1983) and water from the resulting distillate was used to produce CO_2 and H_2 . Gas source isotope ratio mass spectrometer was used to analyse the isotope ratios of ^{18}O : ^{16}O and ^2H : ^1H . The samples were run alongside high enrichment standards that were used to correct the raw data to these standards. For each lactating mouse, initial ^2H and ^{18}O dilution spaces were calculated by the intercept method and then converted to mass assuming a molecular mass of body water of 18,020 and expressed as a percentage of body mass before injection. The intercept method was used since the actual body water pool estimated by desiccation using the intercept method is more accurate than the plateau method in small mammals (Speakman and Król, 2005b). The final ^2H and ^{18}O dilution spaces were inferred from the final body mass, assuming the same percentage of body mass as measured for the initial dilution spaces. For calculation of E_{DEE} based on CO_2 production, single pool model Eqn 7.17 (Speakman, 1997) was used. Energy equivalents of rates of CO_2 production were calculated using a conversion factor of $24.03 \text{ J ml}^{-1} \text{ CO}_2$, derived from the Weir equation (Weir, 1949).

Female total water turnover was calculated by multiplying the fractional turnover rate by the total body water. It was assumed that 25% of the water leaving the body was fractionated (Speakman, 1997). Therefore, a fractionation factor of 0.9366 was applied for deuterium turnover (Speakman, 1997). This approach assumes that rates of water influx and efflux are constant, so the water turnover rate $r\text{H}_2\text{O} = \text{total water influx} = \text{total water efflux}$ (Nagy and Costa, 1980).

Milk collection and analysis

On day 16 of lactation, a sample of 18 females (HF, $N=6$; MF, $N=4$; and LF, $N=8$), with litter sizes ranging from 6 to 16 pups, were separated from their pups for approximately 3 h. After this separation, which was not long enough to affect milk production (Johnson et al., 2001; Krol and Speakman, 2003b), milking was performed manually from all teats after intraperitoneal injection of oxytocin (1 IU) under light isoflurane anaesthesia (Abbot Laboratories Ltd, Queensborough, UK). Oxytocin was used to stimulate milk let-down. Each mammary gland was palpated towards the nipple area and droplets of milk were collected in capillary tubes. Milk collection continued until no milk could be expressed. All the milk samples (0.4–0.6 ml from each female) were snap frozen and kept at -80°C until further analysis for fatty acids.

All analyses (Rowett Research Institute Analytical Services, Aberdeen, UK) were made on duplicate dried samples.

Fatty acid analyses of diets, milk and pup tissues

Samples of the different diets, and total pups weaned from mothers fed on HF, MF and LF diets, respectively, were thawed, individually weighed (± 0.001 g, Ohaus Analytical Plus), and dried in a convection oven at 60°C for 14 days to constant mass. Dried samples of total pups for each of the diets were homogenised and a subsample was used for further analysis ($n=1$ per diet group). The total lipid fractions of diets and pup tissues were extracted using a modification of the methods of Bligh and Dyer (1959). Each dry sample was ground and dissolved in 24 ml chloroform/methanol (2:1). After mixing the dissolved mouse tissues and each of the diets with water (6 ml), the aqueous and chloroform/methanol phases were separated by centrifugation (2500 g) for 5 min. The aqueous layer was then discarded and the lipid layer of each sample was filtered through a Whatman IPS filter paper. The solvents were evaporated at 40°C under vacuum and the resulting lipid extracts (~ 50 mg) were saponified with 1 ml 0.5 mol l^{-1} potassium hydroxide in 95% ethanol for 90 min at 100°C . After adding diethyl ether (9 ml) and water (3 ml), the aqueous phase (containing saponified material) and ether phase (containing non-saponified material) were separated by centrifugation (2500 g) for 5 min. The aqueous layer was then acidified with 5 mol l^{-1} sulphuric acid and free fatty acids extracted with 9 ml hexane/diethyl ether (19:1). The fatty acid extracts were stored at -20°C until further analysis.

Dry milk samples (~ 0.1 g) from lactating mice fed each of the three diets were defrosted at room temperature and thoroughly mixed on a vortex mixer. The lipid fraction was extracted from milk using a method modified from that of Bligh and Dyer (1959). Each milk sample was dissolved in chloroform, methanol and water at a ratio of 2:2:1.8. After this process, the aqueous and chloroform/methanol phases were separated by centrifugation (2500 g) for 5 min. The aqueous layer (top) was then discarded and the lipid layer of each sample was filtered through a Whatman IPS filter paper. The solvents were evaporated at 40°C under vacuum and the resulting lipid extracts (~ 50 mg) were saponified with 1 ml of 0.5 mol l^{-1} potassium hydroxide in 95% ethanol for 90 min at 100°C . After cooling, the samples were acidified to pH 1 with 2 mol l^{-1} hydrochloric acid (HCl). Saponified lipids were extracted into hexane, washed with distilled water and then dried over anhydrous sodium sulphate. All lipid samples were stored at -20°C under nitrogen in a glass vial secured with an aluminium-lined screw cap until further analysis.

Fatty acid composition

The lipid extracts of the three diets, milk samples and pups tissues were trans-esterified to produce fatty acid methyl esters (FAMES). FAMES were prepared by reacting total lipids (10–20 mg) with dry methanol (0.5 ml) containing 2 mol l^{-1} HCl for 2 h at 100°C , and then dissolving the lipids in 1 ml hexane/diethyl ether (19:1). After mixing the dissolved FAMES with water (0.5 ml), the aqueous and hexane/diethyl ether phases were separated by centrifugation (2500 g) for 5 min and the lipid layer dried by passing through anhydrous sodium sulphate. The solvents were evaporated at 35 – 40°C under a stream of nitrogen and the residue FAMES were taken up in hexane containing 0.02% butylated hydroxytoluene (BHT).

Analysis of the FAMES was by capillary gas chromatography (GC) using a Hewlett Packard 5890A (Hewlett Packard, Sunnyvale,

CA, USA) fitted with a 50 m×0.25 mm CP-SIL 88 column coated with a 0.25 µl film thickness (J & W Scientific, Folsom, CA, USA). The GC temperature was programmed from 160°C, held for 1 min, increased by 10°C min⁻¹ to 190°C, held for 3 min, again increased by 2°C min⁻¹ to 230°C and finally held for 15 min. Samples (1 µm) run in duplicates were injected into a split injection system (1:15) and carried through the GC column with helium as the carrier gas.

The GC was linked to a computerised integration system (Unicam 4880 software) to identify the peaks by comparison with absolute retention time (RT) from a standard mixture (Supelco UK, Poole, Dorset, UK). The standard was run daily to determine accurate RTs. Individual fatty acids are designated in International Union of Pure and Applied Chemistry (IUPAC) shorthand nomenclature by carbon chain length:number of double bonds. A total of 10, 11 and 10 fatty acids were identified in the HF, MF and LF diets, respectively. In the milk samples from lactating mice fed on HF, MF and LF diets, 18, 20 and 18 fatty acids, respectively, were identified. Finally, 16, 15 and 14 fatty acids were identified in tissues of pups weaned from lactating females fed on HF, MF and LF diets, respectively. Each fatty acid from diets, milk samples and tissues of pups was expressed as a percentage of the total fatty acids identified in each sample. All fatty acids identified in the diets, milk samples and tissues of pups were used in the comparison of total fatty acid profile.

Body composition and organ morphology

Body composition (fat and fat-free mass) was derived from organ morphology data. On day 18 of lactation, the 56 mothers were weighed and killed by CO₂ inhalation, and immediately dissected. The brain, intrascapular brown adipose tissue, thyroid gland, liver, kidneys, lungs, heart, spleen, gonadal fat, gonads, abdominal fat, mesenteric fat, subcutaneous fat, pancreas, stomach, small intestine and large intestine were removed. The remaining parts were divided into tail, pelage and carcass. The wet mass of tissues was recorded (±0.0001 g; Ohaus Analytical Plus) and tissues dried (Gallenkamp oven at 60°C) for 14 days, and re-weighed to determine dry mass.

Statistical analyses

Body mass and food intake were measured daily throughout lactation, and differences between the dietary groups in body mass and food intake were tested using repeated measures general linear models (RM GLM) with group (different dietary groups; HF, MF and LF) as a fixed factor and day of lactation as the repeated factor. Where significant effects of day or diet were found, *post hoc* Tukey tests were used to assess differences between days and groups. Asymptotic food intake across the three dietary treatment groups in late lactation was compared using one-way ANOVA and *post hoc* Tukey tests. The asymptotic food intake in late lactation was defined as the period during which no significant differences in food intake between days were detected (day 12–17 of lactation). Linear regression analysis was used to examine relationships between variables. Changes in body mass and organ morphology between the dietary groups were compared using one-way ANOVA and *post hoc* Tukey tests. The effects of maternal body mass on wet and dry tissue masses were also assessed using GLM. A Bonferroni correction was applied (significance level divided by the number of comparisons) in assessing the wet and dry organ masses in lactating mice. Data are represented as means±s.d. unless stated otherwise. All data were tested for normality prior to analysis and all statistical analyses were performed using Minitab for Windows (version 14; Minitab Inc., State College, PA, USA). *Post hoc* power analysis using Minitab 18 were performed to ascertain that 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

Maternal body mass

No significant difference between the body mass of HF females (29.51±1.29 g, $N=20$), MF females (29.52±1.33 g, $N=20$) and LF females (29.52±1.42 g, $N=20$; ANOVA: $F_{2,57}=0.01$, $P=0.99$) were observed before mating and during pregnancy (at parturition body mass was 53.96±4.18 g, 55.83±5.06 g and 56.28±5.24 g for HF, MF and LF females, respectively; ANOVA: $F_{2,57}=0.87$, $P=0.419$) or during lactation ($F_{2,53}=0.78$, $P=0.46$; Fig. 1A, Table 1).

Food intake

Gross food intake did not differ significantly between dietary groups before mating (5.28±0.51, 5.28±0.27 and 5.27±0.36 g day⁻¹ for HF, MF and LF females, respectively; ANOVA: $F_{2,9}=0.01$, $P=0.999$) and during pregnancy (6.54±1.0, 6.29±0.94 and 6.57±1.24 g day⁻¹; ANOVA: $F_{2,57}=1.78$, $P=0.169$).

Diet manipulation started on day 4 of lactation, so there were no food intake data for days 1–4 of lactation when animals were fed a mix of rodent chow and the target diets. RM GLM over days 5–18 of lactation showed that there was a highly significant effect of day of lactation ($F_{13,689}=278.2$, $P<0.001$) and diet ($F_{2,53}=5.2$, $P=0.009$) on maternal gross food intake (Table 1). Between days 5 and 11 of lactation, food intake increased steadily in HF, MF and LF females and, over the next 6 days (days 12–17), food intake reached an asymptote and remained constant at an average of 14.95±1.14 g day⁻¹, 16.30±0.61 g day⁻¹ and 16.57±0.26 g day⁻¹ for mice fed HF, MF and LF diets, respectively; i.e. asymptotic food intake (Table 1, Fig. 1B).

The gross energy content of the food (GE_{food}) was measured at 23.11 kJ g⁻¹, 22.89 kJ g⁻¹ and 17.80 kJ g⁻¹ for HF, MF and LF diets, respectively, and gross energy content of the faeces (GE_{faeces}) when feeding on the three diets was 20.91±1.26, 20.96±0.59 and 15.93±0.70 kJ g⁻¹ dry mass, respectively. These values were used to calculate E_{mei} at peak lactation as 306.52±25.03, 340.52±13.49 and 266.67±4.45 kJ day⁻¹ for HF, MF and LF diets, respectively, which were significantly higher in mice on MF and HF diets versus mice on the LF diet (Table 1, Fig. 1B, $P<0.05$).

Litter size, litter mass and pup mass

Mice on all diets gave birth to, and weaned, a similar number of pups [Fig. 1C, Table 1; 10.7±2.7 pups on average (Fig. 1B, Table 1; RM GLM: day of lactation: $F_{17,901}=4.7$, $P<0.001$, group: $F_{2,53}=1.4$, $P=0.26$]. There was a significant difference in M_{litter} between the dietary groups, with mice feeding on HF or MF diets weaning 30–35% larger litters compared with mice fed a LF diet (RM GLM: day of lactation: $F_{17,901}=708.2$, $P<0.001$, group: $F_{2,53}=2.4$, $P=0.099$, lactation×group: $F_{34,901}=9.3$, $P<0.001$; Fig. 1D, Table 1). Similarly, pup mass (M_{pup}) at weaning, but not at birth, was significantly increased in the HF and MF groups compared with the LF group. M_{litter} and M_{pup} of offspring raised by mothers fed HF and MF diets did not differ significantly from each other (Table 1, $P>0.05$).

Daily energy expenditure (E_{DEE}) and milk energy output (E_{milk})

E_{DEE} measured on day 16 of lactation was not significantly different between dietary groups (Table 1). In line with the results on E_{mei} , E_{milk} was significantly increased in females fed HF or MF diets

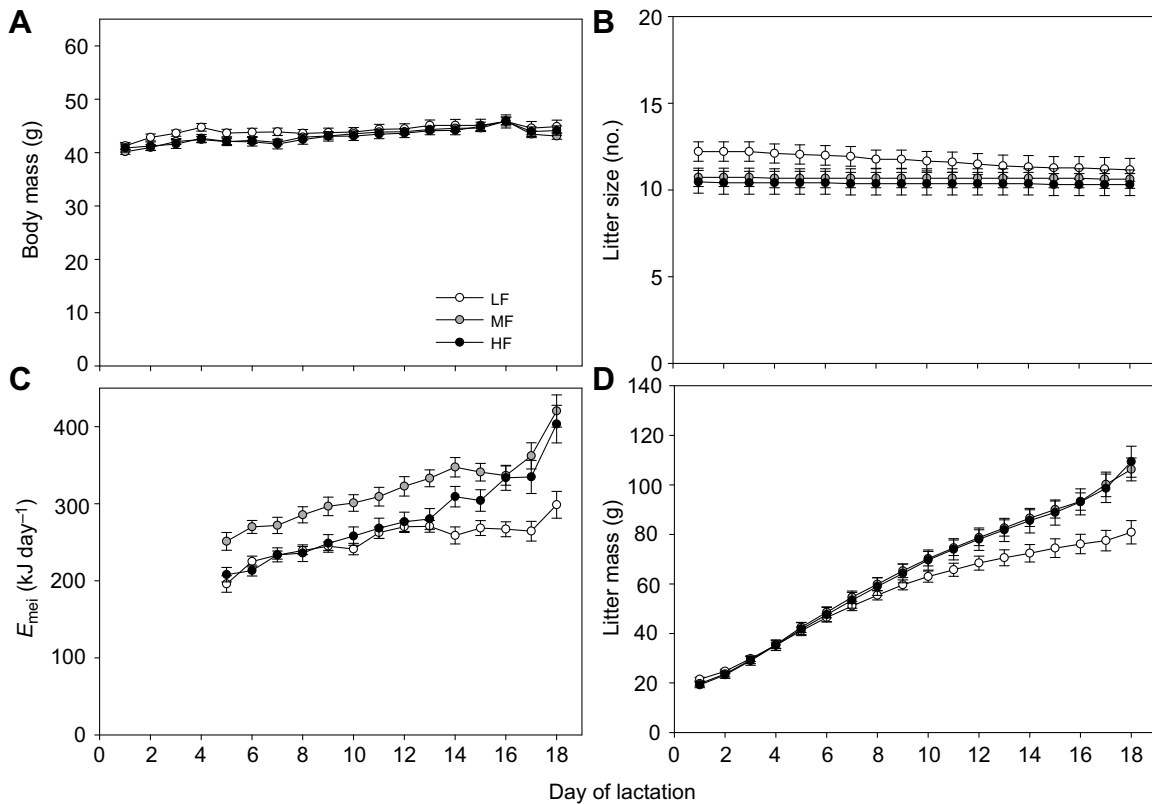


Fig. 1. Body mass, litter size, metabolisable energy intake and litter mass of mice fed on different diets. Means \pm s.e.m. body mass (A), metabolisable energy intake (E_{mei}) (C), litter size (B) and litter mass (D) throughout lactation in mice fed high fat (HF, $n=19$), medium fat (MF, $n=19$) or low fat (LF, $n=18$) diets. Mice were swapped to their respective diets on day 4 of lactation and therefore data on metabolisable energy intake is not available for day 1–4 of lactation.

compared with females fed LF diets, and on average was increased by approximately 30%. Linear regression revealed significant relationships between E_{mei} , litter growth and E_{milk} , and a highly significant effect of fat intake on E_{mei} and E_{milk} (Fig. 2).

Fatty acid composition

To examine whether fat from the diet fed to each group of lactating mice was transferred into milk for suckling pups, comparison of corresponding total lipid fatty acid profiles between the diets, milk and pup tissues was performed on all fatty acids that constituted more than or equal to 1% of the identified fatty acids (Table S1, for

HF, MF and LF diets). This chemical analysis showed that the most abundant fatty acids in the diets corresponded to those in the milk, and the most abundant fatty acids in the milk corresponded to those in the tissues of the pups (Fig. 3). For instance, the most abundant fatty acids in the HF diet were C18:1/oleic acid (50.4%), followed by C18:2/linoleic (24.2%), C16:0/palmitic (20.7%), C16:1/palmitoleic (2.1%) and C20:1 (1.2%) acids (Table S1; Fig. 3A). The most abundant fatty acid in milk was oleic acid (30.78%), followed by palmitic (21.91%), linoleic (21.72%) and palmitoleic (2.13%) acids (Table S1; Fig. 3A,B), and, similarly, the most abundant fatty acid in offspring tissues was oleic acid (38.11%),

Table 1. Descriptive statistics for variables measured in lactating mice fed diets with different fat content

Variables	HF	MF	LF	ANOVA	
				$F_{2,53}$	P
Body mass (g; d1)	41.3 \pm 2.8	40.1 \pm 2.0	40.9 \pm 3.4	0.1	0.918
Asymptotic food intake (g day ⁻¹)	14.9 \pm 1.0 ^b	16.3 \pm 0.6 ^a	16.5 \pm 0.3 ^a	7.8	<0.01
Metabolisable energy intake (kJ day ⁻¹)	306.5 \pm 25.0 ^b	340.5 \pm 13.5 ^a	266.6 \pm 4.5 ^c	29.7	<0.001
Milk energy output (kJ day ⁻¹)	203.2 \pm 49.9 ^a	229.3 \pm 42.2 ^a	164.6 \pm 30.6 ^b	11.6	<0.001
Daily energy expenditure (kJ day ⁻¹)	103.3 \pm 12.6	111.2 \pm 9.7	102.1 \pm 9.7	2.6	0.083
Litter size (d1)	10.5 \pm 2.8	10.7 \pm 2.3	12.2 \pm 2.4	6.4	0.088
Litter size (d18)	10.3 \pm 2.8	10.6 \pm 2.4	11.2 \pm 2.8	0.5	0.614
Litter mass (g; d1)	19.2 \pm 4.9	19.7 \pm 4.3	21.5 \pm 3.2	–	–
Litter mass (g; d18)	109.3 \pm 27.3 ^a	106.2 \pm 20.0 ^a	80.8 \pm 20.0 ^b	8.7	0.001
Pup mass (g; d1)	1.85 \pm 0.18	1.84 \pm 0.13	1.78 \pm 0.17	–	–
Pup mass (g; d18)	10.98 \pm 2.59 ^a	10.26 \pm 2.10 ^a	7.68 \pm 2.56 ^b	5.83	<0.01

Descriptive statistics for lactating mice fed high fat (HF; 60% energy from fat), medium fat (MF; 45% energy from fat) or low fat (LF; 10% energy from fat) diets from day 4–18 of lactation. Values shown are means \pm s.d. Sample sizes were 19, 19 and 18 for HF, MF and LF diets, respectively. All data were analysed using one-way ANOVA with diet as a fixed factor. Where a significant effect of diet was found, results of *post hoc* Tukey tests are indicated using superscript a, b and c; i.e. groups that have a similar letter did not differ significantly and groups with a different letter differed significantly ($P<0.05$). d, day of lactation.

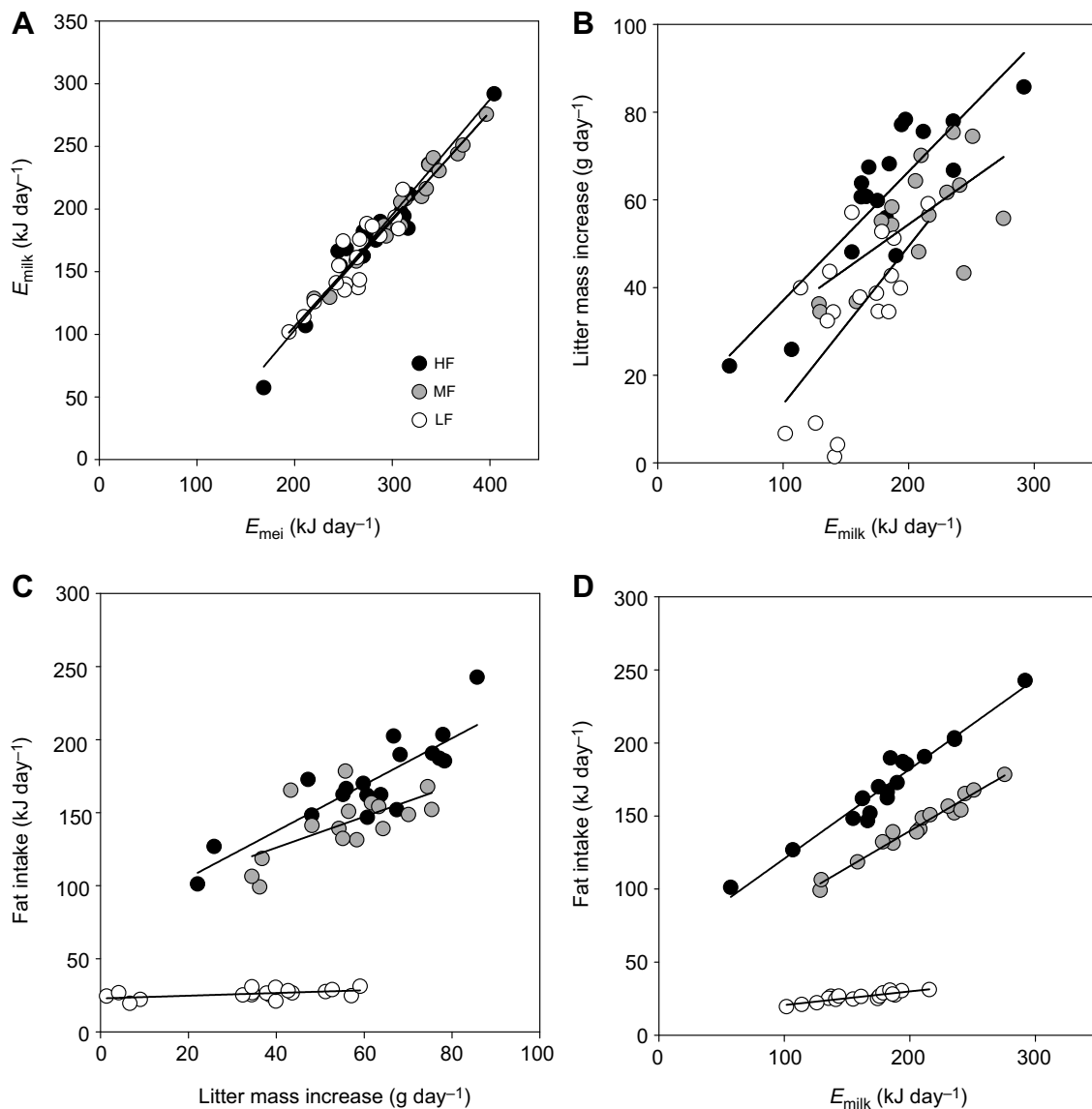


Fig. 2. Relationships between milk energy output (E_{milk}), metabolisable energy intake (E_{mei}), fat intake and litter mass increase. Litter mass increase was calculated over days 4–18 of lactation. (A) Linear regression between E_{mei} and E_{milk} . LF: $R^2=0.80$, $P<0.0001$, $y=0.86x-65.57$; MF: $R^2=0.97$, $P<0.0001$, $y=0.87x-68.88$; HF: $R^2=0.94$, $P<0.0001$, $y=0.92x-80.70$. (B) Linear regression between E_{milk} and litter mass increase. LF: $R^2=0.38$, $P=0.0063$, $y=0.36x-22.65$; MF: $R^2=0.44$, $P=0.0053$, $y=0.20x+13.78$; HF: $R^2=0.73$, $P<0.0001$, $y=0.29x+7.66$. (C) Linear regression between litter mass increase and fat intake. LF: $R^2=0.27$, $P=0.027$, $y=0.09x+22.85$; MF: $R^2=0.40$, $P=0.0008$, $y=1.06x+83.62$; HF: $R^2=0.74$, $P<0.0001$, $y=1.59x+73.64$. (D) Linear regression between E_{milk} and fat intake. LF: $R^2=0.80$, $P<0.0001$, $y=0.09x+11.25$; MF: $R^2=0.97$, $P<0.0001$, $y=0.50x+38.96$; HF: $R^2=0.94$, $P<0.0001$, $y=0.61x+59.34$.

followed by palmitic (20.74%), linoleic (17.50%), palmitoleic (2.32%) and C20:2/ (1.01%) acids (Table S1; Fig. 3B). The fatty acid profiles in the HF or MF diets were significantly related to those in the milk produced ($R^2=0.86$, $P=0.07$ and $R^2=0.92$, $P=0.04$ in HF and MF diet, respectively), but not in the LF diet ($R^2=0.48$, $P=0.13$). Similarly, significant relationships were found between fatty acid profiles in the milk and pups on the HF or MF diets, but not on the LF diet (Fig. 3B; HF: $R^2=0.86$, $P=0.07$; MF: $R^2=0.73$, $P=0.03$; LF: $R^2=0.19$, $P=0.39$).

Effect of diet on maternal organ morphology

To evaluate the effects of the HF, MF and LF dietary treatments on maternal morphology, the dry masses of 20 organs were compared. There were significant differences between dietary groups in the masses of gonadal fat, stomach and liver (Table S2), but not in any

of the other tissues. When compared with the mice fed an LF diet, the HF and MF mothers deposited an extra fat mass of 0.21 g (equivalent to 0.57 kJ) and 0.191 g (equivalent to 0.53 kJ) into the gonadal fat, respectively.

Mathematical model predicting intake of HF and MF diets at peak lactation based on SDA and heat dissipation limits

We built a mathematical model based on the HDL theory to predict the exact food intake and milk production of the mice fed MF and HF diets based on the lower SDA of these two diets (Table 2, Fig. 4A; Kagya-Agyemang et al., 2010). The model is as follows.

Define the maximum heat production that an animal can sustain under the HDL theory as E_{max} . This will be equal to the maximum DEE (E_{DEE}). Define also an obligatory energy expenditure that the animal must have to survive independent of its milk production and

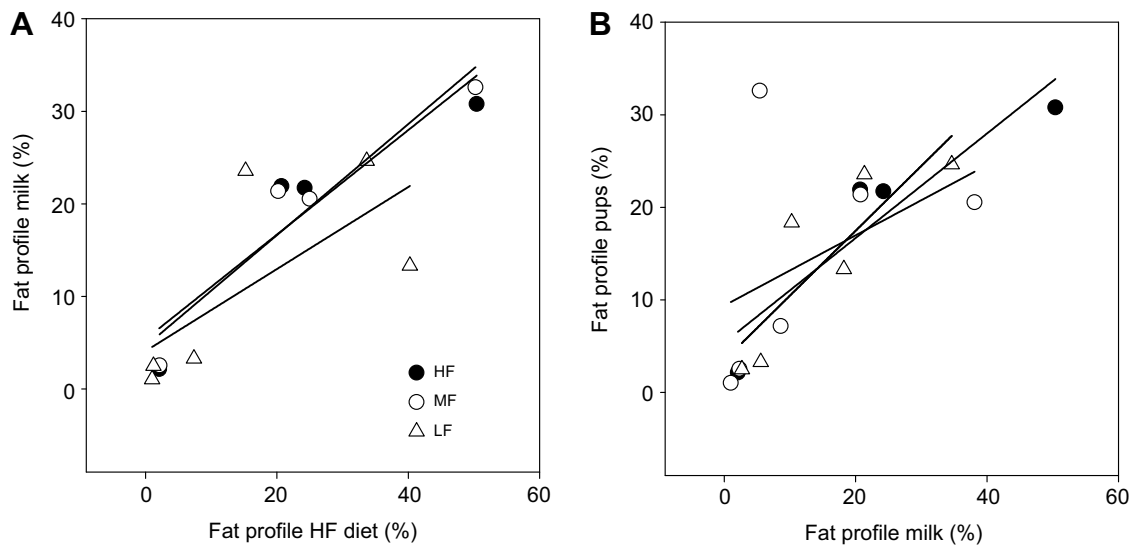


Fig. 3. Linear regressions between fat profiles measured in the three different diets (HF, MF and LF), the milk produced by lactating female mice on those diets and in the tissues of their pups. Fatty acids identified in the diet, milk or pups were expressed as a % of the total fat content. (A) Linear regressions were performed between all the fatty acids identified that constituted 1% or more of the total fat content in both the diets and milk samples. HF diet: $y=0.57x+5.37$, $R^2=0.86$, $P=0.07$; MF diet: $y=0.60x+4.61$, $R^2=0.92$, $P=0.04$; and LF diet: $y=0.44x+4.08$, $R^2=0.48$, $P=0.13$. (B) Similarly, linear regressions were performed between the fatty acids identified in milk and tissues of pups. HF diet: $y=0.57x+5.37$, $R^2=0.86$, $P=0.07$; MF diet: $y=0.70x+3.44$, $R^2=0.73$, $P=0.03$; and LF diet: $y=0.38x+9.38$, $R^2=0.19$, $P=0.39$.

the SDA resulting from food as E_{ob} . The energy expenditure due to SDA can be defined as:

$$E_{SDA} = E_{mei} \times s, \quad (1)$$

where E_{mei} is the metabolisable energy intake and s is the proportion of net intake devoted to SDA. Define also the energy expenditure consequent of milk production (lactogenesis) as:

$$E_{lact} = E_{milk} \times l, \quad (2)$$

where E_{milk} is the milk energy exported and l is the efficiency of lactation.

The gap between the maximum possible expenditure of energy under the HDL constraint and the obligatory expenditure (E_{ob}) is equal to the combined energy devoted to SDA (E_{SDA}) and lactogenesis (E_{lact}). Hence:

$$E_{max} - E_{ob} = E_{SDA} + E_{lact}. \quad (3)$$

Substituting Eqns 1 and 2 into Eqn 3 gives:

$$E_{max} - E_{ob} = (E_{mei} \times s) + (E_{milk} \times l). \quad (4)$$

For mice on the LF diet, we can substitute known values of the parameters to estimate E_{ob} and hence the size of the gap between E_{max} and E_{ob} . Specifically, from the DLW method we know that

E_{DEE} at peak lactation (E_{max}) in the LF mice was $102.1 \text{ kJ day}^{-1}$. We also know that the net energy intake (E_{mei}) was $266.7 \text{ kJ day}^{-1}$ and the E_{milk} was $164.6 \text{ kJ day}^{-1}$. The LF diet has an SDA (s) of 0.061 (Kagya-Agyemang et al., 2010), and Krol et al. (2007) estimated the efficiency of lactation (l) on a diet with very similar composition as 0.3. Substituting these values into Eqn 4 gives:

$$E_{ob} = 102.1 - [(266.7 \times 0.061) + (164.5 \times 0.3)] \\ = 102.1 - (16.3 + 49.3) = 36.5 \text{ kJ day}^{-1}. \quad (5)$$

We can then quantify the heat gap between the maximal heat dissipation and the obligatory heat production as:

$$E_{max} - E_{ob} = 102.1 - 36.5 = 65.6 \text{ kJ day}^{-1}. \quad (6)$$

Substituting this back into Eqn 4 shows that, for the other two diets:

$$(E_{mei} \times s) + (E_{milk} \times l) = 65.6 \text{ kJ day}^{-1}. \quad (7)$$

To find the maximal net energy intake that the mice could eat under the heat dissipation limit, we need to solve Eqn 7 for E_{mei} . Unfortunately, even if we keep l constant at 0.3 and substitute in the known value of s for a given diet, we still have an equation with two unknowns. However, the milk energy export (E_{milk}) is equal to the

Table 2. Model for using specific dynamic action (SDA) results to predict the intake and milk production of females when fed HF and MF diets if intake and milk production are limited by heat dissipation capacity

Parameter	LF actual	MF predicted	HF predicted	HF actual	Difference to predicted	MF actual	Difference to predicted
F_i	266.7	278.9	283.8	306.5	22.6	340.5	61.6
E_{milk}	164.6	176.8	181.8	203.2	21.4	229.3	52.4
E_{max} ($=E_{DEE}$)	102.1	102.1	102.1	103.3	1.20	111.2	9.10
SDA (s)	16.3	12.5	11.1				
Lactogenesis (l)	49.3	53.0	54.5				
HDL model prediction: $s+l=65.6 \text{ kJ day}^{-1}$							

All values are kJ day^{-1} . SDA of the HF (high fat) diet was 0.039; SDA of the MF (medium fat) diet was 0.045; SDA of the LF (low fat) diet was 0.061; LF intake= 16.6 g day^{-1} . F_i , metabolisable energy intake; E_{milk} , milk energy output; E_{DEE} , daily energy expenditure; HDL, heat dissipation limitation.

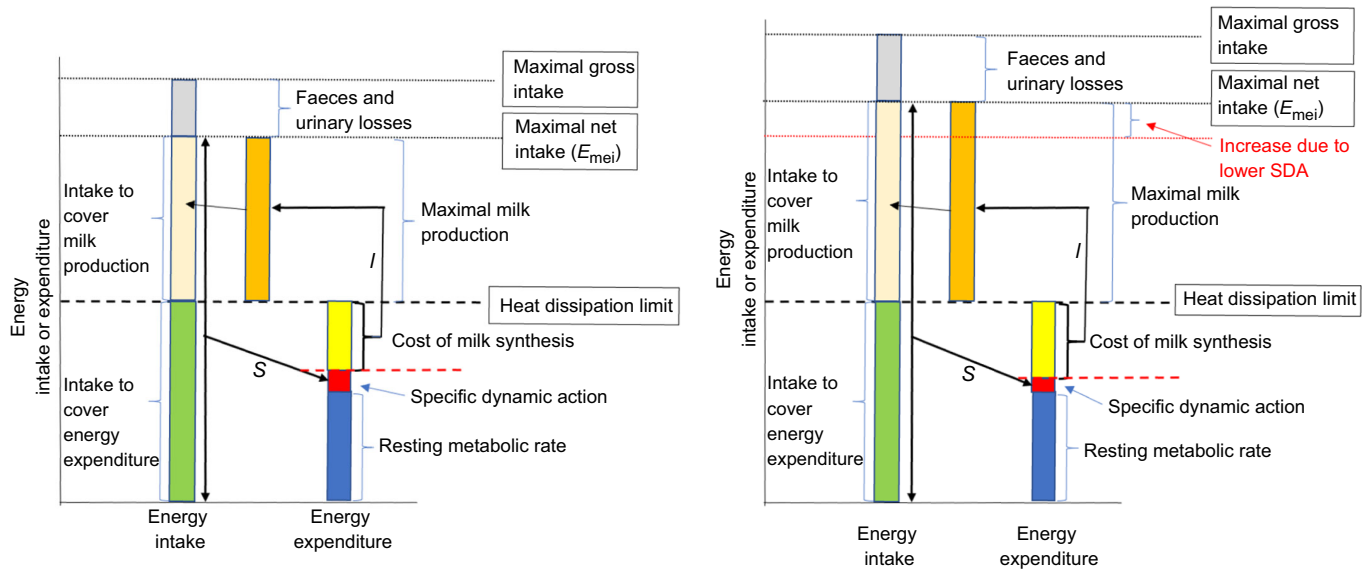


Fig. 4. Schematic diagrams illustrating the mathematical model for predicting maximal energy intake and energy expenditure based on the heat dissipation limitation (HDL) theory incorporating changes in specific dynamic action. (A) Schematic to show the basis of the mathematical model for predicting maximal energy intake and energy expenditure based on the HDL theory. The energy expenditure is made up of the resting metabolic rate (blue bar), which is obligatory, and the specific dynamic action (SDA; red bar). The SDA is a fixed % of the metabolisable energy intake (E_{mei}) defined by the parameter s . The sum of these two costs (dashed red line) is lower than the maximal capacity to dissipate heat (shown as a dashed black line marked 'heat dissipation limit'). The distance between the dashed red and dashed black lines can be filled with expenditure for synthesising milk. The size of this gap then defines the maximal milk production (orange bar) via an efficiency of lactation term (l). The animal needs to eat food to cover the sum of its energy expenditures (green bar) plus the energy exported in milk (light orange), plus any losses that occur in faeces and urine (grey bar). (B) This schematic shows how these predictions alter when s changes because of the change in macronutrient content of the diet. Specifically, this schematic concerns the situation when s is reduced because the animal feeds on a diet containing increased fat, which has a lower s value. In this case, the SDA (red bar) gets smaller and this then makes the gap between the red dashed and black dashed lines greater. The animal can then engage in more milk synthesis (yellow bar), leading to greater milk production (larger orange bar). The previous level of maximal milk production is indicated by the dotted red line. However, this then increases the maximal net food intake (green plus light orange bars), which in turn leads to increase the SDA. There is consequently an optimisation of the level of intake that allows the animal to maximise its milk production at any given value of s . That optimisation problem is solved by the mathematical model in the text. Generally, as s falls, the maximal potential milk production increases and the model allows this effect to be exactly quantified. The key aspect of the model is the fixed nature of the HDL (dashed black line), which defines the envelope within which the expenditure components must be optimised.

difference between the net food intake and the maximal heat dissipation, i.e.:

$$E_{milk} = E_{mei} - E_{max}. \quad (8)$$

Hence, substituting Eqn 8 into Eqn 7 and inserting the values under the LF diet for E_{max} and l gives:

$$(E_{mei} \times s) + [0.3 \times E_{mei} - (30.6)] = 65.6 \text{ kJ day}^{-1}. \quad (9)$$

Simplifying and solving this equation for E_{mei} gives:

$$E_{mei} = 96.2 / (s + 0.3) \text{ kJ day}^{-1}. \quad (10)$$

Hence, for any diet with known s we can calculate from Eqn 10 the maximal metabolisable energy intake under the HDL theory (E_{mei}). Further, substituting this value of E_{mei} and the known value of E_{max} (102.1) into Eqn 8 gives us the predicted maximal milk energy export (E_{milk}). We used this model to generate predicted values of E_{mei} and E_{milk} for the MF and HF diets (Table 2, Fig. 4B).

These predictions allow a direct comparison to the actual figures for these parameters in mice fed the HF and MF diets to evaluate whether the responses of the mice was quantitatively consistent with the prediction of the HDL theory (Table 2). These comparisons show that the mice were able to export substantially more milk when feeding on the MF and HF diets than predicted by the model based on the HDL theory. Specifically, the model predicted the E_{mei} in MF-fed mice should not exceed $278.9 \text{ kJ day}^{-1}$, with a corresponding E_{milk} of $176.8 \text{ kJ day}^{-1}$, yet they took in

61.6 kJ day^{-1} more, and exported as milk 52.5 kJ day^{-1} more, than the model predictions. In a similar manner, the E_{mei} and E_{milk} of the HF-fed mice were 22.6 kJ day^{-1} and 21.4 kJ day^{-1} higher, respectively, than the model predictions (Table 2).

DISCUSSION

Feeding diets high in fat to lactating mice impacted positively on reproductive performance, i.e. the HF- and MF-fed mice consumed more energy at peak lactation, so the energy available for milk production was greatly increased. As a result, pups from mothers fed HF and MF diets were heavier at weaning than those from LF-fed mice. These findings qualitatively agree with expectations from the HDL theory, as diets high in fat content have lower SDA and lactating mice on these diets would be expected to be able to consume more food before reaching the limit on heat dissipation. However, a model constructed using predictions from the HDL hypothesis and measurements in the LF mice showed that mice on the MF and HF diets increased their food intake and milk production more than the HDL theory predicted they should be capable of (Table 2).

Beneficial effects of HF feeding on energy content of the milk, growth and survival of offspring has been shown in several species. For instance, in sows, increased dietary fat intake elevated milk fat concentration and promoted faster growth in piglets throughout lactation (Averette et al., 1999; Van den Brand et al., 2000). Similarly, in rats, HF feeding resulted in increased milk fat and

higher weight gain in pups compared with rats on LF (high carbohydrate) diets (Del Prado et al., 1997; Loh et al., 2002; Rolls et al., 1984). These results, together with the results from this study, suggest that increased dietary intake of fat in lactating animals increases the fat (and energy) content in milk, which in turn increases the energy supply to offspring, thereby improving their survival and growth rate. This is consistent with the idea that energy is the most important limiting factor during lactation, in that all the energy provided to the young must first be consumed by the mother in addition to her own energy requirements to ensure reproductive success (Kenagy et al., 1990; Speakman et al., 2001) or must be supplied by the mother's energy reserves (Valencak et al., 2009).

Using data for performance of the LF-fed mice, we predicted the expected impacts of feeding the HF and MF diets to lactating mice, assuming the mice were limited by their heat dissipation capacity (Table 2). The mathematical model was constructed using assumptions derived from the HDL theory (Krol and Speakman, 2003a,b) and parameterised using data from the mice on the LF diet. For both the HF and MF diets, the metabolisable energy intake (E_{mei}) and the E_{milk} exceeded the model predictions. The E_{DEE} of the HF- and MF-fed mice were 103.3 and 111.20 kJ day⁻¹, respectively, which did not differ significantly from that of mice on the LF diet (102.11 kJ day⁻¹). This suggested that the animals somehow did not manage to elevate their heat production (and break the heat dissipation limit). The positive effects on E_{mei} and E_{milk} occurred, therefore, because the HF- and MF-fed mice consumed much more energy (306.52 and 340.52 kJ day⁻¹, respectively) than predicted at peak lactation. The energy available for milk production was thus greatly increased (to 203.2 kJ day⁻¹ on HF and 229.3 kJ day⁻¹ on the MF diet). This raises the question of how they were able to do that without incurring the associated costs of lactogenesis.

Fatty acids are the major form in which fat is made available as fuel for energy generation. The main ways by which lactating mice can increase the fat content of their milk to support faster growth of their offspring is through increased dietary fat intake and increased *de novo* lipogenesis (i.e. conversion of dietary carbohydrate to fat). At weaning, litters from HF- and MF-fed mice were significantly heavier than pups on the LF diet. This was evidenced by the fact that the HF- and MF-fed mice not only consumed more energy at peak lactation but also delivered more milk energy to their pups than the LF-fed mice. The ability of the HF- and MF-fed mice to directly transfer absorbed dietary fat into the milk might have increased the efficiency of lactation (l) and hence reduced the heat production of lactogenesis (E_{lact}), allowing for higher than expected increases in E_{milk} . This finding is supported by the observation that high dietary fat intake could result in the reduction of metabolic heat production due to the strong suppressive effect of HF diets on lipogenesis in adipose tissue (Mercer and Trayhurn, 1984). It is also well established that feeding rats a HF diet depresses the rate of mammary gland lipogenesis (Del Prado et al., 1999; Grigor and Warren, 1980).

In the present study, the LF diet contained 70% energy as carbohydrate. Conversion of carbohydrate to fat prior to oxidation is thermogenically costly and about 20–25% of metabolisable energy intake is lost as heat (Chwalibog and Thorbek, 2001; Hellerstein, 1996). The LF-fed mice thus had to expend a part of the energy consumed for *de novo* lipogenesis to produce enough milk to support the growth of their offspring. Around 90–95% of the fatty acid profiles of the diets used was made up by three fatty acids – oleic acid (C18:0), linoleic acid (C18:2) and palmitic acid (C16:0) – and these were the dominant fatty acids in the milk profiles as well as making up 61, 75 and 74% of fatty acids in the

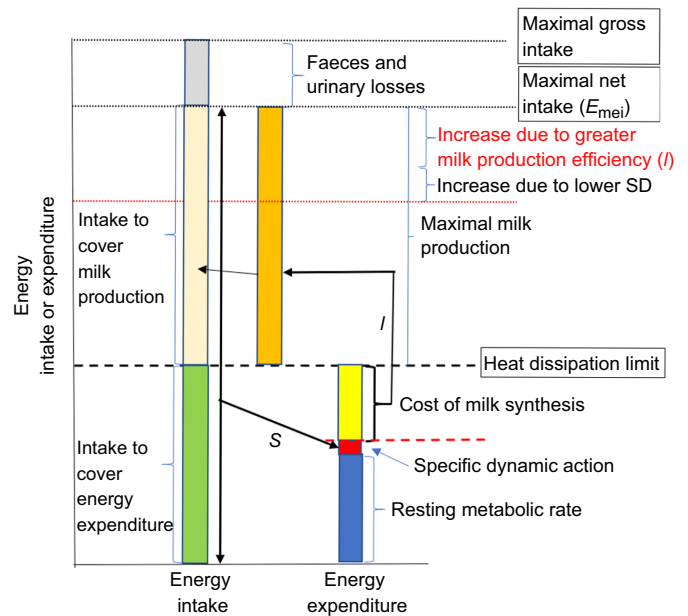


Fig. 5. Schematic diagram illustrating a revised mathematical model – based on experimental data – that includes alterations to the efficiency of lactation. This schematic shows what we think happened in the actual experiment. In our predictions from the HDL theory, we assumed that the value of s changed when the diet was changed, but that the value of l (the efficiency of lactation), would be independent of the diet. However, it seemed that this was not the case because the mice were able to take fats directly from the diet and export them into the milk without the need to synthesise them *de novo*. Hence, the diet had two effects. First, because of the lower s , the SDA fell. This was predicted to increase the net energy intake from the dotted red to the dotted blue line. However, because there was also an increase in l , the mice were able to synthesise much more milk (orange bar) for a given heat production linked to milk synthesis (yellow bar). Therefore, within the same heat dissipation limit they were able to increase their maximal net intake and their milk production much more than we initially predicted (from the blue to the black dotted line). The fixed heat dissipation limit remains key to understanding these responses, and as such we suggest that these data are consistent with the original HDL theory, if not with the precise predictions based on the assumption that diet would only affect s , and not as it turned out both s and l .

milk of LF, MF and HF mothers, respectively (Table S1). Linear regressions comparing the fatty acid profiles in the diets and milk showed a strong positive relationship between the type of fatty acids present in the diet and milk for HF and MF diets, but this relationship was not significant for the LF diet (Fig. 3). These results provide support for the idea that HF and MF mice directly transferred the ingested fatty acids to the milk, whereas mice fed a LF diet used *de novo* lipogenesis, generating a greater heat burden. Despite large differences in E_{milk} at peak lactation, the mice on the different dietary treatments did not show significant differences in body mass and E_{DEE} . This provides further support that the positive effects of HF and MF diets on lactation performance were due to the ability of the HF- and MF-fed mice to transfer fatty acids directly into milk without the need for *de novo* synthesis, thereby reducing the metabolic heat generated as a by-product of milk production and altering l . Hence, while the data did not match the original mathematical model predictions where it was assumed that HF feeding would only affect s , the overall pattern was consistent with the HDL theory (see Fig. 5 for a detailed schematic explaining this). However, why MF mice were able to increase their energy intake and milk production above that of HF mice remains unclear. Given that the fatty acid profiles of the MF and HF diets were not the same,

one possibility is that certain fatty acids are more readily transferred directly into milk than others (see Table S1).

In summary, MF1 mice fed on HF, MF and LF diets reached an asymptote in their daily food intake at peak lactation. At weaning, the pups from HF- and MF-fed mothers were significantly heavier than pups from LF-fed mice. This was because the peak lactation (E_{milk}) of HF- and MF-fed mice was significantly higher than LF-fed mice. The positive effects of feeding fat to mice were in part due to the low SDA but more likely linked to lower heat production for milk synthesis. The mice made morphological changes to cope with the high dietary fat intake by way of increased mass of the stomach and liver and also deposition of fat around the gonads. Dietary fat allowed the mice to elevate their milk production without consequent heat production, which was consistent with the HDL model for lactation limits. However, the reason why mice fed the MF diet were able to elevate their intake much higher than the mice on the HF diet remains unclear.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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Supplementary information

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