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LIMITS TO SUSTAINED ENERGY INTAKE

V. EFFECT OF COLD-EXPOSURE DURING LACTATION IN *MUS MUSCULUS*

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Summary

We have previously observed that female MF1 mice appeared to reach a limit in their food intake and milk production during late lactation, reaching a plateau between days 13 and 16 of lactation and between litter sizes of 9 and 15. These mice did not increase their food intake when forced to raise more offspring or when manipulated to be concurrently pregnant during late lactation, yet they did eat significantly more food at the peak of their second sequential lactation or when challenged with food of reduced energy content. These data suggest that apparent limits on sustained energy intake in this strain may not reflect central limitations but rather peripheral constraints at the mammary glands. In this study, we aimed to determine whether these were indeed limits by increasing the demands on the females during late lactation by cold-exposure (8 °C). Females responded to this manipulation by significantly increasing their food intake ($F_{1,73}$=77.53, P<0.001) above that of lactating females kept in warmer conditions (21 °C). In addition, there was a significant reduction in the number of pups raised in the cold (t=2.36, d.f.=18, P=0.03), with the majority of the mortality occurring within the first 2 days of cold-exposure. The mean mass of the pups raised in the cold was significantly lower ($F_{1,74}$=13.8, P<0.001) than that of those raised in the warm. Despite the cold-exposure and the increased food intake, there was no difference in the resting metabolic rates of the two groups of mothers or in the lengths of their small intestine. The greater food intake of lactating mice during cold-exposure supported our previous observations that they were capable of eating more food than the previously suggested limit of 23.1 g day^{-1}. However, the milk energy output of females in the cold was also significantly higher than in the warm ($F_{1,15}$=11.99, P=0.003), indicating that the asymptotic food intake of females in the warm was not mediated by limitations in their milk production. Sustained energy intake in these mice does not appear to be centrally or peripherally limited. Rather, the mice may restrain their use of energy during their first lactation because of life-history consequences for future reproductive attempts.

Key words: energetics, maximal metabolic rate, sustained metabolic rate, pregnancy, lactation, reproduction, mouse.

Introduction

Many studies have examined the limits to sustainable metabolic rate (SusMR) in small mammals (Kirkwood, 1983; Peterson et al., 1990; Weiner, 1992; Hammond and Diamond, 1992; Hammond and Diamond, 1994; Hammond and Diamond, 1997; Hammond et al., 1994; Hammond et al., 1996; Konarzewski and Diamond, 1994; Koteja et al., 1994; Koteja, 1996a; Speakman and McQueenie, 1996). These have mainly been concerned with the effect of a single stressor, e.g. cold or exercise. For example, in non-reproductive animals, SusMR has been measured by prolonged cold-exposure (Hayes, 1989; Konarzewski and Diamond, 1994; Koteja, 1996a) or by exposure to forced exercise (Hayes and Chappell, 1986; Westerterp et al., 1986; Hinds et al., 1993). In addition to these studies, lactation has frequently been used as a model for measuring SusMR (Hammond and Diamond, 1992; Hammond and Diamond, 1994; Hammond et al., 1994; Hammond et al., 1996; Koteja et al., 1994; Speakman and McQueenie, 1996).

However, in the wild, animals face combinations of stressors (exercise, cold, lactation) simultaneously. Few studies have challenged animals with a combination of stresses similar to those they experience in the wild. Perrigo (Perrigo, 1987) investigated the combined effects of exercise and lactation on deer mice *Peromyscus maniculatus* and house mice *Mus musculus*. Female mice were made to run to obtain food. In response to increasing the amount of running needed to obtain food, the two species responded in different ways. The deer mice increased their feeding effort to raise their young, but the resultant pups were not as large. In contrast, the house mice responded by culling pups to reduce their energy demands.

In a previous study on the effects of cold-exposure (5 °C)

during lactation, Hammond et al. (Hammond et al., 1994) manipulated the litter sizes of Swiss Webster mice *Mus musculus* to five, eight or 14 pups. These mice increased their food intake in the cold above that previously thought to be a limit during normal lactation. Hammond et al. (Hammond et al., 1994) suggested that these latter results indicated that the previous limit was unlikely to be imposed by the capacity of the gut, but was rather a limit acting on peripheral tissues, in this case the mammary tissue.

In a study on the milk production of cotton rats *Sigmodon hispidus* in the cold, Rogowitz (Rogowitz, 1998) found that although the food intake of the females increased when lactating in the cold, compared with the warm, there was no such increase in milk energy output. Females lactating in the warm exported as much energy in milk as those lactating in the cold. Females raising larger litters did produce a greater volume of milk, but this was more dilute than that produced by females with smaller litters. Rogowitz (Rogowitz, 1998) therefore concluded that the cotton rats were also limited peripherally by the capacity of the mammary tissue.

In the study of Hammond et al. (Hammond et al., 1994), the manipulation of litter size could have affected the response of the mice to the added stress of cold-exposure. The reduction of litter size to five or eight pups could have enabled females to raise their litters in the cold, whereas they might have been limited with their natural higher litter sizes, as indicated by females not maintaining the higher litter size of 14 throughout lactation (K. A. Hammond, personal communication). It might also have reduced the amount of food they needed. We have previously found (Johnson et al., 2001a) that MF1 mice differ markedly from Swiss Webster mice in their food intake during lactation. Over a range of litter sizes from five to 15 pups, MF1 mice ate a mean of 23.1 g daily (Johnson et al., 2001a), whereas the maximum food intake of the Swiss Webster mice was 19 g (Hammond et al., 1994) when raising the largest litters of 14 pups. It appears that the Swiss Webster mice were operating at well below the capacity achieved by the MF1 mice. This may afford the Swiss Webster mice the capacity to increase their food intake further under cold stress. Swiss Webster mice also do not reach an asymptote in food intake towards the end of lactation (Hammond and Diamond, 1992). In contrast, MF1 mice exhibit no further increases in food intake between days 13 and 16 of lactation or when raising litter sizes of 9–15 pups (Johnson et al., 2001a). Even when females were forced to raise artificially enlarged litters of up to 18 offspring (Johnson et al., 2001a) or were made concurrently pregnant while lactating (Johnson et al., 2001c), they did not increase their food intake above that associated with lactation alone. However, mice raising the second of two sequential litters (Johnson et al., 2001c) and mice in their first lactation given food of lower energy content (Speakman et al., 2001) did increase their food intake above the supposed limit of 23.1 g day^{-1}, suggesting that the observed asymptote in food intake was peripherally mediated, perhaps by the performance of the mammary glands.

In this study, we aimed to examine the effects of cold-exposure during lactation (as in Hammond et al., 1994) in MF1 mice *Mus musculus* L., but allowing the mice to raise natural litters (i.e. the litter sizes were not manipulated prior to cold-exposure). We measured the effects of cold-exposure on litter size, food intake, resting metabolic rate (RMR), the quantity and quality of milk produced and maternal morphology. The effects of cold-exposure on the body mass and food intake of non-reproductive females were also investigated.

Materials and methods

Animals and housing

Virgin female mice (outbred MF1), 10–11 weeks old, were housed individually in cages (44 cm×12 cm×13 cm) with sawdust and paper bedding. Rodent chow [CRM(P), Special Diet Services, BP Nutrition, UK] and water were available *ad libitum*. Prior to breeding and throughout pregnancy, the mice were kept at 21 °C on a 12 h:12 h L:D photoperiod. Females were paired with males for 6 days, after which the males were removed. Pregnancy was detected by an increase in mass over the following 7 days. On day 10 of lactation, when the pups had grown fur, the females (N=16) and their litters were transferred to a room at 8 °C on the same photoperiod, where they remained until the end of lactation. Lactating females in the cold were given enough paper bedding to cover themselves and the litter (approximately 3 g), as they were in the warm. One lactating female in the cold stopped eating on day 16 of lactation and her litter died. Since this also happens occasionally in mice in the warm, this could not necessarily be attributed to the cold-exposure. Data for this female were removed from the entire analysis, leaving a sample size of N=15. Non-reproducing females (N=15) were kept at 21 °C for 10 days before being transferred to 8 °C for a further 10 days. Data for lactating females exposed to the cold were also compared with data for lactating females that remained in the warm throughout reproduction as controls (N=71: Johnson et al., 2001a).

Body mass and food intake

The following measurements were made between 09:00 h and 11:00 h each day. Female body mass was measured prior to breeding and then daily throughout lactation. Maternal food intake was measured daily throughout lactation as the mass of food missing from the hopper each day. The bedding was checked for large pieces of uneaten food, which were weighed and returned to the hopper. In a separate experiment, only 1.7±0.41 % (mean ± S.E.M.) of the food missing from the hopper was found in the bedding (Johnson et al., 2001a). Following parturition, the number of pups and the mass of the litter were recorded daily. The food intake and body mass of the non-reproductive females were also measured daily, in the same way as for breeding females. All masses were accurate to 0.01 g (Sartorius top-pan balance). To determine the assimilation efficiency, faeces were collected from nine non-breeding females and from 12 lactating females between days 10 and 15. These were weighed, dried (in a Gallenkamp oven)

at 60 °C for 14 days and reweighed. Total food intake over this time was also measured. Gross energy determination was obtained for faeces from non-breeding females (N=5) and lactating females (N=6) and for the food by adiabatic bomb calorimetry (Gallenkamp Autobomb, Rowett Research Institute Analytical Services). The total energy excreted in the faeces was expressed as a percentage of the total energy consumed in food.

Resting metabolic rate (RMR)

Resting metabolic rate (RMR) was quantified as oxygen consumption, using an open-flow respirometry system (as described previously; Hayes et al., 1992; Speakman and McQueenie, 1996). Air was pumped (Charles Austin Pumps Ltd) through a sealed Perspex chamber within a constant-temperature incubator (INL-401N-010, Gallenkamp) set at 30 °C (within the thermoneutral zone; Speakman and Rossi, 1999). A flow rate of 500–700 ml min^{-1} was metered using an Alexander Wright flowmeter (DM3A) upstream of the chamber. A sample of air (approximately 150 ml) in the excurrent stream was dried (silica gel) and directed through a paramagnetic oxygen analyser (Servomex 1100A) (as described previously; Johnson et al., 2001b). We did not absorb CO_2 in the outflow stream prior to gas analysis as this minimised error in the conversion of oxygen consumption to energy expenditure when respiratory quotient is unknown (Koteja, 1996b; Speakman, 2000). The oxygen uptake of the female mice were measured prior to breeding (RMR_{PB}) and at peak lactation (RMR_L) (day 18).

Energy expenditure of the litters

The respiration of the litters was measured using the same procedure as for the females. However, the litters were measured for only 1 h, using a flow rate of 1000–1500 ml min^{-1}, and at 8 °C (the temperature at which they were housed). We extrapolated these estimates of RMR to quantify the total equivalent daily energy expenditure (DEE), which consequently excluded the energy costs associated with variations in activity. The total energy requirement (TER) of the litters was estimated as the sum of the daily energy expenditure from respirometry and the energy diverted to growth, as measured by increases in litter mass. The increase in the mass of the litter from day 13 to day 14 was converted to energy (kJ day^{-1}) using the calorific value of pups (2.14 kcal g^{-1} from Brisbin, 1970). This estimate therefore also excluded any costs associated with litter activity.

Milk production

Milk production by lactating females was estimated using a protocol described previously (Johnson et al., 2001a). Briefly, whole-animal water export was measured in nine non-lactating females and 12 lactating females (days 14–15 of lactation) using the turnover of tritiated water (HTO) (for full details, see Johnson et al., 2001a). The contribution to this water export of losses in the faeces, urine and evaporation was estimated, and the remaining water export was assumed to equal the water exported in milk. The total milk production could then be evaluated from estimates of the water content of milk samples used to assess milk quality.

Milk quality

Ten of the females were separated from their pups for approximately 3 h on day 15 of lactation. After this separation, the females were injected with 0.25 ml of oxytocin to stimulate milk let-down. The teats were manually palpated, and the milk was collected in capillaries. Each teat that was milked was emptied as far as possible because it has been shown that the fat content is atypically low in the first portion of the milk extracted (Oftedal, 1984). In total, 0.5 ml of milk was collected and analysed for water content and for gross energy from the fat, lactose and protein content (Rowett Research Institute Analytical Services) as described previously (Johnson et al., 2001a).

Morphology

The cold-exposed female mice (N=15) were killed at peak lactation after their RMR had been measured (day 18). A complete dissection was performed immediately, with all organs being removed and weighed before they were dried (Gallenkamp oven at 60 °C) for 14 days and reweighed. The masses were accurate to 0.0001 g (Ohaus Analytical Plus) except for the carcass, which was accurate to 0.01 g (Sartorius top-pan balance). The stomach and intestines were rinsed with Ringer's solution to eliminate all contents before being weighed. The small and large intestines were straightened, but not stretched (method after Hammond and Diamond, 1992; Koteja, 1996a), and their lengths were measured to the nearest 5 mm.

Statistical analyses

Repeated-measures analysis of variance (ANOVA) was used to examine changes in both body mass and food intake throughout lactation. Two-sample t-tests were used to compare the litter size, litter mass, maternal mass, RMR and food intake of the cold-exposed mice with those of lactating females kept at 21 °C throughout lactation (Johnson et al., 2001a). Two-sample t-tests were also performed to compare body mass and food intake on each day of lactation, with the significance level adjusted to account for the number of comparisons (Bonferroni correction). Analysis of covariance (ANCOVA) was performed on each organ mass with maternal mass as the covariate and group (warm or cold) as a factor. All statistical analyses were performed using commercially available software (Minitab versions 7.3 and 11; Ryan et al., 1985). Results are presented as means ± S.E.M.

Results

Body mass

Non-breeding females had a mean mass of 26.4±0.4 g (N=15) in the warm, and this increased significantly (paired t=9.28, P<0.0001) during cold-exposure to a mean of

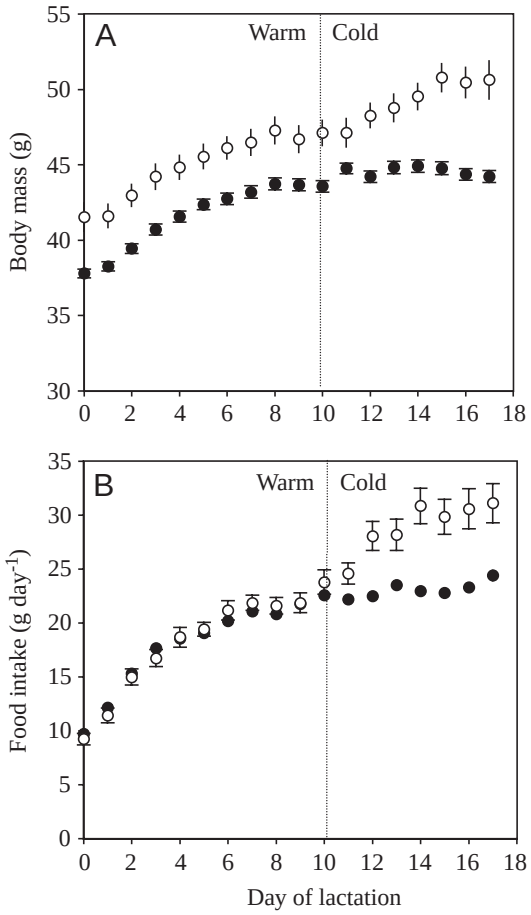

Fig. 1. (A) Mean body mass and (B) mean daily food intake of female mice throughout the period of lactation. Individuals continuously exposed to warm conditions (21 °C) are shown as filled circles (*N*=71), while individuals initially exposed to the warm but at day 10 switched to cold conditions (8 °C) are shown as open circles (*N*=15). Values are means ± S.E.M.

28.8±0.32 g (*N*=15). The pre-breeding body mass of the cold-exposed female mice averaged 32.3±0.63 g (*N*=15). During lactation, body mass increased significantly (repeated-measures ANOVA: $F_{17,270}$=61.04, *P*<0.001) from 41.5±0.95 g on the day of parturition to a maximum of 50.8±0.92 g on day 15 of lactation (Fig. 1A). Although the cold-exposed females were already heavier than the warm females (*N*=71) at the start of lactation (prior to the time they were exposed to the cold), the difference increased a few days after their exposure to cold (Table 1; Fig. 1A).

Assimilation efficiency

The mean assimilation efficiency was 79.8±1.17 % for the non-lactating females (*N*=9) and 82.2±1.01 % for the lactating females (*N*=12). There was no significant difference in the assimilation efficiencies in the warm and the cold in either the non-lactating (*t*=0.9, *P*=0.390) or the lactating (*t*=−1.3, *P*=0.220) females. For every gram of food (16.26 kJ g^{-1} wet mass) that the females consumed, they assimilated 13.4 kJ.

Table 1. *Mean body mass of the warm- (*N=*71) and cold-exposed (*N=*15) lactating females*

	Body mass (g)			
Day	Warm	Cold	*t*	*P*
0	37.81±0.29	41.52±0.95	3.72	0.0017*
1	38.20±0.30	41.60±0.78	4.09	0.0006*
2	39.27±0.32	42.97±0.74	4.61	0.0001*
3	40.52±0.36	44.20±0.83	4.05	0.0006*
4	41.42±0.37	44.83±0.80	3.88	0.0009*
5	42.12±0.35	45.54±0.80	3.90	0.0008*
6	42.72±0.37	46.11±0.72	4.19	0.0003*
7	43.00±0.41	46.47±0.85	3.68	0.0013*
8	43.44±0.41	47.28±0.89	3.91	0.0008*
9	43.49±0.40	46.68±0.89	3.30	0.0034
10	43.84±0.38	47.10±0.84	3.53	0.0020*
11	44.20±0.36	47.10±0.96	2.85	0.0100
12	44.19±0.39	48.26±0.82	4.49	0.0002*
13	44.48±0.41	48.76±0.91	4.31	0.0003*
14	44.91±0.42	49.53±0.87	4.78	0.0001*
15	44.75±0.42	50.77±0.93	5.97	<0.0001*
16	44.59±0.39	50.45±1.01	5.40	<0.0001*
17	44.21±0.40	50.62±1.27	4.83	0.0001*

Values are means ± S.E.M.

The results of two-sample *t*-tests between cold- and warm-exposed females on each day of lactation are also shown.

Because of the large number of comparisons made, the Bonferroni correction was applied to the significance level. Significant differences at the 95 % confidence level are represented by an asterisk.

Food intake

Non-reproducing females (*N*=15) ate a mean of 5.1±0.10 g day^{-1} (82.92 kJ day^{-1} gross intake equivalent to 68.2 kJ day^{-1} assimilated) in the warm. This increased significantly (paired *t*=15.61, *P*<0.0001) during cold-exposure to a mean of 7.8±0.18 g day^{-1} (126.8 kJ day^{-1} gross intake equivalent to 104.3 kJ day^{-1} assimilated). Cold-exposure thus resulted in a mean increase of 2.7±0.18 g day^{-1} (equivalent to 36.1 kJ day^{-1} assimilated) for non-lactating females.

Maternal food intake increased significantly during lactation (repeated-measures ANOVA: $F_{1,269}$=73.18, *P*<0.001) (Fig. 1B). The asymptotic food intake (calculated as the mean daily food intake over days 13–16 of lactation, when there was no further significant increase in food intake in control animals; Johnson et al., 2001a) averaged 30.0±1.55 g (487.8 kJ day^{-1} gross energy intake, 401.1 kJ day^{-1} assimilated energy intake) in the cold-exposed mice (*N*=15), which was significantly higher than that of the warm mice (*t*=4.33, *P*=0.0005), which averaged 23.1 g (369.6 kJ day^{-1} gross intake equivalent to 310.2 kJ day^{-1} assimilated). There was no significant difference between the food intakes of the lactating and cold-exposed mice and the lactating females in the warm until day 12, 2 days after they had been exposed to the cold (Table 2). For the remainder of lactation, the cold-exposed mice continued to eat significantly more than the warm-exposed

Table 2. *Mean food intake of both the warm- (*N=*71) and cold-exposed (*N=*15) lactating females*

Day	Food intake (g) Warm	Cold	*t*	*P*
0	9.68±0.29	9.21±0.53	–0.77	0.4500
1	12.14±0.28	11.40±0.69	–0.99	0.3300
2	15.16±0.30	14.97±0.75	–0.23	0.8200
3	17.54±0.37	16.73±0.78	–0.94	0.3600
4	18.44±0.31	18.65±0.91	0.23	0.8200
5	19.14±0.33	19.40±0.63	0.37	0.7200
6	20.04±0.34	21.16±0.89	1.18	0.2500
7	20.97±0.40	21.85±0.70	1.09	0.2800
8	20.79±0.34	21.58±0.79	0.93	0.3600
9	21.70±0.35	22.85±0.93	1.16	0.2600
10	22.42±0.38	23.77±1.13	1.13	0.2700
11	22.05±0.36	24.57±0.99	2.40	0.0270
12	22.40±0.35	28.05±1.33	4.12	0.0007*
13	23.46±0.39	28.17±1.46	3.12	0.0063*
14	22.93±0.36	30.84±1.65	4.69	0.0002*
15	22.73±0.41	29.84±1.63	4.24	0.0006*
16	23.21±0.39	30.57±1.85	3.79	0.0018*
17	24.39±0.50	31.1±1.81	3.58	0.0023*

Values are means ± S.E.M.

The results of two-sample *t*-tests between cold- and warm-exposed females on each day of lactation are also shown.

Because of the large number of comparisons made, the Bonferroni correction was applied to the significance level. Significant differences at the 95% confidence level are represented by an asterisk.

mice (Table 2). This represented an increase of 30% above the food intake of lactating mice at 21°C. The asymptotic food intake was significantly positively related to litter size (ANCOVA $F_{11,73}$=7.11, P<0.001) and was significantly higher in the cold mice than the warm mice (ANCOVA $F_{1,73}$=77.53, P<0.001). However, asymptotic food intake was not significantly related to body mass ($F_{1,14}$=0.69, P=0.421). There was no significant relationship between the increase in food intake due to cold-exposure and litter size ($F_{1,13}$=1.24, P=0.285) (Fig. 2). The smallest increase of 5.0±0.75 g day^{-1} was observed in females raising six pups, and the greatest mean increase was 13.8±1.91 g day^{-1} for females raising 10 pups.

Litter size and mass

The females exposed to the cold gave birth to a mean of 11.2±0.65 pups and weaned a mean of 9.4±0.75 pups. Litter mass increased from 17.8±0.88 g at birth to 69.8±4.78 g at weaning. There was no significant difference in either the number of pups born (t=0.73, P=0.48) or the mass of the litter at birth (t=0.03, P=0.97) between the warm and cold litters. However, by peak lactation, the warm females had raised significantly more pups (t=2.36, P=0.03) and, hence, heavier litters (t=3.47, P=0.0029). The mean mass of the pups was significantly negatively related to litter size (ANCOVA

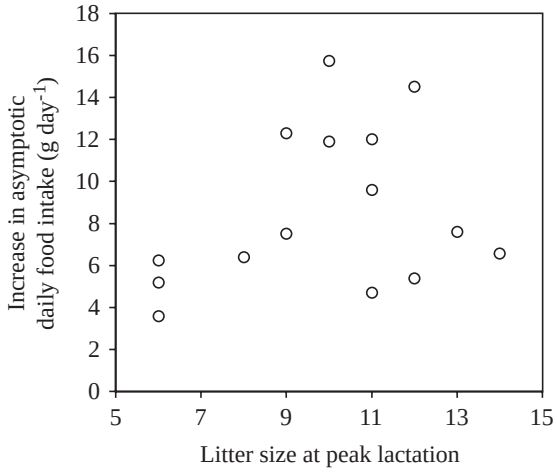

Fig. 2. The increase in asymptotic food intake during late lactation when mice were moved from warm to cold conditions as a function of litter size. The increase was calculated as the actual intake minus the predicted intake from litter size for mice raising equivalent-sized litters continuously in the warm. Each point refers to a separate litter.

$F_{11,74}$=9.22, P<0.001) and was significantly different between the warm and cold mice (ANCOVA $F_{1,74}$=13.8, P<0.001). After accounting for the effect of litter size, litter mass was still significantly lower in the cold litters (ANCOVA $F_{1,74}$=10.16, P=0.002).

Mortality

Mortality in the litters transferred to the cold (N=15) was represented as the number of pups that died on any given day as a proportion of the total number of pups. The pattern of pup mortality differed between the warm- and cold-exposed females (Fig. 3). The major mortality of pups of females in the warm occurred within the first few days of lactation. This is in

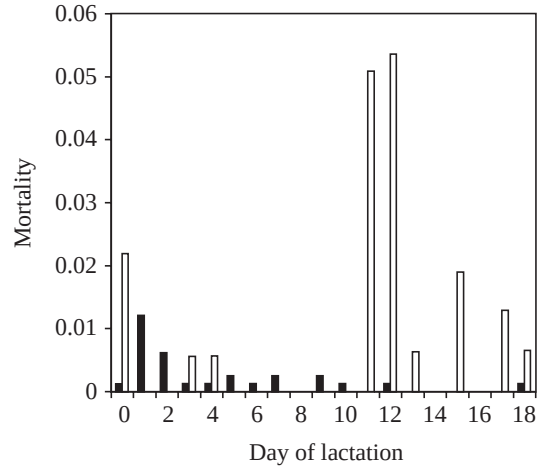

Fig. 3. The mortality rate of pups (the proportion of pups alive that died on any given day) for litters maintained continuously in the warm (filled columns) and litters transferred to the cold on day 10 of lactation (open columns).

contrast to the cold mice, for which the peak of mortality occurred within 2 days of the litters being exposed to cold, with further mortality evident on days 15–18. As expected, there was no significant difference in mortality (t=0.18, P=0.85) when both groups were at 21 °C (days 0–10), but there was significantly greater mortality in the cold-exposed litters than in the warm-exposed litters (one-tailed t=–1.59, P<0.05) between days 10 and 18.

Resting metabolic rate (RMR)

The females exposed to the cold during lactation (N=15) had a mean RMR of 22.46±0.70 kJ day^{-1} prior to breeding, and this increased to 51.84±3.72 kJ day^{-1} at peak lactation. This represented a 2.3-fold increase from pre-breeding to peak lactation. Although the equivalent estimates of RMR for mice breeding in the warm were 21.51±0.72 kJ day^{-1} and 47.05±1.64 kJ day^{-1} in the pre-breeding period and at peak lactation, respectively (Johnson et al., 2001b), the differences between cold-exposed and warm mice did not reach statistical significance at either time point (pre-breeding t=–1.73, d.f.=49, P=0.091; peak lactation RMR t=–1.66, d.f.=20, P=0.11). Even after accounting for the differences in body mass (ANCOVA), there was still no significant difference between warm and cold mice either in pre-breeding RMR ($F_{1,84}$=0.93, P=0.336) or in peak lactation RMR ($F_{1,84}$=2.57, P=0.113). From the peak-lactation RMR in the cold-exposed mice of 51.8 kJ day^{-1}, the sustained daily energy intake at peak lactation was 9.4×RMR if the gross energy intake was used and 7.7×RMR if the assimilated energy intake was used.

Energy expenditure of the litters

Using the data obtained from both the warm (Johnson et al., 2001a) and the cold litters, there was a significant relationship between litter mass and the predicted DEE of the litter (ANCOVA $F_{1,32}$=7.54, P=0.010) and also a significant difference between those measured in the warm and the cold ($F_{1,32}$=6.42, P=0.016). As a result of the difference in growth rates between those litters whose energy expenditure was measured and those that were just weighed ($F_{1,22}$=4.93, P<0.001) (see also Johnson et al., 2001a), the following equation was used to predict the DEE of the original litters (both warm and cold) on day 13 from the litter mass (M, g) and the temperature (T, °C) at which they were kept:

$$\mathrm{DEE} = -45.9 + 1.30M + 30.3T .$$

The predicted daily energy expenditure of the litters was significantly greater (approximately 35 %) in the cold (mean 112±2.54 kJ day^{-1}) than in the warm (mean 82.8±1.26 kJ day^{-1}) ($F_{1,75}$=89.87, P<0.001). When the energy used in growth was included, there was still a significant difference (20.3 %) between the total energy requirement (TER) of the litters in the cold (mean 133±6.99 kJ day^{-1}) and in the warm (mean 110.6±1.82 kJ day^{-1}) ($F_{1,75}$=18.73, P<0.001).

Milk production

The volume of milk produced by the lactating females in the cold was positively related to both litter size (r^2=0.554, $F_{1,10}$=12.44, P=0.005) (Fig. 4A) and litter mass (r^2=0.421, $F_{1,10}$=7.28, P=0.022) (Fig. 4B). There was no significant relationship between milk production and maternal body mass ($F_{1,10}$=4.27, P=0.066). The estimates of volume of milk produced in the warm (Johnson et al., 2001a) and the cold were combined for the following analysis. There was a significant relationship between the volume of milk produced and litter mass (ANCOVA $F_{1,27}$=11.04, P=0.003), but not between the volume of milk produced and maternal body mass (ANCOVA $F_{1,27}$=3.68, P=0.066) or litter size (ANCOVA $F_{1,27}$=0.13, P=0.721). The volume of milk produced by the females in the cold was significantly greater than the volume of milk produced in the warm (ANCOVA $F_{1,27}$=20.67, P<0.001) (Fig. 4B).

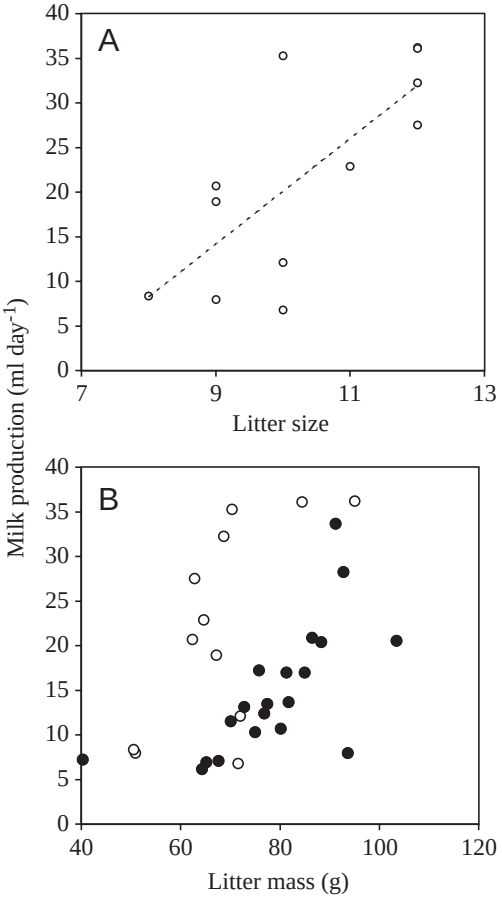

Fig. 4. (A) Relationship between maternal milk production (volume of milk produced on day 15) and litter size for litters in the cold. The line (y=5.93x–39.2) describes the best-fit least-squares regression. (B) Relationship between maternal milk production (volume of milk produced on day 15) and litter mass for litters raised continuously in the warm (filled symbols) and litters transferred to the cold on day 10 of lactation (open symbols) (y=0.596x–18.7).

Milk quality

The relationship between the energy content (kJ g^{-1}) and the volume of milk produced marginally failed to reach

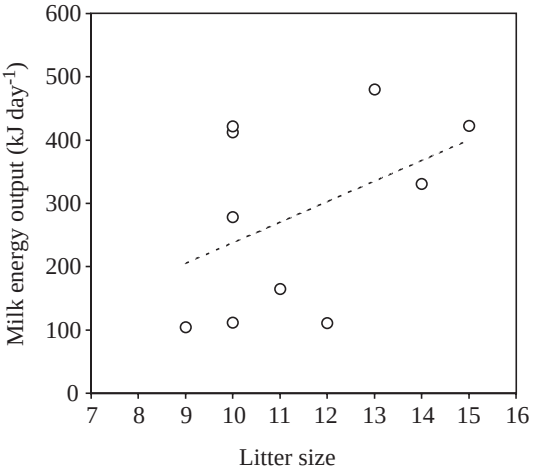

Fig. 5. Relationship between milk energy output at peak lactation (kJ day^{-1} on day 15) and litter size for litters transferred to the cold on day 10 of lactation. The line (y=32.53x–87.3) represents the best-fit least-squares regression equation.

significance ($F_{1,8}$=4.62, P=0.064). The milk energy output was calculated as the product of the energy content of the milk and the volume produced. There was a positive relationship between milk energy output and litter size (r^2=0.529, $F_{1,8}$=8.99, P=0.017) (Fig. 5). The relationship between milk energy output and litter mass marginally failed to reach significance (r^2=0.373, $F_{1,8}$=4.76, P=0.061). There was also no significant relationship between the mean energy supplied per pup and the litter size (r^2=0.309, $F_{1,8}$=3.58, P=0.095). The gross energy content of the milk produced by females in the warm (mean 11.97±0.38 kJ g^{-1}) was significantly lower than the gross energy content of milk produced by females in the cold (mean 13.4±0.44 kJ g^{-1}) ($F_{1,18}$=6.07, P=0.024). Therefore, females in the cold with larger litters produced a greater quantity of milk that contained a greater amount of energy than those with smaller litters.

There was no relationship between the dry matter content of the milk and either litter size ($F_{1,8}$=2.63, P=0.163) or litter mass ($F_{1,8}$=0.33, P=0.580). Fat content was also not significantly related to either litter size ($F_{1,8}$=0.96, P=0.355) or litter mass ($F_{1,8}$=0.83, P=0.389). However, the relationship between litter mass and protein content closely approached significance (r^2=0.396, $F_{1,8}$=5.24, P=0.051) (Fig. 6A), and litter mass was significantly related to lactose content (r^2=0.399, $F_{1,8}$=5.31, P=0.050) (Fig. 6B).

To compare the milk energy output of the females in the warm and in the cold, the present data were combined with those from Johnson et al. (Johnson et al., 2001a). Milk energy output was not significantly related to litter size (ANCOVA, $F_{1,15}$=0.60, P=0.450) or litter mass (ANCOVA, $F_{1,15}$=0.75, P=0.399). The milk energy output in the cold was significantly greater than that in the warm ($F_{1,15}$=11.99, P=0.003) (Fig. 7). On average, females in the cold exported 242 kJ day^{-1} as milk at peak lactation compared with only 164.7 kJ day^{-1} for females at the same stage of lactation in the warm. This milk

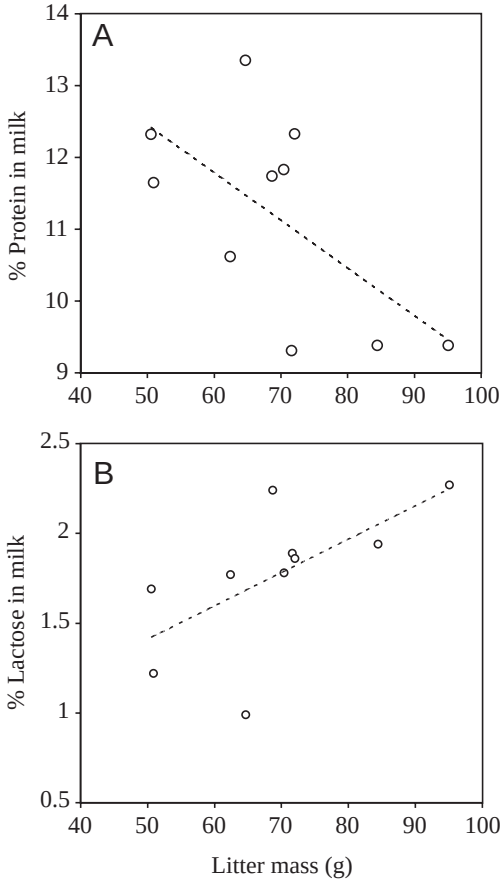

Fig. 6. Relationship between litter mass and (A) protein content (y=–0.07x+15.8, P=0.051, not significant) and (B) lactose content (y=0.02x+0.5, P=0.05) of the milk at peak lactation (day 15) for females raising litters transferred to the cold on day 10 of lactation.

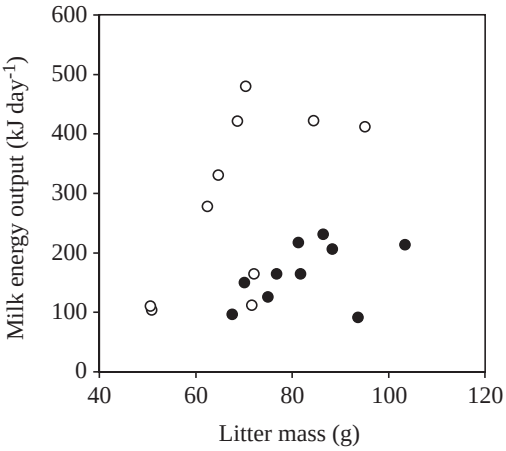

Fig. 7. Milk energy output (on day 15) in relation to litter mass (g) for litters raised continuously in the warm (filled circles) and litters transferred to the cold on day 10 of lactation (open circles).

export represented 49.6±7.83 % of the gross food intake and 60.4±8.78 % of the assimilated energy intake. After exporting this milk, the mice had 158.8±29.5 kJ day^{-1} remaining to

Table 3. *Mean wet organ masses of cold- (*N*=15) and warm-exposed (*N*=35) lactating females*

Organ	Wet organ mass (g)		$F_{1,48}$	P
	Warm	Cold		
Carcass	14.73±0.18	16.87±0.41	0.37	0.546
Pelage	3.34±0.07	4.12±0.14	2.43	0.131
Heart	0.26±0.005	0.32±0.02	0.54	0.467
Lungs	0.36±0.02	0.75±0.08	9.22	0.005
Liver	3.14±0.07	3.47±0.13	2.83	0.104
Spleen	0.09±0.003	0.18±0.02	6.30	0.018
Uterus	0.25±0.02	0.30±0.04	0.00	0.978
Pancreas	0.69±0.02	0.89±0.06	0.13	0.723
Tail	0.82±0.008	0.95±0.02	2.67	0.114
Stomach	0.31±0.01	1.26±0.17	3.62	0.068
Small intestine	1.38±0.05	1.68±0.09	0.05	0.834
Large intestine	0.77±0.03	1.05±0.06	0.26	0.612
Mesenteric fat	0.04±0.007	0.11±0.02	1.76	0.195
Abdominal fat	0.09±0.008	0.66±0.15	3.00	0.094
Mammary	3.94±0.12	6.38±0.55	0.54	0.469
BAT	0.12±0.005	0.25±0.02	3.09	0.090
Brain	0.46±0.005	0.46±0.005	1.92	0.177
Kidney	0.56±0.008	0.67±0.02	0.60	0.446

Values are means ± S.E.M.

BAT, brown adipose tissue.

The results of ANCOVAs are also shown.

Because of the large number of comparisons made, the Bonferroni correction was applied to the significance level. There were no significant differences at the 95% level between the two temperatures after the effect of body mass had been removed.

support their metabolism, which was 3.06 times the measured RMR of the same group of animals. The energy exported to the litters was 2.6±0.41 times greater than their predicted total energy requirement (TER).

Morphology

Wet masses

The mean wet masses of all the organs measured are given in Table 3. The following results refer to estimates of residual organ masses accounting for differences in body mass using least-squares regression. After applying the Bonferroni correction, there were no significant differences in the wet masses of any of the organs between the warm- and cold-exposed females (Table 3).

Dry masses

Mean dry masses of all the organs are listed in Table 4. The following results refer to estimates of residual organ masses accounting for the effects of body mass. The dry masses of the pelage (ANCOVA; $F_{1,48}$=16.51, P>0.001) and the tail ($F_{1,48}$=12.15, P=0.002) were significantly greater in the cold-exposed females than in the warm-exposed females. There were no significant differences in dry mass between any of the other organs measured in the warm and the cold (Table 4). There were also no significant differences between the lengths of the small and large intestine of mice in the warm and cold (Table 4).

Table 4. *Mean dry organ masses, the lengths of the small and large intestines and mass/length of the small intestine of the cold- (*N*=15) and warm-exposed (*N*=35) lactating females*

Organ	Dry organ mass (g)		$F_{1,48}$	P
	Warm	Cold		
Carcass	4.43±0.07	5.46±0.16	3.40	0.076
Pelage	**1.29±0.02**	**1.85±0.08**	**16.51**	**>0.001**
Heart	0.06±0.002	0.08±0.003	0.09	0.764
Lungs	0.08±0.01	0.19±0.02	9.03	0.006
Liver	0.86±0.02	1.03±0.04	0.75	0.393
Spleen	0.02±0.001	0.04±0.003	6.17	0.019
Uterus	0.06±0.003	0.12±0.03	0.64	0.429
Pancreas	0.19±0.01	0.29±0.04	0.39	0.536
Tail	**0.32±0.01**	**0.42±0.01**	**12.15**	**0.002**
Stomach	0.08±0.003	0.33±0.05	6.36	0.018
Small intestine	0.31±0.01	0.40±0.02	0.20	0.658
Large intestine	0.16±0.01	0.24±0.01	0.84	0.367
Mesenteric fat	0.01±0.002	0.05±0.01	2.33	0.138
Abdominal fat	0.04±0.01	0.46±0.13	2.72	0.110
Mammary	1.12±0.04	2.45±0.25	1.46	0.238
BAT	0.05±0.003	0.14±0.02	4.98	0.034
	Length (cm)			
	Warm	Cold		
Small intestine	59.9±0.60	61.0±1.28	5.02	0.033
Large intestine	13.1±0.19	14.7±0.46	0.03	0.874
	Mass/length (mg cm^{-1})			
	Warm	Cold		
Small intestine	5.24±0.17	6.50±0.25	1.20	0.283

Values are means ± S.E.M.

BAT, brown adipose tissue.

The results of ANCOVAs are also shown.

Because of the large number of comparisons made, the Bonferroni correction was applied to the significance level. Significant differences at the 95% level between the two temperatures after the effect of body mass had been removed are in bold type.

Discussion

There were four main effects of exposing lactating MF1 mice to cold during the second half of lactation. These were an increased food intake by the females, a reduction in litter size due to pup mortality, an increase in the volume of milk produced and an increase in the energy content of the milk. Until the day the females were placed in the cold, there was no difference between their daily food intake and that of lactating MF1 mice that remained in the warm throughout lactation (Johnson et al., 2001a). However, food intake increased above the levels of mice kept in the warm after 2 days in the cold and remained significantly elevated from day

12 of lactation onwards. This increase in food intake during cold-exposure in lactation mirrors a previous study using Swiss Webster mice (Hammond et al., 1994). However, the size of the increase differed, with MF1 mice increasing their intake more. This could have been a consequence of the Swiss Webster mice being acclimated to the cold for longer than the MF1 mice. Hammond et al. (Hammond et al., 1994) also found that the increase in food intake due to cold-exposure was similar between non-breeding and breeding females and between the three manipulated litter sizes of five, eight and 14 pups. This was not the case for MF1 mice. Lactating females increased their food intake on average by 8.4 g compared with an average increase of only 2.6 g in the non-reproductive animals. The increase in the reproductive females was, therefore, more than 2.5 times the increase in the non-reproductive females, and there was considerable variation in the increase in food intake with litter size.

The sustained gross energy intake during the asymptotic phase of late lactation was 9.4 times the measured RMR of the cold-exposed females. This is approximately 35 % higher than the postulated limit of 7×RMR proposed previously (Peterson et al., 1990; Hammond and Diamond, 1997) [% increase=100(9.4–7.0)/7.0]. Even using the assimilated energy intake rather than the gross energy intake resulted in a sustained energy intake of 7.7×RMR, which is 10 % above the postulated limit. These are among the highest sustained food intakes as multiples of RMR ever reported. As we have highlighted previously (Speakman and McQueenie, 1996; Speakman, 2000; Johnson et al., 2001a), during late lactation, much of the ingested energy is diverted to milk production and, consequently, there is a mismatch between the sustained energy intake and the sustained metabolic energy expenditure. In these mice, the sustained energy expenditure was much lower, at approximately 3.1×RMR, which does not breach the suggested limit on sustained metabolic rate of approximately 4.0×RMR postulated by Drent and Daan (Drent and Daan, 1980). These data do not support the idea of a limit on sustained energy intake at approximately 7.0×RMR (Peterson et al., 1990; Hammond and Diamond, 1997) but are consistent with a limit on sustained energy expenditure of approximately 4.0×RMR (Drent and Daan, 1980).

An increased mortality of the pups in the cold was also found in the study of Hammond et al. (Hammond et al., 1994), but to a lesser extent than in the present study. There are two possibilities for how the pups died. The mother may have killed them or they may have wandered out of the nest and died of hypothermia. Separating these effects is complex. When the pups were younger, the mother returned them to the nest when they wandered out. Hence, if the pups did wander out of the nest and die from hypothermia, it is still possible that the female indirectly culled them if she left them out of the nest or refused to let them back into the nest. At present, we cannot distinguish between these alternatives. Not all the females had reduced litter sizes in the cold, but of those that did, the majority of pups died within 2 days of exposure to the cold. The remainder died at peak lactation, when the demand on the females was greatest. It is possible that the initial mortality in the cold was caused by pups wandering out of the nest and that the later deaths were due to culling.

When faced with increased demands in the cold, the mice acted in a similar way to house mice *Mus musculus* made to work for their food during lactation (Perrigo, 1987). When forced to work harder for food during lactation, the mice culled pups to reduce the energy burden rather than working harder (Perrigo, 1987). It appears that, at least in some mice, increasing their food intake was not sufficient to meet the increased energy demands. Therefore, these mice combined increasing their intake of energy with a decreased demand from the pups. Some females did succeed in raising their entire original litter by increasing their food intake and by having smaller pups. The litters of three females decreased by between six and eight pups whilst in the cold, but the females managed to raise a much-reduced litter.

In addition to a reduction in litter size after cold-exposure, the total mass of the litter and the mean mass of the pups were also lower in the cold. This is in contrast to Hammond et al. (Hammond et al., 1994), who found no effect of cold temperatures on either the total litter mass or the individual masses of the pups. This could be attributed to the manipulation of the litter sizes of the Swiss Webster mice, whereby females given fewer pups to raise had the capacity to increase their food intake sufficiently to maintain the mass of the pups, or that they were not working at their limit and, hence, had the scope to increase investment in the litters.

It has been shown that some animals increase their RMR in response to cold-exposure (Konarzewski and Diamond, 1994; McDevitt and Speakman, 1994); however, that of others remains unchanged (Hayes and Chappell, 1986; Weiner and Heldmaier, 1987). The mice in the present study increased their RMR during lactation (as in Speakman and McQueenie, 1996; Johnson et al., 2001b), but there was no further significant increase when lactating in the cold.

Exposure to cold in non-reproductive animals often results in morphological changes such as longer gastrointestinal tracts and larger livers (Hammond and Wunder, 1995; Toloza et al., 1991; Koteja, 1996a; Konarzewski and Diamond, 1994). This was also found in cold-exposed lactating Swiss Webster mice (Hammond et al., 1994). In the present study, there were morphological changes, but these were restricted to heavier tails and heavier pelts. The former may have reflected elevated vascularisation of the tail in the cold to prevent cold damage and, presumably, the latter resulted in increased insulation. This change in pelage mass, in combination with an increase in mass of the brown adipose tissue that approached significance (P=0.033, but not significant because of the number of tests made and altered significance criterion using the Bonferroni correction), strongly suggests that these animals were attempting to reduce heat loss in the cold. The increase in their food intake (and milk production) cannot, therefore, be viewed as the consequence of a release from a constraint imposed by the capacity to dissipate heat when animals were in the warm. Neither the length nor the mass per unit length of

the small intestine was significantly different between females lactating in the warm and cold. This could mean that either the length in the warm was sufficient to meet the increased demand in the cold or that a limit was acting on the length of the small intestine, which could not increase further upon cold-exposure. Alternatively, gross measurements of intestine mass and length may be only poor indicators of the maximum potential uptake capacity of the organ, which presumably depends critically on the activity of transport systems at the cell surfaces.

In comparison with mice lactating in the warm (Johnson et al., 2001a), MF1 mice in the cold did increase the volume of milk produced with increasing litter size. The negative relationship between the energy available per pup and litter size observed previously (Russell, 1980; Fiorotto et al., 1991; Rogowitz and McClure, 1995; Rogowitz, 1998) marginally failed to reach significance in the sample of animals we measured in the cold.

Rogowitz (Rogowitz, 1998) also found that, despite increasing their food intake in the cold, cotton rats *Sigmodon hispidus* did not increase their milk energy output and therefore concluded that they were limited peripherally by the mammary glands. This conclusion was also reached by Hammond et al. (Hammond et al., 1994; Hammond et al., 1996), although they made no direct measurements of milk production. In contrast to these previous studies, we found that the cold-exposed MF1 females were able to respond to cold-exposure by increasing both the volume and the energy content of their milk, resulting in greater total milk energy output.

The estimate of milk energy intake by the litters was much greater (2.6 times) than their estimated TER extrapolated from their resting energy expenditure measured at 8 °C (the temperature at which they were housed). The discrepancy between the milk energy intake and the TER estimate reflects partly the fact that not all the gross energy exported to the offspring is assimilated. The majority of this difference is probably accounted for by the fact that our estimate of TER was based on an extrapolation of resting energy requirements and, therefore, excluded any costs of activity.

This study has shown that, during lactation, MF1 females are not limited either centrally, by the gut, or peripherally, by the mammary glands, because they were able to increase both food intake and milk production when faced with increased demands in the cold. These observations are consistent with our previous observations that the same strain of mouse was able to elevate its food intake between the first and second lactations (Johnson et al., 2001c) and also during the first lactation when provided with food of a lower energy content (Speakman et al., 2001). In addition, during their second lactation, the mice produced offspring that were larger than the offspring produced during their first lactation at the same litter sizes. This would be consistent with mice in their second lactation also increasing their milk production (although there are alternative explanations; see Kunkele and Kenagy, 1997). It appears that these mice do have scope for increasing both food intake and milk production during their first lactation. The limits we observed in asymptotic daily food intake and milk production (Johnson et al., 2001a) do not therefore reflect fundamental physiological central or peripheral constraints. Why do these mice routinely limit their food intake and milk production at submaximal levels during their first lactation? One possible explanation is that the mice are selected to maximise reproductive output over their entire lifetime. By restraining their performance during the first lactation, the mice may maximise their performance in later lactations. Such trade-offs between early fecundity and both late fecundity and maternal survival have been demonstrated in several other species (for a review, see Stearns, 1992), although we are unaware whether they are also apparent in MF1 mice. A direct consequence of the restraint we have uncovered is that the first lactation may be an inappropriate system in which to search for fundamental physiological constraints on performance, certainly in this strain of mice, and perhaps more generally.

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