# IS MAMMARY OUTPUT CAPACITY LIMITING TO LACTATIONAL PERFORMANCE IN MICE?

KIMBERLY A. HAMMOND[1],*, K. C. KENT LLOYD[2] AND JARED DIAMOND[1]

[1]*Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90095-1751, USA* and [2]*Department of Medicine and Center for Ulcer Research and Education, UCLA School of Medicine, Los Angeles, CA 90095-1792, USA*

## Summary

Using lactation in mice as a model, we sought to determine whether ceilings on sustained energy expenditure reside in the capacities of energy-acquiring and input organs (such as the intestine) or of energy-expending and output organs (such as the mammary glands). To distinguish between these possibilities experimentally, we surgically varied the teat number of lactating mother mice while simultaneously varying their litter size. The energy burden on each teat (i.e. the pup/teat ratio) could thus be varied independently of the energy burden (i.e. litter size) on the mother herself or on her intestine. At each teat number, pup mass proved to be maximal at intermediate litter sizes. At a given pup/teat ratio, mothers with five teats weaned pups no larger than the pups of normal (10-teat) mothers, even though the total energy burden on the former mothers was only half as large. Mothers with only two teats could not wean any pups. Litter size controlled maternal food intake, which in turn controlled intestinal mass and nutrient uptake capacity. Disproportionately high food intake for the smallest litters appears to reflect capital start-up costs of lactation. Pup mass is evidently limited by inadequate suckling stimulation of mammary glands.

Key words: lactation, energy output, mammary glands, mouse, *Mus musculus.*

## Introduction

What limits metabolic output in animals experiencing high sustained energy demands, such as demands of lactation, heat production and physical activity? Possible explanations include the following: limits on energy acquisition (e.g. on availability, gathering, ingestion or digestion of food); limits on energy utilization (e.g. on milk output, heat production, muscle performance or growth); limits at steps intermediate between energy acquisition and utilization (e.g. neural or hormonal control of nutrient assimilation); or limits on processes shared among different forms of energy utilization (e.g. liver nutrient processing or kidney waste production) (Drent and Daan, 1980; Peterson *et al.* 1990; Weibel *et al.* 1991; Weiner, 1993; Hammond and Diamond, 1992).

To study this question, we previously carried out three studies of lactating mice (*Mus musculus*), which permitted us to manipulate energy demand while measuring potentially limiting physiological and anatomical variables (Hammond and Diamond, 1993, 1994; Hammond *et al.* 1994). Food intake, gut mass and intestinal nutrient absorptive capacity of mother mice increased as lactation proceeded and as the pups grew larger, reaching a peak at day 15 when the pups began to nibble solid food and thereby to relieve the burden on the mother. The mother's increasing food intake served to meet the increasing energy demand of milk production for the growing pups. When we experimentally varied litter number from 5 to 14 pups, the mother's food intake, gut mass and intestinal absorptive capacity reached a plateau for litters of eight or more pups, and individual pup mass at weaning decreased for larger litters (Hammond and Diamond, 1992). When we experimentally prolonged lactation beyond day 15, thereby increasing litter mass without a change in litter number, the mother's food intake, gut mass and intestinal absorptive capacity increased no further beyond day 15 (Hammond and Diamond, 1994). Both of these studies thus indicated some limit on lactational performance. However, when we increased energy demand further by placing lactating females in cold environments (5 °C), they increased food intake, gut mass and intestinal absorptive capacity (to fuel the heat production necessary to maintain body temperature) to even higher levels than they had been able to sustain during lactation at 23 °C (Hammond *et al.* 1994).

The first two of these three studies suggested that some limit on lactational performance exists. The third study suggested that, although food intake, gut mass and intestinal absorptive

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*Present address: Department of Biology, University of California, Riverside, CA 92521, USA.

capacity might appear to be limiting, they might not set ultimate limits, since all three increased even further under another simultaneous energy demand. The ultimate limits must lie elsewhere. For instance, the mammary glands might limit lactational performance, and food intake, gut mass and intestinal absorptive capacity might then adjust to the associated nutrient requirements. A mammary gland limit might involve milk production capacity (the product of teat number times production capacity of each teat), or else problems of pup access to teats. By access problems, we mean that, since female *Mus musculus* have 10 teats and since our litter sizes were up to 14 pups, access time of each pup to teats might have been on the average insufficient in the largest litters.

Hence, in the present study, we experimentally manipulated milk production capacity and teat number by surgically decreasing the number of teats. This test permits us unequivocally to distinguish limits associated with mammary glands from limits associated with the gut, liver and other organs that supply the nutrients exported into milk. In our previous studies, when we increased litter number, we thereby increased both the pressure on each mammary gland (i.e. the number of pups per teat) and the total burden on the mother's gut and other nutrient supply organs. By reducing teat number and pup number in parallel, we can maintain pressure on each mammary gland (i.e. maintain the pup/teat ratio), while nevertheless reducing the total energy burden on the mother and on her gut and associated organs.

For example, normal mother mice (with 10 teats) digest enough food to raise a litter averaging little more than seven pups. They can also raise more pups by digesting more food, but individual pup mass is considerably reduced in litters of 14 pups (1.4 pups teat$^{-1}$), and the mothers cannot raise more than 14 pups (Hammond and Diamond, 1992, 1994). In the present study, we surgically produced experimental females with five teats; these females can still raise a normal litter of seven pups. Mammary pressure is thus still 1.4 pups teat$^{-1}$, but the total burden on the mother is only about half of the burden for normal 10-teat mothers with 14 pups and with that same ratio of 1.4 pups teat$^{-1}$. We can envision three alternative outcomes, suggesting the following three alternative conclusions. (1) Experimental mothers may eat as much or more food than control mothers, but individual pup mass of experimental mothers may just be equal to or less than pup mass of control mothers. This would suggest that teat number and/or milk output limits pup mass. (2) Experimental mothers may eat as much or more food than control mothers, but individual pup mass of experimental mothers may exceed that of control mothers. This would suggest that milk output per teat is now higher than under control conditions, and that teat number and milk output are not limiting pup mass under control conditions. (3) Experimental mothers may eat less food than control mothers, suggesting that litter size (i.e. total energy burden on the lactating mother) controls food intake.

In practice, we varied both teat number and litter size much more widely than in this example. We shall report results from

mothers with two, five or 10 teats (we also studied mothers with six or seven teats but omit the results to save space, since they do not add significantly to the conclusions). We also report results from up to eight different litter sizes for each teat, corresponding to mammary pressures of 0.1–1.8 pups teat$^{-1}$. We measured food intake, digestive efficiency, maternal and pup body masses, masses of the intestine and other significant internal organs, and intestinal brush-border uptake capacity for the sugar glucose. The latter is potentially a physiologically limiting variable that can be assessed by comparing it with dietary glucose intake. We did not attempt to measure milk volume or composition, because technical problems make such measurements in mice difficult to interpret (Oftedal, 1984). Instead, litter mass and individual pup mass at weaning serve as indirect surrogates of milk output.

## Materials and methods

### Mice and their maintenance

We started with 86 virgin female Swiss-Webster mice (*Mus musculus* L.), 90–160 days old, from a colony originating from Charles River Laboratories stock (Wilmington, MA, USA). Mice were divided into two main groups based on teat number: a control group with 10 teats, and an experimental group with five teats (see below for surgical methods of preparing five-teat mice). Within these two groups, mothers were subdivided by litter size: for 10-teat mothers, litter sizes were one, two, four, five, eight, 10, 13 or 14 pups (respective sample sizes were $N=7, 6, 6, 5, 8, 5, 3$ and 4 litters); for five-teat mothers, litter sizes were one, two, five, seven, eight or nine pups ($N=3, 5, 5, 7, 4$ and 2 litters, respectively). Mammary pressure thus ranged from 0.1 to 1.4 pups teat$^{-1}$ for control 10-teat mice and from 0.2 to 1.8 pups teat$^{-1}$ for five-teat mice. We chose these ranges of litter sizes because, as we shall describe below, 10-teat and five-teat mothers could maintain up to only 14 and nine pups, respectively. We also used six virgin 10-teat females and six virgin five-teat females with no pups. Besides the two main groups of mice (10-teat and five-teat), we used four two-teat mothers with one, six, nine and 13 pups, respectively, all those litter sizes resulting from unmanipulated natal litters. No pups of two-teat mothers survived past day 4 of lactation; hence, they could not be studied further.

Females were paired with males for at least 2 weeks to ensure insemination. In our colony, the most frequent litter sizes at birth are 8–10 pups, uninfluenced by teat number. We achieved smaller litters by culling pups, larger litters by cross-fostering at day 4 of lactation (see Hammond and Diamond, 1992, for details). All mice were housed individually in the UCLA Health Sciences Vivarium on a 12 h:12 h L:D cycle at 23 °C. They had continuous access to *ad libitum* quantities of water and food (the 55 % sucrose, 15 % protein, 7 % fat, 15 % fiber diet described by Diamond and Karasov, 1984).

For each female mouse (both mothers and virgins), we collected the following data at the time they were killed: body mass, gut morphology, small intestinal brush-border glucose uptake and wet and dry masses of five internal organs (see

below). We killed all lactating mother mice at day 15 of lactation, the day of maximal energy demand on the mother because it is the last day on which pups are still totally dependent on their mother's milk (they begin to nibble solid food on day 15 or 16). Total litter mass and mother's body mass were measured every 2 days from day 7 to day 11 of lactation, and daily from day 12 to day 15. Body mass of all females, as well as food intake, scattered ort mass, fecal output mass and apparent dry-matter digestive efficiency [defined as (food intake minus fecal output)/food intake)], were measured daily for the last 3 days before they were killed (see Hammond and Diamond, 1992, for details). Most measurements were by methods described in previous papers, to which we refer (Karasov and Diamond, 1983*a*; Diamond and Karasov, 1984; Hammond and Diamond, 1992, 1994). We now describe our surgical method and briefly describe our measurements.

### Surgery and recovery

Surgery on each female was performed while she was a virgin. Swiss-Webster mice typically have 10 teats: six (three on each side) thoracic teats and four (two on each side) abdominal teats. It has been speculated that abdominal teats have a higher milk output than do thoracic teats (Bolander, 1990). To avoid a bias in milk output that might result from the postulated differing capacities of thoracic and abdominal teats, we removed teats in the following fashion: for five-teat mothers, we removed the three thoracic teats from one side (chosen at random) of the ventrum and the two abdominal teats from the other side; and for two-teat mothers, we removed all teats except one abdominal teat from each side.

Females were fasted overnight from food but not from water. On the day of surgery, anesthesia was introduced with pentobarbital ($50 \, \mathrm{mg \, kg^{-1}}$ intraperitoneally) and was maintained by inhalation of halothane (2–4 % in 100 % $O_2$ at a flow rate of $3-51 \, \mathrm{min^{-1}}$). The abdomen and thorax were covered with a depilatory cream (Nair, Carter-Wallace, New York, NY, USA). After 5 min, the cream and hair were gently wiped away with saline. The bare abdomen and thorax were then aseptically prepared by gently cleansing with providone iodine and 70 % alcohol. Individual teats were identified visually and marked for excision according to a pre-arranged pattern. Each teat was excised by gently grasping the teat, tenting the skin, and excising the cutaneous and subcutaneous regions of each gland with scissors. The remaining excisional defect was closed by a skin staple. After surgery, mice were returned to their home cages and allowed to recover from anesthesia. Recovery appeared rapid, because mice began to groom and behave normally upon awakening from surgery (within 1 h), and because their body mass and food intake remained the same after as before surgery. After they were killed, females with resected teats were examined for any remaining mammary tissue. We found that our surgeries were completely successful in removing all of the intended mammary gland. Mice were allowed to recover from surgery for at least 2 weeks before their staples were removed and they were then paired with males or used as virgins.

### Experimental measurements

#### Gut morphology

A mouse was weighed and anesthetized between 08:00 and 12:00 h by injecting $0.12 \, \mathrm{mg}$ Nembutal $\mathrm{g^{-1}}$ body mass intraperitoneally. The small intestine was washed out *in situ* with cold mammalian Ringer's solution (see Karasov and Diamond, 1983*a*, for composition) and excised from the body cavity, and the remainder of the gut was then removed. We divided the gut into four compartments: stomach (from cardiac sphincter to pyloric sphincter), small intestine (from pyloric sphincter to ileocecal valve), cecum and large intestine (from the distal cecum, just below the end of the sacculent portion of the cecum, to the anus). Each compartment was straightened along a ruler, its length was measured, and it was then washed out with cold Ringer's solution. Wet and dry masses of the stomach, cecum and large intestine were measured as in Hammond and Diamond (1992).

The small intestine was divided into three regions (proximal, mid- and distal) of equal lengths. Each region was lightly blotted to remove adherent Ringer's solution, all liquid was drained from its lumen, and its wet mass was measured within 30 s of removal from the mouse. The wet mass of the entire small intestine was taken as the sum of the measured wet masses of the three regions. One segment 1–2 cm long was removed from the distal end of each region, weighed after blotting, dried in an oven for 48 h at 60 °C, and weighed again to obtain the dry mass/wet mass ratio of the segment. We calculated the dry mass of the entire small intestine as the sum of the products (from the three regions) of the region's wet mass multiplied by the dry mass/wet mass ratio measured for the segment from that region. Two 2 cm long segments were removed from each region (one each from near the proximal and distal ends) for estimating mucosal mass (see Hammond and Diamond, 1992, for details). The remainder of the small intestine was used to measure glucose uptake rates as described below. Animals remained under anesthesia for the removal of the gut and were then killed by cutting the diaphragm.

#### Organ masses

The heart, liver, spleen, paired kidneys and paired lungs were removed and weighed wet and again after drying for 48 h at 60 °C.

#### Brush-border glucose uptake

Nutrients transported from the intestinal lumen into the bloodstream traverse two cell membranes in series: the brush-border membrane separating the epithelial cell interior from the intestinal lumen, and the basolateral membrane separating the epithelial cell interior from the serosa and bloodstream. These two membranes have different sets of transport proteins for different nutrients. We measured the maximal transport velocity ($V_{\max}$) of the brush-border D-glucose transporter *in vitro* by the everted-sleeve technique described in detail previously (Karasov and Diamond, 1983*a*; Diamond and Karasov, 1984). Briefly, the small intestine was excised, everted so that the transporting mucosa faced the outside, and

maintained in cold oxygenated Ringer's solution. From the middle of each intestinal region we cut four adjacent sleeves, each 1–2 cm long: two for measuring mucosal mass as mentioned above, and two for measuring glucose uptake. Uptake was measured by mounting an everted sleeve on a glass rod in a solution containing 50 mmol l$^{-1}$ D-glucose and trace amounts of D-[$^3$H]glucose plus an adherent fluid marker (L-[$^{14}$C]glucose), incubating at 37 °C for 2 min, determining the amount of isotope taken up by the tissue, and correcting for tracer in the adherent fluid. This method yields the carrier-mediated uptake of D-glucose. Glucose uptake capacity of the entire length of the small intestine was obtained by calculating the product, for each small intestinal region, of uptake rate per milligram tissue times regional mass and summing these products over the three regions. Further details can be found in Karasov and Diamond (1983a), Diamond and Karasov (1984) and Hammond and Diamond (1992, 1994).

### Statistics

Two types of comparisons are especially relevant. First, we are interested in the effects of mammary pressure (number of pups per teat). Second, for a given mammary pressure, there are also differences in litter size associated with different teat numbers because, for a given mammary pressure, mothers with five teats have half as many pups (and hence face a potentially smaller energy burden) compared with mothers with 10 teats. We carried out these comparisons using three types of statistical tests: analysis of variance (ANOVA), analysis of covariance (ANCOVA) and linear regression. We summarize the ANOVA and ANCOVA tests here and provide further details of individual tests as they arise (see Results and Discussion). Throughout, we take $P=0.05$ as the critical level of statistical significance. Values are presented as means ±1 S.E.M.

### ANOVA

Our data consist of two main independent variables (mammary pressure and teat number, coding virgin females as having a mammary pressure of 0) and many dependent variables [food intake, digestive efficiency, body mass, litter mass, mass provisioned (see below), pup mass, gut morphological variables, other organ masses and glucose uptakes]. We used a 2×11 two-factor ANOVA to test for significant effects of teat number and mammary pressure on most dependent variables: two levels of teat number (five or 10) and 11 levels of mammary pressure (0, 0.1, 0.2, 0.4, 0.5, 0.8, 1.0, 1.3, 1.4, 1.6 or 1.8 pups teat$^{-1}$). Unless otherwise stated, the $F$ and $P$ values cited throughout the text are from these ANOVAs. Treatment and error degrees of freedom are given as subscripts for each $F$ value (denominator degrees of freedom vary between 44 and 60 because some measurements were missing for some individual mice). For analysis of the dependent variables of litter mass, mass provisioned and pup mass, we instead used a 2×10 two-factor ANOVA (two levels of teat number, 10 of mammary pressure), dropping virgin females from the analysis since they had no litters.

### ANCOVA

Because maternal body mass differed between treatment groups, we used covariate analysis in an attempt to eliminate possible confounding effects of differences in body mass, since our goal was to test for effects of teat number and litter size. We found that the mother's body mass acted as a significant covariate for the dependent variables of food intake, liver, kidney, heart, lung and gut masses, and glucose uptake capacity. Hence, we report body-mass-corrected (least-squared) means for all of these variables. Mother's body mass was not a significant covariate for pup and litter masses, digestive efficiency, gut length, mucosal and serosal masses, and glucose uptake rates.

### Litter size differences

We also analyzed some data with litter size rather than mammary pressure as an independent variable. In this case, the ANOVA and ANCOVA were still 2×11 two-factor models, but litter size replaced mammary pressure as the second factor; teat number remained as the first factor.

### Comparison between means

We used the ANOVAs and ANCOVAs described above to compare mean values associated with different mammary pressures or teat numbers. Individual *a priori* pairwise mean comparisons will be expressed by reporting *post-hoc t*-statistics corresponding to the two-tailed *P*-values. In these comparisons, we used the root mean square (corrected for the sample sizes of the two means in question) as the denominator for the total ANOVA or ANCOVA model, so that the comparison is in the context of the ANOVA or ANCOVA model itself (Zar, 1984; SAS Institute, 1987).

## Results

### Maximum litter sizes and mammary pressures

We previously found that the maximum litter size consistently reared to weaning is 14 pups, and maximum mammary pressure is 1.4 pups teat$^{-1}$, for normal 10-teat mothers (Hammond and Diamond, 1992, 1994). In the present study, we sought the corresponding limits for five-teat mothers. Of the three five-teat mothers that we started with 10 pups (2.0 pups teat$^{-1}$), none succeeded in maintaining this litter size: six, three and one pup, respectively, from these three litters died by day 7–9 of lactation. Of the two five-teat mothers that we started with nine pups (1.8 pups teat$^{-1}$), and of the four that we started with eight pups (1.6 pups teat−1), respectively, one and two mothers (50 % in each case) maintained the whole litter; the other mothers lost several pups by day 10. Of eight five-teat mothers started with seven pups (1.4 pups teat−1), seven maintained their litters, a proportion similar to our success rate for control (10-teat) females with the same pup/teat ratio. Hence, for both five-teat and 10-teat mothers, the maximum mammary pressure that can be regularly weaned is 1.4 pups teat$^{-1}$, and the absolute maximum we recorded is 1.8 pups teat$^{-1}$.
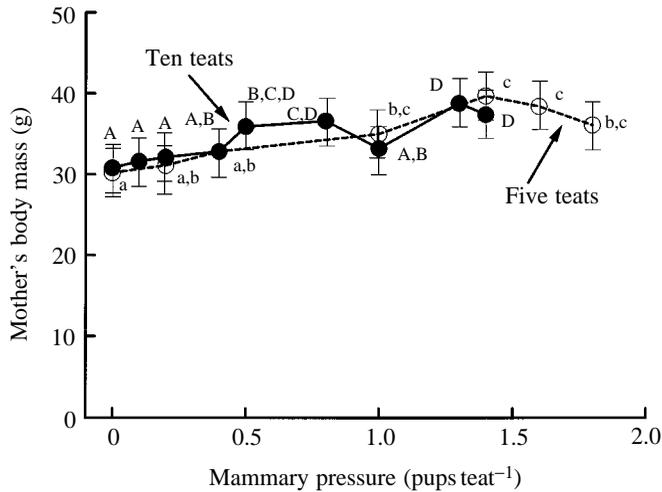
Fig. 1. Body mass as a function of mammary pressure for virgins and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles). In this and subsequent figures, vertical bars represent ±1 S.E.M. Within each set of points at the same teat number (points joined by a solid or dashed line), points labelled with different lower-case or upper-case letters differ significantly from each other as a function of mammary pressure or litter size. Within pairs of points arranged vertically above each other (same mammary pressure or letter size), asterisks denote any means that differ significantly from each other as a function of teat number. Values of $N$ are given in the Materials and methods section.

### Body mass

Body mass of female mice increased by an average of 13–15 % as they progressed from the virgin state to peak lactation (as previously illustrated for control 10-teat mothers in Fig. 2 of Hammond *et al.* 1994), and by 23–28 % during lactation as mammary pressure increased from small to larger numbers of pups per teat ($F_{10,60}=8.0$, $P=0.0001$) (Fig. 1). Between mothers with different teat numbers, there were no differences in body mass at the same mammary pressure (see Fig. 1) or at the same litter size (as may be inferred from Fig. 1 by re-expressing the abscissa as litter size).

### Food intake

As mammary pressure increased, daily food intake of lactating females increased by 109–133 % ($F_{10,58}=63.3$, $P=0.0001$) (Fig. 2A). The asymptotic maximum food intake of both the five-teat and the 10-teat mothers occurred at around 1.3–1.4 pups teat$^{-1}$. The food intake of five-teat or 10-teat mothers with a mammary pressure of 1.4 was 4.7 or 3.9 times the respective intakes of five-teat or 10-teat virgin females. For a given mammary pressure, 10-teat females ate an average of 23 % more than did five-teat females ($F_{1,58}=64.2$, $P=0.0001$), presumably related to their larger litters.

When food intake was plotted against litter size rather than against mammary pressure (Fig. 2B), lactating mothers with 10 teats proved to eat significantly more than did mothers with five teats for a given number of pups ($F_{1,58}=11.84$, $P=0.001$). Planned comparisons showed that this difference was due mainly to differences in food intake of mothers with five and
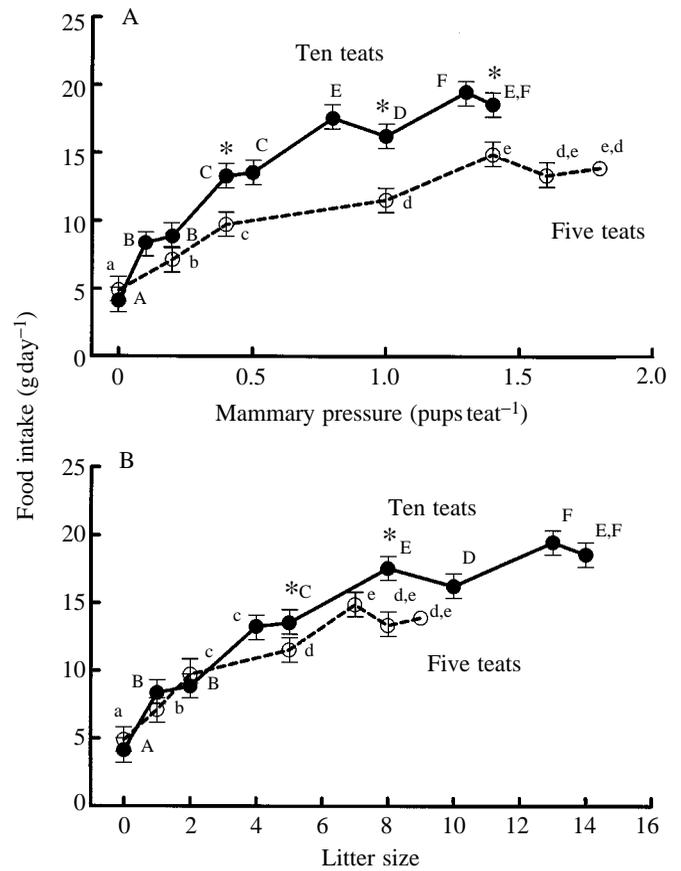


Fig. 2. Least-squared means of daily food intake for virgins (denoted by 0 mammary pressure or litter size) and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure (A) or litter size (B). Letters and asterisks indicate significant differences, as in Fig. 1.

eight pups; there was no significant effect of teat number at other litter sizes.

### Digestive efficiency

Apparent dry-matter digestive efficiency (results not shown) declined as mammary pressure increased ($F_{10,59}=3.3$, $P=0.002$), from an average of 85 % in virgins to 81 % in lactating females. Most of this effect arose from differences between 10-teat virgin and lactating females, but there was also a decline in digestive efficiency with mammary pressure among lactating females. Digestive efficiency did not differ between females with different teat numbers.

### Pup mass, litter mass and food intake per pup

Variation in pup mass presents three interesting features (Fig. 3). First, for both 10-teat and five-teat mothers, pup mass in large litters decreases with increasing litter size (Fig. 3B), as we had previously shown for 10-teat mothers (Hammond and Diamond, 1992, 1994).

Second, for both groups of mothers, pup mass in small litters also decreases with *decreasing* litter size, so that pup mass is maximal at an intermediate litter size. The mammary pressure for maximum pup size (0.4 pups teat$^{-1}$; Fig. 3A) is the same for
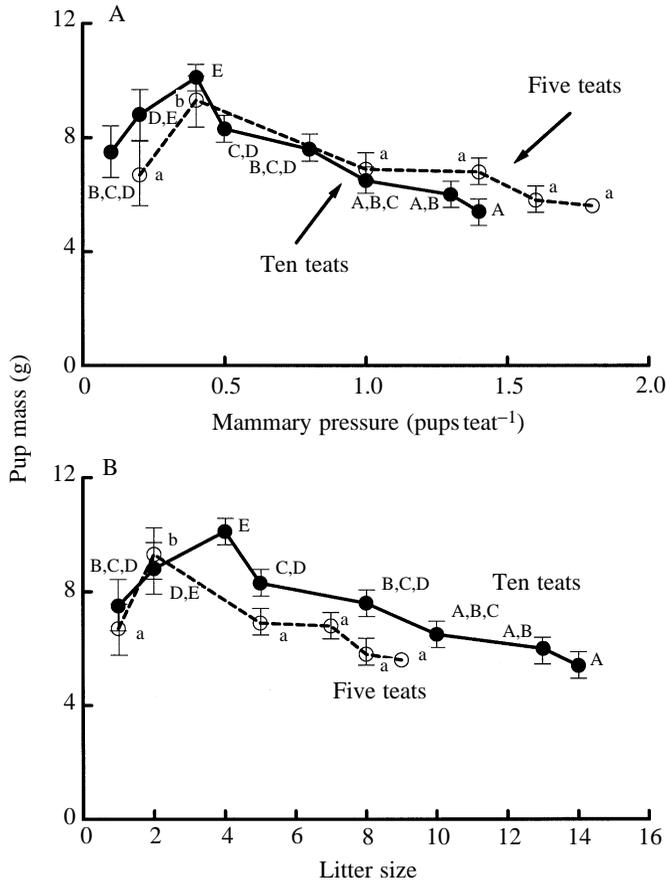
Fig. 3. Average pup mass at peak lactation for mother mice with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure (A) or litter size (B). Letters and asterisks indicate significant differences, as in Fig. 1.
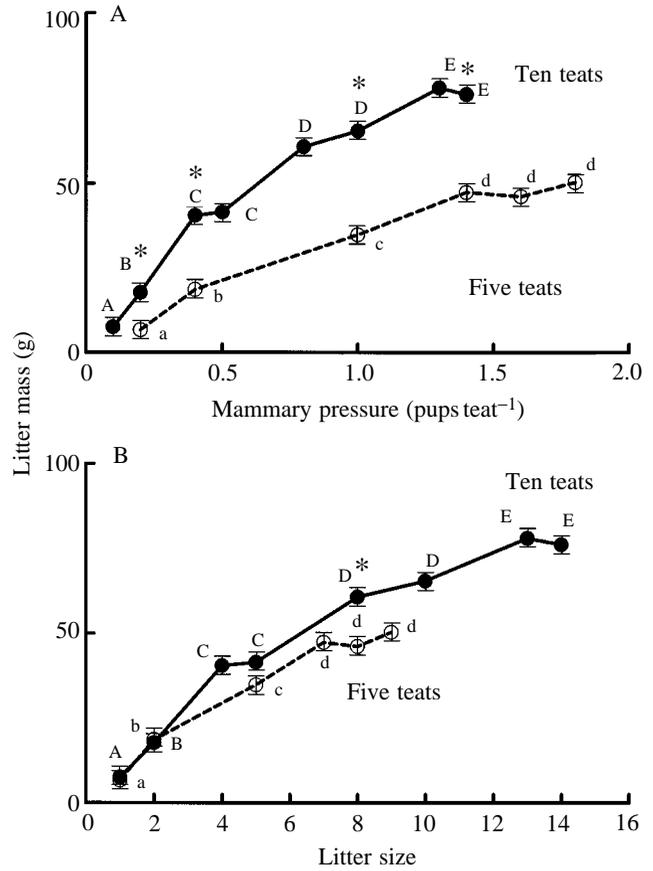
Fig. 4. Litter mass at peak lactation for mother mice with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure (A) or litter size (B). Letters and asterisks indicate significant differences, as in Fig. 1.

10-teat and five-teat mothers. Hence, the litter size for maximum pup size is higher for 10-teat mothers (four pups) than for five-teat mothers (two pups) (Fig. 3B). The average pup mass at this maximum [10.1±0.3 g ($N$=6) for 10-teat mothers, 9.3±0.9 g ($N$=5) for five-teat mothers] is 67–87 % larger than average pup mass in the largest litters [only 5.4±0.1 g ($N$=4) for 10-teat mothers, 5.6 g ($N$=1) for five-teat mothers].

Finally, a striking finding is that even though the energy burden on five-teat mothers is barely half of that on the 10-teat mothers, the five-teat mothers do not respond by producing larger pups. For a given mammary pressure, there is no difference in pup mass between five-teat and 10-teat mothers (Fig. 3A). In fact, for a given litter size, pups of five-teat mothers are smaller than those of 10-teat mothers (Fig. 3B), although the difference falls short of statistical significance ($F_{1,50}$=2.7, $P$=0.11).

The additional energy burden on the lactating mother is determined by litter mass, the product of average pup mass (Fig. 3) times litter size. Hence, litter mass increases with litter size, and also with mammary pressure (because mammary pressure reflects differences in litter size) (Fig. 4). Naturally, litter masses of 10-teat mothers are much bigger (43 % larger) than those of five-teat mothers at the same mammary pressure ($F_{1,50}$=145.9, $P$=0.0001), because 10-teat mothers have twice

as many pups (Fig. 4A). In addition, litter masses of 10-teat mothers are slightly larger (because of slightly larger pup masses) than for five-teat mothers with the same litter size ($F_{9,50}$=87.4, $P$=0.0001), and the difference is significant for litters of eight pups ($P$=0.002).

The total energy burden on the lactating mother is the sum of the additional energy burden imposed by the litter mass plus the burden of the mother's own mass. Graphs showing total mass provisioned (litter mass plus mother's mass) against mammary pressure or litter size are very similar to the corresponding graphs for litter mass (Fig. 4), and hence are omitted, because maternal mass is the same for 10-teat and five-teat mothers and varies only modestly with mammary pressure (Fig. 1).

Fig. 2 showed that maternal food intake increases with mammary pressure or litter size. However, since pup mass varies non-linearly with mammary pressure or litter size (Fig. 3), the changes in food intake cannot be perfectly matched to changes in pup mass. Fig. 5 explores this question by depicting the *extra* food intake associated with lactation (the lactating mother's food intake minus the 3.6 g day$^{-1}$ food intake of a virgin) divided by litter mass. This ratio represents the mass of food consumed (in g) per gram of pup produced, but it consists *both* of food translated into pup mass *and* of
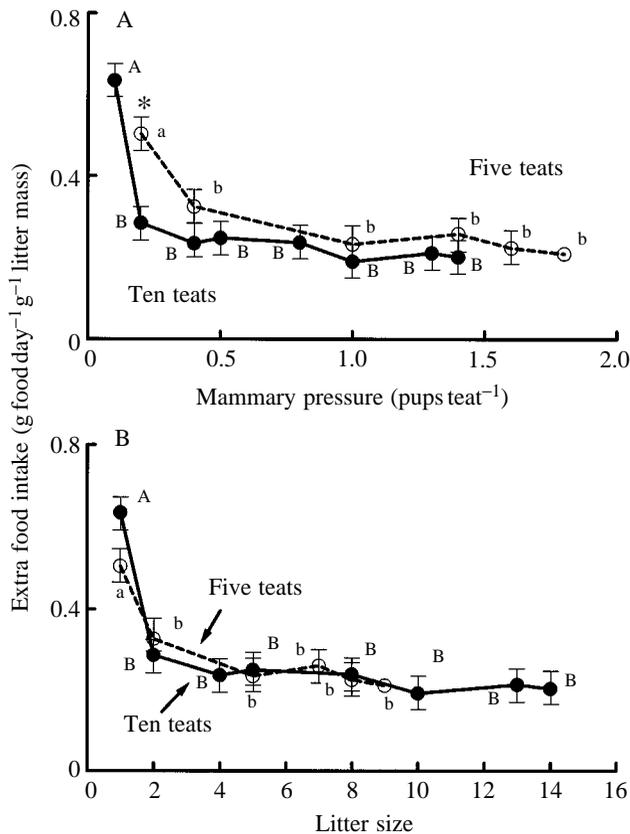
Fig. 5. The extra daily food intake associated with peak lactation [calculated as measured food intake minus the food intake of virgins (3.6 g day$^{-1}$)], per gram of litter mass, for mother mice with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure (A) or litter size (B). Letters and asterisks indicate significant differences, as in Fig. 1.
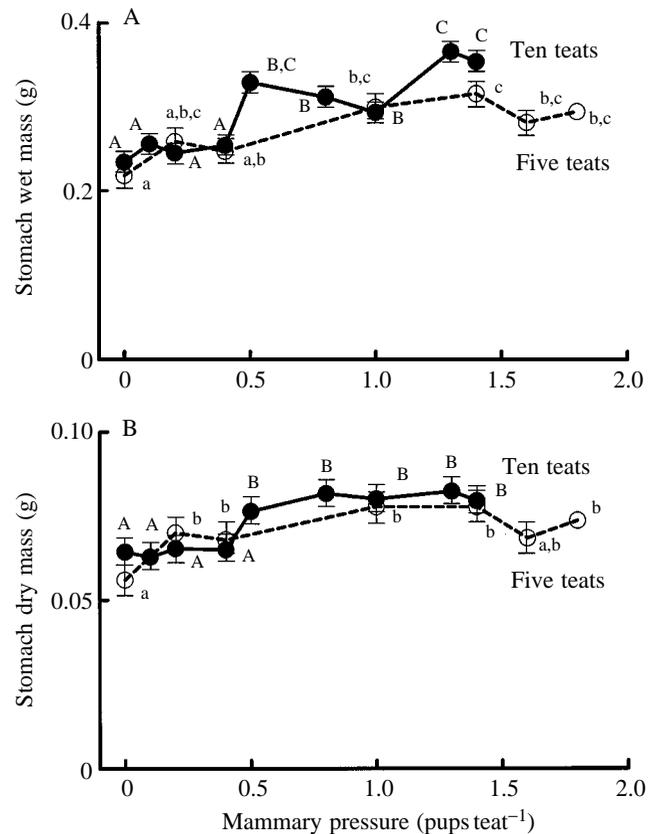


Fig. 6. Least-squared means of stomach wet and dry mass for virgins and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure. Letters and asterisks indicate significant differences, as in Fig. 1.

food required to maintain maternal tissue associated with lactation, especially the mammary glands and, as we shall below (see Fig. 7), the small intestine.

Fig. 5 yields two conclusions. First, extra food intake per gram litter mass (henceforth termed 'normalized intake') decreases with litter size (Fig. 5B: $F_{9,48}=10.9$, $P=0.0001$) and with mammary pressure (Fig. 5A: $F_{9,48}=15.4$, $P=0.0001$) for both 10-teat and five-teat mothers. In all cases, the effect is due entirely to the higher normalized intakes of the smallest litter sizes or mammary pressures; there is no further change in normalized intake from the second-lowest litter size or mammary pressure to the highest value. Second, normalized intake of five-teat mothers is higher than that of 10-teat mothers with the same mammary pressure (Fig. 5A: $F_{1,49}=12.5$, $P=0.001$), but there is no such effect of teat number for mothers with the same litter size (Fig. 5B: $F_{1,49}=0.9$, $P=0.34$). The effect is only significant at the lowest mammary pressure (0.2 pups teat$^{-1}$), when normalized intakes of five-teat mothers are 79 % larger than those of 10-teat mothers (Fig. 5A).

### *Organ morphometrics*
#### Stomach

Stomach wet mass and dry mass increased by 24–51 % with both increasing mammary pressure ($F_{10,56}=6.3$, $P=0.0001$ and $F_{10,56}=4.0$, $P=0.0004$, respectively) and increasing litter size ($F_{10,56}=6.4$, $P=0.0001$ and $F_{10,56}=3.5$, $P=0.002$, respectively). All four relationships were similar in form and in magnitude of effect (see Fig. 6 for examples). There were no changes in stomach mass with teat number and no changes in stomach length with mammary pressure, litter size or teat number.

#### *Small intestine*

Small intestinal length, wet mass and dry mass all increased significantly ($P=0.0001$–0.002) by 20–111 % as a function of either mammary pressure or litter size (Fig. 7). Small intestinal wet masses of 10-teat females were 5–7 % larger than those of five-teat females for a given mammary pressure ($F_{1,56}=7.9$, $P=0.007$). This difference is related to the larger litter sizes of the 10-teat mothers, because small intestinal masses did not differ between 10-teat and five-teat mothers when compared at the same litter size.

The small intestine can be separated into two layers: the epithelium (termed the mucosa) responsible for absorption and hydrolysis, and the supporting connective tissue and muscle (termed the serosa). Mucosal mass increased by 70–133 % ($F_{10,53}=18.5$, $P=0.0001$), serosal mass by 99–150 % ($F_{10,53}=6.1$, $P=0.0001$), with increasing mammary pressure
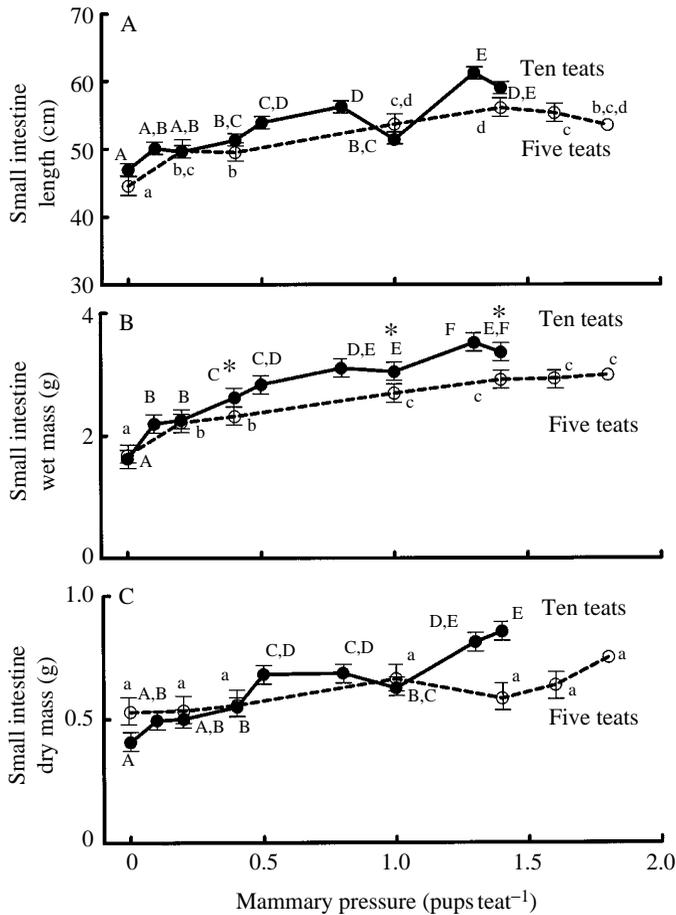
Fig. 7. Length and least-squared means of wet and dry mass of the small intestine for virgins and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure. Letters and asterisks indicate significant differences, as in Fig. 1.
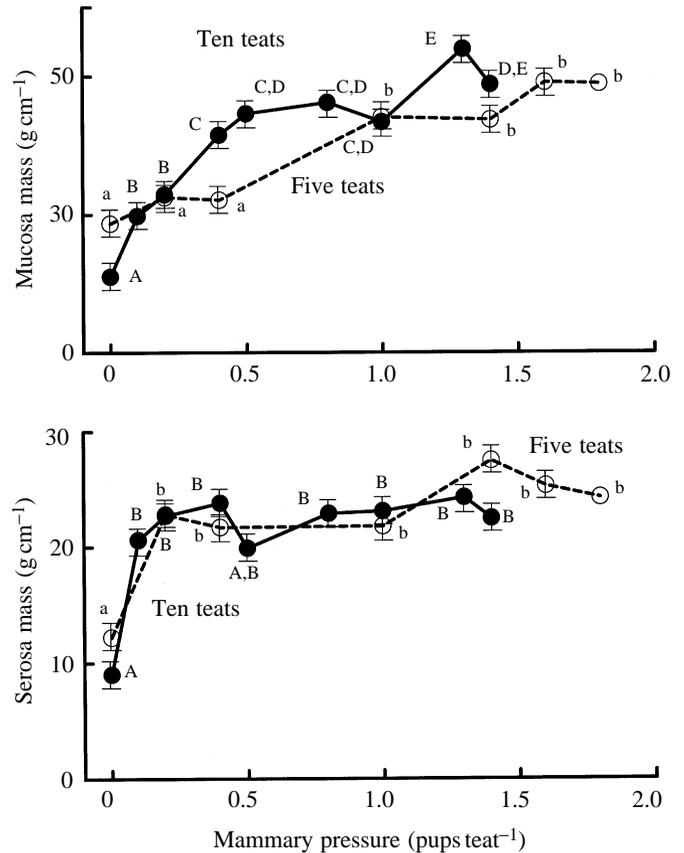


Fig. 8. Wet mass of the small intestinal mucosa and serosa (g per cm length of small intestine) for virgins and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure. Letters and asterisks indicate significant differences, as in Fig. 1.

(Fig. 8) or litter size. For serosal mass, the effect was due primarily to differences between virgins and lactating females, but for mucosal mass there were also significant increases with increasing mammary pressure among lactating females themselves. Neither mucosal nor serosal mass differed between 10-teat and five-teat females.

### Hind gut

Cecum wet mass ($F_{10,56}=3.5$, $P=0.001$) but not dry mass or length, and large intestinal wet mass ($F_{10,56}=7.1$, $P=0.0001$) and length ($F_{10,57}=3.8$, $P=0.0006$) but not dry mass, increased with increasing mammary pressure (Fig. 9). The wet mass increase from virgins to lactating females at the highest mammary pressures was 44–109 % for the cecum, 94–102 % for large intestine wet mass and 14–25 % for length. There were no differences between 10-teat and five-teat lactating mothers in any measurement of the cecum or large intestine.

### Other organ masses

Because wet and dry organ masses or mammary pressure and litter size yielded the same conclusions, we present the results

only for dry masses and mammary pressure. Liver dry mass increased by 30–35 % ($F_{10,56}=5.8$, $P=0.0001$) and kidney dry mass by 14–31 % ($F_{10,55}=4.6$, $P=0.0001$) between virgins and lactating females. Dry masses of heart, lungs and spleen showed statistically significant variation (using ANCOVA) with mammary pressure, but the changes were small and not in a consistent direction with changes in mammary pressure (for all three dry masses, $P=0.004$–$0.04$). There were no differences in liver, kidney, heart or lung masses, and only small and marginally significant ($F_{1,55}=4.5$, $P=0.04$) differences in spleen mass, between 10-teat and five-teat females.

### Glucose uptake

Glucose uptake per milligram of intestinal tissue did not vary significantly with mammary pressure, litter size or teat number. Uptake per centimeter length of intestine did increase with mammary pressure ($F_{10,46}=4.3$, $P=0.0003$) and litter size ($F_{10,46}=4.0$, $P=0.0001$), but only because of the increase in intestinal mass per centimeter length. Glucose uptake capacity of the entire length of the small intestine was 42 % higher for 10-teat lactating females than for virgins ($F_{10,45}=3.7$, $P=0.001$) but did not differ between five-teat lactating females and
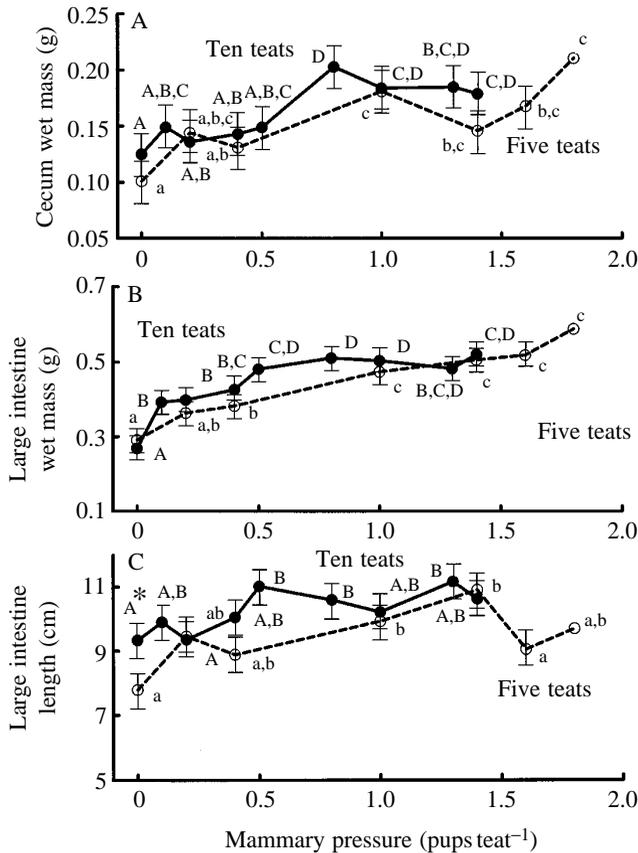
Fig. 9. Least-squared means of cecum (A) and large intestinal (B) wet mass, and large intestinal length (C), for virgins and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure. Letters and asterisks indicate significant differences, as in Fig. 1.
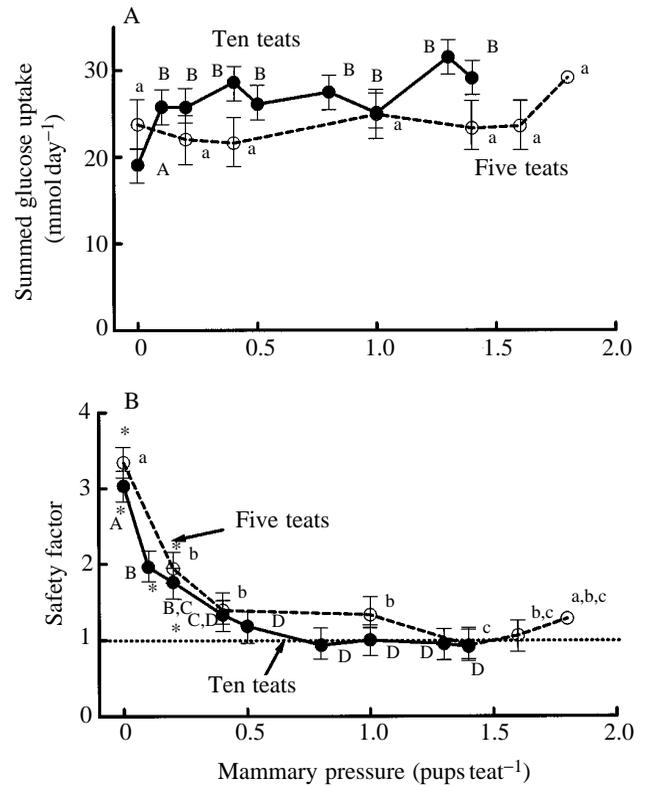


Fig. 10. Least-squared means of summed glucose uptake (A) and safety factor (see text for explanation) for glucose uptake (B) across the small intestinal brush-border membrane of virgins and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles), as a function of mammary pressure. Letters indicate significant differences, as in Fig. 1. In B, asterisks denote differences between the safety factors shown and a safety factor of 1.0 (dotted line).

virgins, nor between 10-teat and five-teat lactating females (Fig. 10A).

Glucose uptake capacity (in mmol day$^{-1}$) may be compared with dietary glucose intake in the same units, calculated from daily food intake (g day$^{-1}$) and dietary content of glucose in the form of sucrose. The excess of uptake capacity over intake represents the unutilized reserve capacity, and the ratio of uptake capacity to intake is termed the safety factor for glucose uptake (Diamond and Hammond, 1992; Diamond, 1993). Safety factors decreased with increasing mammary pressure or litter size ($F_{10,44}$=10.1, $P$=0.0001), were largest (3.0–3.4) for virgins, were also significantly ($P$<0.05, $t$-test) greater than 1.0 at mammary pressures of 0.1 and 0.2 pups teat$^{-1}$, but did not differ significantly from 1.0 for higher mammary pressures (Fig. 10B).

## Discussion

We shall discuss four questions: the significance of five-teat mice for understanding limits on lactational performance; control of intestinal mass by food intake; the evidence for capital start-up costs in lactation; and the dependence of pup mass on mammary pressure.

### Can five-teat mice maintain normal food intake to produce larger pups?

Our initial motivation in preparing five-teat mice was to separate maternal food-processing limits from milk-producing limits on lactational performance. Our previous studies (Hammond and Diamond, 1992, 1994) had shown that the lactational performance of mother mice ultimately reaches a limit, as reflected by the failure to wean more than 14 pups, the decreasing pup mass in large litters, the plateau in maternal food intake for large litters, and the plateau in maternal food intake as pups prevented from weaning grow beyond day 15. However, our previous studies could not distinguish whether these limits on lactational performance arose from limits on the ability to produce milk or from limits on the ability to process the food intake necessary to acquire the nutrients for milk production.

Comparisons of five-teat and 10-teat mice help to distinguish between these two types of limits. By simultaneously reducing teat number as well as litter size, we were able to maintain mammary pressure (number of pups per teat) constant while reducing the total energy burden on the mother. If the five-teat mothers had consumed the same

amount of food as the 10-teat mothers, they would have had more nutrients potentially available per pup. Larger pups under those conditions would have proved that mothers were capable of converting more potentially available nutrients per pup into more milk per pup. But the mothers did not maintain their food intake, or increase the mass of their pups, or increase the ratio of mass of pup produced to mass of food consumed. Instead, these mothers decreased their food intake, while maintaining pup mass and the mass of pup produced to mass of food consumed ratio approximately unchanged for a given litter size. This indicates that the ultimate limit to lactational performance (as reflected in pup mass) does not lie in the intestine or other organs responsible for acquiring nutrients from food, but in the mammary glands or related biological machinery responsible for exporting nutrients into milk. Rather than food intake and acquisition controlling lactational performance, pup demand evidently controls food intake.

### Control of intestinal mass by food intake

An increase in intestinal growth is the usual intestinal adaptive response associated with increased energy requirements and increased needs for all nutrients (Karasov and Diamond, 1983*b*). The reason is that, as intestinal mass increases, the capacities of all nutrient hydrolytic enzymes and transporters integrated over the length of the intestine increase by the same factor, provided that enzyme or transporter activities per milligram intestine remain unchanged (as we found for the glucose transporter in the present study).

In previous studies, we had found that the mass of mouse small intestine increases with increasing food intake during pregnancy, lactation (Hammond and Diamond, 1992, 1994) and cold exposure (Toloza *et al.* 1991; Konarzewski and Diamond, 1994; Hammond *et al.* 1994). The present study verified this result and added the observation that the relationship between intestinal mass and food intake is far more precise than suspected previously. As Fig. 11 illustrates, the regression of intestinal mass on food intake is linear, with a high explained variance ($r^2=0.88$, $P=0.0001$). Furthermore, examination of Fig. 11 makes it clear that points for five-teat and 10-teat mothers fall on the same regression line, even though the highest values (largest intestines and highest food intakes) are confined to 10-teat mice (because they had larger litters). Thus, energy demand controls food intake, which in turn controls intestinal mass.

Since maternal body mass also increases during lactation and is a significant covariate for intestinal mass, we investigated whether intestinal growth was secondary to body growth. However, even when we removed the effect of body mass by ANCOVA, intestinal mass still increased with mammary pressure or litter size. In an additional ANCOVA using food intake together with body mass as covariates for intestinal mass, the effects of both teat number ($F_{1,54}=0.14$, $P=0.71$) and mammary pressure ($F_{10,54}=0.87$, $P=0.57$) disappeared, and body mass ceased to be a significant covariate ($F_{1,54}=2.9$, $P=0.10$), but food intake was a highly significant covariate ($F_{1,54}=10.7$, $P=0.002$). That is, lactation does not control
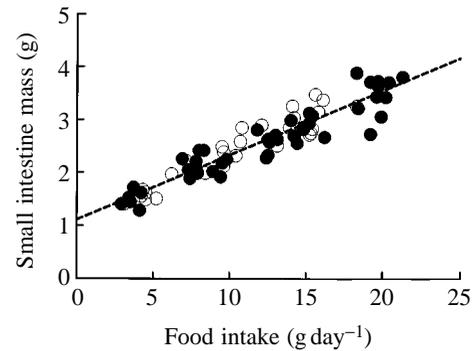


Fig. 11. Regression of small intestinal wet mass ($m$) against food intake ($F_{in}$) for virgins and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles). The best-fit regression line is: $m=0.12F_{in}+1.11$; $r^2=0.88$, $P=0.0001$. Best-fitting lines for five-teat and 10-teat mothers do not differ significantly.

intestinal mass directly but only through the intermediary of food intake. Conversely, lactating mice are not limiting their food intake because of an intestinal bottleneck but because of other reasons, such as pup demand and/or milk production.

Lactating mice increase the mass not only of the intestine but also of the liver and kidneys, suggesting that these organ masses too are controlled by food intake and metabolic rate. The burden on the liver and kidneys is less heavy during lactation than during other energetically stressful conditions (such as cold exposure and physical activity), because some of the ingested nutrients pass intact to the mammary glands without further metabolism. Nevertheless, the liver is still used to break down body stores for the purpose of milk production, and both milk production and maintenance of mammary glands and other tissues yield waste products for excretion by the kidney.

### Capital start-up costs in lactation

Mothers with the largest sustainable litters consumed 4.5 times more food than virgins (Fig. 2). Even mothers with only a single pup (both five-teat and 10-teat mothers) consumed 45–102 % more food than virgins (Fig. 2B). For litters of two or more pups, the increase in food intake is constant, at 0.24 g of food consumed per day per gram pup produced (Fig. 5). For the first pup, however, the increase is far higher, $0.5$–$0.6\,\mathrm{g\,day^{-1}\,g^{-1}}$ (Fig. 5). This suggests that lactation involves capital start-up costs distinct from the operating costs of milk production and nearly equal to the entire energy budget of virgin mice. Obvious candidates as components of these start-up costs are development of the mammary glands, of the hormonal mechanisms involved in lactation and of the intestine itself.

### Dependence of pup mass on mammary pressure

Fig. 3 shows that the dependence of pup mass on mammary pressure is strikingly biphasic. From a low value of around 7 g in one-pup litters, pup mass increases to a maximum of approximately 10 g at a mammary pressure of 0.4 pups teat$^{-1}$ (four pups for 10-teat mothers, two pups for five-teat mothers).

From that maximal value, pup mass then decreases with further increases in mammary pressure to a value of 5.4–5.6 g at the highest sustainable mammary pressures, beyond which any further cross-fostered pups starve and die. Fig. 3 also suggests that the relevant independent variable is mammary pressure rather than litter size, since the peaks in pup masses of 10-teat and five-teat mothers occur at the same mammary pressure but at different litter sizes.

Knight *et al.* (1986) previously noted the same biphasic relationship in mice, with a peak in pup mass for four-pup litters of 10-teat mothers, the same litter size at which we observed a peak for those mothers. Many previous studies on rodents have noted the decrease in pup mass in exceptionally large litters (e.g. Wurtman and Miller, 1976; Epstein, 1978; Russel, 1980; Mattingly and McClure, 1982; König *et al.* 1988), while a few studies have noted a decrease for exceptionally small litters (Wurtman and Miller, 1976; Epstein, 1978; Russel, 1980). The explanation of this biphasic curve seems to involve separate limitations operating at high and low mammary pressures.

### Limits operating at high mammary pressure

Many studies have shown that the smallest individuals of a species are often at a severe disadvantage, even under normal ecological conditions (Fleming and Rauscher, 1978; Fuchs, 1982; Myers and Master, 1983). One must therefore wonder why mouse mothers 'allowed' pup mass to decline with increasing mammary pressure beyond 0.4 pups teat$^{-1}$. In particular, we already know that mother mice at peak lactation increase their food intake even further when transferred to low ambient temperatures, thereby achieving food intakes considerably higher than when lactation is the sole added energy burden. Mouse intestine is thus perfectly capable of digesting more food than lactating mothers digest. Why do lactating mothers with high mammary pressures not 'just' eat more food and thereby wean normal-sized pups that will not be at a severe competitive disadvantage?

The likely explanation is that mice subject to high mammary pressures reach a ceiling on lactational performance, of which low pup mass is one expression. Three of our other findings point to such a ceiling. First, the relationship between litter mass and mammary pressure or litter size increases asymptotically and is highly predictable, with explained variances of 99 % for both 10-teat and five-teat litters (Fig. 12). Second, when we increased the number of pups cross-fostered, both 10-teat and five-teat mothers reached a limit beyond which further cross-fostered pups died. That limit lay at a mammary pressure of 1.4 pups teat$^{-1}$ for 10-teat mothers and 1.8 pups teat$^{-1}$ for five-teat mothers. Finally, while half of the five-teat mothers given cross-fostered pups to achieve mammary pressures of 1.6 or 1.8 pups teat$^{-1}$ succeeded in rearing those litters, the other mothers failed. This failure rate of 50 % is much higher than the failure rate of 12 % observed for mammary pressures of 1.4 pups teat$^{-1}$. All these observations indicate a limit on lactational performance itself.

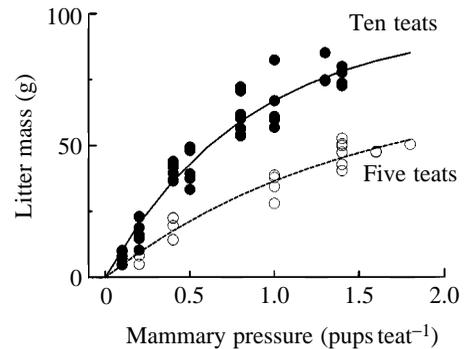The limit is likely to reside at least partly in the mammary



Fig. 12. Non-linear regression of litter mass against mammary pressure for virgins and mother mice at peak lactation with ten teats (filled circles) or five teats (open circles). Data fit the equation $y=a(1-e^{-bx})$, where $a$ is the asymptotic litter mass and $b$ is the rate of litter mass gain with increasing mammary pressure. For five-teat mice, $a=73$, $b=0.69$, $r^2=0.99$, $P=0.0001$; for 10-teat mice, $a=96$, $b=1.2$, $r^2=0.99$, $P=0.0001$. The corresponding relationship with litter size rather than mammary pressure as the abscissa is very similar.

glands themselves. While there are technical obstacles to sampling milk production by mice without introducing unphysiological distortions, studies of milk production by mother rats have shown both quantitative and qualitative ceilings on milk production at high demand (König *et al.* 1988; Fiorotto *et al.* 1991; Rogowitz, 1996). As rat pups grow and as their demand for milk increases, mothers respond with increasing milk volume, but volume hits a ceiling at the time of peak lactation for litters of 10 or more pups. At high volumes, the energy content of rat milk declines because of decreases in both fat content and carbohydrate content. In effect, mothers of large litters attempt to compensate by diluting their milk and dividing it among more pups.

If milk production reaches similar limits in mice, then this would explain most of our observations, suggesting a ceiling on lactational performance arising within each mammary gland. However, one observation suggests the possibility of an additional limit outside the mammary glands. The calculated asymptotic litter mass at weaning is 73 g for five-teat mothers, i.e. 15 g weaned pup per teat, but 96 g for 10-teat mothers, i.e. only 10 g weaned pup per teat (Fig. 12). Apparently, each teat in a 10-teat mother cannot produce as much milk as in a five-teat mother. The same conclusion is suggested by the 50 % success rate of five-teat mothers at mammary pressures of 1.6–1.8 pups teat$^{-1}$, contrasted with the 0 % success rate of 10-teat mothers under the same conditions. These findings suggest an additional limit, related to the total energy burden on the mother, that can be reached in 10-teat mice but that is never reached in five-teat mice (because of their lower total energy burden). Such a limit could reside in the liver's ability to mobilize the nutrients necessary for milk production, or the hormonal mechanisms involved in lactation or elsewhere in the body.

### Limits operating at low mammary pressure

Why are pups in litters of few pups smaller than pups in

litters with normal pup numbers? The suggested physiological mechanism is that low mammary pressure (few pups per teat) means insufficient suckling to activate the neural reflex and hormonal pathways involved in milk secretion, let-down and ejection (Epstein, 1978; Russel, 1980; Knight *et al.* 1986). There are also possible autocrine controls that result in a local negative feedback on milk delivery at low suckling rates and affect secretion rates rather than milk composition (Wilde and Peaker, 1990; Peaker, 1991). Suckling by pups stimulates mammary tissue and thereby results (*via* nerve and hormonal signals) in stimulation of the paraventricular nucleus and supraoptic nerve of the hypothalamus. This releases the hormone oxytocin from the pituitary gland and, within a few seconds, causes milk let-down. Simultaneously, a negative feedback on tuberoinfundibular dopamine neurons (which normally inhibit lactotrophs in the anterior pituitary) releases the hormone prolactin (Amenomori *et al.* 1970), which stimulates the alveolar cells to produce milk for the next meal. There is also a positive feedback between prolactin and growth hormone that stimulates milk production (Barber *et al.* 1992).

For the suckling-induced stimulation of milk production to continue, pups must continue to suckle. When pups cease suckling, milk is no longer manufactured in the mammary glands, prolactin (Amenomori *et al.* 1970) and oxytocin (Grosvenor and Turner, 1958; Fuchs, 1969) levels decline, and lactation ultimately ceases (Hanwell and Linzell, 1972). These are not all-or-nothing phenomena. Instead, the mammary glands respond in greater fashion to stimulation by suckling, such that more suckling results in more milk let-down until a ceiling of milk output is reached (Amenomori *et al.* 1970).

Mother mice with litters of only 0.1–0.2 pups teat$^{-1}$, and mothers with only two teats, receive low suckling pressure and are stimulated to a low milk output. Their milk may be sufficient for small pups in the first few days of life but milk supply may become marginal after the pups grow. This interpretation would explain our observation that pups in litters with low mammary pressure become stunted. It would also explain our observation that our two-teat mothers lost all their pups by day 4 of lactation, even though their mammary pressures (pup/teat ratios of 0.5–6.5) would have been optimal or even super-optimal in mothers with more teats.

In short, pup mass appears to be limited by inadequate suckling stimulation of mammary glands in small litters, by ceilings on mammary gland milk output in large litters of five-teat mothers, and possibly by ceilings elsewhere in the body in large litters of 10-teat mothers. Interestingly, the litter size of wild *Mus musculus*, the ancestor of laboratory mice, averages 5.2 pups, with a range of 4–6 pups (Asdell, 1964; Hayssen *et al.* 1993). That natural litter size compares well with our observed litter size of four pups for maximum pup mass. Artificial selection by animal breeders within the last century has quickly selected for larger litters in domesticated mouse strains, without the other adjustments necessary to maintain maximum pup mass in these larger litters.

## References

AMENOMORI, Y., CHEN, C. L. AND MEITES, J. (1970). Serum prolactin levels in rats during different reproductive states. *Endocrinology* **86**, 506–510.

ASDELL, S. A. (1964). (ed.) *Patterns of Mammalian Reproduction*. Ithaca: Comstock Publishing Associates.

BARBER, M. C., CLEGG, R. A., FINLEY, E., VERNON, G. AND FLINT, D. J. (1992). The role of growth hormone, prolactin and insulin-like growth factors in the regulation of rat mammary gland and adipose tissue metabolism during lactation. *J. Endocr.* **135**, 195–202.

BOLANDER, F. F. (1990). Differential characteristics of the thoracic and abdominal mammary glands from mice. *Expl Cell Res.* **189**, 142–144.

DIAMOND, J. (1993). Evolutionary Physiology. In *The Logic of Life; The Challenge of Integrative Physiology* (ed. C. A. R. Boyd and D. Nobel), p. 89. Oxford: Oxford University Press.

DIAMOND, J. AND HAMMOND, K. (1992). The matches, achieved by natural selection, between biological capacities and their natural loads. *Experientia* **48**, 551–557.

DIAMOND, J. M. AND KARASOV, W. H. (1984). Effect of dietary carbohydrate on monosaccharide uptake by mouse small intestine *in vitro*. *J. Physiol., Lond.* **349**, 419–440.

DRENT, R. H. AND DAAN, S. (1980). The prudent parent: energetic adjustments in avian breeding. *Ardea* **68**, 225–252.

EPSTEIN, H. T. (1978). The effect of litter size on weight gain in mice. *J. Nutr.* **108**, 120–123.

FIOROTTO, M. L., BURRIN, D. G., PEREZ, M. AND REEDS, P. J. (1991). Intake and use of milk nutrients by rat pups suckled in small, medium and large litters. *Am. J. Physiol.* **260**, R1104–R1113.

FLEMING, T. H. AND RAUSCHER, R. J. (1978). On the evolution of litter size in *Peromyscus leucopus*. *Evolution* **32**, 45–55.

FUCHS, A. R. (1969). Ethanol and the inhibition of oxytocin release in lactating rats. *Acta endocr., Copnh.* **62**, 546–554.

FUCHS, S. (1982). Optimality of parental investment: the influence of nursing on reproductive success of mother and female young house mice. *Behav. Ecol. Sociobiol.* **10**, 39–51.

GROSVENOR, C. E. AND TURNER, C. W. (1958). Pituitary lactogenic hormone concentration and milk secretion in lactating rats. *Endocrinology* **63**, 535–539.

HAMMOND, K. A. AND DIAMOND, J. M. (1992). An experimental test for a ceiling on sustained metabolic rate in lactating mice. *Physiol. Zool.* **65**, 952–977.

HAMMOND, K. A. AND DIAMOND, J. (1994). Limits to dietary nutrient and intestinal nutrients uptakes in lactating mice. *Physiol. Zool.* **67**, 282–303.

HAMMOND, K. A., KONARZEWSKI, M., TORRES, R. AND DIAMOND, J. (1994). Metabolic ceilings under a combination of peak energy demands. *Physiol. Zool.* **68**, 1479–1506.

HANWELL, A. AND LINZELL, J. (1972). A simple technique for measuring the rate of milk secretion in the rat. *Comp. Biochem. Physiol.* **43**, 259–270.

HAYSSEN, V., VAN TIENHOVEN, A. AND VAN TIENHOVEN, A. (1993). (eds) *Asdell's Patterns of Mammalian Reproduction*. Ithaca: Comstock Publishing Associates.

KARASOV, W. H. AND DIAMOND, J. M. (1983*a*). A simple method for measuring intestinal solute *in vitro*. *J. comp. Physiol.* **152**, 105–116.

KARASOV, W. H. AND DIAMOND, J. M. (1983*b*). Adaptive regulation of sugar and amino acid transport by vertebrate intestine. *Am. J. Physiol.* **245**, G443–G462.

KNIGHT, C. H., MALTZ, E. AND DOCHERTY, A. H. (1986). Milk yield and composition in mice: effects of litter size and lactation number. *Comp. Biochem. Physiol.* **84**, 127–133.

KONARZEWSKI, M. AND DIAMOND, J. (1994). Peak sustained metabolic rate and its individual variation in cold-stressed mice. *Physiol. Zool.* **67**, 1186–1212.

KÖNIG, B., RIESTER, J. AND MARKL, H. (1988). Maternal care in house mice (*Mus musculus*). II. The energy cost of lactation as a function of litter size. *J. Zool., Lond.* **216**, 195–210.

MATTINGLY, D. K. AND MCCLURE, P. A. (1982). Energetics of reproduction in large-littered cotton rats (*Sigmodon hispidus*). *Ecology* **63**, 181–195.

MYERS, P. AND MASTER, L. L. (1983). Reproduction by *Peromyscus maniculatus*: size and compromise. *J. Mammal.* **64**, 1–18.

OFTEDAL, O. T. (1984). Milk composition, milk yield and energy output at peak lactation: a comparative review. *Symp. zool. Soc. Lond.* **51**, 33–85.

PEAKER, M. (1991). Production of hormones by the mammary gland: short review. *Endocr. Rev.* **25**, 10–13.

PETERSON, C. C., NAGY, K. A. AND DIAMOND, J. M. (1990). Sustained metabolic scope. *Proc. natn. Acad. Sci. U.S.A.* **87**, 2324–2328.

ROGOWITZ, G. (1996). Trade-offs in energy allocation during lactation. *Am. Zool.* (in press).

RUSSEL, J. A. (1980). Milk yield, suckling behavior and milk ejection in the lactating rat nursing litters of different size. *J. Physiol., Lond.* **303**, 403–415.

SAS INSTITUTE (1987). *SAS/STAT Guide for Personal Computers*. Version 6 Edition. Cary, NC: SAS Institute Inc.

TOLOZA, E., LAM, M. AND DIAMOND, J. (1991). Nutrient extraction by cold-exposed mice: a test of digestive safety margin. *Am. J. Physiol.* **261**, 608–620.

WEIBEL, E. R., TAYLOR, C. R. AND HOPPELER, H. (1991). The concept of symmorphosis: a testable hypothesis of structure–function relationship. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10357–10361.

WEINER, J. (1993). Physiological limits to sustainable energy budgets in birds and mammals: Ecological implications. *Tree* **7**, 384–388.

WILDE, C. J. AND PEAKER, M. (1990). Autocrine control in milk secretion. *J. agric. Sci.* **114**, 235–238.

WURTMAN, J. J. AND MILLER, S. A. (1976). Effect of litter size on weight gain in rats. *J. Nutr.* **106**, 697–701.

ZAR, J. H. (1984). *Biostatistical Analysis*. Englewood Cliffs, NJ: Prentice-Hall.