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Limits to sustained energy intake

VII. Milk energy output in laboratory mice at thermoneutrality

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Summary

The limits to sustained energy intake at peak lactation could be imposed peripherally, by the capacity of the mammary glands, or centrally, by the capacity of the animal to dissipate body heat generated as a by-product of processing food and producing milk. To distinguish between the two hypotheses, we examined milk energy output at peak lactation in MF1 laboratory mice exposed to 30°C (*N*=12), 21°C (*N*=10; published data) and 8°C (*N*=10; published data). The peripheral limitation hypothesis predicts that milk energy output will remain constant at different temperatures, while the heat dissipation limit hypothesis predicts a decline in milk energy output as temperature increases. Since estimates of milk energy output in small mammals can vary depending on the calculation method used, we evaluated the milk energy output of mice (*N*=24) using four different methods: (1) as the difference between metabolizable energy intake and daily energy expenditure of the female, (2) from female water turnover, (3) from pup water turnover and (4) from the energy budget of the litter. We assessed these four methods by comparing their accuracy, precision and sensitivity to changes in parameters involved in the calculations. Methods 1, 3 and 4 produced similar estimates of milk energy output, while those derived from female water turnover were significantly lower and more variable. On average, mice at 30°C exported significantly less energy as milk (87.7 kJ day^{-1}) than mice at 21°C (166.7 kJ day^{-1}) and 8°C (288.0 kJ day^{-1}). This reduction in milk energy output at 30°C was caused by a significant decline in both milk flow (20.0 g day^{-1}, 12.9 g day^{-1} and 8.5 g day^{-1} at 8°C, 21°C and 30°C, respectively) and gross energy content of milk (14.6 kJ g^{-1}, 13.1 kJ g^{-1} and 10.5 kJ g^{-1} at 8°C, 21°C and 30°C, respectively). Milk produced at 30°C contained significantly less total solids (34.4%) than milk at 21°C (40.9%) and 8°C (41.5%) and significantly less fat (20.0%) than milk at 21°C (26.4%) and 8°C (30.3%). The reduced milk energy output in mice exposed to 30°C, paralleled by their reduced food intake and low reproductive output, argues against the peripheral limitation hypothesis and provides strong support for the heat dissipation limit hypothesis.

Supplementary data available on-line.

Key words: doubly labelled water, daily energy expenditure, water turnover, water balance, milk composition, peripheral limit, heat dissipation limit, laboratory mouse, *Mus musculus*.

Introduction

The factors that limit the maximal rate of food intake are important because they determine the upper limit to the ability of animals to survive and reproduce (e.g. Drent and Daan, 1980; Peterson et al., 1990; Hammond and Diamond, 1997; Speakman, 2000). One model system that has provided a rich avenue for research in this field is lactation energetics in small rodents (e.g. Perrigo, 1987; Weiner, 1987; Hammond and Diamond, 1992, 1994; Rogowitz and McClure, 1995; Koteja, 1996a; Speakman and McQueenie, 1996; Johnson et al., 2001a,b,c). Small rodents elevate their food intake dramatically during lactation when compared with non-reproductive controls, but they appear to reach a limit during peak lactation above which they are reluctant, or unable, to increase their food intake. In addition to precipitating an apparent limit in food intake, lactation is also the key period for reproductive investment. Hence, failure to upregulate food intake at this time of life may have immediately tangible and measurable impacts on components of fitness (Stearns, 1992).

Early studies on food intake at peak lactation suggested that the limits reflected the capacity of the alimentary tract and associated organs such as the liver to process the ingested food (the ‘central limitation hypothesis’; Kirkwood, 1983; Perrigo, 1987; Hammond and Diamond, 1992, 1994; Koteja, 1996a). However, mice and hispid cotton rats (*Sigmodon hispidus*)

forced to lactate at low ambient temperatures are able to increase their food intake to above the supposed centrally mediated limit (Hammond et al., 1994; Rogowitz, 1998; Johnson and Speakman, 2001). This suggested that the limit was not mediated centrally but was controlled by the ability to expend or export the energy at the sites of utilisation (the 'peripheral limitation hypothesis'). Several lines of evidence support this idea. For example, surgical manipulation of the number of teats in lactating mice demonstrated that females with five or ten teats (but with the same number of pups per teat) weaned pups that did not differ in body mass (Hammond et al., 1996). These results suggest that the mammary tissue remaining after surgery was unable to increase milk production to compensate for the lost production of the tissue that had been removed, presumably because mammary tissue before surgery was already at maximal performance. This conclusion is also consistent with the observation that hispid cotton rats lactating in the cold, despite the increased thermoregulatory demands of their offspring, did not increase milk energy output (*MEO*) and therefore produced smaller pups (Rogowitz, 1998). Again, this implies that the mammary glands of females in the warm were already working at maximal capacity.

However, several other studies are not compatible with the peripheral limitation hypothesis. Johnson et al. (2001c), for example, made mice simultaneously pregnant while lactating. In theory, these mice should upregulate their food intake above that of mice that were only lactating, because the demands for pregnancy do not require elevated milk production. Yet the mice did not do this. Similarly, lactating mice forced to run to obtain food also failed to upregulate their energy intake to meet the demands of milk production and exercise (Perrigo, 1987). Perhaps most critically, Johnson and Speakman (2001) found that milk production in the cold (8°C) was elevated relative to mice housed at 21°C. This shows that animals can increase their milk production above the level thought to be maximal.

Król and Speakman (2003) have suggested a novel hypothesis to explain these conflicting data. According to this hypothesis, the limits to food intake are imposed centrally but at a location different from the alimentary track. We suggest that this central limitation is the maximal capacity of the animal to dissipate body heat, generated as a by-product of processing food and producing milk. This hypothesis predicts that lactating mice kept at 21°C would not elevate their food intake, whatever the additional demands placed on them (Perrigo, 1987; Johnson et al., 2001c), because ingesting additional food would have made them dangerously hyperthermic. However, when females were transferred to the cold (Johnson and Speakman, 2001), the increased driving gradient between body temperature and ambient temperature relaxed the heat dissipation constraint, and the animals were able to elevate their food intake and use that energy for greater milk production. The critical difference between this viewpoint and previous interpretations is that the heat dissipation limit hypothesis views cold as a factor allowing the animals to overcome a constraint on food intake, while previous interpretations have considered exposure to the cold as an additional burden.

To test the heat dissipation limit hypothesis, Król and Speakman (2003) exposed reproducing MF1 laboratory mice (*Mus musculus* L.) to their thermoneutral temperature (30°C) and measured their food intake and reproductive output. Consistent with the heat dissipation limit hypothesis, food intake at peak lactation was lower than observed previously in the same strain of mice lactating at either 21°C (Johnson et al., 2001a) or 8°C (Johnson and Speakman, 2001). Taken alone, this reduction in food intake is also consistent with the peripheral limitation hypothesis as it could be interpreted as a consequence of a reduced demand for thermoregulatory energy expenditure. However, mice exposed to 30°C had a smaller litter size, pup body mass and litter mass as well as a lower rate of litter mass increase than those exposed to 21°C and/or 8°C. This might indicate that milk production was reduced at 30°C, which is consistent with the heat dissipation limit hypothesis but not the peripheral limitation hypothesis. An alternative explanation, however, is that milk production was the same at each temperature but that there were differences in the abilities of the offspring to translate milk into growth.

In the present paper, we provide a further test of the heat dissipation limit hypothesis by measuring the *MEO* of MF1 laboratory mice (*Mus musculus* L.) lactating at 30°C compared with those measured previously in mice at 21°C (Johnson et al., 2001a) and 8°C (Johnson and Speakman, 2001). The heat dissipation limit hypothesis predicts that at the higher ambient temperature *MEO* will be lower because of the lower driving gradient for heat loss. Conversely, the peripheral limitation hypothesis predicts that milk production should not be reduced relative to that observed at lower temperatures.

MEO in small mammals has been evaluated using a variety of methods. These include timed milking (e.g. Harris et al., 1966; Hanrahan and Eisen, 1970; König et al., 1988), mass differences of the litter before and after a suckling bout (e.g. Morag, 1970; Mepham and Beck, 1973), isotope transfer from mother to pups (e.g. Rath and Thenen, 1979; Kunz et al., 1983) and isotope dilution in the body water of the mother (e.g. McLean and Speakman, 1999; Johnson et al., 2001a; Johnson and Speakman, 2001) or the pups (e.g. Stern et al., 1997; Ahlstrøm and Wamberg, 2000; Tardif et al., 2001). Estimates of *MEO* have also been derived from the metabolizable energy intake of the mother (e.g. McClure, 1987; Künkele and Kenagy, 1997; Künkele and Trillmich, 1997) and the litter energy budget (e.g. Knight and Peaker, 1982; Oftedal, 1984; Gittleman and Oftedal, 1987). Data obtained using these methods, however, are not necessarily comparable due to potential differences in the precision of each method and the validity of assumptions on which they are based. Thus, differences in *MEO* between studies could be attributed to variation in the methodology used (Knight et al., 1986) rather than reflecting real biological differences. Only a few studies have compared the estimates of *MEO* evaluated by different methods, and most of these have been performed on humans (e.g. Fjeld et al., 1988; Butte et al., 1991) and large animals such as marine mammals (e.g. Costa, 1987; Arnould et al., 1996) or farm animals (e.g. Holleman et al., 1975; Coward et al., 1982). Therefore, our second aim was

Table 1. *Timing and sample size for measurements of energy and water budget components in reproductive (*N=*55) and non-reproductive (*N=*28) female mice exposed to 30°C*

Measurement	Days of reproduction[a]	Sample size[b]			
		Repr.	Non-repr.	Litters	Pups
DEE by DLW method	14	24 BC	17[c]	–	–
Water turnover by DLW method	14	24 BC[d]	24[d]	–	43 B
Total body water by desiccation[e]	14	8[f]	9[f]	–	46
Faecal and urinary water loss	14 and 15	5 C	5	–	–
Evaporative water loss[g]	15	5 C	5	–	–
RMR by respirometry	14	–	–	23 C	10 BC
Milk composition	15	12 C	–	–	–

Non-reproductive females were measured on the same days as reproductive females.

[a]Numbers indicate days after parturition (day 0 of lactation); [b]B and C indicate the same groups of reproductive females as in Król and Speakman (2003); [c]for 24 females injected with DLW, the blood samples from only 17 individuals were sufficiently large to run both ^{2}H and ^{18}O analyses; [d]from ^{2}H turnover during measurements of *DEE*; [e]measured from separate groups of mice, from which no other measurements were taken; [f]from Król and Speakman (1999); [g]measured in the same individuals as faecal and urinary water loss.

DEE, daily energy expenditure; DLW, doubly labelled water; *RMR*, resting metabolic rate; Repr., reproductive females; Non-repr., non-reproductive females.

to compare the methods commonly used to calculate *MEO* in small mammals by making simultaneous measurements of components of the energy and water budgets in lactating female mice and their pups.

Materials and methods

Animals and experimental protocol

Experiments were conducted on 55 lactating and 28 non-reproductive female mice (*Mus musculus* L.: outbred MF1) that were 12–14 weeks old. Housing, acclimation and breeding protocols are described in Król and Speakman (2003). The timing and sample size for measurements are detailed in Table 1.

Doubly labelled water measurements

We used the doubly labelled water (DLW) method to measure daily energy expenditure (*DEE*; respiratory energy metabolism as carbon dioxide production) from the elimination rates of ^{2}H (deuterium) and ^{18}O in lactating and non-reproductive females. We also calculated total water turnover (rH_2O) from the elimination rate of ^{2}H in lactating females, non-reproductive females and pups. The estimates of *DEE* and rH_2O in adult mice were based on the same ^{2}H turnover data.

The DLW measurements were conducted on 24 lactating females (litter size 1–15), 24 non-reproductive females and 43 pups from seven litters of size 10 and 12 (3–7 pups were labelled in each litter and raised by a non-labelled mother). On day 14 of lactation (between 10:00 h and 12:00 h), mice were injected intraperitoneally with approximately 0.2 g (adult mice) and 0.05 g (pups) of water containing enriched ^{2}H (4.6 atom%) and ^{18}O (9.4 atom%). The syringe used to inject the DLW was weighed (±0.0001 g; Ohaus Analytical Plus) immediately before and after the injection. Mice were replaced in their cages during the 1 h equilibration period (Speakman, 1997). Initial and final blood samples were taken by tail tipping 1 h and 25 h after injection, respectively. Blood samples (30–100 μl for adult mice and 15–20 μl for pups) were immediately flame sealed into pre-calibrated Vitrex pipettes (Modulohm A/S, Herlev, Denmark) and stored at 4°C until analysis. Each adult mouse was also blood sampled on the day before injection to determine background isotope levels. Background ^{2}H enrichment for the pups labelled in each litter was determined from a blood sample of a non-labelled pup that was taken 5–10 min before the first pup was labelled in that litter. The same non-labelled pup was blood sampled 24 h later to allow correction for any incidental ^{2}H uptake or recycling. The body mass of each animal was measured before injection and before taking the final blood sample.

Samples of blood were vacuum distilled into glass Pasteur pipettes (Nagy, 1983) and the resultant distilled water used for mass spectrometric analysis of ^{2}H and ^{18}O. The ^{2}H analysis was performed on hydrogen gas, produced from the distilled water after reaction with $LiAlH_4$ (Ward et al., 2000). For the ^{18}O analysis, distilled water was converted to carbon dioxide gas using the small sample equilibration technique (Speakman et al., 1990). The ^{2}H:^{1}H and ^{18}O:^{16}O ratios were established using dual inlet gas source isotope ratio mass spectrometers (Optima, Micromass IRMS; Manchester, UK), as described previously (Król and Speakman, 1999). Measurements of isotope enrichment in blood samples were based on analysis of either two sub-samples (adult mice), in which case further calculations were performed on the mean values, or one sample (pups) of distilled water.

For each adult mouse, we calculated initial ^{2}H and ^{18}O dilution spaces by the intercept method (Coward and Prentice, 1985). Final ^{2}H and ^{18}O dilution spaces were inferred from the final body mass, assuming the same percentage of body mass as measured for the initial dilution spaces. The isotope elimination rate was calculated following Nagy (1975). For

calculation of *DEE* based on CO_2 production, we used single-pool model equation 3.14 (Lifson and McClintock, 1966) with a pooled fractionation factor of 0.0249 (Speakman, 1997), as presented in equation 10 in Visser and Schekkerman (1999). We assumed a fractional evaporative water loss of 0.64 for non-reproductive mice (3.37 g day^{-1} of 5.24 g day^{-1}; Table 3) and 0.38 for lactating mice. The latter value was derived from direct measurements of evaporative, faecal and urinary water loss of lactating mice (10.94 g day^{-1}, 4.10 g day^{-1} and 5.94 g day^{-1}, respectively; Table 3) combined with the amount of water exported in milk (7.96 g day^{-1}, as estimated from water turnover of pups) and calculated as 10.94 g day^{-1} of (10.94+4.10+5.94+7.96) g day^{-1}. Energy equivalents of CO_2 production were calculated using a conversion factor of 24.026 J ml^{-1} CO_2, derived from the Weir equation (Weir, 1949) for a respiratory quotient (RQ) of 0.85 (Speakman, 1997). Female total water turnover (rH_2O_{fem}) was calculated according to Lifson and McClintock (1966). We assumed that 64% (non-reproductive mice) or 38% (lactating mice) of the water leaving the body was fractionated. We applied a fractionation factor for ^2H of 0.9366 (Speakman, 1977). This approach assumes that rates of water influx and efflux are constant, so rH_2O_{fem} = total water influx = total water efflux (Nagy and Costa, 1980).

We calculated the initial and final ^2H dilution spaces of pups in the same way as for adult animals. The elimination rate of ^2H was calculated according to Nagy (1975) and corrected for uptake of ^2H from the environment or isotope recycling (Baverstock and Green, 1975; Friedman and Bruno, 1976). The estimation of pup total water turnover (rH_2O_{pup}) was based on the assumption of linear pup growth during the measurement period and was calculated according to Coward et al. (1982). We assumed the same fractional evaporative water loss as for non-reproductive mice (0.64). rH_2O_{pup} represents the total water influx according to this approach (Coward et al., 1982).

Faecal, urinary and evaporative water loss

Measurements of faecal water loss (*FWL*), urinary water loss (*UWL*) and evaporative water loss (*EWL*) were conducted on five lactating females (litter size 6–11) and five non-reproductive females. On day 14 of lactation, lactating females and their offspring, or non-reproductive females, were placed individually in metabolic cages (code 3700MO-000, Tecniplast Gazzada, Buguggiate, Italy), provided with water and a weighed portion of food. After 24 h, the food was reweighed, and all faeces and urine were collected and dried at 60°C to constant mass. Sorting through the faeces samples revealed that contribution of pups to faecal and, presumably, urine production was negligible. On day 15 of lactation, we placed the same females on a smooth non-absorbant surface and collected fresh faeces and fresh urine within 2 s of them being produced. These samples were immediately weighed before drying at 60°C to constant mass. *FWL* and *UWL* (g day^{-1}) were calculated by multiplying the amount of faeces (or urine) produced (g dry mass day^{-1}) by the ratio of water to dry mass content of fresh faeces (or urine).

EWL was measured gravimetrically on day 15 of lactation. Mice were placed individually in a respirometry chamber for 1 h, with an ambient air flow of 649–701 ml min^{-1}. The same flow rate was used for the chamber without a mouse, as a control. During the measurements, mice sat on a wire mesh grid through which faeces and urine fell into mineral oil, trapping water from these sources. Water in the excurrent air was absorbed by silica gel. The increase in mass of silica gel was corrected for water content of incurrent air by subtracting water loss from the control chamber measurements. *EWL* (g day^{-1}) was calculated by multiplying the corrected increase in mass of silica gel (g h^{-1}) by 24.

Total body water by desiccation

The measurements of total body water were conducted on 46 pups (5–8 pups from eight litters of size 5–13) on day 14 of lactation. Pups were weighed and killed by cervical dislocation. The carcasses were split open along the midline and transverse cuts across the body were made to increase exposure of the tissues for drying. The carcasses were dried in a convection oven at 60°C for 14 days (Król and Speakman, 1999). Total body water was calculated as the difference between the fresh and dry mass and was expressed as a percentage of the body mass prior to desiccation.

Resting metabolic rate measurements

We assessed resting metabolic rate (*RMR*) of individual pups and whole litters from their rate of oxygen consumption at 30°C, using a modification of the protocol for adult mice (Król et al., 2003). The measurement period was 1 h, the chamber volume was 81 ml (pups) or 885 ml (litters) and the flow rate was 194–208 ml min^{-1} (pups) or 487–592 ml min^{-1} (litters).

For individual pups, we required accurate estimates of oxygen consumption (see equations 3, 4 in Appendix A of supplementary material), while in litters we aimed for accurate estimate of energy expenditure (see equation 6 in Appendix A of supplementary material). Therefore, *RMR* expressed as oxygen consumption was calculated according to equation 1b in Koteja (1996b), assuming an RQ of 0.74 derived from the composition of milk (present study). The *RMR* expressed as energy expenditure was calculated in the same way as for adult mice (Król et al., 2003).

The *RMR* of 10 pups (2–3 pups from four litters of size 2–12) and 23 litters (size 4–15) was measured on day 14 of lactation. Measurements of *RMR* in pups and litters were made from individuals from different families.

Milk collection and analysis

On day 15 of lactation, 12 females (litter size 9–15) were separated from their pups for 3 h and then injected intraperitoneally with 1 IU of oxytocin to stimulate milk flow. Each mammary gland was palpated towards the nipple area and droplets of milk were collected in capillary tubes. Milk collection continued until no more milk could be expressed. Samples of milk (0.4–0.6 ml from each female) were frozen at

–20°C prior to analysis for water, fat, protein and sugar content.

All analyses (Rowett Research Institute Analytical Services, Aberdeen, UK) were made on duplicate samples. Water content was determined by drying milk samples (50 µl) in a convection oven at 100°C for 3.5 h. Fat content (100 µl milk samples) was measured by the Rose–Gottlieb gravimetric method (Kirk and Sawyer, 1991). Crude protein content was calculated as 6.38 × total nitrogen content (Kirk and Sawyer, 1991), determined from 15 µl samples of milk by a micromodification of the Kjeldahl technique (Davidson et al., 1970). No correction was made for nonprotein nitrogen content. Total sugar (20 µl milk samples) was measured by the Anthrone method using lactose monohydrate as the standard (Southgate, 1976) and is therefore expressed as the monosaccharide equivalent.

Gross energy content of milk (GE_{milk}; kJ g^{-1} whole milk) was calculated by multiplying fat, protein and sugar content (g g^{-1} whole milk) by 38.12 kJ g^{-1}, 24.52 kJ g^{-1} and 16.53 kJ g^{-1}, respectively (modified from Perrin, 1958).

Milk energy output

We evaluated *MEO* using four different methods: (1) as the difference between metabolizable energy intake and daily energy expenditure of the female, (2) from female water turnover, (3) from pup water turnover and (4) from the energy budget of the litter. To compare the methods, we calculated *MEO* for the 24 females for which we had individual measurements of asymptotic food intake, litter size and litter mass (Król and Speakman, 2003) as well as individual measurements of *DEE* and rH_2O_{fem} (present study). We examined the sensitivity of each method by determining how an independent 1% change in each parameter influenced the estimate of *MEO*. The maximum potential decrease and increase in estimate of *MEO*, resulting from a 1% change in all parameters, were also computed. The method that gave the most accurate, precise and robust estimate of *MEO* was then applied to 67 reproductive females with individual measurements of asymptotic food intake, litter size and litter mass (Król and Speakman, 2003). All estimates of *MEO* refer to day 14 of lactation. For full details of the four methods, see Appendix A of supplementary material.

Statistics

Data are reported as means ± S.D. (*N* = sample size). The relationships between energy and water budget components and body mass were examined by least-squares linear regression analysis. The regression lines were compared using analysis of covariance (ANCOVA). To test the differences in *DEE*, rH_2O, *FWL*, *UWL* and *EWL* between reproductive and non-reproductive females we used two-sample *t*-tests. Comparisons of the parameters measured in the same individuals [*DEE vs MEI* (metabolizable energy input) and *FWL+UWL+EWL vs* rH_2O] were made using paired *t*-tests. The four methods of evaluating *MEO* were compared using repeated measures analysis of variance (ANOVA), followed by a Tukey *post-hoc* test. Relationships between *DEE* and *MEI*, between *FWL+UWL+EWL* and rH_2O and between the four estimates of *MEO* were described using Pearson product-moment correlation coefficients. All statistical analyses were conducted using Minitab for Windows (version 13.31, Minitab Inc., State College, PA, USA; Ryan et al., 1985). Statistical significance was determined at $P<0.05$. All tests were two-tailed.

Table 2. *Results of the DLW measurements of energy expenditure and water turnover in lactating (*N*=24) and non-reproductive (*N*=17) female mice and pups (*N*=43) at 30°C*

Trait	Females		Pups
	Lactating	Non-reproductive	
BM (g)[a]	37.2±2.7	32.2±3.1	5.6±0.7
k_d (h^{-1})[b]	0.032±0.006	0.009±0.001	0.010±0.002
k_o (h^{-1})[c]	0.040±0.006	0.015±0.002	–
k_o/k_d	1.260±0.053	1.752±0.091	–
N_d (% of *BM*)[d]	77.7±3.6	69.2±3.0	76.7±2.4
N_o (% of *BM*)[d]	74.0±4.0	65.6±3.3	–
TBW (% of *BM*)[e]	73.4±1.1[f]	63.4±3.2[g]	71.4±1.8[h]
N_d/N_o	1.050±0.022	1.055±0.022	–
DEE (kJ day^{-1})[i]	70.4±16.2	45.1±5.7	–
rH_2O (g day^{-1})[j]	22.63±4.82	5.32±1.24[k]	1.09±0.25

Values are means ± S.D.

Lactating females and pups were injected with doubly labelled water (DLW) on day 14 of lactation; non-reproductive females were measured on the same days as lactating mice.

[a]Body mass before injection; [b]deuterium elimination rate; [c]^{18}O elimination rate; [d]deuterium (N_d) and ^{18}O (N_o) dilution spaces (moles) were converted to g assuming a molecular mass of body water of 18.02 and were expressed as % of body mass before injection; [e]total body water measured by desiccation from separated groups of mice, from which no other measurements were taken; [f]data for eight mice (Król and Speakman, 1999); [g]data for nine mice (Król and Speakman, 1999); [h]data for 46 pups (present study); [i]daily energy expenditure; [j]total water turnover; [k]data for 24 mice (17 mice for which *DEE* was measured and seven mice for which only the ^2H enrichments were analysed).

Results

Daily energy expenditure and total water turnover of adult mice

The *DEE* of reproductive females measured on day 14 of lactation was 70.4±16.2 kJ day^{-1} (range 38.9–110.8 kJ day^{-1}, *N*=24; Table 2). For the 12 females for which both *DEE* and *RMR* were measured at peak lactation (Król et al., 2003), *DEE* was 2.6× *RMR* (range 1.6–3.8). There was a weak but significant increase in *DEE* with increasing body mass for lactating females (regression, $r^2=0.23$, $F_{1,22}=6.5$, $P=0.018$; Fig. 1).

The *DEE* of non-reproductive females was 45.1±5.7 kJ day^{-1} (range 35.9–55.9 kJ day^{-1}, *N*=17; Table 2).

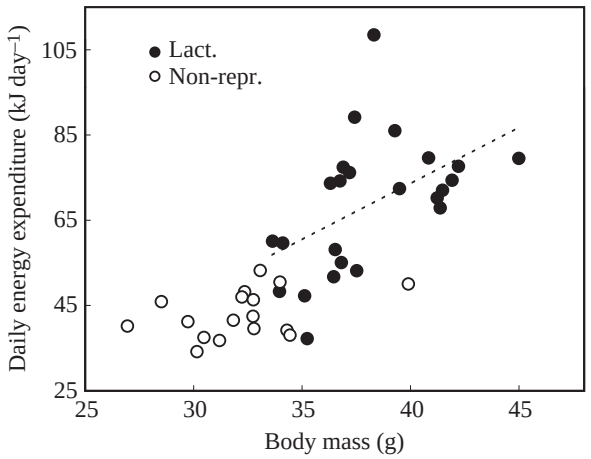

Fig. 1. Daily energy expenditure (*DEE*) as a function of body mass for lactating (filled circles, *N*=24) and non-reproductive (open circles, *N*=17) female mice exposed to 30°C. *DEE* was measured when the reproductive females were at day 14 of lactation. Body mass is the mean value for days 9–13 of lactation (reproductive females) or the mean value for eight consecutive days before the day of doubly labelled water (DLW) injection (non-reproductive females). The relationship for lactating females is described by y=–28.18+2.58x; the relationship for non-reproductive females failed to reach significance.

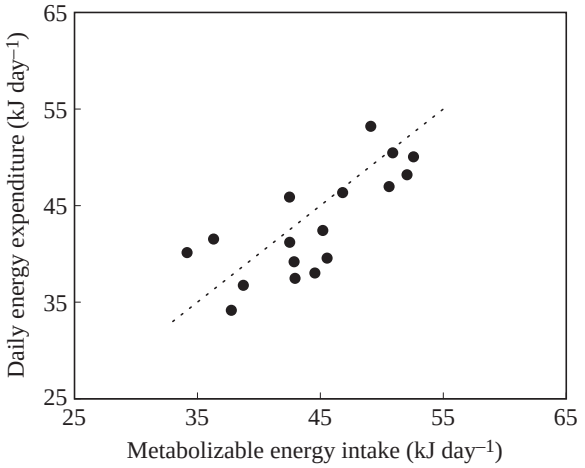

Fig. 2. Correlation between daily energy expenditure and metabolizable energy intake in non-reproductive female mice at 30°C (r=0.75, P=0.001, N=17). The line of equality is shown.

On average, *DEE* of non-reproductive mice was 2.0±10.0% higher than their metabolizable energy intake, with individual differences ranging from –10.4% to 22.9% (*N*=17). However, the differences between *DEE* and *MEI* were not significant (paired t=0.7, P=0.49, N=17). The values of *DEE* and *MEI* were highly correlated (r=0.75, P=0.001, N=17; Fig. 2). *DEE* was 2.5× *RMR* (range 1.9–3.1) for the 11 non-reproductive mice for which both *DEE* and *RMR* were measured (Król et al., 2003). *DEE* was not related to body mass (regression, r^2=0.16, $F_{1,15}$=2.8, P=0.11; Fig. 1). Non-reproductive mice had significantly lower *DEE* than lactating females, both when the comparison was made on the raw data (t_{30}=7.0, P<0.001) and when corrected for the differences in body mass (ANCOVA: interaction body mass × reproductive status, P=0.19; body mass effect, $F_{1,38}$=8.6, P=0.006; reproductive status effect, $F_{1,38}$=6.8, P=0.013).

The total water turnover of lactating and non-reproductive females averaged 22.63±4.82 g day^{-1} (range 10.92–30.99 g day^{-1}, N=24) and 5.32±1.24 g day^{-1} (range 3.74–8.18 g day^{-1}, N=24), respectively (Table 2). In both groups of mice, $r\mathrm{H_2O_{fem}}$ was positively related to female body mass (lactating females, r^2=0.46, $F_{1,22}$=19.0, P<0.001; non-reproductive females, r^2=0.46, $F_{1,22}$=18.8, P<0.001; Fig. 3). There was a significant interaction between body mass and the reproductive status (ANCOVA: $F_{1,44}$=11.0, P=0.002), indicating a steeper slope of the regression line for lactating than for non-reproductive females. For a mouse with a body mass of 35.6 g (mean value for both groups of mice), the predicted $r\mathrm{H_2O_{fem}}$ would be 19.73 g day^{-1} and 5.92 g day^{-1} for lactating and non-reproductive females, respectively. Analyses

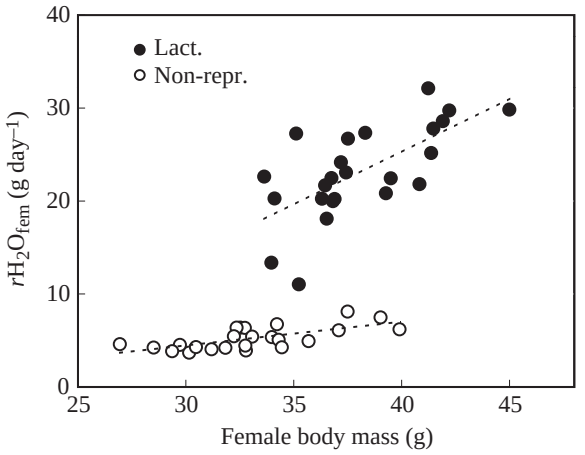

Fig. 3. Total water turnover ($r\mathrm{H_2O_{fem}}$) as a function of body mass for lactating (filled circles, y=–19.07+1.09x, N=24) and non-reproductive (open circles, y=–3.34+0.26x, N=24) female mice at 30°C. $r\mathrm{H_2O_{fem}}$ was measured when the reproductive females were at day 14 of lactation. Body mass is the mean value for days 9–13 of lactation (reproductive females) or the mean value for eight consecutive days before the day of doubly labelled water (DLW) injection (non-reproductive females).

of mass-corrected $r\mathrm{H_2O_{fem}}$ (the residuals from the regression lines on body mass presented in Fig. 3, added to the values of predicted mean $r\mathrm{H_2O_{fem}}$) showed that lactating females had higher total water turnover than non-reproductive females (t_{26}=18.6, P<0.001).

Total water turnover of pups

The total water turnover of individual pups measured on day 14 of lactation was 1.09±0.25 g day^{-1} (range 0.50–1.52 g day^{-1}, N=43; Table 2). The $r\mathrm{H_2O_{pup}}$ increased with pup body mass (regression, r^2=0.57, $F_{1,41}$=54.4, P<0.001; Fig. 4).

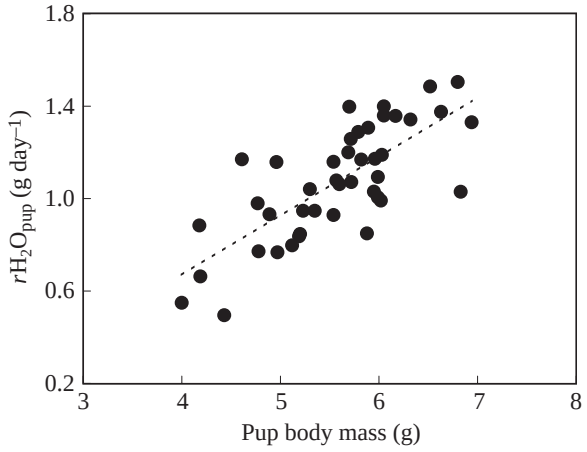

Fig. 4. Total water turnover of pups (rH_2O_{pup}; N=43) at 30°C as a function of body mass. The measurements were taken on day 14 of lactation. The relationship is described by y=–0.36+0.26x.

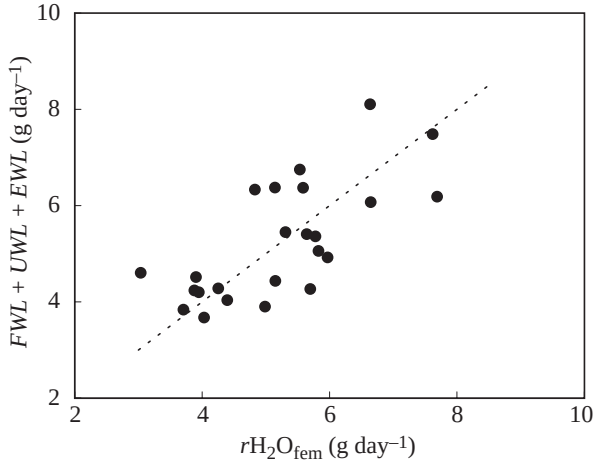

Fig. 5. Correlation between the sum of faecal (*FWL*), urinary (*UWL*) and evaporative water loss (*EWL*) and total water turnover (rH_2O_{fem}) in non-reproductive female mice at 30°C (r=0.71, P<0.001, N=24). The line of equality is shown.

Faecal, urinary and evaporative water loss

Faecal water loss averaged 4.10±1.06 g day^{-1} in lactating females (N=5) and 1.14±0.21 g day^{-1} in non-reproductive females (N=5; Table 3). These values were significantly different (t_4=6.1, P=0.004) and were a consequence of differences in the amount of faeces produced (2.48±0.24 g dry mass day^{-1} and 0.64±0.12 g dry mass day^{-1} for lactating and non-reproductive females, respectively; t_5=15.2, P<0.001). The water content of faeces in lactating (61.7±4.3%) and non-reproductive (64.0±3.4%) mice was not significantly different (t_7=0.9, P=0.37).

The urinary water loss of lactating females (5.94±1.70 g day^{-1}; N=5) was higher than in non-reproductive females (0.73±0.23 g day^{-1}; N=5) (t_4=6.8, P=0.002; Table 3).

Table 3. *Faecal, urinary and evaporative water loss in lactating (*N*=5) and non-reproductive (*N*=5) female mice at 30°C*

	Females	
Trait	Lactating	Non-reproductive
Body mass (g)	40.7±4.1	33.8±3.3
Food consumed (g day^{-1})	13.2±1.7	2.8±0.7
Faeces produced (g dry mass day^{-1})	2.48±0.24	0.64±0.12
Urine produced (g dry mass day^{-1})	0.34±0.07	0.09±0.01
Water content of faeces (%)	61.7±4.3	64.0±3.4
Water content of urine (%)	93.9±2.9	88.4±3.4
Faecal water loss (g day^{-1})	4.10±1.06	1.14±0.21
Urinary water loss (g day^{-1})	5.94±1.70	0.73±0.23
Evaporative water loss (g day^{-1})	10.94±1.25	3.37±1.14
FWL+UWL+EWL (g day^{-1})[a]	20.98±2.42	5.24±1.39

Values are means ± S.D.

[a]Calculated as the sum of faecal, urinary and evaporative water loss.

Lactating mice produced more urine than non-reproductive females (0.34±0.07 g dry mass day^{-1} and 0.09±0.01 g dry mass day^{-1}, respectively; t_4=7.4, P=0.002). Furthermore, the urine of lactating females had higher water content than the urine of non-reproductive mice (93.9±2.9% and 88.4±3.4%, respectively; t_7=2.9, P=0.023).

Evaporative water loss averaged 10.94±1.25 g day^{-1} in lactating females (N=5) and 3.37±1.14 g day^{-1} in non-reproductive females (N=5; Table 3). These values were significantly different (t_7=10.0, P<0.001). *EWL* in lactating females was positively related to litter mass (y=–1.51+0.19x, r^2=0.79, $F_{1,3}$=11.2, P=0.044). In non-reproductive mice, heavier females had a greater *EWL* (y=–7.12+0.31x, r^2=0.79, $F_{1,3}$=11.1, P=0.044).

Using the data presented above, we predicted the water loss for the 24 non-reproductive females for which we had individual measurements of body mass and food intake (Król and Speakman, 2003), as well as individual measurements of total water turnover (rH_2O_{fem}; present study). *FWL* was calculated from the food intake, mean dry mass content of the food (94.4%; Król and Speakman, 2003), the relationship between dry mass food intake and dry mass faecal production (Król and Speakman, 2003), and the mean water content of faeces (64.0%; present study). *UWL* was assumed to be 0.73 g day^{-1} (present study). We predicted *EWL* from body mass using the relationship established for five non-reproductive females (present study). The sum of *FWL*, *UWL* and *EWL* predicted for 24 non-reproductive females averaged 5.22±1.20 g day^{-1} and was not significantly different from the directly measured rH_2O_{fem} (5.32±1.24 g day^{-1}; paired t=0.5, P=0.60). The values of predicted and actual water turnover were highly correlated (r=0.71, P<0.001, N=24; Fig. 5).

Resting metabolic rate of pups and litters

The *RMR* of individual pups and whole litters, measured on

day 14 of lactation, was positively related to mass (pups, r^2=0.94, $F_{1,8}$=124.6, P<0.001, N=10; litters, r^2=0.74, $F_{1,21}$=58.6, P<0.001, N=23). The relationships between *RMR* (ml O_2 min^{-1}) and mass (g) are described by y=–0.03+0.04x (pups) and y=0.24+0.02x (litters) (see Appendix B of supplementary material). After conversion of the oxygen consumption data to energy equivalents, the relationships between *RMR* (kJ day^{-1}) and mass (g) are described by y=–0.79+1.11x (pups) and y=7.28+0.71x (litters).

Milk composition

Milk samples collected on day 15 of lactation contained 65.6±1.9% water, 20.0±2.5% fat, 10.3±0.7% crude protein and 2.1±0.4% total sugar (N=12). Variation in milk composition was not correlated with litter size or litter mass (in all cases P>0.05). The gross energy content of whole milk, calculated from the milk composition, averaged 10.5±1.0 kJ g^{-1} (N=12).

Comparison of methods for evaluating milk energy output

The milk energy output of 24 females determined from (1) the difference between *MEI* and *DEE* of the female, (2) female water turnover, (3) pup water turnover and (4) the litter energy budget averaged 79.5±22.5, 57.0±42.5, 93.7±27.0 and 80.3±20.0 kJ day^{-1}, respectively (Fig. 6). The *MEO* determined from female water turnover was significantly lower than the other three estimates (repeated measures ANOVA, $F_{3,92}$=13.7, P<0.001; Tukey pairwise comparisons between the female water turnover method and the other methods, P<0.05). Evaluation of *MEO* using the difference between *MEI* and *DEE*, pup water turnover and litter energy budget produced similar results (all Tukey pairwise comparisons, P>0.05). The estimates of *MEO* from female water turnover were also approximately twice as variable (i.e. less precise) as those yielded by the other methods.

Analysis of the sensitivity of the four methods showed that the evaluation of *MEO* from the litter energy budget was the most robust to changes in the measured parameters (see Appendix C of supplementary material). None of the parameters, which increased or decreased by 1%, caused an increase or decrease in the estimate of *MEO* from the litter energy budget by greater than 1%. The maximum potential change in *MEO* from the litter energy budget, resulting from combining a 1% change in all six parameters, ranged from –3.5% to 3.8%. The sensitivity of the *MEI–DEE* method and the pup water turnover method were similar. For both methods, increases or decreases of 1% in any of the parameters did not change the estimate of *MEO* by more than 2%. The maximum potential change in estimated *MEO* ranged from –10.0% to 11.1% (the *MEI–DEE* method) and from –7.2% to 8.1% (the pup water turnover method). The method that was most sensitive to errors in the component variables was based on female water turnover. Four of 10 parameters, when changed individually by 1%, had an impact on the estimate of *MEO* larger than 2%, while a 1% change in female water turnover (rH_2O_{fem}) changed *MEO* by 6.0%. Female water turnover, therefore, contributes most of the imprecision in this method.

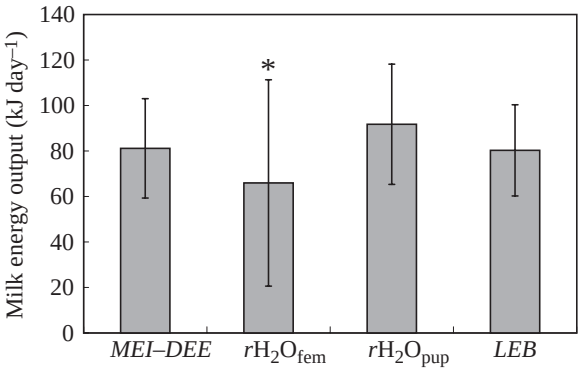

Fig. 6. Milk energy output (*MEO*) of 24 female mice at 30°C, determined as the difference between metabolizable energy intake and daily energy expenditure (*MEI–DEE*), from female water turnover (rH_2O_{fem}), from pup water turnover (rH_2O_{pup}) and from litter energy budget (*LEB*). All estimates of *MEO* refer to day 14 of lactation. *MEO* determined from female water turnover was significantly lower than the three other estimates, as denoted by the asterisk (for statistical details, see Results). Bars are means ± 1 S.D.

The maximum potential change in estimates of *MEO*, when all 10 parameters varied by 1%, ranged from –20.5% to 21.6% when *MEO* was calculated from female water turnover.

Among the three methods that yielded similar estimates of *MEO*, the *MEI–DEE* method involved the minimum number of assumptions (i.e. parameter values taken from the literature) and predictions (i.e. parameter values predicted from relationships for mice not included in the estimate of *MEO*). Apart from the urinary energy loss (assumed from the literature), all other physiological parameters were either measured individually in the 24 females (*FI* and *DEE*) or measured in a similar group of lactating mice (e.g. apparent digestibility of energy; Król and Speakman, 2003). We believe, therefore, that the *MEI–DEE* method provides the most accurate estimate of *MEO* for the 24 mice and can be treated as a reference method.

Individual estimates of *MEO* from the pup water turnover method and the *MEI–DEE* method were highly correlated (r=0.89, P<0.001, N=24; Fig. 7B), as were the estimates from the litter energy budget method and the *MEI–DEE* method (r=0.94, P<0.001, N=24; Fig. 7C). Thus, the pup water turnover method, the litter energy budget method and the reference *MEI–DEE* method appear to provide accurate and precise estimates of *MEO*. The estimates of *MEO* produced by the female water turnover method were not correlated with those yielded by the *MEI–DEE* method (r=0.20, P=0.34, N=24; Fig. 7A).

To calculate *MEO* for the 67 reproductive females with individual measurements of food intake, litter mass and litter size (Król and Speakman, 2003), we used the litter energy budget method. This approach is not only as accurate and precise as the reference method but also the most robust to changes in parameters.

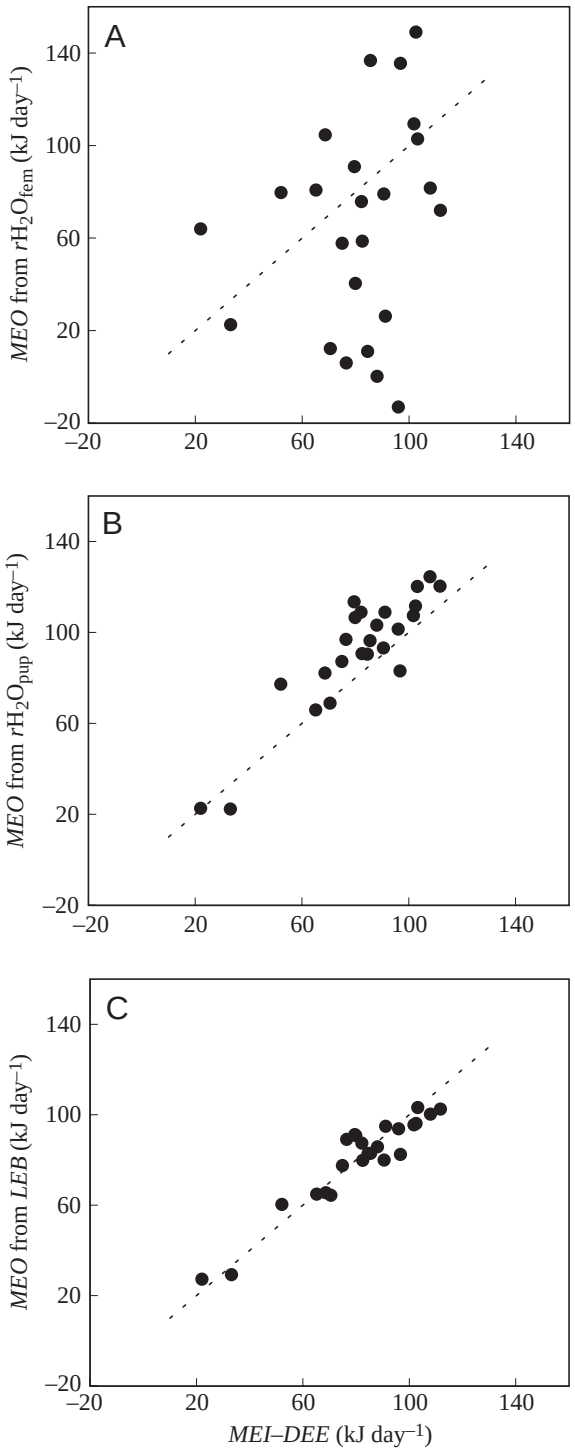

Fig. 7. Milk energy output (*MEO*) of 24 female mice at 30°C, evaluated from (A) female water turnover (rH_2O_{fem}), (B) pup water turnover (rH_2O_{pup}) and (C) litter energy budget (*LEB*) as a function of *MEO* calculated from the difference between metabolizable energy intake (*MEI*) and daily energy expenditure (*DEE*). Only correlations B (r=0.90, P<0.001) and C (r=0.95, P<0.001) are significant. The lines of equality are shown.

Milk energy output at 30°C

Milk energy output on day 14 of lactation calculated for 67 females using the litter energy budget method ranged from 27.2 kJ day^{-1} to 117.9 kJ day^{-1}, with a mean value of 85.3±19.0 kJ day^{-1}. *MEO* was related to litter size on day 14 of lactation (ANOVA, $F_{14,52}$=5.8, P<0.001, N=67). Females raising six pups exported more energy in milk than females raising 1–3 pups (Tukey pairwise comparisons between litter sizes 6 and 1, 2 or 3, P<0.05; Fig. 8). For litter size increasing from 6 to 15, no further increase in *MEO* was observed (all Tukey pairwise comparisons for litters size 6–15, P>0.05). The mean *MEO* for females raising 6–15 pups was 89.4±13.5 kJ day^{-1} (N=61). This value corresponds to 40.8±3.4% of gross energy intake and to 54.6±4.6% of *MEI*.

The effect of temperature on milk composition and milk energy output

We compared milk composition and milk energy output of mice that were raising their first litters in hot (30°C; present study), warm (21°C; Johnson et al., 2001a) and cold (8°C; Johnson and Speakman, 2001) temperatures. The hot and the warm mice were exposed to 30°C and 21°C, respectively, prior to breeding and were kept in those temperatures through the whole course of pregnancy and lactation. By contrast, mice from the cold group were maintained at 21°C until the pups had grown fur and were then exposed to 8°C from day 10 of lactation onwards. The sample sizes for hot, warm and cold groups were 12, 10 and 10, respectively.

All milk samples were collected on day 15 of lactation, using the same protocol. Their composition was analysed using the same methods, and the gross energy content of milk was calculated from the composition using the same formula.

On the day when the milk samples were collected, the body mass of the hot, warm and cold mice averaged 40.0±1.8 g, 49.9±3.0 g and 50.1±3.3 g, respectively (ANOVA, $F_{2,29}$=50.0, P<0.001; mean for the hot mice significantly lower than for both warm and cold mice, Tukey pairwise comparisons, P<0.05). The hot, warm and cold mice raised, on average, 12.0±1.9, 12.4±1.7 and 10.3±1.4 pups, respectively (ANOVA, $F_{2,29}$=4.4, P=0.021; mean for the warm mice significantly greater than for the cold mice, Tukey pairwise comparison, P<0.05). The litter mass of the hot, warm and cold mice averaged 66.7±6.6 g, 82.4±11.0 g and 69.0±13.6 g, respectively (ANOVA, $F_{2,29}$=6.8, P=0.004; mean for the warm mice significantly greater than for both hot and cold mice, Tukey pairwise comparisons, P<0.05).

The three groups differed in the dry mass content of milk (ANOVA, $F_{2,29}$=10.7, P<0.001), with the hot mice having, on average, less total solids in milk (34.4±1.9%) than both the warm (40.9±4.4%) and the cold (41.5±5.3%) mice (Fig. 9A). The effect of temperature on the dry mass content of milk remained significant after adjusting for the differences in maternal body mass (ANCOVA: interaction body mass × temperature, P=0.07; body mass effect, $F_{1,28}$=9.1, P=0.005; temperature effect, $F_{2,28}$=12.5, P<0.001). Dry mass content of milk across temperature was not affected by litter size (ANCOVA, P=0.47) or litter mass (ANCOVA, P=0.21).

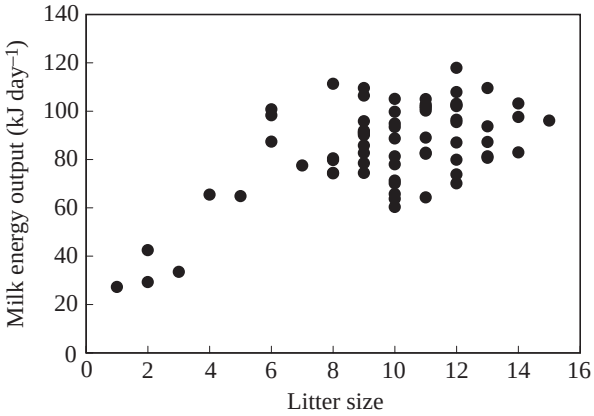

Fig. 8. Milk energy output (*MEO*) determined from litter energy budget of female mice at 30°C (N=67) as a function of litter size. Both *MEO* and litter size are those on day 14 of lactation. Females with six pups exported more energy in milk than those with 1–3 pups. For litter sizes of 6–15, no further increase in *MEO* was observed (for statistical details, see Results).

Milk produced by mice exposed to 30°C, 21°C and 8°C contained 20.0±2.5%, 26.4±3.0% and 30.3±3.8% fat, respectively (ANOVA, $F_{2,29}$=31.6, P<0.001; all three means significantly different, Tukey pairwise comparisons, P<0.05; Fig. 9B). The fat content of milk across temperature was not affected by maternal body mass (ANCOVA, P=0.30). The effect of temperature on milk fat content was still significant when corrected for the differences in litter size (ANCOVA: interaction litter size × temperature, P=0.66; litter size effect, $F_{1,28}$=4.6, P=0.041; temperature effect, $F_{2,28}$=28.6, P<0.001) and litter mass (ANCOVA: interaction litter mass × temperature, P=0.37; litter mass effect, $F_{1,28}$=11.1, P=0.002; temperature effect, $F_{2,28}$=49.1, P<0.001).

The hot, warm and cold mice had similar milk protein content (ANOVA, $F_{2,29}$=3.0, P=0.07), which averaged 10.3±0.7%, 11.3±1.1% and 11.2±1.4%, respectively (Fig. 9C), as well as similar sugar content ($F_{2,29}$=2.8, P=0.08), which averaged 2.1±0.4%, 1.7±0.3% and 1.8±0.4%, respectively (Fig. 9D). Neither milk protein content nor sugar content were affected by maternal body mass (ANCOVA, P=0.50 and 0.61, respectively), litter size (ANCOVA, P=0.10 and 0.89, respectively) or litter mass (ANCOVA, P=0.07 and 0.10, respectively).

Gross energy content of milk produced by the hot, warm and cold mice averaged 10.5±1.0 kJ g^{-1}, 13.1±1.3 kJ g^{-1} and 14.6±1.5 kJ g^{-1}, respectively (ANOVA, $F_{2,29}$=30.9, P<0.001; all three means significantly different, Tukey pairwise comparisons, P<0.05; Fig. 10A). Gross energy content of milk across temperature was not affected by maternal body mass (ANCOVA, P=0.24). The effect of temperature on gross milk energy content remained significant after adjusting for the differences in litter size (ANCOVA: interaction litter size × temperature, P=0.60; litter size effect, $F_{1,28}$=5.7, P=0.023; temperature effect, $F_{2,28}$=28.4, P<0.001) and litter mass (ANCOVA: interaction litter mass × temperature, P=0.40; litter mass effect, $F_{1,28}$=16.3, P<0.001; temperature effect, $F_{2,28}$=55.3, P<0.001).

Milk energy output was calculated for the same individuals for which milk composition data were also available. The estimates of *MEO* for the mice exposed to 21°C and 8°C were derived from the female water turnovers as the product of the rate of milk flow and gross energy content of milk (Johnson et al., 2001a; Johnson and Speakman, 2001). In the present paper we have shown that this method is less accurate and less precise than the others, but we had insufficient data to recalculate the *MEO* of the warm and the cold mice using the *MEI–DEE*, pup water turnover or litter energy budget methods. To allow comparison between the three groups, the estimates of *MEO* for the hot mice were also derived from the female water turnovers. All estimates of milk flow and *MEO* refer to day 14 of lactation.

The rate of milk flow at 30°C, 21°C and 8°C averaged 8.5±1.8 g day^{-1}, 12.9±2.7 g day^{-1} and 20.0±5.0 g day^{-1}, respectively (ANOVA, $F_{2,29}$=27.9, P<0.001; all three means significantly different, Tukey pairwise comparisons, P<0.05; Fig. 10B). The effect of temperature on milk flow was still significant when corrected for the differences in maternal body mass (ANCOVA: interaction body mass × temperature, P=0.32; body mass effect, $F_{1,28}$=4.5, P=0.046; temperature effect, $F_{2,28}$=12.2, P<0.001), litter size (ANCOVA: interaction litter size × temperature, P=0.08; litter size effect, $F_{1,28}$=13.1, P=0.001; temperature effect, $F_{2,28}$=46.5, P<0.001) and litter mass (ANCOVA: interaction litter mass × temperature, P=0.89; litter mass effect, $F_{1,28}$=14.4, P=0.001; temperature effect, $F_{2,28}$=43.2, P<0.001).

The three groups differed significantly in the milk energy output (ANOVA, $F_{2,29}$=28.5, P<0.001). The hot mice exported less energy in milk (87.7±17.2 kJ day^{-1}) than the warm mice (166.7±22.7 kJ day^{-1}), while the cold mice, after 5 days of exposure to 8°C, increased their *MEO* to 288.0±60.7 kJ day^{-1} (Fig. 10C). The *MEO* across temperature was not affected by maternal body mass (ANCOVA, P=0.09). The effect of temperature on amount of energy exported in milk remained significant after adjusting for the differences in litter size (ANCOVA: interaction litter size × temperature, P=0.06; litter size effect, $F_{1,28}$=7.6, P=0.012; temperature effect, $F_{2,28}$=85.9, P<0.001) and litter mass (ANCOVA: interaction litter mass × temperature, P=0.73; litter mass effect, $F_{1,28}$=4.8, P=0.043; temperature effect, $F_{2,28}$=80.6, P<0.001).

Discussion

Comparison of methods for evaluating MEO

Estimates of milk energy output in small mammals can vary depending on the method used to measure energy or water budget components (Knight et al., 1986). Therefore, we evaluated the *MEO* of 24 female mice using four different methods: (1) as the difference between metabolizable energy intake and daily energy expenditure of the female, (2) from female water turnover, (3) from pup water turnover and (4) from the energy budget of the litter. We assessed these four

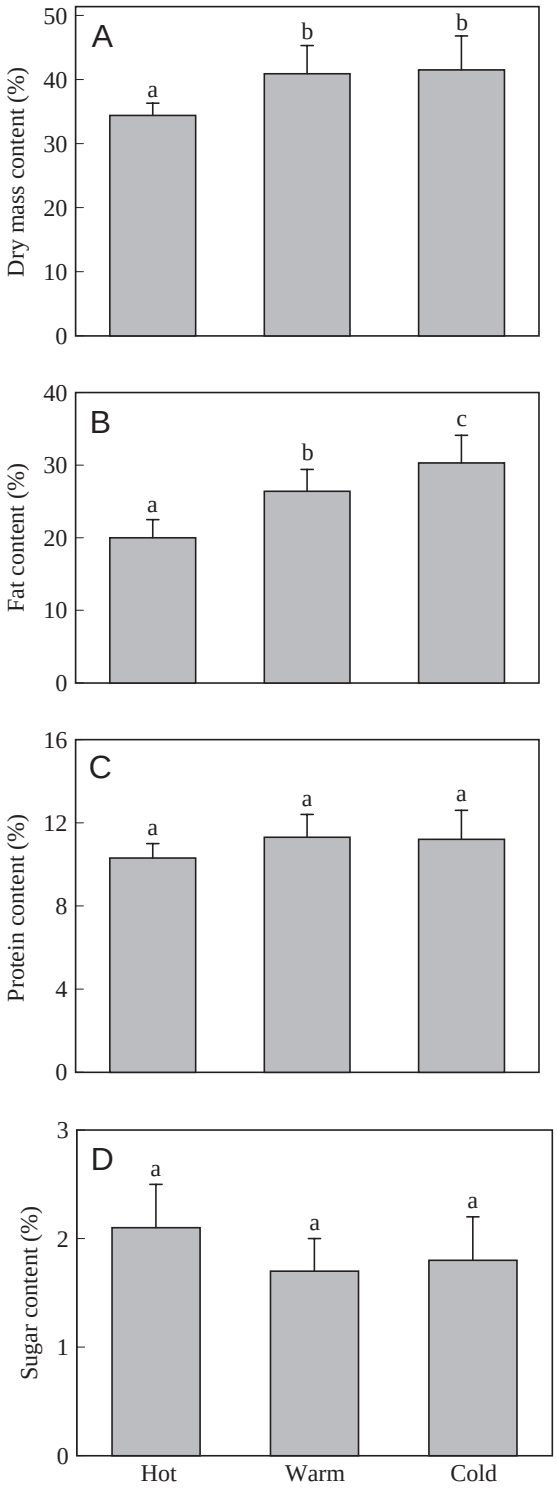

Fig. 9. Dry mass (A), fat (B), protein (C) and sugar (D) content of milk produced by mice exposed to hot (30°C), warm (21°C) and cold (8°C) temperatures. Milk composition data refer to day 15 of lactation. Bars are means + 1 S.D. (*N* as in text). Different letters above bars indicate significant differences between the temperatures ($P<0.05$), as assessed by ANOVA followed by Tukey pairwise comparisons.

methods by comparing their accuracy, precision and sensitivity to changes in parameters involved in the calculations.

The first method is based on an assumption that the difference between energy assimilated (= metabolizable energy intake) and energy used for maternal maintenance represents energy that is allocated for milk. The metabolizable energy intake is relatively easy to measure, but maternal maintenance expenditure is not. For this reason, milk energy output is frequently calculated as an increase in metabolizable energy intake above the non-reproductive level, i.e. as the difference between the *MEI* of lactating and non-reproductive females (e.g. McClure, 1987; Künkele and Kenagy, 1997; Künkele and Trillmich, 1997). However, the assumption that the *MEI* of non-reproductive individuals provides a good estimate of maternal maintenance expenditure may be incorrect, since lactation is frequently associated with an increase in body mass. Furthermore, lactating and non-reproductive females are also likely to differ in their mass-specific maintenance expenditure because of additional heat losses incurred during milk production. Therefore, we suggest that maternal maintenance expenditure should be measured directly, using the doubly labelled water technique.

We validated the DLW technique in non-reproductive mice by simultaneous measurements of *MEI* in a feeding trial (Fig. 2) and demonstrated that *DEE* measured from DLW turnover was, on average, 2.0% higher than *MEI*, with individual errors ranging from –10.4% to 22.9%. A similar range of individual errors is likely to be observed in estimates of *MEO*, since a 1% change in *DEE* would change *MEO* by 0.9% (Appendix C of supplementary material). The *MEI–DEE* method uses a relatively small number of assumptions and predictions (equation 1 in Appendix A of supplementary material) and involves minimal experimental interactions with the animals. However, since the measurements of *DEE* must be conducted immediately after the measurements of *MEI*, the use of this method is limited to the laboratory.

The second method assumes that the difference between the total water turnover of a lactating female and the water she loses through faeces, urine and evaporation represents the water exported in milk (equation 2 in Appendix A of supplementary material). The calculation of total water turnover following an injection of deuterium or tritium into the female body water could be subject to error since the method does not include any protocol to correct for isotope recycling between the mother and the pups (Baverstock and Green, 1975). Furthermore, a 1% change in the female water turnover would change *MEO* by 6.0% (Appendix C of supplementary material). Since the water turnover measured by isotope dilution is expected to be within ±10% of actual flux rates (Nagy and Costa, 1980), estimates of *MEO* could have a potential error of ±60%. Indeed, we demonstrated that the estimates of *MEO* derived from female water turnover were significantly lower and more variable than those from other methods (Fig. 6).

The third method relies on the difference between total water

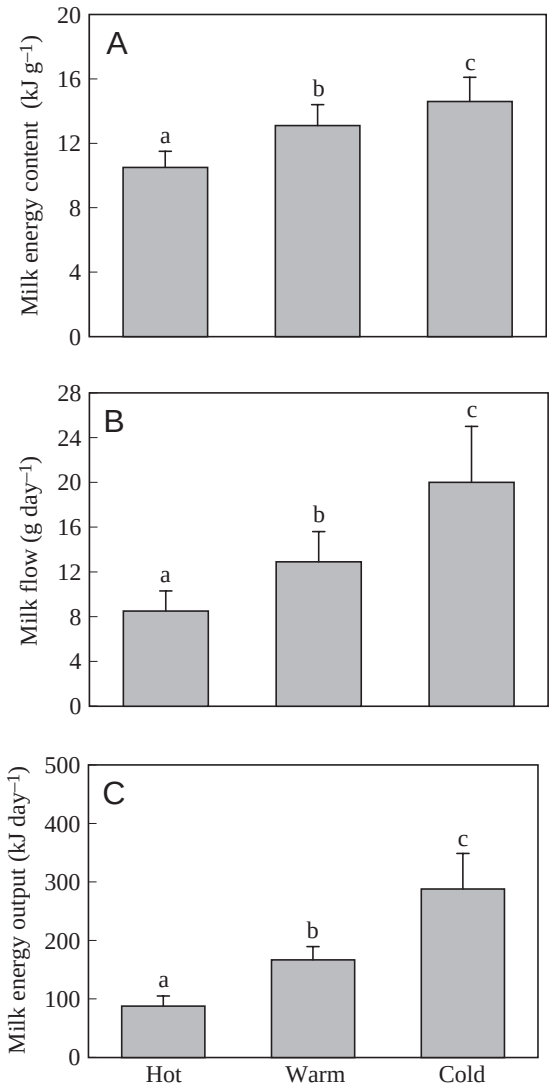

Fig. 10. Gross energy content of milk (A), the rate of milk flow (B) and milk energy output (C) in mice exposed to hot (30°C), warm (21°C) and cold (8°C) temperatures. Milk energy content refers to day 15 of lactation. Milk flow and milk energy output are calculated from female water turnover and refer to day 14 of lactation. Bars are means + 1 S.D. (*N* as in text). Different letters above bars indicate significant differences between the temperatures ($P<0.05$), as assessed by ANOVA followed by Tukey pairwise comparisons.

turnover of the pup and the influx of atmospheric and metabolic water, representing the milk water intake (equation 5 in Appendix A of supplementary material). The total water turnover is calculated from the turnover of deuterium or tritium injected into the pup. The main sources of error associated with this technique include: (1) recycling of isotopes from pups to mother (*via* maternal ingestion of pup urine and faeces) and from the mother back to the pups by uptake of the isotope in milk (Baverstock and Green, 1975), (2) reduction of isotope concentration due to increasing size of the body water pool (Dove and Freer, 1979) and (3) incorporation of isotopes into non-exchangeable hydrogen sites in newly synthesized tissue (Oftedal and Iverson, 1987). To correct for the isotope recycling, we measured accumulation of deuterium in control pups that had not been dosed with the isotope (Baverstock and Green, 1975; Friedman and Bruno, 1976). We eliminated the error related to changing body water pool of growing pup by use of the appropriate flux equation (Coward et al., 1982). However, due to lack of the relevant data, we did not correct for deuterium sequestration. Although the quantitative importance of these errors is difficult to assess, many of them appear to cancel each other out (Oftedal et al., 1993). The pup water turnover method requires a lot of parameters to be measured, but variation in most of them (apart from the pup body mass and the pup water turnover) has little effect on the evaluation of *MEO* (Appendix C of supplementary material). The parameters to which the method is most sensitive (pup body mass and water turnover) are relatively easy to measure in the laboratory as well as in the wild. In our study, the estimates of *MEO* produced by the pup water turnover method were similar to those derived from the *MEI–DEE* and litter energy budget methods (Fig. 6).

Because the energy demands of pups for growth and respiration are met entirely by energy of milk, it is possible to calculate *MEO* from the litter energy budget (equation 6 in Appendix A of supplementary material). The amount of energy accumulated as new tissues is relatively easy to measure, but the measurements of pup respiration are more difficult to perform. The pup respiration is the sum of resting metabolic rate (including the heat increment of feeding and energy costs of biosynthesis), costs of thermoregulation and costs of activity. Since all our measurements were conducted on mice exposed to 30°C (thermoneutrality), we assumed that there were no thermoregulatory costs and that the respiration of the pups was the same as the *RMR* of the litter, corrected for the cost of pup activity (equation 3 in Appendix A of supplementary material). These assumptions significantly simplified our measurements, but it is important to note that at sub-thermoneutral temperatures the thermoregulatory costs also need to be taken into account. Thus, the best approach would be to measure the respiration of the pups directly using the DLW technique. However, the applicability of this technique might be limited by the size of the blood sample. In our study, for example, for 45 pups injected with DLW, the blood samples from only two individuals were sufficiently large to run both ^{2}H and ^{18}O. Recent advances in mass spectrometry technology (Begley and Scrimgeour, 1996) may enable measurements on much smaller samples removing this constraint.

In summary, we compared four methods of measuring *MEO* in laboratory mice and showed that significant differences exist between the various methods that have been employed. The *MEI–DEE* method, pup water turnover method and litter energy budget method produced similar estimates of *MEO*, while the estimates of *MEO* derived from the female water turnover were significantly lower and more variable.

Peripheral versus *heat dissipation limit hypotheses*

We measured milk energy output in MF1 mice exposed to

30°C to test whether limits to lactational energy intake are imposed peripherally by the capacity of mammary glands to produce milk (Hammond et al., 1994, 1996; Rogowitz, 1998) or centrally by the capacity of the animal to dissipate body heat (Król and Speakman, 2003). According to the peripheral limitation hypothesis, mammary glands at peak lactation would work at maximal capacity regardless of ambient temperature, and therefore *MEO* measured at 30°C (present study) should not differ significantly from that measured at 21°C (Johnson et al., 2001a) or 8°C (Johnson and Speakman, 2001). The heat dissipation limit hypothesis predicts that reducing the driving gradient between body temperature and environment by exposing mice to 30°C would lead to a decrease in food intake and milk production, since both these processes contribute greatly to metabolic heat production. We have already demonstrated the decrease in asymptotic food intake following exposure to 30°C (Król and Speakman, 2003), but this behaviour, without concurrent measurements of *MEO*, is inconclusive because the decline in food intake at 30°C is predicted by both hypotheses.

Comparison of *MEO* between mice exposed to 30°C (present study), 21°C (Johnson et al., 2001a) and 8°C (Johnson and Speakman, 2001) showed that females lactating at 30°C exported less energy as milk than those at 21°C, which in turn had a lower *MEO* than mice exposed to 8°C (Fig. 10C). This decline in *MEO* was caused by a decline in both milk flow (Fig. 10B) and gross energy content of milk (Fig. 10A). Milk produced at 30°C contained less total solids (Fig. 9A) and less fat (Fig. 9B) than milk produced at 21°C and 8°C. Milk protein and sugar content, however, did not vary with ambient temperature (Fig. 9C,D). Thus, the data presented in the present study indicate that mice exposed to 30°C responded by reducing their milk flow, milk energy content and consequently milk energy output. These results are consistent with the heat dissipation limit hypothesis.

Reduction in food intake and milk production associated with heat stress is well documented in domestic ruminants and pigs (e.g. Legates, 1960; Abdalla et al., 1993; Silanikove, 2000; Renaudeau and Noblet, 2001). Albright and Alliston (1972) demonstrated that heat stress in dairy cows reduces food intake *via* effects in the hypothalamus. However, the mechanisms by which heat stress affects milk yield are unknown. A heat-induced reduction in milk yield is frequently reported to be similar in magnitude to concurrent decrease in food intake (reviewed by Silanikove, 2000; Renaudeau and Noblet, 2001). In our study, for example, mice exposed to 30°C reduced their food intake and milk energy output by 47% when compared with mice lactating at 21°C (Johnson et al., 2001a). Because of the similarity in food intake and milk yield depression, several authors have suggested that the effect of high temperature on milk production could simply be explained by a decline in nutrient supply due to reduced food intake. However, changes in the amount of nutrients in the milk do not necessarily reflect changes in the availability of dietary nutrients, since most mammals, including laboratory mice, exhibit homeorhetic (preferential) partitioning of nutrients to the mammary glands (Vernon, 1989; Vernon et al., 1999). The uptake of nutrients by mammary glands depends not only on nutrient availability but also on blood flow through the tissue, and suppression of mammary blood flow may compromise mammogenesis as well as milk secretion (Linzell, 1974; Ota and Peaker, 1979). Consequently, it has been suggested that heat stress reduces milk yield by redistributing the blood flow from mammary glands to the skin to improve conductive heat loss (Black et al., 1993). Indeed, this effect has been demonstrated in non-pregnant rabbits during early lactation but not at peak lactation or when lactating females were simultaneously pregnant (Lublin and Wolfenson, 1996).

Milk production is a function of the number and activity of mammary secretory cells. The number of secretory cells increases exponentially during pregnancy (prepartum mammogenesis), as a consequence of very high rates of cell division. In many eutherian mammals, including laboratory mice, prepartum mammogenesis is stimulated by placental lactogen and placental oestrogen (Nagasawa and Yanai, 1971; Jameson, 1998). Both hormones are produced in proportion to the number of placentae, and therefore the number of secretory cells is adjusted to the number of neonates to be fed. After parturition, the mammary cell division drops dramatically, but a limited amount of proliferation continues (postpartum mammogenesis; Knight, 2000). In the laboratory mouse, the maximum number of secretory cells is achieved on day 5 of lactation, at least seven days before peak milk production (Knight and Peaker, 1982). Thus, when mammogenesis is completed, further adjustments in milk production are likely to occur through changes in activity of the secretory cells. Indeed, we demonstrated that mice exposed to 30°C and 21°C, despite the differences in milk production (present study), did not differ in the dry mass of mammary glands at peak lactation (Król et al., 2003). Assuming that the mass of mammary glands correlates with the number of secretory cells, our data suggest that heat stress might constrain milk production by reducing secretory cell activity. However, without direct measurements of the number and activity of secretory cells, this remains unproven (Król et al., 2003).

After the onset of lactation, the maintenance of milk synthesis and secretion requires regular removal of milk. The suckling stimulus induces the release of oxytocin and prolactin from the pituitary gland *via* a neuroendocrine reflex. Oxytocin is responsible for milk let-down and ejection, whereas prolactin, which is the most important lactogenic hormone, activates the transcription of RNAs for milk proteins and enzymes involved in the synthesis of milk fats and sugars (Russel, 1980; Barber et al., 1992; Flint and Knight, 1997). Consequently, frequent suckling (or milking) increases milk production, whilst forced weaning, or a gradual cessation of milk removal that occurs during natural weaning, initiates the process of mammary gland involution (e.g. Peaker, 1995; Quarrie et al., 1996; Capuco et al., 2002). Although the frequency at which each mammary gland is sucked depends primarily on the number of pups per teat, the regular suckling pattern is likely to be disrupted when the mother is not in the nest. It has been demonstrated that the

females lactating at high ambient temperature are at risk of developing prolonged maternal hyperthermia, and therefore they frequently interrupt pup contact and leave the nest area to dissipate the heat load (Croskerry et al., 1978; Adels and Leon, 1986; Scribner and Wynne-Edwards, 1994). Thus, the detrimental effect of heat stress on milk production in mice exposed to 30°C (present study) could be related to a reduced stimulation of mammary glands by offspring, caused by a decrease in the frequency and duration of maternal nest attendance (but see Stern and Azzara, 2002).

Our results suggest that laboratory mice at peak lactation are limited centrally by their capacity to dissipate body heat generated by processing food and producing milk (Król and Speakman, 2003). Mice exposed to 30°C decreased their food intake (Król and Speakman, 2003) and their milk production (present study), presumably because both these processes contribute to metabolic heat production. Consequently, mice exposed to 30°C had a lower reproductive output than mice lactating at cooler temperatures (Król and Speakman, 2003). Taken together, these results argue against the peripheral limitation hypothesis (Hammond et al., 1994, 1996; Rogowitz, 1998) and support the heat limit dissipation hypothesis.

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