RESEARCH ARTICLE

Limits to sustained energy intake. XXIII. Does heat dissipation capacity limit the energy budget of lactating bank voles?

Edyta T. Sadowska[1,*], Elżbieta Król[2], Katarzyna M. Chrzascik[1], Agata M. Rudolf[1], John R. Speakman[2,3] and Paweł Koteja[1]

ABSTRACT

Understanding factors limiting sustained metabolic rate (SusMR) is a central issue in ecological physiology. According to the heat dissipation limit (HDL) theory, the SusMR at peak lactation is constrained by the maternal capacity to dissipate body heat. To test that theory, we shaved lactating bank voles (*Myodes glareolus*) to experimentally elevate their capacity for heat dissipation. The voles were sampled from lines selected for high aerobic exercise metabolism (A; characterized also by increased basal metabolic rate) and unselected control lines (C). Fur removal significantly increased the peak-lactation food intake (mass-adjusted least square means±s.e.; shaved: 16.3±0.3 g day^{-1}, unshaved: 14.4± 0.2 g day^{-1}; *P*<0.0001), average daily metabolic rate (shaved: 109± 2 kJ day^{-1}, unshaved: 97±2 kJ day^{-1}; *P*<0.0001) and metabolisable energy intake (shaved: 215±4 kJ day^{-1}, unshaved: 185±4 kJ day^{-1}; *P*<0.0001), as well as the milk energy output (shaved: 104±4 kJ day^{-1}; unshaved: 93±4 kJ day^{-1}; *P*=0.021) and litter growth rate (shaved: 9.4±0.7 g 4 days^{-1}, unshaved: 7.7±0.7 g 4 days^{-1}; *P*=0.028). Thus, fur removal increased both the total energy budget and reproductive output at the most demanding period of lactation, which supports the HDL theory. However, digestive efficiency was lower in shaved voles (76.0±0.3%) than in unshaved ones (78.5±0.2%; *P*<0.0001), which may indicate that a limit imposed by the capacity of the alimentary system was also approached. Shaving similarly affected the metabolic and reproductive traits in voles from the A and C lines. Thus, the experimental evolution model did not reveal a difference in the limiting mechanism between animals with inherently different metabolic rates.

KEY WORDS: Artificial selection, Doubly labelled water, Food consumption, Maternal investment, Milk energy output, Thermal conductance

INTRODUCTION

The rate at which animals acquire and use energy is an important factor affecting many aspects of animal performance, including reproductive output and thermoregulatory capabilities (e.g. McNab, 2002; Anderson and Jetz, 2005; Speakman and Król, 2005a). Therefore, understanding the factors limiting long-term, sustained energy budgets has remained one of the central issues in ecological and evolutionary physiology. In mammals, particularly small ones, lactation is the most energetically expensive period (Thompson and

[1]Institute of Environmental Sciences, Jagiellonian University, ul. Gronostajowa 7, Kraków 30-387, Poland. [2]Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK. [3]Institute of Genetics and Developmental Biology, State Key Laboratory of Molecular Developmental Biology, Chinese Academy of Sciences, Beichen Xi Lu, Chaoyang, Beijing 100101, People's Republic of China.

*Author for correspondence (edyta.sadowska@uj.edu.pl)

Nicoll, 1986; Speakman, 2008). Lactation often results in an increase in caloric intake by more than 100%, increased metabolic rate (Zhang and Wang, 2007) and elevated body temperature (Gamo et al., 2013). Thus, the discussion on limits to sustained metabolic rate (SusMR) has been largely focused on lactating females. Here, we tested a hypothesis that the upper limit to the energy budget of lactating bank voles (*Myodes glareolus*) is imposed by the capacity to dissipate excess heat.

Two explanations of limitation have been discussed extensively: the central and peripheral limitation theories (Drent and Daan, 1980; Weiner, 1987a; Peterson et al., 1990; Hammond and Diamond, 1992, 1994; Weiner, 1992; Hammond et al., 1994; Koteja, 1996a,b; Rogowitz, 1998). According to the central limitation theory, the capacity of the digestive system to process ingested food limits the total stream of available energy, and thus constrains SusMR, irrespective of the major source of energy demand (e.g. lactation or thermoregulation). Alternatively, according to the peripheral limitation theory, SusMR is limited by the capacities of the organs utilizing the energy. In the case of lactation, the peripheral limitation would be imposed by the capacity of mammary glands to produce milk (e.g. Hammond et al., 1994; Rogowitz, 1998). Several experiments designed to resolve which of the two mechanisms limits females' energy budgets at peak lactation provided contradictory results (Hammond and Diamond, 1992; Hammond et al., 1994; Speakman and McQueenie, 1996; Rogowitz, 1998; Hammond and Kristan, 2000; Johnson and Speakman, 2001; Johnson et al., 2001a,b; Wu et al., 2009; Duah et al., 2013).

More recently, the discussion has been revitalized by introducing the heat dissipation limit (HDL) theory, according to which peak-lactation SusMR is limited by a female's ability to dissipate excess heat (Król and Speakman, 2003a,b; Speakman and Król, 2005a, 2010). Such excess heat results from the increased rate of metabolism associated with the increased rate of food processing, transport of nutrients and milk synthesis, possibly combined with a diminished capacity to dissipate heat because of the suckling offspring adjacent to the mother (Leon et al., 1978).

The HDL theory has been motivated by an observation, paradoxical in the light of the previous ideas, that laboratory mice lactating at a temperature lower than the typical room conditions showed not only increased food consumption but also increased milk production and pup growth rates, whereas reproductive performance was decreased at the ambient temperature of 30°C (Johnson and Speakman, 2001; Król and Speakman, 2003a,b). Similar effects of decreased or increased temperature on lactation performance were observed in common vole (*Microtus arvalis*), Mongolian gerbil (*Meriones unguiculatus*) and Brandt's vole (*Lasiopodomys brandtii*, only in females with a litter size exceeding seven) (Wu et al., 2009; Simons et al., 2011; Yang et al., 2013). Contrary to the predictions of the HDL theory, however, females of European hares (*Lepus europaeus*) were able to

List of abbreviations

ADE	apparent digestive efficiency
ADE_{peak}	peak-lactation apparent digestive efficiency
ADMR	average daily metabolic rate
$ADMR_{peak}$	peak-lactation average daily metabolic rate
A-lines	lines selected for high swim-induced aerobic metabolism
BMR	basal metabolic rate
C-lines	lines unselected, control
DLW	doubly labelled water
FC	food consumption
FC_{peak}	peak-lactation food consumption
GL_{peak}	litter growth rate in the peak-lactation period
HDL	heat dissipation limit
LSM	adjusted least square mean
MEI	metabolisable energy intake
MEI_{peak}	peak-lactation metabolisable energy intake
MEO_{peak}	peak-lactation milk energy output
ML_{end}	mass of litter at the end of the experiment
MP_{end}	mean mass of a pup at the end of the experiment
RMR	resting metabolic rate
SusMR	sustained metabolic rate

reach their maximum energy turnover under thermoneutral conditions (Valencak et al., 2010) and lactating striped hamster (*Cricetulus barabensis*) females increased their food intake in the cold, but were not capable of producing more milk (Zhao, 2011). Such manipulations of ambient temperature affect not only the mother but also the offspring and their growth, which makes the results of such experiments difficult to interpret. Valencak et al. (2013) attempted to disentangle these effects by giving laboratory mice access to two connected cages maintained at distinct temperatures, but the results were also inconclusive.

An alternative and methodologically elegant approach to test the HDL theory is fur removal, which allows direct manipulation of heat dissipation in a lactating female without directly affecting the thermal balance of her offspring (Król et al., 2007). The fur coat can be helpful in maintaining positive energy balance during peak-lactation energy demand if ambient temperatures are low. However, an excess insulation may also result in hyperthermia during nursing, which could decrease the duration of nursing bouts (Croskerry et al., 1978; Leon et al., 1978) and thereby impair pup development. Consistent with the HDL predictions, fur removal in lactating MF1 mice elevated their rates of food consumption and milk production, and accelerated the growth of their litters (Król et al., 2007). In shaved Swiss mice and Siberian hamsters (*Phodopus sungorus*), food intake increased, but milk production and pup growth were not significantly affected (Zhao and Cao, 2009; Paul et al., 2010; Zhao et al., 2010). In common voles, shaving mothers resulted in significantly increased pup growth, but not significantly increased food intake or milk production (Simons et al., 2011). Thus, the fur-removal experiments provided only a mixed support for the HDL theory.

Here, we applied the fur-removal approach to test predictions of the HDL theory in lactating females from a unique experimental evolution model system: lines of bank voles selected for high aerobic exercise metabolism (A) and unselected, control lines (C) (Sadowska et al., 2008). Voles from the A-lines have a higher basal metabolic rate (BMR), daily food consumption and home-cage locomotor activity than animals from unselected C-lines (Koteja et al., 2009, 2011; Sadowska et al., 2015). An analysis of complete transcriptomes from heart and liver revealed modified SNP (single nucleotide polymorphism) allele frequencies and modified expression levels of several genes encoding enzymes involved in carbohydrate and lipid metabolism (Konczal et al., 2015). Importantly, the differentiation of the expression profile was more profound in the liver than in the heart, which confirmed that the selection affected not only exercise-related traits but also overall metabolism.

We predicted that if the HDL theory is correct, shaving should increase reproductive performance, measured as milk production and litter growth at peak lactation. We also explored whether the mechanism limiting SusMR is related to the routine level of metabolism, a question which, to our knowledge, has not yet been addressed. The inconsistent results of previous experiments can reflect a simple fact that the mechanisms do differ among species or even within species tested under different conditions (Speakman and Król, 2011). For obvious reasons, variation in the rate of metabolism can be a major factor responsible for such inconsistencies. If the response to fur removal differed between the A- and C-lines, this would indicate that different mechanisms impose the limit on SusMR in animals of basically the same physiology, but characterized by different levels of metabolism.

MATERIALS AND METHODS
Animals and selection experiment
We used bank voles, *Myodes* (*Clethrionomys*) *glareolus* (Schreber 1780), from generation 14 of an ongoing artificial-selection experiment, comprising four lines selected for high swim-induced aerobic metabolism (A-lines) and four unselected, control (C-lines) lines. The rationale of the selection experiment, detailed breeding and selection protocols, and results of the selection have been presented in our earlier work (Sadowska et al., 2008, 2015; Chrząscik et al., 2014; Ołdakowski et al., 2015). In generation 14, voles from the A-lines achieved a 48% higher mass-adjusted swim-induced maximum rate of oxygen consumption than voles from the unselected C-lines (Fig. S1).

The voles were kept in same-sex groups of three individuals in standard mouse cages (267×207×140 mm, polypropylene; Tecniplast, Italy) with sawdust bedding, at 20±1°C and constant photoperiod (16 h:8 h light:dark). Food (Labofeed H, Morawski Co, Kcynia, Poland) and water were provided *ad libitum*. All animal care and treatment procedures were approved by the Local Bioethical Committee in Kraków, Poland (no. 99/2006; no. 10/2009; 21/2010).

Experimental protocol
For this project, adult voles were randomly sampled from the first and second litters from each available family of the eight lines. The animals were housed individually in large cages (425×266×155 mm, polypropylene; Tecniplast, Italy) with perforated polypropylene plates suspended over the floor, a shelter (ceramic pot), and sawdust as nest material. In total, 184 females were paired with males (A-lines: 83, C-lines: 101), and 30 females served as non-mated controls (A-lines: 16, C-lines: 14), on which the same measurements were performed as on the reproducing females.

Overall, 146 females successfully weaned first litters (A-lines: 74, C-lines: 72). However, as the first litters are often small and maternal care is erratic, we decided to perform the project on the second litters. Therefore, males were kept with females until day 14 postpartum. Day of parturition of the second litter (day 0 of lactation) was determined by daily observations. A second litter was produced by 142 females (A-lines: 70 and C-lines: 72), but six females from the A-lines and one from the C-lines killed their young

and were excluded from further procedures. At the second parturition, the females were on average 135 days old (109–192 days; the age did not differ between selection directions or shaving treatment; Table S1).

On day 5 of lactation, all females were randomly assigned to one of two groups: shaved or unshaved (A lines: 36 unshaved and 34 shaved; C lines: 39 unshaved and 33 shaved; the sample size is as large or larger than used in previous experiments that demonstrated the effect of increased heat loss on reproductive output). Shaving was repeated on day 9 of lactation to minimise fur regrowth. All females were anaesthetised by injection of ketamine (40 mg kg^{-1}) followed by xylazine (8 mg kg^{-1}). Females from the shaved group were shaved dorsally with a Wella Contura Hair Clipper (Król et al., 2007). Sham-shaving was performed on females from the non-shaved group: the animals were anaesthetised and handled in the same way, and for about the same time, as the shaved ones.

Female body mass, litter size and litter mass were recorded daily, between 07:00 h and 09:00 h, throughout the entire lactation period. Daily food intake was estimated by subtracting the mass of food left in the hopper (and on the bottom of cages if there were visible pieces of food) from the mass of food given. Both the animals and the food were weighed to the nearest 0.01 g (Kern 440-33N, KERN & Sohn GmbH, Balingen, Germany). At the peak of lactation (days 11–15 of lactation; Kaczmarski, 1966), 2 day feeding trials were performed to obtain accurate estimates of the rate of food consumption, digestibility and metabolisable energy intake (days 11–13), followed by 24 h measurement of the average daily metabolic rate (ADMR) quantified by the doubly labelled water (DLW) technique (days 14–15). The same measurements were performed at approximately the same time in non-mated females.

In our breeding colony, the voles are weaned on day 17 of lactation, and our observations show that on day 16 most of the young still rely on their mother's milk. However, at least a day earlier the young begin to consume considerable amounts of food and their consumption cannot be separated from that of the mothers. Therefore, because our aim was to measure performance of the females, the experiment was terminated on day 15 of lactation.

Food consumption and metabolisable energy intake

At the beginning of the 2 day feeding trials, the females were transferred to new cages with fresh sawdust and a known mass of food (same as used in regular maintenance, about 30–40 g per cage, weighed to ±0.01 g). To determine the dry mass content of the food given, three samples of fresh food were taken on each day when the feeding trials were started. After 48 h, the remaining food and faeces were collected, separated and dried (samples of fresh food were dried at the same time) at 60°C to constant mass (±0.001 g; Radwag PS 200/2000.R2 Precision Balance, Radom, Poland). For the amount of uneaten food, we distinguished large pieces of pellets left in the feeder or found on the bottom of cages from orts, i.e. the food chewed by the animals to powder, which would not be collected during regular daily estimations of food intake.

The energy content of dry food (5 samples) and of dry faeces (92 samples, chosen randomly from each selection and shaving group) was measured by bomb calorimetry (Model 6100, Parr Instrument Company, Moline, IL, USA). Mean (±s.e.) energy content of dry food was 17.80±0.13 kJ g^{-1}. In non-reproducing females, the energy content of dry faeces (16.52±0.24 kJ g^{-1}) did not differ between shaving or selection groups ($F_{1,24}<0.15$, $P>0.7$; see below for statistical analyses). In lactating females, the energy content in A-lines (16.69±0.10 kJ g^{-1}) was slightly lower than that in C-lines

(16.92±0.09 kJ g^{-1}; $F_{1,57}=3.01$, $P=0.08$), but it did not differ between shaving groups ($F_{1,57}=0.23$, $P=0.64$), and the selection×shaving interaction was not significant ($F_{1,57}=0.24$, $P=0.62$), Therefore, in energy budget calculations, we used separate values for lactating females from A- and C-lines, but a common value for non-reproducing females.

Food consumption rate (FC, g day^{-1}), apparent digestive efficiency (ADE, %) and metabolisable energy intake (MEI, kJ day^{-1}) were calculated from data obtained in the 2 day feeding trials according to the following equations:

$$FC = [(\text{fresh food given} \times \text{dry mass content})$$
$$- \text{dry food uneaten}]/2, \tag{1}$$
$$ADE = 100 \times [(FC \times E_{\text{food}}) - ((\text{faeces mass}/2)$$
$$\times E_{\text{faeces}})]/(FC \times E_{\text{food}}), \tag{2}$$
$$MEI = [(FC \times E_{\text{food}}) - ((\text{faeces mass}/2) \times E_{\text{faeces}})] \times 0.97, \tag{3}$$

where E_{food} and E_{faeces} are respective energy content of the food and faeces, and the 0.97 multiplier accounts for an assumed 3% urinary energy loss (Drożdż, 1968).

We also calculated the proportion of food wasted as orts (PO, %) relative to the entire food intake (i.e. the amount that disappeared from feeders):

$$PO = 100 \times (\text{dry amount of orts})/(\text{dry food consumed}$$
$$+ \text{dry mass of orts}) \tag{4}$$

and used the PO values to obtain corrected estimates of food consumption during the remaining days of lactation, based on the measurements of daily food intake:

$$FC = (\text{dry food intake}) \times (1 - PO/100). \tag{5}$$

Such a correction is justified because the proportion of orts produced by an individual is highly repeatable (in house mice: Koteja et al., 2003; in bank voles: A. Stanisz, E.T.S. and P.K., unpublished observations). The proportion of orts produced in lactating females tended to be higher in the shaved than in the unshaved voles ($P=0.072$) and in the selected than in the control lines ($P=0.052$; Table S2). Thus, the correction was necessary to avoid biases in the comparisons across the selection and shaving groups.

The results of daily food intake measured across the entire lactation showed that the maximum was achieved later than the feeding trials were performed (Fig. 1). Therefore, we used ADE obtained in the feeding trials performed on days 11–13 to estimate the peak-lactation metabolisable energy intake (MEI$_{\text{peak}}$) based on mean daily food consumption (corrected for orts production) recorded on days 12–15 (FC$_{\text{peak}}$):

$$MEI_{\text{peak}} = FC_{\text{peak}} \times ADE \times 0.97. \tag{6}$$

ADMR and milk energy output

ADMR was measured using the DLW method (Butler et al., 2004). Animals were weighed (±0.01 g) and injected intraperitoneally with a known mass of DLW, enriched with ^{18}O (29.2 atom%) and ^{2}H (18.4 atom%). The amount of DLW injected was approximately 0.62% of the body mass of lactating females, and about 0.41% that of non-reproducing ones. The exact dose was quantified by weighing the syringe to the nearest 0.0001 g before and after injection. Two initial blood samples of 50 µl were collected 1 h after the injection by retro-orbital puncture and stored in flame-sealed glass capillaries. Females
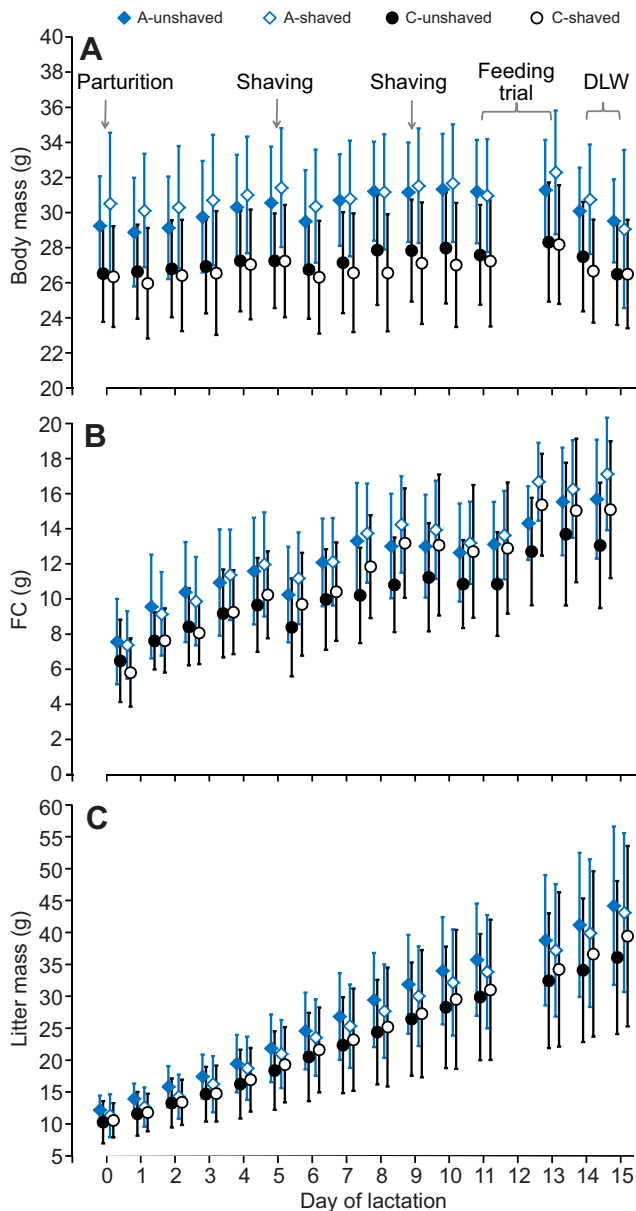
Fig. 1. Effect of shaving on lactation traits in voles from the control (C) and selected (A) lines. (A) Body mass, (B) food consumption (FC) and (C) litter mass (means±s.e.) in lactating females from shaving manipulation (shaved and unshaved) and selection (C- and A-lines) groups plotted against lactation day. DLW, the timing of the doubly labelled water trial. The means were not adjusted for differences in body mass or litter size, and were calculated for all litters of a constant size (after day 5), rather than only the large litters included in the final analysis (sample size: unshaved A-line, 31, C-line, 39; shaved A-line, 25, C-line, 25).

were immediately returned to their cage and litter. A second (final) blood sample was collected 25 h after the injection to estimate isotope elimination rates. Blood was collected from 10 additional voles that had no litter and had not been injected with DLW to estimate the background isotope enrichment in the body water pools of the animals (method C of Speakman and Racey, 1987).

Capillaries with the blood samples were vacuum distilled and the distilled water was used to produce CO_2 and H_2 (Speakman et al., 1990; Speakman and Król, 2005b). The isotope ratios ^{18}O:^{16}O and ^{2}H:^{1}H were analysed using gas source isotope ratio mass spectrometry (ISOCHROM μGAS system and IsoPrime IRMS,

Micromass, Manchester, UK) (Speakman and Król, 2005b). Isotope enrichments were converted to the values of ADMR using a single pool model as recommended for small rodents (see eqn 7.17 of Speakman, 1997).

Peak-lactation milk energy output (MEO_{peak}, kJ day^{-1}) was calculated as the difference between MEI_{peak} and $ADMR_{peak}$ (for a full description of the method, see Król and Speakman, 2003b; Speakman and Król, 2005b).

The effect of shaving on thermal conductance

To confirm the effect of fur removal on thermal conductance, we measured resting metabolic rate (RMR) at 10 and 20°C (in random order, with a 2 day interval between the measurements) in 16 unshaved and 16 shaved non-reproducing females. The metabolic rates were measured as the rate of oxygen consumption (ml O_2 h^{-1}) similar to Sadowska et al. (2015), with an open-flow, positive pressure respirometry system (design 1b in Koteja, 1996c) based on FC-2 and CA2-2A analysers (Sable Systems, Las Vegas, NV, USA). Oxygen consumption was recorded for about 4 h (after a 2 h period of food deprivation), sequentially on four channels. About 14–18 cycles (13 min each) were recorded for each vole. Two trials were performed during 1 day (06:00–12:00 h and 12:00–18:00 h), but the timing had no effect on the results. Activity detectors (MAD-1, Sable Systems) showed that 13 voles were active during the entire trial in one or both temperatures, and further analyses were performed only for resting animals (10 unshaved and 9 shaved). RMR was defined as the minimum rate of oxygen consumption observed in an individual during any of the cycles at a given temperature. The thermal conductance (ml O_2 h^{-1} °C^{-1}) was calculated as the difference between RMR measured at 20°C and 10°C divided by the difference between the temperatures (McNab, 2002).

Statistical analyses

To analyse the relationship between peak lactation metabolic traits and the litter size, and particularly to determine whether the traits' values increase linearly with litter size or achieve an upper limit, we applied a stage-regression model, implemented in SAS (version 9.3, SAS Institute) mixed non-linear procedure (NLMIXED). The model had four main estimable parameters: (1) intercept, (2) the slope of the regression line below a 'breaking point', (3) the value of the breaking point (litter size at which the slope of the relationship changes) and (4) the slope above the breaking point. Body mass was included in the model as a covariate, and residual variance was the single random effect. To check whether a limit was achieved, and whether a model with the breaking point fits the data better than a linear one, likelihood ratio tests were applied, based on results from appropriately reduced models (with the second stage slope fixed to zero or a simple regression model). Because the number of females with small litters (below four) was small, the analysis could be performed only for pooled individuals from all the selection and shaving groups, and therefore could not be used to test whether the upper limits differ between the groups.

For the main analyses, we used SAS MIXED procedure (with REML method) to estimate two-way cross-nested ANCOVA models. All the models included fixed effects of selection (selected versus control), shaving manipulation (shaved versus unshaved), selection×shaving interaction and maternal body mass (except for analyses with body mass as the dependent variable), and random effects of replicate lines (nested in selection groups) and line×shaving interaction. The models also included female age and the litter number from which the female was sampled (first or

second) as the elements of statistical control. However, age did not significantly affected any of the analysed traits, and the litter number affected only digestibility (ADE was slightly higher in females from the second litter), so results concerning these cofactors are presented only in Tables S2 and S3.

The analyses of peak-lactation traits, aimed at testing predictions of HDL theory, were restricted to litters larger than the breaking point estimated by the non-linear model described above. However, because traits' values still depended on litter size above the breaking point, litter size was included as an additional covariate. Thus, results of the analyses showed how shaving affected the performance of females nursing a given litter size. Body mass measured on day 11 (i.e. just before the onset of the peak of lactation) was included as a covariate, rather than body mass in the middle of the peak lactation period (days 12–15), because the latter could be affected by the individual's capacity to acquire energy during the peak demand, and therefore could be confounded with the dependent variables in the model.

For analysing the effect of shaving on the costs of maintenance in non-reproducing females, the models also included body mass change during the trial as an additional covariate. Because the number of individuals in this supplementary experiment was small, the models could not effectively include random effect of replicate lines. Thus, inferences concerning the effect of selection on the cost of living are not general.

In the mixed models, variance was constrained to non-negative values (default approach in SAS), and Satterthwaite approximation for non-orthogonal models was applied to calculate the denominator degrees of freedom (*Satterthwaite* option in the *Model* command of SAS MIXED procedure). Significance of the fixed effects was tested with *F*-tests. Significance of the random effects for which the variance estimate was positive was tested with the likelihood ratio test (Lynch and Walsh, 1998) in models of the same structure, but with variance not constrained (*nobound* option). Before estimating the final models, we checked the assumption of homogeneity of slopes by testing models that included interactions between body mass and the categorical effects. None of those interactions were significant, and we present results from the final models only. Assumptions of the parametric tests were verified by inspection of the distribution of residuals from the models. Because the residuals for the proportion of orts (PO) were severely right-skewed, the analysis was performed on log-transformed values. Observations with absolute values of studentised residuals higher than 3.0 were treated as outliers, and the analyses were repeated after excluding these observations. In the main text, we present results of the models excluding the outliers, but results from both versions are given in Tables S2 and S3. In most cases, the models led to the same conclusions, and if the outcome differed we provided the information in the Results. Here, we present only the main results; complete sets of descriptive statistics (means±s.d.) and results of the statistical models (least square means, LSM±s.e., regression coefficients, test statistics and significance levels of the fixed and random effects) are presented in Tables S2 and S3.

RESULTS
Energy budget of non-mated females
Body mass of the non-mated females was higher in the A-lines than in the C-lines (LSM±s.e., A: 23.0±0.7 g, C: 19.7±0.8 g; $F_{1,24}$=8.7, P=0.007). The mass did not differ between voles assigned to shaved and unshaved groups before the treatment (which reflects proper randomisation) or 7 days after the treatment, when feeding trials started (Table S3).

FC, MEI and ADMR increased with body mass; FC and MEI (but not ADMR) also increased with body mass change during the trial (Table S3). The values of FC, MEI and ADMR, adjusted for both body mass and body mass change, were much higher in shaved than in unshaved voles (FC: shaved 6.7±0.2 g day^{-1}, unshaved 5.06±0.2 g day^{-1}, $F_{1,22}$=44, P<0.001; MEI: shaved 91±2 kJ day^{-1}, unshaved 69±2 kJ day^{-1}, $F_{1,22}$=46, P<0.001; ADMR: shaved 74±3 kJ day^{-1}, unshaved 57±3 kJ day^{-1}, $F_{1,22}$=17, P=0.001). ADE was lower in shaved voles than in unshaved ones (shaved: 78.8±0.5%, unshaved: 80.3±0.4%, $F_{1,21}$=5, P=0.036; with one severe outlier retained P=0.30). The effect of selection was significant only for ADE (A-lines: 78.0±0.4%, C-lines: 81.1± 0.5%, $F_{1,21}$=21, P≤0.001; with one severe outlier retained P=0.02), and the selection×shaving interaction was not significant for any of the traits (P≥0.25).

Consistent with the above results, thermal conductance was nearly twice as high in the shaved (−8.02±0.46 ml O$_2$ h^{-1} °C^{-1}, N=10) than in the unshaved voles (−4.23±0.49 ml O$_2$ h^{-1} °C^{-1}, N=9; $F_{1,14}$=29.9, P<0.001; Table S3).

Lactation overview
The litter size at parturition (day 0 of lactation) ranged from 1 to 9, and it did not differ between the selection directions (A: 5.4±0.3, C: 5.4±0.2; $F_{1,21}$<0.01, P=0.96). During lactation, 45 females (9–13 from each of the selection/shaving groups) reduced their litters, mostly during the first 5 days, before shaving (35 females). Such reductions are common under our regular breeding conditions, and because all our analyses are focused on peak lactation period, we did not exclude these females from further analyses. After shaving, litter reductions occurred in 14 shaved and 7 unshaved females, and the proportion of reduced litters differed between the groups (test of independence with 1 d.f.: χ^2=5.55, P=0.019). The reductions were not associated with energy balance at peak lactation, because after day 11 of lactation only one shaved and one unshaved female reduced their litters. However, the change of litter size shortly before the focal period of the peak of lactation could affect the performance traits estimated for that period. Therefore, we excluded from the final analyses all females that reduced their litters after day 5 of lactation.

Body mass of virgin females on the day of first mating (37–107 days before the second parturition) was higher in A- than in C-lines (A: 22.2±0.6 g, C: 20.1±0.6 g; $F_{1,6}$=6.4, P=0.042). During the entire period of the second lactation, A-line females were about 3 g heavier than C-line ones (separate ANCOVA for each day: P<0.002; Fig. 1A). The mass did not differ between females assigned to the shaving groups either before (P≥0.32) or after the treatment (P≥0.23; Fig. 1A). The selection×shaving interaction was never significant (P≥0.11).

Body mass increased steadily until day 13, but at the end of lactation (days 13–15) the voles lost on average 0.90 g day^{-1} (Fig. 1A). The loss of mass increased with female mass ($F_{1,113}$=35, P<0.001). The adjusted mass loss was smaller in A- than in C-lines ($F_{1,113}$=5.4, P=0.022), but was not affected by shaving ($F_{1,113}$<0.01, P=0.97) or the selection×shaving interaction ($F_{1,113}$=0.04, P=0.85), and it did not depend on total litter mass at the end of lactation or litter growth rate on days 11–15 (P≥0.24).

In all groups, daily FC increased during the course of lactation (on average from 6.8±0.2 g day^{-1} on day 0 to 15.2±0.3 g day^{-1} on day 15; Fig. 1B). The declines of FC on days 5–6 and 10–11, i.e. following the shaving manipulations, occurred in both the shaved and unshaved groups. Thus, they were due not to the shaving itself, but to the associated manipulation (anaesthesia and handling). FC

was always positively correlated with current body mass ($P \leq 0.015$). Mass-adjusted FC did not differ significantly between A- and C-lines during the entire lactation period ($P \geq 0.12$), or between voles assigned to shaved and unshaved groups before the treatment was applied ($P \geq 0.19$; Fig. 1B). However, the adjusted FC was significantly increased in the shaved voles on day 7 of lactation (shaved: 12.7 ± 0.3 g day^{-1}, unshaved: 11.7 ± 0.3 g day^{-1}; $F_{1,12}=4.6$, $P=0.05$) and in all subsequent days. Total litter mass, adjusted for current maternal mass, did not differ significantly between A- and C-lines ($P \geq 0.3$) or between shaved and unshaved females ($P \geq 0.11$; Fig. 1C) on any of the lactation days (note that these analyses included all litters, independent of their size).

Peak-lactation performance

To find out whether at some litter size the females approached a physiological limit, and to determine which litters should be included in the analysis aimed at testing the HDL theory, we analysed the relationship between peak-lactation traits and litter size with non-linear stage-regression models (Tables S4 and S5, Fig. 2). The mass-adjusted FC_{peak} increased rapidly with litter size (slope$\pm$ s.e.: 2.6 ± 0.6 g day^{-1} pup^{-1}; $P<0.0001$) up to a litter size of 3.2 ± 0.4, and above that size further increases were small, although statistically significant (0.43 ± 0.18 g day^{-1} pup^{-1}; $P=0.022$; Fig. 2A). The non-linear model with the breaking point and two slopes fitted the data better than a simple linear regression model ($\chi^2=14.5$, d.f. $=2$, $P=0.001$), and only marginally significantly better than a model with a constant level forced above a breaking point ($\chi^2=4.0$, d.f.$=1$, $P=0.046$). The results were qualitatively the same for MEI_{peak} and MEO_{peak}: the traits increased rapidly with litter size up to a litter size of about 3.2, but above that point further increases were much smaller (MEI_{peak}: $P=0.027$; MEO_{peak}: $P=0.039$; Fig. 2B,D; Table S4). Peak-lactation average daily metabolic rate

($AMDR_{peak}$) was only weakly related to litter size for litter sizes below 3.4 ± 1.0 (8.6 ± 4.5 kJ day^{-1} pup^{-1}, $P=0.058$), and above that value the slope did not differ significantly from zero (1.1 ± 1.3 kJ day^{-1} pup^{-1}; $P=0.39$; Fig. 2C). Thus, females nursing four or more pups were approaching a limit to their energy budgets.

Only four females nursed eight pups and only one nursed nine pups, and the extremely large litters were not representative for the selection and shaving groups (all were from the selected lines and only one was from the shaved group; Fig. 2). Therefore, to avoid bias in the inferences concerning the main hypothesis (comparison between the shaved and unshaved groups), females with the extremely large litters were excluded from further analyses. The stage-regression models after the exclusion gave similar results to those for the full data set, but increases of FC_{peak}, MEI_{peak} and MEO_{peak} with litter sizes above the breaking size of about 3.2–3.3 pups were not statistically significant (Table S5).

The analyses aimed at testing the HDL hypothesis, i.e. testing the effect of shaving on the peak lactation performance, carried out for females nursing litters of 4–7 pups (Table 1; Table S3), showed that FC_{peak}, MEI_{peak}, $ADMR_{peak}$, peak-lactation apparent digestive efficiency (ADE_{peak}) and the final total litter mass (ML_{end}) increased with female's body mass, whereas the relationship was not significant for MEO_{peak}, litter growth rate (in days 11–15: GL_{peak}) or the final mean pup mass (MP_{end}; Table 1, Fig. 3). The values of FC_{peak}, MEI_{peak}, GL_{peak} and ML_{end} increased with litter size, whereas MP_{end} decreased with litter size, and the relationship was not significant for ADE_{peak}, $ADMR_{peak}$ and MEO_{peak} (Table 1).

The mass- and litter size-adjusted FC_{peak}, MEI_{peak} and $ADMR_{peak}$ were significantly higher in the shaved than in the unshaved group ($P \leq 0.0001$; Table 1, Fig. 4A,C,D), whereas ADE_{peak} was lower in shaved than in unshaved voles ($P<0.0001$, or $P=0.001$ before excluding four severe outliers; Fig. 4B). More
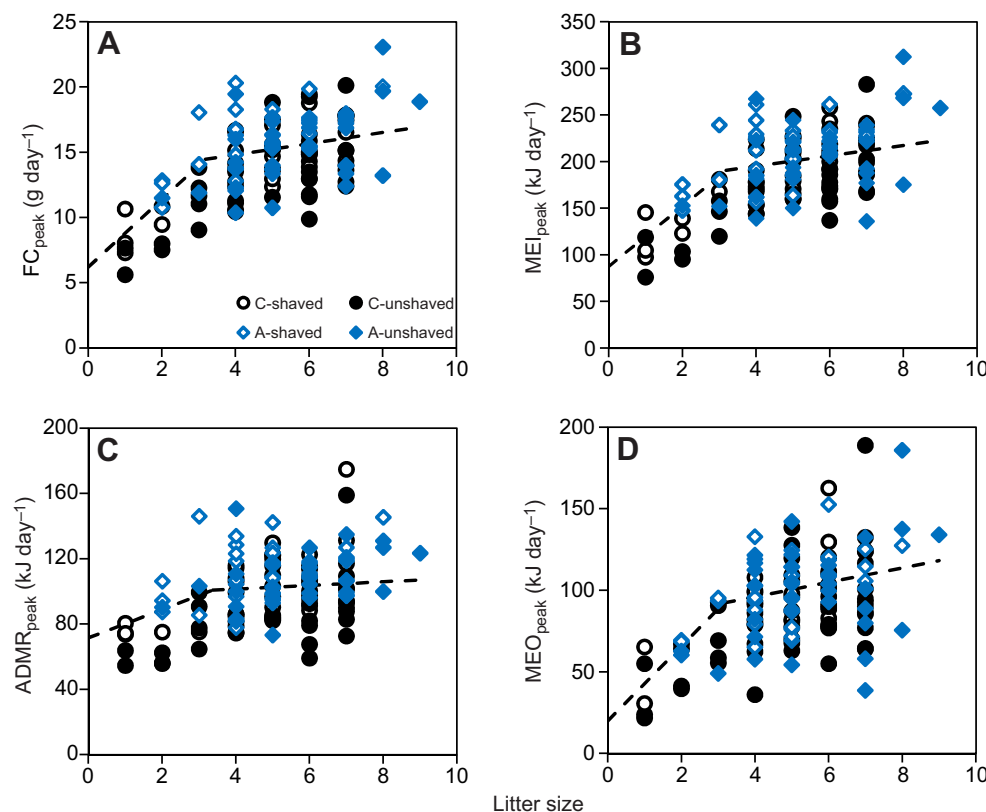

Fig. 2. The effect of litter size on peak-lactation performance traits. Peak-lactation (A) food consumption (FC_{peak}), (B) metabolisable energy intake (MEI_{peak}), (C) average daily metabolic rate ($ADMR_{peak}$) and (D) milk energy output (MEO_{peak}) in bank voles from shaving manipulation (shaved and unshaved) and selection (C- and A-lines) groups plotted against litter size. The dashed line shows predictions from a non-linear regression model, calculated for a female of average body mass (29.1 g; see Table S4 for parameters of the models).

Table 1. The effect of shaving and selection on peak-lactation performance traits of bank voles nursing large litters (4-7 pups)

| | Effect of shaving | | | | Effect of selection | | | | Interaction | | Effect of covariates | | | | | |
| | Adjusted means (±s.e.) | | Significance | | Adjusted means (±s.e.) | | Significance | | Selection×shaving | | Body mass at peak lactation | | | Litter size | | |
Variable	Unshaved	Shaved	F (ddf)	P	C-lines	A-lines	F (ddf)	P	F (ddf)	P	b (±s.e.)	F (ddf)	P	b (±s.e.)	F (ddf)	P
MF	29.4±0.36	29.2±0.42	0.2 (10.8)	0.663	28.2±0.37	30.5±0.42	16.9 (12)	0.002	0.02 (11)	0.905						
$ADMR_{peak}$	97±1.7	109±2.0	24.8 (91)	<0.001	102±1.9	104±2.1	0.62 (91)	0.432	0.62 (91)	0.434	3.16±0.55	32.4 (91)	<0.001	0.49±1.22	0.16 (91)	0.688
FC_{peak}	14.4±0.24	16.3±0.27	34.6 (87)	<0.001	15.2±0.27	15.5±0.30	0.66 (8.5)	0.439	0.23 (87)	0.625	0.45±0.07	41.2 (92)	<0.001	0.34±0.16	4.84 (91)	0.030
ADE_{peak}	79±0.25	76±0.28	46.1 (90)	<0.001	77±0.27	78±0.30	3.49 (90)	0.065	1.84 (90)	0.178	0.17±0.08	4.9 (90)	0.030	−0.06±0.17	0.13 (90)	0.723
MEI_{peak}	193±3.6	215±4.2	19.3 (90)	<0.001	202±4.2	207±4.6	0.61 (8.8)	0.455	0.22 (90)	0.639	5.71±1.05	29.8 (94)	<0.001	3.64±2.35	2.39 (94)	0.126
MEO_{peak}	93±3.5	104±3.9	5.55 (88)	0.021	95±4.1	102±4.4	1.18 (7.8)	0.309	0.01 (88)	0.955	1.28±0.95	1.83 (93)	0.180	2.29±2.13	1.16 (93)	0.285
ML_{end}	41.6±0.90	43.8±1.02	4.05 (90)	0.047	41.1±1.09	44.3±1.7	3.71 (8.6)	0.088	0.22 (90)	0.644	0.58±0.24	5.98 (93)	0.016	4.61±0.53	75 (94)	<0.001
MP_{end}	7.72±0.16	8.16±0.18	4.24 (90)	0.042	7.62±0.18	8.26±0.20	5.39 (9.7)	0.043	0.38 (90)	0.540	0.12±0.05	6.94 (94)	0.010	−0.58±0.10	33 (94)	<0.001
GL_{peak}	7.75±0.67	9.37±0.73	5.07 (88)	0.027	7.97±0.85	9.14±0.89	0.89 (7.3)	0.376	0.09 (88)	0.764	0.08±0.15	0.27 (91)	0.607	0.84±0.35	5.95 (92)	0.017

Adjusted least square means for the shaving and selection groups (LSM±s.e.), test statistics (F with denominator degrees of freedom, ddf) and significance levels (P-values) for the main factors and their interaction, and common regression slopes (b±s.e.) for the main covariates, from the linear mixed ANCOVA models (sample size: not shaved: A-lines, 26; C-lines, 32; shaved: A-lines, 21; C-lines, 23). See Table S2 for complete results, with adjusted means of selection×shaving groups and significance of other cofactors and covariates.
MF, female mass at the onset of peak lactation (day 11, g). $ADMR_{peak}$, daily energy expenditure (kJ day^{-1}, g). $ADMR_{peak}$, daily energy expenditure (kJ day^{-1}); FC_{peak}, food consumption (g day^{-1}); ADE_{peak}, digestive efficiency (%); MEI_{peak}, metabolisable energy intake (kJ day^{-1}) and MEO_{peak}, milk energy output (kJ day^{-1}) in the period of peak lactation. ML_{end}, litter mass and MP_{end}, mean pup mass (g) at day 15 of lactation. GL_{peak}, litter growth at peak lactation (days 11–15; g 4 days^{-1}).

importantly, shaving also resulted in increased MEO_{peak} ($P=0.021$), GL_{peak} ($P=0.027$), ML_{end} ($P=0.047$) and MP_{end} ($P=0.042$; Table 1, Fig. 4E,F).

Absolute values of the metabolic and reproductive output traits were higher in A-lines than in C-lines (Table S1; Fig. 1), but the difference was mostly attributable to the larger body mass of A-line females. The mass- and litter size-adjusted MP_{end} was significantly higher ($P=0.043$), and ML_{end} ($P=0.088$), in A-lines than in C-lines (Table 1). However, neither GL_{peak} nor the metabolic traits differed between the selected and control lines ($P≥0.3$; Fig. 4, Table 1). The exception was ADE_{peak}, which tended to be higher in A-lines ($P=0.065$; $P=0.21$ before excluding four outliers; Fig. 4B). The selection×shaving interaction was not significant for any of the traits.

DISCUSSION

Fur removal in bank voles resulted in increased FC_{peak}, MEI_{peak} and $ADMR_{peak}$. The analysis restricted to litters of 4–7 pups (i.e. excluding both small and abnormally large litters) showed that shaving also resulted in an increased MEO_{peak}, GL_{peak} and ML_{end}. The results support the HDL theory, according to which the upper limit to a female's reproductive energy budget is set by female's capacity to dissipate excess heat (Król and Speakman, 2003a,b; Speakman and Król, 2005a, 2010). In the following text, we will discuss the reliability and generality of this conclusion.

Energy intake in lactating bank voles was previously reported to be about 120% higher than in non-reproducing females (Kaczmarski, 1966; Trebaticka et al., 2007). In our study, the increase was even higher. In unshaved voles from the C-lines, MEI was about 220% higher at the peak of lactation (186 kJ day^{-1}) than in non-reproducing females (59 kJ day^{-1}). The increased energy demand was realised by an even larger (280%) increase of food consumption (lactating: 14.1 g day^{-1}, non-lactating: 5.1 g day^{-1}). About 50% of the increased energy intake of lactating females was exported in milk (90 kJ day^{-1}). However, the remaining part was responsible for a more than 60% increase of metabolic heat production, measured as ADMR (lactating: 96 kJ day^{-1}, non-lactating: 59 kJ day^{-1}). This excess heat, combined with heat generated by the offspring (not measured in this study), indeed imposes a heavy thermoregulatory burden on the lactating female, which supports the rationale of the HDL theory (see Speakman and Król, 2010).

We should ask, however, whether the lactating voles were actually approaching a metabolic ceiling. Because of logistical limitations, we could not manipulate litter size, which would provide a strong test for the presence of a metabolic ceiling (Hammond and Diamond, 1992, 1994; Hammond et al., 1994; Johnson et al., 2001a,c; Paul et al., 2010; Simons et al., 2011; Duah et al., 2013). However, in a parallel experiment on voles from the same colony, adding two extra young to the natural litters resulted in a weaned litter size increased by only one, and a decreased mass of the weaned pups (Ołdakowski et al., 2015), which indicates that females could not adequately nurse the enlarged litters. The results of our study showed that the values of all the metabolic traits measured at the peak lactation did not increase linearly with increasing litter size. For litter sizes above 3, the increases, even if statistically significant, were only small (Fig. 4). Also, the analysis limited to the large litters (4–7 pups), i.e. as large as or larger than the mean litter size in bank voles (about 4 pups; Ołdakowski et al., 2012), showed that final mean pup mass decreased with litter size (Table 1). Thus, females nursing large litters were at least close to a ceiling limiting their reproductive performance. Therefore, if the
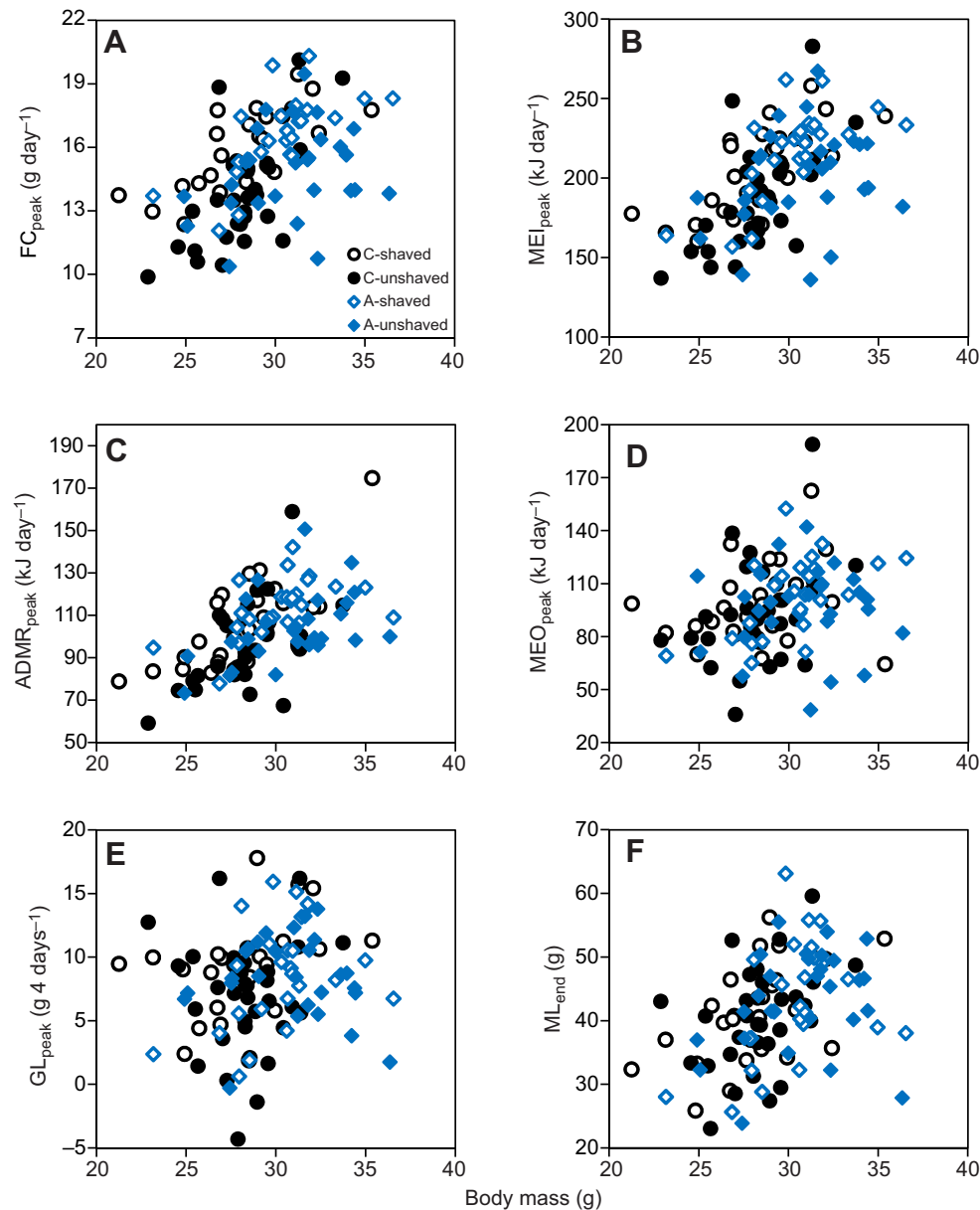
Fig. 3. Peak-lactation metabolic and reproductive output traits in females nursing large litters (4–7 pups). Peak-lactation (A) food consumption rate (FC$_{peak}$), (B) metabolisable energy intake (MEI$_{peak}$), (C) average daily metabolic rate (ADMR$_{peak}$), (D) milk energy output (MEO$_{peak}$) and (E) litter growth (mass change between days 11 and 15, GL$_{peak}$), and (F) final litter mass (ML$_{end}$) in bank voles from shaving manipulation (shaved and unshaved) and selection (C- and A-lines) groups plotted against female body mass (see Table 1 and Table S2 for sample sizes and results of the mixed-model ANCOVA).

HDL theory holds, releasing the females from the burden of excess heat should have resulted in an increased reproductive output.

As expected, fur removal resulted in a large increase in thermal conductance. The estimates were based on only a few individuals (non-reproducing) and did not account for possible variation in body temperature, and therefore should be treated with caution. However, the estimate for unshaved voles (4.41 ml O$_2$ h^{-1} °C^{-1} for a 22.6 g mean body mass) is similar to that reported for bank voles in previous studies (about 4.6 ml O$_2$ h^{-1} °C^{-1} for 18.1 g voles in summer; Hissa and Tarkkonen, 1969). Thus, the nearly twofold increase of thermal conductance in shaved voles can also be treated as a reliable estimate. The increased thermal conductance led to considerably increased costs of maintenance in the non-mated shaved voles maintained at 20°C, manifested as increased rates of FC (29%), MEI (28%) and ADMR (22%).

Fur removal in lactating voles led to significantly increased ADMR$_{peak}$ (13%), FC$_{peak}$ (14%) and MEI$_{peak}$ (12%; Fig. 4). From a traditional perspective, the increases would be interpreted as representing an additional burden imposed on the energy budget,

due to the increased heat loss that had to be compensated for by increased heat production. Consequently, we would predict that the manipulation should result in decreased reproductive output. However, our results showed that shaving resulted in an 11% increase of the MEO$_{peak}$ and a 20% increase in GL$_{peak}$, leading to the increased ML$_{end}$. Thus, the results supported the HDL theory, according to which the increased heat loss should be considered as releasing the females from an overheating thermoregulatory burden, rather than imposing an additional energy burden.

The HDL theory originated from observations on the laboratory mouse MF1 outbred strain (Król and Speakman, 2003a,b; Król et al., 2007), which is known to have exceptionally large litters, as a consequence of a long selection for high reproductive demand under conditions of no food restriction. Therefore, the generality of the HDL as a physiological mechanism limiting energy budgets of lactating females was doubtful. Previous experiments with shaving or temperature manipulation provided inconsistent results. In another strain of laboratory mouse (an unspecified 'Swiss' strain) maintained at 20°C, shaving resulted in elevated food consumption
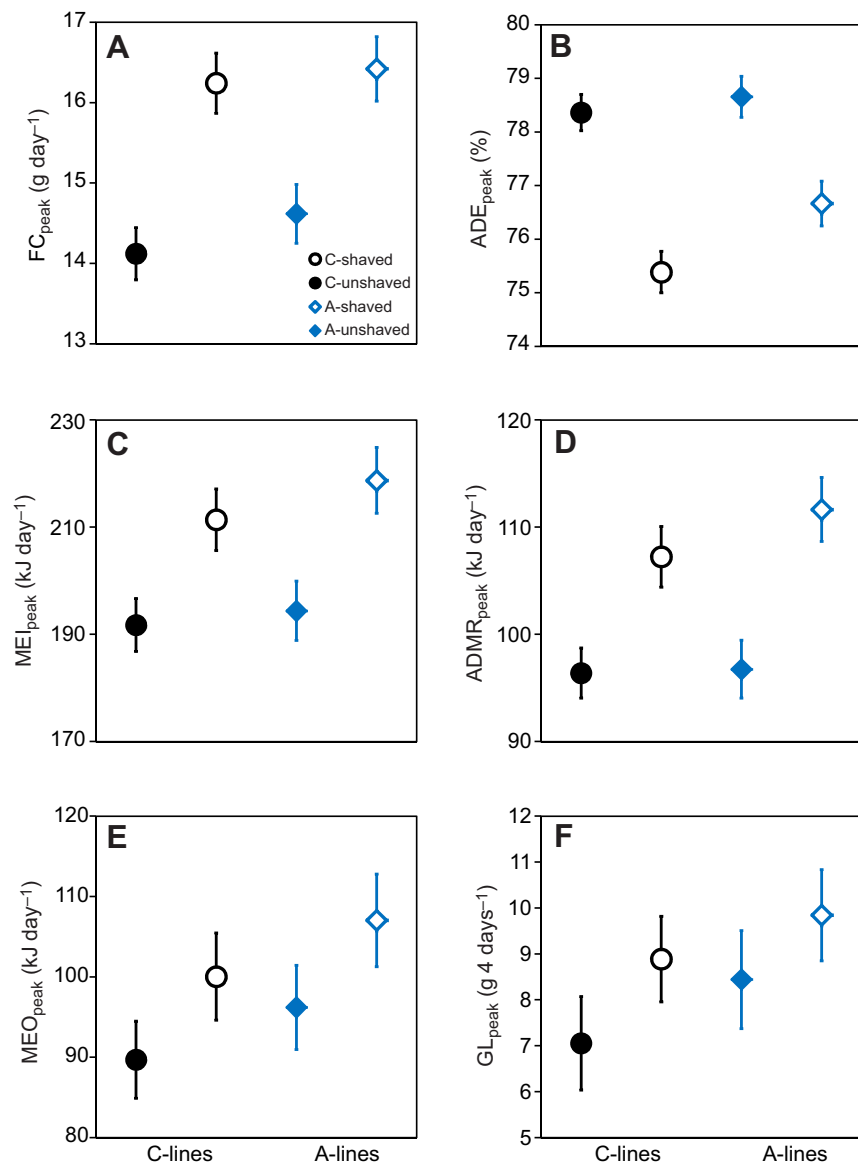
Fig. 4. Peak-lactation metabolic and reproductive output traits in females nursing large litters (4–7 pups). Peak-lactation (A) food consumption (FC_{peak}), (B) apparent digestive efficiency (ADE_{peak}; days 11–13 of lactation), (C) metabolisable energy intake (MEI_{peak}), (D) average daily metabolic rate ($ADMR_{peak}$), (E) milk energy output (MEO_{peak}) and (F) litter growth (mass change between days 11 and 15, GL_{peak}) in bank voles from shaving manipulation (shaved and unshaved) and selection (C- and A-lines) groups. Data are adjusted LSM±s.e. (see Table 1 and Table S2 for sample sizes and results of the mixed-model ANCOVA).

but not in increased pup growth (Zhao and Cao, 2009; Zhao et al., 2010). In common voles at 30°C, shaving did not significantly increase food consumption or milk production, but did increase growth of pups (Simons et al., 2011). Similarly, Mongolian gerbils maintained at 30°C had a lower MEO_{peak} and a lower litter mass compared with those at 10 or 21°C (Yang et al., 2013). However, animals exposed permanently to a sufficiently high temperature will surely be constrained by overheating, which may not necessarily apply to ecologically more relevant temperatures (Speakman and Król, 2011). Our experiment showed all the results predicted by HDL theory in females of a non-laboratory rodent species nursing litters of a modest size of 4–7 at 20°C. Thus, the limitation by the capacity to dissipate excess heat concerns not only rodents with exceptionally large litters or rodents maintained at elevated temperatures.

A separate question is whether the HDL theory applies to free-living animals. Certainly, animals can be limited by food availability, but in the classical studies concerning the problem of limits to energy budgets it has already been argued that food may be not the limiting factor for herbivores during the reproductive season (e.g. Weiner, 1992). However, the commonly assumed 'normal' room temperature of 20°C may still not be relevant in the ecological

context. The bank vole lives across a wide latitudinal range from the Mediterranean to Fennoscandia, and in large parts of the range the summer air temperatures are well above 20°C. However, the voles inhabit forests, spend the day mainly in underground shelters, and are active mostly at night (Petrusewicz, 1983; Bujalska and Hansson, 2000). Therefore, we cannot exclude the possibility that the constant 20°C in the laboratory represents a hotter thermal habitat than that actually experienced by the voles under natural conditions (note, however, that nocturnality and habitat choice may be partly due to selection pressure for avoiding the heat dissipation limit). Thus, further experiments aimed at testing the HDL theory should place more emphasis on modelling relevant thermal conditions.

A decreased digestive efficiency observed in the shaved group of lactating females could be interpreted as supporting the classical concept of energy budgets limited centrally by the capacity of the alimentary system to digest food or absorb nutrients (Weiner, 1987a,b, 1992; Koteja, 1996a,b; Bacigalupe and Bozinovic, 2002; Valencak and Ruf, 2009; Ruf and Grafl, 2010). However, digestibility was also decreased in the shaved group of non-lactating females. Thus, we cannot exclude that the effect resulted from behavioural consequences

of the manipulation (e.g. increased stress levels). Certainly, this aspect should also be considered in further studies applying such a manipulation.

We expected that voles from the A-lines, which are characterised by increased BMR and daily FC rates compared with unselected C-lines (Koteja et al., 2009; Sadowska et al., 2015), would also have increased peak-lactation energy budgets, which could lead to a distinct reaction to the shaving manipulation. Interpretation of the results was complicated by the fact that A-line females were about 3 g heavier than C-line ones. ANCOVA with body mass included as covariate showed that ML_{end} and MP_{end} were indeed higher in the selected lines (Table 1). However, the mass-adjusted FC_{peak}, MEI_{peak}, MEO_{peak} or GL_{peak} did not differ significantly between the selection groups (Table 1, Fig. 4). Thus, the increased ML_{end} in the A-lines apparently results from increased investment throughout lactation, rather than increased performance in the peak-lactation demand. The observation that selection for high aerobic exercise metabolism resulted in increased maternal investment is by itself interesting, especially in the context of the hypothesis linking the evolution of mammalian endothermy with the evolution of both locomotor performance and intensive parental care (Koteja, 2000). However, the lack of a significant effect of selection in the period of peak-lactation demand makes the model not suitable for testing the hypothesis that the limiting mechanism depends on the level of metabolism in that critical period. Consequently, the lack of a significant interaction between the effects of selection and shaving is not informative in that context.

In summary, the results of our experiment showed that lactating voles approach a physiological ceiling limiting their reproductive output, and that the limitation can be released by fur removal, which led to increased heat loss. Thus, the results support the HDL theory and extend it to more ecologically relevant species than laboratory mice. A significantly decreased digestive efficiency suggests that the shaved voles, released from the heat dissipation limit, can face another ceiling, set 'centrally' at a slightly higher level by the capacity of the alimentary system to efficiently digest and absorb nutrients. However, such an interpretation requires further validation, to exclude the possibility that the decreased digestibility results from a stress response to the shaving manipulation.

Competing interests
The authors declare no competing or financial interests.

Author contributions
E.T.S., E.K., J.R.S. and P.K. contributed to study conception and design; E.T.S. collected and analysed the data; J.R.S. and E.K. contributed to the daily metabolic rate analysis; E.T.S., A.M.R. and K.M.C. performed the selection experiment and provided the voles; E.T.S. and P.K. wrote the paper; E.K. and J.R.S. contributed to interpretation of the results and drafting of the article; A.M.R. and K.M.C. commented on the manuscript.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.134437/-/DC1

References
Anderson, K. J. and Jetz, W. (2005). The broad-scale ecology of energy expenditure of endotherms. *Ecol. Lett.* **8**, 310-318.

Bacigalupe, L. D. and Bozinovic, F. (2002). Design, limitations and sustained metabolic rate: lessons from small mammals. *J. Exp. Biol.* **205**, 2963-2970.

Bujalska, G. and Hansson, L. (2000). Bank vole biology: recent advances in the population biology of a model species. *Polish J. Ecol.* **48**, 5-7.

Butler, P. J., Green, J. A., Boyd, I. L. and Speakman, J. R. (2004). Measuring metabolic rate in the field: the pros and cons of the doubly labelled water and heart rate methods. *Funct. Ecol.* **18**, 168-183.

Chrzascik, K. M., Sadowska, E. T., Rudolf, A. and Koteja, P. (2014). Learning ability in bank voles selected for high aerobic metabolism, predatory behaviour and herbivorous capability. *Physiol. Behav.* **135**, 143-151.

Croskerry, P. G., Smith, G. K. and Leon, M. (1978). Thermoregulation and the maternal behaviour of the rat. *Nature* **273**, 299-300.

Drent, R. H. and Daan, S. (1980). The prudent parent: energetic adjustments in avian breeding. *Ardea* **68**, 225-252.

Drożdż, A. (1968). Digestibility and assimilation of natural foods in small rodents. *Acta Theriol.* **13**, 367-389.

Duah, O. A., Monney, K. A., Hambly, C., Król, E. and Speakman, J. R. (2013). Limits to sustained energy intake. XVII. Lactation performance in MF1 mice is not programmed by fetal number during pregnancy. *J. Exp. Biol.* **216**, 2339-2348.

Gamo, Y., Bernard, A., Mitchell, S. E., Hambly, C., Al Jothery, A., Vaanholt, L. M., Król, E. and Speakman, J. R. (2013). Limits to sustained energy intake. XVI. Body temperature and physical activity of female mice during pregnancy. *J. Exp. Biol.* **216**, 2328-2338.

Hammond, K. A. and Diamond, J. (1992). An experimental test for a ceiling on sustained metabolic rate in lactating mice. *Physiol. Zool.* **65**, 952-977.

Hammond, K. and Diamond, J. (1994). Limits to dietary nutrient intake and intestinal nutrient-uptake in lactating mice. *Physiol. Zool.* **67**, 282-303.

Hammond, K. A. and Kristan, D. M. (2000). Responses to lactation and cold exposure by deer mice (*Peromyscus maniculatus*). *Physiol. Biochem. Zool.* **73**, 547-556.

Hammond, K. A., Konarzewski, M., Torres, R. M. and Diamond, J. (1994). Metabolic ceilings under a combination of peak energy demands. *Physiol. Zool.* **67**, 1479-1506.

Hissa, R. and Tarkkonen, H. (1969). Seasonal variations in brown adipose tissue in two species of voles and the common shrew. *Ann. Zool. Fennici* **6**, 443-447.

Johnson, M. S. and Speakman, J. R. (2001). Limits to sustained energy intake V. Effect of cold-exposure during lactation in Mus musculus. *J. Exp. Biol.* **204**, 1967-1977.

Johnson, M. S., Thomson, S. C. and Speakman, J. R. (2001a). Limits to sustained energy intake. I. Lactation in the laboratory mouse *Mus musculus*. *J. Exp. Biol.* **204**, 1925-1935.

Johnson, M. S., Thomson, S. C. and Speakman, J. R. (2001b). Limits to sustained energy intake. III. Effects of concurrent pregnancy and lactation in Mus musculus. *J. Exp. Biol.* **204**, 1947-1956.

Johnson, M. S., Thomson, S. C. and Speakman, J. R. (2001c). Limits to sustained energy intake. II. Inter-relationships between resting metabolic rate, life-history traits and morphology in *Mus musculus*. *J. Exp. Biol.* **204**, 1937-1946.

Kaczmarski, F. (1966). Bioenegetics of pregnancy and lactation in the bank vole. *Acta Theriol.* **11**, 409-417.

Konczal, M., Babik, W., Radwan, J., Sadowska, E. T. and Koteja, P. (2015). Initial molecular-level response to artificial selection for increased aerobic metabolism occurs primarily through changes in gene expression. *Mol. Biol. Evol.* **32**, 1461-1473.

Koteja, P. (1996a). Limits to the energy budget in a rodent, *Peromyscus maniculatus*: the central limitation hypothesis. *Physiol. Zool.* **69**, 981-993.

Koteja, P. (1996b). Limits to the energy budget in a rodent, *Peromyscus maniculatus*: does gut capacity set the limit? *Physiol. Zool.* **69**, 994-1020.

Koteja, P. (1996c). Measuring energy metabolism with open-flow respirometric systems: which design to choose? *Funct. Ecol.* **10**, 675-677.

Koteja, P. (2000). Energy assimilation, parental care and the evolution of endothermy *Proc. R. Soc. B Biol. Sci.* **267**, 479-484.

Koteja, P., Carter, P. A., Swallow, J. G. and Garland, T., Jr (2003). Food wasting by house mice: variation among individuals, families, and genetic lines. *Physiol. Behav.* **80**, 375-383.

Koteja, P., Baliga-Klimczyk, K., Chład, A., Chrząścik, K. M., Damulewicz, M., Dragosz-Kluska, D., Morawska-Ploskonka, J. and Sadowska, E. T. (2009). Correlated responses to a multidirectional artificial selection in the bank vole: activity metabolism, and food consumption. *J. Physiol. Sci.* **59**, 541.

Koteja, P., Chrzascik, K., Dheyongera, G., Rudolf, A. and Sadowska, E. T. (2011). Correlated responses to a multidirectional artificial selection in the bank vole, *Myodes glareolus*. *Comp. Physiol. Biochem.* **28S**, 94.

Król, E. and Speakman, J. R. (2003a). Limits to sustained energy intake VI. Energetics of lactation in laboratory mice at thermoneutrality. *J. Exp. Biol.* **206**, 4255-4266.

Król, E. and Speakman, J. R. (2003b). Limits to sustained energy intake VII. Milk energy output in laboratory mice at thermoneutrality. *J. Exp. Biol.* **206**, 4267-4281.

Król, E., Murphy, M. and Speakman, J. R. (2007). Limits to sustained energy intake. X. Effects of fur removal on reproductive performance in laboratory mice. *J. Exp. Biol.* **210**, 4233-4243.

Leon, M., Croskerry, P. G. and Smith, G. K. (1978). Thermal control of mother-young contact in rats. *Physiol. Behav.* **21**, 793-811.

Lynch, M. and Walsh, J. B. (1998). *Genetics and Analysis of Quantitative Traits*. Sunderland, MA: Sinauer.

McNab, B. K. (2002). *The Physiological Ecology of Vertebrates: A View from Energetics*. Ithaca, New York: Comstock Publishing Associates, Cornell University Press.

Ołdakowski, Ł., Piotrowska, Ż., Chrząścik, K. M., Sadowska, E. T., Koteja, P. and Taylor, J. R. E. (2012). Is reproduction costly? No increase of oxidative damage in breeding bank voles. *J. Exp. Biol.* **215**, 1799-1805.

Ołdakowski, Ł., Wasiluk, A., Sadowska, E. T., Koteja, P. and Taylor, J. R. E. (2015). Reproduction is not costly in terms of oxidative stress. *J. Exp. Biol.* **218**, 3901-3910.

Paul, M. J., Tuthill, C., Kauffman, A. S. and Zucker, I. (2010). Pelage insulation, litter size, and ambient temperature impact maternal energy intake and offspring development during lactation. *Physiol. Behav.* **100**, 128-134.

Peterson, C. C., Nagy, K. A. and Diamond, J. (1990). Sustained metabolic scope. *Proc. Natl. Acad. Sci. USA* **87**, 2324-2328.

Petrusewicz, K. (1983). *Ecology of the Bank Vole. Acta Theriologica* Vol. 28 of supplement. Warsaw: Polish Scientific Publishers

Rogowitz, G. L. (1998). Limits to milk flow and energy allocation during lactation of the hispid cotton rat (*Sigmodon hispidus*). *Physiol. Biochem. Zool.* **71**, 312-320.

Ruf, T. and Grafl, B. (2010). Maximum rates of sustained metabolic rate in cold-exposed Djungarian hamsters (*Phodopus sungorus*): the second wind. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **180**, 1089-1098.

Sadowska, E. T., Baliga-Klimczyk, K., Chrząścik, K. M. and Koteja, P. (2008). Laboratory model of adaptive radiation: a selection experiment in the bank vole. *Physiol. Biochem. Zool.* **81**, 627-640.

Sadowska, E. T., Stawski, C., Rudolf, A., Dheyongera, G., Chrzascik, K. M., Baliga-Klimczyk, K. and Koteja, P. (2015). Evolution of basal metabolic rate in bank voles from a multidirectional selection experiment. *Proc. R. Soc. Lond. B Biol. Sci.* **282**, 20150025.

Simons, M. J. P., Reimert, I., Van Der Vinne, V., Hambly, C., Vaanholt, L. M., Speakman, J. R. and Gerkema, M. P. (2011). Ambient temperature shapes reproductive output during pregnancy and lactation in the common vole (Microtus arvalis): a test of the heat dissipation limit theory. *J. Exp. Biol.* **214**, 38-49.

Speakman, J. R. (1997). *Doubly Labelled Water: Theory and Practice*. London: Chapman & Hall.

Speakman, J. R. (2008). The physiological costs of reproduction in small mammals. *Philos. Trans. R. Soc. B Biol. Sci.* **363**, 375-398.

Speakman, J. R. and Król, E. (2005a). Limits to sustained energy intake IX: a review of hypotheses. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **175**, 375-394.

Speakman, J. R. and Król, E. (2005b). Comparison of different approaches for the calculation of energy expenditure using doubly labeled water in a small mammal. *Physiol. Biochem. Zool.* **78**, 650-667.

Speakman, J. R. and Król, E. (2010). Maximal heat dissipation capacity and hyperthermia risk: neglected key factors in the ecology of endotherms. *J. Anim. Ecol.* **79**, 726-746.

Speakman, J. R. and Król, E. (2011). Limits to sustained energy intake. XIII. Recent progress and future perspectives. *J. Exp. Biol.* **214**, 230-241.

Speakman, J. R. and McQueenie, J. (1996). Limits to sustained metabolic rate: the link between food intake, basal metabolic rate, and morphology in reproducing mice, *Mus musculus*. *Physiol. Zool.* **69**, 746-769.

Speakman, J. R. and Racey, P. A. (1987). The equilibrium concentration of oxygen-18 in body water: implications for the accuracy of the doubly-labelled water technique and a potential new method of measuring RQ in free-living animals. *J. Theor. Biol.* **127**, 79-95.

Speakman, J. R., Nagy, K. A., Masman, D., Mook, W. G., Poppitt, S. D., Strathearn, G. E. and Racey, P. A. (1990). Interlaboratory comparison of different analytical techniques for the determination of oxygen-18 abundance. *Anal. Chem.* **62**, 703-708.

Thompson, S. D. and Nicoll, M. E. (1986). Basal metabolic rate and energetics of reproduction in therian mammals. *Nature* **321**, 690-693.

Trebaticka, L., Ketola, T., Klemme, I., Eccard, J. A. and Ylönen, H. (2007). Is reproduction really costly? Energy metabolism of bank vole (*Clethrionomys glareolus*) females through the reproductive cycle. *Ecoscience* **14**, 306-313.

Valencak, T. G. and Ruf, T. (2009). Energy turnover in European hares is centrally limited during early, but not during peak lactation. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **179**, 933-943.

Valencak, T. G., Hackländer, K. and Ruf, T. (2010). Peak energy turnover in lactating European hares: a test of the heat dissipation limitation hypothesis. *J. Exp. Biol.* **213**, 2832-2839.

Valencak, T. G., Wright, P., Weir, A., Mitchell, S. E., Vaanholt, L. M., Hambly, C., Król, E. and Speakman, J. R. (2013). Limits to sustained energy intake. XXI. Effect of exposing the mother, but not her pups, to a cold environment during lactation in mice. *J. Exp. Biol.* **216**, 4326-4333.

Weiner, J. (1987a). Limits to energy budget and tactics in energy investments during reproduction in the Djungarian hamster (*Phodopus sungorus sungorus* Pallas 1770). *Symp. Zool. Soc. Lond.* **57**, 167-187.

Weiner, J. (1987b). Maximum energy assimilation rates in the Djungarian hamster (*Phodopus sungorus*). *Oecologia* **72**, 297-302.

Weiner, J. (1992). Physiological limits to sustainable energy budgets in birds and mammals: ecological implications. *Trends Ecol. Evol.* **7**, 384-388.

Wu, S.-H., Zhang, L.-N., Speakman, J. R. and Wang, D.-H. (2009). Limits to sustained energy intake. XI. A test of the heat dissipation limitation hypothesis in lactating Brandt's voles (*Lasiopodomys brandtii*). *J. Exp. Biol.* **212**, 3455-3465.

Yang, D.-B., Li, L., Wang, L.-P., Chi, Q.-S., Hambly, C., Wang, D.-H. and Speakman, J. R. (2013). Limits to sustained energy intake. XIX. A test of the heat dissipation limitation hypothesis in Mongolian gerbils (*Meriones unguiculatus*). *J. Exp. Biol.* **216**, 3358-3368.

Zhang, X.-Y. and Wang, D.-H. (2007). Thermogenesis, food intake and serum leptin in cold-exposed lactating Brandt's voles *Lasiopodomys brandtii*. *J. Exp. Biol.* **210**, 512-521.

Zhao, Z.-J. (2011). Energy budget during lactation in striped hamsters at different ambient temperatures. *J. Exp. Biol.* **214**, 988-995.

Zhao, Z.-J. and Cao, J. (2009). Effect of fur removal on the thermal conductance and energy budget in lactating Swiss mice. *J. Exp. Biol.* **212**, 2541-2549.

Zhao, Z.-J., Chi, Q.-S. and Cao, J. (2010). Milk energy output during peak lactation in shaved Swiss mice. *Physiol. Behav.* **101**, 59-66.