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RESEARCH ARTICLE

Milk output and composition in mice divergently selected for basal metabolic rate

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ABSTRACT

From an evolutionary perspective, the high basal metabolic rate (BMR) of homeotherms is hypothesised to be a by-product of natural selection for effective parental care. We estimated daily milk output during two consecutive lactation bouts in mice divergently selected for high/low BMR and applied a cross-fostered design to control for potential differences in the between-line suckling abilities of nursed juveniles. Additionally, to remedy the potential limitation imposed by the ability of mother mice to dissipate excess heat, we exposed them to an ambient temperature of 17°C during the most energetically demanding second week of lactation. We found that the mice selected for high BMR produced significantly more milk in a 24 h period in both reproductive bouts. The milk samples obtained from the high BMR females had lower protein concentration and did not differ with respect to fat. However, the concentration of the primary milk carbohydrate – lactose – was higher. Although all the above between-line differences were statistically significant, their magnitude was too small to unambiguously ascribe them as stemming from a positive genetic correlation between the physiological traits underlying BMR and lactation performance. Nevertheless, our study lends such support at least at the level of phenotypic variation.

KEY WORDS: Milk output, Milk composition, Basal metabolic rate, Selection experiment, Endothermy evolution

INTRODUCTION

From a physiological perspective, milk production ability should be determined by (i) the capacity of the mother's alimentary tract for food intake and nutrient absorption, and/or (ii) the size and/or efficiency of the mammary glands (Hammond et al., 1996; Hurley, 2001). Elevated demands for energy acquisition and turnover during lactation are realised through the hypertrophy of key internal organs: small and large intestines, liver and kidneys (Hammond, 1997; Casirola and Ferraris, 2003). All of these processes, from food processing to milk synthesis, manifest themselves as a systematic elevation of metabolic rates in reproducing animals severalfold above what is most often quantified as the basal (measured at rest, in a post-absorptive state and within the thermoneutral zone, in non-reproducing individuals) or resting (measured at rest) metabolic rate (BMR or RMR). This phenomenon of lactation-induced elevation of metabolic rate in various mammal species has been well documented (Duarte et al., 2010) and can be regarded as the manifestation of the metabolic costs of lactation.

From a broader evolutionary perspective, an increase in BMR/RMR elicited by reproductive effort in mammals can be considered as a by-product of selection for intense parental care (Farmer, 2000; Koteja, 2000; Koteja, 2004). Parental care, especially in the form of providing offspring with high-quality food, such as milk in mammals, decreases mortality and improves offspring development (i.e. pup mass, litter size), and therefore should be favoured by natural selection. This scenario assumes that the higher levels of metabolism characteristic of reproduction are closely linked to the actual parental effort and the parents' direct investment in offspring production. This relationship, in the case of mammals, should result in a positive association between mothers' BMR and the quantity and/or quality of produced milk. To test this hypothesis, we compared the milk output and milk composition in mother mice from two lines divergently selected for high and low BMR. These lines are an exceptional model for testing evolutionary hypotheses on physiological traits related to reproduction because they conspicuously and consistently diverge in BMR and internal organ masses over the course of subsequent reproductive bouts (Sadowska et al., 2013).

In a previous study, we showed that females from the high BMR (H-BMR) line are capable of raising heavier offspring than mothers from the low BMR (L-BMR) line (Sadowska et al., 2013). Controlling for the effect of nursing mother type, we also found that pups originating from the high BMR line gained mass more rapidly during their first 2 weeks of life. In this study, we aimed to determine whether the observed between-line differences in growth rate and offspring quality are consistent with the corresponding between-line differences in milk composition and milk production ability. We expected that milk output and/or the concentration of major milk components – protein, fat and lactose – would be higher in the H-BMR line. We placed special emphasis on lactose, as it determines the volume of produced milk (Jensen, 1995; McSweeney and Fox, 2009). We measured milk output based on the female's water turnover and collected milk from mother mice from both lines nursing cross-fostered litters of mixed origin. By applying a cross-fostering design, we were able to control for the possible differences in the suckling abilities of the nursed pups and their stimulation of lactation (Hammond et al., 1996).

In our previous study, we found that the between-line differences in parental effort are most evident under the conditions resulting from energy demands that have been increased by the exposure of lactating mice to cold (Sadowska et al., 2013). For this reason, we also applied cold exposure in this experiment. Furthermore, because milk composition and production ability differ between consecutive lactations in mice (Zhao, 2011), we extended our study across two consecutive lactations.

RESULTS

BMR and morphometrics – background results

Body mass-corrected BMR measured prior to reproduction differed significantly between the low and high line in all generations used in this study ($F_{1,153}$=465.84; P<0.001; H-BMR: 62.7±1.2 ml O_2 h^{-1}

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Table 1. ANCOVA results and fresh carcass mass-corrected masses of visceral organs of females selected for H-BMR and L-BMR in their first and second lactation

	Line (1,56)	Lactation order (1,23)	Carcass mass (1,23)	Line×lactation order (1,23)	First lactation		Second lactation	
					H-BMR (g)	L-BMR (g)	H-BMR (g)	L-BMR (g)
Intestine	F=33.19	F=3.45	F<0.01	F=0.49	2.35±0.07	2.01±0.07	2.54±0.07	2.11±0.06
	P<0.001	P=0.076	P=0.968	P=0.491				
Liver	F=23.72	F=0.24	F=1.79	F=0.09	4.03±0.12	3.46±0.11	3.94±0.11	3.43±0.10
	P<0.001	P=0.630	P=0.194	P=0.773				
Kidneys	F=68.39	F=8.99	F=4.08	F=0.60	0.72±0.01	0.60±0.01	0.66±0.01	0.57±0.01
	P<0.001	P=0.006	P=0.055	P=0.447				
Heart	F=24.68	F=0.92	F=11.41	F=0.09	0.22±0.01	0.20±0.01	0.22±0.004	0.20±0.004
	P<0.001	P=0.348	P=0.003	P=0.771				
Mammary glands	F=0.18	F=9.00	F=4.25	F=1.16	4.71±0.23	4.57±0.21	5.20±0.21	5.50±0.19
	P=0.671	P=0.006	P=0.051	P=0.292				

H-BMR, high basal metabolic rate line type; L-BMR, low basal metabolic rate line type.
Degrees of freedom are presented in parentheses. Data are presented as adjusted means ± s.e.m.

and L-BMR: 45.9±0.9 ml O_2 h^{-1} in the 35th generation; H-BMR: 62.5±0.9 ml O_2 h^{-1} and L-BMR: 47.6±0.8 ml O_2 h^{-1} in the 37th generation; H-BMR: 64.0±0.8 ml O_2 h^{-1} and L-BMR: 45.1±0.8 ml O_2 h^{-1} in the 43rd generation). There was no effect of generation on BMR ($F_{2,153}$=0.33; P=0.722) and no line×generation interaction ($F_{2,152}$=2.57; P=0.080).

Body mass at peak lactation was not affected by the selection line of lactating mother ($F_{1,56}$=1.69; P=0.200); however, lactation order had a significant effect on body mass ($F_{1,24}$=37.59; P<0.001) (first lactation: H-BMR: 42.6±0.7 g; L-BMR: 42.1±0.7 g; second lactation: H-BMR: 47.0±0.7 g; L-BMR: 45.7±0.6 g). There was no line×lactation order interaction ($F_{1,24}$=0.39; P=0.540).

Carcass mass-corrected mass of intestines, liver, kidneys and heart was affected by the line affiliation in animals from both lactations, with all organs found to be significantly heavier in H-BMR females (Table 1). The order of lactation only affected kidney mass, as the first-lactation females had heavier organs (Table 1). Mammary gland mass remained unaffected by the line affiliation. However, in comparison with the first reproductive bout, we found significantly higher mammary gland masses in the second lactation (Table 1).

Milk output

Water budget estimated for validation of the method in non-lactating females showed that the total water influx and efflux balanced out at the average value of −0.412±0.52 g per 24 h ($F_{1,5}$=0.21, P=0.65), with influx/efflux significantly higher in the H-BMR mice ($F_{1,5}$=13.2; P=0.004).

Milk output estimated on the basis of water budget differed significantly between the two lines, with higher milk production in the H-BMR females (Fig. 1; $F_{1,32}$=5.10; P=0.031) in both consecutive lactation bouts (Fig. 1; $F_{1,10}$=16.76; P=0.002; first lactation H-BMR: 17.66±2.84 g per 24 h; L-BMR: 10.76±2.47 g per 24 h; second lactation H-BMR: 25.08±1.87 g per 24 h; L-BMR: 21.19±1.60 g per 24 h). The pre-reproductive body mass significantly affected milk output ($F_{1,10}$=8.33; P=0.020). The line×lactation order interaction was not significant ($F_{1,10}$=0.47; P=0.508).

In both lactations the differences in milk output did not exceed those expected to arise from genetic drift alone (the difference (d) expressed as a multiple of phenotypic SD, first lactation: d=1.21 was lower than that expected under genetic drift (d_{drift})=1.44; second lactation: d=0.59 versus d_{drift}=1.14). Thus even though the differences were statistically significant, they cannot be unequivocally interpreted as a manifestation of genetic correlation between milk output and BMR.

The between-line differences in daily milk energy output were not statistically significant ($F_{1,24}$=1.18; P=0.287), with higher output in the second lactation ($F_{1,6}$=13.58; P=0.010; first lactation: H-BMR: 208.28±45.85 kJ per 24 h, L-BMR: 165.21±39.36 kJ per 24 h; second lactation H-BMR: 351.97±32.56 kJ per 24 h, L-BMR: 301.10±38.09 kJ per 24 h). The line×lactation interaction was not significant ($F_{1,6}$=0.01; P=0.921) as well as the pre-reproductive body mass as a covariate ($F_{1,6}$=2.35; P=0.176).

Milk composition and sample composition consistency

The composition analysis of milk samples revealed that protein and lactose content were significantly affected by the selection line, with higher lactose and lower protein content in the H-BMR milk (Table 2). Fat content did not differ between the two lines, as well as the dry mass of milk (Table 2). Lactation order affected the content of lactose and the amount of dry mass of collected milk, which were higher in the milk samples collected in the second lactation (but not protein and fat content; Table 2). Milking order, however, significantly affected all of the studied components (Table 2) with their concentration fluctuating between the two milk evacuations.

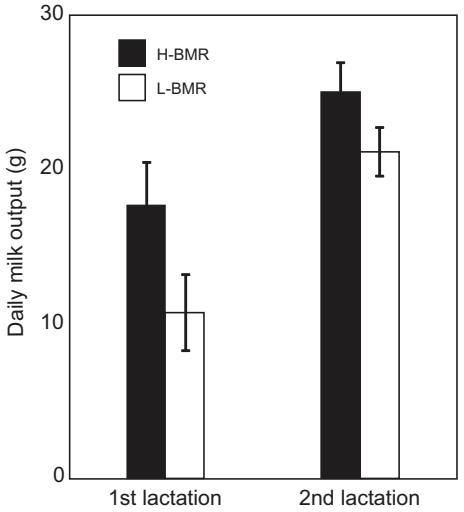

Fig. 1. Daily milk output in H-BMR and L-BMR mice in the two consecutive lactations.

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Table 2. ANOVA results on milk composition and concentration of milk components in all samples collected from H-BMR and L-BMR females in the first and second lactation

					Concentration of milk components (mg g^{-1})				
						First lactation		Second lactation	
	Line	Lactation order	Milk collection order	Line×lactation order×milk collection order	Sample no.	H-BMR	L-BMR	H-BMR	L-BMR
Protein	$F_{1,35}$=6.49	$F_{1,4}$=1.73	$F_{1,34}$=8.40	$F_{4,1}$=1.16	1	110.10±8.17	119.50±8.17	111.20±5.96	121.10±6.97
	P=0.015	P=0.258	P<0.006	P=0.593	2	124.10±8.17	152.90±8.73	120.20±5.96	126.20±7.31
Fat	$F_{1,37}$=3.04	$F_{1,8}$=0.98	$F_{1,36}$=4.10	$F_{4,5}$=0.60	1	304.50±18.14	313.8±19.12	279.10±14.81	292.9±15.33
	P=0.089	P=0.350	P=0.050	P=0.679	2	249.60±18.14	297.3±19.12	265.9±14.81	279.5±16.56
Lactose	$F_{1,37}$=4.76	$F_{1,8}$=13.08	$F_{1,35}$=22.51	$F_{4,3}$=0.63	1	19.94±1.30	15.71±1.37	15.51±1.06	13.26±1.10
	P=0.035	P<0.006	P<0.001	P=0.677	2	13.56±1.37	13.19±1.56	10.83±1.06	9.85±1.19
Dry mass	$F_{1,38}$=0.83	$F_{1,8}$=64.58	$F_{1,37}$=3.70	$F_{4,5}$=2.82	1	382.90±23.18	442.50±23.18	574.40±18.93	527.20±19.59
	P=0.367	P<0.001	P=0.062	P=0.143	2	367.00±23.18	430.8±24.44	516.20±18.93	495.70±20.33

Data are presented as means ± s.e.m.

DISCUSSION

In this study we demonstrated that mice with genetically determined high BMR are characterized by higher milk output at peak lactation than females selected for low rates of basal metabolism. However, despite their statistical significance, the magnitude of the observed between-line differences was too small to unequivocally interpret them as a manifestation of a positive, genetic correlation between milk output and BMR. Nonetheless, at the phenotypic level, our findings are consistent with the existence of a positive association between the maternal basal rate of metabolism and her actual abilities of investment in the offspring production, and provide support for the assimilation capacity model for endothermy evolution (Koteja, 2000).

The between-line differences in milk production were more distinct in the second reproductive bout, which concurs with several studies showing that the later lactation is more effective in terms of milk output as well as offspring growth (Fischbeck and Rasmussen, 1987; Rasmussen and Fischbeck, 1987; Casirola and Ferraris, 2003; Zhao, 2011). Higher milk production of the H-BMR mothers was not caused by higher demands of the nursed pups, as the applied cross-fostered protocol eliminated such potential bias, by providing mothers with equal numbers of the H-BMR and L-BMR juveniles. Additionally, we alleviated the potential limitation imposed by the ability of lactating mother mice to dissipate excess heat (Król and Speakman, 2003a; Król and Speakman, 2003b; Król et al., 2007; Speakman and Król, 2010; Speakman and Król, 2011) by exposing them to an ambient temperature of 17°C during the most energetically demanding second week of lactation. Thus we can safely assume that at the phenotypic level our results are not influenced by confounding factors related to the pup quality or uncontrolled physiological and/or physical constraints.

The maximal daily milk output of our selected mice ranged from 11 to 18 g, which is comparable with the daily milk production of 8–20 g estimated by means of the doubly labelled water (DLW) technique in an unselected MF1 mouse strain (Król and Speakman, 2003a; Król and Speakman, 2003b). However, Riley et al. (Riley et al., 2006) reported much lower daily milk yield (weight-suckle-weight method) at the fourteenth day of lactation of ~3 g per day in the CBA mouse strain and 8 g in the highly fecund QSi5 mouse strain. Jara-Almonte and White (Jara-Almonte and White, 1972) found an even lower milk output (also by the weight-suckle-weight method), of ~3 g on the fourteenth day of lactation in the imprinting control region (ICR) mouse strain nursing a litter of eight pups – a litter size we used in our study. Some variation in milk output may of course stem from differences between used strains of animals, but the precision of the applied measurement technique is most likely to be decisive. Our results are in good agreement with those reported by Król and Speakman (Król and Speakman, 2003a; Król and Speakman, 2003b), who applied by far the most accurate method available, the DLW technique.

The higher concentration of lactose in the H-BMR mouse milk appears to be the essential factor underpinning differences between the lines in the quantity of milk as well as the difference in effectiveness between the two reproductive events. Lactose is not only a prime energy source for the suckling offspring, but also the key component determining the amount of secreted milk. Due to its high osmotic load, lactose draws water into the Golgi vesicles of mammary epithelial cells, thus affecting the volume of milk produced (Jensen, 1995; McSweeney and Fox, 2009; Zhao et al., 2012). The question arising from this finding is: what, exactly, enables H-BMR mothers to synthesise more lactose and, consequently, produce more milk? Also, why did our animals perform better, i.e. show higher milk production ability, in the second reproductive bout?

From a physiological perspective, the ability of milk production should be determined either by the capacity of the alimentary tract for food turnover and nutrient absorption and/or the ability of mammary tissue to secrete milk. Measuring the size/weight of the internal organs involved in the lactation process has been considered a simple, yet effective proxy for estimating the functional capacity of these organs in a range of species (Hurley, 2001; Karasov and McWilliams, 2005; Bauchinger et al., 2009). Our selection lines differ not only in their BMR but also in the mass of viscera essential for nutrient and energy turnover (intestines, liver, kidneys and heart); all are significantly heavier in non-reproducing H-BMR mice (Książek et al., 2004; Książek et al., 2009; Brzęk et al., 2007; Gębczyński and Konarzewski, 2009a). In this study, we showed that differences in organ masses are preserved after the first as well as the second reproductive bout, with H-BMR females having heavier viscera (particularly intestines and liver; Table 1). Additionally, as we have demonstrated elsewhere, lactating H-BMR females are characterised by higher rates of food consumption than L-BMR females (Sadowska et al., 2013). Higher nutrient assimilation abilities, supported by a larger gut size, enabled the H-BMR females to achieve overall a higher nutrient and energy transfer to their pups.

Because most milk components, including the water-drawing lactose (Jensen, 1995; McSweeney and Fox, 2009), are synthesised in the mammary epithelial cells from substrates transported by the blood, it may be reasonable to assume that mammary gland size is of equal importance in determining the ability of the animal to produce milk, as is the size of the alimentary tract. In fact, post-partum mammary gland growth occurs throughout lactation until its

The Journal of Experimental Biology (2015) doi:10.1242/jeb.111245

Table 3. Mass of milk obtained the two consecutive milk evacuations from the females selected for H-BMR and L-BMR in their first and second lactation

	Mass of milk obtained per milking (mg)			
	First lactation		Second lactation	
Milking order	H-BMR	L-BMR	H-BMR	L-BMR
First	841.70±130.86	745.80±130.86	984.27±106.84	893.43±110.59
Second	939.10±130.86	653.90±130.86	969.80±106.84	738.57±110.59

Data are presented as least square means ± s.e.m.

peak is reached to meet the energy requirements of a growing litter (Hurley, 2001). In view of the greater parental investment of H-BMR mothers (measured as pup growth rate) (Sadowska et al., 2013), higher milk production ability and the persistent differences in visceral mass, one would also expect significant differences in mammary tissue mass. It has been demonstrated a number of times that the larger the litter's energy demands get, the higher the suckling pressure and as a result, the higher the rate of milk flow and greater the mass of mammary tissue (Hammond et al., 1996; Zhao, 2012). In our case the higher milk output of H-BMR animals was not accompanied by an increase in mammary gland mass (Table 1; the glands did not differ between the lines) and could not have been caused by a greater suckling pressure due to the cross-fostered protocol, as we explained earlier.

These results would imply energy acquisition capacity as the underlying cause of higher lactation performance (Bacigalupe and Bozinovic, 2002; Riley et al., 2006), at least in the case of the first lactation. In contrast, we found higher mammary tissue mass in both lines in the second reproductive bout, more effective in terms of milk yield (Tables 1, 3). Such increase of reproductive effectiveness (i.e. offspring growth, litter size, milk yield) in subsequent reproductive events is common in rodents (Rasmussen and Fischbeck, 1987; Zhao, 2011) and has been attributed to the increase in intestine size and to the incomplete involution of mammary tissue after the previous reproductive event (Casirola and Ferraris, 2003). In our case the higher milk yield and mammary mass of the second lactation was associated with a decreased lactose content in comparison with the first reproductive bout in both lines. As lactose synthesis is strongly dependent on the immediate blood glucose supply (Arthur et al., 1994; Bussmann et al., 1984; Rigout et al., 2002; Zhao et al., 2012), the higher gut size and thus acquisition abilities in the second lactation should enable higher lactose production than during the first lactation. This was, however, not the case, and we can hypothesise that mammary tissue limits lactation output in the consecutive reproductive bout, possibly by means of glucose transport effectiveness through the gland cells. Nonetheless, to pinpoint with absolute certainty the lactation limiting tissues/organs we would need to place a much higher burden (e.g. larger litters) on the females to ensure that they are operating under maximum energy demands, which was not the focus of this study.

In conclusion, the results of this study demonstrated proximate factors underlying our earlier findings on the correlation between parental care abilities and BMR in mice. We showed that the H-BMR mothers achieve their higher parental outcome primarily by producing more milk of higher instantaneous lactose (but lower protein) content than that of L-BMR females. Higher milk output ability appears to result from the greater capacity for food intake and nutrient absorption of the mice with genetically determined high levels of BMR. It is also important, as we demonstrated in the multiparous animals, that the divergence between lines in viscera mass is retained after reproduction, whereas in non-manipulated populations, BMR (RMR) repeatability is lost after a reproductive bout (Duarte et al., 2010). Together, these findings provide support for the mechanism behind the assimilation capacity hypothesis for endothermy evolution in mammals (Koteja, 2000; Koteja, 2004) and highlight the significance and potential of long-term selection experiments in testing evolutionary scenarios. In contrast to an approach involving the examination only of phenotypic correlations, which may differ not only in strength but even in sign from genetic correlations (Roff, 2002; Sadowska et al., 2005), the use of animals whose divergence in the selected trait (BMR) is maintained over subsequent reproductive bouts allows for credible evolutionary inferences (Garland and Rose, 2009).

MATERIALS AND METHODS

Animals and their maintenance during experiments

This study was approved by the Local Ethical Committee on Testing Animals at the Medical University of Białystok (permit no. 45/2011, 46/2011, 53/2013). We used outbred Swiss Webster mice from generation 43 of a long-term selection experiment carried out in the Institute of Biology, University of Bialystok. The selection is designed to generate two lines of animals with divergent levels of BMR. In each generation, we maintained 15–17 families per line, depending on the current level of reproductive success. Briefly, three randomly chosen males and females from each family were subjected to BMR measurements at the age of 12 weeks. BMR was measured after 4 h fasting in an open respirometry system (S-3A/II Applied Electrochemistry, Pittsburgh, PA, USA), during the final 2 h of a 3 h trial at 32°C, a temperature falling within the thermoneutral zone of our mice. The lowest oxygen concentration that did not change by more than 0.01% for at least 4 min was defined as BMR. No more than three individuals per family with the highest (H-BMR line) and lowest (L-BMR line) body-mass corrected BMR were chosen as progenitors for further selection and mated outside their families (for details, see Książek et al., 2004; Książek et al., 2009; Gębczyński and Konarzewski, 2009b).

For the present study 54 females (24 H-BMR and 30 L-BMR) were paired outside their families and placed together with males in plastic cages for a 2 week period. When pregnancy was detectable by an increase in body mass the males were removed. After weaning the first experimental litter, ~5 weeks after the first reproductive bout, animals were bred for the second time and the same experimental protocol was repeated for the second lactation.

In both lactation bouts, we applied the same procedure as follows: 2 days after birth, we cross-fostered the young between cages, so that each foster mother received four pups from the H-BMR and four from the L-BMR line (i.e. a total of eight pups, none of which was the mother's own). Cross-fostering allowed us to discriminate statistically between the effect of the mother's quality of parental care and the effect of line-specific differences in the growth rate of offspring, as reported by Sadowska et al. (Sadowska et al., 2013). Our experiment lasted until the fourteenth day of life for the pups, including the period when the offspring rely solely on maternal milk (Hammond et al., 1996). For the first week of lactation (days 2–8), all families were kept at an ambient temperature (T_a) of 23°C. For the second week (days 8–14, when the pups grew fur), the families were exposed to a T_a of 17°C. This approach enabled us to increase the mother's energy demands while alleviating potential heat stress to mothers and without

The Journal of Experimental Biology (2015) doi:10.1242/jeb.111245

risking increased offspring mortality due to hypothermia (Johnson and Speakman, 2001; Sadowska et al., 2013).

Evaluation of daily milk production

At peak lactation (for days 12–14) we measured the milk output based on the lactating female's water budget. For that we measured the amount of drinking water as the weight of water disappearing from the pre-weight water bottle in a 2 day period. Metabolic rate (MR), evaporative water loss (EWL) and urinary water loss were measured on day 13 using an open flow respirometry system during a 2 h measurement period (MR measured as oxygen consumption for 2 h in non-fasted animals at 17°C and extrapolated for 24 h; EWL measured as the weight of water vapour captured by Drierite desiccant from the air stream pushed out from the metabolic chamber with the animal). Urine was collected at the bottom of the metabolic chamber during the 2 h MR measurement (successful measurements were performed for 12 individuals in the first lactation, and 14 individuals in the second lactation). Animals were placed in the metabolic chamber equipped with an elevated mesh floor, the bottom of the chamber was coated with a thin layer of mineral oil under which urine was trapped, then collected and weighed at the end of the measurement. Based on the collected measurements, we calculated the percentage of total water income that is lost with urine (39%) and used it for the water budget calculations. To estimate the amount of water consumed with food and lost with faeces we also measured food consumption for which we provided each lactating mouse with an excessive, known amount of food (murine laboratory chow, Labofeed H, Kcynia, Poland). After 2 days we collected the food remains and faeces from the bottom of the cage, then dried them and weighed to the nearest 0.01 g. For precise determination of water content in faeces we collected freshly excreted faeces samples to a pre-weight test tube, weighed the sample, then dried it to a constant mass at 60°C and weighed it again. We calculated the food consumption for each adult mouse during two consecutive days as the mass of food disappearing from the food dispenser minus food remains. Water content of the murine chow was also determined by drying pre-weighed food samples to a constant mass at 60°C. To estimate metabolic water gain of each individual animal we used the MR measured at day 13 of the experiment and extrapolated the volume of oxygen consumed during the 2 h of MR measurement to a 24 h period. We assumed that metabolism produces 1.1 g and 0.55 g of water per 1 g of metabolised fat and carbohydrates, respectively (Schmidt-Nielsen, 1964). They constituted the bulk of the murine chow we used, with carbohydrate/fat ratio of 7 to 1. As oxidation of 1 g of fat and carbohydrate requires 1.9 g and 1.07 g of oxygen, respectively, each 1 g of metabolised food required 1.17 g (or 819 ml) of oxygen and generated 1.79 g of water. Thus 1 l of consumed oxygen yielded 2.19 g of water, which was the value we used to estimate individual metabolic water production.

The milk output for each animal was calculated for a 24 h period as follows: first we calculated the water budget as the sum of all water income (drinking water + metabolic water + water gained with eaten food) minus the water lost through evaporation, and water lost with faeces and urine. The obtained value was then divided by water content of milk (mean of water content from the morning and afternoon samples weighed by sample masses). Using the same protocol we also estimated the water budget for eight non-lactating control animals of the same age and generation to validate our method of choice for measuring milk output.

We calculated energy content of milk by multiplying fat, protein and sugar concentration in milk samples by 38.12, 24.52 and 16.53 kJ g^{-1}, respectively (from Król and Speakman, 2003b) and further we multiplied the daily milk production by the energy content of milk samples to obtain the daily milk energy output.

Milking procedure

The milking procedure was performed twice on the fourteenth day of lactation, first in the morning (3 h after separation from the litter) and second in the afternoon (~8–9 h after the first milk collection; litters were not returned to their mothers in between milk collections). Directly before milking, the animals were anaesthetised subcutaneously with ketamine (100 mg kg^{-1}) and xylazine (20 mg kg^{-1}). After ~5–10 min, the mice received a subcutaneous dose of oxytocin (1 i.u.), which induced milk flow. Immediately after oxytocin injection, milk was collected with a modified laboratory precision liquid dispenser pump (Unipan 336B, Poland) from each of the ten teats. The pump was fitted with a custom-made ending shaped to snugly fit the teat. Milk let-down was induced by a sucking force (same for all animals) determined in a pilot trial and continued until no more milk could be obtained. Milk was then frozen at −20°C for further analyses.

Milk analyses

Fat content (80 µl samples of milk) was determined by the Rose–Gottlieb gravimetric method (Kirk and Sawyer, 1991). Lactose content (20 µl samples of milk) was estimated by the Somogyi–Nelson method, used for the determination of reducing sugar content (Sadasivam and Manickam, 1996). Crude protein content (20 µl samples of milk) was determined by the total nitrogen test (Merck Nitrogen test, 1.14537.0001) and calculated as 6.36×nitrogen content. We did not make any correction for non-protein nitrogen content. Dry mass of milk was determined by drying milk samples (20 µl) in a convection oven at a temperature of 60°C for 24 h.

Morphometrics

For comparative purposes we used mice from generation 35 (47 females after two reproductive bouts, 21 H-BMR and 26 L-BMR) and generation 37 (37 females after a single lactation, 18 H-BMR and 19 L-BMR) that underwent the same experimental protocol as described above. After the final milk evacuation (day 14) mice were killed by cervical dislocation, and the metabolically active organs (liver, kidneys, intestines, heart and mammary glands) were dissected, cleaned of blood and food remains, and weighed to the nearest 0.001 g. As the results on the primary selected trait showed no differences between the used generations, the traits correlated with BMR (organ size, parental investment) should be comparable across generations.

Statistics

BMR and fresh masses of female visceral organs were analysed with an ANCOVA with generation and selection line as fixed factors, family nested within line as a random factor and body mass as a covariate. Body mass was analysed with an ANOVA, with line and generation as fixed factors, and family affiliation nested within the line as a random factor.

Milk output differences measured based on the water budget were analysed with repeated measures ANCOVA with selection line and lactation order as fixed factors, and the pre-reproductive body mass as a covariate. The pre-reproductive body mass was used instead of the momentary body mass because at peak lactation it is strongly correlated with milk production ability, and therefore should not be used as a covariate. As the animals used for the water budget estimation came from different families, the family affiliation (as a random factor) was dropped from the model. Milk sample composition consistency over the course of the day in both lactations was analysed with a repeated measures ANCOVA with line, lactation order and milking order as fixed factors. Water budget of the non-lactating animals was analysed with ANOVA with the flux (two levels: influx and efflux) and line as the fixed factors, and an individual mouse nested within the line.

Because our selection lines are not replicated, we considered the possibility that the between-line differences in milk output might be due to genetic drift rather than a genuine effect of selection. Therefore, we additionally analysed milk output according to Henderson's guidelines (Henderson, 1997; Konarzewski et al., 2005). Briefly, we expressed the magnitude of separation between the high and low lines for the milk output as the difference between the within-line mean trait values divided by the weighted phenotypic s.d. (d) (for details, see Gębczyński and Konarzewski, 2009; Sadowska et al., 2013). To estimate the confidence intervals (denoted d_{drift}) for d, we used a modified equation 16 from Henderson (Henderson, 1997):

$$d_{\text{drift}} \cong 4\sqrt{(h_X^2 F + 1/n)}\,, \quad (1)$$

where h^2 is the narrow sense heritability for milk output (0.17) (Jara-Almonte and White, 1973) and F is the inbreeding coefficient [F=0.29, calculated from equation 3.5 from Falconer and Mackay (Falconer and Mackay, 1996) for the effective population size of generation 43].

The assumptions of parametric tests were verified before data computation (Sokal and Rohlf, 1995). All statistical analyses were performed with SAS software (SAS Institute 1990, Cary, NC, USA).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
J.S. and A.K.G. designed the experiment, J.S., A.K. and conducted the experiment and K.P. assisted. J.S., A.K.G. and M.K. analysed the data; J.S. wrote the paper. J.S., A.K.G and M.K. contributed to data interpretation and revision of the manuscript.

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