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RESEARCH ARTICLE

Negative correlation between milk production and brown adipose tissue gene expression in lactating mice

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SUMMARY

It has been proposed that the performance of lactating animals is limited by the capacity of the female to dissipate body heat – the heat dissipation limit (HDL) theory. This theory predicts that milk production might be constrained not by intrinsic properties of the mammary glands but rather by competitive heat production such as thermogenesis in brown adipose tissue (BAT). To test this prediction, we measured the expression of genes linked to thermogenesis in BAT of lactating laboratory mice. The applicability of BAT gene expression to reflect thermogenic activity of BAT was confirmed by a positive relationship between expression levels of several BAT genes (summarised by the first principal component following principal component analysis) and daily energy expenditure in virgin mice. Milk production at peak lactation was strongly and negatively associated with the expression of thermogenic genes in BAT. Downregulation of these genes during lactation was correlated with low levels of circulating leptin and high levels of circulating prolactin. Our results are consistent with the HDL theory. However, we cannot discount the converse interpretation that milk production may reduce BAT activity. If the reduction in BAT activity does facilitate increased milk production, then reducing the heat generated by competitive processes may be a more productive route to increase lactational performance than attempts to improve mammary gland performance in isolation from the other body systems.

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Key words: heat dissipation limit, lactation, leptin, prolactin.

INTRODUCTION

Lactation is the most energy-demanding process for small mammals (Loudon and Racey, 1987; Speakman, 2008). Food consumption increases rapidly in early lactation to two to four times the level of non-breeding individuals, but then reaches a plateau despite the continued rise in energy requirements of the offspring (e.g. Hammond and Diamond, 1992; Król and Speakman, 2003a; Laurien-Kehnen and Trillmich, 2003). The nature of the limits to milk production is central to understanding many aspects of animal performance, and has been a subject of debate for at least 20 years (reviewed in Speakman and Król, 2005b; Speakman and Król, 2011). Recent attempts to understand how milk production is regulated have focused on intrinsic properties of the mammary glands, using gene expression microarray analyses (Rudolph et al., 2007; Bionaz and Loor, 2008; Maningat et al., 2009). Comparison of the transcriptional data from the mammary glands of model species (e.g. mouse, rat, sheep and cow) has been considered effective for identifying key candidate genes that signify unique and conserved molecular processes important for milk synthesis and secretion (International Milk Genomics Consortium, www.milkgenomics.org). However, the downside of such an approach is that by considering mammary gland performance in isolation from the other body systems, the contribution of organs and tissues that impact milk production indirectly is neglected.

Milk production generates substantial amounts of metabolic heat. This 'lactogenic' heat originates from three main sources (Blaxter, 1989). Some of the excess heat is associated with the enlarged digestive system, which is necessary to process the larger quantities of ingested food (Speakman, 2008) and which has a high mass-specific metabolic rate (Cant et al., 1996). Another important process contributing to lactogenic heat production is the heat increment of feeding, averaging 10% of gross energy intake (Secor, 2009). Finally, some of the lactogenic heat reflects the metabolic costs of maintaining the mammary glands and synthesizing milk (Prentice and Prentice, 1988). These three processes contribute to substantial inefficiencies in converting food to milk, resulting in 24–43% of metabolizable energy intake during lactation being released as heat (Prentice and Prentice, 1988; Blaxter, 1989; Król et al., 2007).

If increased heat production during lactation is not balanced by heat loss, then lactating females are likely to face potentially fatal hyperthermia. Sustained increases in maternal body temperature by 0.5–1.5°C above the non-breeding levels have been reported in several species of lactating rodents and also in large domestic animals (Speakman, 2008). Even such non-fatal elevations in body temperature can have negative consequences related to the disruption of membrane fluidity and protein structure as well as electrolyte and fluid loss, which can subsequently lead to malfunctions and perturbations in physiological homeostasis (Hansen, 2009).

We have previously postulated that lactating animals are limited in their performance by the capacity of the female to

dissipate body heat – the heat dissipation limit (HDL) theory (Król and Speakman, 2003a; Król and Speakman, 2003b; Król et al., 2007; Speakman and Król, 2010). If this theory is correct, then increases in milk production and the associated lactogenic heat would require decreases in the heat generated by competitive processes such as thermogenesis in brown adipose tissue (BAT) (Cannon and Nedergaard, 2004). During lactation, BAT undergoes morphological, physiological and biochemical changes that appear to be consistent with the HDL theory. These changes include tissue hypotrophy, lowered mitochondrial content, decreased activity of cytochrome oxidase, reduced gene expression of uncoupling protein 1 (UCP1), lower protein levels of UCP1, diminished sympathetic activity measured as noradrenaline turnover, and reduced rates of mitochondrial respiration (Trayhurn et al., 1982; Isler et al., 1984; Trayhurn and Wusteman, 1987; Martin et al., 1995; Pedraza et al., 2001; Cannon and Nedergaard, 2004; Xiao et al., 2004). As a result, the capacity for thermogenesis in BAT of lactating females is greatly reduced (Trayhurn, 1983; Isler et al., 1984). However, the majority of these findings are based on group comparisons of lactating and non-breeding animals, and therefore do not provide evidence for potential competition between lactogenic and non-lactogenic heat production at the level of the individual. An example of the problem of extrapolating from the group level to the individual level is provided by the changes in morphology and resting metabolic rate (RMR). When group comparisons are made between lactating and virgin mice, there are large increases in the sizes of the alimentary tract and associated organs such as the liver that parallel the elevated RMR during lactation (Johnson et al., 2001). However, when comparisons are made between lactating individuals, there is no significant association between the individual variation in gut morphology and individual variation in RMR (Speakman et al., 2004).

Here we present data on milk production at peak lactation and its relationship to gene expression linked to BAT thermogenesis (Table 1) in individual lactating laboratory mice. We also measure circulating levels of leptin and prolactin, both of which have received considerable attention as endocrine mediators that may coordinate the lactational changes in BAT activity and food intake to facilitate milk production (Cannon and Nedergaard, 2004; Crowley et al., 2004; Naef and Woodside, 2007; Woodside, 2007; Smith et al., 2010).

MATERIALS AND METHODS

Animals and experimental protocol

We used 63 virgin female mice (*Mus musculus* L., outbred MF1) exposed to a 12 h:12 h light:dark cycle (lights on 07:00 h) at an ambient temperature of 21°C. Standard rodent chow (CRM, Pelleted Rat and Mouse Breeder and Grower Diet, Special Diets Services, BP Nutrition, Witham, UK) and water were available *ad libitum*. At the age of 10–12 weeks, 47 randomly selected females were paired with males for 11 days. The remaining 16 females went through the protocol as non-breeding individuals. Pregnant mice were checked regularly to determine the day of parturition (day 0 of lactation). Body mass, food intake, litter size and litter mass were recorded from day 4 to the end of lactation (day 18). Milk production was evaluated at peak lactation from measurements of metabolizable energy intake (MEI) and daily energy expenditure (DEE) (details below). On day 18 of lactation, all adult females were killed by CO_2 inhalation between 12:00 and 14:00 h. Blood samples were obtained by cardiac puncture, clotted on ice and centrifuged. Serum was stored at –80°C until assayed. Immediately after cardiac puncture, interscapular BAT depots were removed, frozen in liquid N_2 and stored at –80°C for later analysis. All procedures were authorized by the College of Life Sciences and Medicine Ethics Review Board at the University of Aberdeen, and carried out under UK Home Office project licence PPL 60/2881.

MEI

Measurements of MEI were made on days 12–14 of lactation. Females and their litters were placed in cages with fresh sawdust, and a weighed portion of food was added to the hopper on day 12 of lactation. Samples of the food were taken to determine dry mass content, and the food remaining in the hopper was reweighed on day 14 of lactation. Any uneaten food and faeces were removed from the cage, dried to a constant mass and weighed. Simultaneous measurements of MEI were also performed on virgin females. The gross energy content of food and faeces were measured with a Parr 6200 calorimeter using an 1109A semi-micro oxygen bomb (Parr Instrument Company, Moline, IL, USA). All intermediate results used to calculate MEI are shown in Table 2.

DEE

We measured DEE using the doubly labelled water (DLW) technique (Speakman, 1997) on days 15–17 of lactation. Animals

Table 1. QuantiTect primer assays and efficiency (mean ± s.d.) of real-time PCR reactions used to evaluate transcript levels of genes expressed in brown adipose tissue (BAT) of female mice (*N*=63)

Genes in BAT grouped by function	Assay	Efficiency[a]
Thermogenesis		
Mitochondrial uncoupling and respiration		
Uncoupling protein 1 (UCP1)	QT00097300	1.73±0.04
Uncoupling protein 3 (UCP3)	QT00115339	1.75±0.07
Cytochrome *c* oxidase subunit IV isoform 2 (Cox4i2)	QT00137844	1.69±0.04
Lipid droplet mobilization		
Patatin-like phospholipase domain containing 2 (Pnpla2)	QT00111846	1.77±0.05
Fatty acid transport		
Fatty acid binding protein 3 (muscle and heart) (Fabp3)	QT00106946	1.81±0.09
Carnitine palmitoyltransferase 1b (muscle) (Cpt1b)	QT00172564	1.65±0.05
Regulation of transcription		
Peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α)	QT00156303	1.77±0.08
Housekeeping (cellular structure)		
β-actin	QT01136772	1.70±0.04
Ribosomal protein L13a (Rpl13a)	QT00156303	1.89±0.07

[a]For details, see Materials and methods.

Table 2. Results of the measurements performed on virgin female (*N*=16) and lactating (*N*=47) mice

Trait	Virgin mice	Lactating mice
Body mass (g)[a]	32±2	50±4
Metabolizable energy intake (MEI)[b]		
Dry food consumption (g day^{-1})[c]	5.1±0.7	20.1±2.6
Dry faecal production (g day^{-1})	1.2±0.2	4.3±0.6
Faecal energy content (kJ g^{-1} dry mass)	16.8±0.2	17.0±0.2
MEI (kJ day^{-1})[d]	71±9	280±37
Daily energy expenditure (DEE)[e]		
k_D (h^{-1})[f]	0.012±0.002	0.038±0.006
k_O (h^{-1})[f]	0.022±0.003	0.050±0.007
k_O/k_D	1.82±0.13	1.31±0.05
N_D (% of body mass)[g]	71±8	74±3
N_O (% of body mass)[g]	65±7	68±3
N_D/N_O	1.10±0.03	1.09±0.02
DEE (kJ day^{-1})[h]	70±11	128±15
Reproductive performance		
Litter size[i]	–	11.4±1.2
Litter mass (g)[i]	–	84±18
Milk production (kJ day^{-1})[j]	–	153±33
BAT gene expression[k]		
β-actin (housekeeping gene)[l]	0.24±0.06	0.26±0.05
Rpl13a (housekeeping gene)[l]	0.06±0.02	0.06±0.02
gm_{HK}[m]	0.11±0.03	0.12±0.03
UCP1[n]	33.62±8.42	11.32±10.95
UCP3[n]	0.50±0.17	0.29±0.16
Cox4i2[n]	0.16±0.04	0.09±0.04
Pnpla2[n]	7.68±2.11	4.31±2.06
Fabp3[n]	1.37±0.46	2.61±1.72
Cpt1b[n]	7.71±1.73	4.48±2.54
PGC-1α[n]	0.12±0.03	0.07±0.04
Circulating hormone levels[i]		
Leptin (ng ml^{-1})	8.2±6.9	3.9±4.2
Prolactin (ng ml^{-1})	7.8±6.2	23.0±26.1

Mice from both groups were measured on the same days, counted from the day of parturition (day 0 of lactation). Data are reported as means ± s.d.

[a]Measured on day 15 of lactation before injection of doubly labelled water.

[b]Measured on days 12–14 of lactation.

[c]Calculated as mass of food removed from hopper × dry mass content of food (90.7±0.1%; *N*=10) – dry mass of uneaten food from the cage floor.

[d]Estimated as the difference between energy consumed and defecated, assuming that urinary energy loss was 3% of the digestible energy intake (Drożdż, 1975); energy consumed was calculated from the dry food consumption × food energy content (18.0±0.2 kJ g^{-1} dry mass; *N*=3); the energy lost through defecation was calculated from dry faecal production × faecal energy content.

[e]Measured on days 15–17 of lactation.

[f]Elimination rates of ^{2}H (k_D) and ^{18}O (k_O) were calculated following Lifson and colleagues (Lifson et al., 1955).

[g]Dilution spaces of deuterium (N_D) and oxygen (N_O) were calculated by the intercept method (Coward and Prentice, 1985) and expressed as % of body mass before injection.

[h]Calculated using the single-pool model equation 7.17 (Speakman, 1997), as recommended for animals weighing under 1 kg (Speakman and Król, 2005a); energy equivalents of the rate of CO_2 production were calculated using a conversion factor of 24.026 J ml^{-1} CO_2, derived from the Weir equation (Weir, 1949) for a respiratory quotient of 0.85 (Speakman, 1997).

[i]Measured on day 18 of lactation.

[j]Calculated as the difference between MEI and DEE.

[k]Measured in BAT on day 18 of lactation.

[l]Quantities of β-actin and ribosomal protein L13a (Rpl13a) in arbitrary fluorescence units.

[m]Geometric mean expression of housekeeping genes β-actin and Rpl13a.

[n]Gene expression of uncoupling protein 1 (UCP1), uncoupling protein 3 (UCP3), cytochrome *c* oxidase subunit IV isoform 2 (Cox4i2), patatin-like phospholipase domain containing 2 (Pnpla2), fatty acid binding protein 3 (Fabp3), carnitine palmitoyltransferase 1b (Cpt1b) and peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), normalized against the expression of housekeeping genes (gm_{HK}).

were weighed and injected intraperitoneally with 0.25 g (lactating mice) or 0.15 g (virgin mice) of water enriched with ^{18}O (28 atom%) and ^{2}H (16 atom%). Initial blood samples were taken after 1 h of isotope equilibration to estimate initial isotope enrichments; final blood samples were taken 48 h later to estimate isotope elimination rates. Blood samples (tail tipping) were immediately heat sealed into 2×50 µl glass capillaries, which were then vacuum distilled. Water from the resulting distillate was used to produce CO_2 and H_2, and 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, 2005a). All intermediate results used to calculate DEE are shown in Table 2.

Milk production

We used the DLW data to evaluate milk production, calculated from the difference between MEI and DEE (Król and Speakman, 2003b). MEI and DEE were both measured during the period of asymptotic food intake at peak lactation but not on the exact same days to avoid possible changes in behaviour or feeding patterns as a result of DLW

injections or bleeding (Król and Speakman, 2003a; Speakman and Król, 2005a). Animals were in energy balance when milk production was evaluated, as indicated by their stable body mass (data not shown).

Hormone assays

Serum leptin levels were measured with the Mouse Leptin ELISA Kit (Millipore UK Limited, Watford, UK). Samples were run in duplicate and all readings were within the range of the assay (0.2–30 ng ml^{-1} of leptin for a 10 µl sample size). Measurements of serum prolactin levels were performed using the Mouse Prolactin DuoSet ELISA Development Kit (DY1445, R&D Systems, Minneapolis, MN, USA). Because the assay was designed for the analysis of cell culture supernates, we modified the manufacturer's protocol by diluting both standards and mouse serum samples with 50% fetal bovine serum in phosphate-buffered saline. This modification was necessary to equalize the effects of serum components and hence control for any potential matrix effects introduced by serum. Samples were run in duplicate and the majority (N=59) had detectable levels of prolactin with concentrations above the range of the standard curve of the assay (20 ng ml^{-1}). These samples were subsequently diluted and re-assayed. Samples with undetectable levels of prolactin (one lactating and three virgin mice) were assigned a concentration equal to the lower limit of detection (0.3 ng ml^{-1}).

RNA isolation and real-time PCR

Total RNA from interscapular BAT was isolated by homogenization in TRI REAGENT (Sigma-Aldrich, St Louis, MO, USA). The RNA was quantified by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA), with integrity confirmed by electrophoresis (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription of the total RNA into cDNA was performed using the QuantiTect RT Kit (Qiagen, Hilden, Germany) with integrated removal of genomic DNA.

Real-time PCR reactions were performed in 96-well PCR plates (Bio-Rad Laboratories, Hercules, CA, USA) using the QuantiTect SYBR Green PCR Kit and mouse gene-specific QuantiTect Primer Assays (Qiagen) (details in Table 1). All reactions were performed in duplicate on separate plates, which were then subjected to thermal cycling in DNA Engine Opticon 2 (MJ Research, Inc., South San Francisco, CA, USA) using MJ Opticon Monitor Analysis Software version 3.1 (Bio-Rad Laboratories). The amplification conditions were 15 min at 95°C, followed by 40 cycles of 94°C (30 s), 55°C (30 s) and 72°C (30 s). Fluorescence readings were taken at the end of each cycle at 75°C. Melting curves (0.5°C steps between 72 and 93°C) ensured that only a single product had been amplified in each reaction. All fluorescence data were corrected for the background by subtracting the lowest fluorescence reading recorded for each well.

The fluorescence readings were imported into R (R Development Core Team, 2010) and analysed using the qpcR package (Spiess and Ritz, 2010). Amplification efficiency (E, fluorescence at the cycle number at second derivative maximum divided by fluorescence at the previous cycle), the threshold cycle (*cpD2*, cycle number at second derivative maximum) and the threshold fluorescence (F_{cpD2}, fluorescence at *cpD2*) were estimated by fitting sigmoidal models to each amplification curve. Individual efficiencies were checked for outliers and then averaged to provide a single estimate of PCR efficiency (E_{avg}) for each amplified gene (Table 1). The initial fluorescence (F_0), which is proportional to the initial amount of cDNA template, was calculated for each sample as $F_0=[F_{\mathrm{cpD2}}/(E_{\mathrm{avg}})^{cpD2}]$ (Livak and Schmittgen, 2001). β-actin and ribosomal protein L13a (Rpl13a) (housekeeping genes) were not affected by the experimental treatment (Table 2). Gene expression was normalized using the geometric mean expression of β-actin and Rpl13a.

Statistical analysis

All data were assessed for normality and homogeneity of variance and were transformed if these assumptions were not met. Circulating levels of leptin and prolactin were square-root transformed prior to analysis. Associations between measured variables were assessed using Pearson correlation coefficients. Gene expression and hormone levels of lactating and virgin mice were compared using Welch's two-sample t-tests. We used linear models to examine the relationships between the circulating levels of leptin and prolactin (treated as independent variables) and the expression levels of genes (treated as the dependent variable) in BAT of lactating and virgin mice. The significance levels of all tests were corrected for Type I errors by the Benjamini–Hochberg procedure for multiple comparisons. Statistical significance was determined at adjusted P<0.05.

Expression levels of genes in BAT were summarized using principal component analysis (PCA). We used a correlation matrix to compute eigenvectors for each gene on the first principal component (PC1) (supplementary material Tables S1, S2). Scores for individual mice were calculated using the eigenvectors from PC1 and standardized gene expression values.

We used path analysis (Grace, 2006) to further examine associations between hormone levels, BAT activity (reflected by the PC1 scores) and milk production in lactating mice. Our path model was defined *a priori* and assumed that: (1) leptin and prolactin have independent direct effects on milk production, which are not associated with BAT activity; (2) the effects of leptin and prolactin on milk production are also mediated by BAT activity; and (3) both BAT activity and milk production are also affected by factors outside the model, including measurement error. The magnitude of direct (BAT-independent) effects is represented by path coefficients (*pc*); the indirect (BAT-mediated) effects are calculated as the product of intermediate path coefficients. Total effects of leptin and prolactin on milk production are calculated as the sum of both the direct (BAT-independent) and indirect (BAT-mediated) effects. Path analysis was performed in Amos 18.0.0 (SPPS, Chicago, IL, USA).

RESULTS

BAT gene expression and DEE

The DEE of virgin mice was positively correlated with the gene expression of UCP1 (r=0.75, P=0.005), cytochrome c oxidase subunit IV isoform 2 (Cox4i2; r=0.61, P=0.029) and carnitine palmitoyltransferase 1b (Cpt1b; r=0.65, P=0.022) as well as with scores on PC1 (r=0.65, P=0.009; Fig. 1). The PC1 scores for BAT gene expression in non-breeding mice explained 66% of the variation in the individual genes; mice with large positive scores had relatively high expression levels of all genes, whereas mice with large negative scores had relatively low expression of these genes (supplementary material Table S1). In lactating mice, none of the associations between DEE and expression of individual BAT genes or PC1 scores was significant (all P>0.05). The PC1 scores for BAT gene expression in lactating mice explained 79% of the total variation in the individual genes; mice with large positive scores had relatively high expression of UCP1, uncoupling protein 3 (UCP3), Cox4i2, patatin-like phospholipase domain containing 2 (Pnpla2), Cpt1b and peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α), and low expression of fatty acid binding protein 3 (Fabp3), whereas mice with large negative

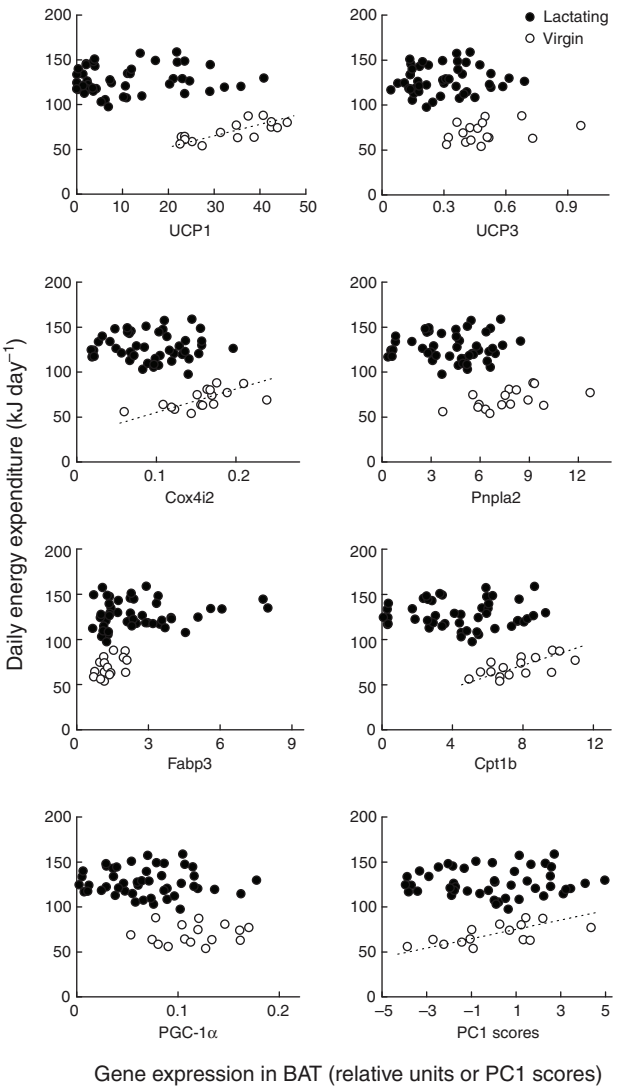

Fig. 1. Daily energy expenditure of lactating (N=47) and virgin female (N=16) mice plotted against the expression of several genes in brown adipose tissue (BAT). Expression of these genes is normalized against the expression of housekeeping genes and presented in relative units. PC1 scores are calculated using principal component analysis to summarize the expression levels of BAT genes in lactating and virgin mice. The lines represent reduced major axis regressions fitted to the associations that are significant (P<0.05).

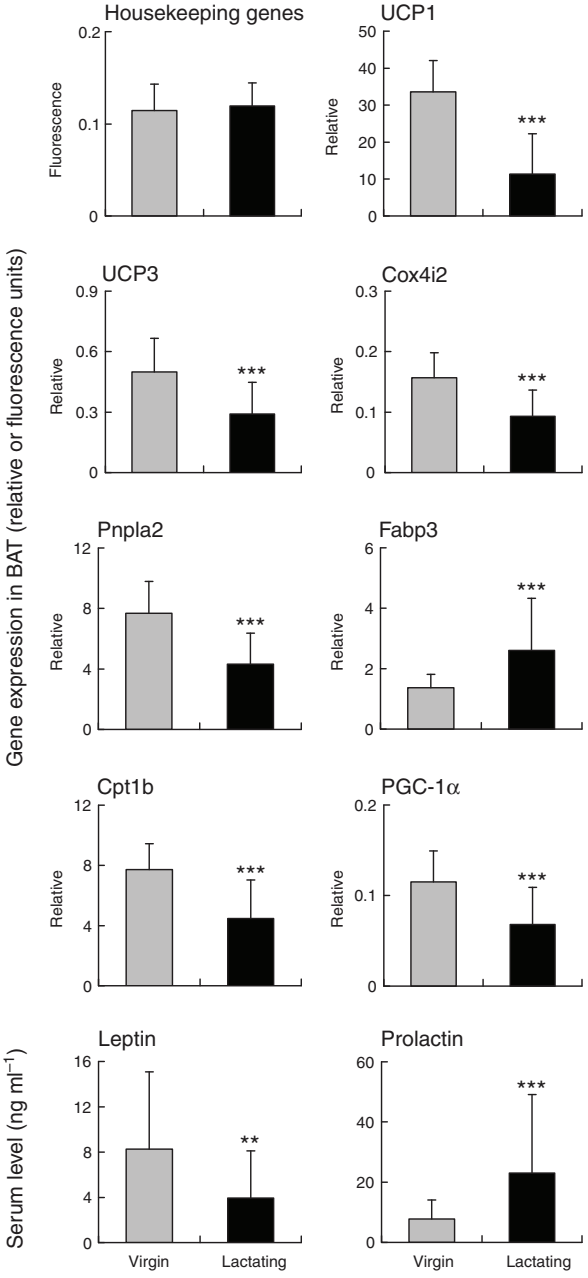

Fig. 2. Expression of genes in BAT and corresponding levels of circulating leptin and prolactin in lactating (N=47) and virgin female (N=16) mice. Expression of housekeeping genes (geometric mean expression of β-actin and Rpl13a) is in arbitrary fluorescence units; expression of thermogenic genes is normalized against the expression of housekeeping genes and is presented in relative units. Data are means + 1 s.d. **P<0.01; ***P<0.001.

scores had relatively high expression of Fabp3 and low expression of the other genes (supplementary material Table S2).

Effects of lactation on BAT gene expression and circulating hormone levels

Lactation had a highly significant effect on the expression levels of all genes in BAT (UCP1, t_{34}=8.4; UCP3, t_{25}=4.4; Cox4i2, t_{27}=5.2; Pnpla2, t_{25}=5.5; Fabp3, t_{59}=4.5; Cpt1b, t_{38}=5.7; PGC-1α, t_{31}=4.5; all P<0.001; Fig. 2). For all the genes except Fabp3, the expression during lactation decreased on average to 34–59% of the levels typical for virgin mice. By contrast, gene expression of Fabp3 increased during lactation to 91% above the control non-breeding level. There was also a significant effect of lactation on circulating levels of leptin (t_{23}=3.0, P=0.007) and prolactin (t_{49}=3.7, P<0.001). During lactation, serum concentration of leptin decreased on average to 48% of the level of non-breeding mice, whereas the concentration of prolactin increased to 197% above the control non-breeding level. Circulating leptin levels were not correlated with circulating levels of prolactin (P>0.05).

Correlations in BAT gene expression

The expression levels of UCP1, UCP3, Cox4i2, Pnpla2, Cpt1b and PGC-1α genes were highly positively correlated with each other not only when the data sets for lactating and virgin mice were pooled, but also when the analysis was restricted to lactating animals (Fig. 3, supplementary material Table S3). Some of these correlations were

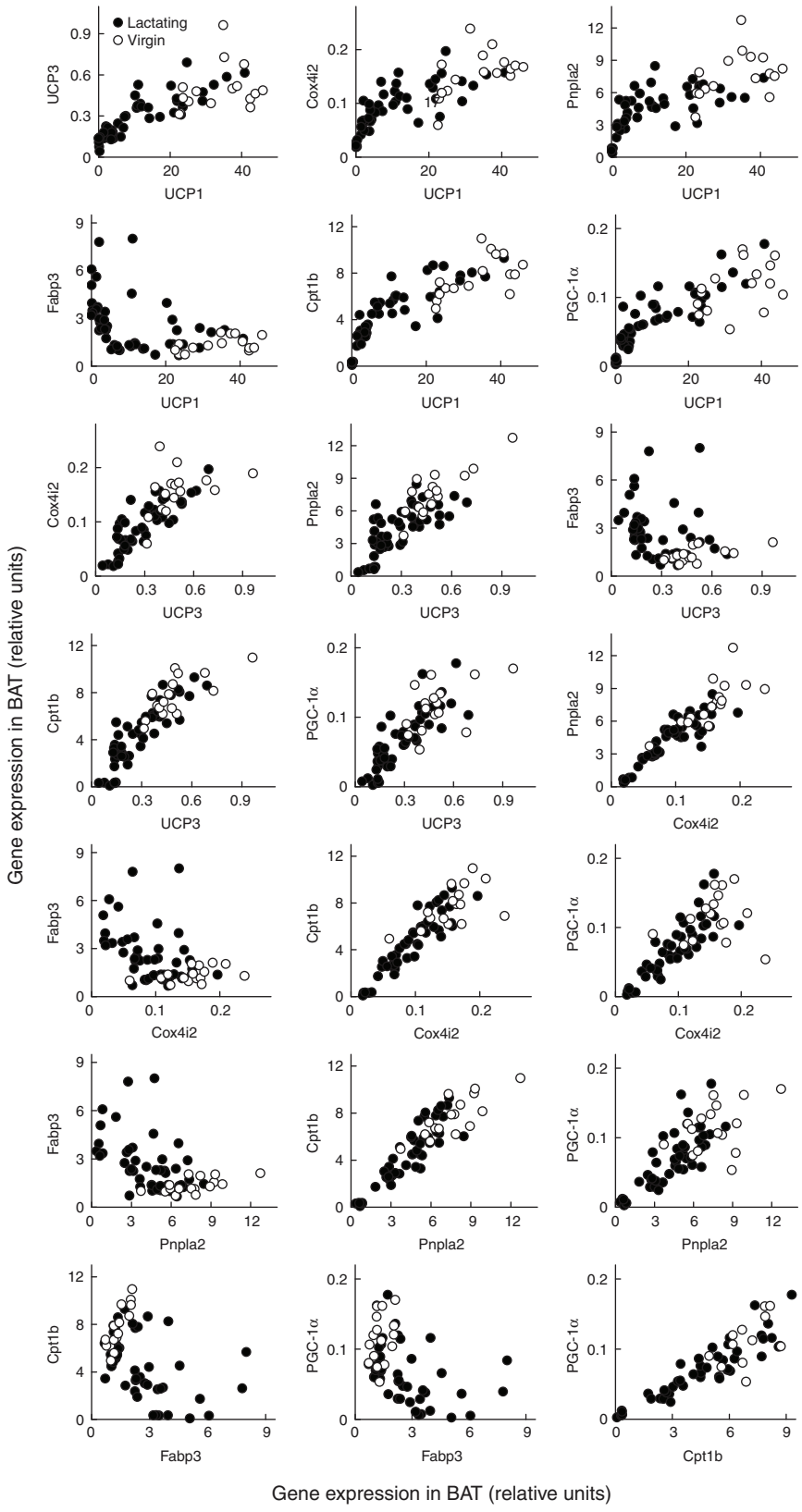

Fig. 3. Correlations between several genes expressed in BAT of lactating (*N*=47) and virgin female (*N*=16) mice. Gene expression is normalized against the expression of housekeeping genes and is presented in relative units. For statistical details, see supplementary material Table S3.

highly significant when the analysis was restricted to the much smaller and less variable data set from non-breeding mice. By contrast, the correlations involving the expression of Fabp3 gene were weaker, more complex and dependent on the reproductive status of mice (positive for virgin and negative for lactating females).

Circulating hormone levels and BAT gene expression

The relationship between the expression of genes in BAT and circulating levels of leptin and prolactin depended on the reproductive status of animals (Table 3). During lactation, the expression of most genes was significantly associated with the levels

Table 3. Results of linear models performed to examine the relationship between circulating levels of leptin and prolactin (treated as independent variables) and the expression levels of several genes (treated as dependent variables) in BAT of lactating and virgin female mice

	Leptin			Prolactin		
Gene	Slope (±s.e.m.)	*t*	*P*	Slope (±s.e.m.)	*t*	*P*
Lactating mice (*N*=47)						
UCP1	8.013±1.191	6.7	<0.001	–0.934±0.482	–1.9	n.s.
UCP3	0.098±0.020	5.0	<0.001	–0.012±0.008	–1.5	n.s.
Cox4i2	0.021±0.006	3.6	0.001	–0.006±0.002	–2.7	0.009
Pnpla2	0.942±0.283	3.3	0.002	–0.217±0.115	–1.9	n.s.
Fabp3	–0.467±0.265	–1.8	n.s.	0.055±0.107	0.5	n.s.
Cpt1b	1.691±0.291	5.8	<0.001	–0.286±0.118	–2.4	0.020
PGC-1α	0.026±0.005	5.6	<0.001	–0.005±0.002	–2.8	0.008
PC1 scores	1.515±0.277	5.5	<0.001	–0.259±0.112	–2.3	0.025
Virgin mice (*N*=16)						
UCP1	0.740±2.032	0.4	n.s.	–1.686±1.778	–1.0	n.s.
UCP3	–0.043±0.040	–1.1	n.s.	0.006±0.035	0.2	n.s.
Cox4i2	0.010±0.010	1.0	n.s.	0.007±0.009	0.8	n.s.
Pnpla2	–0.323±0.522	–0.6	n.s.	0.079±0.456	0.2	n.s.
Fabp3	0.023±0.113	0.2	n.s.	–0.048±0.099	–0.5	n.s.
Cpt1b	0.045±0.430	0.1	n.s.	–0.177±0.376	–0.5	n.s.
PGC-1α	–0.009±0.008	–1.1	n.s.	–0.002±0.007	–0.3	n.s.
PC1 scores	–0.111±0.600	–0.2	n.s.	–0.071±0.492	–0.1	n.s.

The interaction between leptin and prolactin was initially included in all models, but was subsequently removed due to non-significance. Hormone levels were square-root transformed prior to analysis.

PC1 scores were calculated to summarize the expression levels of several genes in BAT of lactating and virgin mice (for details, see Materials and methods).
n.s., not significant (*P*>0.05).

of either leptin (positive effects of leptin with no effects of prolactin for UCP1, UCP3 and Pnpla2) or both leptin and prolactin (positive effects of leptin and negative effects of prolactin for Cox4i2, Cpt1b and PGC-1α). For Fabp3, the relationship between gene expression and both hormone levels was not significant. The overall relationship between BAT gene expression and both leptin and prolactin during lactation was significant when the expression levels of individual genes were replaced with the PC1 scores. By contrast, neither leptin nor prolactin was significantly correlated with the expression levels of individual genes in BAT of virgin mice or when the analyses were performed on the PC1 scores.

Milk production and BAT gene expression

Milk production was significantly correlated with BAT gene expression (Fig. 4). These associations were negative for UCP1 (r=–0.58, P<0.001), UCP3 (r=–0.52, P<0.001), Cox4i2 (r=–0.39, P=0.008), Pnpla2 (r=–0.42, P=0.005), Cpt1b (r=–0.54, P<0.001) and PGC-1α (r=–0.55, P<0.001), indicating that mice with greater downregulation of these genes produced more milk. By contrast, the correlation between milk production and the transcript levels of Fabp3 was positive (r=0.37, P=0.012). When the expression levels of thermogenic genes were summarised by PCA, the correlation between milk production and PC1 scores was highly significant and negative (r=–0.58, P<0.001).

Path analysis

Goodness-of-fit measures obtained from the path analysis indicated that the proposed model (Fig. 5) described the data well (χ^2=0.34, P=0.56; Bollen–Stine bootstrap test, P=0.48). Circulating levels of leptin and prolactin together explained 45% of the variation in BAT activity, while both hormones and BAT activity accounted for 51% of the observed variation in milk production. The overall path coefficient of leptin (pc=–0.67) to milk production was greater than that of prolactin (pc=–0.08). Leptin and prolactin differed also in the relative contribution of direct (BAT-independent) and indirect (BAT-mediated) pathways by which each hormone was correlated with milk production. There was a strong negative BAT-independent correlation of leptin with milk production (pc=–0.47), whereas we detected no significant BAT-independent correlation of prolactin with milk production (pc=–0.16). Moreover, leptin had a strong positive correlation (pc=0.62) with the expression of genes associated with BAT activity (except Fabp3, which decreased), resulting in a negative BAT-mediated correlation of leptin and milk production (pc=–0.20). By contrast, prolactin had a significant negative correlation (pc=–0.26) with the expression of genes in BAT (except Fabp3, which increased), resulting in a small positive BAT-mediated correlation with milk production (pc=0.08). The overall correlation between BAT activity and milk production was significant and negative (pc=–0.32). Taken together, the factors that were correlated with high milk production included low levels of circulating leptin (correlated *via* BAT-independent and BAT-mediated pathways), high levels of circulating prolactin (correlated *via* BAT-mediated pathway) and low levels of BAT activity (correlated with leptin, prolactin and factors outside the model).

DISCUSSION

BAT gene expression in virgin and lactating mice

A primary aim of this work was to test whether individual mice with greater milk production had reduced expression of genes linked to thermogenesis in BAT, indicative of reduced BAT thermogenesis. The applicability of BAT gene expression to reflect thermogenic activity of BAT was confirmed by a positive relationship between BAT gene expression (summarized as the PC1 scores) and DEE in virgin mice (Fig. 1), which is consistent with the positive association between BAT activity and RMR in humans (van Marken Lichtenbelt et al., 2009). Previous evaluations of the effects of lactation on BAT gene expression at the group level have examined the transcript

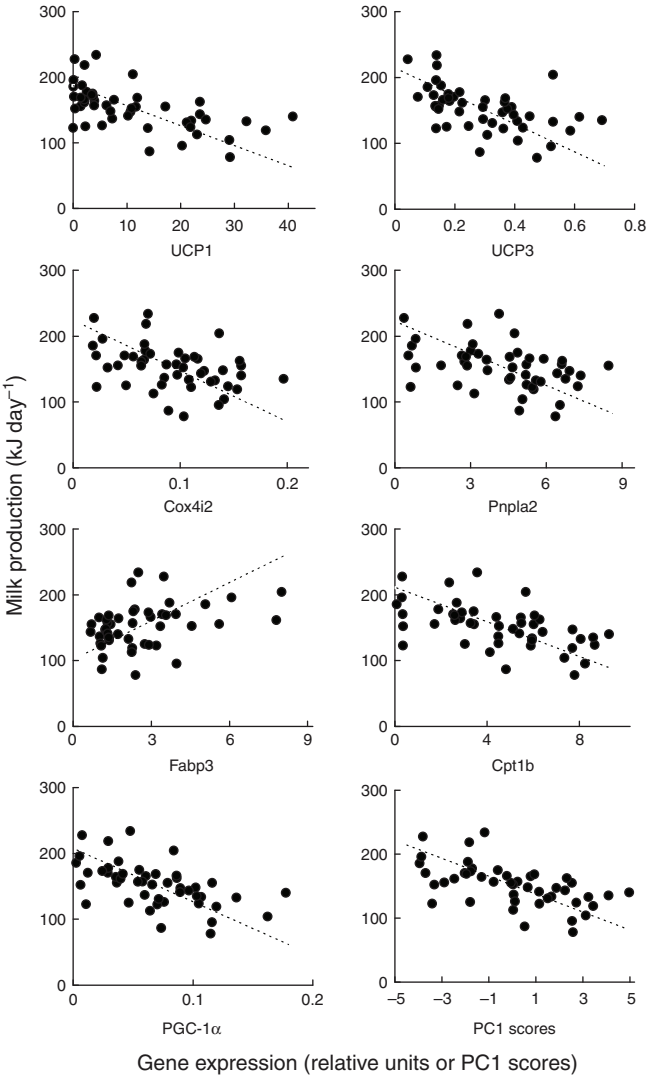

Fig. 4. Milk production plotted against the expression of several genes in BAT of lactating mice (N=47). Gene expression is normalized against the expression of housekeeping genes and presented in relative units. PC1 scores summarize the expression levels of thermogenic genes in BAT of lactating mice. All associations are significant (P<0.05) and fitted with the lines that represent reduced major axis regressions.

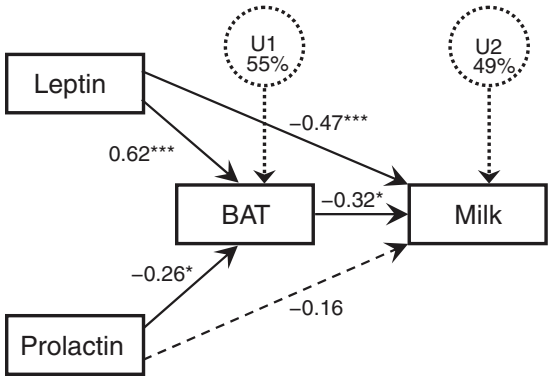

Fig. 5. Path model describing the association of leptin and prolactin with milk production *via* direct (BAT-independent) and indirect (BAT-mediated) pathways. BAT activity is represented by PC1 scores that summarize the expression levels of several genes linked to thermogenesis in BAT of lactating mice. The effects are described by standardized path coefficients and asterisks indicate statistical significance (*P<0.05; ***P<0.001); the dashed arrow reflects a non-significant path. U1 and U2 represent the contribution of unexplained sources of variation to BAT activity (55%) and milk production (49%), respectively.

levels of UCP1, UCP3 and Cox4i2 (Martin et al., 1995; Pedraza et al., 2001; Xiao et al., 2004), which code for proteins on the mitochondrial inner membrane and are directly involved in uncoupling and respiration. Changes in the rate of mitochondrial respiration require coordinated changes in other processes such as lipid droplet mobilization and transport of fatty acids into the mitochondria. The activity of these systems, represented in our study by gene expression of Pnpla2, Fabp3 and Cpt1b, has not been studied previously in the context of lactation. As predicted by the HDL theory, most genes linked to thermogenesis in BAT were downregulated during lactation, to approximately half of the value typical for non-breeding females (Fig. 2). Our study is the first to show that differences in the expression of genes in BAT during lactation are significantly related to milk production at the individual level (Fig. 4). The downregulation of BAT activity during lactation has been previously considered as an energy-sparing mechanism to maximise energy available for milk production (Trayhurn et al., 1982; Xiao et al., 2004; Smith et al., 2010). However, an equally plausible explanation is that downregulation of BAT activity is crucial for milk production because it reduces the amount of non-lactogenic heat that the lactating animal is required to dissipate.

Milk production and BAT gene expression

The causality of the relationship between milk production and BAT activity was not addressed in our study. It is therefore impossible to predict whether the amount of milk produced by individual mice depends on the level to which they are able to downregulate their heat production in BAT (as predicted by the HDL theory) or whether the activity of BAT is downregulated in response to the level of milk production and consequential heat production (e.g. Cannon and Nedergaard, 2004). The mechanisms responsible for maintaining the dynamic balance between BAT activity and milk production are currently unknown. It has been suggested that BAT thermogenesis may decrease directly as a consequence of the thermogenic heat production due to milk synthesis (Cannon and Nedergaard, 2004). However, suckling-induced activation of the dorsomedial hypothalamus may also play an important role in a suppression of sympathetic drive to BAT, most likely through dorsomedial hyphothamalus projections to the medullary raphé in the brainstem (McAllen et al., 2010; Smith et al., 2010). Establishing these relationships will greatly advance our knowledge about how lactogenic and non-lactogenic sources of heat production are integrated.

Fabp3 gene expression: an unexpected result

Previous studies have shown that gene expression of Fabp3 in the BAT of non-breeding rats is induced by cold exposure and reduced at high ambient temperatures, closely resembling the expression profile of UCP1 (Watanabe et al., 2008; Yamashita et al., 2008). These changes in Fabp3 expression were confirmed at the protein level, indicating an essential role of Fabp3 in fatty acid transport and its oxidation through the activity of UCP1 in cold-induced thermogenesis (Yamashita et al., 2008). Similar involvement of Fabp3 in the fatty acid flux has been demonstrated in the skeletal muscle of rats, with relatively low expression of this gene during

lactation when the lipid usage by muscle is reduced and an elevated expression when fat is mobilized as a fuel during fasting (Xiao et al., 2004). These data suggest that Fabp3 reflects the capacity of metabolic tissues to utilise fatty acids (Furuhashi and Hotamisligil, 2008). We therefore expected expression of Fabp3 in BAT during lactation to be downregulated in parallel with UCP1 and other genes linked to thermogenesis. Contrary to our expectations, the expression of Fabp3 in BAT of lactating mice was increased (Fig. 2) and the magnitude of this increase was proportional to milk production (Fig. 4). It remains possible that Fabp3 in BAT during lactation is involved in activities other than the transport of fatty acids to the mitochondria for subsequent β-oxidation (Furuhashi and Hotamisligil, 2008).

Leptin and prolactin as correlates of milk production

Lactation is characterized by profound changes in the levels of circulating hormones, including leptin and prolactin. Leptin levels during lactation are typically very low, linked to depleted fat stores (Speakman, 2008) and/or active inhibition of leptin production by factors associated with the energy drain of milk withdrawal (Xiao et al., 2004; Smith et al., 2010). Circulating levels of prolactin during lactation are typically high and depend on the intensity and duration of suckling (Mattheij et al., 1979; Chan and Swaminathan, 1990). Both suppression of leptin and induction of prolactin have been suggested to contribute to lactational hyperphagia, facilitating milk production (Crowley et al., 2004; Naef and Woodside, 2007; Woodside, 2007; Smith et al., 2010). Importantly, both of these hormones have also been demonstrated to modify the gene and protein expression of UCP1 in BAT (Chan and Swaminathan, 1990; Pearce et al., 2003; Cannon and Nedergaard, 2004; Xiao et al., 2004; Cui et al., 2011). These hormones may therefore influence milk production *via* BAT-mediated and BAT-independent effects. Although our analysis is based on correlations, it clearly shows that low levels of circulating leptin are correlated with increased milk production *via* both BAT-independent and BAT-mediated pathways (Fig. 5). The most likely BAT-independent route of leptin action (if the causality is indeed in that direction) is an alteration of orexigenic and anorexigenic neuropeptide systems in the arcuate nucleus of the hypothalamus to increase the drive to eat (Smith et al., 2010). The BAT-mediated effects of leptin on milk production could be explained by downregulation of BAT thermogenesis due to central suppression of sympathetic drive to brown fat (Haynes et al., 1997; Elmquist, 2001). Leptin may also have direct effects on BAT (Siegrist-Kaiser et al., 1997), consistent with presence of the signalling form of leptin receptor in brown adipocytes (Fei et al., 1997). Indeed, administration of exogenous leptin to lactating rats and Brandt's voles (*Lasiopodomys brandtii*) reversed some of the orexigenic drive provided by the arcuate nucleus of the hypothalamus and increased the gene and protein expression of UCP1 in BAT (Crowley et al., 2004; Xiao et al., 2004; Smith et al., 2010; Cui et al., 2011). Despite these changes, exogenous leptin decreased food intake only when administered for longer than 48 h (Stocker et al., 2007; Cui et al., 2011), and the decrease in food intake was modest, suggesting that other hormones and systems may be crucial for lactational hyperphagia and associated milk production (Smith et al., 2010).

Path analysis indicated that the total correlation between prolactin and milk production was weaker than that of leptin. In addition, the high levels of circulating prolactin are correlated with increased milk production more by downregulation of BAT activity than by BAT-independent processes (Fig. 5). The link between prolactin and BAT activity is consistent with the administration of exogenous prolactin (Pearce et al., 2003) and pharmacological stimulation of endogenous prolactin secretion (Chan and Swaminathan, 1990) in non-breeding rats promoting the loss of UCP1. These effects are likely to be mediated *via* prolactin receptors on brown adipocytes (Pearce et al., 2003). However, the lack of a BAT-independent correlation between prolactin and milk production (Fig. 5) contradicts the accumulated evidence that circulating prolactin re-enters the brain, interacts with distinct populations of hypothalamic neurons that express prolactin receptors (Grattan and Kokay, 2008) and acts in female rats to induce and maintain hyperphagia (Woodside, 2007). Some of these prolactin-responsive neurons co-express leptin receptors, suggesting that prolactin and leptin in rats may act together to regulate food intake (Naef and Woodside, 2007). The discrepancy between our results and the previous research may be due to either differences between animal models (mice *versus* rats) or differences in the blood sampling protocols. In many studies, lactating females have been separated from their pups before blood sampling to standardize their levels of circulating prolactin (e.g. Voloschin et al., 1998; Callahan et al., 2000). In our study, the females were kept with the pups to preserve putative inhibitory effects of suckling on thermogenesis in BAT.

Perspectives and significance

In summary, our results provide novel insights into the relationship between BAT function and milk production during lactation. We have shown that the expression levels of UCP1, UCP3, Cox4i2, Pnpla2, Fabp3, Cpt1b and PGC-1α genes in BAT are strongly correlated with each other, and in non-breeding mice they provide reliable measures of BAT thermogenic activity. These measures (BAT gene expression summarized as the PC1 scores) were then used to describe the activity of BAT in lactating mice. We have demonstrated that the individual variability in the amount of energy exported as milk is negatively associated with the activity of BAT. A positive correlation between the expression of Fabp3 gene in BAT and milk production was unexpected and requires further investigation. Low levels of circulating leptin were correlated with and may contribute to the increase in milk production by reducing BAT activity and by BAT-independent mechanisms. High levels of circulating prolactin were correlated with increased milk production *via* associations with the activity of BAT rather than by BAT-independent mechanisms, but the relationships with prolactin were weaker than those with leptin. Taken together, our results are consistent with the prediction of the HDL theory that downregulation of BAT thermogenesis reduces competitive heat production, permitting greater production of lactogenic heat within the overall heat dissipation capacity limit (Król and Speakman, 2003a; Król and Speakman, 2003b; Król et al., 2007; Speakman and Król, 2010). However, we cannot discount an alternative explanation that the causality in the associations is the reverse, i.e. that greater milk production, directly *via* thermogenic mechanisms alone (Cannon and Nedergaard, 2004) or *via* endocrine and physiological processes, reduces BAT activity. Nevertheless, our findings indicate that the performance of mammary glands may depend on the activity of other systems in the body, suggesting that improvements in milk production might be facilitated by decreases in the heat generated by competitive processes rather than by attempting to increase mammary gland performance in isolation. Manipulations that reduce competitive heat production during lactation may provide new avenues to increase milk production in domesticated livestock and to overcome the problems with initiating and sustaining breastfeeding in humans.

LIST OF ABBREVIATIONS

BAT	brown adipose tissue
Cox4i2	cytochrome *c* oxidase subunit IV isoform 2
Cpt1b	carnitine palmitoyltransferase 1b
DEE	daily energy expenditure
DLW	doubly labelled water
Fabp3	fatty acid binding protein 3
HDL	heat dissipation limit
MEI	metabolizable energy intake
PC1	first principal component
PCA	principal component analysis
PGC-1α	peroxisome proliferator-activated receptor gamma co-activator 1 alpha
Pnpla2	patatin-like phospholipase domain containing 2
RMR	resting metabolic rate
Rpl13a	ribosomal protein L13a
UCP1	uncoupling protein 1
UCP3	uncoupling protein 3

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