

## RESEARCH ARTICLE

# Limits to sustained energy intake. XXX. Constraint or restraint? Manipulations of food supply show peak food intake in lactation is constrained

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## ABSTRACT

Lactating mice increase food intake 4- to 5-fold, reaching an asymptote in late lactation. A key question is whether this asymptote reflects a physiological constraint, or a maternal investment strategy (a 'restraint'). We exposed lactating mice to periods of food restriction, hypothesizing that if the limit reflected restraint, they would compensate by breaching the asymptote when refeeding. In contrast, if it was a constraint, they would by definition be unable to increase their intake on refeeding days. Using isotope methods, we found that during food restriction, the females shut down milk production, impacting offspring growth. During refeeding, food intake and milk production rose again, but not significantly above unrestricted controls. These data provide strong evidence that asymptotic intake in lactation reflects a physiological/physical constraint, rather than restraint. Because hypothalamic neuropeptide Y (*Npy*) was upregulated under both states of restriction, this suggests the constraint is not imposed by limits in the capacity to upregulate hunger signalling (the saturated neural capacity hypothesis). Understanding the genetic basis of the constraint will be a key future goal and will provide us additional information on the nature of the constraining factors on reproductive output, and their potential links to life history strategies.

**KEY WORDS:** Food restriction, Daily energy intake (DEI), Heat dissipation limit (HDL) theory, Milk energy output (MEO), Gene expression profile, Hunger signalling, Peripheral limitation, Swiss mice, Neuropeptide Y, RNAseq

## INTRODUCTION

The maximal rate of sustained energy intake or expenditure is the rate at which an organism can sustain its metabolic performance for periods of days or weeks without the need to resort to utilization of stored energy reserves (Drent and Daan, 1980; Weiner, 1989; Peterson et al., 1990). There has been considerable interest in the factors that may limit this trait because it provides an upper boundary within which all aspects of animal performance are contained. There has been a range of different theories proposed for

the factors that might impose limits on sustained energy intake, which have included the idea that the limit is imposed by the alimentary tract (Tolosa et al., 1991; Hammond and Diamond, 1992, 1997; Koteja, 1996; Sadowska et al., 2015; Thurber et al., 2019), the metabolic capacity of sites where the energy is expended (Hammond et al., 1996; Zhao et al., 2010), the signalling system in the brain that regulates hunger (Speakman and Król, 2005) or the capacity of the individual to dissipate body heat (Król et al., 2007; Speakman and Król, 2010).

In small rodents, lactation is the most energy-demanding period of their lives (Millar, 1977; Loudon and Racey, 1987; Speakman, 2008). Studies of the limitations on lactation performance therefore provide an ideal testing ground for these alternative ideas, and there has been a rich vein of research focused on lactation energy limits stretching back at least 25 years (Hammond and Diamond, 1992; Hammond et al., 1994, 1996; Speakman and McQueenie, 1996; Rogowitz, 1998; Johnson et al., 2001a; Król et al., 2003; Zhao and Cao, 2009a,b; Wu et al., 2009; Paul et al., 2010; Speakman and Król, 2010; Valencak et al., 2010, 2013; Zhao et al., 2010, 2013; Sadowska et al., 2015; Gamo et al., 2016; Thurber et al., 2019). Food intake during lactation in small mammals accelerates over 7 days, but then reaches an asymptote. It is widely presumed that this asymptote reflects a constraint that the animal cannot breach (Hammond and Diamond, 1997; Johnson et al., 2001a,b; Król et al., 2007; Speakman and Król, 2005, 2011), and hence understanding what imposes such a limit will give us insights more broadly into the limits on sustainable intake.

The basis for presuming this is a constraint is as follows. First, if litter size is experimentally increased, then females do not respond by elevating their intake or milk production (Johnson et al., 2001a; Duah et al., 2013), resulting in smaller offspring as the limited milk has to be shared by more individuals. Second, if lactation is combined with increased energy demands owing to exercise, then mice are unable to elevate their intake to match both the exercise and lactation demands (Perrigo, 1987; Duah et al., 2013; Zhao et al., 2013; Gamo et al., 2016). Similarly, animals challenged by lactation and infection, or lactation and pregnancy also cannot increase their intake to deal with both challenges (Johnson et al., 2001b; Hammond and Kristan, 2000). However, contrasting these data is the fact that if animals are placed in the cold when lactating, then they do seem easily capable of eating more food (Hammond and Diamond, 1992; Johnson and Speakman, 2001; Zhao and Cao, 2009a,b), and in some cases this translates to elevated milk production and pup growth (Johnson and Speakman, 2001). This effect of cold exposure was the basis for the formulation of the heat dissipation limit (HDL) hypothesis (Król et al., 2003, 2007; Speakman and Król, 2010, 2011). The basis of this idea is that there is a constraint, but this constraint is temperature dependent, and

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**List of symbols and abbreviations**

<i>Agpr</i>	agouti-related peptide
<i>Cart</i>	cocaine- and amphetamine-regulated transcript
Con	control group
CR	caloric restriction
DEE	daily energy expenditure
DEI	digestive energy intake
DLW	doubly labelled water
<i>Drd2</i>	dopamine receptor D2
FR	food restriction
GE	gross energy
GEI	gross energy intake
<i>Htr2a</i>	5-hydroxytryptamine receptor 2A
<i>Lepr</i>	Long form leptin receptor
<i>Mc3r</i>	melanocortin-3 receptor
<i>Mc4r</i>	melanocortin-4 receptor
MDS	multidimensional scaling
MEI	metabolisable energy intake
MEO	milk energy output
<i>Npy</i>	neuropeptide Y
<i>Pomc</i>	proopiomelanocortin
SDA	specific dynamic action
<i>Socs3</i>	suppressor of cytokine signalling 3
<i>Stat3</i>	signal transducer and activator of transcription 3
<i>Stat5b</i>	signal transducer and activator of transcription 5 beta
$T_b$	body temperature
TMM	trimmed mean of M values
<i>Tnf-<math>\alpha</math></i>	tumor necrosis factor $\alpha$
UEL	urinary energy loss

reflects the capability of the animals to dissipate body heat and hence avoid potentially fatal hyperthermia. Hence, manipulations that aim to elevate expenditure/intake at a single temperature are unsuccessful, but lowering temperature alleviates the constraint allowing intake to rise, while increasing ambient temperature tightens the constraint causing lactation investment to fall (Król et al., 2003; Wu et al., 2009; Zhao et al., 2016).

Attempts to test the HDL theory by shaving lactating females to alleviate their heat burden have had mixed results (Król et al., 2007; Zhao and Cao, 2009a,b; Paul et al., 2010; Zhao et al., 2010; Simons et al., 2011; Sadowska et al., 2016), as have attempts to expose females and their offspring to different ambient temperatures (Valencak et al., 2010, 2013; Zhao et al., 2016). These ambiguous results mean that it is entirely plausible that the difference in the response of lactating animals to manipulations at a single temperature (where they generally do not increase intake and investment) and those where temperature is manipulated (where the animals generally do respond by varying intake and investment) is not because of a limit that varies with temperature (e.g. heat dissipation), but because of an investment strategy that reflects the animals' deeper selection history. That is the strategy they are following to maximize lifetime reproductive output that has been moulded by the process of evolution. For example, it has been suggested that the reproductive value of offspring may vary with time of year, such that those born earlier in the breeding season when it is colder may have greater reproductive value than those born later (Speakman and Król, 2005). Animals may then be willing to elevate their investment when it is colder, and lower it when it is hotter, because of these underlying selection pressures, rather than any physical process related to heat loss capacity. Thus, at a fixed temperature, females may 'choose' not to elevate their investment even though they are physiologically capable of doing so. In other words, the limit reflects 'restraint' rather than a 'constraint'. There is

some evidence for such a trade-off between litters in mice (strain MF1). When litter sizes were experimentally manipulated, those mice raising smaller litters showed greater investment in the subsequent litter (Vaanholt et al., 2018). However, this observation only shows that the conditions necessary for a restraint to exist are present, but this does not necessarily imply that the asymptotic consumption observed when raising the first litter was restrained (Vaanholt et al., 2018).

Separating whether animals are constrained in their intake capacity or restraining intake as part of a broader investment strategy is extremely difficult. In this study, we have tried to perform such a test. The rationale of the approach is relatively simple. If one takes a non-breeding small mammal and completely deprives it of food for 24 h (or partially restricts it for a longer period), then when food is again provided *ad libitum* (normally called the 'refeeding day'), the animals eat more than their normal 24 h intake to compensate for the day of intake that they missed (Wilson and Osbourn, 1960; Bartness, 1997; Cameron and Speakman, 2011; Zhang et al., 2012; Zhao et al., 2014). The animals in this situation are strongly motivated to eat more food on the re-feeding day. We hypothesized that lactating animals would likely have the same sorts of motivations in the face of variable food supply. Food availability in the wild varies from day to day. If lactating animals have a target intake that defines their restrained asymptotic investment level, they might occasionally find insufficient food to meet this requirement. This would provide them with a strong motivation to eat more the next day to compensate for the deficit and keep their average investment on the restrained track. However, if the asymptotic intake reflects a physiological or physical constraint, then the day after a period where they failed to find enough food, they would be physiologically incapable of eating more to compensate. This difference provides the setting for a potential test of whether the asymptotic intake at peak lactation is constrained or restrained.

Studies of mice under caloric restriction suggest that the post-restriction hyperphagia is driven by a complex network of hypothalamic neuropeptides that regulate food intake (Barsh et al., 2000a,b; Schwartz et al., 2000) and are stimulated or inhibited during the restriction phase (Hambly et al., 2007, 2012; Speakman and Mitchell, 2011; Deros et al., 2016a) or alter their network topology (Deros et al., 2016b). Topology is the structure of the network of interacting genes. If mice in lactation do not elevate their intake following restriction, one reason may be that the hunger signalling pathway is already maximally stimulated during lactation (Hambly and Speakman, 2015), and hence there is no additional scope in the system to drive additional intake. Conversely, if this system is further stimulated, yet additional intake does not occur, this would indicate other physiological or physical constraints were at play.

The design we used was to expose Swiss mice at peak lactation (after day 10) to days when they received only 25% or 50% of their asymptotic intake. They were followed to see how they responded to this shortfall during the event, and in particular the following day when they were returned to *ad libitum* feeding. In some mice, this included characterization of the hunger signalling network in the hypothalamus by RNAseq, at the end of the day of restriction, and measurement of circulating hormone levels in the same individuals. Mice responded to restriction by cutting down their milk secretion to their pups and lowering their body temperature. These responses were made in proportion to the level of restriction and had direct impacts on pup growth and at the highest level of restriction. Mice did not elevate their intake or milk production on *ad libitum*

refeeding day, consistent with the asymptote being imposed by a constraint.

## MATERIALS AND METHODS

### Ethical review

All the experiments were reviewed and approved by the Institutional Review Board of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (approval number AP2016052).

### Animals

Female Swiss mice were obtained at 9–10 weeks old from a colony that was maintained in the animal house of Wenzhou University. Animals were housed individually in plastic cages (29×18×16 cm) with sawdust bedding, and kept on a 12 h:12 h light:dark photoperiod (lights on at 08:00 h) at a constant temperature of 21±1°C. Food (standard rodent chow; produced by Beijing KeAo Feed Co.) and water were provided *ad libitum*.

One hundred and eighty-nine virgin female mice were paired with males for mating for 11 days and then males were removed. One hundred and seventy-seven females became pregnant and gave birth. Pups were moved between females on the day of parturition, by which all mothers were experimentally adjusted to raise 12 pups, counted as day 0 of lactation hereafter. The mothers were randomly assigned into one of three groups following litter size adjustment: one control group (control,  $n=56$ ), in which the females were fed *ad libitum* throughout lactation, and two food restriction (FR) groups, within which females were provided with 50% or 25% of their *ad libitum* food intake on days 13, 15 and 17 of lactation (referred to as 50%-FR,  $n=81$ , and 75%-FR,  $n=40$  groups, respectively), but fed *ad libitum* on the days excluding the three days. Note that levels of restriction generally refer to the amount of food missing rather than the amount of food provided, and hence those provided with 25% of *ad libitum* intake were called 75%-FR. The restricted food intake was calculated based on the average of *ad libitum* food intake during days 11 and 12. Twelve females were randomly selected from each group and were killed at the end of day 13 of lactation. Another 12 females were randomly selected from the control group and were killed at the beginning of day 13. The rest of the females ended lactation when pups were weaned on day 18 of lactation. Body temperature, body mass, litter size and litter mass were measured throughout lactation on a daily basis.

### Body temperature

Body temperature ( $T_b$ ) (control,  $n=23$ ; FR-50%,  $n=34$ ; FR-75%,  $n=16$ ) was recorded using encapsulated thermo-sensitive passive transponders (diameter 2 mm, length 14 mm; Destron Fearing, South St Paul, MN, USA). We implanted transponders subcutaneously in the dorsolateral hip region of the females after the parturition day, according to the manufacturer's specifications. A Pocket Reader was used to approach the cage until the  $T_b$  data were taken. The reader did not touch the females and did not affect the behaviour of the mother and pups in the cage.

### Body mass, food intake, litter size and litter mass

Body mass, litter size and litter mass during lactation were measured on a daily basis following the  $T_b$  measurements (control,  $n=23$ ; FR-50%,  $n=34$ ; FR-75%,  $n=16$ ). These measurements were performed at 14:00 h during the period of lactation, but at a 3-h interval across 24 h on day 13 of lactation (to 0.1 g, Sartorius, Beijing). Food intake was also determined on day 13 at a 3-h interval, which was calculated based on the difference of food mass on the hopper over the 3 h, and was expressed as  $g\ 3\ h^{-1}$ .

### Gross energy intake and digestibility

Gross energy intake (GEI), digestive energy intake (DEI) and digestibility were measured (control,  $n=12$ , FR-50%,  $n=17$ ; FR-75%,  $n=8$ ) on day 13 (the day of food restriction) and day 16 (*ad libitum* feeding day). In detail, food was provided at 14:00 h, and any uneaten food or food mixed within the bedding was collected along with any faeces from each animal after 24 h (again at 14:00 h the next day). Food orts and faeces were separated manually after they were dried at 60°C to constant mass. Gross energy contents of the food ( $GE_{\text{food}}$ ;  $\text{kJ}\ g^{-1}$ ) and faeces ( $GE_{\text{faeces}}$ ;  $\text{kJ}\ g^{-1}$ ) were determined using an IKA C2000 oxygen bomb calorimeter (IKA, Germany). GEI ( $\text{kJ}\ \text{day}^{-1}$ ), DEI ( $\text{kJ}\ \text{day}^{-1}$ ), digestibility (%) and gross energy of faeces ( $\text{kJ}\ \text{day}^{-1}$ ) were calculated using the following equations (Zhao et al., 2010):

$$\text{GEI} = [\text{food provided} \times \text{dry matter content of food} - \text{dry spillage of food and uneaten food}] \times \text{GE}_{\text{food}}, \quad (1)$$

$$\text{DEI} = \text{GEI} - \text{GE}_{\text{faeces}}, \quad (2)$$

$$\text{Digestibility} = \text{DEI}/\text{GEI} \times 100\%, \quad (3)$$

$$\text{Gross energy of faeces} = M_{\text{faeces}} \times \text{GE}_{\text{faeces}}, \quad (4)$$

where food provided is in  $g\ \text{day}^{-1}$ , dry matter content is in %, dry spillage is in  $g\ \text{day}^{-1}$ , and  $M_{\text{faeces}}$  is the dry mass of faeces ( $g\ \text{day}^{-1}$ ). Gross energy content of faeces is in  $\text{kJ}\ g^{-1}$  (the energy content per gram faeces) and gross energy of faeces is in  $\text{kJ}\ \text{day}^{-1}$  (gross energy of faeces produced by the female per day).

### Daily energy expenditure and milk energy output

Daily energy expenditure (DEE) of females was measured on day 13 (the days of food restriction, control,  $n=19$ , FR-50%,  $n=26$ ; FR-75%,  $n=11$ ) and day 16 (*ad libitum* feeding day, control,  $n=7$ , FR-50%,  $n=9$ ; FR-75%,  $n=14$ ) of lactation, using the doubly labelled water (DLW) technique as described previously (Speakman, 1997; Król and Speakman, 2003). Briefly, females were weighed at the start of days 13 and 16, followed by the intraperitoneal injection of approximately 0.2 g of the DLW containing enriched  $^2\text{H}$  and  $^{18}\text{O}$ . The syringe was weighed before and immediately after the injection, using a Sartorius balance (to the nearest 0.1 mg). The initial blood samples were taken after 1 h of isotope equilibration to estimate initial isotope enrichments. The final blood samples were taken 24 h after the initial blood collection to estimate isotope elimination rates. Blood sample collections were performed by tail tipping, and immediately sealed into two 60  $\mu\text{l}$  glass capillaries at both sides using a butane torch, which were sealed again with sealing wax. DEE of the females was calculated based on  $\text{CO}_2$  production as described previously (Speakman, 1993; Król and Speakman, 2003). MEO was calculated from the difference between metabolizable energy intake (MEI) and DEE, during which MEI was calculated as  $\text{DEI} \times (100 - 3\%)$  because urinary energy loss was assumed to be 3% of DEI (Drożdż, 1975; Król and Speakman, 2003).

### Body composition and body fat content

Twelve females from each group were killed by decapitation at the end of day 13 of lactation, which started at 14:00 h on that restriction day. Twelve females from the control group were killed at the start of day 13. The heart, liver, lungs, spleen and kidneys, as well as the mammary glands, were removed and weighed (to the nearest 1 mg). The gastrointestinal tract was also separated, and weighed (to the nearest 1 mg) after the contents were removed. The remaining carcass (including head and tail) was weighed (to the nearest 1 mg)



to obtain the wet mass. All the materials were dried in an oven at 60°C for at least 2 weeks and then reweighed to obtain the dry mass (to the nearest 1 mg).

### Serum leptin, insulin, corticosterone, tumor necrosis factor $\alpha$ and prolactin measurements

Serum leptin, insulin, prolactin and corticosterone concentrations were determined by ELISA (control,  $n=6$ ; FR-50%,  $n=6$ ; FR-75%,  $n=6$ ). For mouse leptin (Leptin Mouse ELISA Kit, EK2972, MultiSciences Biotech, Co, LTD, China), the minimum detectable level was 2.48 pg ml<sup>-1</sup>, and the intra- and inter-assay coefficients of variations were 2.1% and 5.9%, respectively. For insulin (Insulin Mouse ELISA Kit, K4271-100, Biovision, Milpitas, CA, USA), the intra- and inter-assay coefficients of variations were 8% and 10%, respectively. For tumor necrosis factor  $\alpha$  (TNF $\alpha$ ; EK2821, MultiSciences Biotech), the minimum detectable level was 2.48 pg ml<sup>-1</sup>, and the intra- and inter-assay coefficients of variations were 6.1% and 7.6%, respectively. For serum prolactin (Mouse Prolactin ELISA Kit ab100736, Abcam, Cambridge, UK), the minimum detectable level was 30 pg ml<sup>-1</sup>. For corticosterone (Mouse Corticosterone ELISA Kit, No. 501320, Cayman Chemical Company, USA), the minimum detectable level was 8.2 pg ml<sup>-1</sup>, and the intra- and inter-assay coefficients of variations were 9.3% and 8.8%, respectively.

### Gene expression profiling of the hypothalamus

Six females from each group were killed by decapitation at the end of day 13 of lactation, the end of the restriction day (control,  $n=6$ ; FR-50%,  $n=6$ ; FR-75%,  $n=6$ ). Blood was collected for analysis of peripherally circulating hormone levels. The whole brain was separated carefully and frozen on aluminium foil on dry ice and stored at -80°C until RNA was extracted for the RNAseq measurements. The hypothalamus was carefully dissected at a later time using a cryostat and landmarks were identified from the mouse brain atlas. RNA was isolated by homogenizing in Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Prior to RNA quantification using the Agilent RNA 6000 Nano Kit, samples were denatured at 65°C.

Extracted RNA from all individuals was sent to the Beijing Genomic Institute (BGI, Shenzhen, China) for RNA sequencing. Library preparation was conducted by enriching total RNA using oligo(dT) magnetic beads. Fragmentation buffer was added to obtain short fragments from the RNA. The mRNA was used as a template for the random hexamer primers, which synthesize the first strand of cDNA. The second strand was synthesized by adding buffer dNTPs, RNase and DNA polymerase. A QiaQuick PCR extraction kit was used to purify the double-stranded cDNA and washed with EB buffer for end repair and single nucleotide A addition. The fragments were ligated with sequencing adaptors, purified using agarose gel electrophoresis and enriched by PCR amplification. As a quality control step, an Agilent 2100 Bioanalyzer and an ABI StepOnePlus Real-Time PCR System were used to qualify and quantify the sample library. The library products were sequenced using an Illumina Hi-seq 2000, resulting in 50 bp single-end reads (standard protocol, BGI). Standard primers and barcodes developed by BGI were used.

Prior to alignment to the reference genome, FASTQ files were quality controlled to identify the presence of adaptors or low quality sequences using fastQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). To ensure high sequencing quality, the reads were trimmed with a cut-off phred score of 28 using Trimmomatic (Bolger et al., 2014). Reads were aligned to the reference genome using HISAT2 (version 2.1.0) with default settings and a prebuild index (*Mus musculus*, GRCm38 release 81 version) (Kim et al.,

2015). Of the 432,335,789 reads, 398,725,650 (92.26%) were successfully aligned to the reference genome. Aligned sequencing reads were counted with featureCounts (Liao et al., 2014) by identification of how many reads mapped onto a single feature (genes containing exons).

### Statistics

Data were analyzed using SPSS 21.0 statistical software. Changes in body mass,  $T_b$ , litter size and litter mass over the period of lactation were examined using repeated-measures one-way ANOVA. Body mass, food intake, litter size and litter mass across 24 h of the restriction day were also examined using repeated-measures ANOVA. The effect of food restriction on body mass, food intake,  $T_b$ , litter size and litter mass, as well as GEI and digestibility, were analyzed using one-way ANOVA, followed by Tukey *post hoc* multiple comparisons where appropriate. The effect of food restriction on organ mass was examined using one-way ANCOVA, with carcass mass as a covariate. Pearson's correlation analysis was used to examine relationships between GEI and MEO, DEE and litter mass. Data are reported as means $\pm$ s.e.m. Statistical significance was determined at  $P<0.05$ .

Differential gene expression was modelled using the edgeR package (Robinson et al., 2009) in R (version 3.4.1) (<https://www.r-project.org/>). To remove any genes that exhibited no or a very low number of mapped reads, only genes that had more than 1 count per million (CPM) in at least two samples across all treatments were retained for further analysis. This resulted in a total of 15,780 unique genes. Read counts were normalized using the trimmed mean of M values (TMM normalization) (Robinson and Oshlack, 2010) to account for highly expressed genes consuming a substantial proportion of the total library size. This composition effect would cause remaining genes to be undersampled (Robinson et al., 2009). Pairwise comparisons were conducted between control and 50%-FR, and between control and 75%-FR groups. Comparisons were corrected for multiple testing using the Benjamini-Hochberg procedure. We next performed a partial least squares discriminant analysis (PLS-DA) on the normalized counts using the library mixOmics to predict whether our classification was representative of the variation observed in the dataset using R version 3.6.1 (Rohart et al., 2017). Volcano plots were used to visualize the statistical significance ( $P$ -value) versus the magnitude of the change [ $\log_2$  fold change (FC)] for the pairwise comparisons (i.e. 50%-FR versus control and 75%-FR versus control). These were made using the library ggplot2, and text labels for genes with an absolute  $\log_2$ FC above 2 were added using the library ggrepel in R version 3.6.1 (Wickham, 2016).

Logged raw expression levels (CPM) were regressed against the levels of peripheral circulating hormones (assay details below) in the same individuals. Pathway analysis was performed using Ingenuity Pathway Analysis (IPA; Qiagen Ltd). To perform the pathway analysis, we first trimmed the data to exclude genes for which the expression log ratio was less than 0.2. This yielded 3616 and 3833 differentially expressed genes (DEGs) in the 50%-FR and 75%-FR groups, respectively, relative to the control unrestricted mice. We overlaid these DEGs on a custom pathway containing the key hypothalamic genes linked to hunger and food intake (Speakman and Mitchell, 2011; Deros et al., 2016a,b). We generated two plots, one based on the log expression fold change and another based on the  $P$ -value of the difference detected. The pathway is available for use by registered users of IPA via the shared pathway function in IPA, by contacting the corresponding authors. In addition, we also explored changes in the canonical pathways available via IPA.

## RESULTS

## Body mass

The three groups did not differ in body mass on days 1 to 12 of lactation (day 1,  $F_{2,70}=1.21$ ,  $P>0.05$ ; day 12,  $F_{2,70}=1.12$ ,  $P>0.05$ ; Fig. 1A). Food restriction had a significant effect on body mass on days 13, 15 and 17, during which food-restricted mice showed significant lower body mass than the control group (day 13,  $F_{2,70}=52.66$ ,  $P<0.01$ ). The lowest body mass of food-restricted mice was observed on day 17 of lactation, and the body masses in the 50%-FR (42.8±0.6 g) and 75%-FR (38.7±0.7 g) groups were lower by 17.0% and 25.1% than that in the control group (51.6±0.8 g) (day 17,  $F_{2,70}=74.56$ ,  $P<0.01$ ). Body mass of food-restricted mice returned to the levels of controls following *ad libitum* refeeding on days 14, 16 and 18 (day 14,  $F_{2,70}=2.46$ ,  $P>0.05$ ; Table S1).

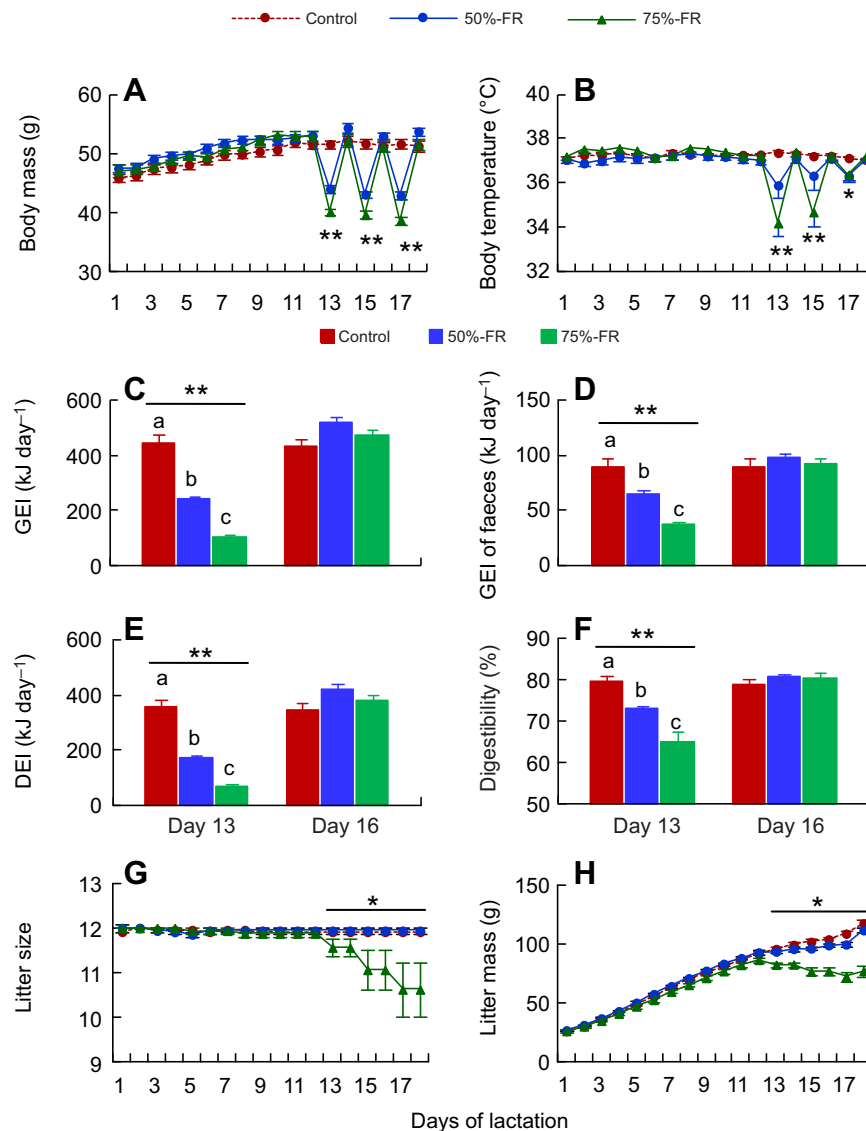
## Body temperature

As observed for body mass,  $T_b$  did not differ among the three groups before the food restriction started (day 1,  $F_{2,70}=0.32$ ,  $P>0.05$ ; day 12,  $F_{2,70}=1.57$ ,  $P>0.05$ ; Fig. 1B).  $T_b$  of the 50%-FR and 75%-FR groups significantly decreased on days 13, 15 and 17 of lactation relative to that of the control group (day 13, control, 37.4±0.1°C,

50%-FR, 35.9±0.2°C and 75%-FR, 34.2±0.6°C,  $F_{2,70}=24.10$ ,  $P<0.01$ ; day 15,  $F_{2,70}=11.06$ ,  $P<0.01$ ; day 17,  $F_{2,70}=3.77$ ,  $P<0.05$ ). No group differences were observed during *ad libitum* refeeding days (day 14,  $F_{2,70}=2.18$ ,  $P>0.05$ ; day 16,  $F_{2,70}=0.18$ ,  $P>0.05$ ; day 18,  $F_{2,70}=0.58$ ,  $P>0.05$ ; Table S1).

## Gross energy intake and digestibility

GEI, GE of faeces and DEI differed significantly among the three groups on the day of food restriction, and food-restricted females had lower GEI and DEI, and produced less faeces than the control group (day 13, GEI,  $F_{2,34}=58.71$ ,  $P<0.01$ ; Fig. 1C; GE of faeces,  $F_{2,34}=17.01$ ,  $P<0.01$ ; Fig. 1D; DEI,  $F_{2,34}=60.78$ ,  $P<0.01$ ; Fig. 1E, Table S1). Digestibility was also significantly different among the three groups, and food-restricted mice showed lower digestibility than that of the controls (day 13,  $F_{2,34}=19.43$ ,  $P<0.01$ , *post hoc*  $P<0.05$ ; Fig. 1F). During the *ad libitum* refeeding day, there were no significant differences among the three groups in GEI, DEI or digestibility (day 16, GEI,  $F_{2,34}=2.71$ ,  $P>0.05$ ; DEI,  $F_{2,34}=2.58$ ,  $P>0.05$ ; digestibility,  $F_{2,34}=0.94$ ,  $P>0.05$ ). The three groups produced a similar amount of faeces (day 16,  $F_{2,34}=0.63$ ,  $P>0.05$ ; Table S1).



**Fig. 1. Body mass, body temperature and energy intake of females, and size and mass of litters in response to food restriction.** Effects of food restriction on (A) body mass, (B) body temperature, (C) gross energy intake (GEI), (D) gross energy of faeces, (E) digestive energy intake (DEI), (F) digestibility of females, (G) litter size and (H) litter mass in lactating Swiss mice. Controls ( $n=23$ ), mice that were fed *ad libitum* throughout the lactation; 50%-FR ( $n=34$ ) and 75%-FR ( $n=16$ ) groups, females that were provided with 50% and 25%, respectively, of *ad libitum* food intake on days 13, 15 and 17 of lactation. The restricted food intake was calculated based on the average food intake during days 11 and 12. Data are means±s.e.m. Asterisks indicate a significant effect of food restriction (\* $P<0.05$ , \*\* $P<0.01$ ).

### Litter size and litter mass

Litter size of the 75%-FR group decreased significantly on day 13 and thereafter compared with that of the control and 50%-FR groups (day 13,  $F_{2,70}=6.19$ ,  $P<0.01$ ; day 18,  $F_{2,70}=8.64$ ,  $P<0.01$ ; Fig. 1G, Table S1). On the day of weaning, litter size in the 75%-FR group ( $10.6\pm 0.6$ ) was lower by 10.8% and 11.2% than that of the control ( $11.9\pm 0.1$ ) and 50%-FR groups ( $11.9\pm 0.0$ ) (day 18, *post hoc*  $P<0.05$ ), respectively, whereas there was no difference among the control and 50%-FR groups (day 18, *post hoc*  $P>0.05$ ). Litter mass did not differ among the three groups before the food restriction treatment (day 12,  $F_{2,70}=2.92$ ,  $P>0.05$ ; Fig. 1H), whereas it significantly decreased in the 75%-FR group ( $82.1\pm 2.5$  g) compared with the control ( $95.5\pm 2.3$  g) and 50%-FR groups ( $93.3\pm 1.5$  g) on day 13 and thereafter (day 13,  $F_{2,70}=10.11$ ,  $P<0.01$ ; day 18,  $F_{2,70}=38.26$ ,  $P<0.01$ ). In detail, litter mass of the 75%-FR group on day 13 was lower by 14.0% and 12.0%, respectively, compared with that of the control and 50%-FR groups (*post hoc*  $P<0.05$ ), while there was no significant differences between the control and 50%-FR groups (*post hoc*  $P>0.05$ ).

### Body mass, food intake, litter size and litter mass on food restriction day

Body mass did not differ among the three groups at the start of the restriction day (0 h,  $F_{2,43}=0.02$ ,  $P>0.05$ ; Fig. 2A, Table S1). Food restriction caused a significant decrease in body mass over a 24-h period, and it decreased by 9.8±1.03% and 21.3±1.18% after 24 h food restriction in the 50%-FR and 75%-FR groups, respectively, compared with 0 h ( $F_{8,360}=39.75$ ,  $P<0.01$ ). Body mass of the 75%-FR group was significantly lower than other two groups at 9 h and thereafter (9 h,  $F_{2,43}=4.74$ ,  $P<0.05$ ; 24 h,  $F_{2,43}=18.98$ ,  $P<0.01$ ).

Food intake was similar between the three groups at the first two 3-h intervals, but it was significantly lower in the 75%-FR group than in the other two groups at 9 h and thereafter (9 h, control,  $3.48\pm 0.33$  g 3 h<sup>-1</sup>, 50%-FR,  $3.86\pm 0.19$  g 3 h<sup>-1</sup> and 75%-FR,  $0.43\pm 0.21$  g 3 h<sup>-1</sup>,  $F_{2,43}=59.22$ ,  $P<0.01$ ; Fig. 2B, Table S1).

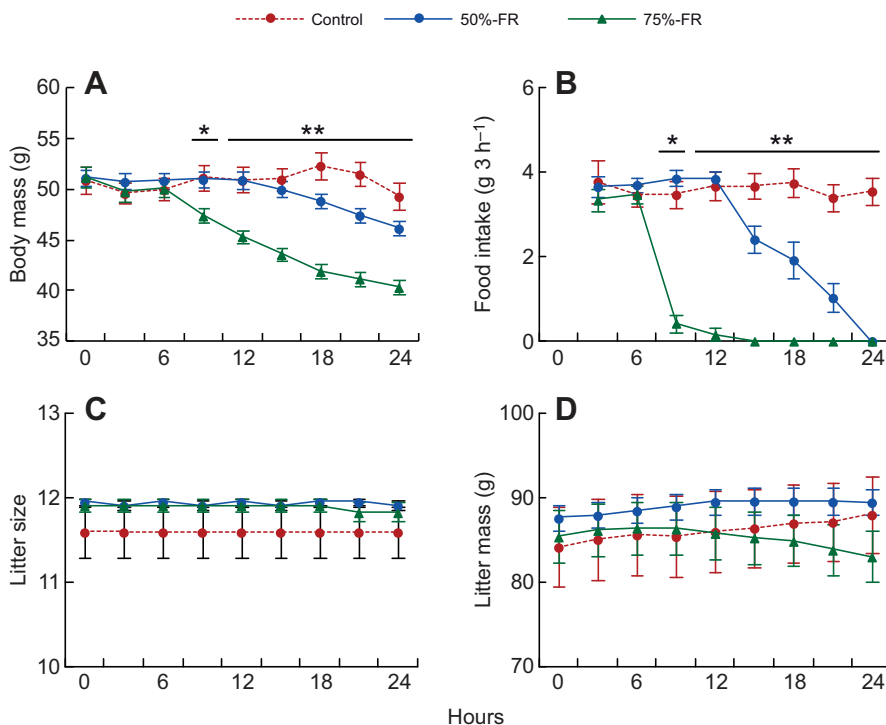
In fact, almost no food was available for the females in the 75%-FR group after 9 h. Food intake of the 50%-FR group was significantly lower from 15 to 24 h (15 h,  $F_{2,43}=23.98$ ,  $P<0.01$ , 24 h,  $F_{2,43}=216.19$ ,  $P<0.01$ ).

No difference in litter size was observed among the groups at any time points of food restriction (0 h,  $F_{2,43}=1.97$ ,  $P>0.05$ ; Fig. 2C, Table S1). Litter mass of the control group increased by  $4.6\pm 0.8\%$  at 24 h compared with the start (repeated-measures ANOVA,  $F_{8,72}=38.35$ ,  $P<0.01$ ; Fig. 2D), and increased by  $2.1\pm 0.3\%$  in the 50%-FR group (repeated-measures ANOVA,  $F_{8,280}=7.39$ ,  $P<0.01$ ), whereas it decreased by  $2.8\pm 0.7\%$  in the 75%-FR group (repeated-measures ANOVA,  $F_{8,28}=22.22$ ,  $P<0.01$ ). However, litter mass was not statistically different among the three groups (24 h,  $F_{2,43}=1.66$ ,  $P>0.05$ ; Table S1). Litter mass was positively correlated with GEI during the restriction day ( $r=0.37$ ,  $P<0.01$ ; Fig. S1A), but no correlation was observed on the refeeding day.

### Daily energy expenditure and milk energy output

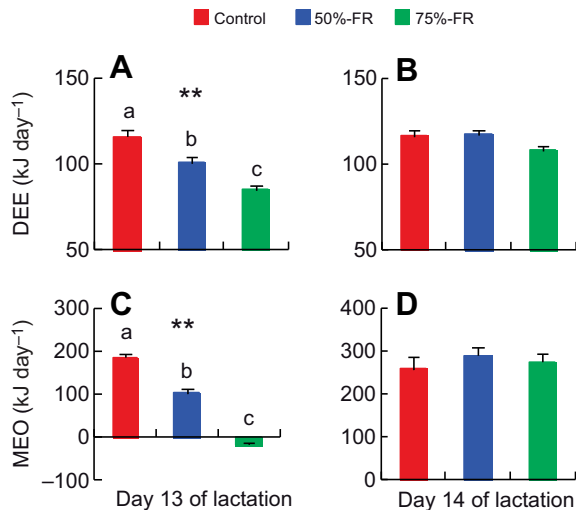
DEE of females on day 13 was significantly affected by food restriction ( $F_{2,53}=15.73$ ,  $P<0.01$ ; Fig. 3A, Table S1), and it decreased by 13.4% and 26.9% in the 50%-FR ( $100.6\pm 3.1$  kJ day<sup>-1</sup>) and 75%-FR ( $84.8\pm 2.3$  kJ day<sup>-1</sup>) groups, respectively, compared with the control group ( $116.1\pm 3.8$  kJ day<sup>-1</sup>) (*post hoc*  $P<0.05$ ). On day 16, the three groups did not differ in DEE ( $F_{2,27}=1.62$ ,  $P>0.05$ ; Fig. 3B). Food restriction had a significant effect on MEO, which decreased notably on day 13 of lactation ( $F_{2,53}=61.86$ ,  $P<0.01$ ; Fig. 3C). In detail, MEO decreased by 44.7% in the 50%-FR group compared with the control group (*post hoc*  $P<0.05$ ). MEO in the 75%-FR group was on average  $-17.1\pm 3.2$  kJ day<sup>-1</sup>, much lower than that in the other two groups (*post hoc*  $P<0.05$ ). MEO on the refeeding day (day 16 of lactation) was not different among the three groups ( $F_{2,27}=0.43$ ,  $P>0.05$ ; Fig. 3D, Table S1).

DEE was positively correlated with GEI on the restriction day (day 13,  $r=0.60$ ,  $P<0.01$ ) and refeeding day (day 16,  $r=0.60$ ,  $P<0.01$ ; Fig. S1B). There was a positive correlation between MEO



**Fig. 2. Body mass and food intake of females, and size and mass of litter on food restriction day.**

Changes in (A) body mass, (B) food intake, (C) litter size and (D) litter mass over a 24 h time course in food-restricted Swiss mice on day 13 of lactation. Controls ( $n=10$ ), mice that were fed *ad libitum* throughout the lactation; 50%-FR ( $n=24$ ) and 75%-FR ( $n=12$ ) groups, females that were provided with 50% and 25%, respectively of *ad libitum* food intake on days 13, 15 and 17 of lactation. Data are means  $\pm$  s.e.m. Asterisks indicate a significant effect of food restriction (\* $P<0.05$ , \*\* $P<0.01$ ).



**Fig. 3. The daily energy expenditure (DEE) and milk energy output (MEO) on restriction day and refeeding day.** Effects of food restriction on (A,B) DEE and (C,D) MEO in lactating Swiss mice. Controls (con,  $n=19$ ), mice that were fed *ad libitum* throughout the lactation; 50%-FR ( $n=26$ ) and 75%-FR ( $n=11$ ) groups, the females that were provided with 50% and 25%, respectively, of *ad libitum* food intake on days 13, 15 and 17 of lactation. Data are means  $\pm$  s.e.m. Asterisks indicate a significant effect of food restriction (\*\* $P<0.01$ ). Different letters (a, b or c) above the columns indicate significant difference between the groups ( $P<0.05$ ).

and GEI (day 13,  $r=0.98$ ,  $P<0.01$ ; day 16,  $r=0.97$ ,  $P<0.01$ ; Fig. S1C). MEO was also correlated with DEE (day 13,  $r=0.44$ ,  $P<0.01$ ; day 16,  $r=0.44$ ,  $P<0.05$ ; Fig. S1D). Litter mass was correlated with DEE ( $r=0.43$ ,  $P<0.01$ ; Fig. S1E) and MEO on day 13 ( $r=0.33$ ,  $P<0.05$ ; Fig. S1F), but the correlations were not significant on day 16. As indicated in more detail in the Discussion, these estimates of MEO are likely compromised to some extent by the strongly dynamic changes in food intake in the restricted animals.

### Body composition

Masses of wet and dry carcass were significantly different among the four groups, and they were lower in the 75%-FR group than in the other three groups (Table S2). Liver mass was significantly lighter in the food-restricted groups than in the two control groups. Masses of spleen and kidneys were significantly affected by food restriction, and the minimum was observed in the 75%-FR group. The four groups differed significantly in the masses of digestive tracts, including empty masses of the stomach, small intestine, large intestine and caecum, which were significantly decreased in the 75%-FR group compared with the control groups (Table S2). Masses of the mammary glands were also significantly different among the four groups, and the wet and dry masses were 38.2% and 39.3% lower, respectively, in the 75%-FR group than in the control group (Table S2).

### Gene expression profiling of the hypothalamus

The multidimensional scaling (MDS) plot indicated that the three groups (unrestricted, 50%-FR and 75%-FR) could be separated based on the major axes of the gene expression profile (Fig. 4A). We explored the changes in gene expression for the two restriction groups (50%-FR and 75%-FR) compared with the control unrestricted group using standard bioinformatics tools. The volcano plots for the contrasts of 50%-FR and 75%-FR to the control are shown in Fig. 4B,C. This shows a number of significantly upregulated and downregulated genes in both conditions. The full list of differentially

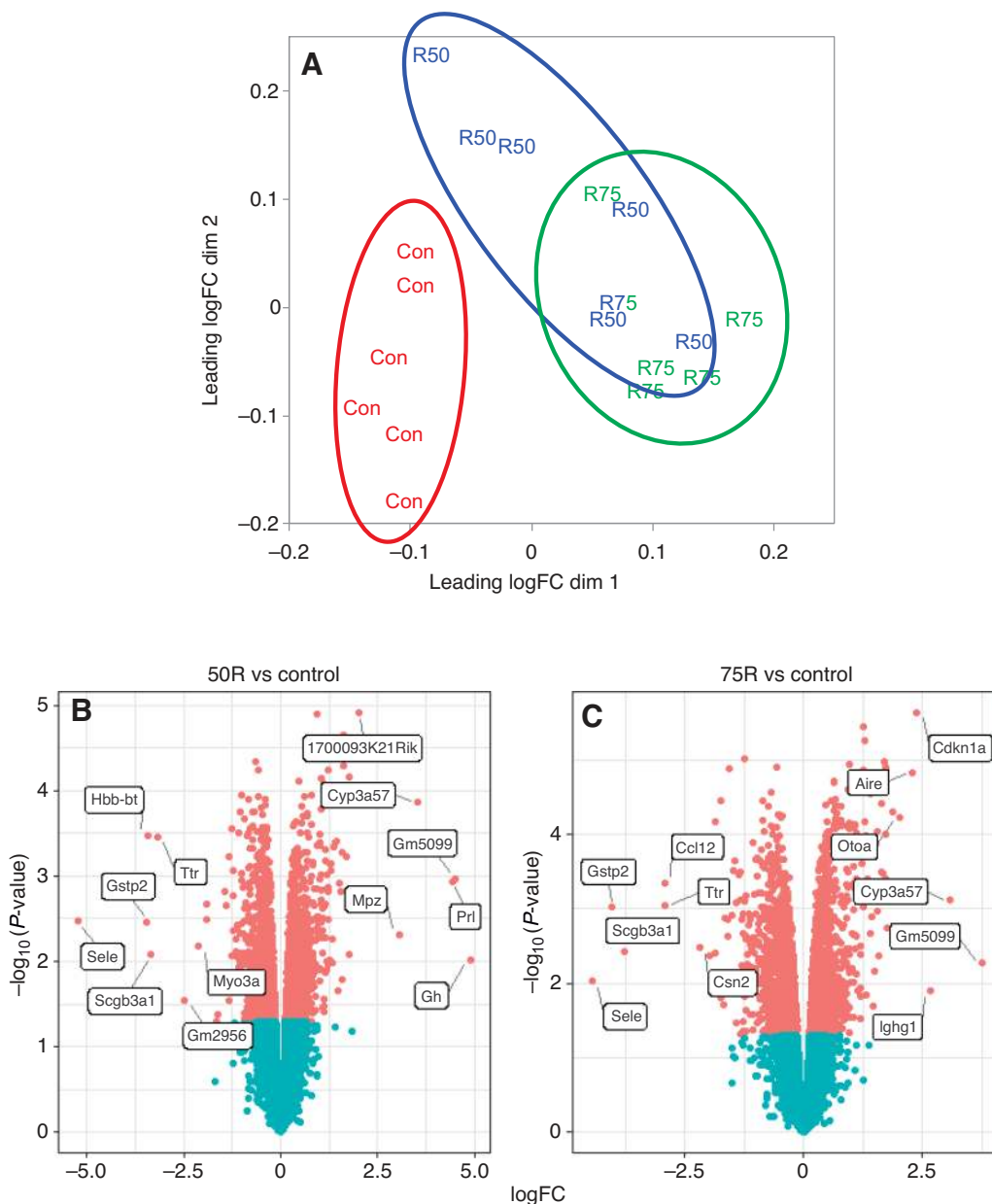
expressed genes comparing the 50%-FR and 75%-FR groups with the control unrestricted group is available in Tables S3 and S4, including  $P$ -values for the contrasts and the false discovery rate (FDR). For the 50% restriction group, the largest log fold changes ( $>2$ ) were for the following upregulated genes, growth hormone (*Gh*), 5099, prolactin (*Prl*), cytochrome p450 family 3 subfamily a polypeptide 57 (*Cyp3a57*) and myelin protein zero (*Mpz*), and for the following downregulated genes, selectin E (*Sele*), glutathione S-transferase pi 2 (*Gstp2*), haemoglobin beta adult t chain (*Hbb-bt*), secretoglobin family 3a member 1 (*Scgb3a1*), transthyretin (*Ttr*), *Gm2956* and myosin 3a (*Myo3a*). For the contrast between the control and 75%-FR groups, the largest fold changes ( $>2$ ) were for the following upregulated genes, *Cyp3a57*, *Gm5099*, cyclin dependent kinase 1a (*Cdkn1a*), otoancorin (*Otoa*), Leucine rich glioma activated 1 (*Lghg1*), and autoimmune regulator (*Aire*), and the following downregulated genes, *Gstp2*, *Ttr*, *Sele*, *Scgb3a1*, *Ccl12* and caesin2 (*Csn2*). Clearly, there was substantial overlap in the responses to the two levels of restriction. A network diagram built in the Ingenuity Pathway Analysis program (Qiagen) was used for the main genes involved in hunger signalling (Derous et al., 2016a,b). When overlaid onto the gene expression profiles on this pathway, we found at 50% restriction there were no changes in the inhibitory arm of the network, but on the stimulation side there was significant upregulation of neuropeptide Y (*Npy*) (expFC=0.79, LR=6.2,  $P=0.0125$ ) but a downregulation of Agouti regulated peptide (*AgRP*) (expFC=-0.654, LR=10.98,  $P=0.00091$ ). There were smaller, mostly non-significant, changes in the main populations of melanocortin, dopamine, serotonin and opioid receptors, except opioid receptor mu1 was significantly downregulated (expFC=-0.46, LR=11.6,  $P=0.00065$ ; Fig. 5). At 75% restriction, there were also no significant changes in the inhibitory arms of the system, coupled with downregulation of *AgRP* (expFC=-0.46, FC=5.56,  $P=0.018$ ) and upregulation of *Npy* (expFC=1.375, FC=18.57,  $P=0.0029$ ; Fig. 6). Levels of the long form of the leptin receptor (*Lepr*) were also significantly upregulated (expFC=0.57, LR=8.83,  $P=0.0029$ ) as well as downregulation of the downstream intracellular signalling molecule Suppressor of cytokine signalling 3 (SOCS3) (expFC=-1.106, FC=10.3,  $P=0.0057$ ), which is a negative regulator of leptin signalling. Neuropeptide Y receptor 1 (*Npyr1*) was also significantly upregulated (expFC=0.485, LR=10.78,  $P=0.052$ ).

We used the IPA software to explore other significantly altered pathways in the hypothalamus linked to the restriction based on the FDR values of the analysed genes. One of the most significantly upregulated pathways in the 50%-FR to control comparison was *Eif2* signalling. This pathway includes 221 components, of which 52 were modulated under 50%-FR ( $P=3.3\times 10^{-5}$ ,  $z=2.26$ ; Fig. 7). At both levels of restriction, the unfolded protein response pathway was downregulated relative to the controls (50%-FR, 19/56 genes altered,  $z=-1.68$ ,  $P=6.4\times 10^{-5}$ ; 75%-FR, 21/56 genes altered,  $z=-1.606$ ,  $P=1.46\times 10^{-5}$ ; Fig. 8). Similarly, at both levels of restriction the neuroinflammation pathway was also downregulated (50%-FR, 61/300 genes altered,  $z=-1.82$ ,  $P=4.06\times 10^{-4}$ ; 75%-FR, 69/300 genes altered,  $z=-5.25$ ,  $P=3.3\times 10^{-5}$ ; Fig. 9).

### Relationship of gene expression in the hypothalamus to circulating hormone levels

Circulating levels of leptin and prolactin were decreased significantly in the 50%-FR and 75%-FR animals (leptin,  $F_{2,15}=14.48$ ,  $P<0.01$ ; prolactin,  $F_{2,15}=57.07$ ,  $P<0.01$ ), while insulin and corticosterone levels were increased. Levels of TNF $\alpha$  were unchanged (insulin,  $F_{2,15}=3.56$ ,  $P<0.05$ ; corticosterone,  $F_{2,15}=4.42$ ,  $P<0.05$ ; Fig. S2).





**Fig. 4. The multidimensional scaling plot and volcano plot in food-restricted mice at peak lactation.** (A) Multidimensional scaling plot showing locations of the individual control ( $n=6$ ), 50% restricted (R50,  $n=6$ ) and 75% restricted (R75,  $n=6$ ) mice in relation to the two major dimensions of gene expression in the hypothalamus. The restricted animals are clearly separated from the controls and also divergent in relation to the level of restriction. (B,C) Volcano plot for the contrasts of 50%-FR and 75%-FR to control. All mice were at peak lactation.

Expression levels of many of the significantly altered genes in the hypothalamus were correlated with the levels of circulating leptin, insulin, prolactin and corticosterone, but less so with circulating levels of *Tnf- $\alpha$*  (Fig. 10). We further explored these correlations for the key genes in the hunger pathway (Fig. 11). Neuropeptide Y (*Npy*) gene expression was negatively correlated with both circulating leptin and prolactin levels. Conversely, *Npy* was positively associated with insulin and corticosterone levels. In contrast, Agouti-related peptide (*AgRP*) showed a positive correlation with circulating leptin and prolactin, and a negative correlation with corticosterone, while insulin showed no relationship. Neither proopiomelanocortin (*Pomc*) nor cocaine- and amphetamine-regulated transcript (*Cart*) had a relationship with the circulating hormone levels. Leptin receptor expression was negatively correlated with circulating leptin and prolactin. Suppressor of cytokine signalling 3 (*Socs3*) expression was positively correlated with leptin and prolactin. Expression levels of signal transducer and activator of transcription (*Stat3* and *Stat5b*) were unrelated to the circulating hormones apart from *Tnf- $\alpha$* ,

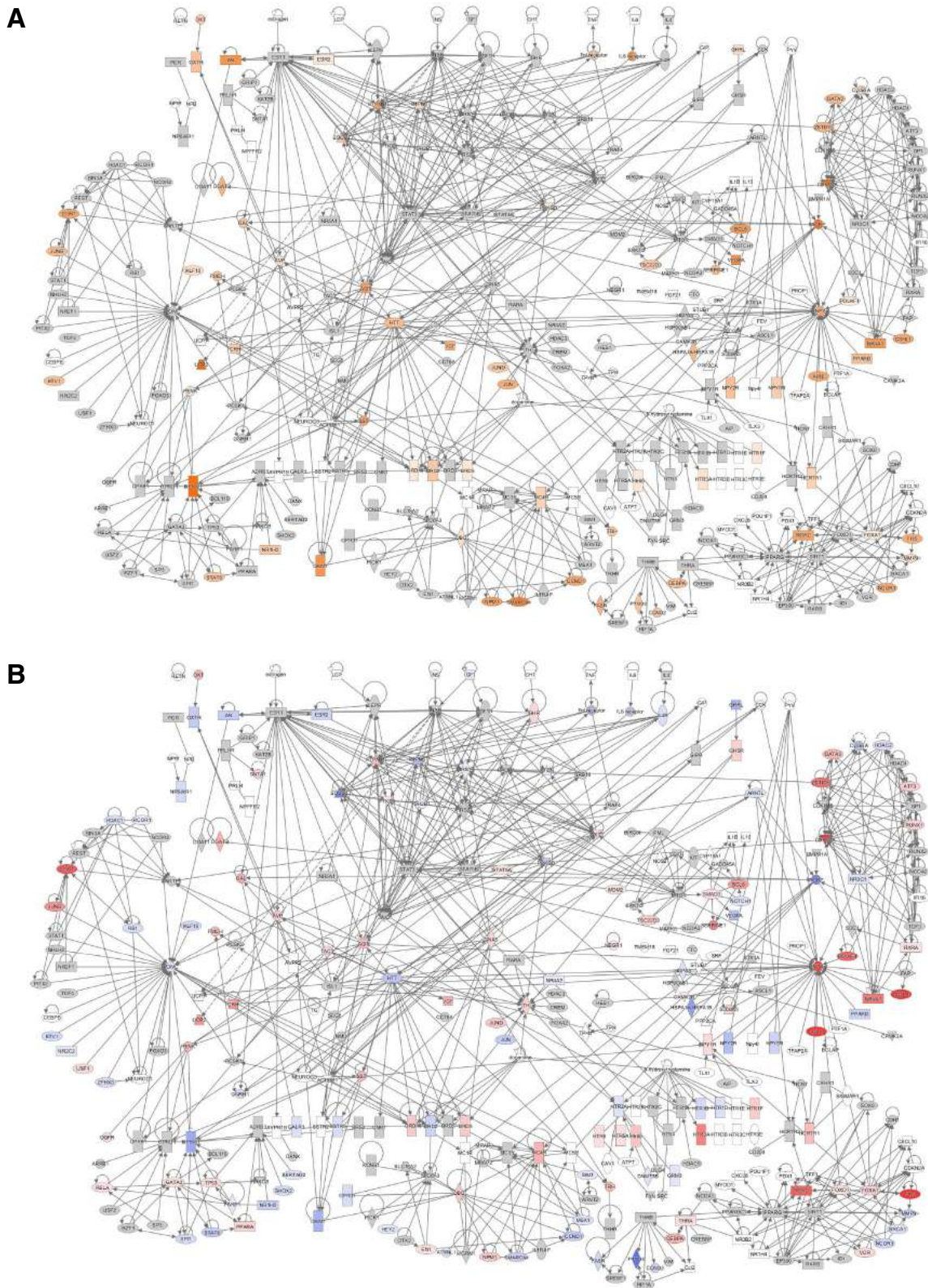
probably reflecting the fact that these intracellular signalling proteins are functionally regulated via phosphorylation rather than transcriptionally (Fig. 11).

## DISCUSSION

### Maternal responses to days of restricted intake

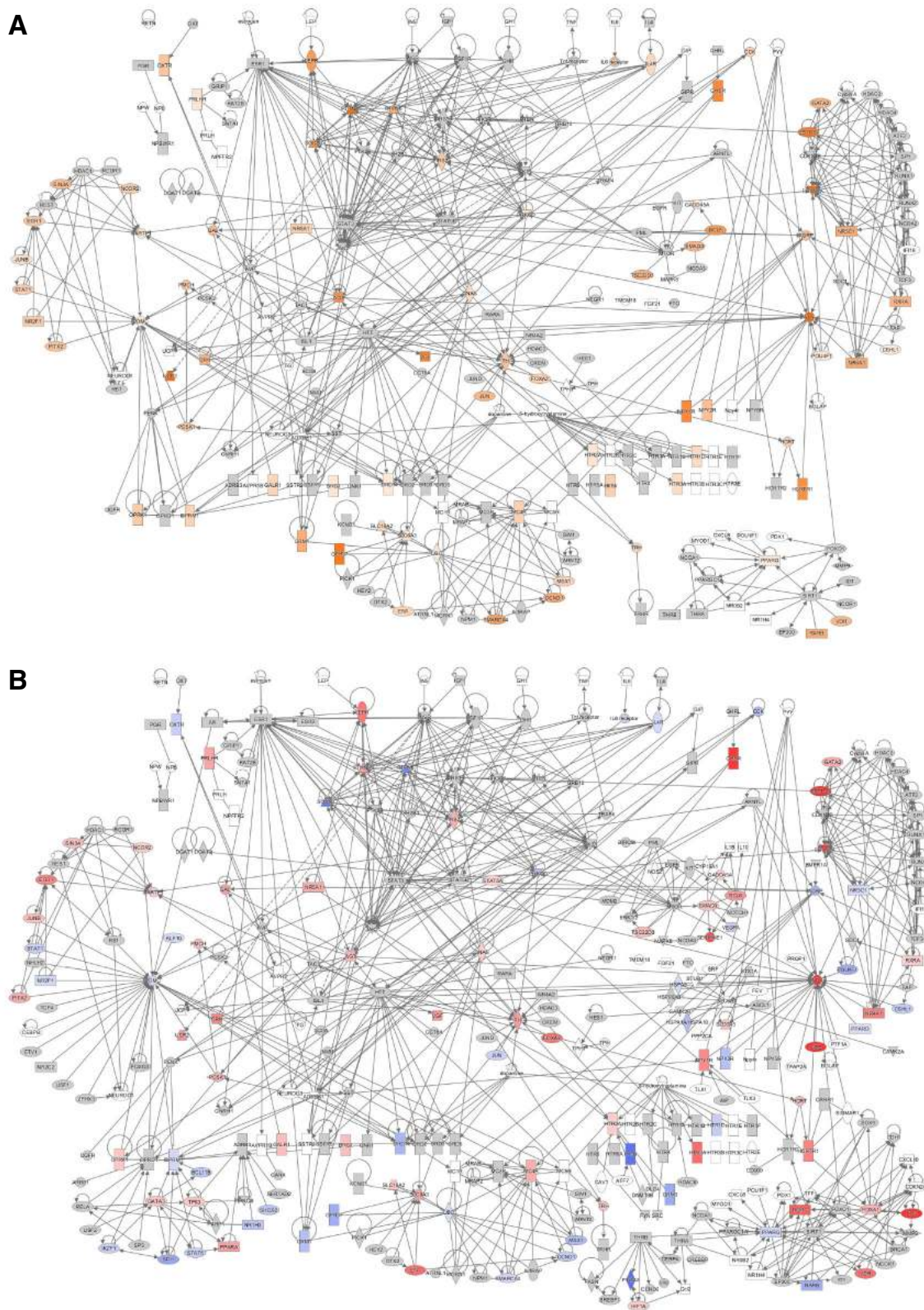
When female mice were given less food during peak lactation, they continued to eat at the same rate as under *ad libitum* conditions until their food ran out. They then starved completely until the food was replenished the next day. In this way, lactating mice were similar to non-lactating individuals that are given restricted food that also eat their entire reduced ration relatively quickly, and then completely starve for the rest of the day (Hambly and Speakman, 2015). Mice made a number of responses to make up for the shortfall in energy. This included a reduction in body temperature, which is also observed in non-lactating individuals under chronic restriction (Duffy et al., 1989, 1997; Rikke et al., 2003; Tabarean et al., 2010; Mitchell et al., 2015a). On the first day of restriction, this body



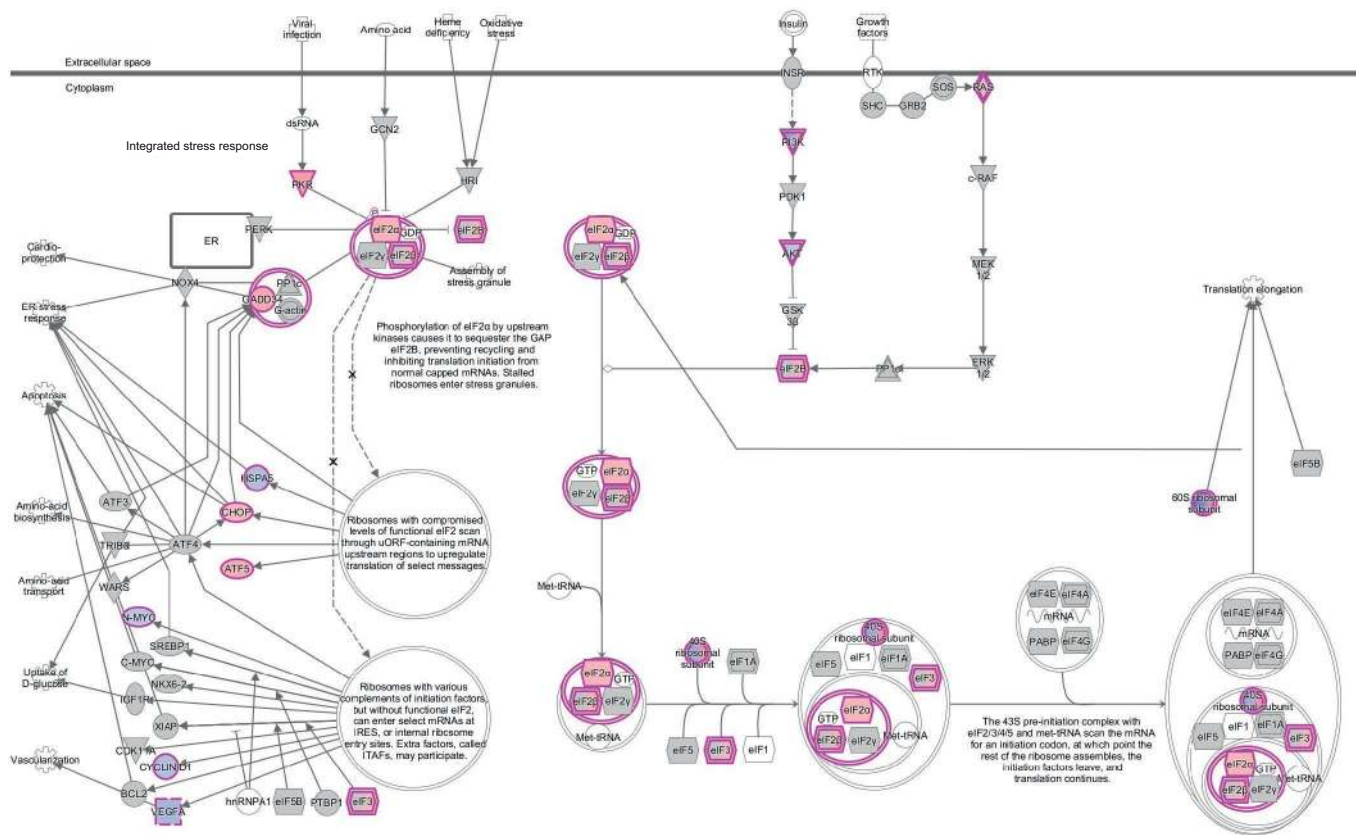


**Fig. 5.** Gene expression profile in the hunger signalling pathway of the hypothalamus (pathway from Deros et al., 2016a) at peak lactation, comparing mice under 50%-FR with those unrestricted. Levels under each node of the network indicate the gene at that node. Edges represent known interactions between genes from the literature as indicated by the IPA software. (A) Significance of the differences. Differences where  $P < 0.05$  are in light orange and greater significance is indicated by a greater depth of colour. (B) Directions of the differences. Red indicates upregulation and blue indicates downregulation in the restricted group. Depth of colour is magnitude of change.





**Fig. 6.** Gene expression profile in the hunger signalling pathway of the hypothalamus (pathway from Deros et al., 2016a) at peak lactation, comparing mice under 75% restriction with those unrestricted. Levels under each node of the network indicate the gene at that node. Edges represent known interactions between genes from the literature as indicated by the IPA software. (A) Significance of the differences. Differences where  $P < 0.05$  are in light orange and greater significance is indicated by a greater depth of colour. (B) Directions of the differences. Red indicates upregulation and blue downregulation in the restricted group. Depth of colour indicates magnitude of change.



**Fig. 7. Changes in the gene expression of the eif2a signalling pathway comparing control lactating mice with lactating mice under 50% food restriction.** Levels under each node of the network indicate the gene at that node. Edges represent known interactions between genes from the literature as indicated by the IPA software. Double-grey lines link together genes the proteins from which complexes form. Blue indicates downregulation in the experimental restricted groups and red indicates upregulation. The pathway was stimulated (for statistics, see Results). Pathway generated by IPA.

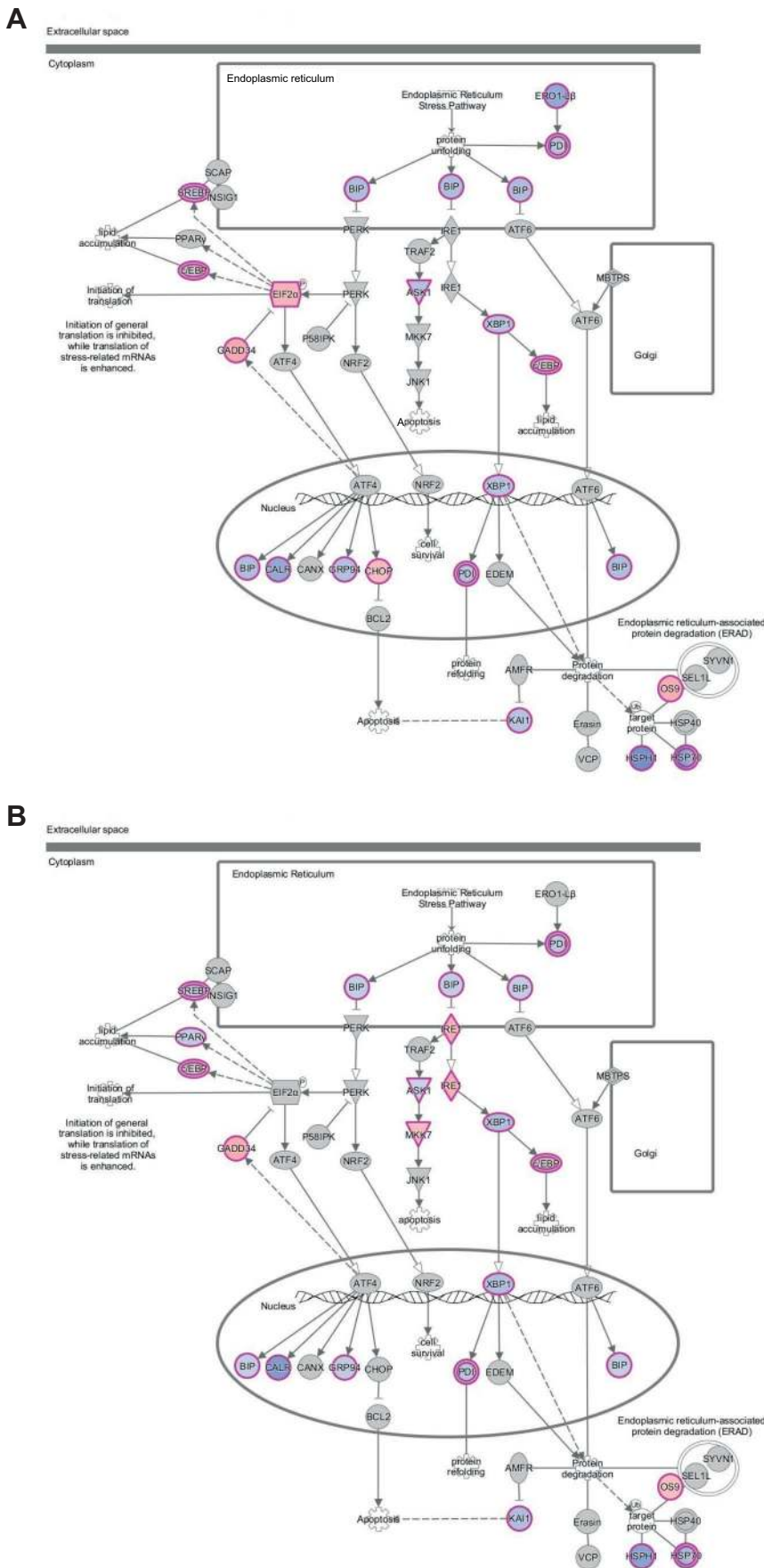
temperature change was much greater in the mice under 75% restriction compared with the mice under 50% restriction (Fig. 1B), which also matches the graded response in non-lactating individuals (Mitchell et al., 2015a). In non-lactating mice under restriction, there was an increase in digestive efficiency (Mitchell et al., 2015b). In contrast, the lactating mice appeared to decrease their digestibility. It seems most likely that this is just an artefact of fecal production being time lagged relative to intake, thus some of the fecal production on the day of restriction would pertain to food taken in the day previous. The fact that apparent digestion decreased more in mice under 75% restriction is consistent with this interpretation.

The most profound changes, however, were in daily energy expenditure and milk production. Mice under 50%-FR scaled back their milk production to approximately 55.3% of that in the controls, and mice under 75%-FR actually had an average calculated negative milk production of 17 kJ day<sup>-1</sup> (Fig. 3C). This negative value is probably a direct consequence of the carried over fecal production, which affected the calculated DEI and thus also the calculated MEO. That is, the digested energy intake was probably slightly underestimated because faeces derived from food ingested the previous day were subtracted from the supplied ration to yield the digested energy intake, and because MEO is calculated as the difference between DEI and DEE (Król and Speakman, 2003), this led to an underestimate of actual milk production. Nevertheless, this suggests that under 75% restriction, milk production was probably almost completely suspended. Because the pups continued to suckle even though the females had ceased to produce milk, the mammary glands were probably completely emptied and this likely explains

most of the difference in mass of the mammary glands between the groups in female mice killed at the end of the first restriction day. The strong correlation between the measured DEE, DEI and MEO indicated that the main mechanism underpinning the reduction of DEE was the reduction in milk synthesis. This is consistent with the fact that mice in lactation have very low levels of physical activity (Gamo et al., 2013; Zhao et al., 2013) and hence the scope to reduce physical activity is limited. Plus, non-lactating mice on chronic restriction generally do not reduce activity anyway (Mitchell et al., 2016).

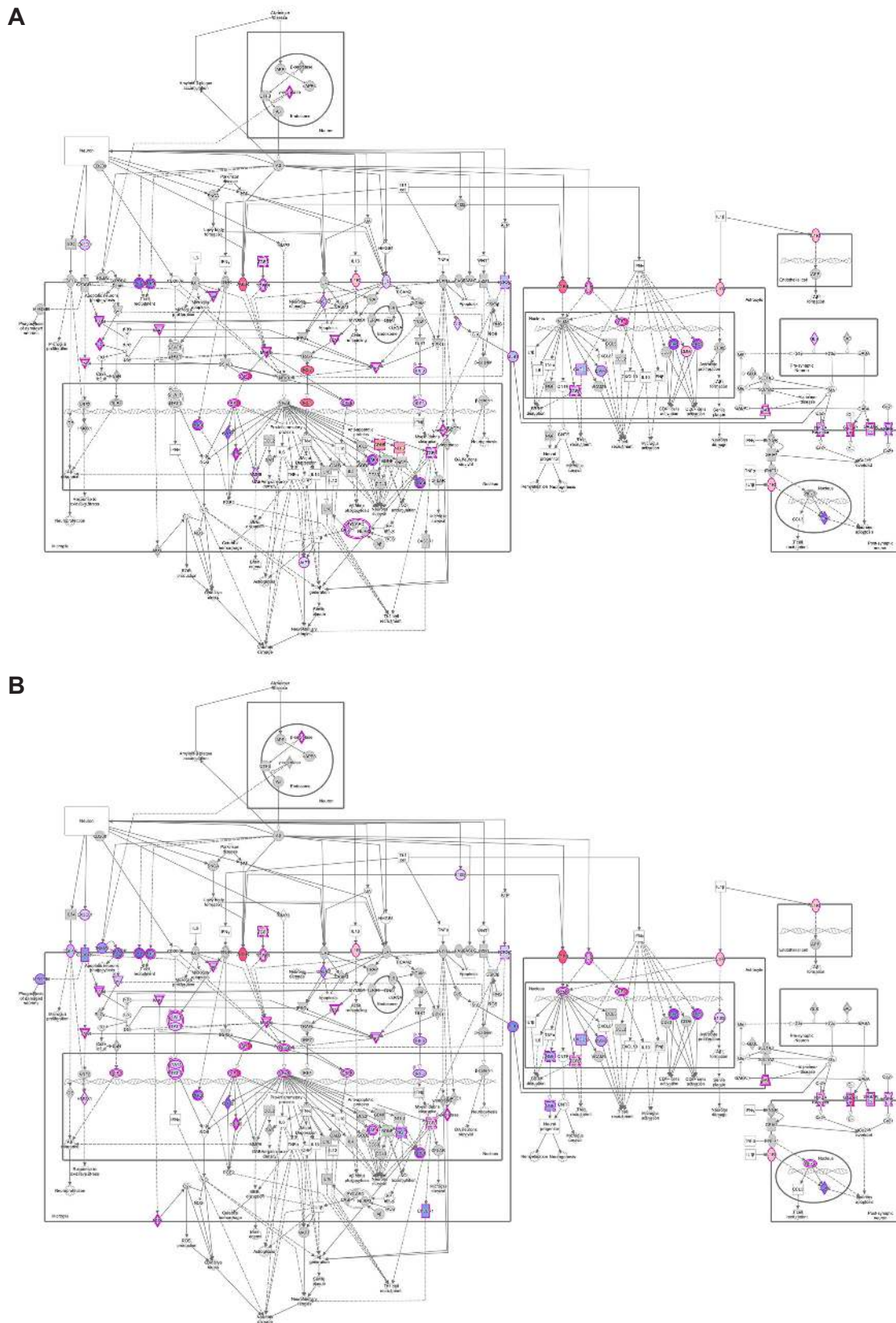
There were two direct consequences of suspending milk production in the 75% restriction group. First, growth of the pups almost completely stopped. This probably meant that the milk they got on the intervening days when the female was feeding normally was only sufficient to cover their accumulated metabolic energy expenditure over the paired restriction and refeeding days, with none left to support growth. Second, the females lost some of their pups. Yet, given the extent to which food was restricted, the losses were relatively slight. Indeed, the mice with 50%-FR did not lose any pups, and their growth was almost normal compared with that of the control animals. This suggests that the pups probably enabled compensation mechanisms to reduce their energy demands and hence make better use (in terms of growth efficiency) of their reduced milk supply. The offspring at 75% food restriction probably did the same, which is why so few of them died, but they were simply unable to compensate enough to sustain their growth. Mothers generally ate the pups that were lost, hence discerning the cause of death was difficult. It is unclear whether mothers waited for



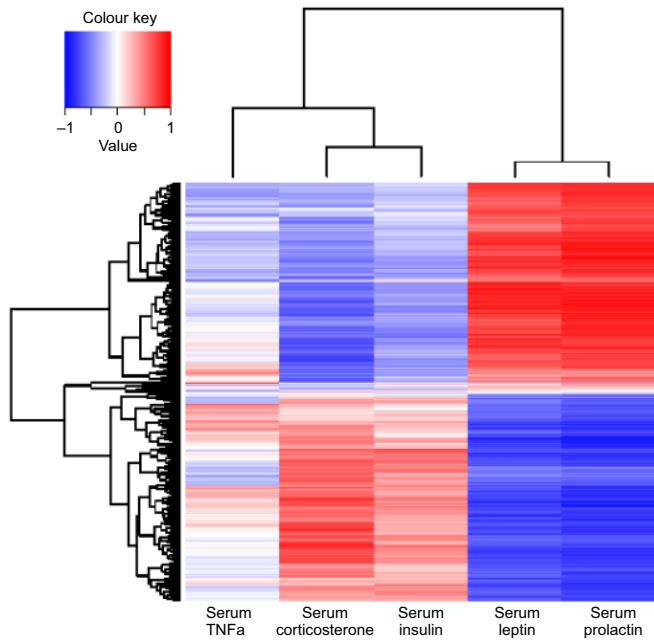


**Fig. 8. Changes in the gene expression (fold change) of the unfolded protein response signalling pathway comparing control lactating mice ( $n=6$ ) with lactating mice under 50% or 75% food restriction. (A) 50% food restriction (R50,  $n=6$ ); (B) 75% food restriction (R75,  $n=6$ ). Levels under each node indicate the gene at that node. Edges represent known interactions between genes from the literature as indicated by the IPA software. Double-grey lines link together genes the proteins from which form complexes. Blue indicates downregulation in the experimental restricted groups and red indicates upregulation. At both levels of restriction, the pathway is inhibited, but more significantly under 75% restriction (for statistics, see Results). Pathway generated by IPA.**





**Fig. 9.** Changes in the gene expression (fold change) of the neuroinflammation signalling pathway comparing control lactating mice ( $n=6$ ) with lactating mice under 50% or 75% food restriction. (A) 50% food restriction (R50,  $n=6$ ); (B) 75% food restriction (R75,  $n=6$ ). Levels under each node of the network indicate the gene at that node. Edges represent known interactions between genes from the literature as indicated by the IPA software. Blue indicates downregulation in the experimental restricted group and red indicates upregulation. The pathway was inhibited under restriction (for statistics, see Results). Pathway generated by IPA.



**Fig. 10. Heat map showing the expression patterning in the hypothalamus of the top 400 altered genes (FDR<0.1) in relation to circulating hormone levels.** Positive correlations are in red and negative in blue. The large-scale patterns of hypothalamic gene expression were associated with the peripheral circulating levels of leptin, insulin, corticosterone and prolactin, but less so TNFa.

them to succumb or whether they actively killed them. It is conceivable that mothers may actively kill pups to reduce demand. The ability of mice to rapidly switch off milk supply to protect themselves, and for their pups to respond to this change by using their milk supply more frugally, is probably an adaptive response to variable food supplies in the wild.

Females also lost considerable body mass during the restriction days, but a large part of this was probably gut fill. Nevertheless, measurements of body composition in mice killed at the end of the restriction day showed that they were also extracting energy from tissues, most notably the liver, skeletal muscle (carcass) and kidneys, with much less from the heart and lungs. Non-lactating mice also withdraw energy from lean tissues when they are under longer-term chronic restriction (Mitchell et al., 2015b,c) with a hierarchy of tissue use, where heart and lungs are protected relative to the liver, muscle and kidneys. In the case of liver and skeletal muscle, a part of this loss over the 24 h of acute restriction observed here is potentially glycogen utilization and associated water loss.

The overall strategy of the females when under restriction appeared to be to protect themselves, and to sacrifice the export of energy to the litter. This impacted growth and, to a small extent in the 75% restriction group, offspring survival. This strategy makes sense in terms of fitness because if the restriction was to be prolonged then it would be better for at least the female to survive to breed again in the future, rather than both the mother and the current litter to perish.

#### Maternal responses on the refeeding days

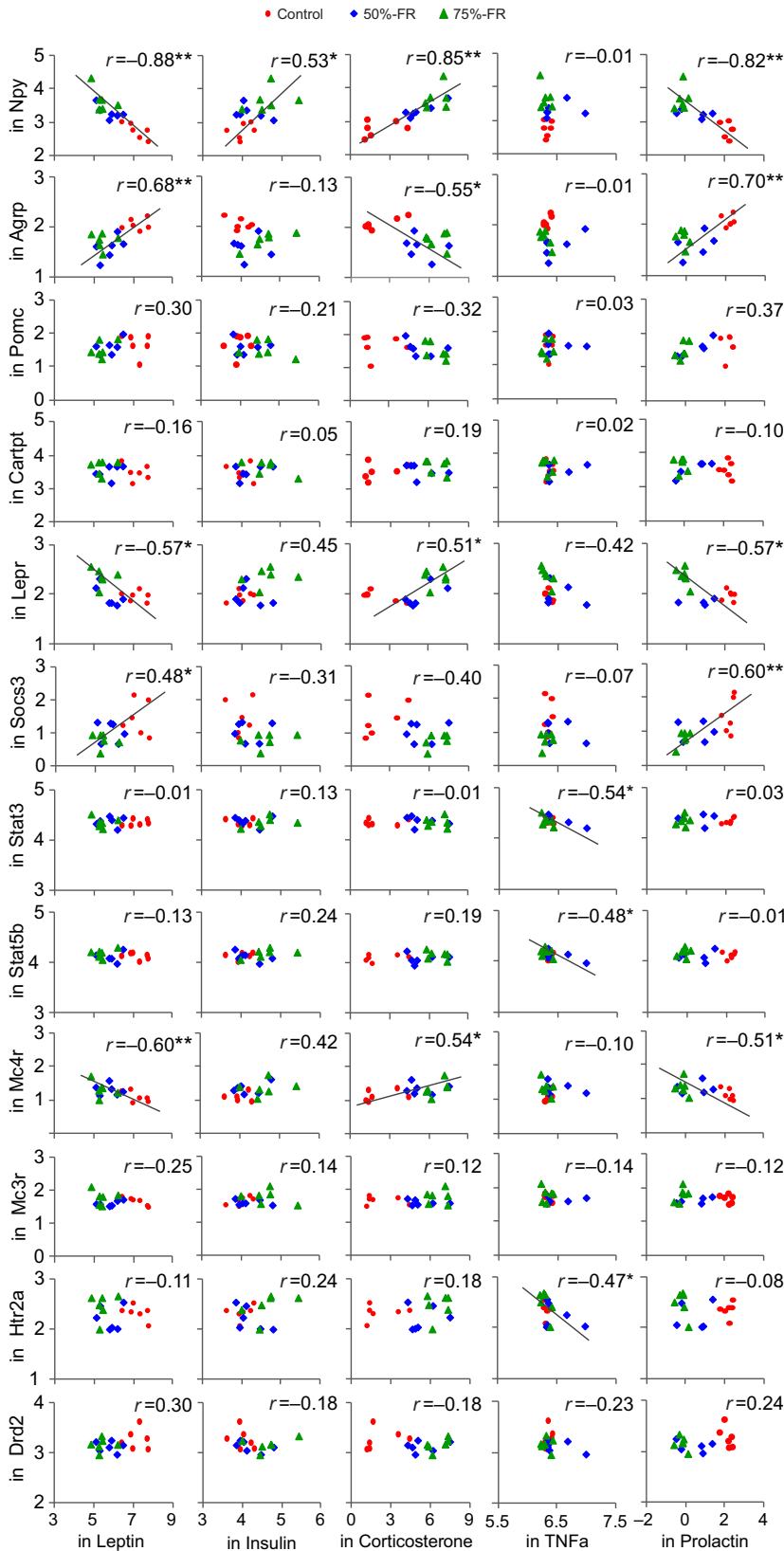
In non-lactating mice that are placed under 24 h starvation, or less intense but more chronic food restriction, there is a period of hyperphagia following the release from restriction (Spydevold et al., 1978; Bartness, 1997; Mercer et al., 1998; Hambly et al., 2007; Zhang et al., 2012; Zhao et al., 2014). The extent of hyperphagia is

greater when the level of restriction is greater (Hambly et al., 2007, 2012). During this hyperphagia, body mass rapidly returns to the level observed pre-restriction, in part reflecting elevated gut fill. In the lactating mice observed here, we also observed a similar return of body mass back to the level on the day preceding the restriction day, when animals were re-fed, supporting the notion that this change is mostly due to gut fill. However, a major difference between the mice observed here and non-lactating individuals was that there was no significant hyperphagia. The level of food intake in the animals released from restriction was no higher than the levels observed in the control mice that had not undergone the restriction day – and this was true independent of the level of restriction they were exposed to. In parallel with this unaffected food intake, the level of milk production was also not significantly different between the three groups (controls, 50% and 75% restricted).

Given the negative impacts of the restriction on the pups, most notably in the mice under the higher level of restriction, one would anticipate that if the females were eating to follow a particular investment strategy, they would have a high motivation to eat more food and produce more milk on the refeeding days to compensate for the shortfalls, if they were physiologically capable of doing so. The fact they did not provides crucial evidence that the intake of the females in late lactation is not due to such restraint, but more likely reflects a physiological or physical constraint on performance.

In non-lactating mice, the post-restriction hyperphagia is driven by stimulation or inhibition of components of a complex network of neuropeptides during the restriction phase (Hambly et al., 2007, 2012; Derosus et al., 2016a) combined with alterations in the network topology (Derosus et al., 2016b). For comparison, the typical response for non-lactating mice under 40% caloric restriction (CR) compared with mice fed *ad libitum* for 12 h per day is shown in Fig. 12 (data from Derosus et al., 2016a,b). This figure illustrates the downregulation of inhibitory pathways on the left side of the diagram (notably for *Cart* and *Pomc* and related genes), and upregulation of the stimulatory side (characterized most notably for *Agrp* and *Npy* and related genes). These contrasting effects are complemented by upregulation of many of the main secondary receptor systems including dopamine, opioid, melanocortin and serotonin signalling receptors. The global transcriptomic screening in lactating mice that had been restricted at 50% and 75% CR compared with the unrestricted lactating mice did not completely replicate these changes (Figs 5 and 6). In particular, for both levels of restriction there was no downregulation of the inhibitory neuropeptides (*Pomc* and *Cart*). Moreover, the main stimulatory neuropeptides for hunger were adjusted in different directions: *Npy* was significantly upregulated, while *Agrp* was significantly downregulated under both the restriction conditions.

Why the inhibition arm of the pathway was not downregulated is uncertain, but perhaps this side of the hunger pathway is already maximally downregulated by the process of lactation itself, and hence there is no further scope to decline to even lower levels, i.e. there is no further ability to reduce inhibitory signals once they are already effectively zero. In contrast, there is potential scope to upregulate the hunger stimulating pathway. The mice under both 50%-FR and 75%-FR responded similarly in this respect. They both upregulated levels of *Npy* but unexpectedly downregulated levels of *Agrp*. It is not exactly clear what this contrasting pattern means with respect to hunger. However, the changes in *Npy* were quantitatively larger than the changes in *Agrp*. This may indicate that hunger was elevated when the mice were restricted. Consequently, it seems unlikely that the reason they did not eat more on the days they were given unrestricted access to food was because the hunger pathway



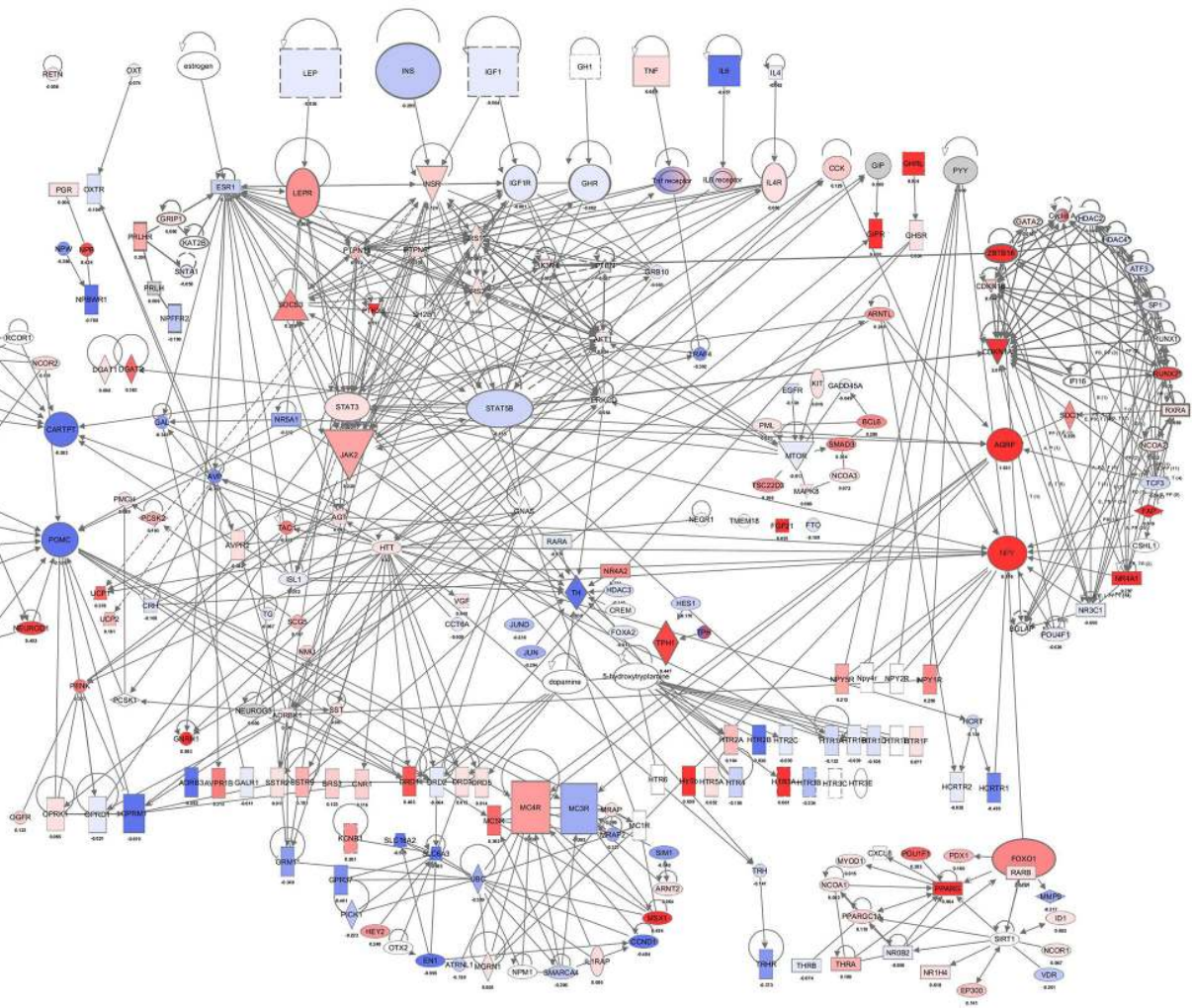
**Fig. 11. Correlations between logged levels of circulating leptin, insulin, corticosterone (coti), TNFα and prolactin and 12 key hunger genes in the hypothalamus (*Npy*, *Agrp*, *Pomc*, *Cart*, *Lepr*, *Socs3*, *Stat3*, *Stat5b*, *Mc4r*, *Mc3r*, *Htr2a*, *Drd2*) in Swiss mice during lactation.** Control mice are in red (con,  $n=6$ ), those on 50%-FR in blue ( $n=6$ ) and those on 75%-FR in green ( $n=6$ ). Asterisks indicate statistical significance (\* $P<0.05$ , \*\* $P<0.01$ ).

was already at maximal capacity; this is known as the saturated neural control hypothesis (Speakman and Król, 2005).

We have previously shown that lowered levels of leptin and *Tnf-α* levels from the periphery contribute to the post-restriction hyperphagia effect in non-lactating individuals (Hambly et al.,

2012) and that differences in the melanocortin and dopamine systems may underpin details of how the animals respond to restriction (Vaanholt et al., 2015; Deros et al., 2016a,b). Circulating leptin levels in lactation are already reduced to less than half of that in virgin females (Zhang and Wang, 2007; Cui





**Fig. 12. Gene expression profile in the hunger signalling pathway of the hypothalamus (pathway from Deros et al., 2016a) for mice under 40% caloric restriction for 3 months compared with mice fed *ad libitum* for 12 h per day. Red indicates upregulation and blue downregulation in the restricted group. Levels under each node of the network indicate the gene at that node. Edges represent known interactions between genes from the literature as indicated by the IPA software. Intensity of colour is correlated with the significance of the difference. High levels of upregulation of the two genes that stimulate hunger (*Npy* and *Agrp*) and downregulation of the two main inhibitory genes (*Pomc* and *Cart*) are apparent.**

et al., 2011; Król et al., 2011) and are correlated with milk production (Cui et al., 2011). Leptin and prolactin declined on the restriction days, while insulin and corticosterone were increased. However, while global gene expression in the hypothalamus was generally responsive to these altered peripheral leptin, insulin and corticosterone levels (Fig. S2), as anticipated from the pathway analysis, both *Pomc* and *Cart* (the main inhibitory signals) were unrelated to the peripheral hormonal signals. However, the key genes stimulatory genes involved in hypothalamic hunger signalling were responsive to the altered peripheral hormone levels (Fig. 11). *Npy* was elevated as leptin fell and corticosterone increased, and was also related to circulating insulin levels. However, as reflected in the pathway analysis, *Agrp* showed the opposite trends to leptin and corticosterone and was unrelated to insulin.

The fact that one arm of the hunger pathway in the brain was stimulated under both levels of restriction, but the mice did not eat more food when derestricted, suggests the constraint on intake does not reside in the brain hunger system (saturated neural control hypothesis; Speakman and Król, 2005). As suggested by earlier work (Hammond and Diamond, 1992, 1997; Hammond et al., 1996;

Zhao et al., 2010, 2013), the main limitation in this mouse strain at 21°C may be the milk production capacity of the mammary glands. Hence on the refeeding day, the mice elevated their milk production back to the maximal level, but despite upregulation of their neuropeptide hunger signalling, they did not eat additional food because this could not be channelled into further milk production to make good the shortfall. An alternative interpretation is that the food intake and milk production limits could reflect a limit on the capacity to dissipate heat generated as a by-product of both the digestive processes (specific dynamic action, SDA) and milk synthesis (both of which generate significant amounts of heat – see above arguments regarding efficiency) and hence the risk of hyperthermia (Speakman and Król, 2010, 2011). This is established to be a significant factor in this strain at 30°C (Zhao et al., 2016; Wen et al., 2017).

A downstream consequence of being unable to upregulate their intake on the unrestricted days was that the females could not, in the face of the first restriction event, eat more food to elevate their fat stores, and thereby provide a buffer that could be drawn on if the food supply failed again. Some mouse strains, when not lactating,



do respond to stochastic variations in food supply by elevating their body fat store (Rozen et al., 1994; Duarte et al., 2012; Zhang et al., 2012; Monarca et al., 2015), but interestingly, similar studies of stochastic food exposure in non-lactating Swiss mice do not evoke such a fat storing response (Zhao and Cao, 2009a,b; Zhao et al., 2009).

A number of additional pathways were stimulated when the mice were under restriction. In particular, at 50%-FR the *Eif2* pathway was upregulated (Fig. 7). This pathway is a classical response to protein restriction. Although the diets we provided at 50%-FR and 75%-FR had greater protein than is required in baseline conditions, the mice clearly have much elevated protein demands when they are lactating. Hence there was a shortfall between supply and demand, which is consistent with upregulation of this pathway. At 75%-FR there was no significant upregulation of this pathway, possibly because the mice in this situation shut down milk production. Hence, their demands for protein may also have declined with the result that they were no longer in protein deficit. Two additional pathways were significantly downregulated at both levels of restriction. These were the unfolded protein (UPF) response (Fig. 8) and the neuroinflammation pathway (Fig. 9). The UPF response is a typical stress response that is stimulated under various situations (Tsai and Weissman, 2010). A major feature of the response is the degradation of unfolded proteins and as such it may be activated under restriction as a mechanism to recycle amino acids to support protein synthesis. Upregulation in these restricted mice was therefore unexpected and remains unexplained. In contrast, neuroinflammation involves activation of the brain's innate immune system and is classically linked to disorders such as infection and degenerative brain diseases. However, in the hypothalamus, it is also observed during dysfunctional weight regulation following exposure to high fat diets, pointing to a link to food intake regulation (Thaler et al., 2012; Duffy et al., 2019). The reduction in neuroinflammation when the mice were restricted parallels the improvements in neuroinflammation when obese mice are similarly restricted (Thaler et al., 2012).

The upregulated genes in common to both levels of restriction were *Sele*, *Gstp2*, *Ttr* and *Scgb3a1*, and the common downregulated genes were *Cyp3a57* and *Gm5099*. Selectin E (*Sele*) codes for a cell adhesion molecule activated by cytokines and linked to inflammation. *Gstp2* is protective against oxidative damage. *Ttr* codes for a protein involved in transport of vitamin A and thyroxine. *Scgb3a1* is also activated by cytokines. Among the downregulated genes, *Cyp3a57* is a cytochrome p450 family member involved in processing of steroid hormones. *Gm5099* is a predicted gene of unknown function. The reasons why these genes were altered under both levels of restriction are unclear.

Although our work demonstrates that these mice were constrained and that this constraint likely has a physiological and hence presumably a genetic basis, we are currently not aware of the underlying genetic factors that regulate the asymptotic food intake. Presumably, the level of the constraint in most mammals is moulded by the process of natural selection, and hence fits into a wider context of the costs of reproduction. In these laboratory mice, in contrast, it may have been shaped by artificial selection pressures (this strain is bred as a good stock breeding mouse), pleiotropic changes owing to selection for domestication, or genetic drift. It will be interesting in future to discern not only the mechanistic basis by which the constraint is imposed, which has been mostly our focus here, but also what the wider implications of the level of the constraint are for the costs of reproducing. Do higher levels of the constraining factor, for example, have negative impacts on somatic

protection, and hence mediate the widely observed negative inter-specific relationship between reproductive output and lifespan? If so, understanding the mechanism by which this trade-off is generated will be a substantive step forwards in our understanding of the physiological basis of life history trade-offs.

## Conclusions

This experiment demonstrates that the asymptotic food intake at peak lactation in the Swiss mouse is constrained, rather than reflecting a restraint by the mother owing to a wider evolutionary context of investment. Gene expression data for the hypothalamic hunger signalling network in response to peripheral levels of circulating hormones suggest the effect was not due to failure to upregulate the hunger signalling pathway (the 'neural saturation hypothesis').

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: Z.Z., J.R.S.; Methodology: D.D., A.G., X.L., C.H., J.R.S.; Data curation: Z.Z., D.D., A.G., J.W., X.L., S.T., C.H., J.R.S.; Writing - original draft: Z.Z., J.R.S.; Writing - review & editing: Z.Z., J.R.S.; Supervision: J.R.S.; Funding acquisition: Z.Z.

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## Supplementary information

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