

Effects of early life adversity on acute metabolic outcomes during neonatal development

Hannah E. Lapp^{a,*}, Melissa G. Salazar^a, Evelyn Deveraux^b, Pawel K. Misztal^b, Frances A. Champagne^a

^a University of Texas at Austin, Department of Psychology, 108 E Dean Keeton St, Austin, TX, 78712, USA

^b University of Texas at Austin, Maseeh Department of Civil, Architectural and Environmental Engineering, 3011E Dean Keeton St, Austin, TX, 78712, USA

ARTICLE INFO

Keywords:

Early life adversity
Metabolism
Stress
Energy expenditure
Maternal behavior
Hypothalamus
Development

ABSTRACT

Early life adversity has lasting effects on neuroendocrine function and increases risk for psychopathology and metabolic disease. Stress and metabolism are closely intertwined, with critical periods for early life stress overlapping those for metabolic programming. Early life adversity models in rodents have the potential to affect development through multiple pathways, including nutrition, parental behavior, and direct physical stress. In the current study, we investigate how a limited bedding and nesting material (LBN) postnatal environment affects maternal behavior, milk composition, and acute metabolic outcomes in rat offspring. We examined the impact of LBN exposure during two postnatal windows (Early: P0–6 and/or Late: P6–13) and effects of key elements of early experiences (maternal behavior and nutrition) on acute metabolic outcomes, with assessment of sex-specific effects in pups. LBN shifted maternal behavioral phenotype, increased dam milk corticosterone and triglyceride concentration, affected serum metabolic and pituitary hormones in pups, and increased pup estimated daily energy expenditure. Differential gene expression analyses of the liver and arcuate nucleus of the hypothalamus showed timing and sex-specific effects of LBN. LBN predicted expression of genes related to amino acid biosynthesis, protein assembly, and developmental transitions. These effects were associated with individual milk components, pup sex, and pup weight. Collectively, these findings demonstrate that early life adversity alters acute metabolic outcomes through multiple, interacting pathways that may contribute to increased vulnerability to endocrine and metabolic dysfunction later in life.

1. Introduction

Adverse early life experiences confer heightened risk for a wide range of poor health outcomes across the lifespan and earlier all-cause mortality (Felitti et al., 1998; Kelly-Irving et al., 2013). There is remarkable overlap in the effects of early adverse experiences related to nutritional extremes (over and under nutrition) and other stressors such as poverty, abuse and neglect, and maternal stress on long-term health outcomes (Boersma et al., 2014). When these stressors are experienced during developmentally sensitive periods, they increase vulnerability to poor metabolic outcomes including obesity, diabetes, cardiovascular disease, and metabolic syndrome as well as psychiatric conditions such as schizophrenia and mood disorders (McMillen and Robinson, 2005; Seal and Turner, 2021). The overlapping effects of early life nutrition

and early life stress on mental and physical health reflect the bidirectional interactions between stress physiology and metabolic regulation (Morris et al., 2015).

Activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress initiates a hormonal cascade beginning in the paraventricular nucleus (PVN) of the hypothalamus and ultimately leading to glucocorticoid (GC) release by the adrenal glands. Circulating GCs mobilize energy reserves by promoting glucose availability with chronic GC elevation affecting appetite, feeding, and visceral adipose deposition (Lee et al., 2014; Sominsky and Spencer, 2014). Metabolic homeostasis, including feeding and appetite, are regulated by an array of hormones that act on the brain and periphery with a central role of the arcuate nucleus of the hypothalamus, lateral hypothalamus, and PVN. Leptin, an anorexigenic cytokine hormone secreted primarily by adipose tissue,

This article is part of a special issue entitled: Gig Levine Special Issue published in Neurobiology of Stress.

* Corresponding author.

E-mail addresses: hannah.lapp@austin.utexas.edu (H.E. Lapp), melissa.salazar@UTDallas.edu (M.G. Salazar), ed2250@princeton.edu (E. Deveraux), misztal@utexas.edu (P.K. Misztal), franceschampagne@utexas.edu (F.A. Champagne).

<https://doi.org/10.1016/j.ynstr.2026.100835>

Received 7 September 2025; Received in revised form 26 April 2026; Accepted 6 July 2026

Available online 9 July 2026

2352-2895/© 2026 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

regulates energy balance and facilitates energy expenditure by acting on the arcuate nucleus (Mainardi et al., 2013; Morton, 2007). GCs and leptin co-regulate each other: leptin acts on several levels of the HPA axis to suppress GC production, while GCs increase leptin expression (Paul and Buchholz, 2023). GCs may also interact with other metabolic hormones, including ghrelin and insulin, to affect food intake and energy balance (Janssen, 2022; Spencer et al., 2012). Collectively, these interactions can account for the significant associations between stress and metabolic dysregulation.

The HPA axis and metabolic systems are immature at birth and undergo substantial developmental changes in the early postnatal period. In rodents, the stress-hypo-responsive period (SHRP) refers to the period from birth through about postnatal day 14 (P14) when the HPA system is actively suppressed to keep adrenocorticotrophic hormone (ACTH) and GC levels low in response to stimuli that would typically elicit a stress response (Levine, 1970; Levine et al., 1967; Schmidt, 2019). Maternal care plays a critical role in maintaining the SHRP and exerts life-long effects on HPA function by affecting GC receptor levels in the brain (Weaver et al., 2004), and pup leptin levels also contribute to the SHRP by lowering GCs and regulating HPA response (Paul and Buchholz, 2023; Salzmann et al., 2004). Hypothalamic circuitry regulating energy balance is similarly immature at birth, with arcuate nucleus projections to feeding-related hypothalamic nuclei emerging around P5 in rats (Matsumoto and Arai, 1976; Sominsky et al., 2018). Leptin, although anorexigenic in adulthood, does not suppress feeding in early development and instead promotes hypothalamic neurocircuitry development (Bouret et al., 2004; Proulx et al., 2002), while ghrelin levels increase progressively over the first two postnatal weeks to reach adult levels by weaning (Sominsky et al., 2018). The liver also undergoes a profound shift at birth, transitioning from its prenatal role as the primary hematopoietic source to a central metabolic regulator through fatty acid synthesis and gluconeogenesis (Böhme et al., 1983; Herrera and Amusquivar, 2000; Innis, 2011).

The developmental trajectory of the HPA axis and metabolic systems is subject to influence by postnatal experience. Prolonged maternal separation induces long-term dysregulation of HPA function, affects metabolic hormones including leptin, ghrelin, and insulin, reduces body weight, and alters neuropeptide expression in the arcuate nucleus in rodents (Paul and Buchholz, 2023; Schmidt et al., 2006; Viveros et al., 2010), with both loss of food intake and loss of maternal tactile care contributing to these effects (Oers et al., 1998; Rosenfeld et al., 1993). Other models of chronic early life stress similarly alter leptin, ghrelin, insulin, adipose tissue, and hypothalamic fiber density (Yam et al., 2017a; Yam et al., 2017b). Beyond the direct effects of parental care, early adverse environments may also affect offspring nutrient intake through changes in milk composition. For example, maternal stress can increase milk GC concentration and affect milk lipid content and immune factors (Wijenayake et al., 2023). Milk composition has long-term effects on offspring emotionality, HPA function, body weight, and hypothalamic feeding circuits (Kirk et al., 2009; Langley-Evans et al., 1996; Vogt et al., 2014), and postnatal diet can affect peripheral metabolic development including liver gene expression (Nakagaki et al., 2018). Corticosterone and leptin transferred through maternal milk may further shape pup stress and metabolic systems during early-life sensitive periods (Casabiell et al., 1997; Stead et al., 2022), suggesting that early adversity affects offspring development through both behavioral and nutritional channels.

The present study used the limited bedding and nesting material (LBN) postnatal environmental manipulation during sensitive periods of HPA and metabolic development to examine effects of early adversity on acute metabolic outcomes in rat pups. The LBN model alters the pup microenvironment by increasing thermal stress, changing pup peer interactions in the litter, and changing maternal behavior, although there is considerable variability in maternal response to LBN in rats (Lapp, Bartlett, et al., 2020a, 2020b; Lapp et al., 2024). The goals of this study were: 1) determine the acute effects of LBN on metabolic outcomes

in offspring, 2) determine how these effects vary with timing of LBN, 3) identify key elements of early experience that affect metabolic outcomes, and 4) examine sex differences in metabolic outcomes. Pup outcomes include serum metabolic and pituitary hormones, energy expenditure, and transcriptomic analysis of the liver and arcuate nucleus of the hypothalamus. We used maternal behavioral phenotype measures and measures of milk macronutrients and hormones to identify key elements of pup experience responsible for metabolic changes.

2. Methods

2.1. Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at Austin and were performed in accordance with IACUC guidelines and regulations (protocol # AUP-2022-00239). Sample sizes were based on previous studies using this model (Lapp et al., 2020a; Moriceau et al., 2009) rather than a formal power calculation. Animals were housed in polycarbonate cages (48 cm × 26.5 cm × 20 cm) with standard wire tops and were kept on a reverse 12:12 h light cycle (lights off at 11am, lights on 11pm). All dams were provided with only Aspen shavings (Nepco) for bedding and nesting material, which can be manipulated to construct nests, with no other enrichment provided. All animals were provided with standard chow (Lab diet 5LL2) and water *ad libitum*. Two cohorts of 30 adult (P60) Long-Evans female rats and 15 adult (P60) Long-Evans male rats were purchased from Charles River Labs and acclimated to the vivarium in housing groups of three animals for at least two weeks before breeding. During breeding, one receptive female was housed with a breeder male overnight. All dams were socially housed throughout pregnancy until they were singly housed starting on gestational day 18–21 before giving birth. Six females did not become pregnant and were excluded from the study.

2.2. Experiment timeline

On P0 (day of birth), pups were culled to five males and five females per litter using anogenital distance to determine sex. Litters were excluded if they had fewer than 5 pups of either sex ($n = 10$) or were born on a day when no other litters were born, precluding cross-fostering on P6 ($n = 5$). Litters included in the study were assigned to Control (225 g of bedding) and LBN (50 g of bedding) conditions in order of birth using a pre-generated random sequence, such that each successive litter born within a cohort was assigned the next condition in the sequence (adapted from Moriceau et al., 2009, Fig. 1A). Sequences were stratified by cohort to ensure balanced group sizes within each experimental group. On P6, milk was collected from dams (5:00am–8:00am) and CO₂ emissions were measured from pups (1:00pm–4:00pm). This resulted in brief maternal separations for up to 1 h during milk collection (dam removed from the home cage) and 5 min during CO₂ emissions testing (1 pup removed at a time). After pup testing on P6 (4:00pm–5:00pm), entire litters were fostered to a new dam in a clean cage in the same or opposite condition resulting in four experimental litter groups reflecting pup exposure to LBN during two exposure periods: LBN-LBN ($n = 10$), LBN-Control ($n = 10$), Control-LBN ($n = 9$), and Control-Control ($n = 10$). Foster dam assignment was not random and was determined by the conditions of the available litters born on the same day such that only litters with P6 pups were fostered, with the goal of achieving balanced representation across the four experimental groups (LBN-LBN, LBN-Control, Control-LBN, Control-Control). P0–P6 LBN exposure occurred prior to the development of axonal projections from the arcuate nucleus to other hypothalamic nuclei involved in feeding regulation, whereas P6–P13 LBN exposure occurred during this period of arcuate development, so these exposure periods reflect distinct developmental stages of metabolic development. Dams remained in the same condition for the duration of the experiment. Milk collection and

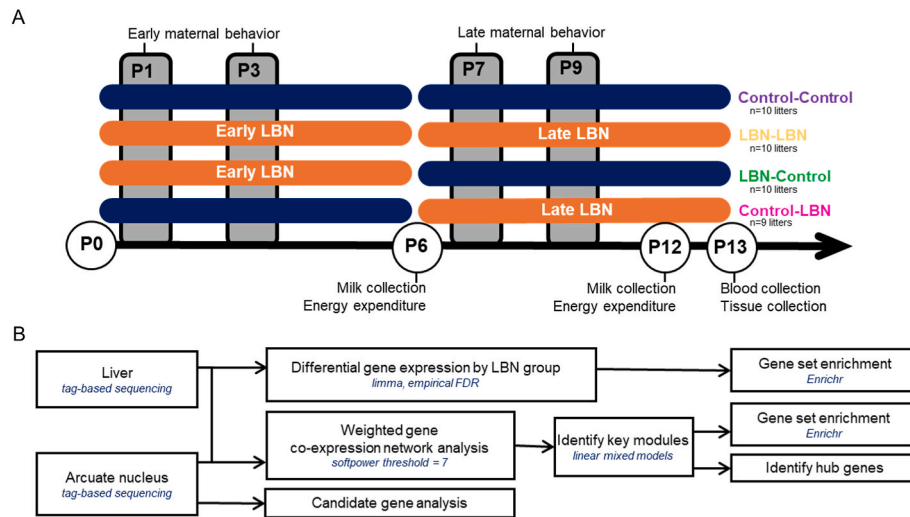


Fig. 1. Experiment methods. A) Litters were exposed to Control and/or LBN conditions. Milk was collected from dams and pup CO₂ emissions were measured on P6 and P12. Pup blood and tissue were collected on P13. Maternal behavior was measured from 24-h home cage recordings on P1, P3, P7, and P9. B) Liver and arcuate nucleus tag-based sequencing data was analyzed for differential gene expression by LBN group. Weighted gene co-expression network analysis was used to identify key modules with eigengenes predicted by LBN exposure, maternal behavior, and milk components and their hub genes. Gene set enrichment analysis was used to determine biological processes associated with gene lists.

CO₂ emission measurements were repeated on P12. On P13, offspring were euthanized via rapid decapitation and brain tissue, liver tissue, and trunk blood were collected from one male and one female pup in each litter (4:00pm to 9:00pm). Male and female pups used for CO₂ testing and tissue collection were selected by taking the first male and female pup selected from the nest in the home cage. Due to limited equipment availability, a subset of pups were not able to undergo CO₂ testing (P6 litters: Control = 3, LBN = 3; P12 litters: Control-Control = 1, Control-LBN = 2, LBN-Control = 1, LBN-LBN = 1), resulting in a smaller sample size for that measure. Because experimental condition was readily apparent from the cage appearance, the researcher selecting the pup was not blind to condition. Pups were then transported to a separate area for CO₂ measurement and tissue collection, where the researcher performing the procedure was blind to condition. Because neonatal pups did not have permanent identification, it was not possible to determine whether pups tested for CO₂ emissions on P6, tested for CO₂ on P12, and tissue collection on P13 were the same individuals. Sample processing and CO₂ measurement calculations were conducted by researchers blind to experimental groups. Statistical analyses were conducted with researchers aware of group assignments.

2.3. Home cage recordings

Home cage behavior was recorded and analyzed as reported in Lapp et al. (2024). Briefly, Raspberry Pi 3B + minicomputers equipped with Raspberry Pi Module 1 NoIR cameras were used to record home cage behavior for 24 h (greyscale, 2 fps) on P1, P3, P7, and P9 (https://github.com/lapphe/raspberry_rat). These recording days provided data on maternal behavior in the beginning and middle of the Early LBN and Late LBN phases while avoiding cage change and testing disruptions (P0, P6, and P12). Videos were screened to ensure the camera was placed at the nest end of the cage and data were excluded if the dam moved the nest to the opposite end (P1 = 7 videos, P3 = 7 videos, P7 = 6 videos, P9 = 10 videos). An additional 9 videos were excluded due to technical failures (e.g. lighting failure; P1 = 3 videos, P3 = 5 videos, P7 = 1 video, P9 = 0 videos) that prevented accurate pose estimation, resulting in a total of 38 excluded videos (24%; Supplemental Table 1 and Supplemental Table 2). Videos were automatically scored using the AMBER pipeline (Lapp et al., 2023, 2024). AMBER produced measurements of total behavior durations, mean bout durations, and mean bout

intervals for nursing (active nursing: crouched above pups with dam supporting her own weight; and passive nursing: dam on her side and not supporting herself with her limbs while pups are seen attached to nipples), pup-directed licking and grooming (licking any part of a pup's body with contact between the dam's nose and mouth and pup), nest attendance (a portion of the dam's body is in the nest and in contact with pups) for each 24 h video recording. Average nursing latency, defined as the average time from the onset of nest attendance to the onset of nursing for each nest attendance bout in each video, was also calculated for each recording. Because nursing is initiated in response to pup cues during nest attendance bouts, nursing latency reflects maternal responsiveness to pup stimulation within the home cage context, with shorter latencies indicating faster responses to pup solicitation. Key settings and parameters for the AMBER pipeline are described in the Supplemental Methods.

2.4. Milk collection

Milk was collected from dams on P6 and P12 to assess milk composition as described in Paul et al. (2015). Milk let down can be induced by oxytocin but is inhibited by stress, so dams were anesthetized with isoflurane (5% induction) prior to the oxytocin injection to collect milk (Lau, 1992). A nose cone was used to maintain anesthesia at 2-3% isoflurane with total anesthesia and recovery time less than 60 min. Once anesthetized, dams were injected with 2IU oxytocin (intra-peritoneal). Dam's nipple regions were cleaned with alcohol wipes and warm water. Milk was collected by squeezing the base of the teat and manually expelling the milk to collect it in capillary tubes. After milk collection, dams were returned to their home cage with their pups once fully conscious. The pharmacokinetic profile of isoflurane suggests that the drug is not absorbed by offspring and that nursing can resume immediately after anesthesia (Lee and Rubin, 1993). The researcher conducting the milk collection was not blind to condition.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Concentration of lactose (Sigma-Aldrich # MAK017, 1:500 sample dilution), triglycerides (Abcam #AB65336, 1:1000 sample dilution), total protein (Pierce BCA Protein Assay # 23225, 1:50 sample dilution), corticosterone (Enzo Life Sciences ADI-900-097, 1:40 sample dilution),

and leptin (Millipore # EXRL-83K, no dilution) in dam milk were measured using ELISA according to the manufacturer's instructions.

2.6. CO₂ emissions

CO₂ exhaled by pups was measured from one male and one female (randomly selected) from each litter to estimate pup total daily energy expenditure. During testing, one pup was placed in a 500 ml flow-through jar for 5 min. Prior to pup measurements, empty jars were sampled to provide background measurements. A flow rate of 800 standard cubic centimeters per minute of filtered sweep air from a hydrocarbon trap (VICI Metronics) was used for the duration of the measurement. The last 15 s of the third minute were used for analysis to allow ample time for the volume to be flushed of any lab air. The CO₂ Analyzer (Model LI-850; LI-COR Environmental, 2018) measured CO₂ concentrations from flow-through effluent at 1 Hz.

2.7. Blood and tissue collection

Following rapid decapitation, 1 mL of pup trunk blood was collected in tubes containing 1:100 protease cocktail inhibitor (Sigma P2714), 1:100 Pefabloc SC (Sigma 11429868001), and DPP inhibitor (Millipore # Dpp4-101). Pup whole livers and brains were extracted and placed in hexanes on dry ice. Blood was allowed to sit for a minimum of 30 min on ice before being spun down at 1000 x g at 4 degrees C for 10 min and serum was aliquoted for metabolic and pituitary assay panels. Tissue and serum were stored at -80 Celsius (C) until processing.

2.8. Blood panels

Serum samples were processed using the Milliplex Rat Pituitary (Millipore RPTMAG-86K) and Metabolic Hormone (Millipore RMHMAG-84K) Magnetic Bead panels by the Bioanalytics and Single-Cell Core at UT-Health San Antonio.

2.9. Tissue processing

2.9.1. Liver extraction

Liver tissue was processed as previously described (Lee et al., 2022). Approximately 30 mg of liver tissue was extracted into reinforced microvials containing zirconia/silica beads (11079110z, BioSpec) and homogenized in 500ul of lysis buffer (Thermo Fisher Scientific #A27828) with 0.7% beta-mercaptoethanol using a beadbeater (607, Bio Spec). 100ul aliquots of homogenates were used for RNA extraction on the KingFisher Flex (Thermo Fisher #5400630) according to the manufacturer's protocol. RNA quality was determined in a subset of samples using the RNA 6000 Nano Assay with BioAnalyzer (Agilent Technologies) and RNA concentration for all samples was determined with Quant-it RNA High Sensitivity assay kit (Thermo Fisher Scientific #Q33140). Two samples (one male Control-Control and one female Control-Control) had insufficient RNA quality (RIN <5) and were not sent for sequencing. Remaining RNA samples were normalized to 100ng/ul and stored at -80C until sequencing.

2.9.2. Arcuate nucleus sectioning and RNA extraction

Brains were cut into 70 µm sections on a cryostat set at -20C. The arcuate nucleus was dissected using a 0.35 mm core sampling tool (Electron Microscopy Sciences, #NC1310088) from brain slices ranging from bregma -1.8 to -3.12 (Paxinos and Watson, 1998). 120ul of lysis buffer containing 0.7% beta-mercaptoethanol was immediately added to tube containing the tissue and vortexed until homogenized. Samples were frozen at -80C until RNA extraction. RNA was extracted from tissue samples using the MagMax Total RNA isolation kit on Kingfisher Flex system according to manufacturer instructions (MagMax #A27828). RNA quality was determined using RNA 6000 Nano Assay and RNA concentration was determined with Quant-it RNA High

Sensitivity assay kit. Samples with a RIN <5 or RNA concentration <10 ng/ul were not sent for sequencing (females: Control-Control = 2, Control-LBN = 1, LBN-Control = 2, LBN-LBN = 1, males: Control-Control = 2, Control-LBN = 1, LBN-Control = 3, LBN-LBN = 2). Remaining RNA samples were normalized to 10ng/ul and stored at -80C until sequencing.

2.9.3. Transcriptomic processing

Liver and arcuate nucleus samples were Tag-sequenced at the Genome Sequence and Analysis Facility at the University of Texas at Austin. Tag-sequencing is designed to measure polyadenylated transcripts yielding data for differential gene expression analysis in well annotated genomes (Lohman et al., 2016; Meyer et al., 2011). Reads were sequenced on the NovaSeq 6000 SR100 with minimum reads of four million and target reads of five million. Raw data were preprocessed to remove adapter sequences, PCR duplicates, and low-quality reads using cutadapt v. 2.8 (Martin, 2011) and FastQC v. 0.11.9 was used to check quality control of the sample data (Andrews, 2010). All samples passed QC checks and were used in the analysis. Preprocessed reads were then aligned to the rat genome (rnor6) using STAR (Dobin et al., 2013) and raw counts were obtained.

2.10. Data analysis

2.10.1. Maternal behavior PCA

Principal component analysis (PCA) was conducted on home cage maternal behavior received by pups during the early (P1 and P3) and late (P7 and P9) recording days as a dimension reduction technique. Maternal behavior measures were first averaged for P1 and P3 (early maternal behavior) and P7 and P9 (late maternal behavior) recordings, resulting in 20 maternal behavior measurements. The majority of dams/litter had 3 or 4 videos with useable data (Supplemental Table 1) and Fisher's exact tests with simulated p-values (Monte Carlo, 10,000 replicates) confirmed that missing videos did not differ significantly by Early LBN exposure ($p = .880$), Late LBN exposure ($p = .149$), or post-natal day ($p = .607$) indicating that missingness was balanced across conditions and recording days (Supplemental Table 2). Missing measurements due to missing videos (10-15% per measure) were imputed using random forest imputation with the missForest function of the missForest package in R. Although imputation may subtly influence the covariance structure underlying the PCA, the low proportion of missing values per measure and the balanced pattern of missingness across conditions minimizes the risk of systematic bias. Prior to PCA, data were z-scored and assumption checks were conducted. Bartlett's test of sphericity confirmed sufficient correlation structure to justify dimension reduction ($\chi^2(190) = 716.11, p < .001$). No multivariate outliers were detected based on Mahalanobis distance, and no variables were removed due to multicollinearity. PCA was run using the prcomp function in R without rotation and PCA loadings are reported as eigenvectors extracted from the component coefficient matrix. Given the diffuse loading structure characteristic of behavioral data, variables with absolute loadings greater than 0.25 were considered meaningful contributors to each component for interpretation purposes.

2.10.2. Energy expenditure analysis

CO₂ emission rates were calculated from the flow rate of the sweep air and from measured volume mixing ratios which were converted to mass concentrations assuming constant temperature and pressure. Incoming CO₂ concentrations were subtracted using background measurements. Exhalation flow rates of CO₂ in units of milliliters per minute were calculated using the following equation:

$$\dot{V}_{CO_2} = ER \times \frac{1}{\rho_{CO_2}} \div 60 \frac{min}{hour}$$

Where ER is the calculated mass emission rate of CO₂ in milligrams per

hour and ρ_{CO_2} is the density of CO_2 at room temperature. Daily energy expenditure (DEE) in units of kilocalories was estimated using the following equation:

$$\text{DEE} = \sum \left(\frac{D \times \dot{V}_{\text{CO}_2}}{E \times R} \right)_i \times 1,440 \frac{\text{min}}{\text{day}} \div 4.184 \frac{\text{kJ}}{\text{kcal}}$$

Where D is the percentage of total macronutrient content of the milk consumed by each pup, E is the volume of O_2 consumed per kilojoule produced, and R is the ratio of CO_2 produced to O_2 consumed for each of i macronutrients (carbohydrates, protein, and lipids; Even and Nadkarni, 2012). Sampling was limited to 5-min measurement periods which were then extrapolated to estimate the total daily energy expenditure for each pup.

2.10.3. Differential gene expression analysis

Differentially expressed gene (DEG) analysis was conducted using the *limma* package in R in male and female datasets separately (Smyth, 2005). First, genes with less than 10 counts across all samples were removed. DEG analysis with voom transformation was used to identify DEGs for three comparisons: LBN-LBN vs. Control-Control, LBN-Control vs. Control-Control, and Control-LBN vs. Control-Control. Raw p-values were adjusted using empirical false discovery rate (eFDR; Storey and Tibshirani, 2003) by permutating sample labels 5000 times to obtain a null distribution. Exploratory DEGs (unadjusted p value < 0.05) and significant DEGs (eFDR < 0.05) are reported as indicated in the results. Gene ontology (GO) enrichment analysis was used to explore biological processes related to DEGs using Enrichr with the full gene list as the background (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021, Fig. 1B).

2.10.4. Candidate gene analysis

A list of 78 candidate genes was compiled *a priori* to examine expression of genes related to neuropeptide and neurotransmitter signaling, synaptic plasticity, axon growth, and neurotrophic factors in the arcuate nucleus (Bedont et al., 2015; Bouret, 2022; Burbidge et al., 2016; Molnár et al., 2016; Tucker et al., 2007; Vernes et al., 2011; Zhang et al., 2024). Only 57 candidate genes were present in the data after filtering low counts.

2.10.5. Weighted gene co-expression network analysis (WGCNA)

WGCNA was performed in R using the WGCNA package (Langfelder and Horvath, 2008) with limma-normalized male and female data (Fig. 1B). For both the liver and arcuate nucleus, the softpower threshold was set to 7 based on the scale-free topology criterion (Zhang and Horvath, 2005) and signed hybrid mode with a minimum model size set of 30 was used to construct modules. Regression models were run in male and females separately to predict module eigengene expression from LBN exposure, maternal behavior, and milk components with batch included as a covariate to determine modules of interest. Two-way ANOVAs (sex and Late LBN) were run as post-hoc tests for modules with LBN exposure as a significant predictor. Module membership was determined and intramodular connectivity was determined for each module of interest. The top 5 genes with the highest intramodular connectivity score are reported as hub genes. GO analysis was run on genes in modules of interest using Enrichr.

2.10.6. Statistical analysis

Statistical analyses were conducted using R 4.3.3. For all mixed models, normality of residuals and homoscedasticity were verified by visual inspection of Q-Q plots and residuals versus fitted value plots, and normality was confirmed using Shapiro-Wilk tests prior to analysis. Litter was considered the unit of analysis for data collected from pups, with data from only one female and one male pup per litter included for each analysis. Mixed effects models on pup data that included data from both sexes had litter ID nested within cohort included as a random factor

to account for non-independence of littermates and cohort variation. Mixed effects models stratified by pup sex (serum hormones, transcriptomic expression) or for dam data (milk measures) included cohort as a random factor. Early LBN, Late LBN, and Early X Late LBN effects on all PCs accounting for at least 5% for variance were evaluated using linear mixed effects models with cohort included as a random factor. Satterthwaite's method was used to estimate degrees of freedom for linear mixed effects models. Model selection was performed using sample-size corrected AIC (AICc) where noted, calculated using the MuMIn package (Burnham and Anderson, 2002). Models differing by less than 2 AICc units were considered equivalent, in which case the simpler additive model was retained. Statistical significance was set at $\alpha = .05$ for all analyses. Error bars in figures are reported as mean $\pm$ SEM. Results are exploratory and were not corrected for multiple comparisons. Therefore, they should be interpreted with caution.

Dam P12 milk corticosterone and P12 triglyceride data violated the assumption of normality ($p < .05$), so log-transformations were applied prior to analysis. Linear mixed model analysis with the assay plate as a random factor was used to assess the effects of dam LBN exposure on milk macronutrient and hormone concentrations separately on P6 and P12 measures. For several models, the plate random effect variance was estimated at zero; these models were retained with the random effect structure intact. Pearson correlations were run to examine relationships between different milk components and between milk components and Early caregiving attenuation, Late caregiving attenuation, and Bout brevity PCs.

Group differences in pup daily energy expenditure and CO_2 emissions were analyzed using separate linear mixed effects models for P6 and P12 data. For P6 outcomes, a single additive model was fit including Early LBN exposure, pup sex, and pup body weight as fixed effects. For P12 outcomes, two models were compared: an additive model including Early LBN exposure, Late LBN exposure, pup sex, and pup body weight, and an interaction model that additionally included an Early $\times$ Late LBN exposure interaction term. Effects of Early caregiving attenuation, Late caregiving attenuation, Bout brevity PCs, and milk hormones were evaluated in separate P6 and P12 linear mixed models with a gaussian distribution with pup sex and pup body weight as fixed effects. Relationships between energy expenditure and milk macronutrients were not explored because energy expenditure estimates are derived from macronutrient measures.

Serum hormones with more than 50% of samples below the limit of detection were not analyzed (ACTH, FSH, LH, $\text{TNF-}\alpha$, prolactin). Hormone concentrations were modeled using generalized linear mixed models (GLMMs) with a gamma distribution and log link, to account for the positive, right-skewed distributions of hormone data. Pup serum hormone concentrations were analyzed in two stages. First, the effects of early and late LBN exposure on each hormone were examined. Each model included Early LBN exposure, Late LBN exposure, pup sex, and pup body weight as fixed effects. For each hormone, an additive model was compared to an interaction model including Early $\times$ Late LBN exposure. Second, to examine whether maternal behavior, milk composition, and metabolic variables predicted pup hormone concentrations, a series of GLMMs was run separately for males and females due to pronounced sex differences identified in the first analysis. Each model included a single predictor of interest and pup body weight as a covariate. Predictors included Early caregiving attenuation, Late caregiving attenuation, Bout brevity, milk corticosterone, leptin, lactose, protein, and triglyceride concentrations at P6 and P12, and pup energy expenditure at P6 and P12. Models were fit for each combination of predictor and hormone outcome.

To assess effects of predictor variables (behavior PCs, LBN exposure, energy expenditure, and milk measures) on module eigengene expression for the liver and arcuate nucleus transcriptomic data and arcuate candidate genes, linear mixed models with a gaussian distribution were run with a single predictor variable in separate models stratified by sex. For modules showing significant effects of LBN exposure, 2x2 ANOVAs

(Sex x LBN exposure) were used as follow-up tests for sex differences and LBN effects. For arcuate candidate gene analysis, results were filtered to only significant relationships and the top 10 genes were selected based on largest absolute estimates.

To examine whether the effects of early-life experience and maternal factors on liver and arcuate gene module expression were direct or mediated through maternal behavior variables, we used path analysis (structural equation modeling) with the lavaan and piecewiseSEM packages (Lefcheck, 2016; Rosseel, 2012). Piecewise SEM estimates each component of the causal model as a separate regression equation, allowing the use of mixed models to account for the nested data structure. Continuous predictors were standardized prior to analysis to allow comparison of effect sizes across predictors on different scales. LBN exposure variables were dummy coded as “0” for Control and “1” for LBN since piecewiseSEM was not designed for categorical variables (Lefcheck, 2024). For each module of interest, we specified a set of *a priori* candidate models representing alternative causal structures based on prior work suggesting LBN effects on pups are through effects on maternal behavior. Specifically, the liver pink and purple modules, which were both predicted by LBN exposure and a maternal behavior measure, were tested to determine whether the effects of Late LBN on eigengene expression were direct, mediated through Late caregiving attenuation, or both. We first assessed whether each model exhibited adequate global fit using the χ^2 statistic ($p > .05$) or Fisher's C statistic if submodels failed to converge and the χ^2 statistic was not reported. Acceptable models were then compared using AICc. Tests of directed

separation were used to evaluate whether excluded paths were justified.

3. Results

3.1. Maternal behavior principal component analysis and relationship to LBN exposure

The effects of LBN on specific maternal behaviors (total duration, bout intervals, and bout durations) have been previously reported (Lapp et al., 2024) and raw data are presented in Supplemental Fig. 1. LBN increased pup-directed maternal behaviors (nest attendance, nursing, licking and grooming), reduced bout intervals, and increased nursing latency on P3 and reduced nursing latency on P6. PCA of maternal behavior received by pups showed seven PCs that each explained at least 5% of the variance (Supplemental Fig. 2A). Principal component 1 accounted for 27.23% of the variance with top loadings that included positive loadings of maternal behavior total durations and negative loadings of bout intervals on P1 and P3 (Fig. 2A–B, Supplemental Fig. 2B). We refer to this PC as “Early caregiving attenuation”, where higher values reflect less pup-directed behavior on P1 and P3. Condition analysis of Early caregiving attenuation scores showed a strong significant effect of Early LBN exposure (Fig. 2C; $\beta = -3.662$, $SE = 0.751$, $t(35.00) = -4.876$, $p < 0.001$). Likewise, principal component 2, which accounted for 16.51% of the variance, had top loadings that included positive loadings of maternal behavior total durations and negative loadings of bout intervals for P7 and P9 pup-directed behaviors and was

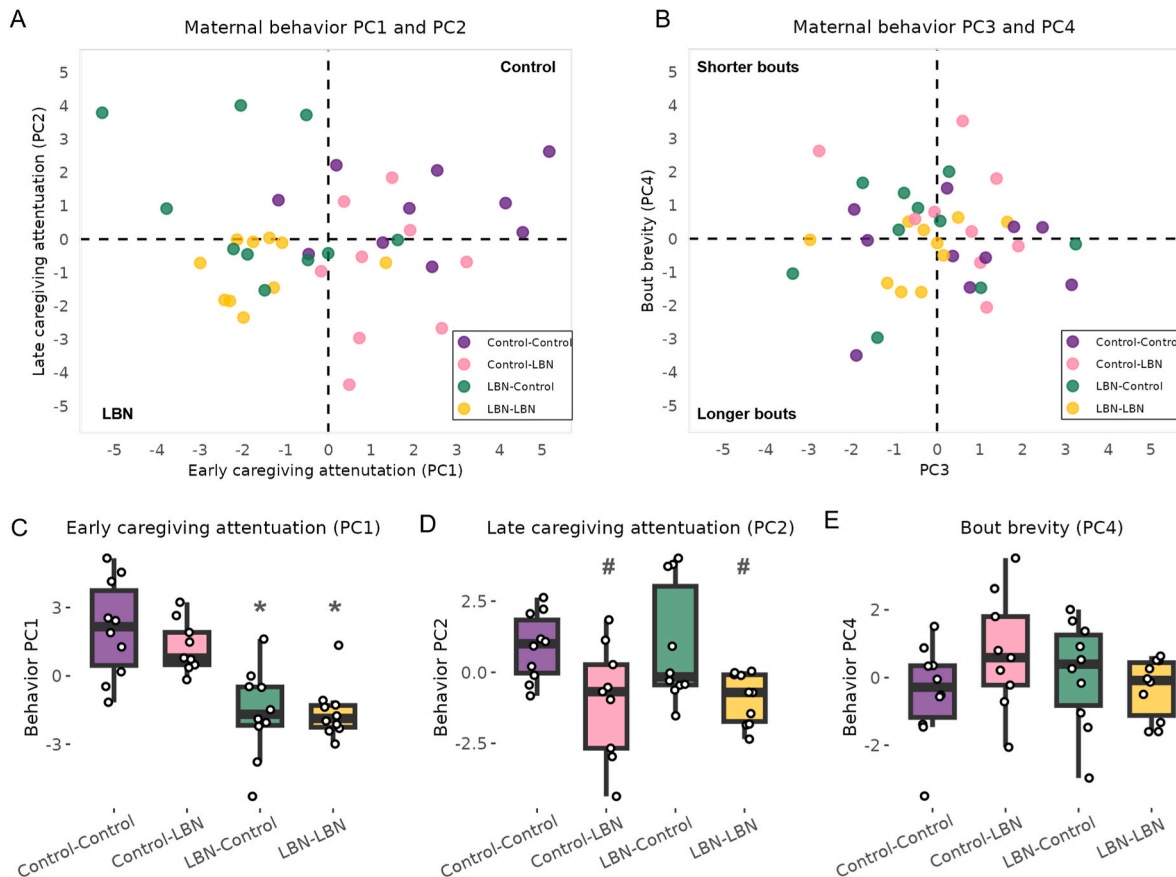


Fig. 2. Maternal behavior principal component analysis. A) PC1 (Early caregiving attenuation; 27.23% of variance) had top loadings of behavior measurements received by litters from P1 and P3 and PC2 (Late caregiving attenuation, 16.51% of variance) had top loadings of behavior measurements received by pups from P7 and P9. Litters with lower scores of both PCs tended to be litters exposed to Early LBN (PC1) and Late LBN (PC2). B) LBN groups did not separate by PC3 (12.11% variance) or PC4 (Bout brevity, 10.36% variance). PC4 top loadings included measurement of bout durations across all recording days, with higher values associated with shorter bout length. C) Early LBN exposure had a strong effect on Early caregiving attenuation (PC1) scores. D) Late caregiving attenuation scores (PC2) were reduced in litters exposed to Late LBN. E) There were no effects of LBN group on Bout brevity PC scores. $n = 10$ LBN-LBN, $n = 10$ LBN-Control, $n = 9$ Control-LBN, $n = 10$ Control-Control litters. *main effect of early LBN $p < .05$, # main effect of Late LBN $p < .05$.

significantly affected by Late LBN exposure (Fig. 2D; Supplemental Fig. 2B; $\beta = -1.882$, $SE = 0.746$, $t(35.00) = -2.523$, $p = .016$). We refer to this PC as “Late caregiving attenuation”, where higher values reflect less pup-directed behavior on P7 and P9. There were no other statistically significant main effects or interactions of Early or Late LBN on PCs1-7 (all $p > .05$). Principal component 4, which accounted for 10.36% of variance, had top loadings primarily comprised of negative loadings of mean bout durations and will be referred to as “Bout brevity”, where high values of Bout brevity correspond to shorter bouts of pup-directed behaviors. Although Early and Late LBN exposure did not significantly affect Bout brevity (Fig. 2E; $p > .05$), shorter bout durations have been proposed as a key mechanism linking fragmented maternal care to adverse offspring outcomes in the LBN model (Molet et al., 2016). The emergence of a PC mapping well onto bout duration therefore provided an opportunity to directly test whether caregiving fragmentation, independent of overall caregiving quantity, predicts pup outcomes. PC3 (12.11% of variance), PC5 (8.07% of variance), PC6 (6.43% of variance), and PC7 (5.47% of variance) had top loadings that included a range of maternal behavior measures over the early and late recording periods and were not significantly affected by Early or Late LBN exposure (Supplemental Fig. 2C–F; all $p > .05$), so they were not explored further.

3.2. LBN effects on dam milk composition

Milk triglyceride ($\beta = 76.43$, $SE = 19.91$, $t(32.09) = 3.84$, $p = .001$; Fig. 3A) and corticosterone ($\beta = 0.71$, $SE = 0.26$, $t(32.19) = 2.70$, $p = .011$; Fig. 3B) concentrations were elevated in LBN dams on P6, but not on P12 (all $p > .05$; Supplemental Table 4). Mixed model analysis did not show any effect of LBN on lactose, total protein, or leptin concentration on P6 or P12 (all $p > .05$; Fig. 3C, D, 3E; Supplemental Table 4). P6 milk corticosterone concentration and Early caregiving attenuation scores were not correlated (Fig. 3F). P12 milk corticosterone concentration (log-transformed) was moderately negatively correlated with Late caregiving attenuation scores ($r(37) = -0.32$, $p = .047$; Fig. 3G) and positively correlated with Bout brevity scores ($r(37) = .39$, $p = .014$;

Fig. 3H). Milk leptin concentration was positively correlated with milk triglyceride concentration on P6 ($r(32) = .40$, $p = .022$) and P12 (log-transformed; $r(33) = .35$, $p = .039$) and negatively correlated with P6 milk protein concentration ($r(34) = -0.45$, $p = .005$).

3.3. Pup energy expenditure

On P6, pups exposed to Early LBN exposure had elevated estimated total daily energy expenditure ($\beta = 0.79$, $SE = 0.23$, $t(31.25) = 3.52$, $p = .001$; Fig. 4) and pup body weight was a significant positive predictor ($\beta = 0.33$, $SE = 0.07$, $t(62.09) = 4.69$, $p < .001$), but pup sex was not significant ($p > .05$). For P12 pup energy expenditure, model selection via AICc favored the additive model over the interaction model (AICc = 253.20 vs. 254.44) of LBN exposure, so the additive model was retained. Early LBN exposure was associated with significantly lower energy expenditure ($\beta = -0.79$, $SE = 0.37$, $t(28.03) = -2.15$, $p = .040$), while Late LBN exposure was associated with significantly higher energy expenditure ($\beta = 0.90$, $SE = 0.36$, $t(27.34) = 2.50$, $p = .018$). Pup body weight was a significant positive predictor of P12 energy expenditure ($\beta = 0.25$, $SE = 0.07$, $t(45.57) = 3.60$, $p < .001$), while pup sex was not significant ($p > .05$). P6 CO₂ emissions were increased by LBN exposure on P6 ($\beta = 0.004$, $SE = 0.001$, $t(36.71) = 2.594$, $p = .014$; no effects of sex or body weight) but were not affected by Early LBN or Late LBN on P12 (interaction model, $p > .05$; Supplemental Fig. 3). Early caregiving attenuation, Late caregiving attenuation, Bout brevity scores, and milk hormone concentrations did not predict energy expenditure on P6 or P12 (all $p > .05$).

3.4. Variation in pup serum hormones

The full results from serum hormone mixed model analysis by LBN group are shown in Table 1 with LBN group means and standard deviations provided in Supplemental Table 4. Insulin, C peptide, leptin, peptide YY, glucagon, glucagon-like peptide-1, and thyroid-stimulating hormone concentrations were lower in males compared to females, while growth hormone concentration was higher in males than females

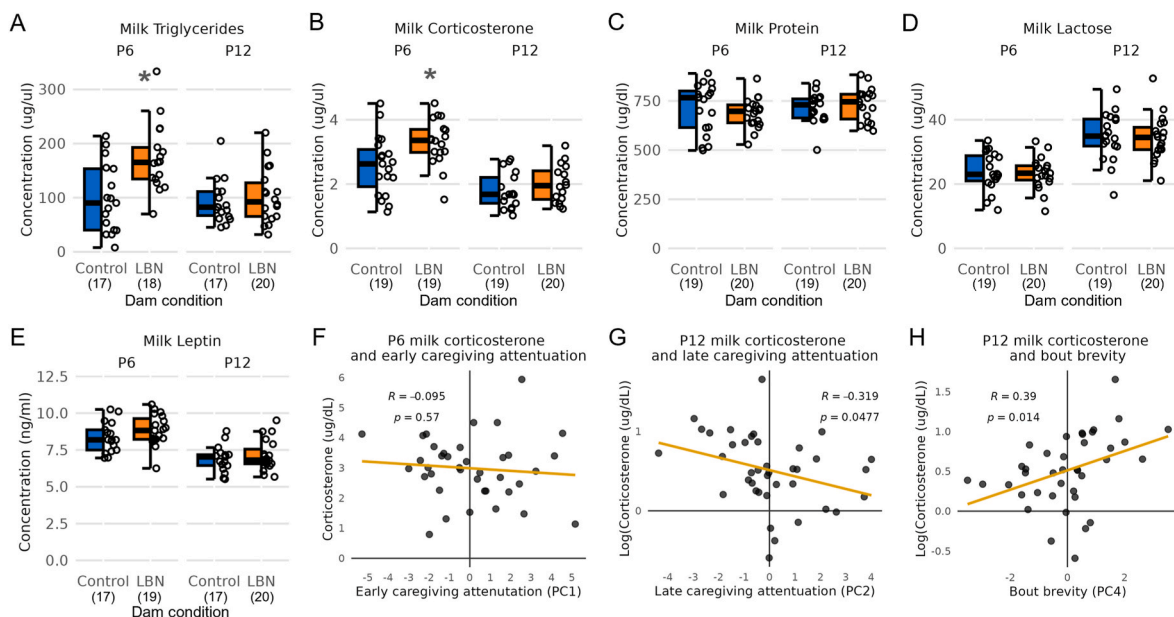


Fig. 3. Milk macronutrients and hormones. A) Milk triglyceride concentration was higher in dams exposed to LBN on P6 but not on P12. B) Milk corticosterone concentration was higher in dams exposed to LBN on P6, but there were no group differences on P12. C) Milk protein concentration was not affected by dam exposure to LBN on P6 or P12. D) Milk lactose concentration was not affected by dam exposure to LBN on P6 or P12. E) Milk leptin concentration was not affected by dam LBN exposure. F) P6 milk corticosterone concentration and Early caregiving attenuation were not correlated. G) P12 milk corticosterone concentration and Late caregiving attenuation was negatively correlated. H) P12 milk corticosterone concentration and Bout brevity were positively correlated where shorter bouts were associated with higher corticosterone concentrations. Samples sizes are noted below each group label. *effect of LBN $p < .05$.

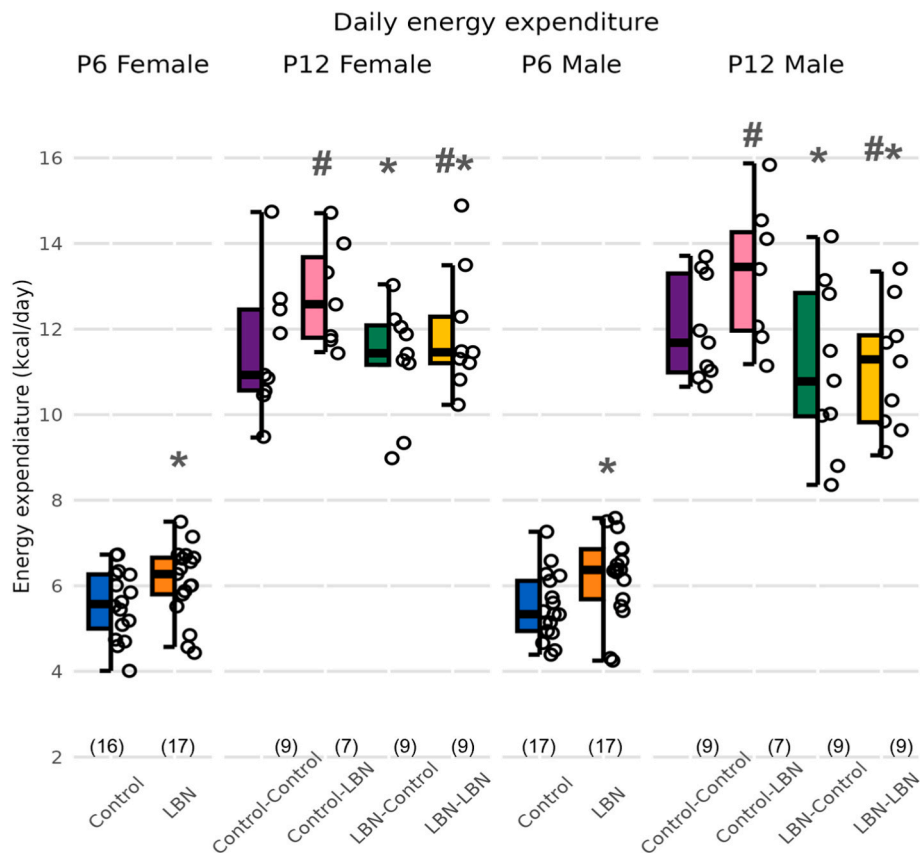


Fig. 4. Pup energy expenditure. Estimated daily energy expenditure was elevated in LBN-exposed pups on P6 in male and female pups. On P12, Early LBN exposure reduced and Late LBN exposure increased energy expenditure in male and female pups. Sample sizes are noted above each group label. *significant effect of Early LBN, #significant effect of Late LBN.

($p < .05$). Early LBN exposure reduced serum concentrations of C-peptide, peptide YY, brain-derived neurotrophic factor, and glucagon-like peptide-1 ($p < .05$). GLMM analysis of maternal behavior, milk, and energy expenditure measures stratified by sex are shown in Supplemental Fig. 4 and reported in Supplemental Table 5. Early caregiving attenuation positively predicted amylin ($\beta = .49$, $SE = .17$, $z = 2.84$, $p = .008$), C peptide ($\beta = .31$, $SE = .12$, $z = 2.48$, $p = .019$), interleukin-6 ($\beta = .38$, $SE = .14$, $z = 2.65$, $p = .013$), leptin ($\beta = .30$, $SE = .14$, $z = 2.21$, $p = .035$), and pancreatic peptide ($\beta = .30$, $SE = .15$, $z = 2.05$, $p = .049$) concentration in males. Bout brevity positively predicted peptide YY in females ($\beta = .38$, $SE = .15$, $z = 2.49$, $p = .019$). P12 milk triglyceride concentration positively predicted leptin concentration in both sexes (females: $\beta = .50$, $SE = .23$, $z = 2.14$, $p = .041$; males: $\beta = .43$, $SE = .17$, $z = 2.52$, $p = .018$) and P13 pup weight predicted C peptide (females: $\beta = .521$, $SE = .16$, $z = 3.36$, $p = .002$; males: $\beta = .33$, $SE = .14$, $z = 2.42$, $p = .022$) and leptin concentration (females: $\beta = .91$, $SE = .13$, $z = 6.85$, $p < .001$; males: $\beta = .64$, $SE = .10$, $z = 5.91$, $p < .001$) in both sexes.

3.5. Liver transcriptomic analysis

3.5.1. Differential gene expression in LBN-exposed male and female pups

Differential gene expression (DEG) analysis of the liver comparing LBN-LBN pups to Control-Control pups revealed 487 genes for females and 473 for males with an unadjusted p value less than 0.05 (Fig. 5A). Of those genes, 346 were differentially expressed in both sexes (71% females, 73% males) with complete agreement in the direction of effects (Fig. 5B). Four overlapping genes had an eFDR of less than 0.05: *Atp9a* (females logFC = -0.31 , eFDR < 0.001 ; males logFC = -0.35 , eFDR < 0.001), *Nop9* (females logFC = -0.47 , eFDR < 0.001 ; males logFC =

-0.49 , eFDR < 0.001), *Acsf4* (females logFC = 0.36 , eFDR < 0.001 ; males logFC = 0.33 , eFDR < 0.001), and *Lamc2* (females logFC = -1.00 , eFDR < 0.001 ; males logFC = -0.93 , eFDR < 0.001). *Mov10* (logFC = -0.62 , eFDR < 0.001) was lower and *Oat* (logFC = 1.35 , eFDR < 0.001) was higher in LBN-LBN females only and *Ak2* (logFC = -0.19 , eFDR < 0.001) was lower in males only (Fig. 5A). The top three GO terms for DEGs for LBN-LBN males and females, LBN-LBN males, and LBN-LBN females are reported in Supplemental Fig. 4A.

3.5.2. Effects of LBN timing on the liver transcriptome

Gene expression in the male and female liver varied depending on timing of LBN exposure (Fig. 5C and D), with the majority of DEGs unique to each exposure condition and similar number of DEGs for each type of exposure in males and females. Only 9-15% of DEGs were differentially expressed in all LBN conditions relative to the same-sex Control-Control pups. *Cfhr1* (females logFC = 1.03 , eFDR < 0.001 ; males logFC = 1.01 , eFDR < 0.001) and *Aass* (females logFC = 0.88 , eFDR < 0.001 ; males logFC = 0.85 , eFDR < 0.001) were higher in male and female pups in Control-LBN compared to same-sex Control-Control pups. *Atp9a* (females logFC = -0.38 , eFDR < 0.001 ; males logFC = -0.40 , eFDR < 0.001) was lower and *Acsf4* (females logFC = 0.49 , eFDR < 0.001 ; males logFC = 0.52 , eFDR < 0.001) was higher in male and female pups in both LBN-Control and LBN-LBN and in Control-LBN and LBN-LBN respectively. The top three GO terms for overlapping and LBN-timing specific DEGs are shown in Supplemental Fig. 5B (females) and 5C (males). GO terms were largely sex-specific, except for Negative regulation of phosphate metabolic process which was a top term for DEGs overlapping in all three LBN exposures in both males and females.

Table 1

Effects of Early LBN and Late LBN exposure on serum metabolic and pituitary hormone concentration. For each sex: Control-Control n = 10, Control-LBN n = 9, LBN-Control n = 10, LBN-LBN n = 9.

Hormone	Factor	Coef. β	SE (β)	t value	p value
Insulin	Early LBN	-0.133	0.493	-0.27	0.787
	Late LBN	0.267	0.505	0.529	0.597
	Sex (Male)	-0.37	0.144	-2.568	0.01
C peptide	Early LBN	-0.245	0.121	-2.015	0.044
	Late LBN	-0.081	0.123	-0.659	0.51
	Sex (Male)	-0.317	0.093	-3.404	0.001
Amylin	Early LBN	-0.341	0.179	-1.905	0.057
	Late LBN	-0.168	0.18	-0.928	0.353
	Sex (Male)	0.033	0.104	0.314	0.754
Leptin	Early LBN	-0.38	0.305	-1.243	0.214
	Late LBN	-0.503	0.306	-1.645	0.1
	Sex (Male)	-0.371	0.095	-3.888	<0.001
Peptide YY	Early LBN	-0.335	0.161	-2.086	0.037
	Late LBN	0.011	0.162	0.066	0.948
	Sex (Male)	-0.379	0.105	-3.615	<0.001
Brain-derived neurotrophic factor	Early LBN	-0.673	0.267	-2.522	0.012
	Late LBN	0.106	0.273	0.389	0.698
	Sex (Male)	0.009	0.142	0.064	0.949
Growth Hormone	Early LBN	-0.169	0.348	-0.487	0.626
	Late LBN	0.245	0.357	0.687	0.492
	Sex (Male)	0.787	0.147	5.349	<0.001
Active Ghrelin	Early LBN	-0.286	0.195	-1.463	0.143
	Late LBN	0.212	0.195	1.086	0.278
	Sex (Male)	-0.01	0.119	-0.084	0.933
Glucagon	Early LBN	-0.09	0.157	-0.57	0.569
	Late LBN	0.189	0.158	1.195	0.232
	Sex (Male)	-0.278	0.086	-3.227	0.001
Monocyte Chemoattractant Protein-1	Early LBN	-0.167	0.134	-1.246	0.213
	Late LBN	0.003	0.135	0.026	0.979
	Sex (Male)	-0.043	0.088	-0.486	0.627
Pancreatic peptide	Early LBN	-0.15	0.16	-0.937	0.349
	Late LBN	-0.1	0.162	-0.614	0.539
	Sex (Male)	-0.097	0.119	-0.81	0.418
Glucagon-like peptide-1	Early LBN	-0.463	0.146	-3.169	0.002
	Late LBN	0.121	0.147	0.821	0.411
	Sex (Male)	-0.249	0.111	-2.233	0.026

Table 1 (continued)

Hormone	Factor	Coef. β	SE (β)	t value	p value
Interleukin-6	Early LBN	-0.436	0.231	-1.886	0.059
	Late LBN	-0.042	0.237	-0.178	0.859
	Sex (Male)	-0.021	0.179	-0.117	0.907
Thyroid-stimulating hormone	Early LBN	-0.307	0.179	-1.718	0.086
	Late LBN	0.014	0.179	0.078	0.938
	Sex (Male)	-0.68	0.118	-5.777	<0.001

3.5.3. Liver WGCNA analysis

WGCNA with liver transcriptome data revealed 11 gene co-expression modules (Supplemental Fig. 5D). Fig. 6A shows results of linear mixed model analysis for eigengene expression predicted by maternal behavior, LBN exposure, milk components, and serum hormones, with full results available in Supplemental Table 6. No module expression was significantly predicted by Early LBN exposure, Late LBN exposure, Bout brevity, milk corticosterone, or energy expenditure at P12 in females. No module expression was predicted by Early LBN exposure, Bout brevity, or milk corticosterone in males.

Pink module eigengene expression was predicted by Late LBN exposure in males ($\beta = 1.23$, $SE = .26$, $t(34) = 4.79$, $p < .001$) and females ($\beta = 1.03$, $SE = .28$, $t(34) = 3.70$, $p < .001$) and ANOVA analysis of the Pink module showed females had higher eigengene expression than males ($F(1, 72) = 6.83$, $p = .011$) and Late LBN pups had increased eigengene expression compared to Late control pups ($F(1,72) = 35.42$, $p < .001$; Fig. 6B). Pink module expression was also negatively predicted by Late caregiving attenuation in both sexes (males: $\beta = -0.33$, $SE = .16$, $t(34) = -2.07$, $p = .046$; females: $\beta = -0.51$, $SE = .14$, $t(34) = -3.59$, $p = .001$). Path analysis indicated that the model with only direct effects of Late LBN and sex on pink module expression was best supported (lowest AICc), indicating that Late caregiving attenuation did not mediate the effect of Late LBN on Pink module expression. Global model fit was good ($\chi^2(2) = 2.58$, $p = .275$; Fisher's C = 4.25, $p = .373$). Late LBN exposure had a significant positive direct effect on pink module eigengene expression ($\beta = 1.10$, $SE = .24$, $t(35.15) = 4.64$, $p < .001$, standardized $\beta = 0.55$), and male pups had significantly lower pink module expression than females ($\beta = -0.49$, $SE = .11$, $t(37) = -4.43$, $p < .001$, standardized $\beta = -0.24$). Late LBN also had a significant negative effect on Late caregiving attenuation ($\beta = -1.01$, $SE = .20$, $t(74) = -5.09$, $p < .001$, standardized $\beta = -0.51$), but Late caregiving attenuation had no direct effect on pink module expression. Fixed effects explained 36% of variance in pink module expression (marginal $R^2 = .36$), with the full model including random effects explaining 78% (conditional $R^2 = .78$). GO-enrichment analysis indicated that genes in the pink module were largely related to hepatic amino acid metabolic processes and nitrogen handling. Pink module hub genes included *Sds*, *Ass1*, *Got1*, *Gls2*, and *Cps1*.

Eigengene expression of the purple module in females was also predicted by Late LBN ($\beta = -0.89$, $SE = .29$, $t(34) = -3.02$, $p = .005$), with follow-up ANOVA analysis showing no sex difference ($p > .05$) in expression and lower expression in Late LBN pups ($F(1,72) = 5.92$, $p = .017$). The purple module was also predicted by Late caregiving attenuation ($\beta = 0.44$, $SE = .15$, $t(34) = 2.92$, $p = .006$) in females and by body weight (females: $\beta = 0.52$, $SE = .14$, $t(34) = 3.65$, $p < .001$; males: $\beta = 0.58$, $SE = .14$, $t(34) = 4.32$, $p < .001$) and P12 milk protein concentration (females: $\beta = -0.35$, $SE = .16$, $t(34) = -2.18$, $p = .037$; males: $\beta = -0.38$, $SE = .16$, $t(34) = -2.41$, $p = .021$) in both sexes (Fig. 6C). Path analysis with female data indicated that the effect of Late LBN exposure on purple module eigengene expression was fully mediated by Late caregiving attenuation. Global model fit was acceptable (Fisher's C

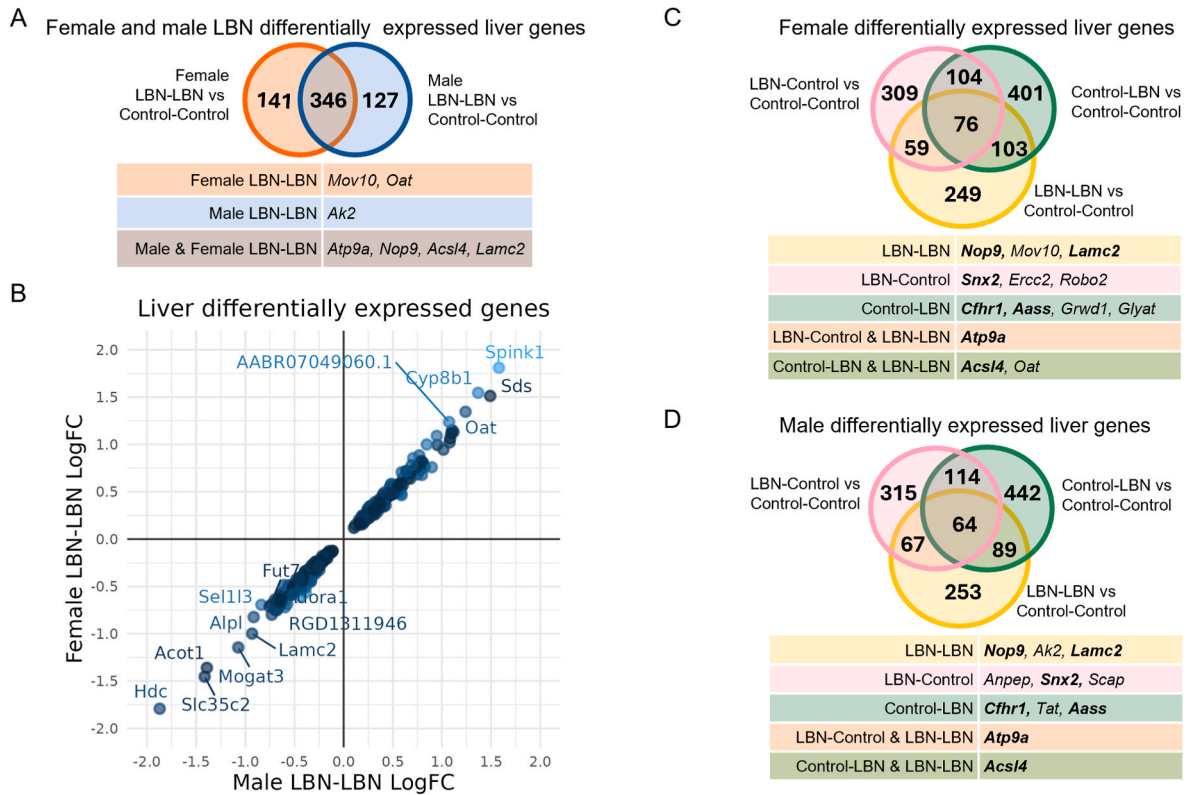


Fig. 5. Differential expression of genes in the liver by LBN group. A) LBN-LBN males and LBN-LBN females had a large percentage of overlapping liver DEGs relative to same-sex Control-Control pups (unadjusted $p < .05$). B) All of the 346 male and female LBN-LBN overlapping liver DEGs had the same direction of expression change. Points and text are colored by the absolute value of the difference in LogFC between sexes (lighter = greater difference). C) Overlap of liver DEGs in female LBN groups relative to Control-Control females (unadjusted $p < .05$). D) Overlap of liver DEGs in male LBN groups relative to Control-Control males (unadjusted $p < .05$). Tables show differentially expressed genes with eFDR < 0.05 for each comparison. Bold genes indicate genes that are differentially expressed in both sexes. For each sex: Control-Control $n = 9$, Control-LBN $n = 9$, LBN-Control $n = 10$, LBN-LBN $n = 10$.

$= 5.10$, $p = .078$; χ^2 statistic was not estimable due to submodel convergence). Late LBN exposure had a significant negative effect on Late caregiving attenuation ($\beta = -1.01$, $SE = .29$, $t(36) = -3.55$, $p = .001$, standardized $\beta = -0.51$), and Late caregiving attenuation in turn had a significant positive effect on purple module eigengene expression ($\beta = 0.04$, $SE = .01$, $t(74) = 2.79$, $p = .007$, standardized $\beta = 0.32$). Late caregiving attenuation explained 9% of variance in purple module expression (marginal $R^2 = .09$).

Eigengene expression of three modules was predicted by Late caregiving attenuation in females (blue: $\beta = -0.45$, $SE = .15$, $t(34) = -3.03$, $p = .005$; turquoise: $\beta = 0.45$, $SE = .15$, $t(34) = 3.00$, $p = .005$; green: $\beta = 0.41$, $SE = .15$, $t(34) = 2.69$, $p = .011$; Fig. 6D–E). Expression of those same three modules was predicted by Early caregiving attenuation in males (blue: $\beta = -0.39$, $SE = .15$, $t(34) = -2.51$, $p = .017$; turquoise: $\beta = 0.34$, $SE = .16$, $t(34) = 2.19$, $p = .035$; green: $\beta = 0.33$, $SE = .16$, $t(34) = 2.09$, $p = .044$). The top GO terms for the turquoise module included translation, mitochondrial translation, mitochondrial gene expression, ubiquitin-dependent protein catabolic process, and macromolecule biosynthetic process with hub genes *Stt3a*, *Dcaf8*, *Mfn2*, *Myl6*, and *Cln4* (Fig. 6D). The top GO terms for the green module were regulation of hematopoietic stem cell proliferation, intracellular protein transport, protein transport, ribosome assembly, and RNA decapping with hub genes *Stk16*, *Eif4a*, *Rps14*, *Ganab*, and *Arf5* (Fig. 6E).

P6 energy expenditure predicted eigengene expression of the green ($\beta = 0.46$, $SE = .16$, $t(29) = 2.80$, $p = .009$; Supplemental Fig. 5E), brown ($\beta = 0.34$, $SE = .15$, $t(29) = 2.25$, $p = .032$; Supplemental Fig. 5F), greenyellow ($\beta = 0.46$, $SE = .16$, $t(29) = 2.83$, $p = .008$; Supplemental Fig. 5G), and red ($\beta = 0.39$, $SE = .13$, $t(29) = 2.92$, $p = .007$) modules in males but not females. Early LBN exposure predicted decreased red module eigengene expression in females ($\beta = -0.87$, $SE = .29$, $t(34) =$

-2.95 , $p = .006$; Supplemental Fig. 5H). P6 milk leptin predicted eigengene expression of the blue ($\beta = -0.42$, $SE = .17$, $t(31) = -2.50$, $p = .018$; Supplemental Fig. 5I) and turquoise ($\beta = 0.43$, $SE = .17$, $t(31) = 2.61$, $p = .014$) modules in males but not females.

3.6. Arcuate nucleus transcriptomic analysis

3.6.1. Differential gene expression in LBN-exposed male and female pups

Differential gene expression analysis of the arcuate nucleus comparing LBN-LBN pups to Control-Control pups revealed 433 genes for females and 393 for males with an unadjusted p value less than 0.05 (Fig. 7A). Of those genes, only 86 were differentially expressed in both sexes (19.9% females, 21.9% males). Using the eFDR cutoff of 0.05, one gene *Abcf1* ($\logFC = 0.22$, eFDR < 0.001) was higher and one gene, *Furin* ($\logFC = -0.46$, eFDR < 0.001), was lower in LBN-LBN males compared to Control-Control males (Fig. 7A). The top GO terms for DEGs for LBN-LBN males and females, LBN-LBN males only, and LBN-LBN females included androgen receptor signaling pathway (females), neuron development (males), and negative regulation of microtubule polymerization (both sexes; Supplemental Fig. 6A).

3.6.2. Effects of LBN timing on transcription in the arcuate nucleus

Similar to the liver, the majority of DEGs (unadjusted $p < .05$) in the male and female arcuate nucleus were unique to the timing of LBN exposure (Fig. 7B and C), although males had more total DEGs in all LBN groups compared to LBN females. There were no DEGs that met the criteria of eFDR < 0.05 in females. *Abcf1* and *Furin* were significantly differently expressed in LBN-LBN males, *Rwdd4* ($\logFC = -0.44$, eFDR < 0.001) was lower in LBN-Control males, and *Pierce2* ($\logFC = -0.84$, eFDR < 0.001) was lower in Control-LBN males compared to Control-

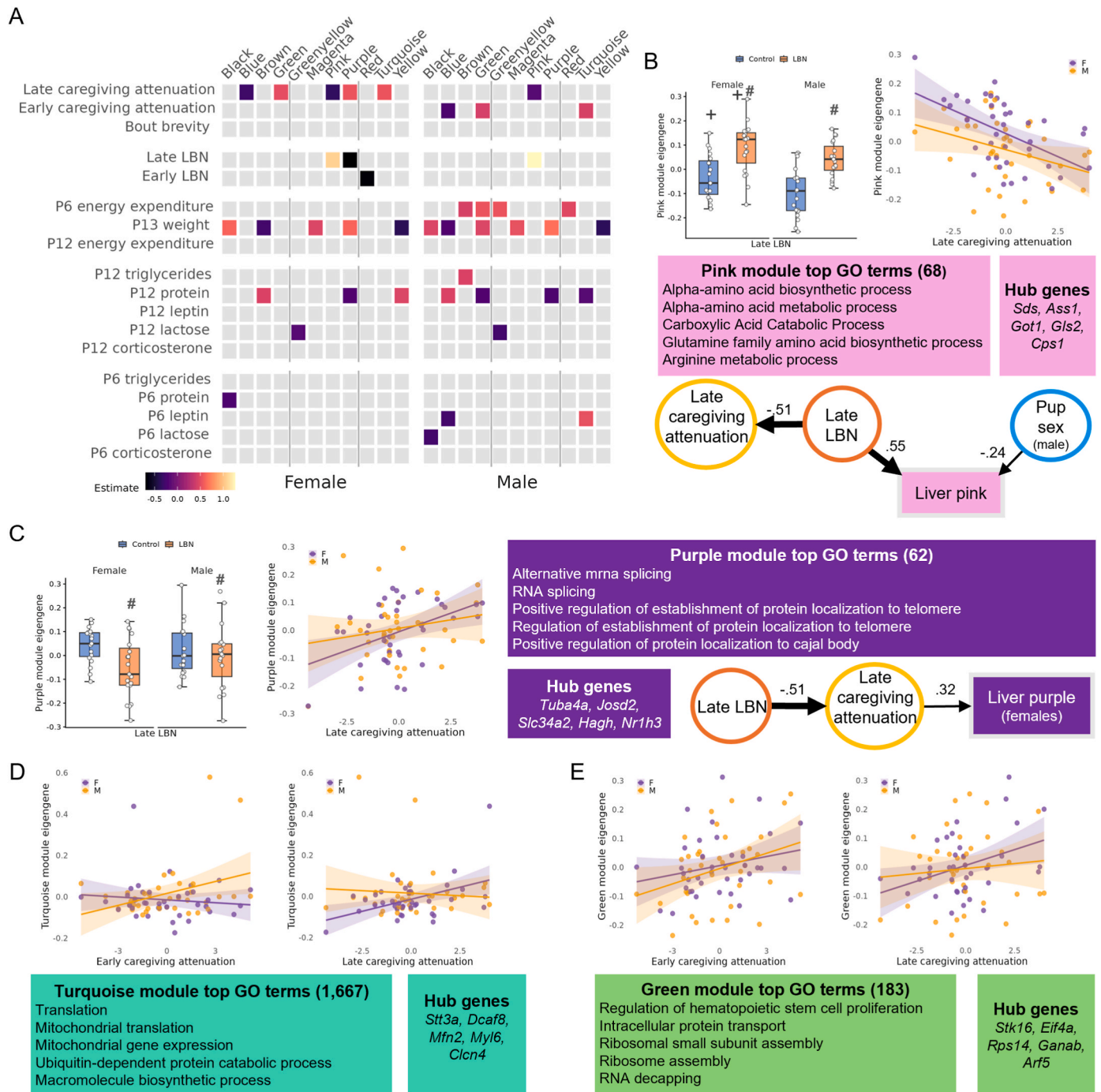


Fig. 6. Liver WGCNA analysis. A) Heatmap showing significant predictors of eigengene expression in the eleven liver co-expression modules from regression analysis. Non-significant estimates are in grey. B) Pink module eigengene expression was predicted by Late LBN and Late caregiving attenuation. Follow up analysis showed lower eigengene expression in males compared to females and higher expression in pups exposed to Late LBN. Path analysis showed a strong effect of Late LBN on eigengene expression and on Late caregiving attenuation and a moderate effect of sex on eigengene expression. There was no direct effect of Late caregiving attenuation on eigengene expression. C) Purple module eigengene expression was predicted by Late LBN and Late caregiving attenuation. Follow up analysis showed lower eigengene expression in pups exposed to Late LBN. Path analysis showed that the effect of Late LBN on eigengene expression was fully mediated by Late caregiving attenuation. D) Turquoise module expression was predicted by Early caregiving attenuation in males and Late caregiving attenuation in females. E) Green module expression was predicted by Early caregiving attenuation in males and Late caregiving attenuation in females. Top significant GO terms and hub genes are shown for pink, green, and magenta modules. Standardized estimates are shown for path analyses with line thickness to show approximate size of estimate. For each sex: Control-Control n = 9, Control-LBN n = 9, LBN-Control n = 10, LBN-LBN n = 10. +main effect of sex $p < .05$, # main effect of Late LBN $p < .05$.

Control males (Fig. 7C). The top three GO terms for overlapping and LBN-timing specific DEGs are shown in Supplemental Fig. 6B (females) and 6C (males). Top GO terms included Androgen receptor signaling pathway (female LBN-LBN only), Regulation of triglyceride metabolic process (female LBN-Control only), Regulation of primary metabolic

process (female Control-LBN only, male LBN-Control only), Regulation of peptidase activity (male LBN-LBN only), and Astrocyte differentiation (male all LBN groups).

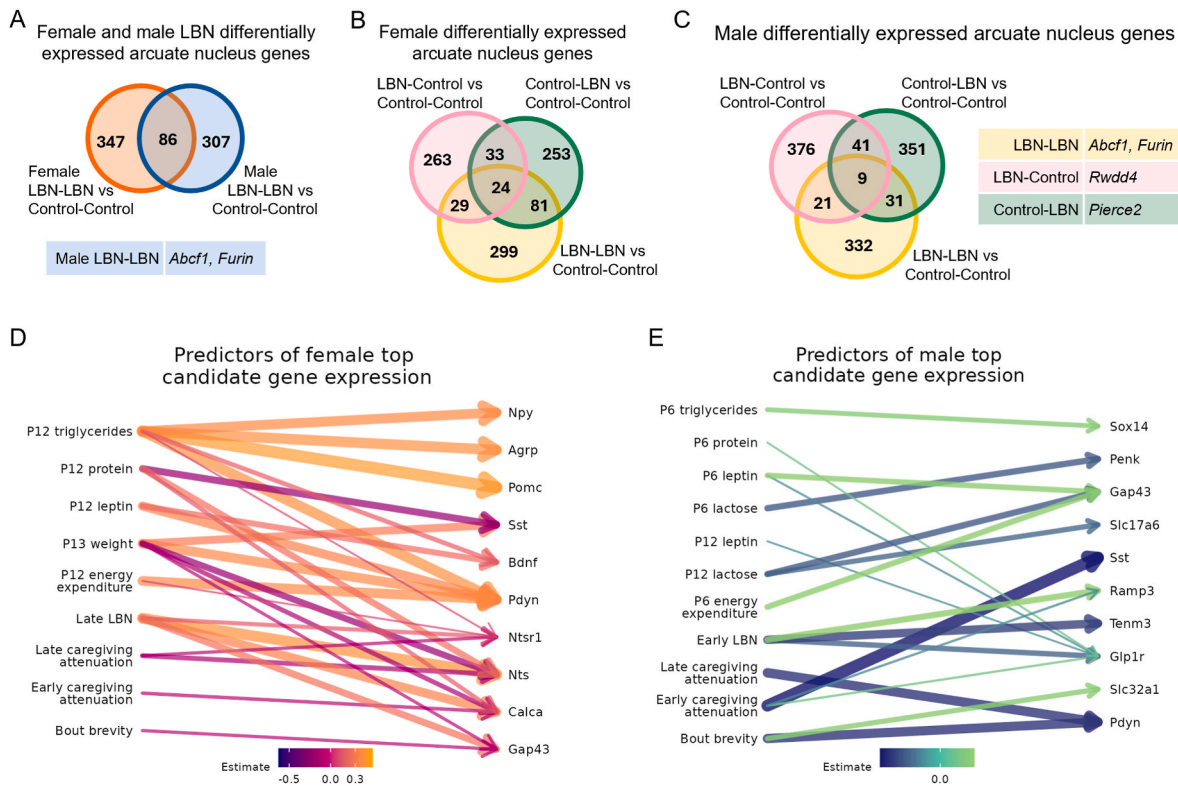


Fig. 7. Differential expression of genes in the arcuate nucleus by LBN group. A) LBN-LBN males and LBN-LBN females had a small percentage of overlapping arcuate nucleus DEGs relative to same-sex Control-Control pups (unadjusted $p < .05$). B) Overlap of arcuate nucleus DEGs in female LBN groups relative to Control-Control females (unadjusted $p < .05$). C) Overlap of arcuate nucleus DEGs in male LBN groups relative to Control-Control males (unadjusted $p < .05$). Tables show differentially expressed genes with eFDR < 0.05 for each comparison. D) Top candidate genes for female arcuate nucleus candidate gene analysis shown with predictor variables. E) Top candidate genes for male arcuate nucleus candidate gene analysis shown with predictor variables. Arrow thickness indicates size of effect and arrow color indicates direction of effect. Females: Control-Control $n = 8$, Control-LBN $n = 8$, LBN-Control $n = 8$, LBN-LBN $n = 9$. Males: Control-Control $n = 8$, Control-LBN $n = 8$, LBN-Control $n = 7$, LBN-LBN $n = 8$.

3.6.3. Candidate gene analysis in the arcuate nucleus

The female top candidate gene counts were predicted by Late LBN, Late and Early caregiving attenuation, Bout brevity, P13 wt, P12 energy expenditure, and P12 milk components (protein, triglycerides, leptin; Fig. 7D; full results in Supplemental Table 7). Late LBN positively predicted *Ntsr1*, *Nts*, *Calca*, and *Gap43*. Male top candidate genes were predicted by Bout brevity (*Slc32a1*, *Pdyn*), Early caregiving attenuation (*Glp1r*, *Tenm3*, *Sst*), Late caregiving attenuation (*Pdyn*), Early LBN (*Ramp3*, *Tenm3*, *Glp1r*), P6 energy expenditure, P12 milk components (lactose, leptin), and P6 milk components (triglycerides, protein, lactose, leptin; Fig. 7E, full results reported in Supplemental Table 7).

3.6.4. Arcuate nucleus WGCNA analysis

Arcuate nucleus WGCNA revealed 17 gene co-expression modules (Supplemental Fig. 6D). Significant effects from linear mixed model analysis for eigengene expression predicted by LBN exposure, milk components, and serum hormones are shown in Fig. 8A and Supplemental Table 8.

Early caregiving attenuation, Late caregiving attenuation, and Early LBN did not significantly predict the eigengene expression of any modules.

Bout brevity predicted tan module eigengene expression ($\beta = 0.45$, $SE = .17$, $t(27) = 2.70$, $p = .011$) in males, which included genes related to mitochondrial processes and ATP synthesis (Fig. 8B). In females, Late LBN negatively predicted grey60 eigengene expression ($\beta = -0.92$, $SE = 0.31$, $t(29) = -2.96$, $p = .006$; Fig. 8C). Follow-up analysis of the grey60 module revealed a sex by Late LBN interaction where eigengene expression was higher in Control females ($F(1, 60) = 7.92$, $p = .006$), but no main effects of sex or Late LBN ($p > .05$). Grey60 module eigengene

expression was also predicted by P6 energy expenditure in males ($\beta = -0.41$, $SE = .18$, $t(24) = -2.29$, $p = .030$; Supplemental Fig. 6E). Grey60 module hub genes include *Ppia*, *Ft1l*, *Rpl32*, *Ndufb4*, and *Rps25l2*.

Late LBN also positively predicted greenyellow eigengene expression in females ($\beta = 0.86$, $SE = .32$, $t(29) = 2.72$, $p = .011$; Fig. 8D). Follow-up analysis of the greenyellow module showed males had higher expression ($F(1, 60) = 4.55$, $p = .037$) than females and Late LBN increased ($F(1, 60) = 6.21$, $p = .016$) eigengene expression. Greenyellow eigengene expression was also predicted by pup body weight ($\beta = -0.55$, $SE = .15$, $t(29) = -3.70$, $p < .001$) and P12 milk protein ($\beta = 0.53$, $SE = .16$, $t(29) = 3.37$, $p = .002$) in females. Greenyellow top GO terms included neuron differentiation, regulation of canonical Wnt signaling pathway, and positive regulation of peptide hormone secretion and top hub genes included *Foxg1*, *Nts*, *Pnoc*, *Hmx2*, and *Lratd1*.

P6 milk corticosterone positively predicted brown eigengene expression in males ($\beta = 0.47$, $SE = .16$, $t(27) = 2.85$, $p = .008$; Fig. 8E). Top GO terms for the brown module were related to cilia, with hub genes including *Cfap43*, *Cdhr4*, *Hydin*, *Dnah12*, and *Ak9*. In females, P12 milk corticosterone negatively predicted purple module eigengene expression ($\beta = -0.38$, $SE = .18$, $t(29) = -2.12$, $p = .044$; Fig. 8F) and positively predicted midnightblue eigengene expression in females ($\beta = 0.39$, $SE = .17$, $t(29) = 2.37$, $p = .024$; Fig. 8G). Purple top GO terms were related to protein synthesis and translational regulation. Midnightblue top GO terms included those related to translation and all top hub genes were mitochondrial genes. P12 milk leptin positively predicted magenta eigengene expression females ($\beta = 0.37$, $SE = .17$, $t(28) = 2.16$, $p = .039$), which had top GO terms related to calcium signaling, ion channel regulation, and neuronal development including axonogenesis (Supplemental Fig. 6F).

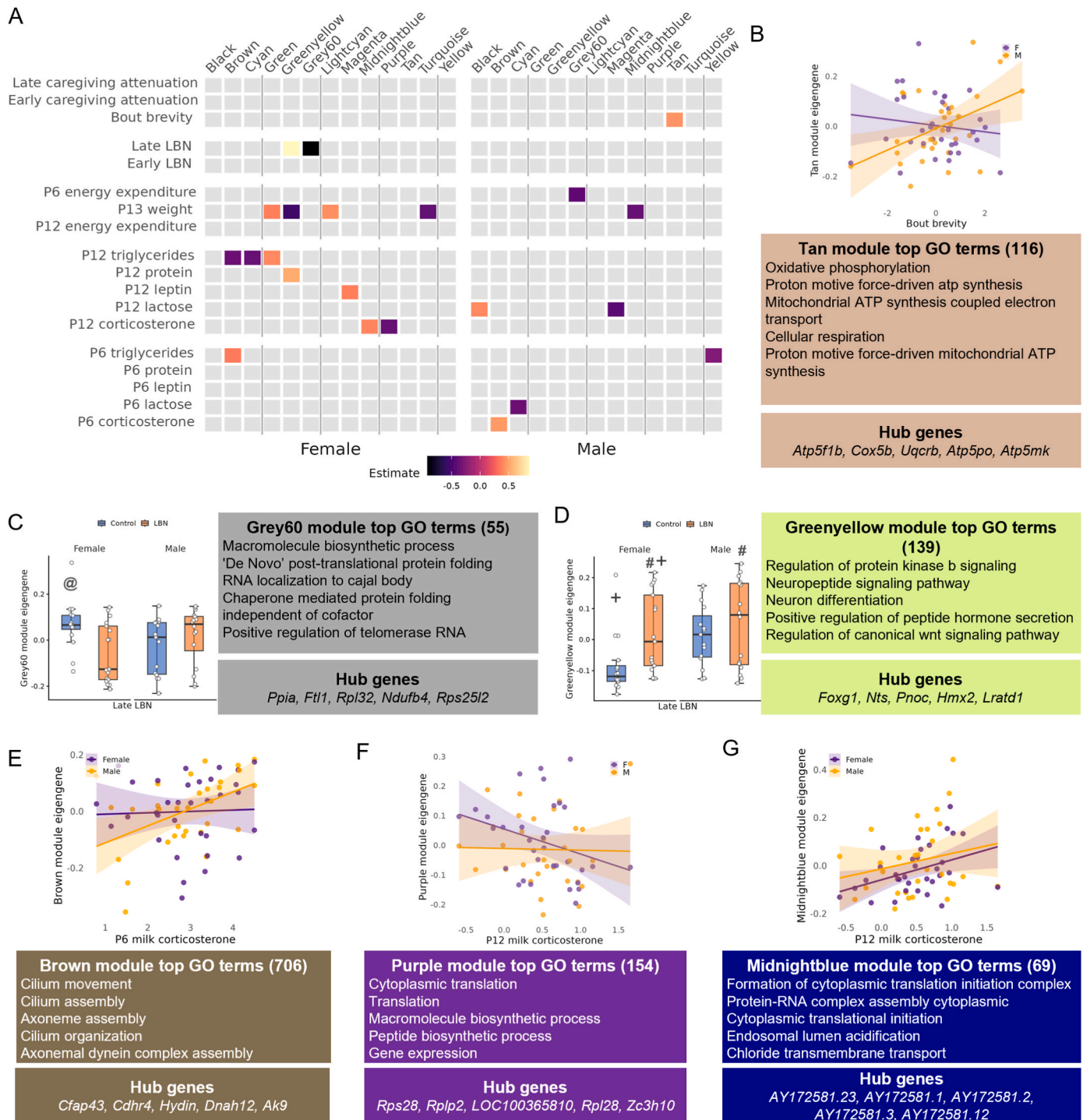


Fig. 8. Arcuate nucleus WGCNA analysis. A) Heatmap showing significant predictors of eigengene expression in the arcuate nucleus co-expression modules from regression analysis. Non-significant estimates are in grey. Red, salmon, and blue modules were not predicted by any factors and are not shown. B) Tan module eigengene expression was predicted by Bout brevity in males. C) Late LBN exposure was a significant negative predictor of grey60 module eigengene expression in females, and follow-up analysis showed a significant sex by Late LBN interaction. D) Greenyellow module eigengene expression was higher in males and increased by Late LBN exposure. E) Milk corticosterone concentration on P6 was a significant positive predictor of brown module eigengene expression in males but not in females. F) P12 Milk corticosterone concentration was a significant negative predictor of purple eigengene expression and G) a significant positive predictor of midnightblue eigengene expression in females. Top significant GO terms and hub genes are shown for each module. Females: Control-Control n = 8, Control-LBN n = 8, LBN-Control n = 8, LBN-LBN n = 9. Males: Control-Control n = 8, Control-LBN n = 8, LBN-Control n = 7, LBN-LBN n = 8. # main effect of Late LBN $p < .05$, + main effect of sex $p < .05$, @: sex by Late LBN interaction $p < .05$.

4. Discussion

Stressful experiences occurring during early life require substantial energy expenditure to maintain homeostasis, potentially diverting

resources away from critical growth and maturation processes during developmentally sensitive periods. In the current study, we found that LBN during the first two postnatal weeks exerted acute effects on rat pup metabolic outcomes, including estimated energy expenditure,

circulating metabolic and pituitary hormones, and transcriptomic profiles of the liver and arcuate nucleus. Many of these effects were specific to timing of LBN, with peripheral measures impacted by exposure occurring both early and later within the postnatal period and the hypothalamus more heavily impacted by the later LBN exposure. Limited bedding had multifaceted effects on dams and pups including changes to dam milk composition, and here we identify several key elements of early experience contributing to the development of metabolic endpoints. Finally, we observed sex differences across many of these LBN-associated outcomes. Sex differences were particularly pronounced in the arcuate nucleus transcriptome, where gene modules in females were more susceptible to influence of early experiences.

4.1. Acute effects of LBN on pup metabolism

Exposure to LBN during P0–13 altered pup estimated energy expenditure, circulating hormone levels, and transcriptomic profiles in both the liver and arcuate nucleus. Estimated daily energy expenditure was elevated when measured proximal to the stressor (Early LBN at P6; Late LBN at P12) but was reduced at later time points relative to LBN exposure (Early LBN at P12). This pattern suggests distinct acute versus delayed responses to the stressor, where initial energy expenditure increases to mobilize resources to restore homeostasis (Seal and Turner, 2021). Maternal corticosterone, transferred via milk, may contribute to this effect since corticosterone increases blood glucose through actions in the liver, pancreas, smooth muscle, and white adipose tissue to increase energy supply during acute stress (Seal and Turner, 2021). However, this early increase in energetic output may incur a cost, leading to subsequent energy depression as reserves are depleted. A similar effect was previously observed where a marker of brown adipose tissue (BAT) activation, an energetically expensive thermoregulatory mechanism, was initially increased in LBN females relative to controls, consistent with mild cold stress induction by LBN. Later measures of BAT activation were reduced despite continued exposure to the LBN thermal environment and reduction in core body temperature until approximately P9 (Lapp et al., 2024; Lapp et al., 2020a). Reduced energy expenditure at later stages could indicate a lower metabolic rate, potentially freeing resources previously devoted to immediate stress responses (Pettersen et al., 2016). This idea is corroborated by a previous study showing decreased P10 blood glucose levels in male and female rat pups exposed to LBN with a wire mesh platform on P2–10 (Moussaoui et al., 2016). This adaptive reallocation of energetic resources may help sustain growth and development under chronic stress conditions. The observed changes in serum hormones and transcriptional profiles of LBN-exposed pups are consistent with this interpretation and analogous work in adult animals demonstrates long-term alterations in hormonal regulation, glucose metabolism, and mitochondrial dysfunction that contribute to heightened risk for metabolic syndrome following chronic stress exposure (Nirupama et al., 2012; van der Kooij, 2020).

After birth, the liver undergoes a functional transformation to become the central regulator of glucose homeostasis, coinciding with the dietary shift to energy derived from maternal milk (Girard, 1990). The onset of gluconeogenesis is critical to maintaining blood glucose levels in the switch to the new relatively low-carbohydrate diet by producing glucose from non-carbohydrate precursors during intervals between feedings (Li et al., 2023). LBN affected expression of genes related to essential processes associated with gluconeogenesis including amino acid biosynthesis, which provides critical non-essential amino acid substrates such as glutamine (Zhang et al., 2018). Expression of genes related to regulation of phosphate metabolism and macromolecule glycosylation, processes which contribute to foundational liver functions critical for health and maintaining energy balance, were also disrupted in both sexes (Supplemental Fig. 5A; Blomme et al., 2009; Yasuda et al., 2023). These transcriptional changes could suggest early impairments in liver-mediated glucose homeostasis that are similar to

chronic stress-induced dysregulation of gluconeogenesis that leads to hyperglycemia and insulin insensitivity in adult animals (Nirupama et al., 2012). Because uncontrolled gluconeogenesis is a major contributor to type 2 diabetes, these transcriptional signatures may represent early markers of increased susceptibility to metabolic syndrome later in life (Petersen & Shulman, 2018). Additionally, alterations in processes such as the regulation of neutrophil migration suggest that LBN disrupts the concurrent postnatal maturation of the liver as an immune-regulatory organ.

LBN also altered the arcuate nucleus transcriptome, although to a lesser extent than in the liver. Generally, LBN impacted transcription of genes contributing to biological processes involved in developmental maturation (i.e. proximal/distal pattern formation, astrocyte differentiation, positive regulation of podosome assembly, negative regulation of microtubule polymerization) as well as those involved in gene regulation (i.e. protein sumoylation, chromatin looping; Supplemental Fig. 6B and 6C). These transcriptional changes may be mediated by LBN-induced alterations in circulating hormones, as arcuate nucleus development is highly sensitive to leptin and ghrelin signaling, which were respectively reduced and elevated following LBN exposure (Bouret et al., 2004; Sominsky et al., 2018; Steculorum et al., 2015). Candidate gene analysis further revealed effects of LBN on genes associated with neuropeptide signaling (*Ntsr1*, *Sst*, *Calca*, *Nts*, *Pdyn*, *Glp1r*, *Ramp3*) and axonal growth (*Term3*). In males, expression of the arcuate “brown” module eigengene, which was enriched for cilia-related genes, was predicted by P6 corticosterone levels. Primary cilia in the arcuate play key roles in nutrient sensing and hormonal responsiveness, and their developmental disruption has been linked to adult obesity (Lee et al., 2020; Leon-Mercado et al., 2017; Yang et al., 2021). Although these effects were specific to LBN timing and pup sex, they nevertheless demonstrate that LBN-induced transcriptional changes for genes critical to energy homeostasis are detectable before arcuate nucleus projections and circuits supporting metabolic homeostasis have matured (Bouret and Simerly, 2007).

4.2. Effects on metabolic measures depend on timing of LBN exposure

Few previous studies have compared the effects of different LBN exposure periods, and none have specifically focused on LBN timing effects on pup metabolic outcomes. After controlling for pup body weight, we found that concentrations of four circulating metabolic and pituitary hormones measured on P13 were more strongly affected by Early LBN than by Late LBN exposure (Table 1). Another study with mice subject to P2–9 LBN with wire mesh found no effect of LBN on active (acetylated) ghrelin and LBN-induced increases in insulin on P10, which were reduced by Early LBN and unaffected by LBN respectively in our study (Yam et al., 2017b). This disagreement in findings may be attributed to methodology differences including species, use of wire mesh platform, and LBN timing. In adults, serum hormone levels are sensitive to nutrient intake, and the observed changes in milk corticosterone and triglyceride levels in LBN dams on P6 (but not P12) may contribute to the greater hormonal effects of Early LBN (Lu et al., 2021; Vella and Camilleri, 2017). Notably, *Atp9a*, a gene involved in protein trafficking and differentially expressed in the liver following Early LBN in both sexes, has been implicated in Neurodevelopmental Disorder with Poor Growth and Behavioral Abnormalities, in which affected individuals display failure to thrive and poor overall growth (Vogt et al., 2022). Consistent with this, Pardo et al. (2025) reported that LBN exposure on P2–4, but not P5–6 or P7–9, reduced litter body weight in Sprague-Dawley rats using a wire mesh floor variation of the LBN paradigm. Effects of LBN on maternal behavior also appear to be weaker during later postnatal days compared to the early postpartum period (Lapp et al., 2024; Pardo et al., 2025). Similarly, the thermal effects of LBN are more pronounced in younger pups, as many physiological thermoregulatory mechanisms do not fully emerge until after P6 (Lapp et al., 2024; Shelton and Alberts, 2018). Together with our findings, this

suggests that early postnatal LBN exposure exerts stronger effects on peripheral metabolic systems undergoing rapid developmental transitions. These systems lack fully developed mechanisms to cope with adverse experiences at a time when maternal behavior and maternal milk are also more susceptible to environmental influence.

Linear mixed model analysis of WGCNA modules in the liver and arcuate nucleus revealed that Late, but not Early, LBN exposure predicted eigengene expression of select modules (liver pink and purple; arcuate greenyellow and grey60). The influence of Late LBN on the arcuate transcriptome may reflect the maturation of arcuate hypothalamic projections during this developmental window, consistent with the regulatory and developmental GO terms enriched in the affected modules (Matsumoto and Arai, 1976; Sominsky et al., 2018). Notably, these effects were observed in only females, suggesting that females may be more susceptible than males to the effects of environmental adversity on neural circuitry underlying metabolic homeostasis during this period. At the level of individual genes, some arcuate candidates were selectively affected by either Early or Late LBN in a sex-specific manner, suggesting that the timing of LBN exposure exerts distinct, non-overlapping effects on transcriptional regulation. A prior investigation of P2-9 LBN effect on P9 hypothalamic expression of 14 genes related to fatty acid metabolism, hormone receptors, and neuropeptides in mice found higher expression of *Lepr* with LBN in both sexes (Ruigrok et al., 2021). In contrast, *Lepr* expression in the arcuate nucleus was unaffected by Early or Late LBN in our study (Supplemental Table 7). Our hormonal and transcriptomic measures were collected shortly after Late LBN exposure and it remains unclear whether Early LBN would have produced stronger effects on these outcomes had parallel measures been obtained at P6.

4.3. Individual variability in LBN effects on maternal behavior and dam milk composition

LBN litters had lower scores on both Early and Late caregiving attenuation, indicating that they received more pup-directed behaviors from dams with shorter intervals between bouts. This is consistent with previously reported LBN-induced increases in nursing, nest attendance, and licking and grooming, and suggests that the stress of limited bedding increased overall quantity of pup-directed care (Eck et al., 2020; Fuentes et al., 2014; Lapp et al., 2020b; Lapp et al., 2024; Shupe and Clinton, 2021). This increase in caregiving quantity may reflect compensatory maternal responses to changes in pup signals and underscores the importance of examining individual variability and specific dimensions of maternal behavior rather than relying solely on group-level comparisons.

The PCA approach builds on a tradition of using PCA to reduce correlated behavioral measures into interpretable components that predict biologically meaningful outcomes across animal species (Budaev, 2010; Tamin et al., 2023; Vannoni et al., 2014). While LBN clearly altered maternal behavior, maternal behavior phenotypes showed considerable overlap between LBN and Control dams. Reducing the dimensionality of correlated maternal behavior measures into orthogonal components allowed us to capture the full spectrum of individual variation in maternal behavior and to identify which aspects of that variation (early versus late caregiving quantity and bout length) were most predictive of pup outcomes. The sensitivity of this approach to account for individual variability is further illustrated by the relationship between maternal behavior and milk corticosterone: although Bout brevity did not differ at the group level, shorter mean bout length was significantly associated with higher milk corticosterone on P12, consistent with expected effect of LBN-induced maternal stress on dam hormones and behavior and demonstrating that individual variability can obscure group-level effects. Notably, we only investigated three maternal behavior PCs that collectively accounted for ~54% of the total variance in maternal behavior, leaving more than half unaccounted for in our analyses. The remaining PCs may capture ethologically

meaningful dimensions of maternal care organization, including temporal contrasts in care across lactation, that were beyond the scope of the present study but represent important directions for future work examining how the fine-grained temporal structure of maternal behavior shapes offspring development.

LBN also altered triglyceride and corticosterone levels in dam milk on P6, implicating maternally derived hormones and macronutrients as additional potential mediators of LBN effects on pups alongside changes in maternal care. Maternal corticosterone can elevate corticosterone levels in serum and the brains (which contain GR) of pups at a developmental stage when endogenous corticosterone is typically low (Brummelte et al., 2010; Rosenfeld et al., 1988). Tight regulation of endogenous GC concentration during the SHRP is thought to protect the developing brain from GC effects on neurogenesis and neural growth; thus, increased exposure via milk may disrupt brain development in regions containing GR, including the hypothalamus (Schmidt, 2019). Fatty acids, in addition to serving as the most energy-dense macronutrient, play essential roles in neural growth, synaptic signaling, and cell differentiation (Uauy and Dangour, 2006). Accordingly, the elevated triglyceride levels in LBN dams may have broad developmental consequences for pups. Future studies should examine whether environmental adversity alters the composition of specific fatty acids, micronutrients, the milk microbiome, and bioactive factors such as milk-derived extracellular vesicles, which could be additional pathways of maternal transmission (Wijenayake et al., 2023).

4.4. Key elements of early experience contributing to metabolic outcomes

By examining maternal behavior and milk measures, we were able to disentangle specific effects of key components of early influential experiences on acute metabolic outcomes. Although LBN-induced changes in maternal behavior are often considered the primary driver of LBN effects on pups, maternal behavior in this study was associated with a minority of outcomes, and these relationships were largely dependent on developmental timing and sex. This more limited influence may reflect our focus on metabolic outcomes, which are likely to be shaped more strongly by other aspects of early experience, such as nutrient intake. Accordingly, milk composition exerted broader effects than maternal behavior on many of our endpoints. The distinct relationships between P6 and P12 milk components and transcriptional profiles in the liver versus arcuate nucleus align with the developmental timing of these tissues: the liver undergoes substantial maturation immediately after birth, making it highly sensitive to nutrient input across the entire developmental period examined here (Patel and Srinivasan, 2006), whereas the arcuate nucleus projections develop starting about a week later, rendering it more likely to be influenced by later milk content or influenced by early milk indirectly through secondary pathways (Cottrell et al., 2009). Milk corticosterone was a significant predictor of eigengene expression of a few arcuate modules, potentially affecting processes including translational regulation, protein synthesis, and ciliary assembly and function in a sex-specific manner. These predictive relationships suggest a potential role for elevated corticosterone exposure, as opposed to variation in maternal care, during the SHRP in shaping some LBN-related outcomes, though mechanistic studies would be required to establish causality. Because corticosterone increases gluconeogenesis and lipogenesis, excess exposure during a period when endogenous levels are typically low could prime metabolic systems for long-term dysfunction (Seal and Turner, 2021). Body weight had especially large effects on arcuate and liver eigengene expression. Effects of body weight likely reflect not only differences in overall size but also in body composition, particularly reduced white adipose tissue following LBN, which plays a central role in glucose and lipid metabolism as well as metabolic hormone secretion (Speakman, 2005; Yam et al., 2017a). These findings underscore the potential for early adversity to shape metabolism via hormonal and nutrition exposure through maternal milk and open opportunities for intervention through maternal diet. Indeed,

prior studies have successfully mitigated some effects of early adversity by manipulating maternal intake of polyunsaturated fatty acids, methionine, and B vitamins (Bernardi et al., 2013; Naninck et al., 2017; Yam et al., 2019).

Path analysis was used to test whether the effects of LBN exposure on module eigengene expression were direct or mediated through maternal behavior variables. For the liver pink module, path analysis revealed that Late LBN had a strong direct effect on eigengene expression that was independent of its effect on Late caregiving attenuation, suggesting that the programming of hepatic amino acid metabolism genes occurs through a pathway other than altered caregiving quality. In contrast, for the liver purple module, the effect of Late LBN on eigengene expression was fully mediated by Late caregiving attenuation, indicating that the influence of Late LBN on genes related to mRNA splicing and gene regulation is contingent on changes in maternal behavior rather than a direct effect of the LBN condition itself. Together, these results demonstrate that LBN exposure programs liver gene expression through at least two distinct mechanisms: one that operates independently of maternal behavior and one that is transmitted through the caregiving environment.

4.5. Sex differences in metabolic outcomes

There are prevalent sex differences in energy balance, energy intake, and metabolic disease susceptibility, so it is not surprising that we observed sex differences in several of our outcomes (Tramunt et al., 2020). Seven metabolic and pituitary hormones in serum were lower in males than females, consistent with previous reports (e.g. Matsuda et al., 1997). Males, however, had higher levels of growth hormone, which is regulated by testosterone and acts on the liver to regulate transcription and contribute to sex differences in primary liver functions (Ramirez et al., 2012). Although there was high agreement among differentially expressed genes by LBN groups between sexes, there were also several DEGs that were sex-specific and differences in predictors of module eigengene expression, which is consistent with studies showing sex differences in liver response to nutritional stress (Maggi, 2022). Late caregiving attenuation predicted eigengene expression of multiple liver modules exclusively in females, while Early caregiving attenuation predicted expression of the same modules exclusively in males, suggesting there may be sex-specific temporal dissociation in the relationship between maternal behavior quantity and hepatic gene expression such that female offspring liver transcriptome is more sensitive to late lactation caregiving whereas male offspring liver transcriptome is more sensitive to early lactation caregiving. Similarly, the effects of LBN on the arcuate nucleus transcriptome were largely sex-specific, with little overlap in the top candidate genes except for *Tenm3*, a gene that is important for establishing synaptic connections. Predictors of arcuate module eigengene expression were entirely sex-dependent, with more associations in females. The arcuate nucleus has testosterone-dependent ER- α expression in the postnatal period and synapse formation has been shown to be modified by neonatal estrogen, which contribute to sex differences in arcuate function and may be related to sex differences in developmental programming of obesity (Arai and Matsumoto, 1978; Cortes et al., 2021; MacKay et al., 2022). Our previous work has shown that LBN exposure increases circulating testosterone in females, which could contribute to the shift in the female LBN eigengene expression of the greenyellow module, which was associated with key neural developmental processes, towards more male-like expression levels (Lapp et al., 2024).

4.6. Strengths and limitations

By assessing a wide range of metabolic, hormonal, and transcriptomic endpoints, we built a multidimensional picture of how early adversity shapes both peripheral and central processes underlying energy balance. Importantly, we examined two key components of early

experience, maternal behavior and milk composition, using approaches that accounted for individual variability between dams. Multiple, complementary measures of metabolic state allowed us to assess relationships between outcomes, and the use of path analyses helped clarify direct and indirect effects on pup physiology. A further strength was the ability to examine the timing of adversity, distinguishing between early and late exposure periods, which aligned with distinct developmental windows.

These strengths are balanced by several methodological limitations. While the maternal behavior principal components included loadings for nursing duration, the time a dam spends in a nursing posture does not necessarily reflect milk transfer, especially given evidence that stress can inhibit milk production (Negoro et al., 1987). Future studies will need to disentangle the relative influence of nursing behavior, milk intake, and total pup-directed care on developmental outcomes. Furthermore, because P13 sampling was more temporally distant from Early LBN than from Late LBN, the observed effects may have differed had Early LBN data also been collected on P6. Milk collection and behavioral testing on P6 and P12 required separation of the dam and pups, so it should be noted that pups were exposed to a form of maternal separation in addition to LBN that may affect the outcomes measured, although this separation applied to all groups. Additionally, although we treated the relationship between pup weight and energy expenditure as unidirectional (weight predicting expenditure) for interpretability, this relationship is likely bidirectional, as energy expenditure also influences weight trajectories over time (Franczak et al., 2024). Our method for estimating daily energy expenditure has inherent constraints: it does not account for the thermic effect of feeding, was based on indirect estimates of oxygen consumption, and did not include pup activity during measurement (Speakman et al., 2013). Finally, while the high-dimensional methods we used are well-suited for generating hypotheses and identifying candidate pathways, the relatively small sample size may limit statistical power, model stability, and generalizability. Specifically, PCA loadings and WGCNA module composition may be unstable at this sample size and should be interpreted as exploratory rather than confirmatory. Independent replication in a larger cohort will be necessary to validate the specific modules, pathways, and mediating relationships identified here. Additionally, because analyses were not corrected for multiple comparisons given the exploratory nature of the study, the probability of false positives is elevated, and all findings should be interpreted with appropriate caution pending replication. These considerations highlight the need for future studies to further parse the complex mechanisms linking early adversity to metabolic development.

4.7. Conclusions and future directions

This study provides evidence that early adversity exerts acute and timing-specific effects on metabolic function, gene expression, and hormones critical for metabolic signaling. Our findings highlight the complex interplay between the environment and foundational elements of mother-offspring interactions. These data suggest that the liver transcriptome is shaped by early stress experiences across both the first and second postnatal weeks, while the arcuate nucleus of the hypothalamus is primarily affected by later experiences (particularly in females), mapping on to the respective periods of maturation for these tissues. These findings underscore the need to consider temporal dynamics and sex-specific nature of developmental sensitivity to environmental stressors. Moreover, we were able to uncover otherwise hidden outcomes by considering individual variability of the litter's early experience rather than solely relying on LBN group differences. Future work should aim to disentangle contributions of specific components of maternal care, milk, and other facets of LBN and understand synergistic effects on offspring outcomes including long-term effects on metabolism, stress response, and emotionality. Understanding these causal mechanisms will inform strategies to mitigate long-term

consequences of early life adversity.

CRediT authorship contribution statement

Hannah E. Lapp: Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Melissa G. Salazar:** Investigation. **Evelyn Deveraux:** Writing – review & editing, Investigation, Data curation. **Pawel K. Misztal:** Writing – review & editing, Supervision, Resources, Funding acquisition, Data curation. **Frances A. Champagne:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Funding sources

This work was funded by institutional funds from the University of Texas at Austin and the National Science Foundation grant no. 2146347.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jynstr.2026.100835>.

Data availability

Data will be made available on request.

References

- Andrews, S., 2010. FastQC: a quality control tool for high throughput sequence data [Computer software]. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.
- Arai, Y., Matsumoto, A., 1978. Synapse formation of the hypothalamic arcuate nucleus during post-natal development in the female rat and its modification by neonatal estrogen treatment. *Psychoneuroendocrinology* 3 (1), 31–45. [https://doi.org/10.1016/0306-4530\(78\)90039-2](https://doi.org/10.1016/0306-4530(78)90039-2).
- Bedont, J.L., Newman, E.A., Blackshaw, S., 2015. Patterning, specification, and differentiation in the developing hypothalamus. *WIREs Dev. Biol.* 4 (5), 445–468. <https://doi.org/10.1002/wdev.187>.
- Bernardi, J.R., Ferreira, C.F., Senter, G., Krolow, R., Aguiar, B. W. de, Portella, A.K., Kauer-Sant'Anna, M., Kapczinski, F., Dalmaz, C., Goldani, M.Z., Silveira, P.P., 2013. Early life stress interacts with the diet deficiency of Omega-3 fatty acids during the life course increasing the metabolic vulnerability in adult rats. *PLoS One* 8 (4), e62031. <https://doi.org/10.1371/journal.pone.0062031>.
- Blomme, B., Van Steenkiste, C., Callewaert, N., Van Vlierberghe, H., 2009. Alteration of protein glycosylation in liver diseases. *J. Hepatol.* 50 (3), 592–603. <https://doi.org/10.1016/j.jhep.2008.12.010>.
- Boersma, G.J., Bale, T.L., Casanuello, P., Lara, H.E., Lucion, A.B., Suchecki, D., Tamashiro, K.L., 2014. Long-term impact of early life events on physiology and behaviour. *J. Neuroendocrinol.* 26 (9), 587–602. <https://doi.org/10.1111/jne.12153>.
- Böhme, H.J., Sparmann, G., Hofmann, E., 1983. Biochemistry of liver development in the perinatal period. *Experientia* 39 (5), 473–483. <https://doi.org/10.1007/BF01965164>.
- Bouret, S.G., 2022. Developmental programming of hypothalamic melanocortin circuits. *Exp. Mol. Med.* 54 (4), 403–413. <https://doi.org/10.1038/s12276-021-00625-8>.
- Bouret, S.G., Draper, S.J., Simerly, R.B., 2004. Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science (New York, N.Y.)* 304 (5667), 108–110. <https://doi.org/10.1126/science.1095004>.
- Bouret, S.G., Simerly, R.B., 2007. Development of Leptin-Sensitive circuits. *J. Neuroendocrinol.* 19 (8), 575–582. <https://doi.org/10.1111/j.1365-2826.2007.01563.x>.
- Brummelte, S., Schmidt, K.L., Taves, M.D., Soma, K.K., Galea, L.A.M., 2010. Elevated corticosterone levels in stomach milk, serum, and brain of male and female offspring after maternal corticosterone treatment in the rat. *Dev. Neurobiol.* 70 (10), 714–725. <https://doi.org/10.1002/dneu.20805>.
- Budaev, S.V., 2010. Using principal components and factor analysis in animal behaviour research: caveats and guidelines. *Ethology* 116, 472–480. <https://doi.org/10.1111/j.1439-0310.2010.01758.x>.
- Burbridge, S., Stewart, I., Placzek, M., 2016. Development of the neuroendocrine hypothalamus. *Compr. Physiol.* 6 (2), 623–643. <https://doi.org/10.1002/j.2040-4603.2016.tb00697.x>.
- Burnham, K.P., Anderson, D.R., 2002. *Model Selection and Multimodel Inference: a Practical information-theoretic Approach*, second ed. Springer-Verlag.
- Casabiell, X., Piñeiro, V., Tomé, M.A., Peinó, R., Dieguez, C., Casanueva, F.F., 1997. Presence of Leptin in Colostrum and/or breast milk from lactating mothers: a potential role in the regulation of neonatal food intake. *J. Clin. Endocrinol. Metabol.* 82 (12), 4270–4273. <https://doi.org/10.1210/jcem.82.12.4590>.
- Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R., Ma'ayan, A., 2013. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinf.* 14 (1), 128. <https://doi.org/10.1186/1471-2105-14-128>.
- Cortes, L.R., Cisternas, C.D., Cabahug, I.N.K.V., Mason, D., Ramlall, E.K., Castillo-Ruiz, A., Forger, N.G., 2021. DNA methylation and demethylation underlie the sex difference in Estrogen receptor alpha in the arcuate nucleus. *Neuroendocrinology* 112 (7), 636–648. <https://doi.org/10.1159/000519671>.
- Cottrell, E.C., Cripps, R.L., Duncan, J.S., Barrett, P., Mercer, J.G., Herwig, A., Ozanne, S. E., 2009. Developmental changes in hypothalamic leptin receptor: relationship with the postnatal leptin surge and energy balance neuropeptides in the postnatal rat. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 296 (3), R631–R639. <https://doi.org/10.1152/ajpregu.90690.2008>.
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)* 29 (1), 15–21. <https://doi.org/10.1093/bioinformatics/bts635>.
- Eck, S.R., Ardekani, C.S., Salvatore, M., Luz, S., Kim, E.D., Rogers, C., Hall, A., Lee, D.E., Famularo, S. T., Bhatnagar, S., Bangasser, D.A., 2020. The effects of early life adversity on growth, maturation, and steroid hormones in male and female rats. *Eur. J. Neurosci.* 52, 2664–2680. <https://doi.org/10.1111/ejn.14609>.
- Even, P.C., Nadkarni, N.A., 2012. Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 303 (5), R459–R476. <https://doi.org/10.1152/ajpregu.00137.2012>.
- Franczak, E., Maurer, A., Drummond, V.C., Kugler, B.A., Wells, E., Wenger, M., Peelor, F. F., Crosswhite, A., McCain, C.S., Koch, L.G., Britton, S.L., Miller, B.F., Thyfault, J.P., 2024. Divergence in aerobic capacity and energy expenditure influence metabolic tissue mitochondrial protein synthesis rates in aged rats. *GeroScience* 46 (2), 2207–2222. <https://doi.org/10.1007/s11357-023-00985-1>.
- Felitti, V.J., Anda, R.F., Nordenberg, D., Williamson, D.F., Spitz, A.M., Edwards, V., Koss, M.P., Marks, J.S., 1998. Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults: the Adverse Childhood Experiences (ACE) Study. *Am. J. Prev. Med.* 14 (4), 245–258. [https://doi.org/10.1016/S0749-3797\(98\)00017-8](https://doi.org/10.1016/S0749-3797(98)00017-8).
- Fuentes, S., Daviu, N., Gagliano, H., Garrido, P., Zelena, D., Monasterio, N., Armario, A., Nadal, R., 2014. Sex-dependent effects of an early life treatment in rats that increases maternal care: vulnerability or resilience? *Front. Behav. Neurosci.* 8, 56. <https://doi.org/10.3389/fnbeh.2014.00056>.
- Girard, J., 1990. Metabolic adaptations to change of nutrition at birth. *Biol. Neonate* 58 (Suppl. 1), 3–15. <https://doi.org/10.1159/000243294>.
- Herrera, E., Amusquivar, E., 2000. Lipid metabolism in the fetus and the newborn. *Diabetes Metabol. Res. Rev.* 16 (3), 202–210. [https://doi.org/10.1002/1520-7560\(200005/06\)16:3<202::aid-dmrr116>3.0.co;2](https://doi.org/10.1002/1520-7560(200005/06)16:3<202::aid-dmrr116>3.0.co;2).
- Innis, S.M., 2011. Metabolic programming of long-term outcomes due to fatty acid nutrition in early life. *Matern. Child Nutr.* 7 (Suppl. 2), 112–123. <https://doi.org/10.1111/j.1740-8709.2011.00318.x>.
- Janssen, J.A.M.J.L., 2022. New insights into the role of insulin and hypothalamic-pituitary-adrenal (HPA) axis in the metabolic syndrome. *Int. J. Mol. Sci.* 23 (15). <https://doi.org/10.3390/ijms23158178>. Article 15.
- Kelly-Irving, M., Lepage, B., Dedieu, D., Bartley, M., Blane, D., Grosclaude, P., Lang, T., Delpierre, C., 2013. Adverse childhood experiences and premature all-cause mortality. *Eur. J. Epidemiol.* 28 (9), 721–734. <https://doi.org/10.1007/s10654-013-9832-9>.
- Kirk, S.L., Samuelsson, A.-M., Argenton, M., Dhonye, H., Kalamatianos, T., Poston, L., Taylor, P.D., Coen, C.W., 2009. Maternal obesity induced by diet in rats permanently influences central processes regulating food intake in offspring. *PLoS One* 4 (6), e5870. <https://doi.org/10.1371/journal.pone.0005870>.
- Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., McDermott, M.G., Monteiro, C.D., Gundersen, G.W., Ma'ayan, A., 2016. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* 44 (Web Server issue), W90–W97. <https://doi.org/10.1093/nar/gkw377>.
- Langfelder, P., Horvath, S., 2008. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinf.* 9, 559. <https://doi.org/10.1186/1471-2105-9-559>.
- Langley-Evans, S.C., Gardner, D.S., Jackson, A.A., 1996. Maternal protein restriction influences the programming of the Rat hypothalamic-pituitary-adrenal axis. *J. Nutr.* 126 (6), 1578–1585. <https://doi.org/10.1093/jn/126.6.1578>.
- Lapp, H.E., Bartlett, A.A., Zup, S.L., Hunter, R.G., Moore, C.L., 2020a. Early experience alters developmental trajectory of central oxytocin systems involved in hypothalamic-pituitary-adrenal axis regulation in Long-Evans rats. *Horm. Behav.* 126, 104822. <https://doi.org/10.1016/j.yhbeh.2020.104822>.
- Lapp, H.E., Mueller, I., Moore, C.L., 2020b. Limited bedding and nesting material changes indices of cellular metabolism and behavioral thermal regulation in Long-Evans rats during the first two weeks of life. *Physiol. Behav.* 222, 112957. <https://doi.org/10.1016/j.physbeh.2020.112957>.

- Lapp, H.E., Salazar, M., Champagne, F.A., 2024. Postnatal rearing environment alters pup cues for caregiver-offspring interactions. *Horm. Behav.* 165, 105630. <https://doi.org/10.1016/j.yhbeh.2024.105630>.
- Lapp, H.E., Salazar, M.G., Champagne, F.A., 2023. Automated maternal behavior during early life in rodents (AMBER) pipeline. *Sci. Rep.* 13, 18277. <https://doi.org/10.1038/s41598-023-45495-4>.
- Lau, C., 1992. Effects of various stressors on milk release in the rat. *Physiol. Behav.* 51 (6), 1157–1163. [https://doi.org/10.1016/0031-9384\(92\)90302-1](https://doi.org/10.1016/0031-9384(92)90302-1).
- Lee, C.H., Song, D.K., Park, C.B., Choi, J., Kang, G.M., Shin, S.H., Kwon, I., Park, S., Kim, S., Kim, J.Y., Dugu, H., Park, J.W., Choi, J.H., Min, S.H., Sohn, J.-W., Kim, M.-S., 2020. Primary cilia mediate early life programming of adiposity through lysosomal regulation in the developing mouse hypothalamus. *Nat. Commun.* 11 (1), 5772. <https://doi.org/10.1038/s41467-020-19638-4>.
- Lee, J.J., Rubin, A.P., 1993. Breast feeding and anaesthesia. *Anaesthesia* 48 (7), 616–625. <https://doi.org/10.1111/j.1365-2044.1993.tb07130.x>.
- Lee, M.-J., Pramyothin, P., Karastergiou, K., Fried, S.K., 2014. Deconstructing the roles of glucocorticoids in adipose tissue biology and the development of central obesity. *Biochim. Biophys. Acta* 1842 (3), 473–481. <https://doi.org/10.1016/j.bbdis.2013.05.029>.
- Lee, W., Milewski, T.M., Dworz, M.F., Young, R.L., Gaudet, A.D., Fonken, L.K., Champagne, F.A., Curley, J.P., 2022. Distinct immune and transcriptomic profiles in dominant versus subordinate males in mouse social hierarchies. *Brain Behav. Immun.* 103, 130–144. <https://doi.org/10.1016/j.bbi.2022.04.015>.
- Lefcheck, J.S., 2016. piecewiseSEM: piecewise structural equation modelling in r for ecology, evolution, and systematics. *Methods Ecol. Evol.* 7 (5), 573–579. <https://doi.org/10.1111/2041-210X.12512>.
- Lefcheck, J.S., 2024. GitHub—Jslfche/sem_book: an introduction to structural equation modeling. https://github.com/jslfche/sem_book.
- Leon-Mercado, L., Herrera Moro Chao, D., Basualdo, M.D.C., Kawata, M., Escobar, C., Buijs, R.M., 2017. The arcuate nucleus: a site of fast negative feedback for corticosterone secretion in Male rats. *eNeuro* 4 (1). <https://doi.org/10.1523/ENEURO.0350-16.2017>.
- Levine, S., 1970. The Pituitary—Adrenal System and the developing brain. *Prog. Brain Res.* 32, 79–85. [https://doi.org/10.1016/S0079-6123\(08\)61521-6](https://doi.org/10.1016/S0079-6123(08)61521-6). Elsevier.
- Levine, S., Haltmeyer, G.C., Karas, G.G., Denenberg, V.H., 1967. Physiological and behavioral effects of infantile stimulation. *Physiol. Behav.* 2 (1), 55–59. [https://doi.org/10.1016/0031-9384\(67\)90011-X](https://doi.org/10.1016/0031-9384(67)90011-X).
- Li, L., Zhou, H., Wang, J., Li, J., Lyu, X., Wang, W., Luo, C., Huang, H., Zhou, D., Chen, X., Xu, L., Li, P., 2023. Metabolic switch from glycogen to lipid in the liver maintains glucose homeostasis in neonatal mice. *J. Lipid Res.* 64 (10), 100440. <https://doi.org/10.1016/j.jlr.2023.100440>.
- Lohman, B.K., Weber, J.N., Bolnick, D.I., 2016. Evaluation of TagSeq, a reliable low-cost alternative for RNAseq. *Mol. Ecol. Resour.* 16 (6), 1315–1321. <https://doi.org/10.1111/1755-0998.12529>.
- Lu, V.B., Gribble, F.M., Reimann, F., 2021. Nutrient-induced cellular mechanisms of gut hormone secretion. *Nutrients* 13 (3), 883. <https://doi.org/10.3390/nu13030883>.
- MacKay, H., Gunasekara, C.J., Yam, K.-Y., Srisai, D., Yalamanchili, H.K., Li, Y., Chen, R., Coarfa, C., Waterland, R.A., 2022. Sex-specific epigenetic development in the mouse hypothalamic arcuate nucleus pinpoints human genomic regions associated with body mass index. *Sci. Adv.* 8 (39). <https://doi.org/10.1126/sciadv.abo3991>.
- Maggi, A., 2022. Sex and liver disease: the necessity of an overarching theory to explain the effect of sex on nonreproductive functions. *Endocrinology* 163 (1). <https://doi.org/10.1210/endo/bqab229>.
- Mainardi, M., Pizzorusso, T., Maffei, M., 2013. Environment, Leptin Sensitivity, and hypothalamic plasticity. *Neural Plast.* 1–8. <https://doi.org/10.1155/2013/438072>.
- Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17 (1). <https://doi.org/10.14806/ej.17.1.200>. Article 1.
- Matsuda, J., Yokota, I., Iida, M., Murakami, T., Naito, E., Ito, M., Shima, K., Kuroda, Y., 1997. Serum leptin concentration in cord blood: relationship to birth weight and gender. *J. Clin. Endocrinol. Metabol.* 82 (5), 1642–1644. <https://doi.org/10.1210/jcem.82.5.4063>.
- Matsumoto, A., Arai, Y., 1976. Developmental changes in synaptic formation in the hypothalamic arcuate nucleus of female rats. *Cell Tissue Res.* 169 (2), 143–156. <https://doi.org/10.1007/BF00214204>.
- Mcmillen, I.C., Robinson, J.S., 2005. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol. Rev.* 85 (2), 571–633. <https://doi.org/10.1152/physrev.00053.2003>.
- Meyer, E., Aglyamova, G.V., Matz, M.V., 2011. Profiling gene expression responses of coral larvae (*Acropora millepora*) to elevated temperature and settlement inducers using a novel RNA-Seq procedure. *Mol. Ecol.* 20 (17), 3599–3616. <https://doi.org/10.1111/j.1365-294X.2011.05205.x>.
- Molet, J., Heins, K., Zhuo, X., Mei, Y.T., Regev, L., Baram, T.Z., Stern, H., 2016. Fragmentation and high entropy of neonatal experience predict adolescent emotional outcome. *Transl. Psychiatry* 6 (1). <https://doi.org/10.1038/tp.2015.200>.
- Molnár, C.S., Sárvári, M., Vastagh, C., Murnyi, C., Fekete, C., Liposits, Z., Hrabovszky, E., 2016. Altered gene expression profiles of the hypothalamic arcuate nucleus of male mice suggest profound developmental changes in peptidergic signaling. *Neuroendocrinology* 103 (3–4), 369–382. <https://doi.org/10.1159/000439430>.
- Moriceau, S., Shionoya, K., Jakubs, K., Sullivan, R.M., 2009. Early-Life stress disrupts attachment learning: the role of Amygdala Corticosterone, Locus ceruleus corticotropin releasing hormone, and Olfactory Bulb norepinephrine. *J. Neurosci.* 29 (50), 15745–15755. <https://doi.org/10.1523/JNEUROSCI.4106-09.2009>.
- Morris, M.J., Beilharz, J.E., Maniam, J., Reichelt, A.C., Westbrook, R.F., 2015. Why is obesity such a problem in the 21st century? The intersection of palatable food, cues and reward pathways, stress, and cognition. *Neurosci. Biobehav. Rev.* 58, 36–45. <https://doi.org/10.1016/j.neubiorev.2014.12.002>.
- Morton, G.J., 2007. Hypothalamic leptin regulation of energy homeostasis and glucose metabolism. *J. Physiol.* 583 (2), 437–443. <https://doi.org/10.1113/jphysiol.2007.135590>.
- Moussaoui, N., Larauche, M., Biraud, M., Molet, J., Million, M., Mayer, E., Taché, Y., 2016. Limited nesting stress alters maternal behavior and in vivo intestinal permeability in Male Wistar pup rats. *PLoS One* 11 (5), e0155037. <https://doi.org/10.1371/journal.pone.0155037>.
- Nakagaki, B.N., Mafra, K., de Carvalho, É., Lopes, M.E., Carvalho-Gontijo, R., de Castro-Oliveira, H.M., Campolina-Silva, G.H., de Miranda, C.D.M., Antunes, M.M., Silva, A.C.C., Diniz, A.B., Alvarenga, D.M., Lopes, M.A.F., de Souza Lacerda, V.A., Mattos, M.S., Araújo, A.M., Vidigal, P.V.T., Lima, C.X., Mahecha, G.A.B., et al., 2018. Immune and metabolic shifts during neonatal development reprogram liver identity and function. *J. Hepatol.* 69 (6), 1294–1307. <https://doi.org/10.1016/j.jhep.2018.08.018>.
- Naninck, E.F.G., Oosterink, J.E., Yam, K.-Y., de Vries, L.P., Schierbeek, H., van Goudoever, J.B., Verkaik-Schakel, R.-N., Plantinga, J.A., Plosch, T., Lucassen, P.J., Korosi, A., 2017. Early micronutrient supplementation protects against early stress-induced cognitive impairments. *FASEB (Fed. Am. Soc. Exp. Biol.) J.: Official Publication of the Federation of American Societies for Experimental Biology* 31 (2), 505–518. <https://doi.org/10.1096/fj.201600834R>.
- Negoro, H., Honda, K., Uchida, K., Higuchi, T., 1987. Facilitation of milk ejection-related activation of oxytocin-secreting neurones by osmotic stimulation in the rat. *Exp. Brain Res.* 65 (2), 312–316. <https://doi.org/10.1007/BF00236303>.
- Nirupama, R., Devaki, M., Yajurvedi, H.N., 2012. Chronic stress and carbohydrate metabolism: persistent changes and slow return to normalcy in male albino rats. *Stress*. <https://www.tandfonline.com/doi/abs/10.3109/10253890.2011.619604>.
- Oers, H. J. van, Kloet, E. R. de, Whelan, T., Levine, S., 1998. Maternal deprivation effect on the infant's neural stress markers is reversed by Tactile stimulation and feeding but not by suppressing corticosterone. *J. Neurosci.* 18 (23), 10171–10179. <https://doi.org/10.1523/JNEUROSCI.18-23-10171.1998>.
- Pardo, G.E., Cuevas, L.B., Vázquez-Lizárraga, J., Pary, A.C., Pacheco-Otalora, L.F., Oruro, E.M., 2025. Temporal variation in maternal behavioral transitions under limited nesting conditions across postpartum windows in rats: implications for neurobehavioral development. *Behav. Brain Res.* 495, 115769. <https://doi.org/10.1016/j.bbr.2025.115769>.
- Patel, M.S., Srinivasan, M., 2006. Metabolic programming as a consequence of the nutritional environment during fetal and the immediate postnatal periods. In: Thureen, P.J., Hay, W.W. (Eds.), *Neonatal Nutrition and Metabolism*, second ed. Cambridge University Press, pp. 76–90. <https://doi.org/10.1017/CBO9780511544712.008>.
- Paul, B., Buchholz, D.R., 2023. Minireview: Glucocorticoid-leptin crosstalk: role of Glucocorticoid-Leptin counterregulation in metabolic homeostasis and normal development. *Integr. Comp. Biol.* 63 (6), 1127–1139. <https://doi.org/10.1093/icb/icad119>.
- Paul, H.A., Hallam, M.C., Reimer, R.A., 2015. Milk collection in the rat using capillary tubes and estimation of milk fat content by creamatocrit. *J. Vis. Exp.* 106, e53476. <https://doi.org/10.3791/53476>.
- Paxinos, G., Watson, C., 1998. *The Rat Brain in Stereotaxic Coordinates*, sixth ed. Academic Press, New York.
- Petersen, M.C., Shulman, G.I., 2018. Mechanisms of insulin action and insulin resistance. *Physiol. Rev.* 98 (4), 2133–2223. <https://doi.org/10.1152/physrev.00063.2017>.
- Petersen, A.K., White, C.R., Marshall, D.J., 2016. Metabolic rate covaries with fitness and the pace of the life history in the field. *Proc. Biol. Sci.* 283 (1831), 20160323. <https://doi.org/10.1098/rspb.2016.0323>.
- Proulx, K., Richard, D., Walker, C.-D., 2002. Leptin regulates appetite-related neuropeptides in the hypothalamus of developing rats without affecting food intake. *Endocrinology* 143 (12), 4683–4692. <https://doi.org/10.1210/en.2002-220593>.
- Ramirez, M.C., Bourguignon, N.S., Bonaventura, M.M., Lux-Lantos, V., Libertun, C., Becu-Villalobos, D., 2012. Neonatal xenoestrogen exposure alters growth hormone-dependent liver proteins and genes in adult female rats. *Toxicol. Lett.* 213 (3), 325–331. <https://doi.org/10.1016/j.toxlet.2012.07.015>.
- Rosenfeld, P., Ekstrand, J., Olson, E., Suchecki, D., Levine, S., 1993. Maternal regulation of adrenocortical activity in the infant rat: effects of feeding. *Dev. Psychobiol.* 26 (5), 261–277. <https://doi.org/10.1002/dev.420260504>.
- Rosenfeld, P., Sutanto, W., Levine, S., De Kloet, E.R., 1988. Ontogeny of Type I and Type II corticosteroid receptors in the rat hippocampus. *Dev. Brain Res.* 42 (1), 113–118. [https://doi.org/10.1016/0165-3806\(88\)90206-4](https://doi.org/10.1016/0165-3806(88)90206-4).
- Rosseeff, Y., 2012. lavaan: an R package for structural equation modeling. *J. Stat. Software* 48 (2). <https://doi.org/10.18637/jss.v048.i02>.
- Ruigrok, S.R., Stöberl, N., Yam, K.-Y., de Lucia, C., Lucassen, P.J., Thuret, S., Korosi, A., 2021. Modulation of the hypothalamic nutrient sensing pathways by sex and early-life stress. *Front. Neurosci.* 15. <https://doi.org/10.3389/fnins.2021.695367>.
- Salzmann, C., Otis, M., Long, H., Roberge, C., Gallo-Payet, N., Walker, C.-D., 2004. Inhibition of steroidogenic response to adrenocorticotropin by Leptin: implications for the adrenal response to maternal separation in neonatal rats. *Endocrinology* 145 (4), 1810–1822. <https://doi.org/10.1210/en.2003-1514>.
- Schmidt, M.V., 2019. Chapter 4—Stress-Hyporesponsive period. In: Fink, G. (Ed.), *Stress: Physiology, Biochemistry, and Pathology*. Academic Press, pp. 49–56. <https://doi.org/10.1016/B978-0-12-813146-6.00004-7>.

- Schmidt, M.V., Levine, S., Alam, S., Harbich, D., Sterlemann, V., Ganea, K., De Kloet, E. R., Holsboer, F., Müller, M.B., 2006. Metabolic signals modulate hypothalamic-pituitary-adrenal axis activation during maternal separation of the Neonatal mouse. *J. Neuroendocrinol.* 18 (11), 865–874. <https://doi.org/10.1111/j.1365-2826.2006.01482.x>.
- Seal, S.V., Turner, J.D., 2021. The ‘Jekyll and Hyde’ of gluconeogenesis: early life adversity, later life stress, and metabolic disturbances. *Int. J. Mol. Sci.* 22 (7). <https://doi.org/10.3390/ijms22073344>. Article 7.
- Shelton, D.S., Alberts, J.R., 2018. Development of behavioral responses to thermal challenges. *Dev. Psychobiol.* 60 (1), 5–14. <https://doi.org/10.1002/dev.21588>.
- Shupe, E.A., Clinton, S.M., 2021. Neonatal resource scarcity alters maternal care and impacts offspring core temperature and growth in rats. *Dev. Psychobiol.* 63, e22144. <https://doi.org/10.1002/czpxy.lib.utexas.edu/10.1002/dev.22144>.
- Smyth, G.K., 2005. Limma: Linear models for microarray data. In: Gentleman, R., Carey, V.J., Huber, W., Irizarry, R.A., Dudoit, S. (Eds.), *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. Springer-Verlag, pp. 397–420. https://doi.org/10.1007/0-387-29362-0_23.
- Sominsky, L., Jasoni, C.L., Twigg, H.R., Spencer, S.J., 2018. Hormonal and nutritional regulation of postnatal hypothalamic development. *J. Endocrinol.* 237 (2), R47–R64. <https://doi.org/10.1530/JOE-17-0722>.
- Sominsky, L., Spencer, S.J., 2014. Eating behavior and stress: a pathway to obesity. *Front. Psychol.* 5. <https://doi.org/10.3389/fpsyg.2014.00434>.
- Speakman, J.R., 2005. Body size, energy metabolism and lifespan. *J. Exp. Biol.* 208 (9), 1717–1730. <https://doi.org/10.1242/jeb.01556>.
- Speakman, J.R., Fletcher, Q., Vaanholt, L., 2013. The “39 steps”: An algorithm for performing statistical analysis of data on energy intake and expenditure. *Disease Models & Mechanisms* 6 (2), 293–301. <https://doi.org/10.1242/dmm.009860>.
- Spencer, S.J., Xu, L., Clarke, M.A., Lemus, M., Reichenbach, A., Geenen, B., Kozicz, T., Andrews, Z.B., 2012. Ghrelin regulates the hypothalamic-pituitary-adrenal axis and restricts anxiety after acute stress. *Biol. Psychiatry* 72 (6), 457–465. <https://doi.org/10.1016/j.biopsych.2012.03.010>.
- Stead, S.M., Bădescu, I., Boonstra, R., 2022. Of mammals and milk: how maternal stress affects nursing offspring. *Mamm. Rev.* 52 (1), 129–147. <https://doi.org/10.1111/mam.12267>.
- Steculorum, S.M., Collden, G., Coupe, B., Croizier, S., Lockie, S., Andrews, Z.B., Jarosch, F., Klusmann, S., Bouret, S.G., 2015. Neonatal ghrelin programs development of hypothalamic feeding circuits. *J. Clin. Invest.* 125 (2), 846–858. <https://doi.org/10.1172/JCI73688>.
- Storey, J.D., Tibshirani, R., 2003. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. U. S. A* 100 (16), 9440–9445. <https://doi.org/10.1073/pnas.1530509100>.
- Tamin, T., Morinay, J., Germain, M., Recapet, C., Doligez, B., 2023. Behavioural syndrome between boldness and aggressiveness and link with reproductive success in a wild bird population. *Anim. Behav.* 197, 27–41. <https://doi.org/10.1016/j.anbehav.2022.12.011>.
- Tramunt, B., Smati, S., Grandgeorge, N., Lenfant, F., Arnal, J.-F., Montagner, A., Gourdy, P., 2020. Sex differences in metabolic regulation and diabetes susceptibility. *Diabetologia* 63 (3), 453–461. <https://doi.org/10.1007/s00125-019-05040-3>.
- Tucker, R.P., Kenzelmann, D., Trzebiatowska, A., Chiquet-Ehrismann, R., 2007. Teneurin: transmembrane proteins with fundamental roles in development. *Int. J. Biochem. Cell Biol.* 39 (2), 292–297. <https://doi.org/10.1016/j.biocel.2006.09.012>.
- Uauy, R., Dangour, A.D., 2006. Nutrition in brain development and aging: role of essential fatty acids. *Nutr. Rev.* 64 (5 Pt 2), S24–S33. <https://doi.org/10.1301/nr.2006.may.s24-s33> discussion S72–91.
- van der Kooij, 2020. The impact of chronic stress on energy metabolism. *Mol. Cell. Neurosci.* 107, 103525. <https://doi.org/10.1016/j.mcn.2020.103525>.
- Vannoni, E., Voikar, V., Colacicco, G., Sánchez, M.A., Lipp, H.P., Wolfer, D.P., 2014. Spontaneous behavior in the social home cage discriminates strains, lesions and mutations in mice. *J. Neurosci. Methods* 234, 26–37. <https://doi.org/10.1016/j.jneumeth.2014.04.026>.
- Vernes, S.C., Oliver, P.L., Spiteri, E., Lockstone, H.E., Puliyadi, R., Taylor, J.M., Ho, J., Mombereau, C., Brewer, A., Lowy, E., Nicod, J., Groszer, M., Baban, D., Sahgal, N., Cazier, J.-B., Ragoussis, J., Davies, K.E., Geschwind, D.H., Fisher, S.E., 2011. Foxp2 regulates gene networks implicated in neurite outgrowth in the developing brain. *PLoS Genet.* 7 (7), e1002145. <https://doi.org/10.1371/journal.pgen.1002145>.
- Vella, A., Camilleri, M., 2017. The gastrointestinal tract as an integrator of mechanical and hormonal response to nutrient ingestion. *Diabetes* 66 (11), 2729–2737. <https://doi.org/10.2337/dbi17-0021>.
- Viveros, M.-P., Díaz, F., Mateos, B., Rodríguez, N., Chowen, J.A., 2010. Maternal deprivation induces a rapid decline in circulating leptin levels and sexually dimorphic modifications in hypothalamic trophic factors and cell turnover. *Horm. Behav.* 57 (4), 405–414. <https://doi.org/10.1016/j.yhbeh.2010.01.009>.
- Vogt, M.C., Paeger, L., Hess, S., Steculorum, S.M., Awazawa, M., Hampel, B., Neupert, S., Nicholls, H.T., Mauer, J., Hausen, A.C., Predel, R., Kloppenburg, P., Horvath, T.L., Brüning, J.C., 2014. Neonatal insulin action impairs hypothalamic neurocircuit formation in response to maternal high-fat feeding. *Cell* 156 (3), 495–509. <https://doi.org/10.1016/j.cell.2014.01.008>.
- Vogt, G., Verheyen, S., Schwartzmann, S., Ehmke, N., Potratz, C., Schwerin-Nagel, A., Plecko, B., Holtgrewe, M., Seelow, D., Blatterer, J., Speicher, M.R., Kornak, U., Horn, D., Mundlos, S., Fischer-Zirnsak, B., Boschann, F., 2022. Biallelic truncating variants in ATP9A cause a novel neurodevelopmental disorder involving postnatal microcephaly and failure to thrive. *J. Med. Genet.* 59 (7), 662–668. <https://doi.org/10.1136/jmedgenet-2021-107843>.
- Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. *Nat. Neurosci.* 7 (8), 847–854. <https://doi.org/10.1038/nn1276>.
- Wijenayake, S., Martz, J., Lapp, H.E., Storm, J.A., Champagne, F.A., Kentner, A.C., 2023. The contributions of parental lactation on offspring development: it's not under nonsense. *Horm. Behav.* 153, 105375. <https://doi.org/10.1016/j.yhbeh.2023.105375>.
- Xie, Z., Bailey, A., Kuleshov, M.V., Clarke, D.J.B., Evangelista, J.E., Jenkins, S.L., Lachmann, A., Wojciechowicz, M.L., Kropiwnicki, E., Jagodnik, K.M., Jeon, M., Ma'ayan, A., 2021. Gene set knowledge discovery with Enrichr. *Curr. Protoc.* 1 (3), e90. <https://doi.org/10.1002/cpz1.90>.
- Yam, K.Y., Naninck, E.F.G., Abbink, M.R., la Fleur, S.E., Schipper, L., van den Beukel, J. C., Grefhorst, A., Oosting, A., van der Beek, E.M., Lucassen, P.J., Korosi, A., 2017a. Exposure to chronic early-life stress lastingly alters the adipose tissue, the leptin system and changes the vulnerability to western-style diet later in life in mice. *Psychoneuroendocrinology* 77, 186–195. <https://doi.org/10.1016/j.psyneuen.2016.12.012>.
- Yam, K.Y., Ruigrok, S.R., Ziko, I., De Luca, S.N., Lucassen, P.J., Spencer, S.J., Korosi, A., 2017b. Ghrelin and hypothalamic NPY/AgRP expression in mice are affected by chronic early-life stress exposure in a sex-specific manner. *Psychoneuroendocrinology* 86, 73–77. <https://doi.org/10.1016/j.psyneuen.2017.09.006>.
- Yam, K.-Y., Schipper, L., Reemst, K., Ruigrok, S.R., Abbink, M.R., Hoeijmakers, L., Naninck, E.F.G., Zarekiani, P., Oosting, A., Van Der Beek, E.M., Lucassen, P.J., Korosi, A., 2019. Increasing availability of ω -3 fatty acid in the early-life diet prevents the early-life stress-induced cognitive impairments without affecting metabolic alterations. *FASEB J.* 33 (4), 5729–5740. <https://doi.org/10.1096/fj.201802297R>.
- Yang, D.J., Hong, J., Kim, K.W., 2021. Hypothalamic primary cilium: a hub for metabolic homeostasis. *Exp. Mol. Med.* 53 (7), 1109–1115. <https://doi.org/10.1038/s12276-021-00644-5>.
- Yasuda, S., Inoue, K., Matsui, I., Matsumoto, A., Katsuma, Y., Okushima, H., Imai, A., Sakaguchi, Y., Kaimori, J., Yamamoto, R., Mizui, M., Isaka, Y., 2023. Hepatic phosphate uptake and subsequent nerve-mediated phosphaturia are crucial for phosphate homeostasis following portal vein passage of phosphate in rats. *Sci. Rep.* 13 (1), 5794. <https://doi.org/10.1038/s41598-023-32856-2>.
- Zhang, B., Horvath, S., 2005. A general framework for weighted gene co-expression network analysis. *Stat. Appl. Genet. Mol. Biol.* 4. <https://doi.org/10.2202/1544-6115.1128>. Article17.
- Zhang, X., Yang, S., Chen, J., Su, Z., 2018. Unraveling the regulation of hepatic gluconeogenesis. *Front. Endocrinol.* 9, 802. <https://doi.org/10.3389/fendo.2018.00802>.
- Zhang, Y., Yang, P., Zhang, X., Liu, S., Lou, K., 2024. Asprosin: its function as a novel endocrine factor in metabolic-related diseases. *J. Endocrinol. Investig.* 47 (8), 1839–1850. <https://doi.org/10.1007/s40618-024-02360-z>.