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RESEARCH REPORT

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Metabolic effects of early life stress and pre-pregnancy obesity are long lasting and sex specific in mice

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Abstract

Early life stress (ELS) is associated with metabolic, cognitive, and psychiatric diseases and has a very high prevalence, highlighting the urgent need for a better understanding of the versatile physiological changes and identification of predictive biomarkers. In addition to programming the hypothalamic–pituitary–adrenal (HPA) axis, ELS may also affect the gut microbiota and metabolome, opening up a promising research direction for identifying early biomarkers of ELS-induced (mal)adaptation. Other factors affecting these parameters include maternal metabolic status and diet, with maternal obesity shown to predispose offspring to later metabolic disease. The aim of the present study was to investigate the long-term effects of ELS and maternal obesity on the metabolic and stress phenotype of rodent offspring. To this end, offspring of both sexes were subjected to an adverse early-life experience, and their metabolic and stress phenotypes were examined. In addition, we assessed whether a prenatal maternal and an adult high-fat diet (HFD) stressor further shape observed ELS-induced phenotypes. We show that ELS has long-term effects on male body weight (BW) across the lifespan, whereas females more successfully counteract ELS-induced weight loss, possibly by adapting their microbiota, thereby stabilizing a balanced metabolome. Furthermore, the metabolic effects of a maternal HFD on BW are exclusively triggered by a dietary challenge in adult offspring and are more pronounced in males than in females. Overall, our study suggests that the female microbiota protects against an ELS challenge, rendering them more resilient to additional maternal- and adult nutritional stressors than males.

Abbreviations: ACEs, adverse childhood experiences; BW, body weight; ELS, early life stress; GTT, glucose tolerance test; HFD, high-fat diet; HPA, hypothalamic–pituitary–adrenal; ITT, insulin tolerance test; LBN, limited bedding and nesting paradigm; MHFD, maternal high fat diet; PCA, Principle Component Analysis; PND, postnatal day; SD, standard diet.

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1 | INTRODUCTION

Early life stress (ELS), in humans referred to as adverse childhood experiences (ACEs), such as abuse, neglect, social stress, and socio-economic position, is an important contributor to the development of physical and psychosocial issues in adulthood, including metabolic, cognitive, and psychiatric diseases (Fogelman & Canli, 2019). A study of frequency estimates of ACEs in 2019 revealed that more than half of the study population (noninstitutionalized US adults) was affected by childhood adversity, highlighting the urgent need for a better understanding of the physiological changes following adverse childhood experiences (Giano et al., 2020). The changes in response to negative environmental stimuli are a phenomenon called *early life programming*, which can either allow an organism to adapt to unfavourable conditions or may turn out maladaptive and increase the susceptibility to pathologies (Nederhof & Schmidt, 2012; Vargas et al., 2016). ELS was shown to programme the hypothalamic–pituitary–adrenal (HPA) axis and thereby increase basal glucocorticoid levels, which are implicated as a central mechanism of stress-related pathologies such as obesity, diabetes, metabolic syndrome, and psychiatric diseases such as depression and posttraumatic disorder (Maccari et al., 2014). Although ELS causes metabolic complications in adulthood, mouse studies investigating the effects of ELS on body weight (BW) report conflicting results. Some studies describe initial weight loss and weight normalization with advancing age in the limited bedding and nesting paradigm (LBN) (Goodwill et al., 2018; Kanatsou et al., 2017; Naninck et al., 2015; Rice et al., 2008) and maternal deprivation and separation model (de Lima et al., 2020), while others report BW gain (Peña et al., 2017, 2019; Ruiz et al., 2018). Sex may also be a crucial factor determining vulnerability or resilience to ELS, but studies directly comparing males and females have so far been scarce. Therefore, it is crucial to understand the underlying mechanisms and related biomarker signatures of early life programming in order to predict metabolic outcomes later in life.

For these reasons, the microbiota and metabolome are ideal candidates in the search for predictive biomarkers for metabolic diseases, such as diabetes type 2, overweight, and obesity. However, apart from the fact that ELS shapes disease phenotypes in adulthood via the neuroendocrine and neuroimmune axes (Hantsoo et al., 2019), little is known about how ELS affects the gut microbiome and metabolome.

The gut microbiome refers to the totality of the microbiota (bacteria, viruses, protozoa, fungi) that inhabit the gut and are believed to be connected to the central nervous system via the gut-brain axis, a bidirectional communication axis (Morais et al., 2020). Studies show that psychosocial stressors in animal models and in humans modulate microbial composition (Crumeyrolle-Arias et al., 2014; De Palma et al., 2015; Hantsoo & Zemel, 2021). Gut microbiota produce microbial metabolites that can travel through the portal circulation and thereby influence host metabolism and other systemic processes (Aarts et al., 2017), interact with the immune system, and affect local tissue/mucosae and environments such as neuronal cells of the enteric nervous system and afferent pathways of the *Vagus* nerve that send signals directly to the brain (Morais et al., 2020).

Another important driver of the gut microbiota and metabolome is diet. A high-carbohydrate diet or high-fat diet (HFD) over a prolonged period alters the physiology of gut microbiota in humans (David et al., 2013) and rodents (Daniel et al., 2013) in a rapid and diet-specific manner, thereby predisposing individuals to the onset of metabolic diseases. Since the microbial colonization of the newborn is shaped by the interplay of neonatal, maternal, and environmental factors, the nutritional and metabolic status of the mother during pregnancy also influence the offspring's microbiome in humans and rodents (Senn et al., 2020). Furthermore, the detrimental effects of maternal obesity before and during pregnancy are widely recognized to have long-term implications on offspring's metabolic and mental health (Godfrey et al., 2017). Although the exact mechanisms are unclear, emerging evidence points to diet-induced shifts in the maternal microbiome, which are passed on to their offspring (Gohir et al., 2014).

The aim of the present study was to investigate the effects of ELS on the metabolic and stress phenotype of both male and female offspring in mice. In addition, we assessed whether an HFD stressor in pregnant maternal mice and in their adult offspring shape ELS-induced phenotypes. To induce ELS, the LBN paradigm was utilized, a widely used rodent model of adverse early-life experiences (Naninck et al., 2015; Rice et al., 2008). We show that ELS has long-term effects on male BW decrease across the lifespan, whereas females cope better with ELS-induced weight differences over time by more quickly regaining a healthy weight. We speculate that this sex-dependent differential response to ELS is possibly explained by adaptations of the gut microbiota, thereby regaining a healthier metabolome. Furthermore, the metabolic effects on BW, microbiota, and metabolome composition of a maternal HFD (MHFD) are exclusively triggered by an additional dietary challenge in adult offspring and are more pronounced in male than in female offspring. Overall, our study suggests that the female microbiota is more capable to adapt to an ELS challenge compared with males, which also makes them more resilient to additional maternal and adult (nutritional) stressors.

2 | MATERIALS AND METHODS

2.1 | Animals and animal housing

All experiments and protocols were performed in accordance with the European Communities' Council Directive 2010/63/EU and were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria. All effort was made to minimize any suffering of the animals throughout the experiments. Female and male inbred C57/BL6n animals of the F_0 generation were obtained from the in-house breeding facility of the Max Planck Institute of Psychiatry. Pups were weaned and segregated according to sex at postnatal day (PND) 24 with a maximum of five animals per cage. Experimental cohorts of the F_1 generation were group housed with their litters throughout the experiments unless indicated otherwise. All animals were kept in individually ventilated cages (IVC; 30 cm × 16 cm × 16 cm; 501 cm^2) serviced by a central airflow system (Tecniplast, IVC Green Line – GM500). Animals had ad libitum access to water (tap water) and food (see Section 2.4) and were maintained under constant environmental conditions (12:12 h light/dark cycle, 23 ± 2°C, and humidity of 55%). All IVCs had sufficient bedding and nesting material as well as a wooden tunnel for environmental enrichment. Animals were allocated to experimental groups in a semirandomized fashion, and data analysis and execution of experiments were performed blinded to group allocation.

2.2 | Early life stress paradigm (ELS)—Limited bedding and nesting (LBN)

Initially introduced in rats (Gilles et al., 1996), the LBN paradigm has been successfully adopted to provoke chronic stress in mice (Naninck et al., 2015). From PND 2 to PND 9 dams and pups were transferred from their home cage into a new IVC (30 cm × 16 cm × 16 cm; 501 cm^2) with a customized wire mesh inlay (30 cm × 16 cm) and half of a cotton nestlet. Throughout these 7 days, dams had ad libitum access to food and water and were left undisturbed. Control litters were transferred to a new cage with sufficient bedding material and two full cotton nestlets. Following the LBN paradigm, all dams and their pups were returned to normal bedding and nesting conditions until weaning at PND 24 (see Figure 1a for experimental procedure).

2.3 | Maternal HFD diet and adult HFD challenge

To assess the impact of a pre-pregnancy MHFD diet combined with an LBN ELS paradigm on metabolic control in the adult offspring, F_0 C57/BL6n females were split into a standard diet (SD) or an HFD group for 10 weeks until their BW was significantly increased compared with the SD control group. Between weeks 10 and 12, females were mated with male C57/BL6n fed either an SD or HFD for 3 days before mating. In this way, we were able to avoid additional stressors for the males due to a sudden change in diet at the beginning of mating. Females that received an HFD were switched to an SD for the remainder of the experiment once they were visibly pregnant as determined by the formation of vaginal plugs. F_1 offspring of HFD and SD mothers were split into ELS and control groups. All groups weaned onto a regular SD diet at PND 24 and remained group-housed throughout the first round of experiments. At 6 months of age, all experimental groups were challenged with an HFD for 4 months to investigate the combinatorial effects of an adult metabolic challenge with the MHFD and ELS in early life.

2.4 | Diets

Baseline metabolic characterization was performed under an SD (standard research diet by Altromin 1318, Altromin GmbH, Germany) with the following nutritional

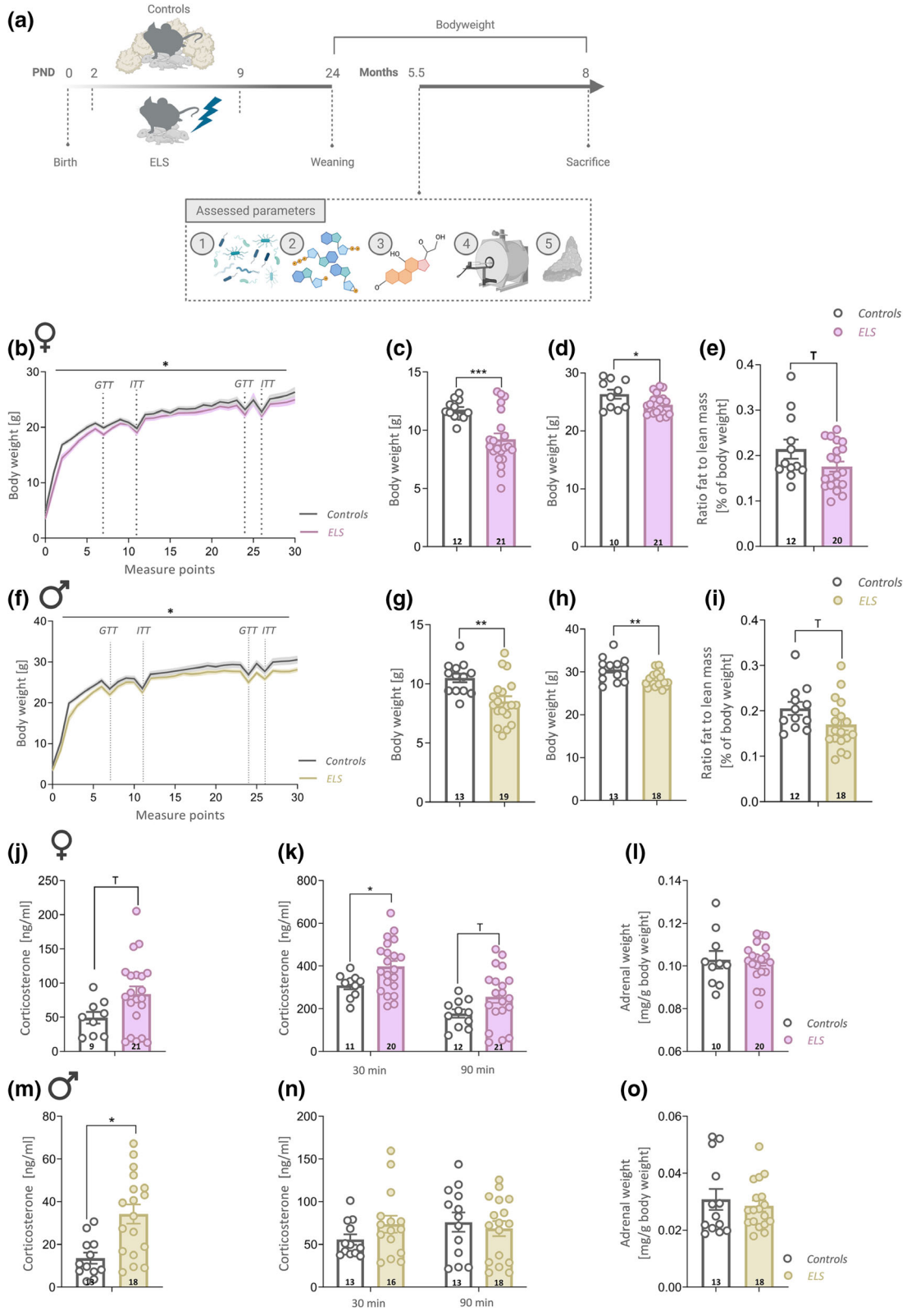

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FIGURE 1 Metabolic and endocrine effects of ELS in male and female mice. (a) Experimental design: Male and female offspring of C57/Bl6n dams underwent the LBN paradigm from PND 2–9. At PND 24, offspring was weaned onto an SD and litters remained group-housed throughout the experiment. BW was assessed on a regular basis from weaning until sacrifice at 8 months of age. At 5.5 months of age, faeces were collected under baseline conditions in the early morning for microbiota (1) and metabolome (2) analysis. CORT (3) was measured 30 and 90 min after acute restraint stress and in the morning under baseline conditions. Further, body composition (4) was assessed with an NMR-body composition scanner at 5.5 months of age. Finally, adrenals were dissected and weighed (5). The BW of female ELS offspring was significantly lower than that of unstressed controls throughout the entire experiment (30 weeks) (b), at weaning (PND 24) (c), and sacrifice (d). (e) A body composition scan confirmed lower fat mass in females that underwent the ELS paradigm. (f–i) Males developed the same BW phenotype as female mice, but differences are more robust over time. (j) CORT at morning baseline was mildly but not significantly increased in ELS females. (k) CORT 30 and 90 min after acute restraint stress was higher in female ELS offspring. Relative adrenal weights in females (l) and males (o) were unchanged. (m) In males, morning baseline CORT was significantly increased in the ELS group. (n) CORT after acute restraint was not affected in male ELS offspring. Data are received from mice between PND2 to 8 months of age and are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** p > 0.001, T < 0.1. ELS early life stress, PND postnatal day.

values: 14% fat and sucrose, 27% protein, and 59% carbohydrates. Animals under dietary challenge received an HFD diet (HFD, D12331, Research Diets, New Brunswick, NJ, USA) over a period of several weeks to induce overweight. Nutritional values HFD: 58% fat and sucrose, 17% protein, 25% carbohydrates. Bodyweight and food intake were measured weekly.

2.5 | Nuclear magnetic resonance (NMR)

In addition to weekly regular measures of BW, the animal's body composition was assessed with a body composition analyser (LF50 BCA NMR Minispec Analyzer, Bruker Optik) after several weeks on chow and HFD. This method applies time domain nuclear magnetic resonance to measure lean tissue mass, fat mass, and free fluids non-invasively and in vivo without the need for anaesthetics in small rodents (Halldorsdottir et al., 2009). Body constituents were normalized to bodyweight for each group and the ratio of fat to lean mass was calculated.

2.6 | Tissue sampling procedure

Fresh faeces for microbiota and metabolome analysis were collected in 1.5-mL Eppendorf tubes (three to five pellets each) in an empty cage in the morning hours between 07:00 and 10:00 AM under sterile conditions. Samples were immediately snap-frozen on dry ice and stored at −80°C until further processing.

On the day of sacrifice, animals were weighed, deeply anaesthetized with isoflurane, and sacrificed by decapitation. Trunk blood (for basal morning CORT measures) was collected in labeled 1.5-mL Heparin-coated microcentrifuge tubes (Kabe Labortechnik, Germany) and kept on ice until centrifugation. After centrifugation (4°C, 8000 rpm for 15 min), plasma was removed and transferred to new labelled 2-mL tubes and stored at −80°C until hormone quantification. Adrenals were dissected and weighed.

2.7 | Acute restraint stress paradigm

The acute restraint stress paradigm is perceived as a severe stressor robustly inducing the entire spectrum of known allostatic responses in rodents and was, therefore, the stress paradigm of choice. At 8:00 AM, 1 h after the lights-on, each animal was placed in a custom-made restrainer (50-mL falcon tube with holes at the bottom and the lid to provide enough oxygen and space for tail movement) for 15 min in their home cage. After 15 min, animals were removed from the tube. Subsequent blood samples were collected at 30 and 90 min post-stress in the home cage via tail cut. The animals were left undisturbed in between sampling procedures.

2.8 | Hormone assessment

Baseline morning (measured between 08:00 and 12:00 AM) and post stress plasma CORT (ng/mL) concentrations were determined by radioimmunoassay using CORT ^{125}I RIA kit (sensitivity: 12.5 ng/mL, MP Biomedicals Inc) following the manufacturers' instructions. The radioactivity of the pellet was measured with a gamma counter (Packard Cobra II Auto Gamma; Perkin-Elmer). Final CORT levels were derived from the standard curve.

2.9 | Intraperitoneal glucose (GTT) and insulin (ITT) tolerance test

After several weeks under a chow diet, an intraperitoneal glucose tolerance test (GTT) was carried out after lights-on. A 20% D-(+)-glucose solution (Sigma Aldrich, Merck, Darmstadt) was prepared, and animals were subjected to

an overnight fast of 14 h (6 PM until 8 AM) prior to the experiment. Every animal was weighed and intraperitoneally injected with 2-g glucose per kg bodyweight. Blood glucose concentrations were measured from tail stitches at 0, 30, 60, 90, and 120 min after the glucose injection using a handheld XT glucometer (Bayer Health Care, Basel, Switzerland).

An intraperitoneal insulin tolerance test (ITT) was performed 14 days after the GTT to ensure a complete recovery from the overnight fast. A similar procedure as for the GTT was applied as follows: An insulin stock solution of 0.5 IU/mL (Actrapid® Penfill®, Novo Nordisk Pharma GmbH, Bagsværd, Denmark) was prepared, and animals were fasted for 4 h (7 until 11 AM) before the onset of the ITT. Every animal was weighed and intraperitoneally injected with 1 IU insulin per kg bodyweight. Blood glucose concentrations were measured at 0, 30, 60, 90, and 120 min after the insulin injection.

2.10 | Microbiota analysis

2.10.1 | DNA extraction and 16S rRNA amplicon sequencing

Total DNA was isolated from faecal pellets (approx. 100 mg) using the MasterPure Complete DNA & RNA Purification Kit (Epicentre, Madison WI, USA) with some modifications as previously described (Sanguinetti et al., 2018). DNA concentrations were measured and normalized using a Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA).

Specific 16SrRNA gene (V3 and V4 region, amplicon ~460 bp) was amplified following the 16S Metagenomic Sequencing Library Preparation Illumina protocol. The multiplexing step was performed using Nextera XT Index Kit (Illumina) and PCR product was checked on a bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA). The samples were pooled in equimolar amounts and sequenced with the MiSeq® Reagent kit v3, on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain), producing 2 × 300 bp paired-end reads. To rule out and control for potential reagent contamination, the reagents for DNA extraction and PCR amplification were also sequenced as controls.

2.11 | Metabolome analysis

Faecal samples were suspended in Milliq water (100 μL) and centrifuged (14,800 rpm, 5 min) for three consecutive times to obtain the extract. Extracts (20 μL) supplemented with D2O (2 μL) were transferred into 1-mm NMR tubes. ^{1}H-NMR spectra (8000 Hz width) were recorded in a Bruker Avance DRX 600 spectrometer (Valencia, Spain), equipped with a triple resonance 1 H/13 C/31 P probe. Samples were measured at 310 K and a single-pulse presaturation experiment was acquired in all samples. The water signal was saturated with weak irradiation during the recycle delay. Data were Fourier transformed after the free induction decay was multiplied by a 0.3-Hz exponential line-broadening function. Spectra were processed using MestReNova 8.1 (Mestrelab Research S.L., Spain) and transferred to MATLAB using in-house scripts for data analysis. The chemical shift region including resonances 0.50–4.70 ppm (the aliphatic region) and 5.20–10.00 ppm (the aromatic region) was investigated. Metabolite spin systems and resonances were identified by literature data and Chenomx resonances database (Chenomx NMR 7.6). Spectra were normalized to total aliphatic spectral area to eliminate differences in metabolite total concentration, binned into 0.01 ppm buckets and then subjected to mean-centring before multivariate analysis. Signals belonging to selected metabolites were integrated and quantified using semiautomated in-house MATLAB peak-fitting routines. Final metabolite levels were calculated in arbitrary units as peak area normalized to total spectral area. More details can be found elsewhere (Monleon et al., 2009).

2.12 | Statistical analysis

2.12.1 | Metabolic and endocrine measures

The data presented are shown as means ± standard error of the mean (SEM) and samples sizes are indicated in the main text. BW, body composition, GTT, ITT, and CORT data were analysed by the commercially available GraphPad Prism 9.0.1 software (GraphPad Software, San Diego, California, USA). When two groups were compared, the unpaired two-tailed Student's t test was applied. Data with more than two groups were tested by the appropriate analysis of variance (ANOVA) model followed by *Bonferroni* post-hoc analysis to determine statistical significance between individual groups. Data based on repeated observations comparing two or more groups were tested by repeated measures ANOVA. Data sets with missing values were analysed with the mixed-effects model. Statistical significance was defined as $p \leq 0.05$. A statistical trend was accepted with a p value of $0.05 \leq p \leq 0.1$ and indicated in the figures with the symbol "T." Outliers were assessed with the online available Graph Pad outlier calculator performing the two-sided *Grubb's* outlier test. As this was an exploratory study, no statistical methods were used to predetermine sample sizes.

2.12.2 | Microbiota sequencing analysis

The DADA2 pipeline was used to analyse the raw data and perform the quality profiles, filtering and trimming, and also to remove Ns, expected errors and low-quality tails in the R environment (R Core Team, 2020). After learning the error rates with the DADA2 algorithm, a dereplication step was used to reduce computation time by collapsing redundant reads into unique ones, but counting them. Next, using the dereplicated data, true amplicon sequence variants (ASV) were inferred. Paired reads were merged by aligning denoised forward and reverse reads and used to construct the ASV table. Chimeric sequences were identified and removed. The taxonomic classification of the 16SrRNA sequences were performed using the SILVAv132 database, including species-level classification. The microbiota analysis was performed in the R environment using different packages: phyloseq (McMurdie & Holmes, 2013) and vegan (Okansen et al., 2020) and ggplot2 (Lahti, 2017; Wickham, 2009) packages. Data were used as total sum scaling normalization (TSS) for the statistical analysis and cumulative sum scaling normalization (CSS) for multivariate tests (redundancy discriminate analysis [RDA]). The alpha diversity metrics applied were Chao1 and Shannon (differences were tested with conventional *T* test statistics), and difference in beta diversity was evaluated with a PERMANOVA analysis, based on the Bray–Curtis distances (i.e., non-phylogenetic) among samples of different contrasts (e.g., between the ELS study groups).

2.12.3 | Metabolite spectra data analysis

Metabolomics data analysis was performed using in-house MATLAB scripts and PLS-Toolbox (Eigenvector Research). Principle component analysis (PCA) was applied to NMR spectra data sets, to find low-dimensional embeddings of multivariate data. PCs were chosen to explain at least 70% of the variance. The loading plots of the corresponding PC were used to detect the position of most discriminative variables in the NMR spectra. p values ≤ 0.05 were regarded as statistically significant.

2.12.4 | Correlation of microbiota and metabolome

For calculating the paired-sample Spearman correlation and p values of 16S microbiota (TSS-normalized values) with metabolite (arbitrary units) omics features, we have used the *rcorr* function from R package Hmisc (version 4.7.0). For the bubble-plot heatmap visualization of this correlation data, we have used R package ggcorrplot (version 0.1.3). These R libraries were run in R version 4.1.2.

3 | RESULTS

3.1 | ELS has long-lasting effects on BW and the endocrine stress phenotype in a sex-dependent manner

To assess the long-term effects of ELS on the stress and metabolic phenotype, eight control and 11 LBN breeding pairs were established. Female and male offspring (females: $n_{\text{Controls}} = 12$, $n_{\text{ELS}} = 21$; males: $n_{\text{Controls}} = 13$, $n_{\text{ELS}} = 18$), we monitored their BW and body composition, CORT responses to acute stress compared with baseline, relative adrenal weights, and analysed their faecal microbiota and metabolome composition at adulthood (5.5 months) (Figure 1a).

In females (group effect: $F_{1,31} = 6.77$, $p = 0.014$, mixed-effects) (Figure 1b), the ELS induced a slight reduction of BW compared with controls. Weight differences were most prominent at younger age (PND 24: $t_{31} = 3.55$, $p = 0.0009$, unpaired t test) and were caught up progressively in adulthood (5.5 months: $t_{28} = 2.33$, $p = 0.027$, unpaired t test), which was reflected in a body composition scan (5.5 months: $t_{30} = 1.78$, $p = 0.085$, unpaired t test) (Figure 1c–e). In males, the ELS induced a long-lasting and stable reduction of BW compared with nonstressed controls (group effect: $F_{1,29} = 7.03$, $p = 0.013$) (Figure 1f). Weight differences between ELS and controls were observed at young age (PND 24: $t_{30} = 3.24$, $p = 0.003$, unpaired t test) and at adulthood (5.5 months: $t_{29} = 3.02$, $p = 0.005$, unpaired t test). The ratio of fat to lean mass in males at 5.5 months of age lay in the same range as for females and is lower in ELS offspring ($t_{28} = 1.81$, $p = 0.082$, unpaired t test) (Figure 1g–i). To assess the effects of ELS on glucose signalling and insulin tolerance, we performed a glucose (GTT) and insulin tolerance test (ITT) at 5.5 months of age. The GTT did not reveal any differences in glucose signalling in females ($t_{30} = 0.54$, $p = 0.6$, unpaired t test) nor in males ($t_{29} = 0.88$, $p = 0.39$, unpaired t test) (Figure S1a,b). Insulin tolerance was unaffected in females ($t_{29} = 1.0$, $p = 0.32$, unpaired t test), whereas ELS males displayed significantly lower glucose levels until 120-min postinsulin injection compared with controls ($t_{29} = 4.14$, $p = 0.0003$, unpaired t test) (Figure S1c,d).

At baseline ($t_{28} = 1.88$, $p = 0.071$, unpaired t test) (Figure 1j) and after an acute restraint stress (Figure 1k)

ELS females (5.5 months old) had elevated CORT levels at 30 ($t_{29} = 2.22$, $p = 0.035$, unpaired t test) and 90 min post stress ($t_{29} = 1.87$, $p = 0.071$, unpaired t test), indicating a disturbed negative feedback regulation of their HPA axis, whereas relative adrenal weights were unaffected ($t_{28} = 0.21$, $p = 0.83$, unpaired t test) (Figure 1l). In males, baseline CORT levels were significantly elevated in ELS offspring ($t_{28} = 3.49$, $p = 0.002$, unpaired t test) (Figure 1m) but their stress response (T30: $t_{25} = 1.45$, $p = 0.16$; T90: $t_{25} = 0.53$, $p = 0.6$, unpaired t test) (Figure 1n) and relative adrenal weights ($t_{29} = 0.59$, $p = 0.56$) (Figure 1o) were unaffected by the ELS.

Overall, these data show that metabolic control and the endocrine stress phenotype of female and male mice are affected in a sex- and age-dependent manner after ELS throughout the entire lifespan.

3.2 | ELS affects microbiota composition in females but not in males

As we observed that ELS-induced alterations in metabolic and stress phenotypes were sex dependent, we were interested in the effects of the ELS on microbiota composition in female and male mice.

At 5.5 months of age, we therefore collected faeces and analysed the faecal microbiota by 16S rRNA marker gene sequencing (16S) and performed an RDA analysis to uncover sex-dependent differences in microbial composition (Figure 2a). In females, RDA analysis revealed a clear distinction in the microbial composition between stressed and nonstressed animals with RDA1 accounting for 8% of the explained variance ($F = 2.52$, $p = 0.006$) (Figure 2b), whereas in males this shift was not detectable ($F = 1.00$, $p = 0.467$; RDA1 accounting for 3.3% of the explained variance) (Figure 2c). Those observations were also obtained by PERMANOVA analysis on beta diversity using the Bray-Curtis distance (PERMANOVA $p = 0.002$ and $p = 0.281$, female and male groups, respectively). Combined RDA analysis of both sexes showed a general difference between female and male microbial content ($F = 2.333$, $p = 0.001$; RDA1 accounting for 5.7% of the explained variance) and confirmed an ELS-induced shift exclusively for females (Figure 2d).

Therefore, observed sex-dependent differences in microbial composition could be responsible for differences in BW, with the ELS-induced microbial shift possibly having a protective effect in females. No differences were observed in alpha-diversity indexes (Shannon and Chao 1).

3.3 | ELS affects metabolite profile in males but not in females

Intrigued by female-specific shifts in microbial composition, we analysed faecal metabolomics via ^{1}H-NMR spectroscopy at 5.5 months of age (Figure 3a). Interestingly, PCA analysis applied to NMR spectra data sets, revealed a stronger shift in metabolome composition in males (PC1 boxplot: $p = 0.086$) compared with females (PC1 boxplot: $p = 0.44$) (Figure 3b–e), indicating ELS-induced changes in metabolome that were more prominent in males. Analysis of relative metabolite levels, calculated as Z scores from normalized metabolite values, revealed that the metabolome content of ELS mice differs from control animals in both females and males (Figure 3f,g). In females (Figure 3f), NACs-glutamate ($p = 0.036$), methionine ($p = 0.012$), and aspartate ($p = 0.0071$) are significantly increased. However, differences were more pronounced in males (Figure 3g), with increases in leucine ($p = 0.047$), alanine ($p = 0.041$), malonate phenylalanine ($p = 0.046$), lysine ($p = 0.048$), and tyrosine ($p = 0.045$).

Together, it is tempting to speculate that adaptive processes in microbial composition in ELS females protect them from ELS-induced maladaptive changes in their metabolome as observed in males.

3.4 | ELS induces sex-specific changes in microbiota–metabolome associations

Since the faecal metabolome is also directly influenced by the faecal microbial content, we were interested in the correlation patterns between the two feature types (Figure 4a). Therefore, we performed Spearman correlation analyses of faecal microbial genera and metabolites. In ELS females, a bubble-plot heatmap visualization of the correlations indicated an overall shift in significant correlations, that is, an increase in number of significant correlations, as indicated by an asterisk (Figure 4b). Further, a number of positive and negative correlation patterns were evident in the female ELS group, most notably with *Ruminiclostridium* species and with *Intestimonas*, with positive correlations with butyrate, leucine, valine, isoleucine, glutamate, and glycine and negative correlations with acetoin, UNK2, acetylcarnitine, and methanol. Most of these significant correlations were completely absent in nonstressed females. In males, we observed a much lower number of significant correlations in both nonstressed and ELS animals compared with females, albeit that the observed significant correlations were mostly negative rather than positive for ELS males compared with ELS females (Figure 4c).

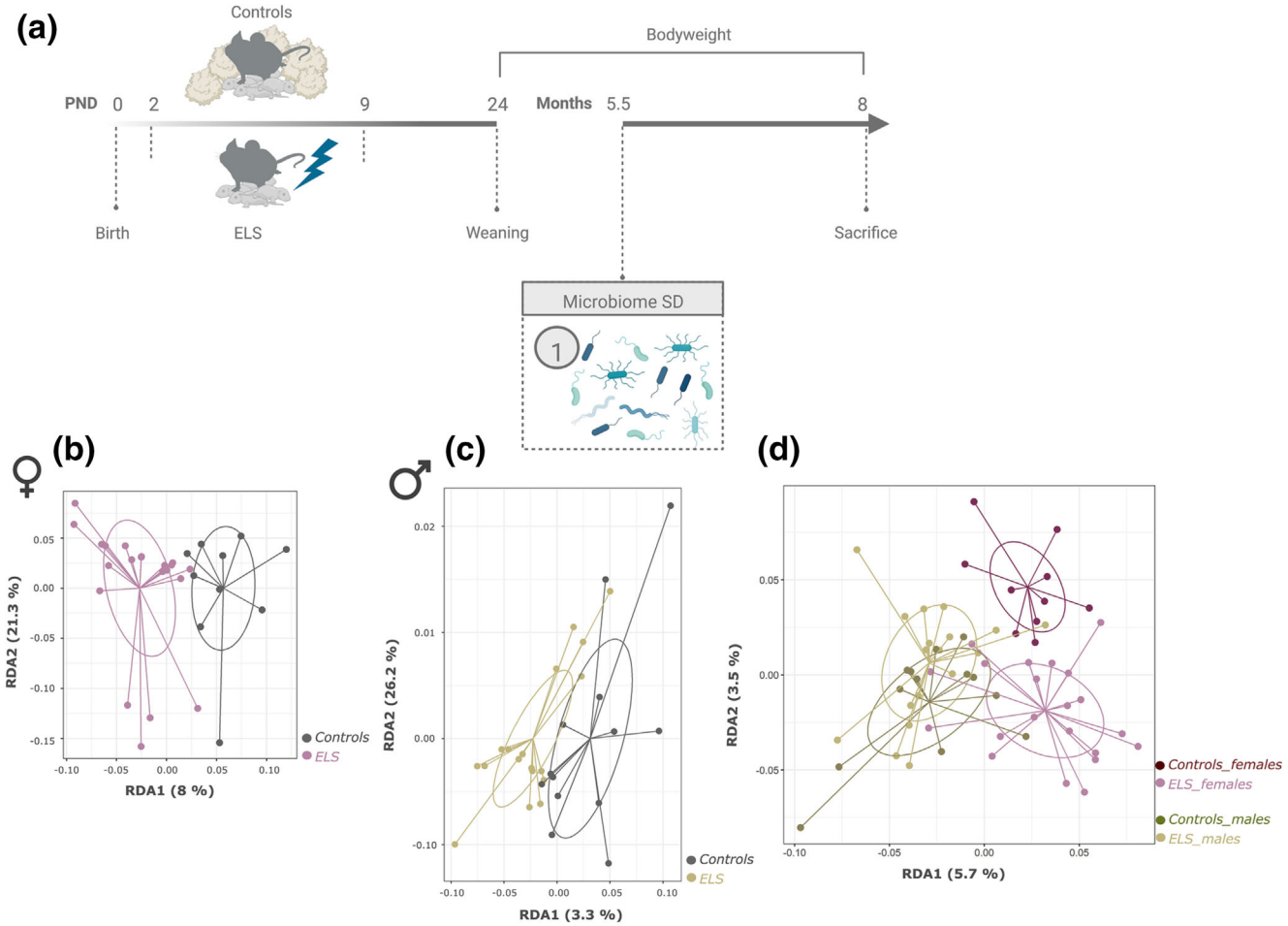

FIGURE 2 Faecal microbiota composition in female and male mice after ELS. (a) Experimental design: Male and female offspring of C57/Bl6n dams underwent the LBN paradigm from PND 2–9 (females: $n_{\text{Controls}} = 11$, $n_{\text{ELS}} = 21$; males: $n_{\text{Controls}} = 13$, $n_{\text{ELS}} = 18$). Bodyweight was assessed on a regular basis from weaning until sacrifice at 8 months of age. At 5.5 months of age, faeces were collected under baseline conditions in the early morning for 16S rRNA microbiota analysis. (b) RDA plot at ASVs in female microbiota samples revealed a clear separation between controls and ELS groups. (c) In males, there was no separation in microbiota composition between controls and ELS mice. (d) RDA analysis of all four groups showed a clear separation between male and female faecal microbiota composition and confirms ELS-induced shift in females. Data are received from male and female C57/Bl6n mice at 5.5 months of age and each symbol represents a single sample coloured according to the respective group. ELS early life stress, RDA redundancy analysis, PND postnatal day.

3.5 | Maternal and adult HFD and ELS have long-lasting effects on BW and the endocrine stress phenotype in a sex-dependent manner

Given the long-lasting and sex-dependent effects of ELS on the metabolic phenotype of the offspring, we investigated how a maternal (MHFD) and adult HFD challenge interact with the ELS in shaping homeostatic control throughout life.

Therefore, the offspring of HFD ($n = 22$) and SD dams ($n = 15$) were divided into ELS and control groups and challenged with an HFD in adulthood (Figure 5a,b). In females ($n_{\text{MSD Controls}} = 18$, $n_{\text{MSD ELS}} = 18$, $n_{\text{MHFD Controls}} = 17$, $n_{\text{MHFD ELS}} = 17$), we observed the same time course of ELS-induced differences in BW as in the first cohort (group: $F_{3,66} = 2.31$; $p = 0.08$, mixed-effects) (Figure 5c), with lighter ELS offspring in early life but no interaction effect of maternal diet and ELS (interaction: $F_{1,65} = 4.7 \times 10^{-5}$; $p = 0.99$, # condition: $F_{1,65} = 21.97$; $p < 0.0001$, two-way ANOVA, Bonferroni post hoc $\text{MSD}_{\text{Control}}$ vs. MSD_{ELS}: $t_{65} = 3.39$; $p = 0.007$, $\text{MHFD}_{\text{Controls}}$ vs. MHFD_{ELS}: $t_{65} = 3.25$; $p = 0.01$) (Figure 5d) and an adjustment of BW in adulthood irrespective of the dam's diet (interaction: $F_{1,66} = 0.64$; $p = 0.43$, two-way ANOVA) (Figure 5e). In males ($n_{\text{MSD Controls}} = 18$, $n_{\text{MSD ELS}} = 12$, $n_{\text{MHFD Controls}} = 20$, $n_{\text{MHFD ELS}} = 16$) however, ELS-induced differences were again more pronounced

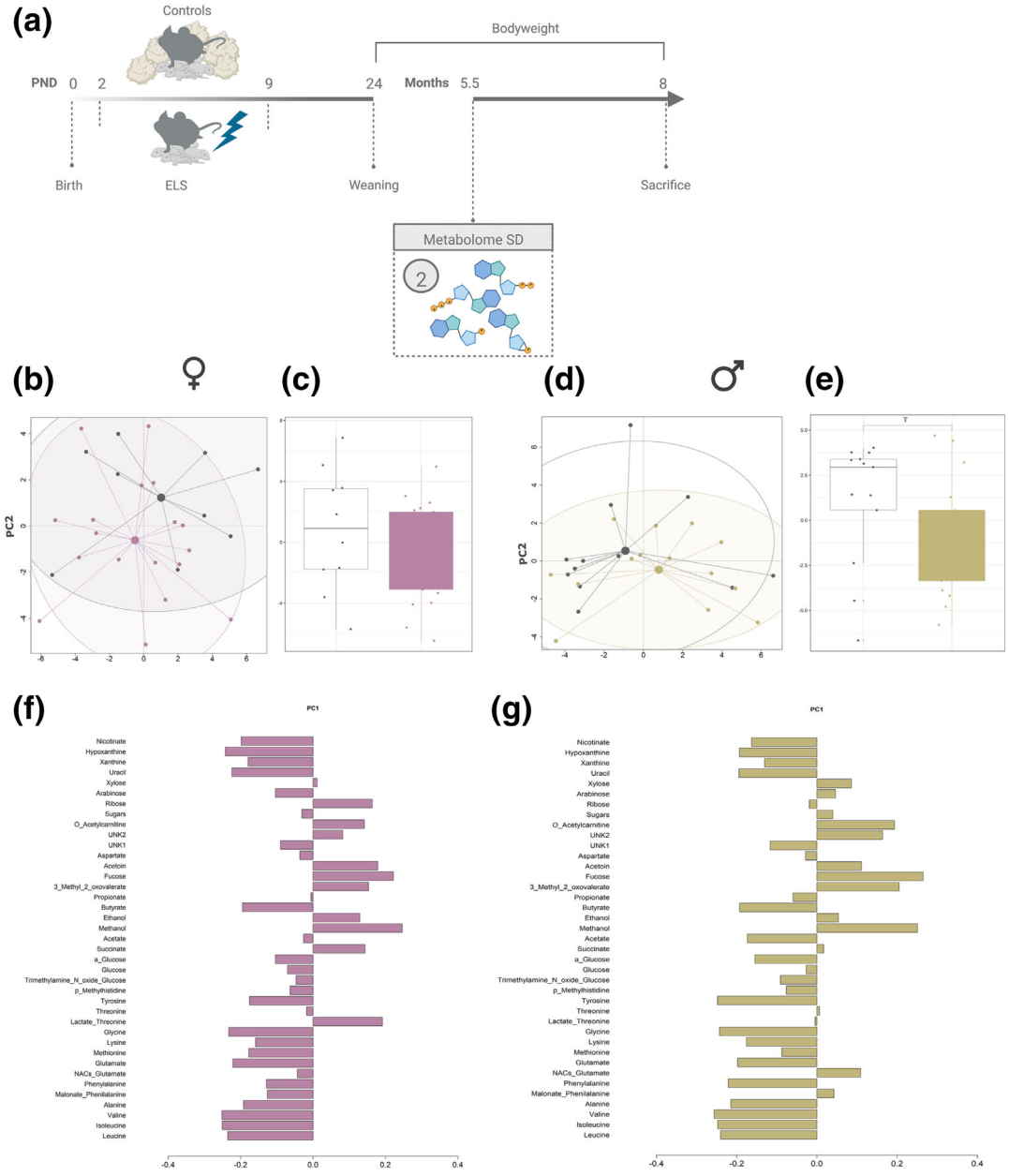

FIGURE 3 Faecal metabolome composition in female and male mice after ELS. (a) Experimental design: Male and female offspring of C57/Bl6n dams underwent the LBN paradigm from PND 2–9 (females: $n_{Controls} = 11$, $n_{ELS} = 21$; males: $n_{Controls} = 13$, $n_{ELS} = 18$). Bodyweight was assessed on a regular basis from weaning until sacrifice at 8 months of age. At 5.5 months of age, faeces were collected under baseline conditions in the early morning for ^{1}H-NMR metabolome analysis. PCA analysis for faecal metabolome in females showing (b) scores plot and (c) PC1 box-and-whisker plot. The box limits represent the lower and upper quartile limits. The whiskers are lines extending from each end of the boxes to show the extent of the rest of the data. The PCA analysis showed no effect of ELS on metabolome signatures in females. (d,e) Metabolome analysis in males, however, shows a slight separation between stressed and nonstressed animals. Analyses of relative metabolite levels (Z scores and 90% CI) of control versus ELS females (f) and males (g) indicate ELS-induced changes in metabolome content in both sexes. Data are received from male and female C57/Bl6n mice at 5.5 months of age and each symbol represents a single sample coloured according to the respective group. *$p < 0.05$, **$p < 0.01$, $T < 0.01$. ELS early life stress, CI confidence interval, SD standard deviation, PC1 principal component 1, PND postnatal day.

and stable throughout life on an SD, which confirmed findings in the first cohort (group: $F_{3,62} = 25.57$; $p < 0.0001$, mixed-effects) (Figure 5f). As in females, two-way ANOVA of male BW at PND24 (Figure 5g) did not reveal a significant interaction effect of maternal diet and ELS but depicted a significant effect of the ELS in both dietary groups (interaction: $F_{1,61} = 0.002$; $p = 0.96$, # condition: $F_{1,61} = 182.3$; $p < 0.0001$, two-way ANOVA,

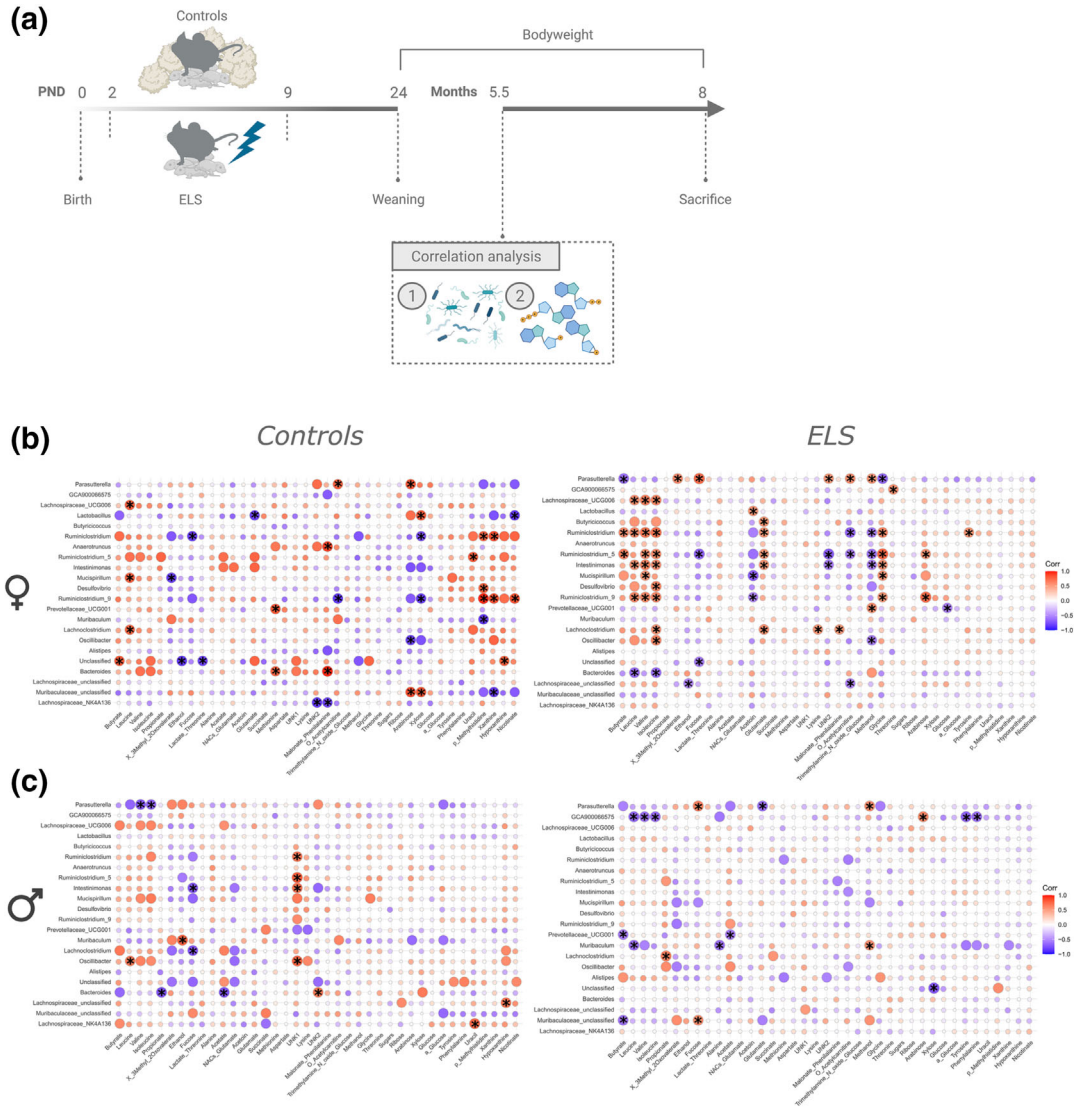

FIGURE 4 Associations between faecal microbiota and metabolome. (a) Experimental design: Male and female offspring of C57/Bl6n dams underwent the LBN paradigm from PND 2–9 (females: $n_{Controls} = 11$, $n_{ELS} = 21$; males: $n_{Controls} = 13$, $n_{ELS} = 18$). Bodyweight was assessed on a regular basis from weaning until sacrifice at 8 months of age. At 5.5 months of age, faeces were collected under baseline conditions in the early morning for ^{1}H-NMR metabolome and 16S analysis. Spearman correlation analysis between faecal microbial genera and metabolites revealed an increase in positive correlations in ELS females (b) and, overall, a lower number of significant correlations in stressed and nonstressed males compared with females (c). *p* values indicating the probability scores of the correlations (*$p < 0.05$). Data are received from male and female C57/Bl6n mice at 5.5 months of age. Size and colour intensity of the bubbles indicate strength of rho correlation value. Negative correlations are indicated in blue and positive correlations in red. ELS early life stress, PND postnatal day.

Bonferroni post hoc $MSD_{Control}$ vs. MSD_{ELS}: $t_{61} = 9.19$; $p < 0.0001$, $MHFD_{Controls}$ vs. $MHFD_{ELS}$: $t_{61} = 10$; $p < 0.0001$). As in the first cohort, the ELS-induced reduction in BW was stable in males (Figure 5h) (interaction: $F_{1,62} = 0.03$; $p = 0.87$, # condition: $F_{1,62} = 20.46$; $p < 0.0001$, two-way ANOVA, Bonferroni post hoc $MSD_{Control}$ vs. MSD_{ELS}: $t_{62} = 2.93$; $p = 0.03$, $MHFD_{Controls}$ vs. $MHFD_{ELS}$: $t_{62} = 3.51$; $p = 0.005$).

To trigger potential long-lasting effects of the maternal HFD in the offspring's adulthood, we challenged all groups with an HFD for 6 months and assessed body composition after 4 months and baseline morning CORT at 12 months of age. In females the additional HFD did not trigger weight differences between the groups (Figure 5i) (interaction: $F_{1,64} = 0.006$; $p = 0.93$, two-way ANOVA), but there was an overall effect of the stress on body composition after the adult HFD (Figure 5j), with the MHFD ELS offspring displaying higher fat content compared with nonstressed controls (irrespective of dam's diet) (interaction: $F_{1,62} = 1.55$; $p = 0.22$, #

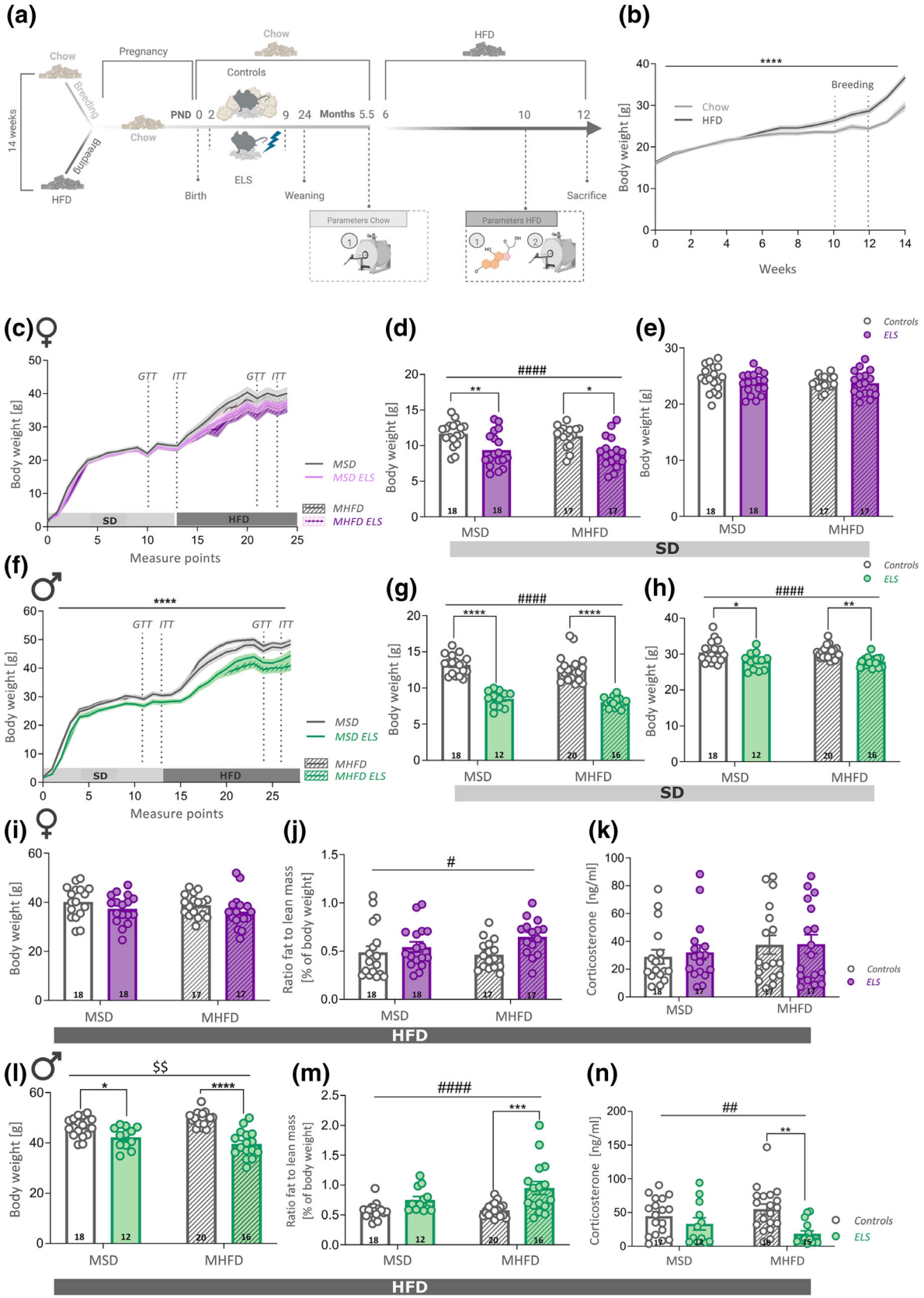

FIGURE 5 Legend on next page.

FIGURE 5 Metabolic effects of a pre-pregnancy maternal and adult HFD challenge in combination with ELS. (a) Experimental design: Female C57/Bl6n were split into HFD and SD groups and remained on their respective diet for 14 weeks until the onset of pregnancy. Once females were visibly pregnant (vaginal plug), the HFD group was switched to a chow diet for the remaining pregnancy and during lactation. Offspring of HFD and SD mothers were split into ELS and control groups and were weaned onto a regular SD diet at PND 24 and remained group-housed throughout the experiment. This experimental design resulted in four experimental groups: MSD = maternal standard diet; MSD ELS = maternal standard diet and ELS; MHFD = maternal high-fat diet; MHFD ELS = maternal high-fat diet and ELS. BW was assessed on a regular basis from weaning until sacrifice at 12 months of age. At 6 months of age, all experimental groups were challenged with an HFD for 4 months. At 5.5 (before adult HFD challenge) and 10 months (after adult HFD challenge) of age a body-composition scan was performed (1) and baseline CORT (2) was assessed at the endpoint. (b) Dams on HFD were significantly overweight before pregnancy. (c) BW overtime was not different between all four groups in female offspring, whereas at PND 24 there was a significant effect of the ELS on BW (d) that disappeared over the course of the experiment (e). Male ELS offspring (irrespective of the mother's diet) had a lower BW throughout life (f) with more pronounced effects at a young age (PND 24) (g) and sustained and robust differences at the end of the SD (h). (i) An adult HFD challenge did not alter BW in female offspring after 4 months on the diet. (j) In the body composition scan, there was an overall effect of the ELS visible, with higher fat to lean mass ratio in the stressed MHFD group. (k) Baseline CORT after 6 months on HFD was not different in females. (l) An adult HFD challenge in males revealed an overall significant effect of the maternal HFD on BW with the highest increase in the nonstressed MHFD group. The ELS effect was stable also under the adult HFD challenge. (m) Fat to lean mass ratio however, was higher in MHFD males that underwent an ELS, despite lower BW in this group. (n) CORT at baseline after 6 months on an HFD showed an overall effect of the stress, with significantly lower CORT levels in the combined group (MHFD plus ELS). Data are received from mice between PND2 to 12 months of age and are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $T < 0.1$, # = significant ELS effect $p < 0.05$, ##$p < 0.01$, ####$p < 0.0001$, $$ significant interaction effect $p < 0.01$. ELS early life stress, MHFD maternal high-fat diet, MSD maternal standard diet, PND postnatal day.

condition: $F_{1,62} = 5.3$; $p < 0.03$, two-way ANOVA). CORT levels were unaffected following the adult HFD challenge and did neither reveal an effect of the dam's diet, nor the ELS (interaction: $F_{1,64} = 0.05$; $p = 0.83$, two-way ANOVA) (Figure 5k). In males, the adult HFD triggered a significant interaction effect between maternal diet and ELS, with a significant ELS-induced BW reduction and a slight, but nonsignificant, increase in BW in the non-stressed MHFD offspring compared with all other groups ($$ interaction: $F_{1,62} = 8.71$; $p = 0.005$, # condition: $F_{1,62} = 49.71$; $p < 0.0001$, two-way ANOVA, Bonferroni post hoc $MSD_{Control}$ vs. MSD_{ELS}: $t_{62} = 2.76$; $p = 0.046$, $MHFD_{Controls}$ vs. $MHFD_{ELS}$: $t_{62} = 7.46$; $p < 0.0001$) (Figure 5l). The body composition scan revealed the same result as for females, with higher fat content in the stressed MHFD offspring group (interaction: $F_{1,62} = 2.32$; $p = 0.13$, # condition: $F_{1,62} = 20.48$; $p < 0.0001$, two-way ANOVA, Bonferroni post hoc $MHFD_{Controls}$ vs. $MHFD_{ELS}$: $t_{62} = 4.52$; $p < 0.0002$) (Figure 5m). Baseline morning CORT measures after an adult HFD challenge were significantly reduced exclusively in ELS MHFD males (interaction: $F_{1,60} = 3.04$; $p = 0.09$, # condition: $F_{1,60} = 11.32$; $p < 0.001$, two-way ANOVA, Bonferroni post hoc $MHFD_{Controls}$ vs. $MHFD_{ELS}$: $t_{60} = 1.11$; $p < 0.002$) (Figure 5n).

Glucose signalling in both sexes (interaction females: $F_{1,66} = 1.29$; $p = 0.26$, interaction males: $F_{1,63} = 0.01$; $p = 0.75$, two-way ANOVA) and insulin signalling in females under an SD (interaction: $F_{1,64} = 1.25$; $p = 0.27$) (Figure S2a–c) were unaffected by maternal diet and ELS. In males, however, there was an overall significant effect of the maternal diet on glucose recovery in stressed MHFD offspring but no interaction effect was observed (interaction: ITT $F_{1,58} = 2.48$; $p = 0.12$, § maternal diet effect: $F_{1,58} = 8.58$; $p = 0.005$, Bonferroni post hoc MSD_{ELS} vs. $MHFD_{ELS}$ $t_{58} = 2.95$ $p = 0.03$) (Figure S2d). After an adult HFD challenge for 4 months there were no differences between groups in both sexes in the GTT (interaction females: $F_{1,61} = 0.21$; $p = 0.65$, interaction males: $F_{1,59} = 2.09$; $p = 0.15$) (Figure S2e,f) and ITT (interaction females: $F_{1,63} = 0.45$; $p = 0.5$, $F_{1,61} = 1.37$; $p = 0.25$) (Figure S2g,h).

The data from this cohort confirmed the results of the first cohort and demonstrated sex-specific effects of ELS on BW in males, whereas females recovered from the initial weight loss during their lifetime. Furthermore, the negative effects of maternal HFD before pregnancy on body composition were exclusively triggered in males by an HFD challenge in adulthood. Interestingly, despite their lower BW, male ELS offspring developed a higher fat mass after an HFD challenge in adulthood, suggesting that the early stress exposure renders the animals more vulnerable to future HFD-induced metabolic complications.

4 | DISCUSSION

Adverse childhood experiences in humans have been shown to impact later metabolic health, and there is evidence that these effects can be sex dependent (Reemst et al., 2022). However, data from animal models to study

the potential underlying mechanisms are scarce. Here, we describe interesting sex-dependent consequences of early life stress exposure on metabolism and stress physiology. The differential effects observed for male and female offspring are correlated with sex-dependent shifts in gut microbiota and metabolome, hinting at a possibly causal relationship.

Our data demonstrate that exposure to an adverse postnatal environment, where maternal care is negatively affected by limited nesting and bedding resources, leads to robust and long-lasting alterations in BW and metabolic control in male offspring. Conversely, female offspring recovered from this early life insult over time and could largely normalize their BW and metabolism. These effects were consistently seen over two independent cohorts of animals, underlining the notion that male mice are more susceptible to long-term programming of metabolism by early life adversity. These data are supported by a few studies in humans, where especially weight loss following early adversity was found in boys, but not in girls. Park and colleagues reported in a large study of 4394 children that antenatal maternal depression was associated with lower weight specifically among boys (Park et al., 2017). Similarly, body fat mass was reported to be lower in boys of mothers with high cortisol levels, whereas in girls, cortisol was associated with marginally higher fat mass (Van Dijk et al., 2012). However, there are also a number of reports in humans where weight gain following adverse childhood experiences are reported (Reemst et al., 2022), and here girls seem to be more often affected than boys. Importantly, most human studies on the interaction of early life adversity and metabolism investigate the effects in children and adolescents, whereas our data show that the sex-dependent differential susceptibility emerges only in middle-aged mice.

There are many possible mechanisms that may lead to differential susceptibility in males and females following early life stress on metabolism, including alterations in stress system function or metabolism-related brain circuits. However, here the observed alterations in gut microbiota and metabolites are also promising candidates that may contribute to the phenotype (Vogel et al., 2020). Interestingly, on the level of gut metabolites, we observed that again females are less affected by early life adversity than males. Surprisingly, the opposite was found for the gut microbiota, where the most pronounced alterations were detected in ELS females. Remarkably, the correlations between microbiota and metabolome were weaker in (nonstressed and ELS) males than in females. Based on the correlation- and microbiota analyses it is tempting to speculate that early adversity induces adaptive changes in the microbiota of female animals, which were associated with strong and stable correlations between their microbiota and metabolome. The weaker correlation observed in males could render them more susceptible to maladaptive changes in their metabolome content and to metabolic consequences of early life stress. This notion is supported by previous studies, indicating a close relationship between adversity early in life, sex, and later outcomes on metabolism, physiology and behaviour (Dandekar et al., 2022; Golubeva et al., 2015; Park et al., 2017; Rincel et al., 2019). However, our experiments do not provide direct evidence whether alterations in gut microbiota and metabolome drive the metabolic effects of early life adversity, if they are a consequence of metabolic alterations or if they are a largely unrelated phenomenon and this warrants further investigations.

While early life adversity can directly shape the metabolism and stress physiology, some of these effects may only be unmasked following additional metabolic challenges. In fact, early life stress has been shown to predispose individuals to be more vulnerable or resilient to subsequent second or third hit challenges (Daskalakis et al., 2013; Gaspar et al., 2021). Interestingly, we here observed that maternal and adult high-fat diet exposure have long-lasting effects on BW and the endocrine stress phenotype in a sex-dependent manner. Again, predominantly in males, the combination of maternal obesity and high-fat diet exposure of the adult offspring resulted in an increased fat to lean mass ratio in ELS animals, despite the significant reduction in total body mass due to ELS. This suggests that the early stress exposure renders male mice more vulnerable to future metabolic consequences mediated by additional metabolic challenges. Intriguingly, maternal obesity alone did not result in robust alterations of the metabolic and stress physiology phenotype. This is in contrast to previous reports (Edlow, 2017; Hasebe et al., 2021; Lagisz et al., 2015; Shrestha et al., 2020) but can be explained by the fact that we switched dams to a standard diet during pregnancy, while most studies on maternal obesity continue the high-fat diet exposure throughout pregnancy and lactation. Thus, our data in comparison to previous studies suggest that long-term detrimental effects in the offspring are especially mediated by maternal intake of high-fat diet during pregnancy and lactation, rather than by the maternal obesity per se, which opens up important intervention possibilities for obese women during their pregnancy and lactation period.

5 | CONCLUSION

In summary, we here provide evidence that exposure to early life stress affects the metabolic and stress

physiological development of the offspring, rendering especially males more susceptible to metabolic dysfunctions. The results correlate with changes in the gut microbiota and metabolome, which could be used as biomarkers for individual metabolic risk as well as potential treatment interventions in the future.

AUTHOR CONTRIBUTIONS

Lea M. Brix and Mathias V. Schmidt conceived the project and designed the experiments. Lea M. Brix managed the breeding and conducted all animal experiments. Lea M. Brix, Irmak Toksöz, Joeri Bordes, Lotte van Doeselaar, Clara Engelhardt, Shiladitya Mitra, and Sowmya Narayan assisted with the experiments. Daniel Monleon performed ^{1}H-NMR spectroscopy and statistical analysis of faecal metabolome. Maria Carmen Collado performed 16SrRNA sequencing and statistical analysis of faecal microbiota. Thomas H.A. Ederveen performed Spearman correlation analysis. Lea M. Brix wrote the initial version of the manuscript. Mathias V. Schmidt supervised the research and all authors revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supporting information. Raw data are available upon request to the corresponding author (MVS).

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16047.

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