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Perinatal exposure to a dietary pesticide cocktail does not increase susceptibility to high-fat diet-induced metabolic perturbations at adulthood but modifies urinary and fecal metabolic fingerprints in C57Bl6/J mice



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ABSTRACT

Background: We recently demonstrated that chronic dietary exposure to a mixture of pesticides at low-doses induced sexually dimorphic obesogenic and diabetogenic effects in adult mice. Perinatal pesticide exposure may also be a factor in metabolic disease etiology. However, the long-term consequences of perinatal pesticide exposure remain controversial and largely unexplored.

Objectives: Here we assessed how perinatal exposure to the same low-dose pesticide cocktail impacted metabolic homeostasis in adult mice.

Methods: Six pesticides (boscalid, captan, chlopyrifos, thiachlopid, thiophanate, and ziram) were incorporated in food pellets. During the gestation and lactation periods, female (F0) mice were fed either a pesticide-free or a pesticide-enriched diet at doses exposing them to the tolerable daily intake (TDI) level for each compound, using a 1:1 body weight scaling from humans to mice. All male and female offsprings (F1) were then fed the pesticide-free diet until 18 weeks of age, followed by challenge with a pesticide-free high-fat diet (HFD) for 6 weeks. Metabolic parameters, including body weight, food and water consumption, glucose tolerance, and urinary and fecal metabolomes, were assessed over time. At the end of the experiment, we evaluated energetic metabolism and microbiota activity using biochemical assays, gene expression profiling, and ¹H NMR-based metabolomics in the liver, urine, and feces.

Results: Perinatal pesticide exposure did not affect body weight or energy homeostasis in 6- and 14-week-old mice. As expected, HFD increased body weight and induced metabolic disorders as compared to a low-fat diet. However, HFD-induced metabolic perturbations were similar between mice with and without perinatal pesticide exposure. Interestingly, perinatal pesticide exposure induced time-specific and sex-specific alterations in the urinary and fecal metabolomes of adult mice, suggesting long-lasting changes in gut microbiota.

Conclusions: Perinatal pesticide exposure induced sustained sexually dimorphic perturbations of the urinary and fecal metabolic fingerprints, but did not significantly influence the development of HFD-induced metabolic diseases.

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1. Introduction

Obesity is considered a pandemic disease, affecting over 650 million adults, and is commonly associated with metabolic syndromes, such as type II diabetes, dyslipidemia, and non-alcoholic fatty liver disease (NAFLD). Although genetic and lifestyle factors contribute to metabolic diseases, emerging evidence suggests that exposure to environmental contaminants also plays a role. This environmental obesogen hypothesis (Grün and Blumberg 2006) is corroborated by accumulating epidemiological and experimental data. Notably, pesticide exposure has been linked to metabolic disorder development in adults (Czajka et al. 2019; Evangelou et al. 2016). In human adult cohorts, pesticide exposure is reportedly associated with diabetes prevalence (Park et al. 2019), and metabolic disease markers (Kongtip et al. 2018). The first compounds found to be obesogenic in adult humans were persistent pesticides from the organochlorine family, including dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE), (Cano-Sancho et al. 2017). Since then, results of *in vivo* and *in vitro* experimental studies have further indicated that non-persistent pesticides (e.g., organophosphorus compounds, pyrethroids, and neonicotinoids) may also contribute to metabolic changes and obesity (De Long and Holloway 2017). In adult mice, long-term exposure to the organophosphate chlorpyrifos induces insulin resistance and obesity (Liang et al. 2019). Other murine studies have demonstrated that the metabolic changes induced in adult animals by a high-fat diet are potentiated by exposure to low doses of permethrin, neonicotinoid, and triazine (Jin et al. 2014; Sun et al. 2017; Xiao et al. 2018).

During conception, gestation, and lactation, maternal nutrition, metabolic and physiologic state, and exposure to various environmental factors play fundamental roles in the health programming of the offspring (Koletzko et al. 2017; Kwon and Kim 2017). This so-called DOHAD “developmental origins of health and disease” notion was first highlighted by Barker (Barker 1998) and has been abundantly reviewed (Heindel et al. 2017; Hoffman et al. 2017; Preston et al. 2018). Studies show that prenatal exposure to organochloride pesticides is associated with being overweight in childhood (Karlsen et al. 2018) and with childhood obesity (Liu and Peterson 2015), and is inversely correlated with adiponectin levels in umbilical cord blood among girls (Debost-Légrand et al. 2016). Within a cohort of children whose mothers were employed in greenhouse horticulture, pesticide exposure during pregnancy was associated with lower birth weight and greater body weight gain between birth and primary school age (Jurewicz et al. 2005). In rodents, early life exposure to low doses of organophosphorus pesticides alters lipid metabolism and worsens metabolic perturbations induced by dietary fat intake (Thayer et al. 2012). However, these results are controversial with other studies showing limited effects of perinatal pesticide exposure on metabolic homeostasis later in life (Svingen et al. 2018).

The European Food Safety Agency (EFSA) recently reported that 42% of fruits and vegetables are contaminated with pesticide residues, suggesting that consumers are exposed to low doses of multiple compounds through the food chain (EFSA report 2020). Despite this knowledge, most experimental studies have assessed the effects of individual compounds, often at doses higher than the likely potential consumer exposures. The consequences of exposure to low-dose pesticide cocktails remain understudied, especially using exposure protocols relevant to humans such as direct incorporation in food. In a recent study in mice, we demonstrated that adulthood dietary exposure to a cocktail of six pesticides at low doses induced sexually dimorphic metabolic perturbations after one year (Lukowicz et al. 2018). The investigated pesticides were selected from those used for apple orchard treatment in the south of France, such that the tested pesticide cocktail may be commonly encountered in apples within the European Union, as recently described by the EFSA (EFSA 2015).

In our present study, we examined the metabolic long-term

modifications associated with perinatal exposure to the same pesticide cocktail tested in our prior work. Our protocol was designed to mimic some important aspects of consumer exposure regarding oral intake of multi-contaminated food and allowed us to gain insights into potential differences arising between perinatal and chronic effects of pesticide exposure. Moreover we aimed to evaluate the consequences of the combination of several ADI levels. Pesticides were incorporated in a standard chow diet, and F0 male and female mice were exposed to the tolerable daily intake (TDI) for each pesticide during the mating period, and throughout the gestation and lactation periods. At weaning, male and female F1 mice from all groups were fed a pesticide-free normal diet for 15 weeks, and then challenged with a pesticide-free high-fat diet for an additional 6 weeks. The adult F1 mice with perinatal pesticide exposure were characterized by significant changes in the urinary and fecal metabolomes, but showed no effects in terms of body weight gain, glucose tolerance, or hepatic metabolism.

2. Materials and methods

2.1. Chemicals

We purchased high-purity analytical standards of boscalid, captan, chlorpyrifos (99.2%), thiacloprid, thiophanate, and ziram from Pestanal Sigma-Aldrich (St. Quentin Fallavier, France). Pesticide toxicity information was obtained from Agritox (<http://www.agritox.anses.fr/>). Liquid chromatography–mass spectrometry (LC-MS) grade methanol was purchased from Thermo Fisher Scientific (Illkirch, France). Ultrapure water from the Milli-Q® system (Millipore S.A.S. Guyancourt and Molsheim, France) was used for mobile phases. All other non-specified products were purchased from Sigma-Aldrich (St Quentin Fallavier, France).

2.2. Pesticide chow

Ziram, thiophanate, captan, chlorpyrifos, boscalid, and thiacloprid are fungicides and insecticides belonging to various chemical families. We calculated the quantity of pesticide to incorporate into rodent pellets, in order to expose the animals to the TDI of each pesticide (published by the EFSA for the Joint Food and Agriculture Organization of the United Nations/World Health Organization Meeting on Pesticide Residues) divided by a mean body weight of 30 g and we considered a daily food intake of 5 g per mouse. This protocol aimed to transpose the TDI exposure values using a 1:1 body weight scaling strategy from humans to mice. It is noteworthy that other authors recommend an additional scaling factor for the transposition of exposure values from human to mouse (Nair and Jacob 2016). When this factor is applied, we found that our mice were exposed to a daily dose of pesticides lower than the human TDI by a 10-fold factor. Table 1 presents the properties and quantities of each pesticide incorporated in the mouse diet.

Pesticides were individually dissolved in a 9:1 volume/volume (v/v) mixture of methanol:acetone, and then mixed together. The solution was dispersed over the vitamin powder [PV 200, Scientific Animal Food Engineering (SAFE, Augy, France), and then homogenized in a rotavapor (Laborota 4000™; BUCHI Switzerland) for 30 min at 45 °C to evaporate the solvents, and for 50 min at room temperature. Control feed was prepared as described above, with the vitamin powder incubated with a 9:1 mixture of methanol:acetone without pesticide. The vitamin powder with or without pesticide enrichment was sent to the Animal and Food Science Unit (SAAJ, Jouy en Josas, France) of the National Research Institute for Agriculture, Food and environment (INRAE), which prepared control and pesticide-enriched pellets from a mixture of our control or pesticide-enriched vitamin powders (1%) with minerals complements (7%) and other diet components (63% carbohydrate, 5% fat, 22% protein, and 2% cellulose).

The pesticides in the pellets were quantified by Eurofins (Nantes, France) using gas chromatography–tandem mass spectrometry and

Table 1

Chemical families, function and tolerable daily intake (mg/kg body weight (BW)/day) of each pesticide and the expected and measured pesticides concentrations (determined level) ($\mu\text{g}/\text{kg}$ food) in the animal pellets.

Pesticide name	Chemical family	Function	Tolerable Daily Intake (mg/kg BW/day)	Expected quantity ^a ($\mu\text{g}/\text{kg}$ food)	Determined level ($\mu\text{g}/\text{kg}$ food)
Ziram	Dithiocarbamate	Fungicide	0.006	36	ND ^b
Chlorpyrifos	Organophosphorus	Insecticide	0.01	60	57
Thiacloprid	Neonicotinoid	Insecticide	0.01	60	63
Boscalid	Carboxamide	Fungicide	0.04	240	200
Thiofanate	Benzimidazole	Fungicide	0.08	480	260
Captan	Dicarboximide	Fungicide	0.1	600	230

Note: BW, Body weight; ND, not determined; (<http://www.agritox.anses.fr/>).

^a Expected quantity refers to the incorporated quantities of pesticides in mice pellets.

^b Ziram was not present at a detectible level (< 0.01 mg/kg).

liquid chromatography–tandem mass spectrometry. The final concentrations were confirmed, except for ziram, which was present at a concentration too low to detect (Table 1). Analysis from Eurofins laboratory confirmed that the control feed did not contain the pesticides of interest, or any other pesticides.

2.3. Animal experiment

Animal experiments were conducted at the Experimental, Breeding, and Conditioning Center for Animal Models (CECEMA) at the university of Montpellier France, in a conventional laboratory animal room. All protocols followed the European Union guidelines for laboratory animal use and care, and were approved by an independent ethics committee (authorization number APAFIS#13145-2018012216217153v3). The animals were treated humanely, with due consideration to alleviating distress and discomfort, and were housed in polycarbonate cages (Charles River Type S, 424 cm²). Eight-week-old female and male C57BL/6J mice were purchased from Charles Rivers Laboratories (L'Arbresle, France), and were allowed to acclimatize for one week while being fed a standard chow diet. Upon receipt, groups of males ($n = 10$) and females ($n = 20$) were randomly housed five or six per cage, with a 12-h light/dark cycle, at a stable temperature of 23 ± 2 °C, and were allowed ad libitum access to food and water. For a mating period, the animals were randomly divided into two groups (exposed and unexposed), and housed with one male and two females per cage (16 cages). During the mating period, the mouse diet was switched to either the pesticide-enriched or the pesticide-free diet (Figure S1). Food consumption was monitored weekly during the mating and gestation period (Figure S5A) but not during the lactation period to avoid cannibalism. For each cage, the food pellets were weighed each week before and after resupplying. Food consumption was evaluated by subtracting these two measurements and dividing the result by the total body weight of mice per cage. The same assessment was made for water consumption.

Litters were changed at least once a week and weaning was performed at PND21 exactly for each litter to minimize direct eating of pellet residues by the offsprings. At PND21, the male and female pups from exposed or non-exposed dams were thus separated, housed 5 or 6 per cage according to their group, and fed the pesticide-free control diet for 15 weeks. From birth to 18 weeks, mouse number per group was: control (C) males $n = 26$, control (C) females $n = 31$, perinatal pesticide (P) males $n = 20$, perinatal pesticide (P) females $n = 20$ (Figure S1). At 18 postnatal weeks of age, animals from both exposed and unexposed mothers were switched to a pesticide-free commercial high-fat diet (HFD, 60% calories from fat, D12492) or a pesticide-free commercial low-fat diet (LFD, D12450J) from Research Diets (New Brunswick, NJ) for 6 weeks (Figure S1). Diet duration was chosen based on previous unpublished data from our lab (Smati et al. personal communication) showing a linear increase in body weight gain between 0 and 9 weeks of HFD feeding in C57BL6/J male and female mice. We hypothesized that six weeks of HFD feeding would allow us to see a

potential interaction between perinatal pesticide exposure and HFD feeding. From 18 to 24 weeks, mouse number per group was: control low-fat diet (C-LFD) males $n = 14$, control low-fat diet (C-LFD) females $n = 17$, control high-fat diet (C-HFD) males $n = 12$, control high-fat diet (C-HFD) females $n = 14$, perinatal pesticide low-fat diet (P-LFD) males $n = 11$, perinatal pesticide low-fat diet (P-LFD) females $n = 11$, perinatal pesticide high-fat diet (P-HFD) males $n = 9$, perinatal pesticide high-fat diet (P-HFD) females $n = 9$. At 4, 11, and 22 postnatal weeks of age, fresh feces were collected from all groups of pups, and immediately frozen in liquid nitrogen. Then 9–10 mice per group were randomly chosen and placed into individual metabolic cages (Tecniplast, Decines-Charpieu, France) for collection of 24-h urine samples, which were stored at -80 °C until analysis.

At the end of the experiment, we collected blood from 24-week-old mice via the submandibular vein, using a lancet (goldenrod, Braintree Scientific Inc, MA) and lithium heparin-coated tubes (BD Microtainer[®]; BD, Dutscher, Brumath, France). Blood collection occurred at Zeitgeber time 17, i.e., in the fed state, during which hepatic activities are optimal (Lu et al. 2013). Then the mice were sacrificed by cervical dislocation. Plasma samples were prepared by centrifugation (1500 g; 10 min; 4 °C) and stored at -80 °C until biochemical analysis. Liver samples were collected, weighed, snap-frozen in liquid nitrogen, and stored at -80 °C until further analyses (liver neutral lipid analyses and metabolomics). We also collected and weighed the subcutaneous and epididymal white adipose tissue (WAT) and caecum.

2.4. Glucose tolerance test and plasma insulin level measurement

All experiments were performed using conscious mice. Twelve mice per group were randomly chosen, then fasted for 6 h before receiving an oral glucose load (2 g/kg BW). Blood glucose was measured at the tail vein using an Accu-Check[®] Performa glucometer (Roche Diabetes Care France, Meylan, France) at 30 min before and 0, 15, 30, 60, 90, and 120 min after the glucose load. At 30 min before and 15 min after glucose injection, we sampled 20 μL blood from the tip of the tail vein for measurement of plasma insulin concentration using HTRF serum kits (Cisbio, Codolet, France). Briefly, insulin was detected with a sandwich assay format, using two specific monoclonal antibodies: one labeled with Terbium Cryptate (donor) and the other with d2 (acceptor). We incubated 5 μL serum overnight at 4 °C with the two corresponding monoclonal antibodies. When the dyes are in close proximity, donor excitation via a light source triggers a fluorescence resonance energy transfer (FRET). We measured the fluorescence energy transfer using a Tecan Infinite 500 plate reader (Tecan, Lyon, France). The results were analyzed against a standard curve fit with the four-parameter logistic (4 PL) model, following the instructions for the kit (GraphPad Prism, USA).

2.5. Serum biochemical analysis

We measured aspartate transaminase (AST), alanine transaminase

(ALT), total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, free fatty acids (FFAs), and triglycerides (TGs) using a Pentra 400 biochemical analyzer (HORIBA, Montpellier, France; Anexplo facility, Toulouse, France).

2.6. Liver neutral lipid analysis

Hepatic lipid contents were extracted as previously described (Bligh, E.G. and Dyer 1959). Briefly, tissue samples were homogenized in 2:1 (v/v) methanol/ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 5 mM). We then extracted lipids corresponding to an equivalent of 2 mg tissue in chloroform:methanol:water (2.5:2.5:2.1, v/v/v) in the presence of the internal standards glyceryltridecanoate, stigmaterol, and cholesterol heptadecanoate (Sigma-Aldrich, St Quentin Fallavier, France). We analyzed triglycerides, free cholesterol, and cholesterol esters by gas chromatography on a Focus Thermo Electron system using a Zebron-1 Phenomenex fused-silica capillary column (5 m, 0.32-mm i.d., 0.50- μ m film thickness; Phenomenex, England), as previously described (Podechard et al. 2018). The oven temperature was programmed to increase from 200 °C to 350 °C at 5 °C/min, and the carrier gas was hydrogen (0.5 bar). The injector and detector were set to 315 °C and 345 °C, respectively.

2.7. Gene expression studies

RNA was extracted from liver samples using TRI Reagent® (Sigma-Aldrich, St Quentin Fallavier, France), following a previously described method (Chomczynski and Sacchi 1987; Lukowicz et al. 2018), and quantified using a NanoDrop™ 1000 (Thermo Fisher Scientific, Illkirch, France). We then reverse transcribed 2 μ g total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). The SYBR Green (Applied Biosystems, ThermoFisher Scientific, Illkirch, France) assay primers are presented in Table S1. Amplification was performed using an ABI Prism 7,300 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, Illkirch, France). Quantitative real-time polymerase chain reaction (qPCR) data were normalized to TATA-box-binding protein mRNA levels, and analyzed using LinRegPCR (version 2015.3; Jan Ruijter) to determine mean efficiency (NO), which was calculated as follows: $NO = \text{threshold}/(\text{Eff mean Cq})$, where Eff denotes mean PCR efficiency, and CQ denotes quantification cycle.

2.8. Metabolomics based on proton nuclear magnetic resonance (^1H NMR)

Urine, liver aqueous extracts, and feces aqueous extracts were prepared for NMR analysis as previously described (Beckonert et al. 2007; Lukowicz et al. 2018; Zhang et al. 2019). Metabolomics analysis were performed on liver samples from all animals ($n = 9$ –17 per group), on urine samples from 9 to 10 animals per group due to availability of the metabolic cages for sample collection and on feces samples either on half of the available samples (at 14 weeks) or on all available samples (at 22 weeks). All ^1H NMR spectra were obtained on a Bruker DRX-600-Avance NMR spectrometer (Bruker) using the MetaToul-AXIOM metabolomics platform (MetaboHUB), operating at 600.13 MHz for the ^1H resonance frequency, with an inverse-detection 5-mm H1-C13-N15 cryoprobe attached to a cryoplatfom (the preamplifier cooling unit). The ^1H NMR spectra were acquired at 300 K using a standard one-dimensional noesypr1D pulse sequence, with water presaturation and a 100-ms total spin-echo delay ($2\pi\tau$). A total of 128 transients were collected into 64,000 datapoints using a spectral width of 12 pulses/s, relaxation delay of 2.5 s, and acquisition time of 2.28 s. We obtained H1-H1 COSY, ^1H - ^1H TOCSY, and ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) for each biological matrix on one representative sample for metabolite identification. Annotated representative spectra originating from one control male from this study can be found in Figure S2 and metabolite annotations are given in Table S2. Raw data

are available upon request to the corresponding authors.

Data were analyzed by applying an exponential window function with a 0.3-Hz line broadening prior to Fourier transformation. The resultant spectra were phased, baseline corrected, and manually calibrated to trimethylsilylpropanoic acid (TSP) (δ 0.00 ppm) using Mnova NMR (version 9.0; Mestrelab Research S.L.). Next, the spectra were imported into MatLab (R2014a; MathsWorks, Inc.), and all data were analyzed using full-resolution spectra. After removing the region containing the water resonance (δ 4.6–5.2 ppm), the spectra were normalized to the probabilistic quotient (Dieterle et al. 2006) and aligned using a previously published function (Veselkov et al. 2009).

Data were mean-centered and scaled using unit variance scaling, followed by analysis with orthogonal projection on latent structure-discriminant analysis (O-PLS-DA). We used ^1H NMR data as independent variables (X matrix), which were regressed against a dummy matrix (Y matrix), indicating the class of samples (mice fed control or pesticide-enriched chow) (Trygg and Wold 2002). We evaluated the O-PLS-derived model for goodness of prediction (Q^2Y value) using n -fold cross-validation, where n depends on the sample size. The reliability of each model was established using a permutation test of the Y vector (1000 permutations) in order to determine a p value for each Q^2Y . All models were fitted with one predictive and none to 3 orthogonal components (Table S3). Final models were fitted with one orthogonal component and the parameters of the final models (Q^2Y and p -value) are indicated in the figures and in Tables S3 and S4. To identify metabolites responsible for discrimination between the groups, we calculated the O-PLS-DA correlation coefficients (r^2) for each variable and back-scaled them into a spectral domain, thus preserving the NMR spectra shapes and the coefficient signs (Cloarec et al. 2005a; Olivier Cloarec et al. 2005b). The variables' weights were color-coded according to the square of the O-PLS-DA correlation coefficients. We filtered the correlation coefficients extracted from significant models, such that correlations were only considered significant if they exceeded the threshold defined by Pearson's critical correlation coefficient ($p < 0.05$; $|r| > 0.7$; for $n = 8$ per group). For illustration purposes, the area under the curve for significant metabolites was integrated and significance tested once again using one-way analysis of variance (ANOVA) followed by Sidak's post-test for multiple comparisons. Peak integration for AUC calculation was performed manually as illustrated on Figure S4. Metabolites with both significant correlation coefficient to the predictive axis on the O-PLS-DA models and significant p -value using univariate statistics were considered as significantly different between groups. Parameters of the O-PLS-DA models and p -values for discriminant metabolites are summarized in Table S4.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 7.00; GraphPad Software). One-way or two-way analysis of variance (ANOVA) was performed, followed by appropriate post-hoc tests (Dunn's or Bonferroni) when statistically significant differences were found. Student's t -test was used for comparisons between two groups. A p value of < 0.05 was considered significant. To account for measurement of multiple genes on the same samples, raw p -values were corrected using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) implemented in the multtest package in Bioconductor (Pollard et al. 2005) using R version 3.6.3.

3. Results

3.1. Pesticide exposure of F0 female mice

After a 2-week acclimation period, F0 female mice were exposed to a cocktail of six pesticides from mating until F1 weaning (Figure S1). The pesticides in the feed pellets were quantified (Table 1) and food consumption was assessed weekly (Figure S5A), enabling us to calculate

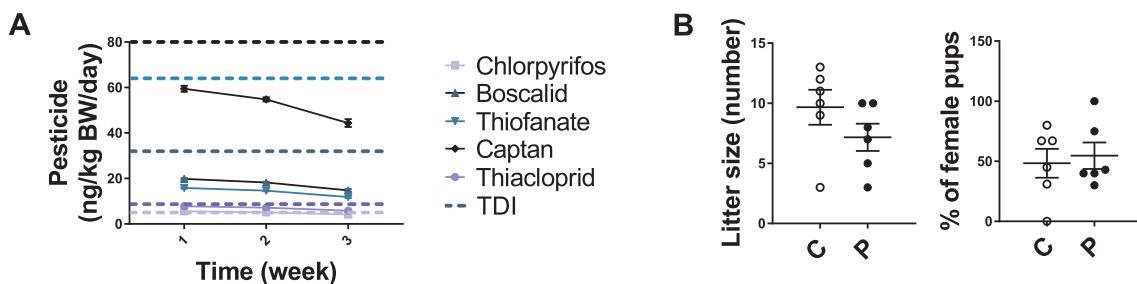


Fig. 1. (A) Characterization of pesticide exposure in F0 mice during gestation. Pesticide exposure was assessed by quantifying each pesticide in the feed pellets, and monitoring the daily food consumption of mice. (B) Litter size and sex ratio in F1 mice. Data are presented as mean ± SEM.

that the F0 mice were exposed to each pesticide at doses close to its human TDI level using a 1:1 body weight scaling from humans to mice (Fig. 1A). Pesticide exposure did not significantly impact the size or sex ratio of litters (Fig. 1B).

3.2. Impact of perinatal pesticide exposure on metabolic parameters

Upon weaning, male and female offspring (F1) were fed a control chow diet that did not contain pesticides for 15 weeks (Figure S1). Food consumption was similar between the perinatally exposed mice and the non-exposed mice (Figure S5B and C). Body weight survey and glucose tolerance assays did not reveal any significant differences between the perinatally exposed mice and the non-exposed mice at 6 or 14 weeks of age (Fig. 2). However, ¹H NMR-based metabolomic analysis of urine from 6- and 14-week-old mice revealed a specific metabolic profile that was associated with perinatal pesticide exposure (Fig. 3, Figure S3). O-PLS-DA statistical models were fitted that could significantly

discriminate perinatally exposed mice from their non-exposed counterparts, among both males (Fig. 3A,C and Figure S3A,C) and females (Fig. 3B,D and Figure S3B,D).

Perinatal pesticide exposure mainly affected the urinary levels of urea and trimethylamine (TMA), and these metabolic changes differed between males and females. Among males, perinatal pesticide exposure was associated with higher urinary urea levels in 6- and 14-week-old animals, and decreased urinary TMA levels in 14-week-old animals (Fig. 3A, C). Among females, the metabolic changes were time-specific. Compared to the corresponding unexposed animals, 6-week-old pesticide-exposed female mice displayed higher urinary urea levels and lower urinary TMA levels (Fig. 3B), while 14-week-old pesticide-exposed females had lower urinary urea levels and higher urinary TMA levels (Fig. 3D). TMA is produced through the gut microbiota metabolism of dietary choline, carnitine, and trimethylamine oxide (TMAO) (Zeisel et al. 1983). Therefore, the observed changes in TMA levels suggested that perinatal pesticide exposure influenced the metabolic

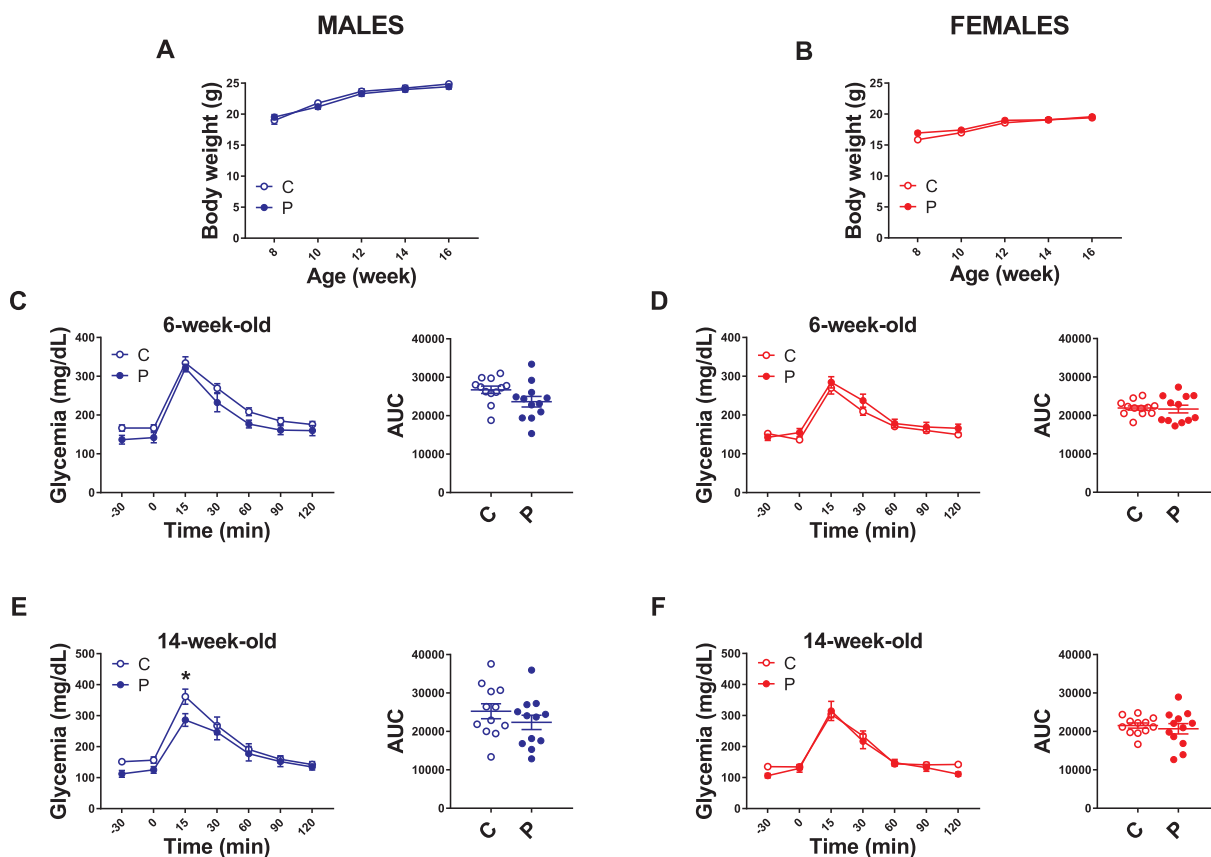


Fig. 2. (A, B) Body weights of F1 male (A) and female (B) mice from 8 to 16 weeks of age (n = 21–36/group, see Figure S1). (C–F) Blood glucose after administration of an oral glucose load (2 g/kg BW) in 6-week-old male (C) and female (D) mice, and in 14-week-old male (E) and female (F) mice (n = 12/group). Graphs shows the area under the curve for F1 control mice (c) and F1 perinatally exposed mice (p). Data are presented as mean ± SEM.

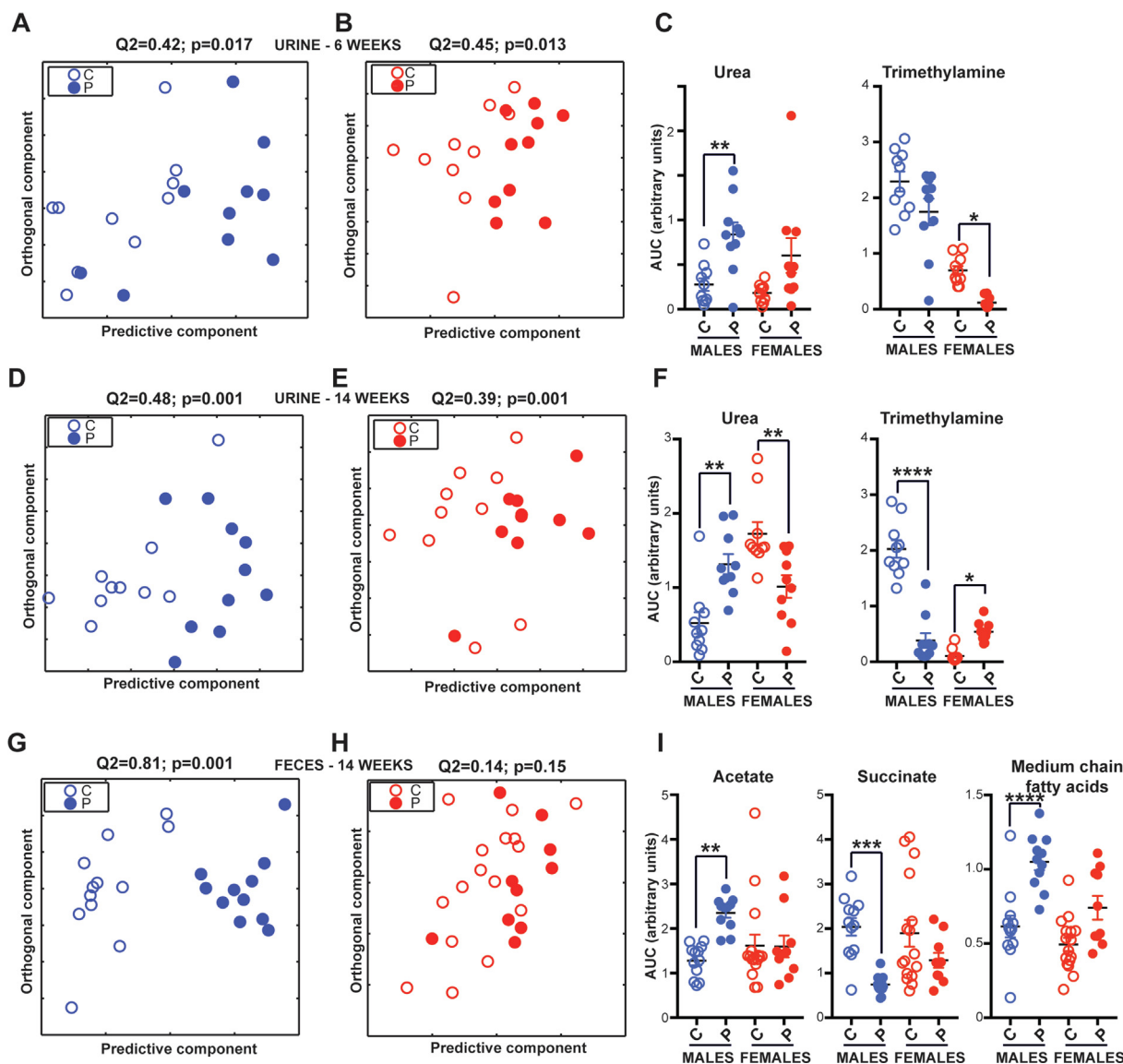


Fig. 3. Effects of perinatal pesticide exposure on urinary and fecal metabolomes. (A) O-PLS-DA score plots related to the discrimination between urinary¹H- NMR spectra for 6-week-old male mice perinatally-exposed to pesticides (P) vs. controls (C). (B) O-PLS-DA score plots related to the discrimination between urinary¹H- NMR spectra for 6-week-old female mice perinatally-exposed to pesticides (P) vs. controls (C). (C) Area under the curve for urinary metabolites that significantly discriminate C vs. P animals. (D) O-PLS-DA score plots related to the discrimination between urinary¹H- NMR spectra for 14-week-old male mice perinatally-exposed to pesticides (P) vs. controls (C). (E) O-PLS-DA score plots related to the discrimination between urinary¹H- NMR spectra for 14-week-old female mice perinatally-exposed to pesticides (P) vs. controls (C). (F) Area under the curve for urinary metabolites that significantly discriminate C vs. P animals. (G) O-PLS-DA score plots related to the discrimination between fecal¹H- NMR spectra for 14-week-old male mice perinatally-exposed to pesticides (P) vs. controls (C). (H) O-PLS-DA score plots related to the discrimination between fecal¹H- NMR (I) Area under the curve for fecal metabolites that significantly discriminate C vs. P animals. Significant metabolites were selected based on significant correlation with the discriminant axis from the O-PLS-DA models, as illustrated in Figure S3. Additional univariate statistics (1-way ANOVA + Sidak's post-tests) were conducted to confirm significant difference in metabolite levels. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to controls. ($n = 10$ /group for urine metabolomics, $n = 11$ – 17 /group for feces metabolomics).

activity of gut microbiota.

We also used ¹H NMR-based metabolomics to investigate the fecal metabolic profiles of exposed and non-exposed mice. The fecal metabolome is considered a relevant functional readout of the gut microbiome (Karu et al. 2018; Zierer et al. 2018). We observed a significant discrimination between the fecal metabolome of perinatally exposed and non-exposed 14-week-old males (Fig. 3E, Figure S3E). This impact was sexually dimorphic, as no difference in fecal metabolic profile was observed among 14-week-old females (Fig. 3F, Figure S3F). Among males, the perinatally exposed and unexposed animals showed significant differences in acetate, succinate and medium-chain fatty acids (MCFAs) (Fig. 3E). These findings suggested that perinatal pesticide exposure had a long-lasting sexually dimorphic impact on microbiota

activity in the adult gut.

Overall, our results demonstrated that perinatal pesticide exposure did not affect adult glucose homeostasis, but led to time- and sex-specific changes of the metabolic fingerprints in urine and feces.

3.3. Effects of perinatal pesticide exposure on metabolic perturbations induced by a High-Fat diet (HFD)

Gut microbiota dysbiosis, and particularly the metabolism of methylamines by gut microbiota, have previously been linked to disruptions of metabolic homeostasis (Dumas et al. 2006, 2017). Therefore, we next challenged the animals with a pesticide-free high-fat diet (HFD, 60% lipids) for 6 weeks (Figure S1). We investigated the hypothesis that

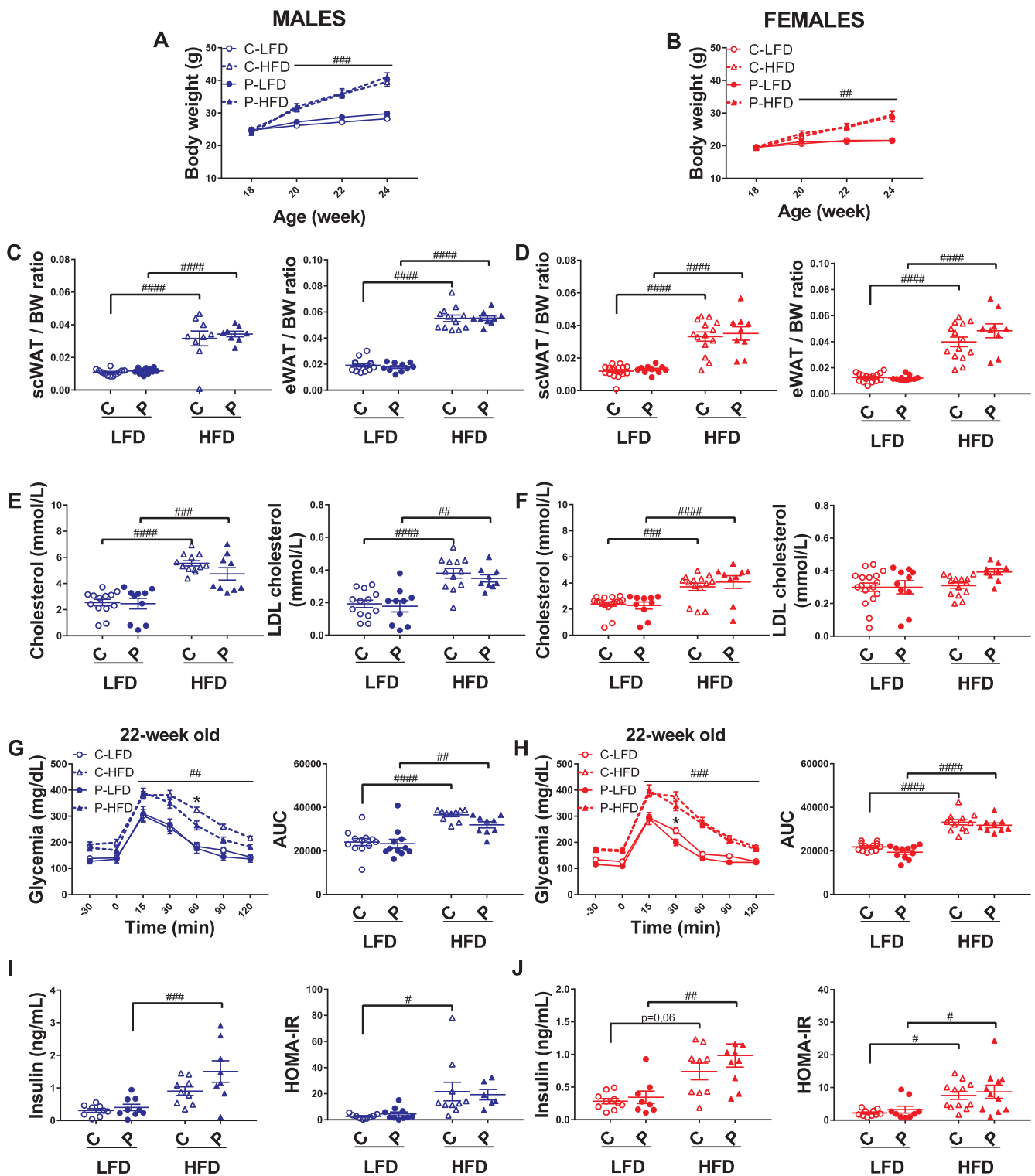


Fig. 4. Effects of perinatal pesticide exposure on HFD-induced metabolic perturbations. (A,B) F1 control (c) and perinatally exposed (p) mice were fed LFD or HFD from 18 to 24 weeks of age, and the body weights were recorded for males (A) and females (B). (C,D) Ratios of subcutaneous (sc) and epididymal (e) white adipose tissue (WAT) to body weight among male (C) and female (D) F1 mice (E,F) Plasma biochemistry in male (E) and female (F) F1 mice. (G,H) Oral glucose tolerance in male (G) and female (H) F1 mice. (I,J) Fasted insulinemia and HOMA-IR in male (I) and female (J) F1 mice ($n = 9-12/\text{group}$). Data are presented as mean \pm SEM for $n = 9-17/\text{group}$, see Figure S1). #, $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ for HFD vs. LFD; *, $p < 0.05$ for P vs. C.

perinatal pesticide exposure would sensitize the animals to diet-induced metabolic perturbations in adulthood.

As expected, in both male and female mice, HFD feeding led to significant increases of body weight (Fig. 4A,B), subcutaneous and epididymal adipose tissue mass (Fig. 4C,D), and plasma total

cholesterol level (Fig. 4E,F). The HFD led to increased plasma LDL-cholesterol in males, but not in females (Fig. 4E,F). Moreover, in both males and females, HFD feeding resulted in significantly decreased glucose tolerance (Fig. 4G,H), and increases of fasted insulinemia and HOMA-IR (Fig. 4I,J). None of these parameters significantly differed

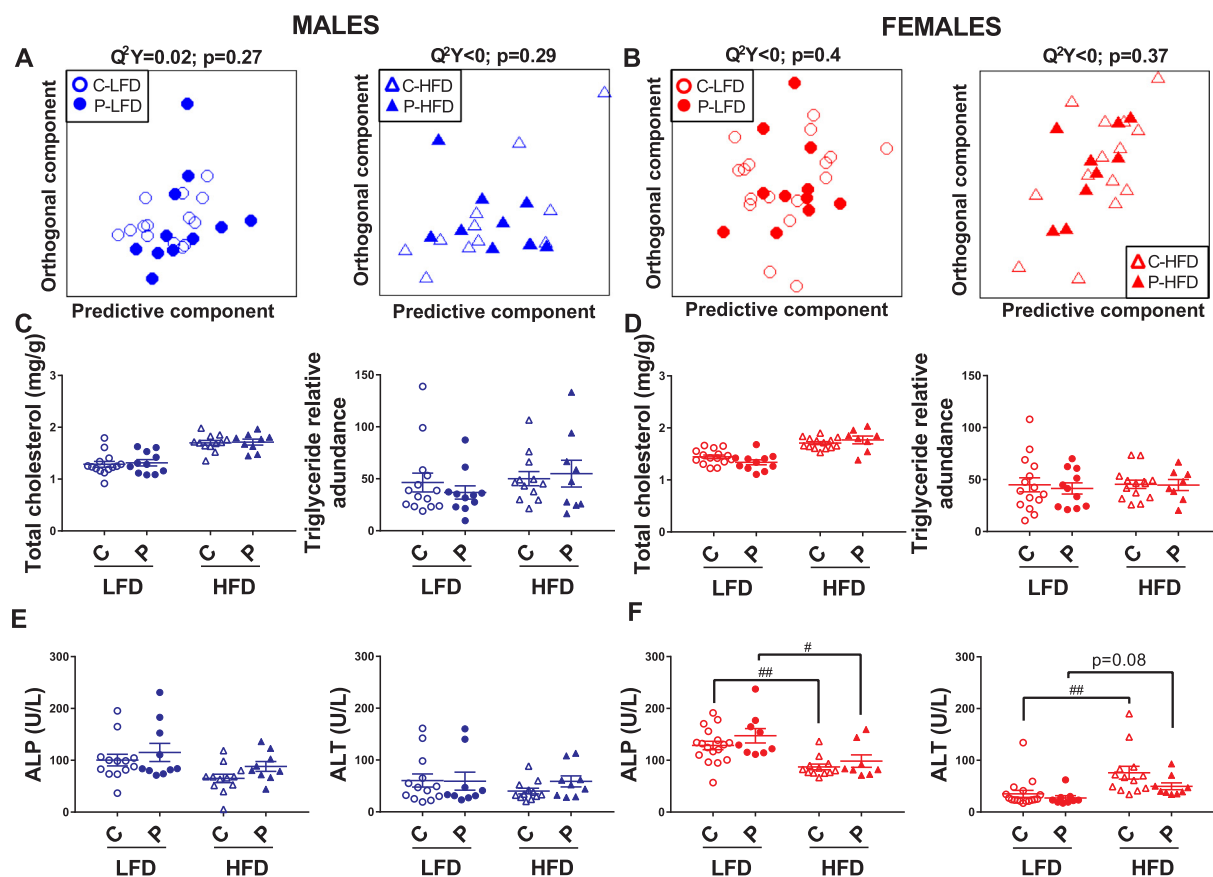


Fig. 5. Effect of perinatal pesticide exposure on hepatic metabolism upon HFD-feeding. (A&B) O-PLS-DA score plots derived from ^1H NMR spectra of aqueous hepatic extracts from 24-week-old males (A) and females (B) ($n = 9\text{--}10/\text{group}$). (C&D) Hepatic lipid quantification in males (C) and females (D) ($n = 9\text{--}17/\text{group}$, see Figure S3). (E&F) Plasma biochemistry in males (E) and females (F) ($n = 9\text{--}17/\text{group}$, see Figure S3). ALT alanine aminotransferase, ALP alkaline phosphatase. Data are presented as mean \pm SEM. #, $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ for HFD vs. LFD; *, $p < 0.05$ for P vs. C.

between the pesticide-exposed mice and their non-exposed counterparts, under either LFD or HFD conditions (Fig. 4A–J). Food intake was also not significantly affected neither by HFD feeding, nor by perinatal pesticide exposure (Figure S5D&E).

Similarly, in the liver, ^1H NMR-based metabolic profiles, total cholesterol, and triglyceride levels were not significantly affected by perinatal pesticide exposure among males or females (Fig. 5). Consistent with this lack of pesticide impact on liver homeostasis, we also found that plasmatic alanine amino-transferases (ALT) and alkaline phosphatase (ALP) did not differ between the perinatally exposed animals and their respective controls (Fig. 5). We also examined hepatic gene expression of enzymes that regulate thyroid hormone levels, and other genes involved in xenobiotic and fatty acid metabolism, and in inflammatory processes (Fig. 6), and found that, although several of these gene expressions were significantly impacted by HFD feeding, none of them differed between the perinatally exposed animals and their respective controls.

Metabolomic analysis of urinary samples from 22-week-old mice did not distinguish between perinatally pesticide-exposed and non-exposed animals, under LFD or HFD feeding (Fig. 7A&B). Similarly, fecal metabolic profiles did not significantly differ between 22-week-old pesticide-exposed and control males under LFD or HFD (Fig. 7C and S6). However, among 22-week-old females, the fecal metabolic fingerprint significantly differed between pesticide-exposed and non-exposed mice, both under LFD and HFD (Fig. 7D). The metabolic profiles were mainly differentiated based on the glucose levels in LFD-fed females, and on the levels of an unknown metabolite in HFD-fed females (Fig. 7E–G). These fecal metabolic changes were not associated with significant differences in the mRNA expression of tight junction proteins in the

colon (Figure S7).

In summary, our results demonstrated that perinatal pesticide exposure did not exacerbate metabolic changes induced by a HFD.

4. Discussion

Xenobiotic exposure occurs across the lifespan, but specific developmental windows are recognized as periods of vulnerability. Notably, gestation is a time of cellular replication and differentiation, and these processes are sensitive to perturbations of the intrauterine milieu. It is now thought that developmental exposure to xenobiotics, such as endocrine disruptors or pesticides, may increase the incidence of metabolic diseases (Braun 2017; Sargis and Simmons 2019; Tang-Péronard et al. 2014; Thayer et al. 2012; Valvi et al. 2012). Several animal studies show that perinatal pesticide exposure induces long-term disruption of metabolic homeostasis (La Merrill et al. 2014; Ruiz et al. 2019), oxidative stress (Ghorzi et al. 2017), or insulin sensitivity (Ribeiro et al. 2016). Notably, most of those studies have assessed the effects of perinatal exposure to rather high doses of individual pesticides, under conditions far different from those experienced by the general population. We previously demonstrated that adult mice exposed to a cocktail of six pesticides at nontoxic doses for one year exhibited sexually dimorphic metabolic changes (Lukowicz et al. 2018). In the present study, we assessed the metabolic impact of perinatal exposure to the same pesticide cocktail in male and female mice upon reaching adulthood.

In the general human population, food is considered the primary source of pesticide exposure. Therefore, we mimicked perinatal pesticide exposure by indirectly exposing fetal/neonatal mice to pesticides

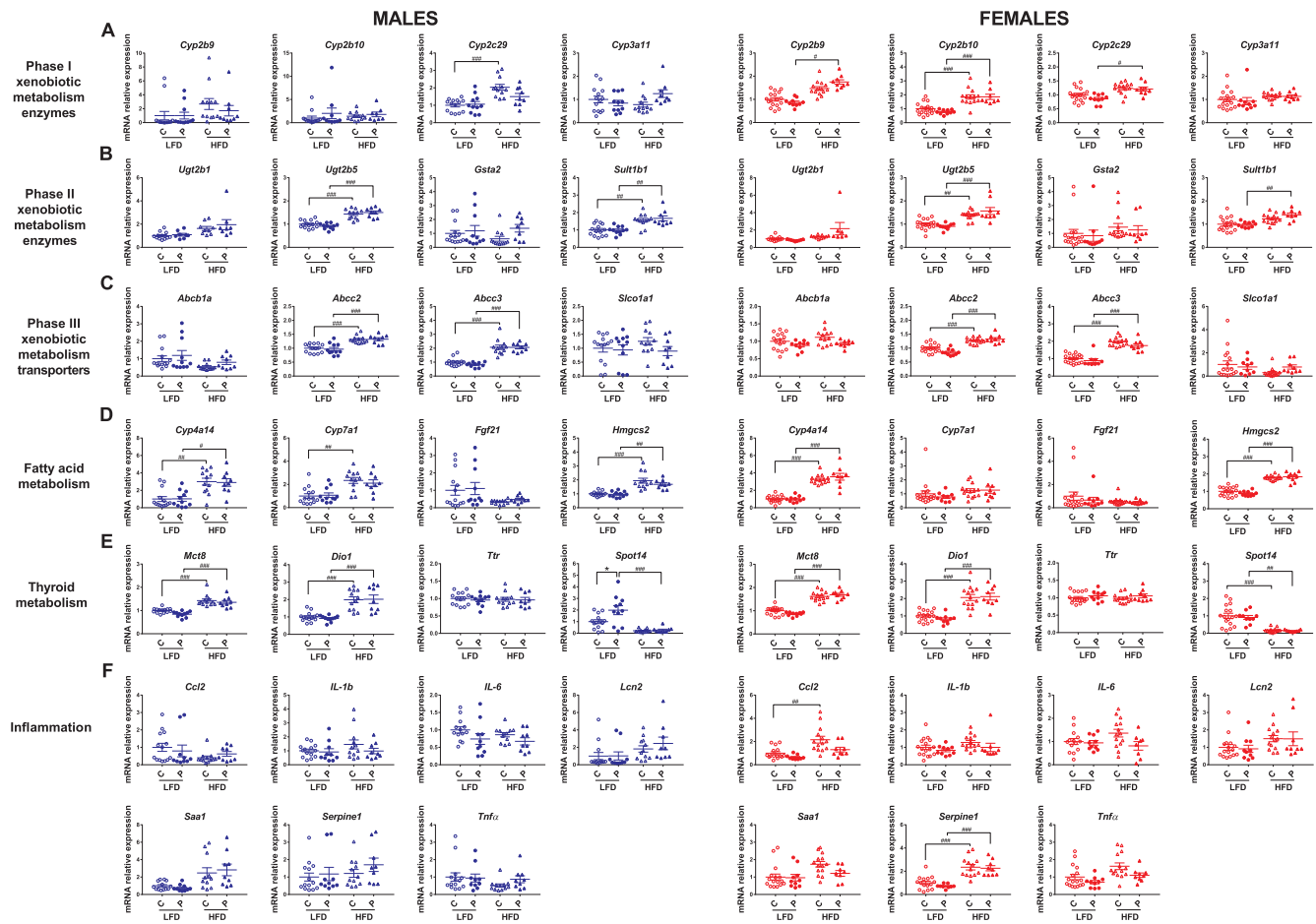


Fig. 6. Effect of perinatal pesticide exposure on hepatic mRNA expression. The mRNA expression levels of genes involved in phase I xenobiotic metabolism enzymes (A), phase II xenobiotic metabolism enzymes (B), phase III xenobiotic transporters (C) genes involved in fatty acid metabolism (D), in thyroid hormone metabolism (E) and in inflammatory processes (F) was assayed by RT-qPCR in liver samples of 24-week-old males (left side) and females (right side). (n = 9–17/group, see Figure S1) Data are presented as mean \pm SEM. #, $p_{\text{adjusted}} < 0.05$; ## $p_{\text{adjusted}} < 0.01$; ### $p_{\text{adjusted}} < 0.001$ for HFD vs. LFD; *, $p_{\text{adjusted}} < 0.05$ for P vs. C. using 1-way ANOVA followed by Tukey's post-tests and Bonferroni-Holm correction for multiple testing.

through dietary exposure of their gestating and lactating dams. To fully discriminate between perinatal and chronic effects, the pesticide exposure was stopped after weaning. Although male and female mice were both exposed to pesticides in food during mating and thus before conception, the exposure of the F0 males during the preconception period was considered negligible compared to that of F0 females during mating, gestation and lactation. Assessment of metabolic homeostasis during adulthood (at 6 and 14 weeks of age) did not reveal significant alterations in body weight or glucose tolerance based on perinatal pesticide exposure.

To further test for potential metabolic perturbations arising from perinatal pesticide exposure, we challenged the F1 mice with a HFD, and assessed body weight, fat accumulation, glucose tolerance, HOMA-IR, and liver metabolism. Again, our results showed no significant effects of perinatal pesticide exposure on any of the measured metabolic parameters in adult mice fed either a LFD or HFD. Thus, we conclude that perinatal pesticide exposure was not sufficient to induce significant changes in metabolism during adulthood, or to sensitize the F1 mice to HFD-induced metabolic perturbations.

Our results are in agreement with recent findings that perinatal exposure to a pesticide mixture (cyromazine, MCPB, pirimicarb, quinoctamine, thiram, and ziram) per gavage, at below the non-adverse effect levels had no effects on the assessed phenotypic changes, including insulin and glucagon regulation, and liver and pancreas histopathological scores (Svingen et al. 2018). However, many other studies have reported that perinatal pesticide exposure triggers metabolic

disruptions resembling prediabetes (Ghorzi et al. 2017; Ribeiro et al. 2016; Slotkin 2011). This discrepancy might arise from differences in the utilized pesticides, doses, or rodent models (C57Bl6/J mice vs. Wistar rats). It is also interesting to compare our present results with those from our previous study, in which adult mice were exposed to the same pesticide cocktail for one year, using the same dietary exposure protocol (Lukowicz et al. 2018). Compared to non-exposed male mice, the pesticide-exposed male mice exhibited greater body weight gain, increased hepatic triglyceride deposition, and were glucose intolerant. On the other hand, pesticide-exposed female mice displayed evidence of modified gut microbiota activity. The phenotypic differences between our two studies suggest that chronic pesticide exposure may be necessary for the development of major metabolic perturbations, although this warrants further investigation. Moreover, it would be interesting to explore further generations to evaluate whether perinatal exposure to our pesticide mixture induces epigenetic changes that has health impact in F3 generation, as reported for other pesticides and reviewed recently in (Van Cauwenbergh et al. 2020).

Although our present results indicated that perinatal pesticide exposure did not induce phenotypical metabolic disruptions, it was associated with significant changes in the metabolic fingerprints, as detected in urine at 6 and 14 weeks of age. Specifically, urinary urea levels were affected in a sex- and time-dependent manner. This modification was unlikely due to altered renal function, since we observed no changes in the urinary albumin/creatinine ratio or histological aspects of the kidneys (not shown). We also observed modified urinary

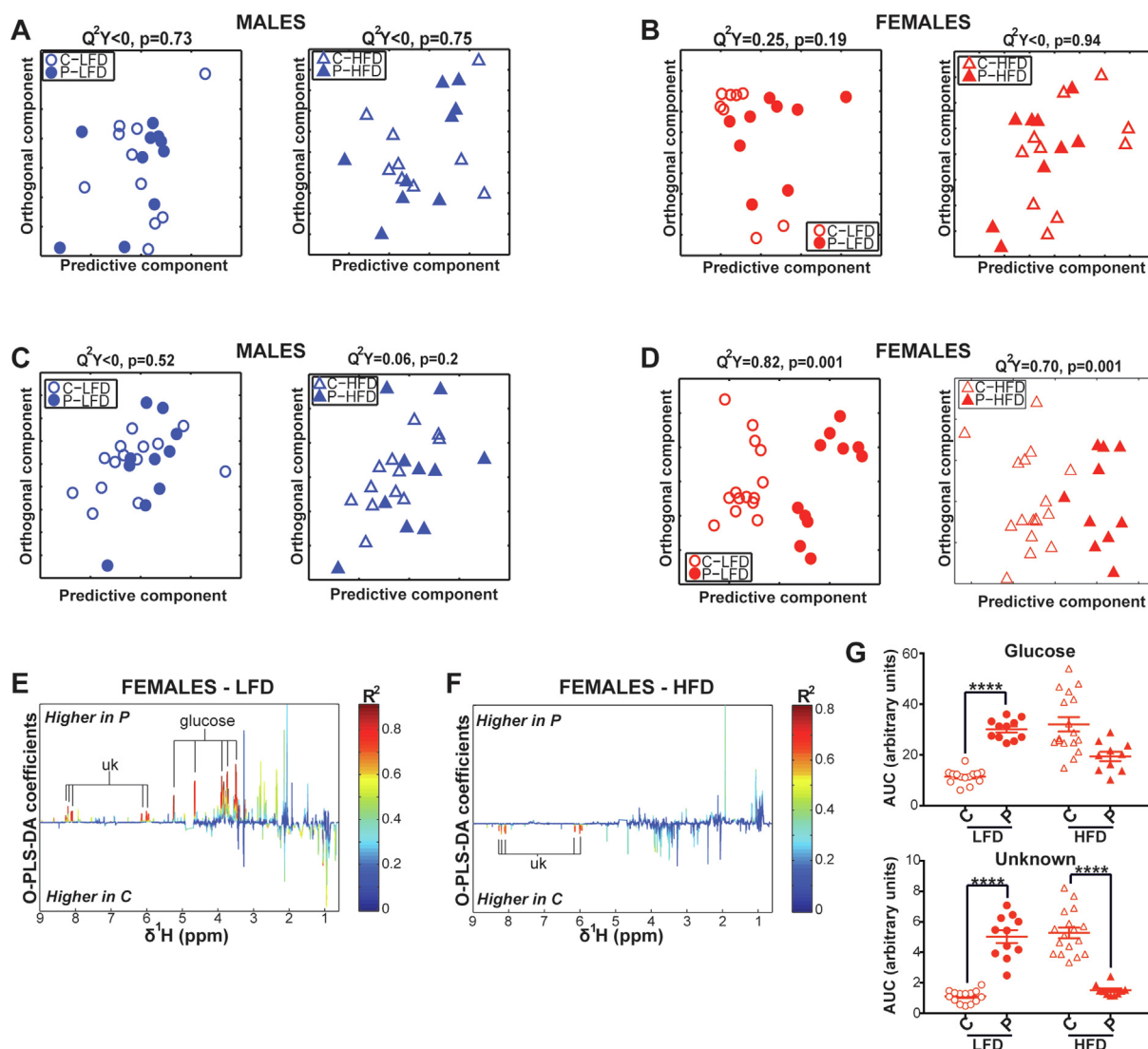


Fig. 7. Impact of perinatal pesticide exposure on ¹H NMR-based fecal metabolic profiles in control (c) and perinatally exposed (p) F1 mice fed LFD or HFD. (A,B) O-PLS-DA score plots derived from 600-MHz ¹H NMR urinary spectra from C-LFD vs. P-LFD (circles) or from C-HFD vs. P-HFD (triangles) male (A) and female (B) 22-week-old F1 mice ($n = 8-10$ /group). (C,D) O-PLS-DA score plots derived from ¹H NMR-based metabolic profiles of feces from male (C) and female (D) 22-week-old F1 mice (9–17/group, see Figure S1). (E,F) Coefficient plots derived from the O-PLS-DA models in 22-week-old female F1 mice under LFD (E) or HFD (F). Metabolites are color-coded according to their correlation coefficient, with red indicating a very strong positive correlation ($R^2 > 0.6$) i.e. glucose (doublet at 5.22 ppm) and for an unknown metabolite (doublet at 5.95 ppm). Data are presented as mean \pm SEM. ****, $p < 0.001$ for P- vs. C using 1 way-ANOVA followed by Sidak's post-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

levels of TMA, which is produced through gut microbial metabolism of dietary choline and L-carnitine, then absorbed in the small intestine (Stremmel et al. 2017), and finally reaches the liver where it is further oxidized by hepatic flavin monooxygenases to form trimethylamine N-oxide (Tang et al. 2013). Therefore, variations in urinary TMA levels are linked to gut microbiota activity (Al-Waiz et al. 1992; Claus et al. 2011; Koeth et al. 2013; Seim et al. 1985) Abnormal levels of TMA have been associated with a number of diseases including renal disorders, cancer, obesity, diabetes, cardiovascular diseases and neuropsychiatric disorders (Chhibber-Goel et al. 2016) and may be linked to environmental changes or microbial dysbiosis. For example, HFD-feeding, dietary choline supplementation and oral pesticide exposure have all been reported to modify urine TMA levels (Barouei et al. 2017; Lukowicz et al. 2018; Stec et al. 2019; Wang et al. 2013; Zeisel et al. 1983, 1989). Thus, we suggest that the observed changes in TMA levels may be related to changes in metabolic homeostasis and gut microbiota activity.

To further investigate this possibility, we performed ¹H NMR-based metabolomic analysis of fecal samples to assess potential changes in gut microbiota metabolism. In 14-week-old mice, the fecal metabolic profiles showed sexually dimorphic alterations upon perinatal pesticide exposure. Compared to their unexposed counterparts, pesticide-exposed males displayed higher fecal levels of acetate and medium-chain fatty acids, and lower levels of succinate. In contrast, no significant change was observed in females. Fecal succinate can be derived from bacterial fermentation of dietary fibers, and dietary succinate reportedly improves glucose and insulin tolerance in mice through induction of intestinal neoglucogenesis (De Vadder et al. 2016). Acetate is the most abundant short-chain fatty acid produced during dietary fiber fermentation, and is also produced during microbial degradation of organophosphate pesticides (Velmurugan et al. 2017). Finally, changes in MCFAs have been associated with inflammatory bowel diseases (Garner et al. 2007) and can exhibit antibacterial properties (De Preter 2015; Hsiao and Siebert 1999; Zentek et al. 2012). In our study, the changes in

these fecal metabolites cannot be attributed to direct pesticide metabolism or to between-group differences in food consumption. Thus, we can hypothesize that they derive from pesticide-induced changes in gut microbiota metabolism.

Indeed, several pesticides present in the mixture used in this study could be suspected or have been shown to affect the gut microbiota *in vivo*. Boscalid inhibits mitochondrial complex II in both eukaryotic and prokaryotic organisms (Bénil et al. 2019; Paris et al. 2020; Yang et al. 2011). Rat pups exposed to chlorpyrifos from gestation to weaning, and during 60 days post weaning presented with gut microbial dysbiosis at weaning and adulthood (Yuan et al. 2019). Methyl thiophanate is a broad-spectrum carbamate benzimidazole fungicide which is metabolized into benzimidazole compounds including carbendazim. This last compound was shown to induce gut microbiota dysbiosis in mice upon a chronic exposure (14 weeks) (Jin et al. 2018).

The gut microbiota-host symbiotic relationship is increasingly recognized as a key factor in maintaining the host's health and pollutant-induced dysbiosis (ie changes in gut microbiota homeostasis) has been found to induce for e.g. intestinal inflammation and metabolic perturbations in mice (Chassaing et al. 2015; Liang et al. 2019). In our present work, further studies are needed to determine if the observed changes in the fecal metabolites are indeed related to changes in gut microbiota communities, as well as the physiological relevance of these changes. Indeed, although the fecal metabolome is increasingly considered as a relevant functional readout for the fecal microbiome and displays high correlation with the gut microbiota composition (Zierer et al. 2018), characterizing the relative distribution of bacterial species in the fecal samples from animals exposed or not to the pesticide mixture during the perinatal period would provide interesting and complementary information. Finally, given the wide differences between rodent and human gut microbe communities, it might also be interesting to test the relevance of our findings in humans by investigating for example the effect of individual and mixed pesticide on a complex *ex-vivo* human gut microbiota as described in (Auchtung et al. 2015).

In our study, several results display significant sexually-dimorphic effects that can not be explained by a large variability within one sex or by a lack of statistical power; including those obtained on the fecal metabolomics signature (Fig. 3I, 7G, Figure S6). In particular, males displayed higher baseline levels of fecal glucose and of the unknown metabolite compared to females, and these were completely suppressed upon HFD feeding. These baseline differences could have prevented both the pesticide-driven increase observed in LFD-fed females and the pesticide-driven decrease observed in the HFD-fed females. Several mechanisms are thought to contribute to sex-specific impact of xenobiotics and can be proposed to explain our present results, among which levels of sexual hormones, in particular estrogens that are known to protect females from metabolic diseases (Gao et al. 2012). Interestingly, estrogen sulfotransferase, that prevents binding of estrogens to the estrogen receptor has been previously described as the target of several pollutants (Naville et al. 2013). Moreover, absorption, distribution, metabolism, and excretion of xenobiotics greatly differ between males and females (Waxman and Holloway 2009), and our own previous results have demonstrated a significantly different metabolism of the 6 pesticides between male and female adult mice (Lukowicz et al. 2018). Finally, gut microbes modulate the enterohepatic recirculation of estrogens and androgens, and generates glucocorticoids, thereby affecting local and systemic levels of steroid hormones (reviewed in (Cross et al. 2018)), while estrogens can in return affect the gut microbiota composition (Kaliannan et al. 2018). The interplay between sex hormones and gut microbes is therefore complex and whether these sex-driven microbiota differences influence the response to xenobiotics in general, and in particular in our study, is still unknown.

Our study has several limitations. First, we only had indirect proof of animal exposure. Due to the short-term exposure protocol used, we were not able to quantify pesticide residues in the urine and milk of the pesticide-fed mothers using the protocols from our previous study

(Lukowicz et al. 2018). However, based on the robust measurements of pellet contamination and food intake, we inferred that the F0 animals were exposed as expected. Second, we did not verify whether the pesticides included in the cocktail crossed the placental barrier and/or were transferred into the milk, as this was not technically feasible given the low doses used. Third, at 18 weeks of age, when our animals were challenged with a HFD, we also changed the diet for our control groups from a regular chow diet to a synthetic low-fat diet, which represents the proper control diet for the HFD. Compared to chow diets, synthetic diets induce significant changes of the gut microbiota (Guerville et al. 2019). This might explain the changes of exposure biomarkers observed in the control groups between 14 and 22 weeks.

5. Conclusion

To our knowledge, our present study is the first to assess the effects of perinatal exposure to a realistic pesticide mixture at non-toxic doses administered through food intake, a protocol that was designed to mimic some important aspects of consumer exposure regarding oral intake of multi-contaminated food and that allowed us to gain insights into potential differences arising between perinatal and chronic effects of pesticide exposure. Our results showed that perinatal pesticide exposure was sufficient to induce specific changes in urinary and fecal metabolite fingerprints, suggesting sustained alterations of gut microbiota activity or composition. However the health-related effects of these changes in metabolite levels are undetermined. Moreover, these changes in the metabolic fingerprints were not paralleled by significant effects on metabolic homeostasis and perinatal exposure did not sensitize the mice to HFD-induced metabolic perturbations later in life.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2020.106010>.

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