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Adult female rats perinatally exposed to perfluorohexane sulfonate (PFHxS) and a mixture of endocrine disruptors display increased body/fat weights without a transcriptional footprint in fat cells

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Running title: Developmental exposure to perfluorohexane sulfonate affects body composition in adult female rats

Key words: obesity, PFAS, PFHxS, metabolic disorders, endocrine disruptors

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Highlights

- Early life exposure to PFHxS and endocrine disruptors increased female body weights
- The retroperitoneal fat pad weight was increased in adult offspring
- Increased fat weight was not reflected in transcriptional footprint of fat cells
- Endocrine disruptors at current exposure levels may contribute towards obesity

ABSTRACT

Obesity is a complex disease with many causes, including a possible role for environmental chemicals. Perfluorohexane sulfonate (PFHxS) is one of many per- and polyfluoroalkyl substances (PFASs) frequently detected in humans and it is suspected to be an obesogenic compound. We examined the potential long-term effects of PFHxS on metabolic parameters in rats after developmental exposure to 0.05, 5 or 25 mg/kg bw/day, with or without co-exposure to a background mixture of twelve endocrine disrupting chemicals (EDmix). Both male and female offspring showed signs of lower birth weight following intrauterine exposure. Female offspring exposed to both PFHxS and EDmix had increased body weight in adulthood. The retroperitoneal fat pad was larger in the PFHxS-exposed female offspring when compared to those exposed to EDmix alone. An attempt to detect putative molecular markers in the fat tissue by performing whole transcriptome profiling revealed no significant changes between groups and there were no significant effects on plasma leptin levels in exposed females. Our results show that early life exposure to endocrine disrupting chemicals can influence body weight later in life, but the effect is not necessarily reflected in changed gene expression in the fat tissue.

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1. INTRODUCTION

The number of overweight and obese individuals has tripled since the mid-1970s and account for at least 2.8 mill deaths per year worldwide (WHO, 2020). The World Health organisation (WHO) has classified it an epidemic and flagged childhood obesity as particularly disconcerting, since it is linked to a higher risk of developing diabetes and cardiovascular disease at a younger age than in non-overweight children (WHO, 2017). Although obesity is the result of prolonged disturbance in homeostasis of energy storage and expenditure in postnatal life (Grün and Blumberg, 2009), fetal programming can also play a role (Braun et al., 2016; Darbre, 2017; Janesick and Blumberg, 2016; Magueresse-Battistoni et al., 2017). Apart from the mother's dietary habits and general health, intrauterine exposure to industrial chemicals can affect early development (Diamanti-Kandarakis et al., 2009) and a causal link between chemical exposure and obesity was suggested two decades ago (Baillie-Hamilton, 2002).

Industrial chemicals can affect metabolic development and homeostasis by, for instance, increasing the number of adipocytes or accumulation of fat in individual cells, or by interfering with hormonal control of appetite and satiety (Heindel et al., 2017). Among the growing number of suspected 'obesogenic' chemicals we find the per- and polyfluoroalkyl substances (PFASs). PFASs are widely used in manufacturing industries because of their ability to repel both oil and water; properties that are tractable for many consumer products such as food contact material, frying pans and clothes (Lindstrom et al., 2011). Unfortunately, these same properties also make them persistent pollutants and bio-accumulative, and they are now ubiquitous in the environment (Lau et al., 2007).
PFASs have been associated with metabolic diseases in humans (Alderete et al., 2019; Christensen et al., 2019; Hartman et al., 2017; Huang et al., 2019; Lauritzen et al., 2018; Maisonet et al., 2015; Matilla-Santander et al., 2017; Mora et al., 2017; Nelson et al., 2010; Timmermann et al., 2014),

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associations that are supported by both *in vivo* (Hines et al., 2009; Ziquan et al., 2011) and *in vitro* (Ma et al., 2018; Watkins et al., 2015) studies. One PFAS of concern, perfluorohexane sulfonate (PFHxS), is widely distributed in the environment and contamination with this chemical has been found in drinking water (Zhu and Bartell, 2020) as well as in samples from polar bears (Glynn et al., 2012; Greaves et al., 2012). In humans, PFHxS has a half-life of 7 years (Axmon et al., 2014; Olsen et al., 2007). It is the third most detected PFAS in humans and has been found in cord blood of newborns (Berg et al., 2014; Glynn et al., 2012; Gützkow et al., 2012; Kato et al., 2011) and in serum (Chang et al., 2020). Yet, very few studies have investigated potential developmental toxicity (Butenhoff et al., 2009; Chang et al., 2018; Ramhøj et al., 2018, 2020) and apart from our study on thyroid hormones and developmental neurotoxicity (Ramhøj et al., 2020) no studies have investigated late-life effects of early exposures.

We recently reported that intrauterine exposure to PFHxS, when combined with a low dose human-relevant background exposure of endocrine disrupting chemicals, resulted in decreased birth weight in rats (Ramhøj et al., 2018). By monitoring these offspring into adulthood, we can now present data showing that the female offspring became heavier than control animals after reaching adulthood. This observation fits with the hypothesis that a low birth weight can increase the risk of becoming obese or developing metabolic disorders later in life (Cottrell and Ozanne, 2007). Hence, we wanted to examine in more detail the metabolic status of these offspring in adulthood, including looking for changes to the fat tissue long after exposure had ceased to see if we could detect a 'transcriptional footprint' of early disruption to adipocyte programming.

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2. MATERIALS AND METHODS

2.1 Chemicals

Perfluorohexane sulfonate (PFHxS) (purity>98%) was purchased from Sigma-Aldrich (Tridecafluorohexane-1-sulfonic acid potassium salt, CAS-No: 3871-99-6, lot #BCBC3545V, Sigma- Aldrich, Copenhagen, Denmark). Corn oil (Sigma-Aldrich) was used as vehicle for all treatments and dosing in the control group. The lowest dose of PFHxS was chosen to result in a rat internal exposure closer to human relevant levels i.e. human PFAS serum levels (Lau, 2012) multiplied by a factor of 2.5 for toxicodynamic differences between rats and humans and 10 for inter-individual differences between humans. PFHxS exposures was given with and without a fixed dose of a background mixture of endocrine disrupting chemicals, the EDmix (Table 1) to mimic a more realistic exposure scenario. The rationale for selecting the compounds, including doses, was based on actual serum levels in humans and is described in (Christiansen et al., 2012) whereas the rational for the present study design and EDmix without acetaminophen (paracetamol) is described in detail in (Ramhøj et al., 2018). Briefly, the mixture consisted of 12 endocrine disruptors given at a dose of 32.11 mg/kg body weight (bw)/day (Table 1), reflecting 100x high-end human intake. The factor of 100 was chosen based on safety factors of 10 for extrapolation from rat to human and a safety factor of 10 for inter-individual differences, thus effects in rats at this dose level indicate a concern for health effects in highly exposed humans.

2.2 Animals and Dosing

A developmental study with 144 time-mated adult Wistar rats was carried out as previously described (Ramhøj et al., 2018). In brief, the study consisted of 8 groups with 16-20 animals in each, exposed to vehicle (controls), or 0.05, 5, and 25 mg/kg bw/day of PFHxS both with and without a background EDmix exposure (total EDmix dose was 32.11 mg/kg bw/day), as well as a

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group who only received the EDmix. The study was performed in 4 blocks with all dose groups equally represented in all blocks. The study design is illustrated in Figure 1.

The day of vaginal plug detection was designated as gestational day 1 (GD 1), whereas the day of expected delivery was designated pup day 1 (PD 1); corresponding to 'GD 23'. The dams were received on GD 3 and dosing by oral gavage was performed daily from GD 7 to GD 22, and again after birth from PD 1 to PD 22. The animals were housed in a room with controlled environmental conditions with reversed light/dark cycles of 12 hours with light intensity 500 lux starting at 9 PM, humidity 55%, temperature at 21 +/- 1°C and ventilation changing air 10 times per hour. All animals had *ad libitum* access to standard diet with ALTROMIN 1314 (soy- and alfalfa-free, ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast). This cohort of weaned offspring was assessed for behavioural changes as described in (Ramhøj et al 2020) prior to the presently reported assessments. The animal experiment was carried out at the National Food Institute (Mørkhøj, Denmark). Ethical approval was obtained from the Danish Animal Experiments Inspectorate: authorization number, 2015-15-0201-00553 C3. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

2.3 Offspring body weight after weaning

Offspring were weighed at weaning, at the time of activity testing (PD 27, 115 and ~340), and approximately every 4 weeks from weaning until the study was terminated: males at 11.5 months and females at 13 months of age.

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2.4 Feed consumption

Since we had observed effects on female body weights during activity testing on PD 115, we recorded their weights and feed consumption for 7 days at ages 38-40 weeks. On the start day offspring and feed were weighed and then again 7 days later (specific start day in the blocks were:PD 286 for block 1, PD 279 for block 2, PD 266 for block 3 and PD 259 for block 4). Registered feed consumption represents the combined consumption of the 2 animals housed together and a mean consumption per rat was calculated. Energy efficiency was determined as weight-gain divided by feed intake. Feed consumption was expressed as feed intake per day per 100 g rat. Specific growth rate (SGR) was defined as weight gain between the two points in time divided by start body weight and calculated for the 7 days where feed consumption was registered.

2.5 Necropsy

Necropsy of one male and one female pup for each litter was carried out on PD 16 and PD 17, respectively, and again on PD 22 when dams were humanely killed (see below). On PD 16/17, pups were weighed, decapitated under CO2/O2 anaesthesia, and trunk blood collected. On PD 16/17, one retroperitoneal fat pad was excised and weighed from each pup and flash frozen and stored at -80 °C until preparation for transcriptional analysis as previous reported (Ramhøj et al., 2018). The weaned cohort of male offspring was necropsied at approximately 11.5 months of age (block 1 and 2 together and block 3 and 4 together, both rounds over the course of 8 days at PD 342-356). Female offspring was necropsied during oestrus at approximately 13 months of age (block 1 and 2 over the course of 11 days at PD 384-401 and block 3 and 4 over the course of 5 days at PD 391-402). Rats were weighed, then decapitated under CO2/O2 anaesthesia and blood collected directly in heparinized tubes. The left retroperitoneal fat pad from each animal was excised and weighed.

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Retroperitoneal fat pad from block 1 and 2 was placed in RNAlater and stored at -80 °C until preparation for transcriptional analysis.

2.6 RNA extraction and gene array analysis

Total RNA was extracted from the left retroperitoneal fat pad using the RNeasy Lipid Tissue Mini kit (Qiagen) including on-column DNase I treatment according to manufacturer's instructions. RNA from the left retroperitoneal fat pad samples from 4 adult female offspring were semi-randomly selected from each group: control, 25-Px, EDmix and 25-Px+ED. RNA quantity and quality was determined using the Agilent 2100 Bioanalyzer system and Eukaryote Total RNA Nano assay (Agilent Technologies, Santa Clara, California) as per manufacturer's instructions. All samples had an RNA integrity number (RIN)-score >8.

2.6.1 Microarray hybridization

Total RNA was amplified and labelled using the Gene Chip™ WT PLUS Reagent Kit according to the manufacturer's instructions (ThermoFischer Scientific). For each sample, RNA pools were hybridized to Rat Clariom™ S GeneChip (ThermoFischer Scientific). After hybridization, false-color pseudo-images of the GeneChips hybridized were reconstructed and revealed no hybridization artefacts. Based on box plots, we also checked that the signal intensity distributions before and after normalization were in the normal range across the dataset. Comparing samples based on their overall expression signals using a distance matrix together with a dendrogram revealed no clear separation of control and exposed samples.

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2.6.2 Data normalization

The gene array data were normalized with the Robust Multi-Array Average (RMA) method (Irizarry et al., 2003) implemented in the statistical software R (version 3.6.0) using the Brainarray custom Chip Description Files (CDF) so that intensity values are not summarized for each probe set but directly for each Entrez Gene ID (Dai et al., 2005). Raw data and transcriptomic signatures were uploaded to the NCBI Gene Expression Omnibus (GEO) (Barrett et al., 2013) repository under the accession numbers GSE146489.

2.6.3 Statistical filtration of differentially expressed genes

The statistical filtration of the genes differentially expressed between control and treated samples was performed using the Annotation, Mapping, Expression and Network suite of tools (Chalmel and Primig, 2008). Briefly, genes showing a signal higher than a given background cut-off (median of the normalized data set, cut-off = 5.95) and at least a 1.5-fold change in at least one pairwise comparison were selected. To define the set of genes displaying significant statistical changes across comparisons, the empirical Bayes moderated t-statistics was performed using the LIMMA package (F-value adjusted using the Benjamini & Hochberg (BH) False Discovery Rate approach, $p \leq 0.05$) (Smyth, 2004).

2.7 RT-qPCR analysis

The protocols were essentially as described previously (Svingen et al., 2015). 300 ng total RNA was used to synthesise cDNA from adult offspring (control, 25-PFHxS, EDmix, 25-PHFxS+EDmix; n=10) and 500 ng from PD16/17 pups (all exposure groups; n=10). RNA quantity and purity was obtained by Nanodrop spectrophotometry. The TaqMan Gene Expression Assays (Life Technologies) were: *Agrp* (Rn01431703), *Il1b* (Rn00580432), *Lep* (Rn00565158), *Lepr*

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(Rn01433205), *Nmb* (Rn01478123), *Nmbr* (Rn00680847), *Npy* (Rn01410145), *Npy1r* (Rn02769337), *Pparg* (Rn00440945), *Ppargc1a* (Rn00580241), *Tnf* (Rn99999017), *Adipoq* (Rn00595250), *Cebp alfa* (Rn00560963), *Fabp4* (Rn04219585), *Lipin1* (Rn01469026), *Arntl* (Rn00577590) and *IL-6* (Rn01410330). RT-qPCR experiments were run on a Quant Studio 7 Flex Fast Real-Time PCR System (ABI) in a 384-well format using 3 µl diluted (1:20) cDNA per reaction. Relative transcript abundance was calculated by the comparative Ct-method with the geometric mean of the reference genes *Hprt* (Rn01527840) and *Rpl13a* (Rn00821946).

2.8 Measurements of plasma leptin levels

Plasma leptin levels from adult offspring (control, 25-Px, EDmix and 25-Px+ED; n=7-10) were determined in duplicate with ELISA kits from Millipore (Rat Leptin article no.EZRL-83K) according to the manufacturer's protocol. Absorbance was measured using an Enspire® Multilable Plate Reader (PerkinElmer). The amount of leptin was derived from assay reference curve without further normalization.

2.9 Statistical analysis

Data with normal distribution and homogeneity of variance were analysed using ANOVA. Where an overall significant treatment effect was observed, Dunnett's test was used for two-tailed comparison with the alpha level for statistical significance set to 5% and the litter as the statistical unit. When relevant, body weight was included as a covariate in the analyses, e.g. when testing terminal organ weights. Body weights over time were analysed by repeated measures in a mixed model ANOVA.

Exposure groups were tested against the vehicle control (termed "simple models") by testing the

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PFHxS exposed groups against the control, all PFHxS+EDmix groups against the EDmix group and by using Student's t-test to compare the EDmix group against the control group. In addition, the study design with dose response exposure to PFHxS and groups exposed to both PFHxS and the EDmix allowed for construction of a statistical model integrating all exposures and control groups into one full linear model the "full model" as described in (Ramhøj et al., 2018). The full model includes PFHxS and EDmix exposures and their interactions parameterized as indicator variables. With this approach we could examine whether, (and to what degree), exposure to the EDmix changed the effects of PFHxS exposure alone, across the different exposure groups both when multiple doses were present (2x4 design) or at a single dose level (2x2 design), e.g. for leptin levels and RT-qPCR on the fat pad of 13 months old females. The full model considers a larger sample size and is thus able to identify smaller effect changes than using a simple pairwise comparison. The model was tested by a main factor for EDmix and dose-dependent interaction terms to account for non-parallellity between the PFHxS and PFHxS + EDmix dose-response patterns. For details regarding the rat gene array refer to that section. SAS Enterprise Guide 4.3 (2010), SAS Institute Inc, Cary, NC 27513, USA, was used for statistical analyses.

3. RESULTS

3.1 Effects on body and retroperitoneal fat pad weight of offspring following pre- and postnatal exposure to PFHxS and EDmix

We have previously reported that male pups born from dams exposed to PFHxS+ EDmix had lower birth weight than male pups born from control dams (Ramhøj et al., 2018). After weaning, body weights were recorded approximately every four weeks. Males exposed to both PFHxS and EDmix were significantly smaller at birth, but the average body weight became similar to control animals later during development and in adulthood (Figure 2B, suppl. Table A2). For the females, the

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picture was different, as they only showed a trend towards lower birth weight in exposed offspring, but in adulthood the EDmix significantly increased the body weight (Fig. 2A and suppl. Table A1; p = 0.0099, full model). There was also a significant effect when comparing the EDmix group directly to the control group, with increased body weight at mid-life: days 83 to 139 (Fig. 2A, suppl. Table A1, simple model).

PFHxS exposure alone did not affect terminal weight of the retroperitoneal fat pad in female offspring from any exposure group. However, in females that had been exposed to an early-life combination of PFHxS and EDmix, the weight of the fat pad was higher than in those exposed to EDmix alone (Fig. 2A). No effect was observed for retroperitoneal fat pad weight in male offspring (Fig. 2B).

3.2 Effect on feed consumption, energy efficiency and blood leptin levels

In late adulthood, feed consumption was recorded for one week (weeks 38-40, PD ~270 for the females). Also, when investigating this period specifically, the EDmix caused a significant increase in body weight (p = 0.0443, full model) across all groups. However, when comparing the body weight in the EDmix group directly to the control group, there was no statistically significant difference (p = 0.0696, simple model, data not shown). Yet, the mean feed intake per animal over the 7 days was increased in the EDmix group when compared to controls, likely a result of the higher body weight of the animals (data not shown).

We observed no effects on SGR (data not shown) or on energy efficiency from either PFHxS or EDmix exposure (Fig. 3A). At termination of the study, blood was collected from the animals and analysed for differences in leptin levels, but no effect of either PFHxS or the EDmix was observed (Fig. 3B).

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3.3 Gene expression profiling of fat tissue

Since PFHxS can affect serum lipids (Butenhoff et al., 2009) we investigated the potential effects of concurrent PFHxS exposure on gene expression in the retroperitoneal fat pads of the pups on PD 16/17. We observed no significant effects within groups or between the sexes (Fig. 4). The same genes, and more, were investigated again in the old female offspring in order to examine if early-life exposure to PFHxS alone or in combination with EDmix could leave a long-lasting transcriptional footprint in the adipocytes, and potentially point towards dysregulated metabolic regulation. Additionally, the fat pad transcriptome could potentially reveal which pathways and processes that were affected and could explain the increased fat pad weight. Older male rats were excluded as they showed no significant change to fat pad weight or body weight.

First, we used a targeted approach analysing target genes known to be affected in fat tissue following exposure to xenobiotics. Gene selection was based on our previous study where we saw transcriptional changes in offspring following developmental exposure to mixtures of pesticides and represent established obesity-related factors (Svingen et al., 2018). In this study, however, only one out of sixteen genes were affected in the adult female rats, where *Pargc1a* was upregulated after early-life exposure to PFHxS (Fig. 5), and one gene, *IL6*, was not expressed. With this lack of persistent effects on expression of the target genes, we next performed a full transcriptional profiling on four animals per group (control, EDmix, Px-25 and Px-25-EDmix). Here too, we detected almost no significant changes in gene expression across the various groups, with only one gene, *Arntl*, showing a slight upregulation in exposed animals; an effect that was not verified in the subsequent RT-qPCR analysis (Fig. 5).

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4. DISCUSSION

Fetal exposure to PFAS can lead to lower birth weight in the offspring, both in rodents (Grasty et al., 2003; Lau et al., 2003; Ramhøj et al., 2018) and in humans (Bach et al., 2015; Callan et al., 2016; Johnson et al., 2014; Starling et al., 2017). With regard to PFHxS, we have previously reported that perinatal exposure, in combination with a background exposure to a mixture of human-relevant endocrine disruptors, also cause pups to be born smaller (Ramhøj et al., 2018). Studies examining any long-term effects on metabolic parameters following developmental PFHxS exposure, however, are lacking. Since a lower birth weight can influence the risk of developing metabolic diseases later in life, we sought to examine our developmentally exposed rats in adulthood for effects on metabolic parameters.

We monitored the body weight of the pups from weaning until about 1 year of age. Although male pups from the exposed group displayed lower birth weight, their body weights were comparable to control males immediately after weaning and throughout adulthood. For the female offspring, however, the exposed animals displayed increased body weights throughout the study, likely augmented by exposure to the EDmix. In addition, the retroperitoneal fat pad weight from exposed females was also increased at 1 year of age when comparing the fat pads from animals exposed to a combination of PFHxS and EDmix with those exposed to EDmix alone. These observations suggest that developmental exposure to PFHxS can predispose offspring for metabolic disease later in life, particularly when considering a realistic scenario of constant background exposure to industrial chemicals.

To our knowledge, this is the first study on gestational and lactational exposure to PFHxS with subsequent analyses on metabolic parameters late in life. Although PFHxS did not cause major

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changes to body weight, there was a significant change to fat storage in exposed females. Others have found pronounced metabolic disruption after exposure to a similar perfluorinated compound, perfluorooctane sulfonate (PFOS), reporting low birth weight along with effects on metabolic parameters such as increased fat pad weight, elevated serum leptin and insulin, and decreased adiponectin serum levels in adult pups (Wan et al., 2014; Ziquan et al., 2011). We also analysed serum leptin levels in the exposed female offspring but did not detect any significant changes compared to control animals. This is in contrast to a mouse study reporting that developmental exposure to PFOA increased body weight and elevated leptin levels in mid-life (Hines et al., 2009). Notably, effects on the mouse serum hormones were reported in fasted animals, whereas we measured leptin levels in non-fasted animals. In hindsight, it would have been sensible to also measure leptin levels in fasted animals. Although speculative, the observed trend towards increased serum leptin levels in the 25-Px+EDmix group (Figure 3B) could have reach statistical significance, as leptin levels fluctuate according to changes in calorie intake (Dalamaga et al., 2013).

It should be noted that we did not directly measure PFHxS levels in maternal milk or in the suckling offspring, so exact exposure levels were not empirically determined; nevertheless, PFHxS has previously been reported in milk. Also, it is quite clear from our data that the pups were exposed via the milk, as there were sizeable reductions in serum T4 levels in the PD 16 pups concomitant with increased liver weights; both event are very unlikely to occur without lactational transfer of PHHxS (Ramhøj et al., 2018, 2020). Furthermore, Butenhoff et al. (2009) measured PFHxS in serum and liver of dams, fetuses and PND 22 offspring and found higher concentrations in PD22 pups than in dams and fetuses, supporting the fact that PFHxS indeed can transfer via mother's milk to offspring.

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Prenatal exposure to PFAS has been associated with overweightness and obesity in children (Chen et al., 2019; Hartman et al., 2017; Lauritzen et al., 2018). Knowing that overweight and obese children also have a higher risk of becoming overweight or obese as adults (Serdula et al., 1993; Singh et al., 2008), and that obesity is a high risk factor for developing a number of diseases and health complications (Afshin et al., 2017), a possible confounding influence from developmental exposure to PFASs is disconcerting. It is also notable that there appears to be sex differences in the response to xenobiotic insult and metabolic disorders. Epidemiological studies have shown different responses to PFAS exposure and metabolic parameters, with females seemingly more susceptible for developing adverse effects (Chen et al., 2019; Halldorsson et al., 2012; Lauritzen et al., 2018; Mora et al., 2017). Indeed, sex differences in metabolic outcomes following exposure to chemicals are reported more often. For example, developmental exposure to certain pesticides increase leptin levels in female, but not male, rat offspring (Svingen et al., 2018). In humans, pesticide exposures have been associated with low birth weight and metabolic biomarkers in girls, but not boys (Andersen et al., 2018; Labayen Goñi et al., 2011). The fact that leptin is regulated differently between males and females in rodents and humans has been known for a long time (Hickey et al., 1996; Landt et al., 1998; Montague et al., 1997), yet to what extent this sexual dimorphism influences metabolic health and disease in response to environmental triggers is poorly investigated.

As we reported previously (Ramhøj et al., 2018), an increase in retroperitoneal fat pad weight of male pups at PD 16 was driven by the exposure to PFHxS. Therefore, we investigated potential effects of PFHxS on gene expression in fat tissue of the young male and female pups PD16/17. Both inflammatory and adipogenic markers were chosen for analysis, several of which have been shown to be upregulated by PFHxS in adipocytes *in vitro*, including: *Adipoq*, a peptide hormone responsible for glucose levels and fatty acid breakdown (Wang and Scherer, 2016); *Lep*, a hormone

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responsible for appetite regulation (Münzberg and Morrison, 2015)*; Lepr,* a leptin receptor*; Plin1*, a lipid droplet protein involved in the mobilisation of lipids (Smith and Ordovás, 2012); *Pparγ,* the master regulator of adipogenesis (Watkins et al., 2015). Also, upregulation of markers for fatty acid metabolism was seen in F_0 males, as well as in F_1 males and females, in CD-1 mice exposed to PFHxS (Chang et al., 2018). In the present study, however, we saw no changes to expression levels of any of the genes across the groups. We also extended our transcriptomics analyses by performing a gene array analysis on fat tissue from adult rats, as we had seen differences in fat pad weights in adult females. This we did to potentially detect a long-lasting transcriptional 'footprint' in developmentally exposed, and thus primed, fat cells. Such findings have the potential to pinpoint biomarkers of effects also relevant for humans. However, we did not find any up- or down-regulated genes in the exposed groups. This was somewhat surprising, as others have shown specific genes to be positively correlated with increased adipose tissue, for instance *Fabp4, Lep* (Friedman and Halaas, 1998; Prentice et al., 2019), or inversely correlated, for instance *Adipoq* (Kawano and Arora, 2009).

We have previously shown several genes to be dysregulated in fat cells following developmental exposure to chemical mixtures (Svingen et al., 2018). One reason for this lack of effect at the transcript level in this current study could be that there is too much inter-variability in control animals making it difficult to detect statistically significant alterations in exposed animals (suppl. Fig. A1). Using our sample size we can thus only conclude that, in this study, we observed no significant changes to the transcriptome in the retroperitoneal fat cells in female rats following developmental exposure to PFHxS or the EDmix, despite the fat pad being larger in these animals. Also, without mechanistic results from the transcript analysis it would be speculative to guess at potential mechanisms responsible for this female specific effect in fat tissue although we note that

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such effects in general are not uncommon with other EDCs as described previously, nor are sex-specific effects surprising.

This, together with our previous PFHxS exposure studies, show that a background exposure to a mix of endocrine disrupting chemicals (EDmix) can augment the effects of PFHxS on various endpoints: anti-androgenic effects, liver toxicity, thyroxine levels, sexual differentiation of the brain, as well as birth weight and adipose tissues (Ramhøj et al., 2020, 2018). This raises concern about the potential effects of endocrine disrupting mixtures and background exposures for risk assessment of single chemicals, but also for PFAS in particular. Even though an increase in body and retroperitoneal fat pad weight was not reflected in other metabolic parameters in the PFHxS-exposed offspring, it is still disconcerting that developmental exposures, when given on a background of a mixture of endocrine disruptors, can cause life-long effects on body weight. In combination with epidemiological studies linking PFAS exposures to increase in childhood overweight and obesity, this calls for further studies specifically designed to detect effects on metabolic parameters of PFAS and should also take potential sex differences into account.

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing interests.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest pertaining to this work.

DECLARATION OF INTEREST STATEMENT

The authors declare that they have no competing interests pertaining to this work.

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TABLES

Table 1. Composition of the mixture consisting of 12 endocrine disrupting chemicals, termed EDmix.

Chemical	CAS Registry Number	Purity (%)	Dose (mg/kg bw/day)
DBP (Dibutyl phthalate)	84-74-2	>99.0	1.00
DEHP (di-2-ethylhexyl phthalate)	117-81-7	>99.5	2.00
Vinclozolin	50471-44-8	>99.5	0.90
Prochloraz	67747-09-5	>98.5	1.40
Procymidone	32809-16-8	>99.5	1.50
Linuron	330-55-2	>99.0	0.06
Epoxyconazole	106325-08-8	>99.0	1.00
4-MBC (4-Methylbenzylidene camphor)	36861-47-9	>98.0	6.00
OMC (Octyl methoxycinnamate)	5466-77-3	>98.0	12.00
p,p'-DDE (Dichlorodiphenyldichloroethylene)	72-55-9	>98.5	0.10
Bisphenol A	80-05-7	>99.5	0.15
Butyl paraben	94-26-8	>99.0	6.00
Total			32.11

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FIGURE LEGENDS

Figure 1. Study design of the developmental toxicity study. The endpoints for dams and offspring are depicted along with times for exposure and tissue collections. Exposure of dams to vehicle, perfluorohexane sulfonate (PFHxS), and/or a mixture of EDCs (EDmix) from GD 7 through to PD 22, except day of delivery. Study design and early-life outcomes was reported in (Ramhøj et al., 2018). Px, perfluorohexane sulfonate (PFHxS); EDmix, mixture of EDCs; Table 1.

Figure 2. Offspring body- and retroperitoneal fat pad weights after developmental exposure to PFHxS alone or in combination with EDmix. (A) Female body weight was recorded approximately every four weeks (n=18). From PD 83-363 the EDmix increased body weight when comparing groups exposed to EDmix with groups not exposed to EDmix (full model). Additionally, from PD 83-129 there was a significant increased body weight in the group exposed only to EDmix when comparing to the control group (simple model), *$p<0.05$ compared to control group, #$p<0.05$ compared with no EDmix exposure (full model). (B) Weight of the retroperitoneal fat pad at approximately 13 months, n=12-20, *$p<0.05$ compared to EDmix (simple model) (C) Male body weight recorded approximately every four weeks except from day 251 and 363 (n=18). (D)Weight of the retroperitoneal fat pad at approximately 12 months, n=14-19. Body weights are expressed as mean and retroperitoneal fat pad is expressed as mean +/- SEM. Px, perfluorohexane sulfonate (PFHxS); EDmix, mixture of EDCs; Table 1.

Figure 3. Energy efficiency, body weight and serum leptin levels in adult female offspring. (A) Feed intake was measured at PD 270, and energy efficiency (change in body weight/feed intake) was calculated and expressed using a violin plot showing means and distribution of data points,

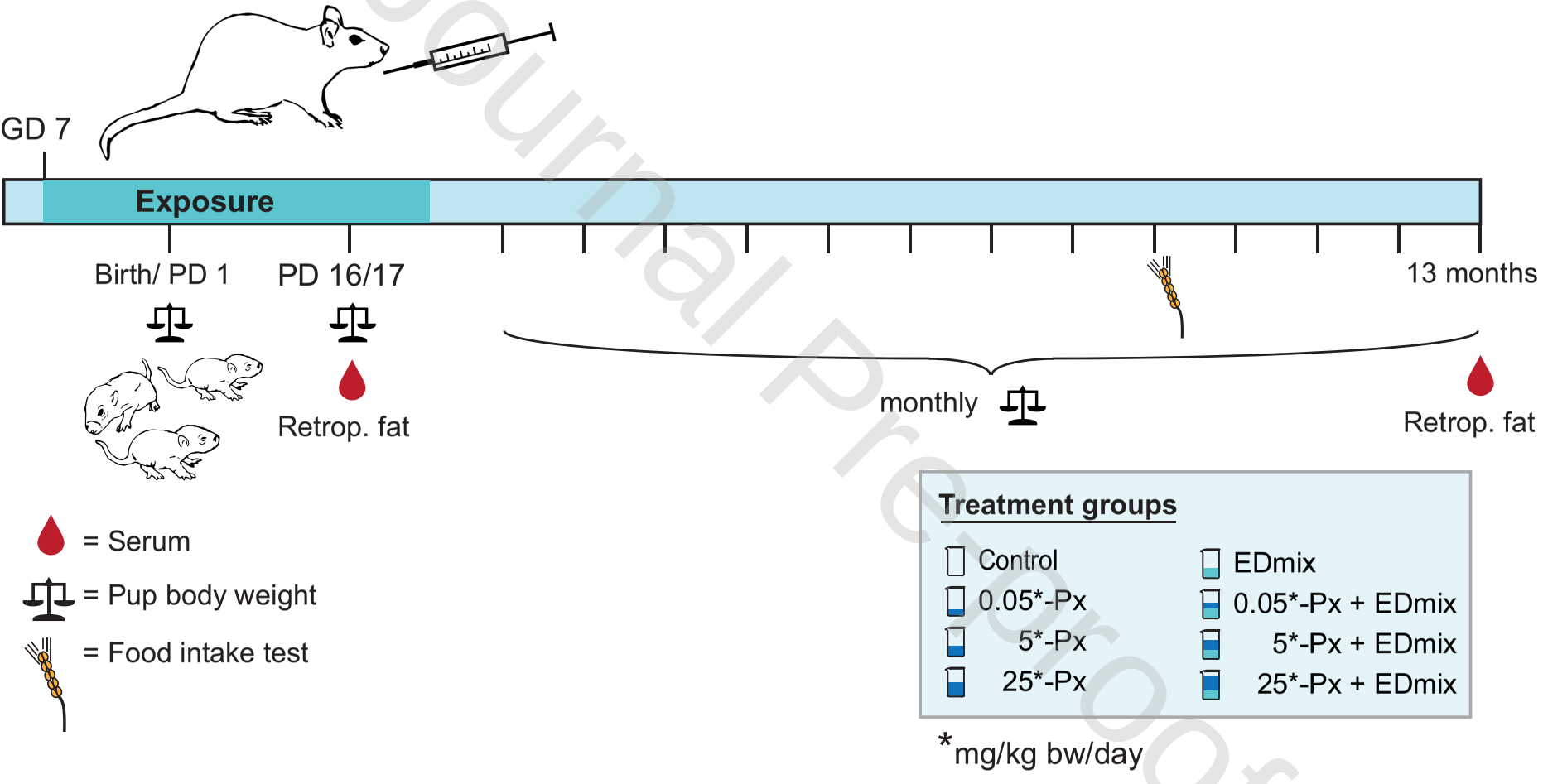
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n=13-20. (B) Body weight at PD 270 when feed intake measurements were started, n = 13-20, *p<0.05 compared with no EDmix exposure (full model). (C) Leptin levels in control and high dose groups at approximately 13 months of age, expressed as mean+/-SEM. n = 7-10. Px, perfluorohexane sulfonate (PFHxS); EDmix, mixture of EDCs; Table 1.

Figure 4. Relative mRNA expression of nine obesity related genes in the retroperitoneal fat pad from PD16 male and PD17 female rats exposed to three different doses of PFHxS. TaqMan RT-qPCR assays was performed for n=10 from each group. The red (left) and blue (right) bars represents female and male offspring, respectively. Data are expressed as mean +/-SEM and show no significant effects (ANOVA) for any of the genes. Px, perfluorohexane sulfonate (PFHxS).

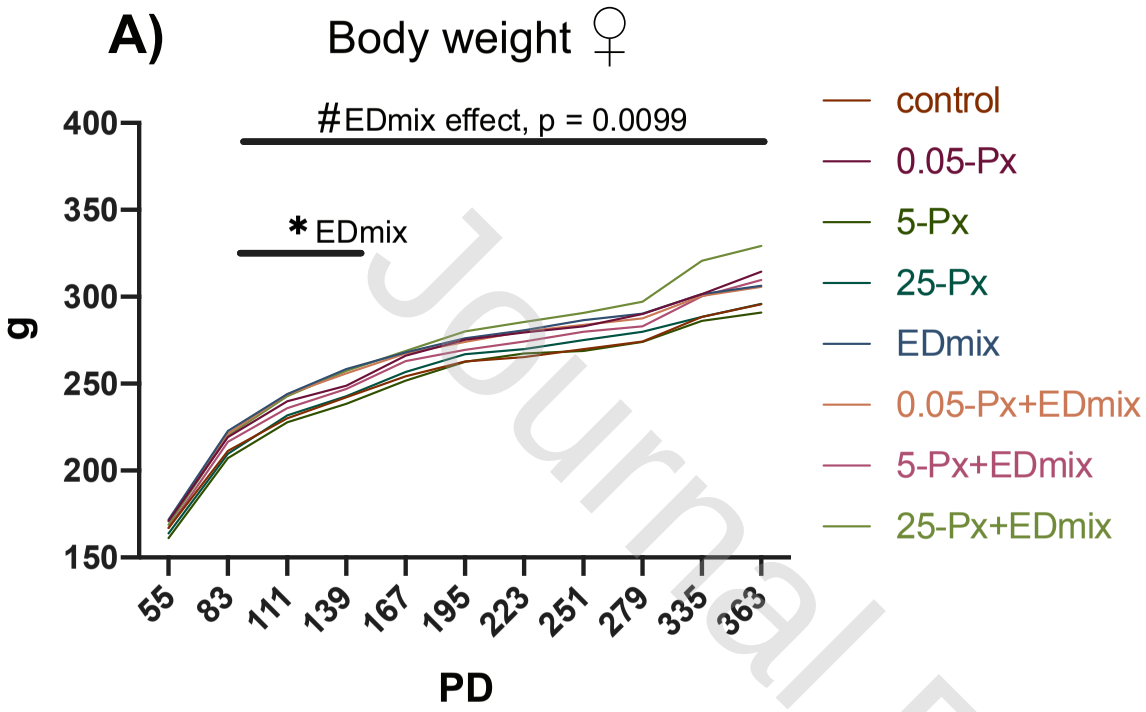
Figure 5. Relative mRNA expression of 15 obesity related genes in the retroperitoneal fat pad from 13-month-old female rats exposed to PFHxS and/or EDmix during development. Relative gene expression was measured using TaqMan RT-qPCR assays, n=7-10. Data show no significant effect with the exception of the overall PFHxS effect on Ppargc1a gene (full model). Data are expressed as mean □SEM. Px, perfluorohexane sulfonate (PFHxS); EDmix, mixture of EDCs; Table 1.

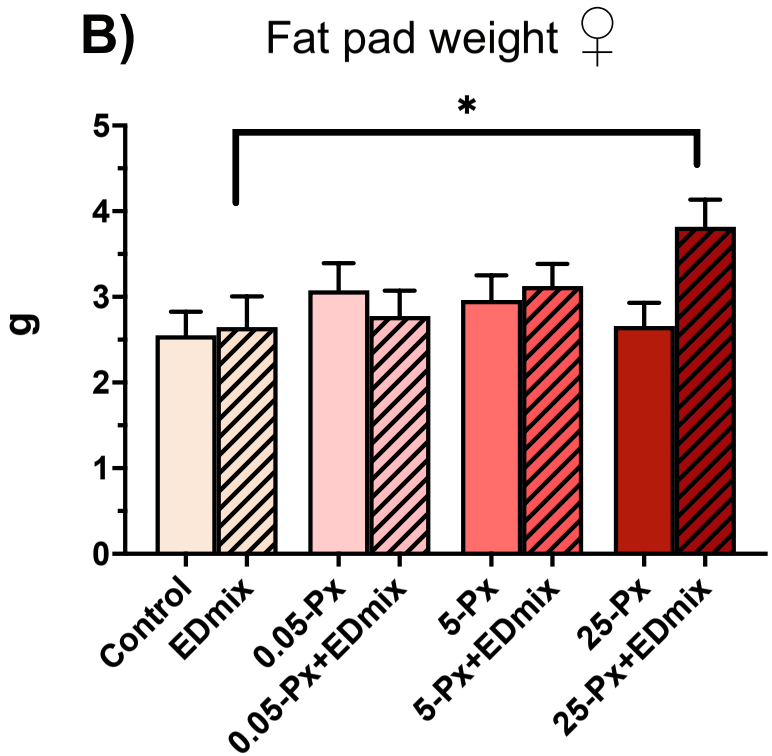
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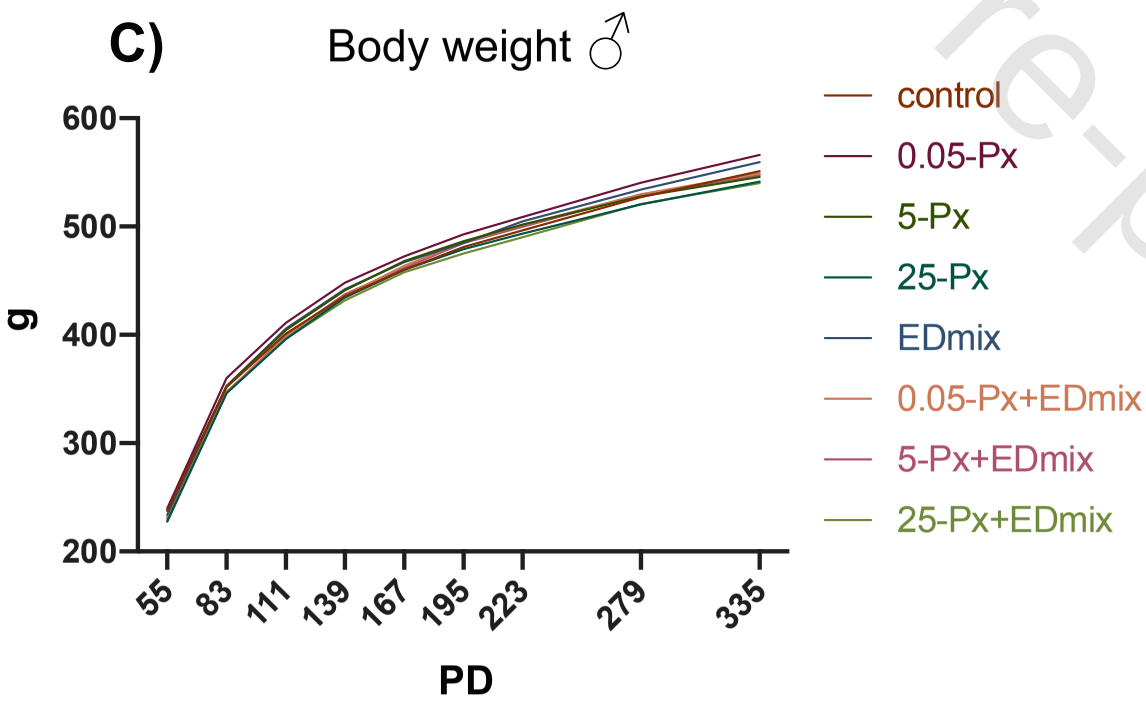


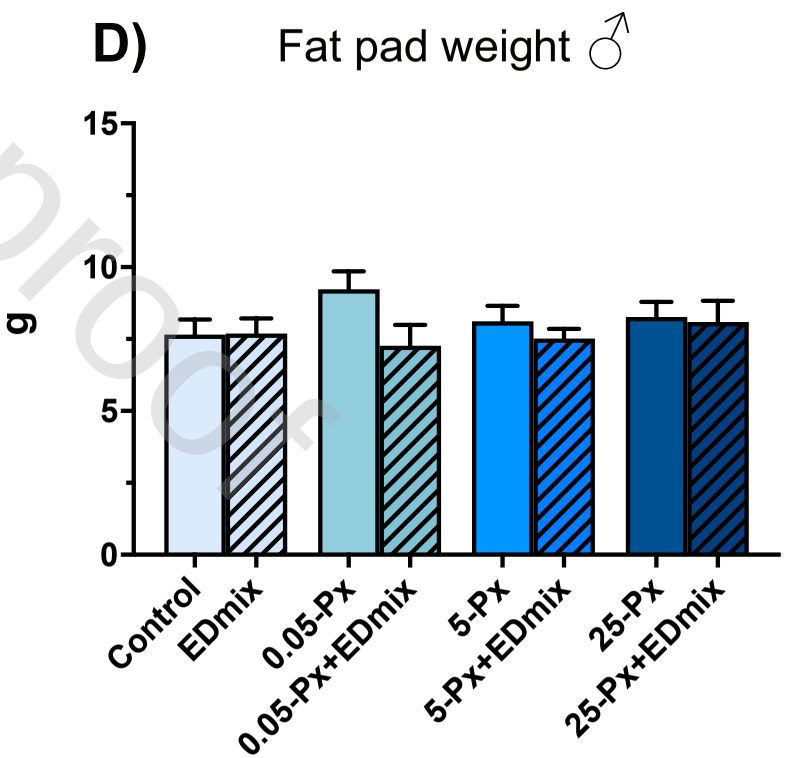
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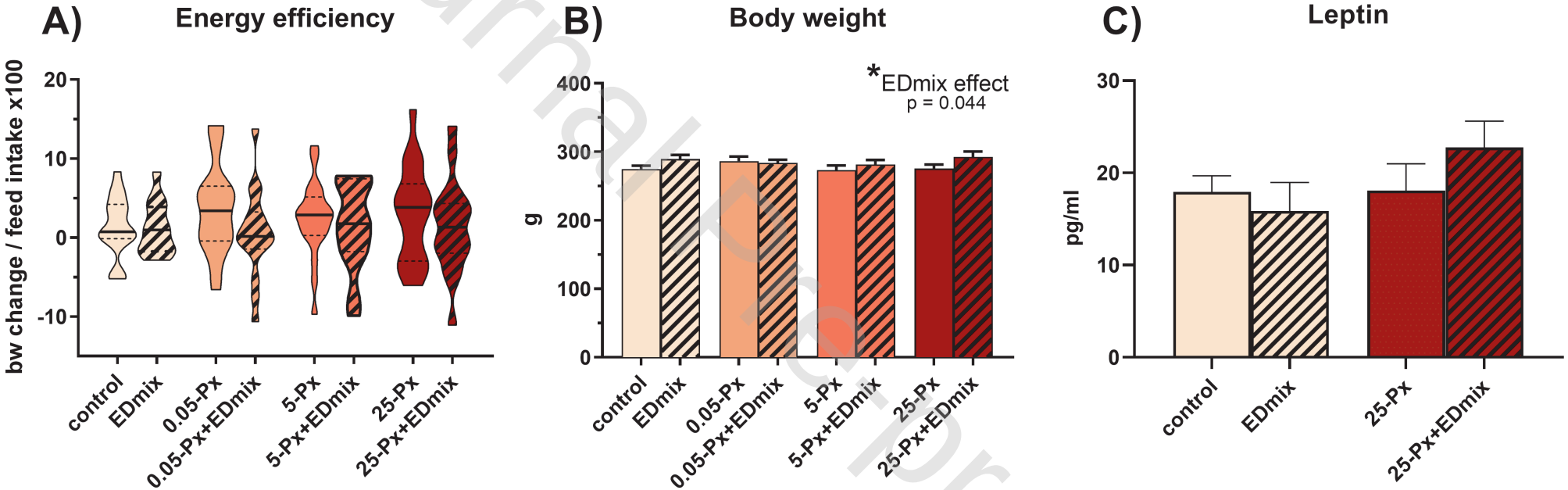






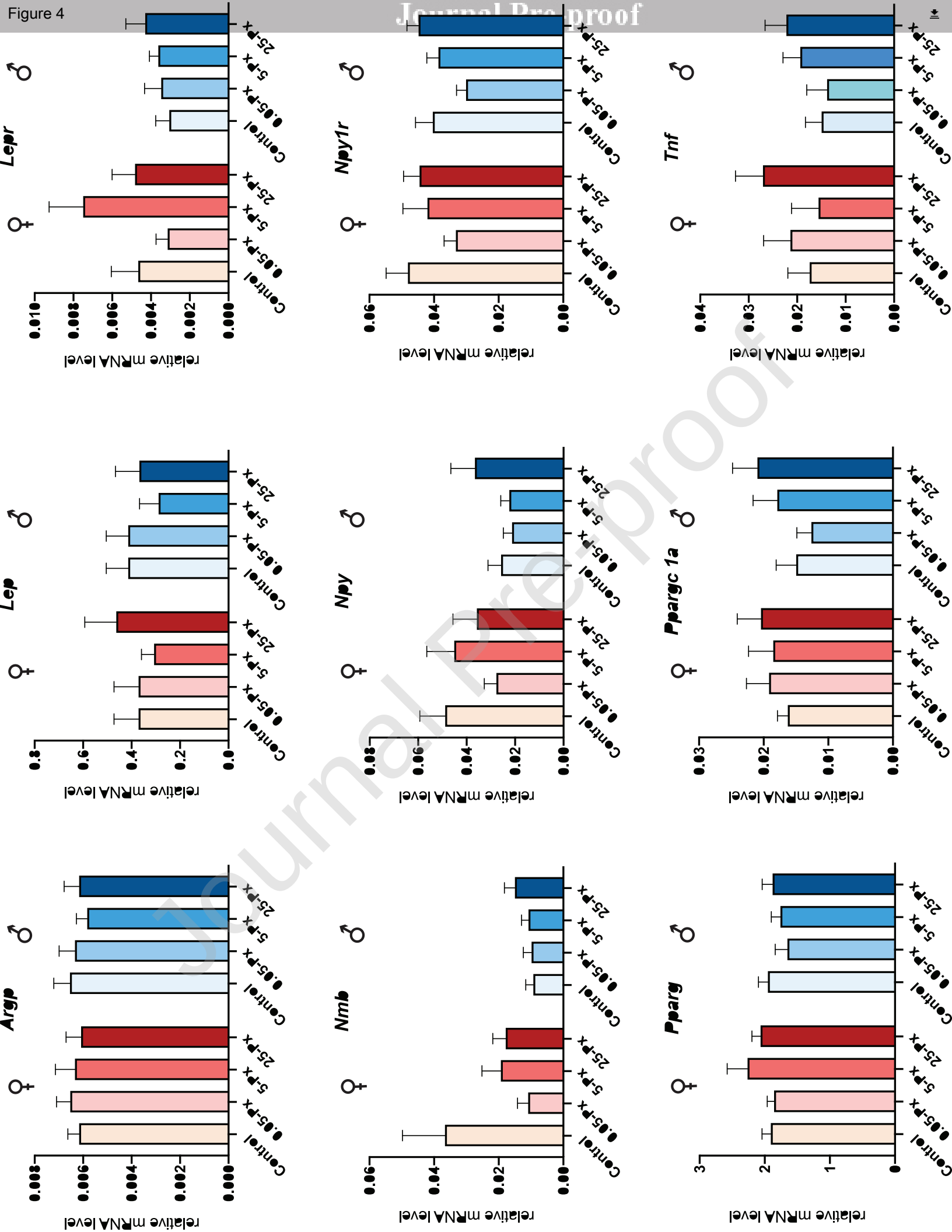


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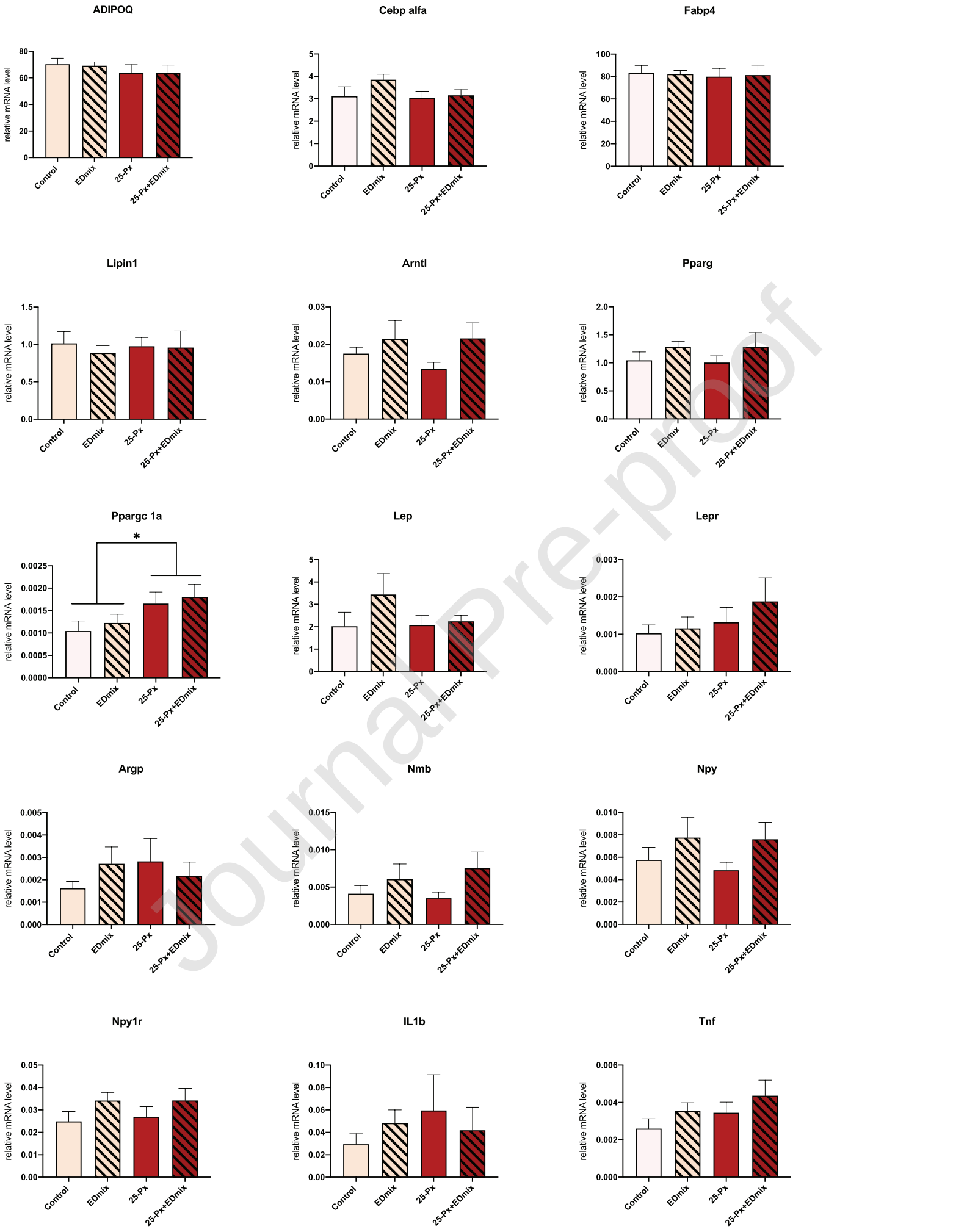
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