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Perinatal phthalate and high-fat diet exposure induce sex-specific changes in adipocyte size and DNA methylation

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Abstract

Environmental factors such as diet and endocrine-disrupting chemicals have individually been shown to mediate metabolic function. However, the underlying mechanism by which the combination disrupts adipocyte morphology and fat storage remains unknown. The current study evaluated early-life programming by diet and phthalate exposure. During gestation and lactation, pregnant Long-Evans hooded rat dams were fed either a control (C) or high fat (HF) diet and were orally administered one of three phthalate dosages (0, 200, or 1000 µg/kg/day), yielding 6 groups of offspring: C-0, C-200, C-1000, HF-0, HF-200, and HF-1000. On postnatal day (PND) 90, gonadal fat pads were collected and analyzed for histology, gene expression, and DNA methylation. Differences in body weight were observed only in males. H&E staining revealed larger adipocyte size in HF-0 versus C-0 females. Exposure to 200 or 1000 µg/kg/day phthalates modulated diet-induced changes in adipose morphology. Compared to C-0 females, HF-0 females also had higher expression of the adipogenesis gene Wnt receptor, frizzled 1 (*Fzd1*), and the triglyceride cleaving enzyme, lipoprotein lipase (*Lpl*). These increases in gene expression were accompanied by lower DNA methylation surrounding the transcription start sites of the two genes. Diet-driven effects were observed in unexposed females, but not in phthalate-treated rats. Results suggest a sex-specific association between perinatal HF diet and body weight, adipocyte size, and DNA methylation. Perinatal phthalate exposure appears to produce a phenotype that more closely resembles HF-fed animals.

Keywords

phthalate; high fat; adipogenesis; adipocyte; DNA methylation; early-life programming

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1. Introduction

With changing lifestyle and dietary patterns in the modern world, obesity and metabolic syndrome are becoming significant public health concerns. The developmental origins of health and disease hypothesis proposes that the etiology of such conditions can often be traced back to early-life and that maternal environment is critical in defining long-term health outcomes of offspring and subsequent generations. The perinatal period is characterized by rapid growth and is thus highly vulnerable to exogenous disruption that can alter developmental progression, resulting in undesirable adulthood diseases.

Several environmental toxicants have been shown to produce detrimental effects in humans. Phthalates are one class of endocrine-disrupting chemical that can be detected in a variety of household plastic products [1, 2] and foods [3]. Epidemiological studies have uncovered associations between phthalate exposure and reduced sperm quality [4], shorter anogenital distance [5], and adverse neurobehavioral outcomes such as impaired executive function [6–9]. Phthalates have been suggested to have metabolic consequences, however, conclusions have been inconsistent across epidemiological reports. While some have suggested a positive association between early life phthalate exposure and body mass index (BMI) [10, 11], others have found no correlation [12] or even a negative correlation [13, 14] between perinatal phthalates, BMI, waist circumference, and body fat percentage. Furthermore, phthalate exposure has been shown to be associated with gestational outcomes [15, 16], thyroid function [17], and body fat distribution [18] in a gender-dependent manner. Additional experimentation is necessary to uncover the role of phthalates in determining metabolic outcomes. Such studies might shed light on how toxicants interact with other environmental factors in a sex-dependent manner during the perinatal period.

Investigation of adiposity and adipocyte function can be used to quantify metabolic health. Adipose tissue serves as a reservoir for excess energy and accommodates extra fat in two ways: by increasing adipocyte size and by increasing adipocyte number. The capacity of an individual adipocyte is determined by how efficiently fatty acids (FA) are imported into the cell. Indeed, adipocyte hypertrophy is an indication of higher body weight [19]. As opposed to increased adipocyte size, elevated adipogenesis can increase the number of adipocytes in adipose tissue, also referred to as hyperplasia. During development, differentiation of mesenchymal stem cells into mature adipocytes is induced by transcription factor signaling [20]. While adipocyte hypertrophy may occur early or late in life, adipocyte hyperplasia typically occurs before puberty [21, 22]. Indeed, adipocyte hypertrophy is characteristic of adulthood obesity, while both hypertrophy and hyperplasia are seen in childhood obesity [23].

The sex-specific consequences of perinatal nutrition and toxicant exposure in adipose dynamics have not been studied extensively, however our group has examined the metabolic impact of perinatal diet and endocrine disruptors separately. We previously demonstrated that perinatal high fat (HF) induces excessive hepatic fat accumulation and disrupts histone modifications specifically in female offspring [24]. Similarly, we also reported that perinatal administration of the toxicant bisphenol A (BPA) produced sex-dependent changes in hepatic DNA methylation, gene expression, and lipid accumulation [25]. Recently we have

also shown that a postnatal HF dietary challenge has sex-specific effects on adipocyte size depending on whether the diet is preceded by prenatal phthalate exposure [26]. In the current study, we expand upon previous findings and examine whether concurrent perinatal HF dietary and phthalate treatment alter adipocyte dynamics. During gestation and lactation, Long-Evans hooded rats were exposed to either a control (C) or high-fat (HF) diet along with one of three phthalate treatments: 0, 200, or 1000 µg/kg/day. On postnatal day (PND) 90 body composition was measured and gonadal fat pads were collected. Histology, gene expression, and DNA methylation analysis point to sex-specific changes in adipose tissue in response to perinatal phthalate and HF exposure.

2. Methods

2.1. Animals

Three-month old Long-Evans hooded female rats (n = 7-11 per group) were obtained from Harlan Laboratories (Indianapolis, IN). For two weeks prior to breeding, rats were housed in same-sex pairs and were given ad libitum access to a low phytoestrogen diet (Harlan 2020X; Taklad Diets, Madison, WI). During breeding dams were placed in suspended wire-bottom cages, and once a sperm plug was detected, dams were removed and individually housed. From gestational day 0 until PND 10, dams received one of two diets, control (C; 15.8% kcal fat, 63.9% kcal carbohydrate, 20.3% kcal protein; D10012G, Research Diets Inc., New Brunswick, NJ) or high-fat (HF; 45% kcal fat, 35% kcal carbohydrate, 20% kcal protein; D12451, Research Diets Inc.), and one of three phthalate treatments, 0, 200, or 1000 µg/kg body weight/day (Fig. 1). This resulted in 6 groups that were referred to by their diet-phthalate combination: C-0, C-200, C-1000, HF-0, HF-200, and HF-1000.

The phthalate mixture used in the current experiment consisted of approximately 35% diethyl (DEP), 21% bis(2-ethylhexyl) (DEHP), 15% dibutyl (DBP), 15% diisononyl (DiNP), 8% diisobutyl (DiBP), and 5% benzyl butyl (BBP) phthalate. This distribution reflects the urinary metabolites of pregnant women in Champaign-Urbana, Illinois (unpublished data) as well as exposure levels in the general U.S. population [27]. The mixture was suspended in tocopherol-stripped corn oil at a concentration of 0, 0.6, or 3 mg phthalates/mL to facilitate daily administration of 0, 200, or 1000 µg/kg, respectively. The phthalate mixture was administered orally. All dams received half a cookie (Newman's Own organic alphabet cookie, vanilla flavor) with the phthalate mixture pipetted onto it. Dams were acclimatized for two days with half a cookie topped with tocopherol-stripped corn oil. From gestational day 2 through PND 10, C-0 and HF-0 dams continued to eat half a cookie overlaid with the tocopherol-stripped corn oil vehicle. The other dams consumed half a cookie overlaid with phthalate mixture at their respective concentrations.

On PND 0 the size and sex ratio of each litter was recorded. On PND 1, litters were culled to 10 pups. On PND 25, one female and one male offspring from each dam were randomly selected, weaned, and pair-housed with a rat of the same sex, diet, and phthalate exposure (C-0 (n=8 males, n=10 females), C-200 (n=9 males, n=7 females), C-1000 (n=9 males, n=10 females), HF-0 (n=7 males, n=8 females), HF-200 (n=8 males, n=7 females), and HF-1000 (n=11 males, n=9 females)). On PND 90, body composition was measured using the EchoMRI-700 Body Composition Analyzer (Echo Medical Systems, Houston, TX). The rats

were then euthanized and gonadal adipose tissue was dissected out. Half the tissue was snap-frozen in liquid nitrogen and stored at -80°C and half was fixed in formalin for future histological analysis.

2.2 Histology

In order to quantify adipocyte size, formalin-fixed samples were embedded in paraffin, sectioned to a thickness of $7\text{ }\mu\text{m}$, stained with H&E, and imaged under $20\times$ magnification. Three independent fields were randomly chosen for each offspring. Contrast was manually adjusted for each frame such that individual adipocyte outline was clearly visible. Adipocyte area was quantified using the ADIPOCYTE software according to previously published methods [28].

2.3. Gene Expression

Frozen adipose tissue was ground in liquid nitrogen using mortar and pestle. Ground tissue was immediately placed in a 1.5 ml tube containing Trizol. Before loading the sample into the column, the tube was spun and the lipid layer was removed. Total RNA was extracted using Direct-zol RNA MiniPrep Plus (Zymo Research, Irvine, CA) with in-column DNase I treatment to eliminate DNA contamination. cDNA synthesis and qRT-PCR were performed as previously described [24]. Primers were designed using VectorNTI software (Life Technologies, Grand Island, NY) and synthesized by IDT (Coralville, IA). Validation and specificity was performed using the Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primers were verified to have no SNPs using the dbSNP track available in Genome Browser. All mRNA primer sequences and amplification efficiencies can be found in Supplementary Table 1. All gene expression was normalized to *Rpl7a* due to its widespread use and proven stability [29, 30].

2.4. DNA Methylation

Genomic DNA was isolated using the ZR Genomic DNA Tissue MiniPrep kit (Zymo Research). Bisulfite conversion was performed using the EZ DNA Methylation Gold Kit (Zymo Research). To perform the conversion reaction, samples were brought to 98°C for 10 minutes, 64°C for 2.5 hours, and held at 4°C until samples were desulphonated and diluted to $5\text{ ng}/\mu\text{l}$. For methylation specific PCR (MSP), bisulfite converted gDNA samples were amplified using Sybr Green (Quanta Biosciences, Beverly, MA). CT values were compared to a serially diluted standard curve for quantification and percent methylation was calculated by dividing quantity of methylated DNA by the sum of methylated and unmethylated DNA. Methylation was normalized to the C-0 group. MSP is the most common locus-specific bisulfite-based technique and was chosen over pyrosequencing, Epityper, and other bisulfite-based methods because of its rapid, cost-effective implementation [31].

Methylated and unmethylated primer pairs were manually designed using the Primer Express 3.0.1 software's Primer Probe Test Tool (Life Technologies). The T_m for each primer was between 68 and 70°C and the CG content was between 30 and 80 percent. Primers were screened in the IDT OligoAnalyzer to minimize dimers and hairpin loops. Unmethylated DNA was measured using both unmethylated forward and reverse primers. Methylated DNA was measured by pairing the methylated forward primer with the

unmethylated reverse primer as well as by pairing the unmethylated forward primer with the methylated reverse primer. The quantity of methylated DNA was normalized to the total amount of DNA (methylated + unmethylated) and presented as a ratio to the control group (C-0). All MSP primer sequences and amplification efficiencies can be found in Supplementary Table 2.

2.5 Statistical Analysis

Male and female data were analyzed separately, yielding a 3 by 2 factorial study design. A full ANOVA model was performed with covariates diet (HF and Control), phthalate (0, 200, and 1000), and their interaction term. If significance was achieved in either of the main effects or interaction, then post-hoc comparisons corrected for multiple testing were made to test the effect of diet within the same phthalate level (C-0 vs HF-0, C-200 vs HF-200, and C-1000 vs HF-1000) as well as the effect of phthalates within the same diet level (C-0 vs C-200, C-0 vs C-1000, C-200 vs C-1000, HF-0 vs HF-200, HF-0 vs HF-1000, HF-200 vs HF-1000). A p-value of less than 0.05 was considered statistically significant. All statistical analysis was performed in R version 3.3.2.

3. Results

3.1 Body weight and composition

The role of early-life environment in adipose morphology and function was studied using Long-Evans rat offspring of dams subjected to various diet and phthalate treatments from gestational day 0 to PND 10. On PND 10, phthalate exposure was negatively correlated with body weight in control-fed but not HF-fed animals. Moreover, HF diet increased body weight only with phthalate exposure. Specifically, HF-200 and HF-1000 female offspring weighed more than their C-200 ($p=0.0030$) and C-1000 counterparts ($p=0.0065$; Fig. 2A), respectively. There was no difference in body weight between C-0 and HF-0 females. Similarly, HF-200 males weighed more than C-200 males ($p=0.0018$; Fig. 2B), however this effect was not observed between C-1000 and HF-1000 males or between C-0 and HF-0 males. Additionally, on PND 10, C-200 males weighed less than C-0 males ($p=0.015$; Fig. 2B). There were no significant effects from litter size or sex ratio [32].

At PND 90, all body weight differences amongst females were abolished, but male body weight was dependent on both HF diet and phthalate challenges (Fig. 2C). HF-0 males weighed more than C-0 males ($p=0.019$; Fig. 2D). This diet effect was not seen with either 200 or 1000 $\mu\text{g/kg/day}$ phthalate administration. In PND 90 males, phthalates had a positive relationship with body weight, as the C-200 group weighed more than the C-0 group ($p=0.0080$). However, when combined with HF diet, the highest phthalate concentration had a negative effect in body composition, as the HF-1000 group weighed less than the HF-200 group ($p=0.044$; Fig. 2D). No differences body composition were observed between any groups at PND 90 (Fig. 1E and 2F).

3.2 Adipocyte size

On PND 90, rats were sacrificed and gonadal adipose tissue was fixed and embedded in paraffin for histological analysis. Upon H&E staining, we observed that HF diet enhanced

adipocyte size in females only. Without phthalate treatment, HF diet increased adipocyte size, as HF-0 females had larger adipocytes than C-0 females ($p=0.046$; Fig. 3A). This diet effect was not observed with 200 or 1000 $\mu\text{g/kg/day}$ phthalate levels. Additionally, the highest phthalate dosage impacted adipocyte size, as C-1000 females had larger adipocytes than C-0 females ($p=0.040$). No differences in adipocyte size were observed in male offspring (Fig. 3B).

3.3 Gene expression

Genes involved in adipogenesis and FA uptake were measured in gonadal adipose tissue. Four genes in the WNT signaling pathway were measured, including frizzled 1 (*Fzd1*), LDL receptor related protein 6 (*Lrp6*), *Wnt5b*, and *Wnt10b*. In females, HF diet and phthalate alone affected gene expression. *Fzd1* was more highly expressed in the HF-0 group than in C-0 group ($p=0.045$; Fig. 4A). Like adipocyte size, this effect was not seen between HF-200 and C-200 or between HF-1000 and C-1000 females but was observed between C-0 and C-1000 groups ($p=0.042$). There was also a modest increase in *Wnt10b* expression in HF-1000 females compared to C-1000 ($p=0.061$; Fig. 4D). In males, gene expression changes were minimal. There was a trending increase in *Lrp6* expression in HF-1000 compared to HF-200 animals ($p=0.080$, Fig. 5B).

FA uptake genes were also measured. These genes included the triacylglycerol-cleaving enzyme lipoprotein lipase (*Lpl*) as well as two FA transporters expressed in adipose tissue, *Cd36* and fatty acid transport protein 4 (*Fatp4*). In females, *Lpl* showed a HF diet-induced increase in expression, as HF-0 had higher levels than C-0 ($p=0.040$; Fig. 4G). This dietary effect was not observed under phthalate treatment because there was no difference in expression between HF-200 and C-200 or between HF-1000 and HF-1000 (Fig. 4G). *Cd36* expression showed a negative association with phthalate treatment under HF conditions. HF-0 females had higher expression of *Cd36* compared to HF-1000 females ($p=0.037$, Fig. 4E). In males, the highest phthalate dosage impacted gene expression in both dietary treatments. The HF-1000 group had higher *Fatp4* expression compared to HF-0 animals ($p=0.035$; Fig. 5F). Males also showed a modest difference in *Lpl* expression between C-0 and C-1000 groups ($p=0.098$; Fig. 5G).

3.4 DNA methylation

Due to diet-induced differences in expression of *Fzd1* and *Lpl*, DNA methylation was measured in regions surrounding the TSS of both *Fzd1* and *Lpl*. HF diet in the absence of phthalates decreased methylation around the *Fzd1* promoter. Compared to C-0 females, HF-0 females had lower DNA methylation at CpG sites located at 384 and 123 base pairs (bp) upstream of the *Fzd1* TSS ($p=0.022$ and $p=0.038$ respectively; Fig. 6A). DNA methylation was also averaged over six loci between 400 bp upstream and 200 bp downstream of the *Fzd1* TSS. HF-0 females had lower average DNA methylation surrounding the *Fzd1* TSS than C-0 rats ($p=0.0035$). A diet effect was also observed at the -283 site, where C-1000 females had higher methylation than HF-1000 animals ($p=0.020$). In order to confirm that differential methylation was specific to differentially expressed genes, MSP was repeated in male rats. Only one site was differentially methylated in males. HF-200 offspring showed decreased DNA methylation at 384 bp upstream of the *Fzd1* TSS

($p=0.023$) and had lower average methylation of the region surrounding the *Fzd1* TSS compared to C-200 males ($p=0.00012$; Fig. 6B).

Also in females, HF diet reduced methylation at a locus in the *Lpl* promoter. Compared to C-0, HF-0 rats had lower DNA methylation at CpG sites located at 508 bp upstream of the *Lpl* TSS ($p=0.018$; Fig. 7A). A phthalate treatment of 200 $\mu\text{g/kg/day}$ yielded higher DNA methylation at the -423 site in C-200 compared to HF-200 females ($p=0.0055$). No DNA methylation differences were observed in CpGs surrounding the *Lpl* TSS in male animals (Fig. 7B).

4. Discussion

The perinatal period marks a time of heightened susceptibility to detrimental stimuli which can alter developmental processes and cause epigenomic modifications that solidify in adulthood to produce undesirable health conditions. Here we looked at a combination of dietary perturbation and toxicant exposure during gestation and lactation in Long-Evans hooded rats. In female offspring, HF feeding was associated with increased adipocyte size, elevated expression of *Lpl* and *Fzd1* mRNA, and reduced DNA methylation around the *Lpl* and *Fzd1* TSSs. This effect was not observed with either 200 or 1000 $\mu\text{g/kg/day}$ perinatal phthalate administration (Fig. 8).

4.1 Perinatal HF diet and phthalate exposure alters body weight in male offspring

On PND 90, we found sex-specific effects of phthalates on body weight. PND 90 females showed no differences in either body weight or body composition. This is consistent with findings from our group and others [26, 33–35]. In PND 90 males, on the other hand, HF diet alone as well as 200 $\mu\text{g/kg/day}$ phthalates increased body weight. Previously, we reported that perinatal phthalate did not change body weight of PND 97 males [26]. It should be noted, however, that the duration and dosage of phthalate administration are very different between this study and our previous work. Previously, 300 mg/kg/day was used, whereas the present experiment used a concentration of 200 and 1000 $\mu\text{g/kg/day}$, which represents a 300 fold daily difference. Additionally, in our past study, dams were exposed to phthalates from gestational day 6 until PND 21; however in the current study, they were exposed from gestational day 2 to PND 10. Given the developmental timeline in rats, this discrepancy could have a major impact on offspring outcomes [36, 37]. Interestingly, despite body weight changes, there were no differences in body fat percentage in PND 90 males, which suggests that increases in body weight were the result of increases in both fat and lean mass.

4.2 Diet-induced changes in female offspring are modulated by phthalate exposure

HF-fed female offspring had larger adipocytes than control-fed rats. Adipocyte hypertrophy, or uptake of excess fat, can be achieved through upregulation of the *Lpl* enzyme which mediates the cleavage and import of circulating triglycerides into the adipocyte for storage (Fig. 8B). In our experiment we found an increase in both adipocyte size and *Lpl* mRNA expression in HF-0 compared to C-0 females. These results are supported by previous work that has directly linked adipocyte size to *Lpl* function and membrane density [38]. These

findings are supported by studies that have shown no difference in *Lpl* activity in postnatal HF-fed male rats [39] and increased *Lpl* mRNA expression in female offspring of HF-fed mouse dams [40].

Interestingly, changes in adipocyte size and FA uptake genes were limited to females. It is known that female adipocytes typically display greater insulin sensitivity, inflammation resistance, and estrogen receptor expression than male adipose tissue [41, 42]. One study showed that estrogen receptor knockout mice had larger adipocyte size and increased expression of inflammatory genes, suggesting an endocrine-mediated, protective role of adipocyte expandability [43]. Thus, in our study, the higher adipocyte size in C-1000 females might be attributed to the endocrine-disrupting properties of phthalates. On the other hand, diet-driven increases in adipocyte size and gene expression were limited to rats given no perinatal phthalate treatment. The clear distinction between female C-0 and HF-0 was not observed between C-200 and HF-200 or between C-100 and HF-1000 (Fig. 8D). This suggests that phthalates potentially act on the same pathways as HF diet.

While there were changes in adipocyte size in female offspring, there were no differences in body weight or body fat percentage. We hypothesize that a decreased number of adipocytes could counterbalance the increase in adipocyte size. As an alternative to an expansion in individual adipocyte volume, excess fat may be accommodated by increasing the total number of adipocytes. This hyperplasia is likely not as relevant in adulthood due to the stability of the adipocyte pool, but in childhood, adipogenesis occurs much more readily [20]. During adipogenesis, receptors such as *Fzd1* and *Lrp6* bind WNT family members and initiate the β -catenin cascade to induce osteoblastogenesis and inhibit transcription of adipogenic genes (Fig. 8A) [44]. At PND 90, we found higher expression of *Fzd1* in HF-0 than in C-0 females. With phthalate treatment, C-1000 females had higher expression than C-0 females, thus abolishing the diet-induced effects and producing *Fzd1* expression levels that more closely resemble HF-exposed animals. The diet and phthalate-mediated increases perhaps reflect adipogenic events that occurred earlier in development, as *Fzd1* is highly expressed in pre-adipocytes [20]. This hypothesis is supported by the observed changes in DNA methylation.

Increased *Lpl* suggests greater lipid capacity per adipocyte; however high *Fzd1* expression might suggest a decreased number of adipocytes available for fat storage. Because these two processes appear to be opposing one another, this may be a reason why we observed no differences in body fat percentage between HF-0 and C-0 females. This study is limited in that only gonadal fat pads were examined, adipocyte hyperplasia was not directly measured by cell counting, and adipose depots were not weighed.

4.3 HF diet and phthalates: potential mechanisms of action

The combined effects of phthalates and diet have not been extensively studied. It is unclear how they impact each other's digestion, absorption, and mediation of cell processes. Previous literature cites phthalates and FAs as ligands of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors [45–47]. Furthermore, PPARs have been shown to alter expression of *Lpl* [48, 49] and *Fzd1* [50] and impact adipogenesis [51].

Thus, it may be the case that HF-induced increases in *Lpl* and *Fzd1* were masked with phthalate treatment through a PPAR-mediated mechanism.

The means by which HF diet and phthalates impact DNA methylation is unknown. HF diet has been shown to alter DNA methylation in liver [52], adipose tissue [53], and muscle [54]; and phthalates have been reported to change DNA methylation in placenta [55, 56], cell lines [57], and blood [58, 59], primordial germ cells [60], and adrenal glands [61]. It has been suggested that both fatty acid metabolism as well as phthalates produce reactive oxygen species that damage DNA and interfere with methyl-CpG binding proteins [62–64]. It is also possible that chromatin remodeling and DNA methylation changes occur via PPAR interactions with the switching defective/sucrose non-fermenting (SWI/SNF) complex, resulting in nucleosome repositioning and altered DNA methylation patterns [65–70]. We observed no changes in mRNA levels of PPAR α or PPAR γ (Supplementary Fig. 1), however, because both phthalates and fatty acids are ligands of the PPAR family, they may activate PPARs without upregulating their transcription. Further experimentation is needed to show that HF diet and phthalate exposure affect DNA methylation through a common PPAR-mediated mechanism.

Phthalates are hypothesized to act in an anti-androgenic manner and have indeed been shown to lead to developmental abnormalities in both the male and female reproductive tracts [71, 72]. The endocrine-disrupting properties of phthalates probably account for sex differences observed in the current study. Perinatal phthalate exposure not only induces reproductive abnormalities, but also elicits sex-specific effects in glucose tolerance [26], anxiety behavior [73], and adrenal function [74]. Although the exact mechanism by which phthalates interact with the hormonal environment in males versus females remains unclear, it is possible that DNA methylation differences are a result of the different developmental time courses or the hormonal milieu between sexes.

5. Conclusion

Here we describe the morphological and epigenetic outcomes of perinatal diet and phthalate exposure in adipose tissue. One strength of the current experiment is the usage of environmentally feasible concentrations of 200 and 1000 $\mu\text{g/kg/day}$ phthalates [75]. Previous studies have used high dosages of phthalates that bear little relevance to typical human exposure, including concentrations that are up to 750 times higher than the ones used in the current study [76, 77]. Furthermore, our study benefits from originality in design and outcome measures. We are the first to examine the concurrent role of phthalates and diet in both male and female offspring, and this is the first study to our knowledge that examines DNA methylation in adipose tissue of phthalate-exposed animals. Furthermore, while male animals have previously received the most attention in the literature, we herein highlighted female response to phthalates.

Future experimentation should expand upon our small subset of measured genes to paint a broader picture of adipocyte dynamics in response to early-life environmental stimuli. Here, we took a candidate gene rather than genome-wide approach. More extensive investigation has already been undertaken in the liver [78], however adipose response to endocrine

disruptors remains to be studied in a transcriptome-wide and epigenome-wide manner. Moreover, the present study only measured changes at one time point. Future work should address multiple developmental stages to assess when changes are occurring and whether they remain stable beyond 90 days.

This study quantified the sex-specific effects of concurrent perinatal HF diet and phthalate treatment. We observed sex-dependent changes in adipose morphology, gene expression, and DNA methylation. We show that perinatal HF-fed female offspring have greater adipocyte size compared to controls. This difference was accompanied by higher *Fzd1* and *Lpl* gene expression and lower DNA methylation around the two TSSs. When phthalates were not present, females displayed two distinct phenotypes in response to C and HF diets (Fig.7D). However, perinatal phthalate exposure appears to modulate adipocyte size and *Fzd1* expression in females, producing a phenotype that more closely resembles HF-treated rats. We conclude that perinatal phthalate and diet act in a sex-dependent manner, possibly through an epigenetic mechanism, to alter adipocyte morphology and gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Experimental design involved diet and phthalates administration during the perinatal period. Body weight was measured at PND 10. Body weight, body composition, gene expression, and DNA methylation were measured at PND 90. GD: gestational day, PND: postnatal day.

Figure 2.

Body weights of (A) female and (B) male offspring at PND 10 (n = 7-10/group). Offspring were weaned onto control diet at PND 25 and given no phthalate treatment for the remainder of the experiment. (C,D) Body weight and (E,F) body composition were measured in female and male offspring at PND 90. Black points represent the averaged adipocyte size over 3 independent fields for one animal. *p < 0.05.

Figure 3.

Adipocyte size in gonadal adipose tissue was measured at PND90 in (A) females and (C) males. Representative images highlight differences in (B) female adipocytes and no change in (D) males. Sections were imaged under 20 $\times$ magnification. * $p < 0.05$.

Figure 4.
Expression of genes involved in (A-D) adipogenesis and (E-G) fatty acid uptake were measured at PND90 in females. * $p < 0.05$.

Figure 5. Expression of genes involved in (A-D) adipogenesis and (E-G) fatty acid uptake were measured at PND90 in males.

Figure 6.

(A) In female and (B) male offspring, Methylation specific PCR (MSP) was used to measure DNA methylation (DNAMe) surrounding the *Fzd1* transcription start site (TSS). CpGs were measured individually and DNAMe was averaged over the entire region. * $p < 0.05$.

Figure 7.

(A) In female and (B) male offspring, Methylation specific PCR (MSP) was used to measure DNA methylation (DNAMe) surrounding the *Lpl* transcription start site (TSS). CpGs were measured individually and DNAMe was averaged over the entire region. *p < 0.05.

Figure 8.

(A) Adipogenesis involves WNT signaling factors that bind to frizzled 1 (*Fzd1*) and LDL receptor related protein 6 (*Lrp6*) to initiate the β -catenin cascade and turn on expression of anti-adipogenic genes. (B) Fatty acid uptake by adipose tissue begins with the cleavage of circulating triacylglycerol (TAG) by lipoprotein lipase (*Lpl*) to yield 3 fatty acids (FA) and the glycerol backbone. The FAs are transported into the adipocyte by CD36 and fatty acid transport protein 4 (*Fatp4*). (C) At PND10, there were sex-specific changes in body weight.

(D) At PND90, sex-specific changes in adipose tissue were observed. Red denotes the diet-induced changes in females between C-0 and HF-0. All listed comparisons have $p < 0.05$.