Developmental Exposures to Phthalates and Phthalate Mixtures and Life-Course Metabolic Outcomes: Using a Mouse Model to Inform Human Studies and Elucidate Mechanisms

by

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ABSTRACT

Nearly 40 percent of US adults and 20 percent of US children are obese. Given obesity's multiple dangerous comorbidities, this presents a significant concern for public health. A growing body of evidence suggests that exposures to environmental chemicals may be contributing to the obesity epidemic. Such chemicals have been termed "obesogens" and among them are phthalates, endocrine disrupting chemicals (EDCs) that are present in food packaging, children's toys, and personal care products. Exposures to phthalates during development have been linked to adverse metabolic health outcomes in both animal and human studies, but findings from human studies are less consistent. One possible reason is humans are co-exposed to many phthalates, and these mixture exposures are difficult to interpret. Additionally, the vast majority of animal studies to date have focused on examining metabolic impacts of diethylhexyl phthalate (DEHP), despite the recent introduction of newer phthalates on the market to replace it, including diisononyl phthalate (DINP). Furthermore, mechanisms linking developmental exposures and later-life health outcomes, such as epigenetic reprogramming via DNA methylation, are still poorly understood.

The overall objective of this dissertation was to utilize an animal model of perinatal phthalate exposures to investigate long-term metabolic impacts in a manner that would inform human studies and infer underlying mechanisms. We incorporated exposures to three individual phthalates (DEHP, DINP, and dibutyl phthalate (DBP)), as

well as two phthalate mixtures (DEHP+DINP and DEHP+DINP+DBP). We then took phenotypic and molecular measurements on the offspring at two time points: at weaning on postnatal day 21 (PND21) at the end of the exposure period and at 10 months of age, >9 months after exposure had ceased. In Aim 1, we investigated early-life metabolic phenotypes by measuring body weight and relative liver weights and examined biomarkers of whole-genome DNA methylation alterations at PND21. In Aim 2, we evaluated metabolic phenotypes longitudinally at two and eight months of age to determine whether developmental exposures to phthalates influenced metabolism across the life course. Finally, in Aim 3, we measured the transcriptome and DNA methylation in liver and white adipose tissue (WAT) at both PND21 and 10 months to elucidate a molecular mechanism.

We found that developmental exposures to individual phthalates and phthalate mixtures were associated with increased body weights in males and females in early postnatal life. Females, but not males, perinatally exposed to DINP-only and a mixture of DEHP+DINP also had increased relative liver weights at PND21. We also observed a sex-specific effect on tail DNA methylation at repetitive elements in mice exposed to individual phthalates and phthalate mixtures, indicating a sexually dimorphic effect on the epigenome. Developmental exposures to DEHP-only and DINP-only resulted in increased body fat percentage and glucose intolerance, respectively, across the life course. However, we did not observe longitudinal adverse metabolic impacts in mice perinatally exposed to phthalate mixtures, suggesting a potential adaptive response in these mice. In females perinatally exposed to DINP, we identified several persistently up-regulated PPAR target genes in the liver that could lead to increased fatty acid

synthesis. Fatty acid synthase (*Fasn*) also exhibited increased promoter region DNA methylation at both PND21 and 10 months of age, implicating a role for epigenetic reprogramming. Taken together, the work here demonstrates short-term and long-term metabolic impacts following perinatal exposures to phthalates, and presents a new potential mechanism describing the underlying biology in the liver.

CHAPTER 1

INTRODUCTION

Phthalates

Phthalates are ubiquitous plasticizing chemicals found in a wide variety of consumer products, including personal-care products, vinyl flooring, clothing, and children's toys.[1] There are two main classes of phthalates: high molecular weight phthalates, and low molecular weight phthalates. High-molecular weight (HMW) phthalates (e.g., diethylhexyl phthalate or DEHP, and diisononyl phthalate or DINP) are typically found in plastic products such as PVC tubing and food packaging, whereas low-molecular weight (LMW) phthalates (e.g., dibutyl phthalate or DBP) are typically found in personal-care products and pharmaceuticals.[1]

Because phthalates are not covalently bound to plastic polymers, the leaching of phthalates into food and drink poses risks for human exposure.[2] Due to the high prevalence in consumer products, humans are ubiquitously exposed to phthalates, and are typically exposed to a mixture of many phthalates. According to recent United States National Health and Nutrition Examination Survey (NHANES) data, metabolites from 13 different phthalates were detected in urine samples with at least one phthalate metabolite detected in all available samples.[3] Some phthalates, such as DEHP and DBP have been banned in the US for use in toys due to adverse health outcomes

observed in mice and humans.[4] Other phthalates that have been used as replacements for banned phthalates, such as DINP, are currently understudied.

Developmental exposures to phthalates have been linked to reproductive toxicity in both males and females in both animal and human studies. Adverse health effects associated with developmental phthalate exposures in males have been the focus of many studies and include fertility, testosterone production, and reproductive tract malformations.[5] The impacts of developmental phthalate exposures on female reproductive health are less clear, but epidemiological studies have found links between phthalate exposures and preterm birth[6] and animal studies have demonstrated impacts on the ovaries.[7] Although historically there has been a focus on reproductive health, more recent studies have suggested a link between phthalate exposures and metabolic health outcomes.

Obesogens and Endocrine Disrupting Chemicals

Obesity occurs in nearly 40 percent of US adults and nearly 20 percent of US children.[8] Comorbidities of obesity include cardiovascular disease, hypertension, type II diabetes, and non-alcoholic fatty liver disease (NAFLD). These comorbidities are commonly referred to together broadly as metabolic syndrome. The high prevalence of obesity and seriousness of its comorbidities present a serious risk to public health. Although obesity risk can largely be attributed to genetics and poor nutrition, a growing body of evidence has implicated exposures to environmental chemicals as contributors to the rising obesity epidemic.[9,10] Chemicals that have been associated with obesity and other metabolic outcomes have been termed “obesogens” and include endocrine disrupting chemicals (EDCs).[11] EDCs interfere with the activities of hormones involved

in metabolism and feeding behaviors, which in turn influences obesity and metabolic syndrome risk.

Phthalates were originally classified as EDCs due to their antiandrogenic effects. High doses of phthalates during development results in reduced testosterone production and reproductive tract malformations in male rodents.[12,13] More recently, phthalates have been linked to obesity and metabolic syndrome risk in human and animal studies, and therefore phthalates have been identified as potential "obesogens."[14,15] In vitro and animal studies have demonstrated that phthalates and their metabolites are capable of binding and activating peroxisome proliferator-activated receptors (PPARs), which are nuclear receptors that are considered to be master regulators of metabolism. [16–20] Thus, PPAR activation presents a potential mechanism linking phthalate exposure and obesity; however, mechanisms are still not fully understood.

Developmental Origins of Health and Disease

Obesogenic effects of phthalates may be greatest when exposures occur during critical periods of development. The Developmental Origins of Health and Disease (DOHaD) theory suggests that the fetal environment early in development has a strong impact on chronic disease in adulthood.[21] The DOHaD theory stems from the idea that the *in utero* environment primes the organism for the environment in which it will live. There is strong evidence for this theory with respect to EDC exposures, with many studies demonstrating that there are critical periods of development during which an organism is most susceptible.[22–24] It has been postulated that exposures to EDCs during these critical periods may have the greatest impact on obesity risk.[25] Human birth cohort studies, however, have yielded mixed results. Although several studies have found

positive correlations between developmental phthalate exposures and obesity-related outcomes in childhood, others found no associations or negative associations.[26–29] Animal studies, on the other hand, have more consistently demonstrated positive correlations between developmental DEHP exposure and increased body weight, fat accumulation, glucose intolerance, and hepatic steatosis.[30–33] A primary difference between human and animal studies is that to date, animal studies on metabolic effects have focused primarily on single exposures to DEHP, whereas humans are co-exposed to multiple different phthalates, including newer phthalates such as DINP. This co-exposure to phthalate mixtures adds to the complexities of interpreting human epidemiological data. Animal studies incorporating newer phthalates and phthalate mixtures could therefore greatly benefit interpretations of human studies.

Epigenetic Gene Regulation

Epigenetic reprogramming is a potential mechanism linking early-life phthalate exposure and later-life obesity. Epigenetics is the study of mitotically heritable changes in gene expression that are independent of DNA sequence.[34] There are several types of epigenetic modifications to DNA, including chemical modifications on histone proteins (e.g., histone acetylation) and DNA methylation, which have been demonstrated to influence gene expression.[22,35] DNA methylation is the most well-characterized epigenetic modification to the genome. DNA methylation is a covalent modification to the 5’ carbon of cytosine residues (5mC) typically adjacent to guanines (CpGs). The addition of a methyl group to cytosine is catalyzed by DNA methyltransferases (DNMTs) while DNA de-methylation is catalyzed by ten-eleven translocase (TET) and base excision repair (BER) enzymes.[36,37] DNA de-methylation is a multi-step process during

which TET enzymes oxidize 5mC to 5'-hydroxymethyl cytosine (5hmC) and further oxidize 5hmC to 5'-formyl cytosine (5fC) and 5'-carboxyl cytosine (5caC), which can then be removed by BER and replaced with an un-methylated cytosine.[38] In general, high levels of DNA methylation in the promoter region of a gene are associated with repressed expression, whereas low levels of promoter DNA methylation are associated with activation of gene transcription.[39]

Epigenetic modifications are reprogrammed early in development.[40,41] In particular, DNA methylation marks are nearly entirely removed from the genome and then added back on as cells begin to differentiate. Exposures to some EDCs during this time period have been demonstrated to cause persistent changes in the epigenome that were correlated with altered phenotypic changes.[22,42,43] Some animal studies have found that developmental phthalate exposures alter DNA methylation in the reproductive tract to influence adverse health effects,[44] and human birth cohort studies have found associations between prenatal phthalate exposures and altered cord blood DNA methylation at PPARα and at repetitive elements.[45,46] Thus, epigenetic re-programming of DNA methylation represents a potential mechanistic link between early-life environment and increased risk of chronic disease in adulthood, including obesity and metabolic syndrome.

Experimental Design

The research in this dissertation utilized a longitudinal isogenic mouse model of perinatal phthalate exposures in order to 1) inform human birth cohort studies investigating developmental phthalate exposures and metabolic health outcomes, and 2) to elucidate molecular mechanisms linking developmental phthalate exposures and

long-term metabolic health outcomes. The longitudinal mouse model is outlined in **Figure 1.1**. To address gaps in the literature, we incorporated a study design that includes exposures to a newer HMW phthalate that is frequently used as a replacement, DINP, a LMW phthalate, DBP, and a well-studied HMW phthalate, DEHP. We also incorporated two mixture exposures; one mixture of the two HMW phthalates (DEHP+DINP) and one mixture of all three phthalates (DEHP+DINP+DBP).

The exposure route chosen was through the diet, which is far less stressful for pregnant mice than oral gavage,[47] and results in a gradual daily dose as opposed to a bolus one-time daily dose to more closely mimic human exposures. To select the exposure level, we targeted a dose that was both likely to result in metabolic syndrome or obese phenotype as well as one that was human-relevant. Based on a review of the animal literature evaluating metabolic effects following perinatal DEHP exposure, we selected a dose of 5 mg/kg-day.[32,33,48] This dose results in amniotic fluid concentrations of phthalate metabolites that are within the range of those measured in human studies, though on the higher end.[49–55] There are very few studies that have examined developmental DBP exposures and metabolic outcomes and no previous studies have examined developmental DINP exposures and metabolic outcomes. Potency estimates from Hannas et al. 2011[13] indicated that DBP has a roughly equivalent potency to DEHP and that DINP is three times less potent than DEHP with respect to antiandrogenic effects. Thus, a target dose of 5 mg/kg-day was used for DBP and 15 mg/kg-day was used for DINP. Since phthalates were administered through the diet, we worked with Envigo to generate customized diets containing 25 mg DEHP/kg chow, 25 mg DBP/kg chow, and 75 mg DINP/kg chow, based on estimates used in previous

studies from the lab that pregnant mice weigh 25 g on average and consume roughly 5 g of chow per day.[56]

Animals were obtained from an in-house colony of viable yellow agouti (A^{vy}) mice with forced heterozygosity through the male line. In the A^{vy} strain, heterozygous A^{vy}/a mice range in coat color from brown to mottled to yellow depending on epigenetic marks, including DNA methylation, in a cryptic promoter region containing an intercisternal-a particle (IAP).[57–59] Thus, distribution of offspring coat color can be used as an epigenetic biosensor for developmental exposures.[60,61] On the other hand, "wild-type" *a/a* mice are black in coat color and are 93% similar to the C57BL/6 strain.[56]

Virgin female *a/a* mice were randomized onto one of six experimental diets: 1) 7% corn oil control (phytoestrogen-free, modified AIN-93G diet), 2) 25 mg DEHP/kg chow, 3) 25 mg DBP/kg chow, 4) 75 mg DINP/kg chow, 5) 25 mg DEHP + 75 mg DINP/kg chow, and 6) 25 mg DEHP + 75 mg DINP + 25 mg DBP/kg chow. Two weeks after the start of exposure, females were mated to A^{vy}/a males and were kept on their assigned chow through pregnancy, gestation and lactation, resulting in an exposure window spanning the entire perinatal period. Measurements were carried out on offspring at two time points: 1) at weaning on postnatal day 21 (PND21) at the end of the exposure period and 2) at 10 months of age in adulthood long after the exposure had ceased. This allowed for us to characterize early-life impacts of phthalate exposures in mice that were still being exposed as well as impacts of early life exposure that persisted into adulthood. Metabolic measures were mostly confined to *a/a* mice since A^{vy}/a mice are epigenetically predisposed to adult-onset obesity, diabetes, and tumorigenesis.

Aim 1

Animal studies have found that developmental exposures to DEHP result in increased body weight and fat mass, impaired glucose tolerance, disrupted insulin signaling, and increased serum triglyceride and cholesterol levels.[15,32,33,48] Few animal studies, however, have evaluated metabolic effects of developmental DBP exposure and no studies have examined metabolic effects following developmental DINP exposure. Additionally, there are no previous studies that have evaluated metabolic effects of developmental exposures to phthalate mixtures, despite ubiquitous co-exposure in humans. Furthermore, human birth cohorts have revealed relationships between prenatal phthalate exposures and DNA methylation at repetitive elements in infant cord blood,[45,46,62] but there is no animal data to corroborate these findings in humans.

The first Aim of this dissertation sought to fill these gaps in the literature. First, we evaluated body weight in female and male *a/a* and *A^{vy}/a* mice perinatally exposed to DEHP-only, DINP-only, DBP-only, a mixture of DEHP+DINP, and a mixture of DEHP+DINP+DBP in early postnatal life at PND21. We also measured relative liver weights and hepatic triglyceride levels in *a/a* males and females at PND21. We hypothesized that perinatal exposures to these phthalates and phthalate mixtures would result in increased body weight, increased relative liver weights, and increased hepatic triglyceride levels at PND21. In the second part of Aim 1, we used coat color distributions in *A^{vy}/a* mice perinatally exposed to phthalates as a biomarker for an altered epigenome and measured DNA methylation at repetitive elements (IAPs and LINE1) globally in tail tips collected from *A^{vy}/a* and *a/a* mice. we hypothesized that mice

perinatally exposed to phthalates and phthalate mixtures would result in altered coat color distributions and DNA methylation at repetitive elements in tail tissue.

Aim 2

Previous animal studies examining metabolic effects of developmental phthalate exposures have reported metabolic effects at PND21, PND60, 12 weeks, and 27 weeks of age.[31–33,48] To date, there are no reports of metabolic effects in animals at older ages following developmental phthalate exposures, despite trends of increased risk of metabolic syndrome with increased age.[63,64] Furthermore, longitudinal trends in metabolic phenotyping of mice developmentally exposed to phthalates have not yet been documented in animal studies, but human birth cohort studies have found associations between early life phthalate exposures and longitudinal metabolic health outcomes.[65,66]

The research in Aim 2 sought to examine long-term, longitudinal metabolic effects of developmental phthalate and phthalate mixture exposures in mice by following mice until 10 months of age, which corresponds to roughly middle aged, and by measuring metabolic phenotypes across the life course. Longitudinal metabolic phenotyping included body weight, body composition analysis, and glucose tolerance testing. We hypothesized that developmental exposures to phthalates would result in longitudinally increased body weight and body fat, and impaired glucose tolerance. We also hypothesized that mice exposed to phthalate mixtures would experience larger magnitudes of effects than those exposed to single phthalates.

PPARs

Phthalates have been demonstrated to activate PPARs,[16,20,67,68] which are nuclear receptors that activate a multitude of transcription factors that control a wide variety of metabolic processes.[69–72] There are three isoforms of PPARs: PPARα, PPARγ, and PPARδ/β. PPARα expression is highest in the liver, PPARγ expression is highest in adipose tissue, and PPARδ/β is ubiquitously expressed across all tissues at low levels.[73,74] Phthalates have been demonstrated to interact with all three isoforms.[16,20,67,68] PPAR signaling is critical during development,[75,76] and thus phthalate interference with PPAR signaling during this critical time could result in detrimental adverse health outcomes.

Upon ligand binding, PPARs heterodimerize with retinoic acid receptor (RXR) and recruit a multitude of co-factors and enzymes. To facilitate gene transcription, PPARs recruit TET enzymes to the promoter region of target genes to locally alter DNA methylation.[77] If PPARs are activated by phthalate metabolite binding in early development during epigenetic reprogramming, then transcription of their target genes may remain activated into adulthood. PPARs, particularly PPAR-α, are highly expressed in the liver and influence multiple metabolic pathways.[78] Thus, understanding whether PPAR target genes can be reprogrammed in the liver would provide insights into the underlying biology linking developmental phthalate exposures and later-life metabolic outcomes.

Aim 3

Many studies have demonstrated that phthalates and their metabolites can activate PPARs,[16,20,67,68] and others have demonstrated that adulthood DEHP exposure

results in PPAR activation in the liver[79,80] and in adipose.[30] However, only one study to date has examined PPAR activation in the liver following developmental exposure to DEHP,[31] and no studies have evaluated hepatic PPAR activation following developmental DINP or phthalate mixture exposures. Furthermore, only one gene was evaluated for DEHP-related alterations in mRNA expression. With respect to adipose tissue, developmental exposure to a phthalate mixture resulted in increased expression and decreased methylation of one PPAR target gene, *Lpl*, in white adipose tissue (WAT) from rodents at PND60.[81] However, gene expression was not measured earlier in life while exposure was ongoing, and it is unclear whether a change in its expression and DNA methylation were due to the exposure or to the observed phenotype of increased adipocyte volume and size. Thus, the underlying molecular mechanisms linking developmental phthalate exposures to early and later-life metabolic health outcomes are still unclear.

To elucidate molecular mechanisms underlying the association between developmental phthalate exposures and metabolic health outcomes, we used transcriptomics to screen for PPAR target genes that were potentially reprogrammed by phthalate exposures via RNA-sequencing in liver and WAT from mice at PND21 at the end of the exposure period, and at 10 months, long after exposure had ceased. We hypothesized that developmental exposure to phthalates and phthalate mixtures would result in persistent PPAR target gene activation in liver and WAT by altering promoter region DNA methylation (**Figure 1.2**).

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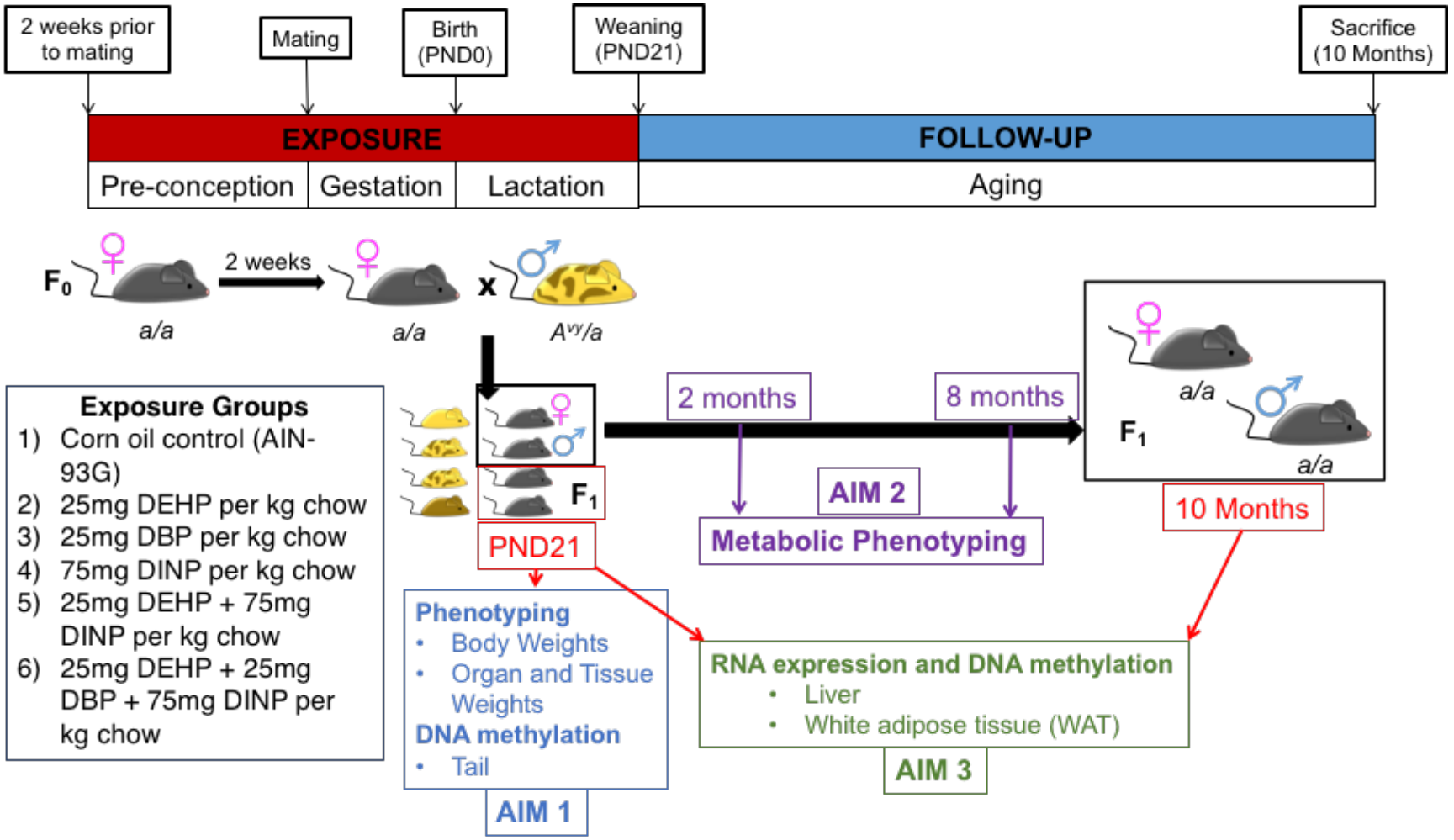

Figure 1.1 Experimental Design: Longitudinal mouse model of perinatal phthalate exposures.

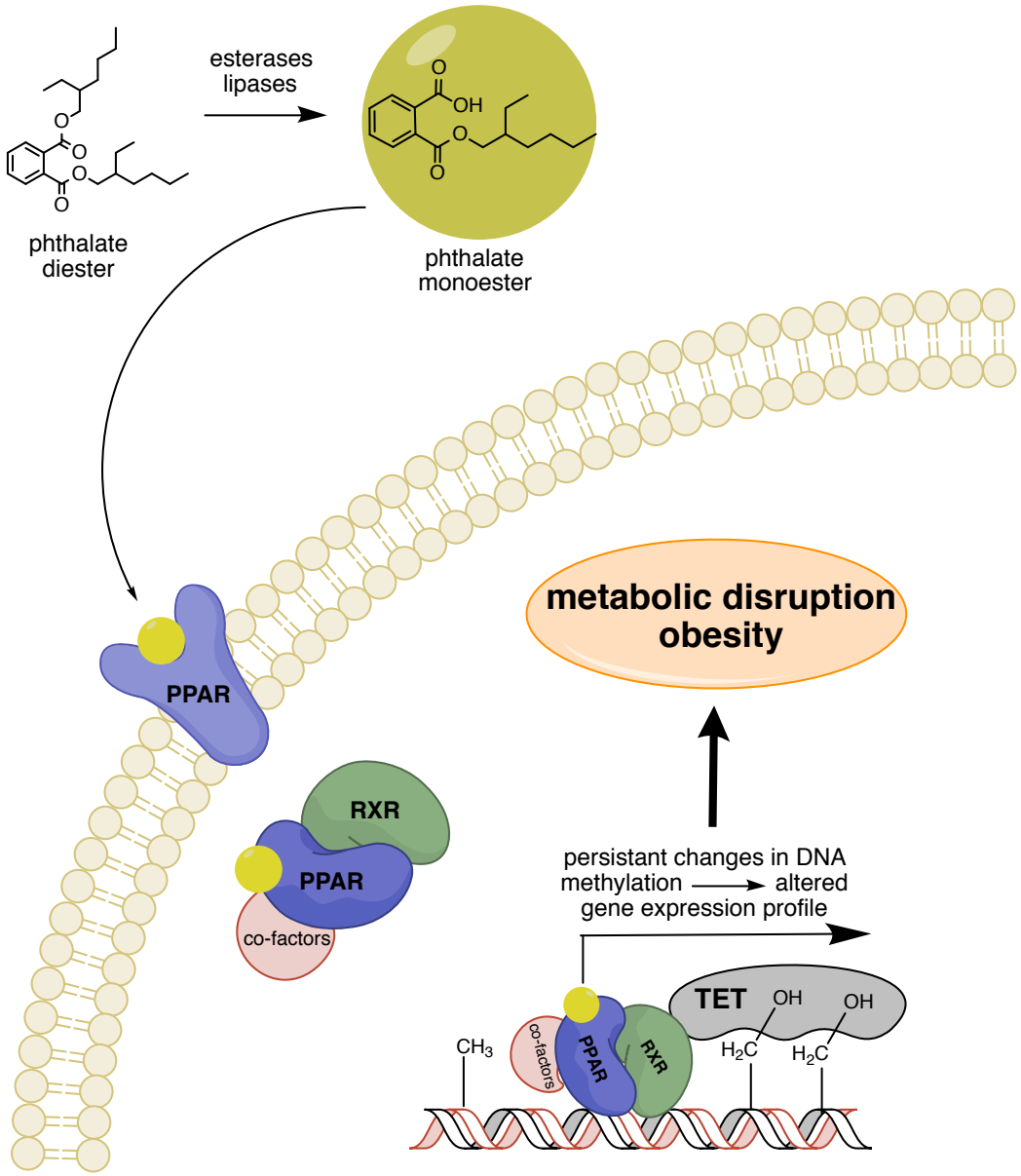

Figure 1.2 Mechanistic hypothesis: developmental exposure to phthalates and phthalate mixtures would result in persistent PPAR target gene activation in liver and WAT by altering promoter region DNA methylation. The primary metabolites of phthalates, phthalate monoesters, have been demonstrated to bind and activate all three PPAR isoforms. Upon ligand binding, PPARs heterodimerize with retinoic acid receptors (RXRs) and recruit tissue-specific transcription cofactors. To facilitate transcription, PPARs recruit ten-eleven translocation (TET) enzymes to locally convert 5'-methylcytosine (5mC) to 5'-hydroxymethylcytosine (5hmC), which is further oxidized and eventually removed by base excision repair machinery (BER) to result in an un-methylated cytosine. Decreased DNA methylation levels are associated with increased gene transcription. Therefore, this local de-methylation in the promoter of PPAR target genes is associated with increased gene expression. If PPARs are activated by phthalates during development, then the resulting alterations in promoter DNA methylation of PPAR target genes may be persist into adulthood, thereby resulting in persistent PPAR target gene activation. Given that PPAR target genes regulate several aspects of metabolism, this disruption in the regulation of their transcription could lead to altered metabolism and obesity later in life.

Chapter 2

Aim 1: Perinatal Exposures to Phthalates and Phthalate Mixtures Result in Sex-Specific Effects on Body Weight, Organ Weights, and IAP DNA Methylation in Weanling Mice

Abstract

Developmental exposure to phthalates has been implicated as a risk for obesity; however, epidemiological studies have yielded conflicting results and mechanisms are poorly understood. An additional layer of complexity in epidemiological studies is that humans are exposed to mixtures of many different phthalates. Here, we utilize an established mouse model of perinatal exposure to investigate the effects of three phthalates, diethylhexyl phthalate (DEHP), diisononyl phthalate (DINP), and dibutyl phthalate (DBP), on body weight and organ weights in weanling mice. In addition to individual phthalate exposures, we employed two mixture exposures: DEHP+DINP and DEHP+DINP+DBP. Phthalates were administered through phytoestrogen-free chow at the following exposure levels: 25mg DEHP/kg chow, 25 mg DBP/kg chow, and 75 mg DINP/kg chow. The A^{vy} mouse strain, along with measurement of tail DNA methylation, was used as a biosensor to examine effects of phthalates and phthalate mixtures on the DNA methylome. We found that female and male mice perinatally exposed to DINP alone had increased body weights at PND21, and that exposure to mixtures did not exaggerate these effects. Females exposed to DINP and DEHP+DINP had increased relative liver weights at PND21, and females exposed to a mixture of DEHP+DINP+DBP

had increased relative gonadal fat weight. Phthalate-exposed *A^{vy}/a* offspring exhibited altered coat color distributions and altered DNA methylation at intracisternal a-particles (IAPs), repetitive elements in the mouse genome. These findings provide evidence that developmental exposures to phthalates influence body weight and organ weight changes in early postnatal life, and are associated with altered DNA methylation at IAPs.

Introduction

Endocrine disrupting chemicals (EDCs) are a class of compounds that are capable of interfering with hormone activity. Exposure to EDCs during development has been linked to increased risk of developing disease in adulthood, following the Developmental Origins of Health and Disease (DOHaD) paradigm.[1] Phthalates are one such example. Phthalates are chemicals added to plastics and personal care products and are present in a wide variety of consumer products, resulting in ubiquitous human exposure.[2,3] Exposure to high levels of phthalates during the period of reproductive tract development leads to malformations in the reproductive tract, including hypospadias and cryptorchidism in males as demonstrated by both animal and human studies,[4,5] and dysregulated ovarian function in females in rodent studies.[6] More recently, human and animal studies have suggested that developmental exposure to lower levels of phthalates may increase the risk of obesity and other features of metabolic syndrome, such as insulin resistance.[7–10] However, epidemiological studies have presented conflicting evidence with some studies demonstrating associations between early-life phthalate exposure and increased risk for obesity and insulin resistance, while other studies found no associations or negative associations.[11,12] One factor that may

contribute to inconsistencies in epidemiological literature is difficulty in interpreting effects of exposures to phthalate mixtures.[13,14] Humans are exposed to mixtures of several phthalates, as evidenced by data from the National Health and Nutrition Examination Survey (NHANES) indicating that metabolites from 13 different phthalates were detected in urine samples from a representative study population from the US.[15] Thus, animal experiments utilizing a mixtures approach to characterize metabolic outcomes resulting from developmental phthalate exposures will aid in the interpretation of human epidemiological studies.

Phthalate exposure during development may influence risk of reproductive and metabolic effects by altering the epigenome. The epigenome consists of heritable factors that influence chromatin structure and gene expression without a change in the underlying DNA sequence. DNA methylation at CpG sites is the most widely studied type of epigenetic factor, and consists of a methyl group attached to the 5' position of a cytosine adjacent to a guanine (C-G dinucleotide). DNA methylation marks are inherited across mitotic cellular divisions and are relatively stable in somatic cells. However, during development, the epigenome is extensively reprogrammed, undergoing waves of DNA de-methylation and re-methylation during pre-implantation and during gametogenesis.[16,17] Thus, exposure to environmental chemicals that interfere with this process may result in an altered epigenome that persists throughout the life course.[18,19] Developmental phthalate exposures have been shown to impact DNA methylation levels at loci near genes that are relevant to hormone signaling, including reproductive and metabolic signaling, in germ cells and offspring in both animal and human studies.[20–24] For example, a recently published birth cohort study identified 27

differentially methylated regions (DMRs) in cord blood from newborns that were associated with gestational exposure to phthalates; many of these DMRs were associated with exposure to multiple phthalates.[23] However, no studies to date have examined the mixture effects of developmental phthalate exposures on DNA methylation.

Repetitive elements are particularly susceptible to epigenetic re-programming via developmental exposures, including intracisternal A-particle (IAP) retrotransposons in mice.[18,25] IAP retrotransposon repetitive elements are present throughout the mouse genome (several thousand IAPs have been detected in the mouse genome),[26,27] and contain long terminal repeats (LTRs) carrying CpG sites. DNA methylation at these CpG sites influences expression and regulation of nearby genes, which could have implications for disease risk. One such IAP is found in the cryptic promoter region of the A^{vy} allele in the viable yellow agouti mouse model (A^{vy}).[28] The A^{vy} mouse model and its use in environmental epigenetics have been thoroughly described in previous studies and literature reviews.[29–31] Briefly, DNA methylation levels at CpG sites within the IAP LTR in the promoter region of the A^{vy} locus are highly correlated with A^{vy} expression and therefore with the coat color of the mouse; yellow fur corresponds to low DNA methylation and high ectopic *agouti* expression, and brown fur corresponds to high DNA methylation and low *agouti* expression. DNA methylation at the A^{vy} locus is stochastic and set during early development. Therefore, the coat colors of mice in a colony are approximately normally distributed. However, coat color distributions can be shifted towards brown or yellow in mice exposed to toxicants or nutrients during early development, acting as a biosensor for altered DNA methylation.[28,29]

Here, we utilized an established mouse model of developmental exposure to evaluate effects of three individual phthalates and two phthalate mixtures on weaning body weight, organ weight, coat color shift, and tail DNA methylation at a specific IAP (*A^{vy}*) and IAPs on a global scale. We chose to examine dietary exposure of two high molecular weight (HMW) phthalates, diethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP), and one low molecular weight (LMW) phthalate, dibutyl phthalate (DBP), as well as a mixture of the two HMW phthalates (DEHP+DINP) and a mixture of all three phthalates (DEHP+DINP+DBP). We evaluated offspring at postnatal day 21 (PND21), which is roughly equivalent to early childhood, in order to provide comparisons to human birth cohort studies that examine metabolic outcomes and DNA methylation in early childhood. To our knowledge, this is the first study to characterize the effects of developmental exposure to phthalate mixtures on weaning body weight, organ weights, and DNA methylation in a mouse model.

Materials and Methods

Animals and Exposures

This study utilized viable yellow agouti (*A^{vy}*) mice as described in Chapter 1.[32,33] *A^{vy}/a* mice range in coat color from yellow to brown based on epigenetic modifications present at the *A^{vy}* locus.[28,31,32,34] Mice were exposed to phthalates perinatally through maternal dietary consumption, with an exposure period that spanned pre-conception to weaning; **Figure 2.1** contains a schematic describing the experimental design used in this Chapter. Two weeks prior to mating, virgin *a/a* dams at six to eight weeks of age were randomly assigned to one of six diets: 1) 7% corn oil control (phytoestrogen-free Teklad diet TD-95092, ENVIGO, Madison, WI), 2) 25mg DEHP/kg chow, 3) 25mg

DBP/kg chow, 4) 75mg DINP/kg chow, 5) 25mg DEHP+75mg DINP/kg chow, or 6) 25mg DEHP+75mg DINP+25mg DBP /kg chow. Dams (*a/a*) were mated with A^{vy}/a males two weeks after being placed on the experimental diets. Dams remained on experimental diets throughout gestation and lactation until weaning. Birth was designated postnatal day 0 (PND0), and pups were weaned three weeks after birth at postnatal day 21 (PND21).

Exposure diets were based on ENVIGO Teklad diet TD-95092, with phthalate diesters (Sigma) mixed into the corn oil used for the chow. Concentrations in chow were intended to result in estimated maternal doses of 5 mg/kg-day for DEHP and DBP and 15 mg/kg-day for DINP, based on the assumption that pregnant and nursing female mice weigh approximately 25 grams and eat approximately five grams of chow per day.[35] These target doses were selected based on studies in the published literature that observed obesogenic effects, including increased body weight, in animals perinatally exposed to phthalates.[36–38] At the beginning of the study, there were no such studies that found metabolic changes consistent with obesogenic effects for DINP; therefore, a three-fold higher target dose of DINP was selected based on its three-fold lower anti-androgenic potency relative to DBP and DEHP.[39] Although fetal exposure was not directly assessed in this study, a previously published study[40] administered 11 mg/kg-day of DEHP to pregnant rats and found mean levels of DEHP metabolites of 68 ng/mL in amniotic fluid. Amniotic fluid phthalate concentrations were likely lower than 68 ng/mL for the present study (estimated daily intake of 5 mg/kg-day), and would therefore be within the range of measured amniotic fluid levels in human populations,[41–47] albeit on the higher end (**Table 2.1**).

A total of 98 litters were generated, with 17 control litters, 16 DEHP litters, 15 DBP litters, 15 DINP litters, 16 DEHP+DINP litters, and 19 DEHP+DINP+DBP litters. Approximately half of the total offspring were *A^{vy}/a* (50.5%, n=312) and half were *a/a* (49.5%, n=296). One DEHP litter (8 pups) and one DEHP+DINP+DBP litter (12 pups) were removed from further analyses based on high pup mortality between PND14 and PND21, which was determined to be due to poor maternal care after animal handlers examined pups. Maternal body weights were recorded at the start of exposure (two weeks prior to mating), at mating, and at time of weaning.

At weaning on PND21, body weights were recorded for all *a/a* and *A^{vy}/a* offspring, and tail tips were collected from all *A^{vy}/a* offspring. A subset of *a/a* mice (n=152) were sacrificed for organ and tissue collection, including tail tips, at PND21. Organs were not collected or analyzed from *A^{vy}/a* mice. For organ and tissue collection, mice were fasted during the light cycle for four hours beginning in the morning, and then underwent CO_2 euthanasia and cardiac puncture in the afternoon, followed by whole-body perfusion with saline (Sigma). Body weights were taken both pre- and post-fasting. Organs were weighed at the time of collection. On postnatal PND21, a single observer classified each *A^{vy}*/a offspring (n=311) into one of five categories based on the proportion of brown fur (yellow, <5% brown; slightly mottled, between 5 and 40% brown; mottled, between 40 and 70% brown; heavily mottled, between 70 and 95% brown; pseudoagouti, >95% brown). Coat color classifications were used as a visual proxy and preliminary assessment of DNA methylation at the *A^{vy}* allele.

All animals were given food and water ad libitum and kept on a 12-hour light/dark cycle. The daily health of the animals was monitored by the University Unit for

Laboratory Animal Medicine (ULAM). Mice were treated humanely, and the guidelines for the use and care of laboratory animals were followed throughout the study. The University of Michigan Institutional Animal Care and Use Committee (IACUC) approved all animal procedures used for this project.

Hepatic Triglyceride Levels

Hepatic triglyceride levels were measured in wildtype non-agouti *a/a* mice that were sacrificed at PND21. Frozen liver tissue (30-35 mg) was homogenized in lysis buffer containing 10% NP-40 (ThermoFisher Scientific), 50 mM Tris-HCl (Sigma), and 100 mM NaCl, and then triglycerides were extracted using chloroform (Fisher). Triglyceride levels were measured using spectrometry; Infinity Triglycerides reagent (ThermoFisher Scientific, Waltham, MA) was added to each sample and then read at 550nm and 660nm and compared to glycerol standards to estimate triglycerides per milligram of liver tissue.

DNA Isolation and Methylation Analysis

Genomic DNA was isolated from PND21 tail tissue, flash frozen, and stored at -80°C from all A^{vy}/a and *a/a* offspring. Approximately 0.5cm of thawed distal tail tissue was placed in a tube containing 173.32 µL of Buffer ATL and 26.67 µL of Proteinase K and then incubated overnight at 50°C on a shaker. Samples were then cooled to room temperature before transferring to the Maxwell® 16 Mouse Tail DNA Purification Kit cartridges (Promega Corporation, Madison, WI) using manufacturers' standard protocol. One modification was made in which only 250 µL of elution buffer was added to the final elution tube to maximize final DNA concentration. DNA concentration was quantified

using the NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA); concentrations ranged from 50-100 ng/μL.

Genomic DNA was bisulfite converted using the EZ-96 DNA Methylation kit protocol (Zymo Research Corp, Irvine, CA), which converts all unmethylated cytosines to uracils and methylated cytosines are left unaffected.[48] For *A^{vy}*/a samples (n=302), 500 ng of DNA was used as input for conversion and 1000 ng was inputted for all *a/a* samples (n=152); both 500 ng and 1000 ng are considered acceptable according to the EZ-96 DNA Methylation Kit, but 500 ng was used for *A^{vy}/a* samples due to lower tissue amounts and subsequent DNA concentrations extracted from some samples. After bisulfite conversion, amplification of *A^{vy}* and global IAP regions was performed by standard PCR in order to attain enough DNA to pyrosequence. Each PCR reaction well included 19.4 μL of HotStarTaq DNA Polymerase (Qiagen Inc., Germantown, MD), 0.12 μL forward primer (Invitrogen, Waltham, MA), 0.12 μL biotinylated-reverse primer (Invitrogen, Waltham, MA), and 13.36 μL H_2O. Two microliters of bisulfite converted DNA were added to each well to complete the 35 μL reaction. Each PCR run included a no-template control and laboratory methylation controls (0%, 25%, 50%, 75%, 100%). Assay specifications are included in **Table 2.2**. PCR amplification lengths were confirmed by the Qiagen QIAxcel Advanced System (Qiagen) and QIAxel ScreenGel software.

Pyrosequencing with the PyroMark Q96 ID (Qiagen) was used to assess DNA methylation percent at four CpG sites of interest for *A^{vy}* and IAP regions on a global scale. The global IAP assay covers all IAPs on the mouse genome with intact LTRs, including the *A^{vy}* IAP. The protocol followed is outlined in the PyroMark Q96 ID User

Manual. PyroMark Software determines the percentage of methylation by calculating the fraction of methylated cytosines (read as cytosine after PCR) over the total sum of methylated and unmethylated cytosines (read as thymines after PCR). The amount of input DNA used for both assays was 16μL. All *A^{vy}* and global IAP assay specifications and pyrosequencing information are available in **Table 2.2**.

Statistical Analysis

All statistical analyses were conducted using R version 3.3.1 (www.R-project.org). Exposure-related differences in sex ratio, genotype ratio, and survival rate were determined by Fisher's exact test, comparing each exposure group to the control group. For sex and genotype ratios, exposure groups were also compared to expected values of 50%, since these are the values expected based on standard genetic probabilities. Coat color distribution across exposure groups was compared via the X^2 goodness-of-fit test with the control coat color distribution as the reference. Coat color distribution analyses could not be stratified by sex because doing so yields some coat color categories within exposure groups having a count of zero, making it difficult to make interpretable statistical comparisons. Differences in litter size across the six experimental groups were compared using an ANOVA with a post-hoc Tukey test. Body weight, organ weights, and DNA methylation for each exposure group were compared to controls using linear mixed effects models with the lme4 and lmeTest packages in R. Mixed effects models were used to account for inherent correlations between littermates, and were also used to account for correlations between DNA methylation levels across nearby CpG sites. Number of pups in each litter was added into models evaluating exposure effects on body weight to control for litter size. Tail *A^{vy}* methylation

levels were bimodally distributed, and we therefore split *A^{vy}* methylation data into high and low methylation groups, based on the median methylation of the control group (i.e., low methylation = methylation level below the control median, and high methylation = methylation level above the control median) in order to make statistical comparisons.[32] All analyses were stratified by genotype so that comparisons were made across isogenic mice. Analyses were also stratified by sex if initial analyses indicated that sex modified exposure effects.

Results

Litter Parameters

Perinatal exposure to phthalates did not significantly alter genotype ratio, sex ratio, or mean pups per litter compared to controls, but did impact mortality rate in pups through weaning at PND21 (**Table 2.3**). Overall mortality rate in controls was 8.3%. Compared to controls, there was a reduced pup mortality rate in the DBP exposure group (mean=1.1%, p=0.02), and increased pup mortality rate in the DEHP exposure group at a level trending towards significance (mean=15.5%, p=0.10). One litter exposed to DEHP and one exposed to a mixture of all three phthalates were removed from subsequent analyses due to high pup mortality between PND14 and PND21 which was attributed to poor maternal care and hyperactive maternal behavior.

Body Weight

Mice that were perinatally exposed to phthalates and phthalate mixtures had increased body weights at PND21 (**Figure 2.2**). Different phthalate exposures exhibited significant impacts on body weight, depending on sex and genotype. Perinatal exposures to DINP and a mixture of all three phthalates were associated with increased

body weights in females (**Figure 2.2A**). Female *a/a* and *A^{vy}/a* mice exposed to DINP weighed more than female *a/a* and *A^{vy}/a* control mice (p = 0.03, p = 0.01, respectively). Wild-type *a/a* and *A^{vy}/a* females exposed to DINP weighed an average of 8.44 and 9.29 grams, respectively, whereas *a/a* and *A^{vy}/a* control females weighed an average of 7.65 grams and 7.77 grams, respectively. Females of both genotypes exposed to a mixture of DEHP+DINP+DBP also had increases in body weight at PND21 that approached statistical significance (p = 0.08 for both genotypes); *a/a* females exposed to all three phthalates weighed 8.42 grams on average and *A^{vy}/a* females exposed to all three phthalates weighed 9.13 grams on average.

In males, phthalate-related body weight effects at PND21 were more pronounced in *A^{vy}/a* mice than in *a/a* mice, with more exposure groups exhibiting significant differences versus controls in *A^{vy}/a* mice than in *a/a* mice (**Figure 2.2B**). Male *A^{vy}/a* mice that were exposed to DEHP, DINP, and DEHP+DINP weighed more than male *A^{vy}/a* controls (p=0.02, p=0.02, and p=0.03, respectively). *A^{vy}/a* males exposed to DEHP weighed an average of 9.38 grams, and those exposed to DINP and DEHP+DINP weighed an average of 9.26 grams, while control *A^{vy}/a* males weighed an average of 8.22 grams. In addition, increased body weights observed in male *A^{vy}/a* mice exposed to DEHP+DINP+DBP (mean = 9.32 grams) approached statistical significance (p=0.06). On the other hand, the only difference in body weight across exposure groups in *a/a* males was an increase in body weight in DINP *a/a* males relative to control *a/a* males that trended towards significance (mean = 8.91 and 7.74 grams, respectively; p=0.07).

Maternal (F_0) body weights were not significantly different in exposure groups versus the control group at the time of exposure, time of mating, or time of weaning,

with or without controlling for number of pups given birth to (p>0.10) (**Appendix Table 2.A1**). However, dams exposed to DINP gained more body weight between mating and weaning than controls at a level near statistical significance (p=0.06) when controlling for the number of pups per litter; dams exposed to DINP gained 9.77 grams whereas control dams gained 8.16 grams. Dams exposed to DINP also gained more weight across the duration of the study (from exposure onset to weaning) than controls (p=0.01), and dams exposed to DBP gained more weight than controls between exposure and weaning at levels nearing statistical significance (p=0.08).

Organ and Tissue Weights

Several organs and tissues were collected from fasted *a/a* mice at PND21; full details on organ and tissue weights can be found in **Appendix Table 2.A2-2.A3**. The most prevalent effect of developmental phthalate exposures on organ weight was observed in female livers (**Figure 2.3; Appendix Table 2.A2-2.A3**). Females exposed to DINP, and DEHP+DINP had increased relative liver weights, expressed as percent of body weight, compared to control females (p=0.02 and p=0.006, respectively). DEHP+DINP+DBP exposed female offspring also exhibited increased relative liver weights vs. controls at a level near statistical significance (p=0.08). Mean relative liver weights of DINP, DEHP+DINP, and DEHP+DINP+DBP exposed females were an average of 4.17%, 4.25%, and 3.99%, respectively, whereas control females had a mean relative liver weight of 3.61%. Hepatic triglyceride levels were measured to determine whether differences in relative liver weights were due to an increase in lipid accumulation in the liver; however, we did not observe any significant differences in hepatic triglycerides across exposure groups (data not shown).

Relative gonadal fat weight was also significantly higher in females exposed to all three phthalates compared to control females (p=0.02); gonadal fat from females exposed to DEHP+DINP+DBP weighed 0.15% of body weight whereas gonadal fat from control females weighed 0.10% of body weight. Relative brain weights were lower in females exposed to DEHP+DINP+DBP compared to controls (p=0.04), and decreases observed in relative brain weights in DINP and DEHP+DINP+DBP exposed males approached statistical significance (p=0.07 for both). Absolute brain weights, however, were not significantly different across exposure groups, with mean brain weights that ranged from 288.7 grams to 305.3 grams for all *a/a* mice. Males exposed to all three phthalates had higher absolute pancreas weights than controls (p=0.05), and females exposed to all three phthalates had higher absolute pancreas weights than controls at nearly statistically significant levels (p=0.07), but alterations observed in relative pancreas weights did not reach statistical significance. An increase observed in absolute spleen weights between females exposed to all three phthalates compared to controls approached statistical significance (p=0.08), but relative spleen weights were not significantly different across exposure groups. Lastly, kidney weights were not impacted by developmental phthalate exposure (p>0.10).

Coat Color Distribution

Developmental exposure to phthalates resulted in altered coat color distributions in A^{vy}/a offspring. Exposure to DEHP, DBP, DEHP+DINP, and DEHP+DINP+DBP significantly altered coat color distributions (p=0.02, p<0.0001, p=0.02, p<0.0001, respectively**; Figure 2.4**), while exposure to DINP resulted in only a modest change in coat color distribution compared to controls (p=0.10). Developmental exposure to DBP

alone (**Figure 2.4C**) and in the mixture (DEHP+DINP+DBP, **Figure 2.4F**) exhibited a pronounced increase in the proportion of pseudoagouti offspring; 6.12% of the control offspring were classified as pseudoagouti, compared with 32.7% of the DBP offspring and 17.9% of the DEHP+DINP+DBP offspring. DEHP exposure (**Figure 2.4B**) showed the most pronounced increase in proportion of slightly mottled offspring, represented by 44.9% compared to 26.5% of the control offspring. There was also a relative increase in slightly mottled offspring among the DEHP+DINP exposed group (32.7%), and the DBP exposed group (36.7%).

A^{vy} DNA Methylation

Molecular analyses of DNA methylation at A^{vy} were used to corroborate the coat color assignments. Mean DNA methylation percent at four CpG sites within the 5' IAP LTR in the promoter of the A^{vy} allele corresponded appropriately to the coat color phenotypes (**Appendix Figure 2.A1**). Mice classified as having a yellow coat color had methylation levels at or approaching 0% (mean=0.73%), and increasing amounts of brown fur corresponded with increasing amounts of methylation. Pseudoagouti offspring had the highest amount of methylation (mean=74.4%). Mean A^{vy} methylation levels across exposure groups, with and without sex stratification, can be found in **Table 2.4**. Distributions of mean methylation for each exposure were not normally distributed, and in many exposure groups followed a bimodal distribution (**Appendix Figure 2.A2**). Thus, methylation for each exposure group was dichotomized at the median methylation value of the control group (≤29.9=low-methylation, >29.9=high-methylation) to more appropriately compare mean methylation values. The high-methylation group in DBP exposed females was significantly greater than control females, with a mean A^{vy}

methylation of 67.37% in highly methylated DBP exposed females and a mean *A^{vy}* methylation of 50.03% in highly methylated control females (p= 0.006). The high-methylation group in females perinatally exposed to DEHP+DINP+DBP also had modestly increased *A^{vy}* DNA methylation compared to control females (60.45% versus 50.03%, respectively; p=0.07). There were no significant differences (p<0.10) observed in males, or among the low-methylation categories.

Global IAP DNA Methylation

After observing phthalate-related differences in DNA methylation patterns at the *A^{vy}* IAP, we measured global IAP methylation levels in tail tips from *A^{vy}/a* (n=302) and *a/a* mice (n=152) to further explore whether developmental phthalate exposures are capable of altering DNA methylation on a global level. Developmental phthalate exposure altered IAP methylation on a global scale, in a sexually dimorphic manner. Developmental phthalate exposure tended to result in decreased IAP DNA methylation in males and increased IAP DNA methylation in females (**Figure 2.5**). Compared to controls, male *A^{vy}/a* mice exposed to DBP had a 1.4% decrease in mean global IAP methylation and DEHP+DINP had a 1.5% decrease in mean global IAP methylation across four CpG sites assayed by pyrosequencing (p=0.03, p=0.01, respectively). In contrast, female *A^{vy}/a* mice exposed to DEHP had a 1.9% increase and DEHP+DINP had a 2.2% increase mean DNA methylation relative to controls (p=0.03, p=0.01, respectively). Female *A^{vy}/a* mice exposed to DINP had increased global IAP DNA methylation at levels approaching statistical significance when compared to controls (p=0.09). Trends in mean and site-specific DNA methylation levels were similar in *a/a*

mice, but were mostly non-significant, possibly due to lower statistical power (**Appendix Figure 2.A3**).

Discussion

Perinatal exposure to phthalates resulted in sex-specific and phthalate-specific effects on weaning body weight, organ weights, and tail DNA methylation at IAPs. Exposure to phthalates in mixture form did not appear to have an exaggerated effect for most outcomes tested, with the exception of certain organ and tissue weights. In general, developmental phthalate exposure was associated with an increase in PND21 body weight, especially in mice exposed to DINP. The DINP exposure group showed the most consistent effects on body weight, with both genotypes and both sexes having increased body weight relative to controls; in *a/a* males, the only group that was notably different compared to controls was the group exposed to only DINP. To our knowledge, this study was the first to report on body weight effects in mice following developmental exposures to DINP. Thus, the consistent increases in PND21 body weight we observed in mice developmentally exposed to DINP is a novel finding. One study in rats reported an association between *decreased* body weight gain in adult rats that were exposed to DINP from preconception to weaning; however, the exposure levels investigated in that study were much higher than those used in the present study (0.5% to 1.5% DINP in chow vs. 0.075% in our study).[49] Another study in rats similarly found that exposure to DINP from GD15 to PND10 resulted in decreased weight gain between PND2 and PND10, but the exposure levels examined were also relatively high (20,000 ppm or 2% in chow).[50] EDCs have been demonstrated to exhibit non-monotonic responses and

low-dose effects.[51] It is possible that increased body weight is only associated with lower-level developmental exposures of DINP, such as the levels used in this study.

Only *A^{vy}/a* males had increased body weight following developmental exposure to DEHP alone. Other studies on developmental DEHP exposure in mice have demonstrated mixed effects on body weight, with some studies showing an increase and some a decrease.[36,52] Mice that were exposed to DBP alone did not have significantly different body weights from controls for either genotype or sex. This study is the first to examine body weight effects following developmental DBP exposure in mice, but studies in rats have also demonstrated mixed effects on offspring body weight.[37,53,54]

This is the first animal study to examine the impact of developmental exposures to mixtures of phthalates on body weight. Exposure to phthalate mixtures did not appear to result in an exaggerated effect on PND21 body weight; females exposed to DEHP+DINP did not weigh significantly more than controls, and those exposed to DEHP+DINP+DBP weighed more than controls to a moderate degree of statistical significance. In addition, male *A^{vy}/a* mice exposed to DEHP+DINP during development had increased PND21 body weights relative to controls, and those exposed to DEHP+DINP+DBP had a modest trend towards increased body weight compared to controls, but male *a/a* mice exposed to phthalate mixtures did not have increased body weight relative to controls. It is possible that mixture effects would be more apparent at lower exposures, especially if increased body weight is more prominent at lower exposures of phthalates. Since the mice exposed to mixtures of phthalates had higher total phthalate exposures, it is possible that any additive effects, or lack thereof, could be due to dose effects rather than mixture effects.

Because body composition was not assessed in this analysis, it is unknown whether the observed increases in body weight in phthalate-exposed mice were due to an increase in body fat, lean mass, or both. Additional studies are needed with inclusion of different dosing levels to confirm whether developmental exposures to phthalate mixtures have exaggerated effects on body weight, as well as on body composition. A follow-up study of a subset of wildtype non-agouti *a/a* mice that were aged to 10 months and includes body composition analysis is currently underway in order to assess long-lasting impacts and to determine whether perinatal phthalate exposures impact body fat or lean mass.

Multiple organ and tissue weights were altered by developmental exposures to phthalates in *a/a* mice, and females appeared to be more sensitive than males in this respect. Relative liver weights were increased in females exposed to DINP and DEHP+DINP compared to controls, and were increased in females exposed to DEHP+DINP+DBP at levels trending towards statistical significance. Phthalates have been demonstrated to activate PPAR-α, the dominant PPAR in the liver, in other animal and *in vitro* studies,[55–57] and PPAR-α activation has been associated with increased relative liver weights in investigations of other chemicals.[58] The observed increases in relative liver weights of phthalate-exposed female offspring were likely not due to an increase in lipid accumulation in the liver since hepatic triglyceride levels did not differ across exposure groups. Thus, these findings are consistent with the idea that developmental exposure to DINP and DINP mixed with DEHP activate PPAR-α in the liver, and indicate that females may be more sensitive at this early developmental time point. The mixture of DEHP+DINP had a slightly larger impact on relative liver weights,

but not at a statistically significant level, and the mixture of DEHP+DINP+DBP had only a modest effect on relative liver weights compared to controls. Thus, phthalate mixtures at the exposure levels tested in this study do not appear to have exaggerated effects on relative liver weights at PND21.

On the other hand, the DEHP+DINP+DBP exposure group was the only group that exhibited an alteration in relative gonadal fat weight at PND21, with females showing an increase relative to controls. This observation is consistent with the theory that phthalate exposure increases the risk of obesity, and indicates that a mixture of HMW and LMW phthalates have the largest impact on body fat at PND21, with females being more sensitive than males. A recent meta-analysis of developmental DEHP exposure in animal studies also indicated that early life phthalate exposures results in increased fat weight.[59] Absolute pancreas weights were increased in mice perinatally exposed to DEHP+DINP+DBP compared to controls, but this difference was mitigated when comparing pancreas weights relative to body weight, and therefore the increased pancreas weights might have been due to the modest increased body weights observed in DEHP+DINP+DBP-exposed females. Decreased relative brain weights were observed in females exposed to a mixture of all three phthalates, and to a lesser degree of significance in males exposed to all three phthalates. However, these findings are skewed by the significant and non-significant increases in body weights observed in the mice in these exposure groups, since absolute brain weights did not differ by exposure.

This study was the first to utilize the A^{vy} mouse model as a biosensor to determine whether developmental exposures to phthalates and phthalate mixtures are capable of altering epigenetic marks at repetitive elements in the genome, such as the

IAP located in the *A^vy^* promoter region. Coat color distributions were significantly altered in all groups exposed to phthalates, except for the group exposed to only DINP. Mixtures did not appear to have an exaggerated effect on offspring coat color. Coat color distributions for DEHP and DEHP+DINP were extremely similar to one another, with marked increases in slightly mottled mice in both exposure groups. Coat color distributions for DBP and DEHP+DINP+DBP were also similar to one another, with prominent increases in pseudoagouti mice in both groups. The shift towards pseudoagouti coat color may indicate a protective effect against the yellow obese phenotype in mice perinatally exposed to DBP and DEHP+DINP+DBP; however, we did not follow the *A^vy^/a* mice into adulthood, when coat color-associated metabolic phenotypes emerge, to confirm this. In addition, developmental exposures to phthalates may influence metabolism through other mechanisms distinctive from altered DNA methylation at the *A^vy^* locus. It should be noted, however, that while *A^vy^* hypermethylation in the viable yellow agouti mouse model is likely protective against obesity, hypermethylation in humans is context dependent, and could result in protective, adaptive, or null effects depending on which regulatory or genic region is affected. These data suggested that DEHP and DBP both influenced shifts in coat color, and that DEHP, a HMW phthalate, had different effects from DBP, a LMW phthalate. Consistent with previous studies utilizing the *A^vy^* mouse model to investigate environmental exposures,[29,60,61] we confirmed that coat colors were representative of methylation at the *A^vy^* locus via tail DNA. Our analyses of DNA methylation at the *A^vy^* locus indicated that there was an increase in methylation in the DBP exposure group, which was consistent with the observed increase in pseudoagouti mice.

Tail DNA methylation levels at IAPs were also altered by developmental phthalate exposures on a global level, and in a sexually dimorphic manner. We observed a general trend towards increased global IAP methylation in females exposed to phthalates, but in males exposed to phthalates we observed a general trend towards decreased global IAP methylation. In addition, different individual phthalates had more pronounced effects on global IAP methylation in females versus males. In *A^{vy}/a* mice, developmental exposure to DEHP, and DINP to a lesser degree of significance, resulted in altered global IAP DNA methylation in females, whereas developmental exposure to DBP resulted in altered DNA methylation in males. Phthalate mixtures effects were complex. The DEHP+DINP mixture group had altered DNA methylation in both sexes, while the DEHP+DINP+DBP mixture group did not exhibit significant differences in either sex. Effect sizes for exposure-related changes in global IAP methylation were between 1.4 and 2.2%, and while small, are comparable to effect sizes for DNA methylation at repetitive elements frequently associated with developmental exposures to other environmental chemicals.[62] Similar trends in tail global IAP DNA methylation were observed in *a/a* mice, but we observed less statistically significant differences across exposure groups, likely due to decreased power resulting from lower sample size. It should also be noted that phthalate-mediated alterations in DNA methylation at repetitive elements are unlikely to be the sole drivers of phthalate-mediated metabolic impacts. Because phthalate metabolites have been demonstrated to activate PPARs,[55,56] additional studies investigating DNA methylation at the promoter region of PPAR-α and PPAR-γ target genes in liver and adipose, respectively, would provide crucial mechanistic insights.

A^{vy} methylation levels were not predictive of global IAP methylation levels in this study (R^2 = 0.001, p=0.22 via Pearson correlation test). However, this is not unexpected. The *A*^{vy} locus is just one IAP out of thousands of IAPs that are present in the mouse genome, and previous studies that have demonstrated high variation in DNA methylation across different IAPs.[27] Another study examining perinatal lead (Pb) exposure in mice found different impacts of Pb on individual IAPs;[63] thus phthalates may also have IAP-specific effects that are not fully detected by examining *A*^{vy} and global IAP methylation levels alone. Based on the findings of this study, evaluation of methylation at additional specific IAPs may be warranted, especially those that are near promoter regions of genes that are known to play a role in metabolism.

Phthalates are ubiquitous chemicals that present a significant risk for human exposure, and recent research has suggested that exposure to physiologically relevant levels may influence risk of obesity and metabolic syndrome. Further, phthalates are known EDCs, and high exposures during development have been linked to permanent reproductive tract abnormalities. However, little is known regarding metabolic health outcomes resulting from developmental exposures to phthalates, and even less is known about risks from developmental exposures to phthalate mixtures. In this study, developmental exposures to phthalates in the perinatal window influenced body weight, liver weight, and gonadal fat weight in mice at weaning. Interestingly, females appeared to be more sensitive than males. Tail IAP DNA methylation at the *A*^{vy} locus and on a global level were altered by developmental phthalate exposures, presenting a possible mechanistic link between early-life exposures and potential later-life health outcomes; research focused on characterizing long-term metabolic impacts on a subset of *a/a* mice

is currently underway in order to evaluate this potential. The results from this study are the first to describe metabolic and epigenetic effects of developmental exposure to phthalate mixtures. Mixture effects were complex in that they did not necessarily result in exaggerated effects, and in some cases, phthalate mixtures exhibited attenuated effects. However, this may be attributed to dose effects rather than mixture effects, since the groups receiving mixtures of phthalates received higher exposures to total phthalates. Future work investigating phthalate mixtures at multiple dose levels is needed to better understand these effects, which will aid in the interpretation of human epidemiological studies and further our understanding of molecular mechanisms.

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Tables and Figures

Population	Daily Intake (mg/kg-day)	Amniotic Fluid Levels (ng/mL)
Human Studies	0.0006 (min) 0.002 - 0.005 (range of medians) 0.042 (max)	<LOD (min) 1.6 - 22.1 (range of medians) 100.6 (max)
Calafat et al. (2006) (Rodents)	11 100	68 766
Present Study	**5**	**<68**

Table 2.1 Comparison of DEHP daily intakes and DEHP metabolites in amniotic fluid in human and rodent studies. This table was adapted from the recent National Academies of Sciences, Engineering and Mathematics (NASEM) report on implementing systematic reviews to evaluate low-dose effects of EDCs.[64] Data from human studies includes a variety of populations.[41–47] One rat study[40] examined concentrations of DEHP metabolites in amniotic fluid following oral doses of 11 and 100 mg/kg-day and was used to estimate potential amniotic fluid concentrations in the present mouse study.

	***A^{vy}* Assay[1]**	**Global IAP Assay[2]**
Primer/ Sequence Location (5' to 3')	Chr2: 154761458 strand = reverse	Repetitive Element
Forward PCR Primer	ATTTTTAGGAAAAGAGTAAGAAGTAAG	GTGTTATTTTTTGATTGGTTGTAGTTT
Reverse PCR Primer (biotinylated)	CTACAAAAACTCAAAAACTCA	ACCAAAAATATCTTATAACTACTTATACT
Sequencing Primer	TAGAATATAGGATGTTAG	ATTTTTTGATTGGTTGTAGTTTA
Sequence to Analyze	YGTTATTTTGTGAYGGYGAATGTGGGGGYGGTT	TYGGTYGAGTTGAYGTTAYGGGGAAAGTAGAGTATAAGTAGTTA
Amplicon Length (bp)	294	87
Annealing Temperature	53°C	56°C
Number of Cycles	54	45
Number of CpG Sites	4	4

Table 2.2 PCR and Pyrosequencing Assay Conditions. [1]=Assay adapted from Faulk et al. (2013); [2]=Assay adapted from Montrose et al. (2017)

Exposure Group	# Litters	Pups born	Pups died	Mean pups/litter (+/- SD)	Pup mortality rate (%)	*A^{vy}/a* offspring (%)	Female offspring (%)
Control	17	120	10	7.06 +/- 2.5	8.3	45.0	51.4
DEHP	16	110	17	6.88 +/- 1.8	**15.45^**	51.1	48.9
DBP	15	93	1	6.20 +/- 1.6	**1.1***	53.3	42.4
DINP	15	101	4	6.73 +/- 2.1	4.0	48.0	48.0
DEHP+DINP	16	119	15	7.00 +/- 2.6	12.6	52.9	47.1
DEHP+DINP+DBP	19	123	15	6.47 +/- 2.2	12.2	53.4	51.5

Table 2.3 Litter outcomes: litter size, mortality rates, genotype ratio and sex ratio across exposure groups. *p ≤ 0.05 vs. control, ^p ≤ 0.10 vs. control. Genotype and sex ratios were determined for pups that survived until PND21 and do not include pups not surviving until PND21.

A^{vy} Mean Methylation Percent Across 4 CpG Sites									
Exposure	All A^{vy} Pups	Male A^{vy}	Female A^{vy}	Low-Methylation	High-Methylation	Male Low-Methylation	Female Low-Methylation	Male High-Methylation	Female High-Methylation
Control	31.8 (25.6)	26.8 (25.8)	37.4 (24.5)	10.2 (9.1)	52.4 (17.9)	11.0 (9.7)	8.5 (7.9)	56.7 (18.8)	50.0 (17.6)
DEHP	28.5 (24.8)	25.0 (25.2)	32.1 (24.5)	11.8 (9.2)	54.9 (17.5)	11.9 (9.6)	11.6 (9.1)	58.9 (20.4)	52.6 (16.0)
DBP	40.5 (30.1)	37.7 (29.6)	43.9 (31.1)	10.1 (7.2)	**65.2 (14.7)***	10.1 (6.2)	10.1 (8.9)	63.3 (16.1)	**67.4 (13.4)****
DINP	34.7 (27.3)	35.6 (26.7)	33.4 (28.8)	11.5 (8.4)	60.1 (14.9)	13.7 (8.5)	8.1 (7.5)	61.1 (14.5)	58.8 (16.2)
DEHP+DINP	36.1 (27.0)	35.3 (27.1)	37.4 (27.6)	11.5 (8.3)	58.8 (15.8)	11.2 (8.2)	12.0 (8.9)	59.3 (14.6)	58.1 (18.1)
DEHP+DINP +DBP	35.2 (26.9)	33.0 (27.3)	37.3 (26.8)	11.2 (8.1)	59.3 (14.3)	9.9 (8.5)	12.5 (7.8)	58.0 (15.5)	**60.4 (13.6)^**

Table 2.4 Methylation Percent for *A^{vy}.* Reported as percent mean methylation (standard deviation). **p<0.01 versus control, *p<0.05 versus control, ^p<0.10 versus control.

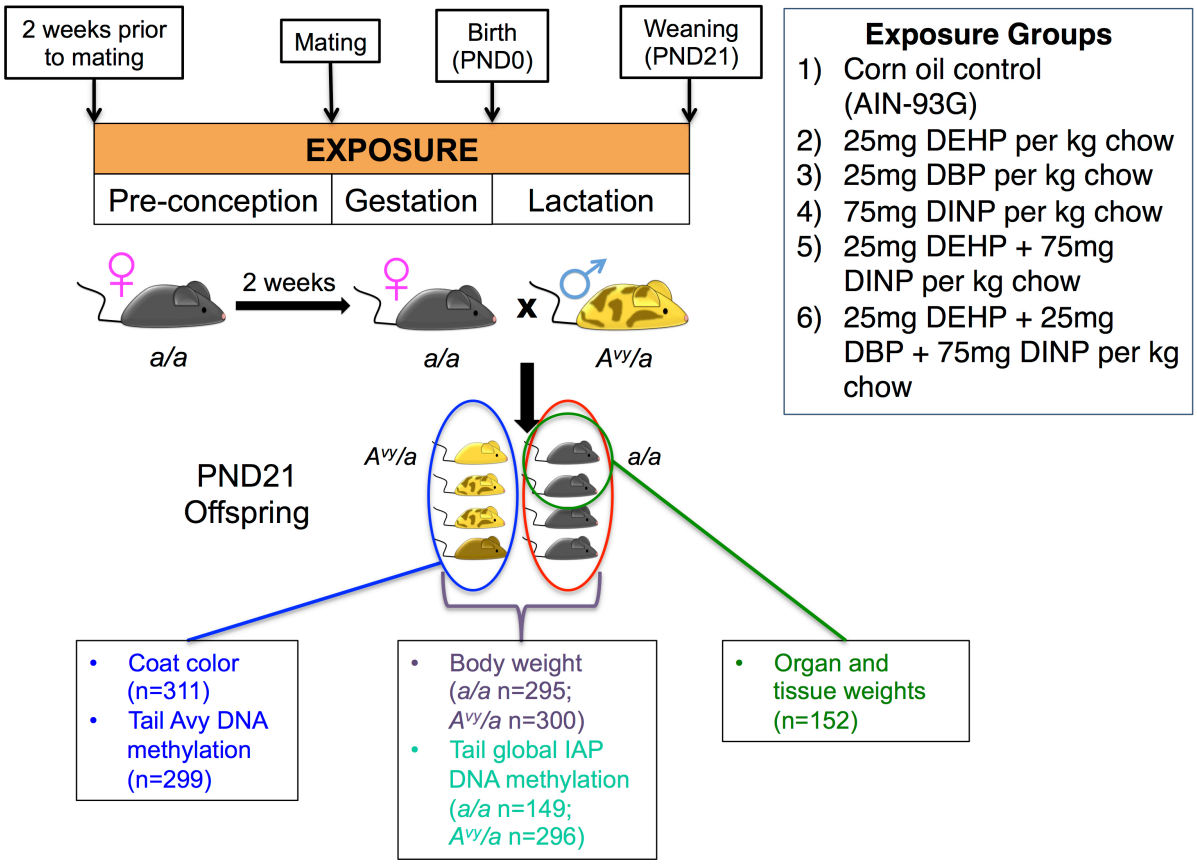

Figure 2.1 Experimental Design. Virgin *a/a* dams were given one of six types of chow containing either corn oil or phthalates dissolved in corn oil two weeks prior to mating with an A^{vy}/a male. They remained on this chow throughout gestation and lactation until weaning at PND21. Body weights at PND21 and tail global IAP DNA methylation were analyzed from both A^{vy}/a and *a/a* offspring. Coat color distribution and A^{vy} DNA methylation from tail tips were analyzed in only A^{vy}/a offspring, and organ and tissue weights were analyzed from a subset of *a/a* mice. A schematic of the Avy and global IAP assays can be found in Faulk et al. (2013)[61] and Montrose et al. (2017)[63], respectively.

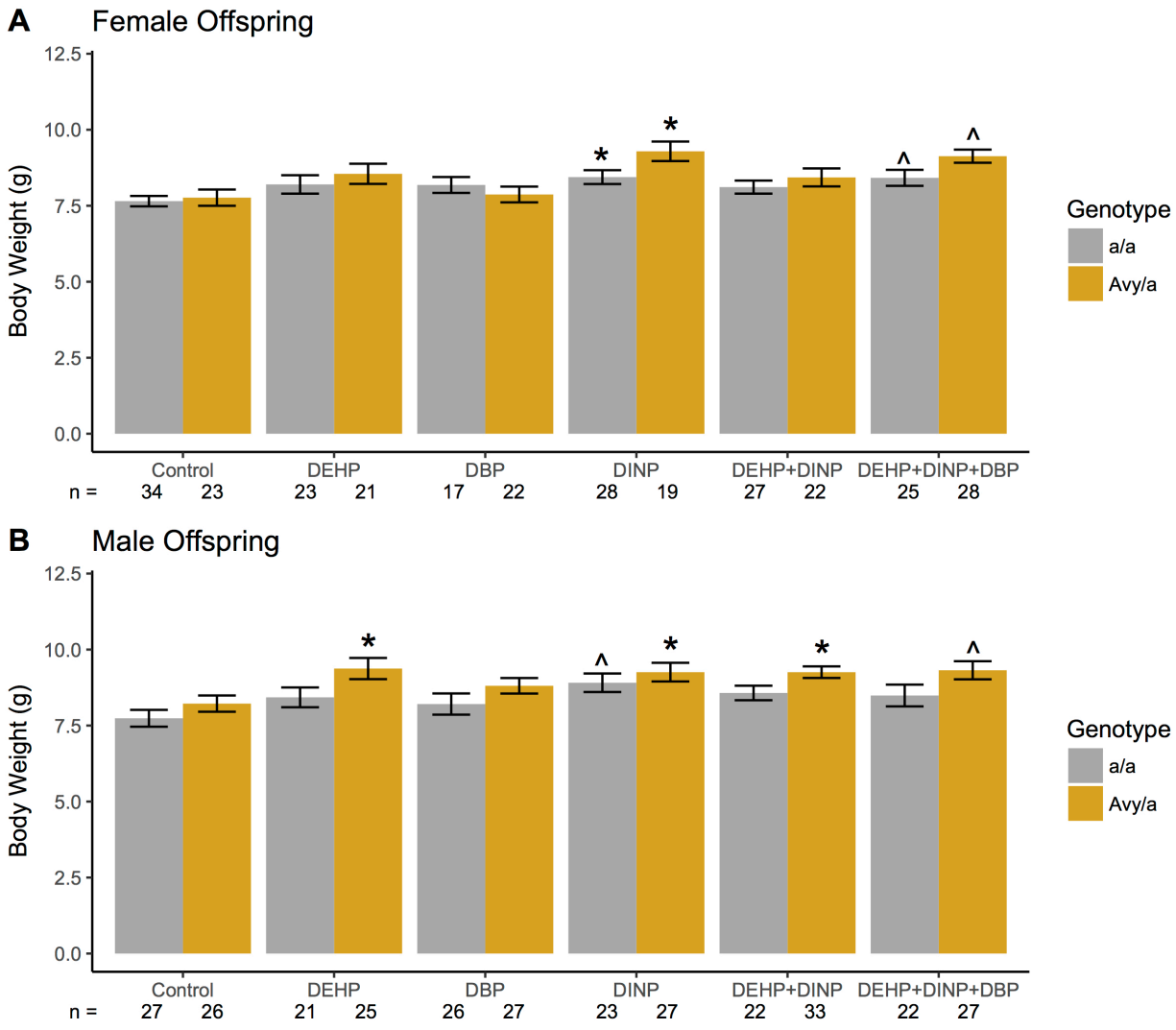

Figure 2.2 PND21 body weights in females (A) and males (B) developmentally exposed to phthalates and phthalate mixtures. Body weights of mice in each exposure group were compared to those of control mice using linear mixed effects models controlling for number of pups per litter and within litter effects. Analyses were stratified by sex and genotype. Bars represent the mean for each group and error bars represent standard error (SE). ^p <0.10, *p < 0.05.

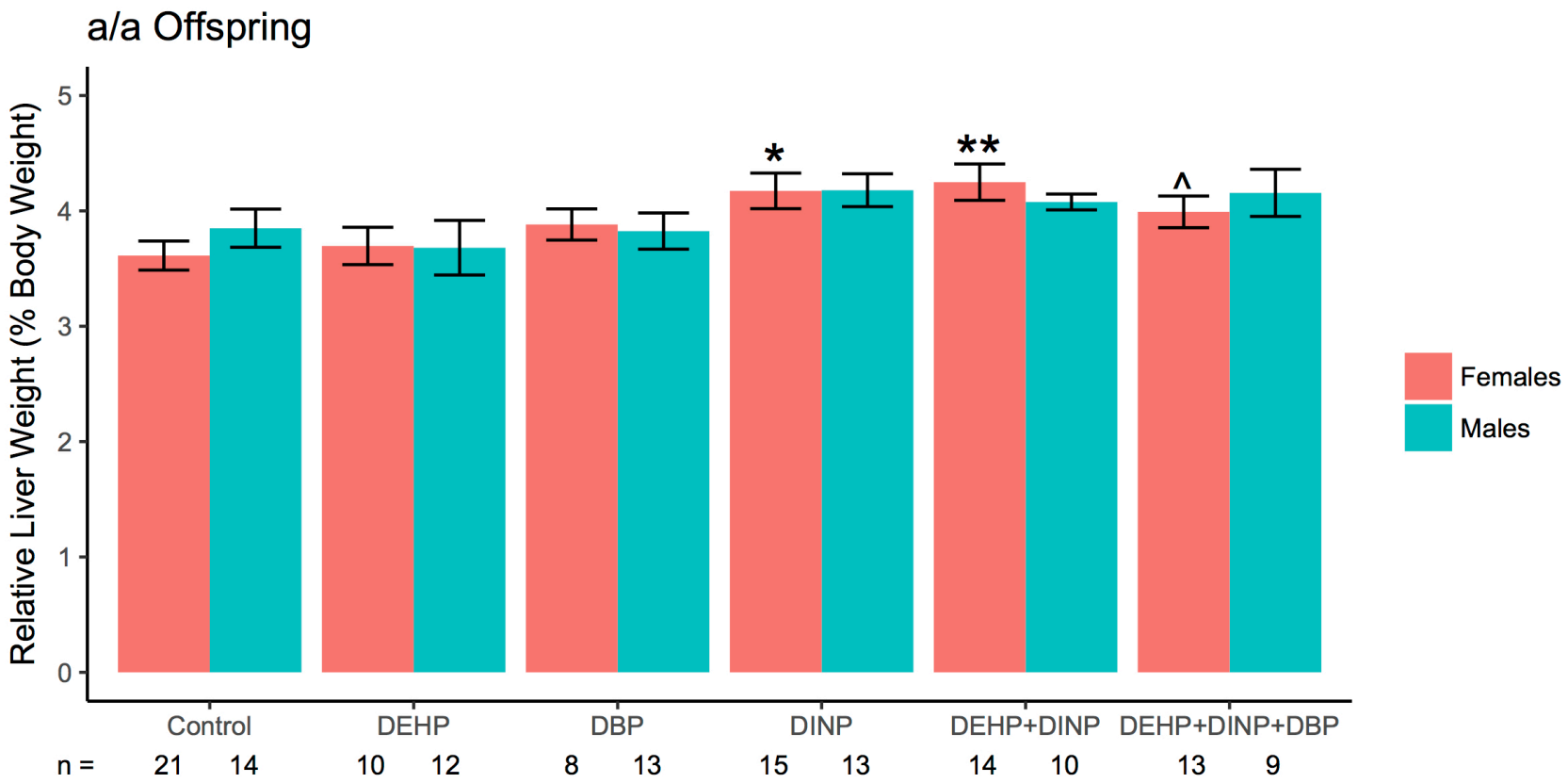

Figure 2.3 Relative liver weights in a subset of PND21 a/a mice (n=152) exposed to phthalates and phthalate mixtures. Relative liver weights are liver weight normalized to body weight, expressed as a percentage. Relative liver weights in exposure groups were compared to controls using linear mixed effects models controlling for within litter effects, and analyses were stratified by sex. Bar height represents mean relative liver weights for each group and error bars represent standard error (SE). ^p <0.10, *p < 0.05, **p < 0.01.

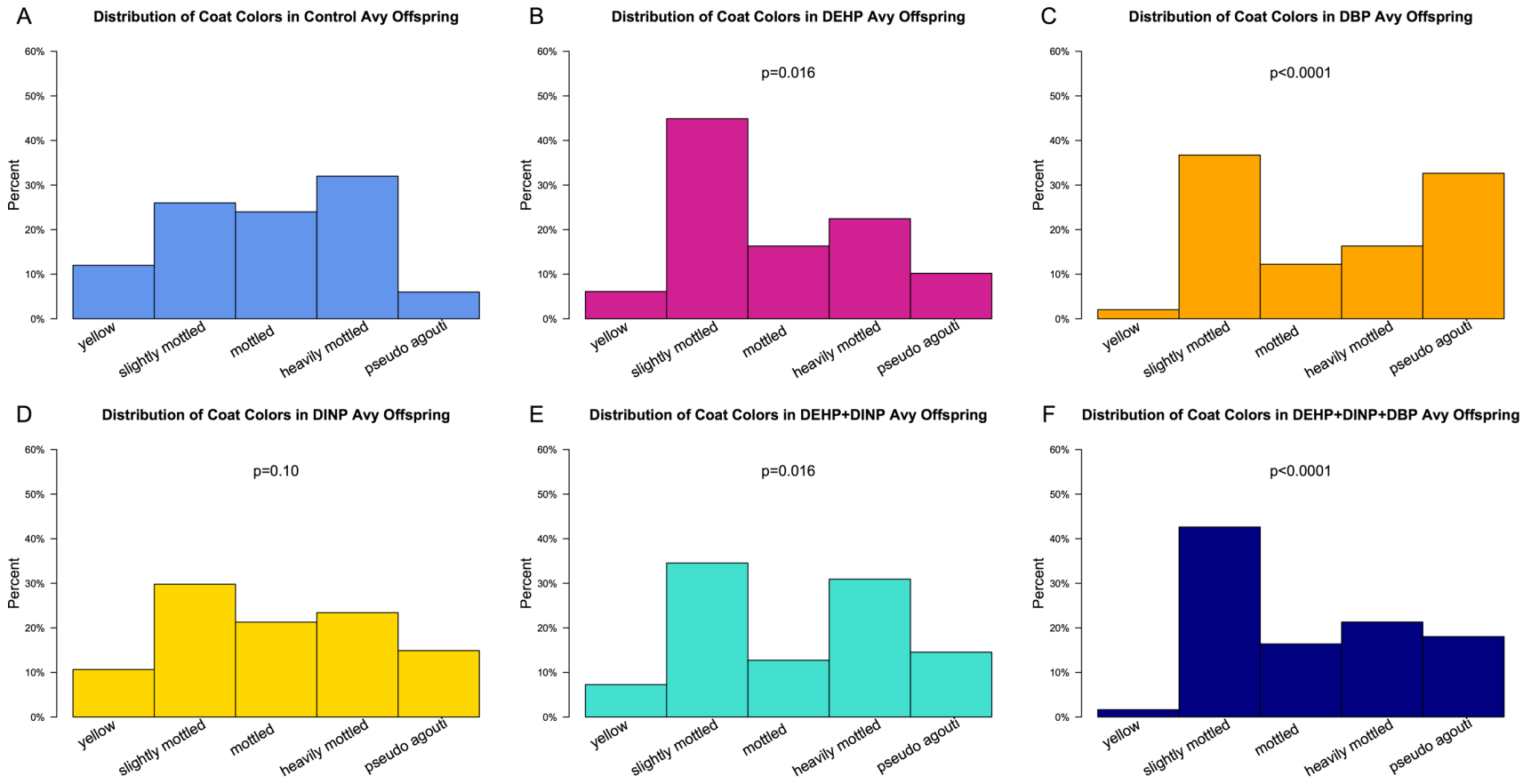

Figure 2.4 Coat color distributions of *A^{vy}/a* offspring across exposure groups. Coat color distributions for each exposure group were compared to the control using a X^2 goodness of fit test. (A) Control coat color distribution. n=16 litters, 50 animals. (B) DEHP coat color distribution. p=0.016. n=14 litters, 49 animals. (C) DBP coat color distribution. p<0.0001. n=14 litters, 49 animals. (D) DINP coat color distribution. p=0.10. n=15 litters, 47 animals. (E) DEHP+DINP coat color distribution. p=0.016. n=16 litters, 55 animals. (F) DEHP+DBP+DINP coat color distribution. p<0.0001. n=17 litters, 61 animals.

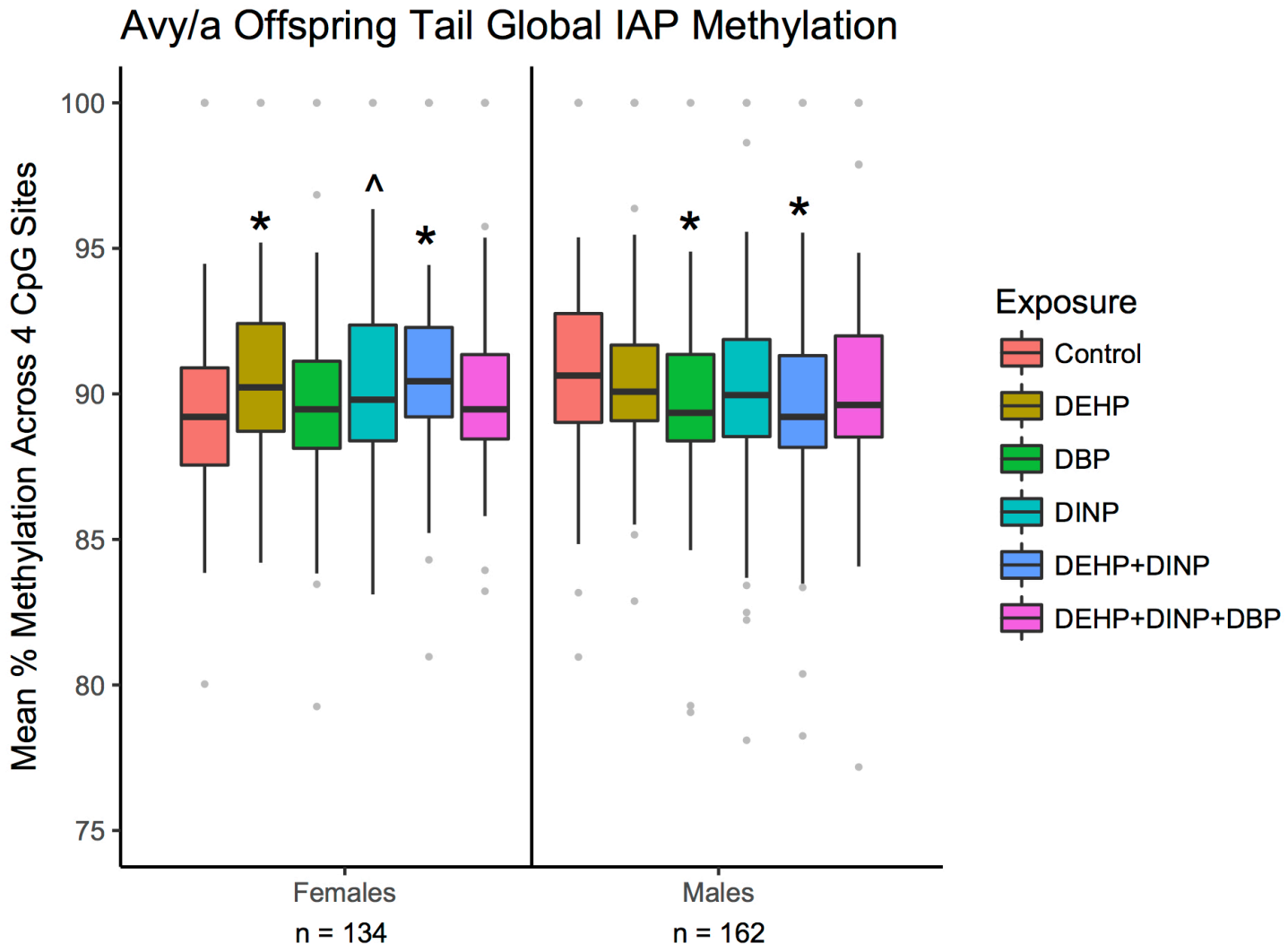

Figure 2.5 Mean tail global IAP DNA methylation across four CpG sites from PND21 *A^{vy}/a* mice across exposure groups. Mean methylation in exposure groups was compared to controls via linear mixed effects models, which take methylation levels at nearby CpG sites into account, as well as within litter effects. Analyses were stratified by sex since initial analyses indicated a significant interaction between exposure and sex. Lines within boxes represent medians and whiskers represent 1.5*IQR. Gray dots are values outside of 1.5*IQR. ^p < 0.10, *p <0.05.

Appendix

	Weight +/- SE (grams)		
Exposure Group	**Exposure Onset**	**Mating**	**Weaning**
Control	18.79 +/- 0.30	18.24 +/- 0.27	26.53 +/- 0.65
DEHP	18.81 +/- 0.35	18.26 +/- 0.33	27.46 +/- 0.91
DBP	18.79 +/- 0.23	18.54 +/- 0.23	27.85 +/- 0.73
DINP	18.53 +/- 0.41	18.30 +/- 0.34	28.04 +/- 0.75
DEHP+DINP	18.65 +/- 0.49	18.04 +/- 0.47	26.31 +/- 0.82
DEHP+DINP+DBP	18.57 +/- 0.29	17.89 +/- 0.31	26.85 +/- 0.70

Table 2.A1 Maternal weight at exposure onset, mating, and weaning. ^p <0.10 versus controls, via multiple linear regression controlling for # pups born. *p<0.05 versus controls, via multiple linear regression controlling for # pups born.

Exposure Group	Organ/Tissue	Sex	N	Mean Organ/Tissue Weight (mg) +/- SE	p-value (vs. controls)	Mean Relative Weight (% Body Weight) +/- SE	p-value (vs. controls)
Control	Liver	Females	21	257.2 +/- 14.3	-	3.61 +/- 0.13	-
		Males	14	265.1 +/- 23.4	-	3.85 +/- 0.17	-
	Pancreas	Females	21	27.0 +/- 1.6	-	0.38 +/- 0.02	-
		Males	13	27.8 +/- 3.9	-	0.41 +/- 0.05	-
	Gonadal Fat	Females	21	7.2 +/- 1.0	-	0.10 +/- 0.01	-
		Males	13	16.0 +/- 4.2	-	0.21 +/- 0.03	-
	Spleen	Females	21	41.4 +/- 5.7	-	0.55 +/- 0.06	-
		Males	14	37.8 +/- 6.0	-	0.52 +/- 0.06	-
	Kidneys	Females	21	114.3 +/- 4.3	-	1.62 +/- 0.05	-
		Males	14	104.4 +/- 5.9	-	1.54 +/- 0.03	-
	Brain	Females	21	294.7 +/- 4.8	-	4.25 +/- 0.13	-
		Males	14	297.5 +/- 7.2	-	4.57 +/- 0.29	-
DEHP	Liver	Females	10	274.1 +/- 25.1	0.53	3.70 +/- 0.16	0.69
		Males	12	275.3 +/- 30.1	0.80	3.68 +/- 0.24	0.80
	Pancreas	Females	10	31.5 +/- 5.1	0.35	0.42 +/- 0.04	0.47
		Males	12	30.1 +/- 2.8	0.65	0.41 +/- 0.03	0.99
	Gonadal Fat	Females	10	10.9 +/- 4.2	0.23	0.13 +/- 0.04	0.27
		Males	12	19.7 +/- 3.9	0.81	0.25 +/- 0.04	0.81
	Spleen	Females	10	55.1 +/- 14.8	0.22	0.69 +/- 0.14	0.23
		Males	11	48.8 +/- 10.8	0.75	0.62 +/- 0.10	0.77
	Kidneys	Females	10	121.1 +/- 8.7	0.50	1.65 +/- 0.04	0.87
		Males	12	109.8 +/- 7.2	0.68	1.49 +/- 0.07	0.50
	Brain	Females	10	305.3 +/- 7.0	0.20	4.29 +/- 0.26	0.87
		Males	12	300.0 +/- 5.4	0.74	4.18 +/- 0.19	0.44
DBP	Liver	Females	8	290.4 +/- 22.2	0.37	3.88 +/- 0.14	0.32
		Males	13	274.1 +/- 27.9	0.52	3.83 +/- 0.16	0.73
	Pancreas	Females	8	34.8 +/- 4.7	0.22	0.46 +/- 0.04	0.24
		Males	13	31.4 +/- 3.6	0.43	0.44 +/- 0.04	0.56
	Gonadal Fat	Females	8	9.6 +/- 1.2	0.46	0.13 +/- 0.01	0.36
		Males	13	15.7 +/- 3.8	0.64	0.20 +/- 0.04	0.66
	Spleen	Females	8	43.8 +/- 6.9	0.75	0.58 +/- 0.09	0.74
		Males	13	39.0 +/- 6.2	0.81	0.53 +/- 0.05	0.85
	Kidneys	Females	8	116.0 +/- 6.5	0.86	1.58 +/- 0.03	0.70
		Males	13	106.9 +/- 6.9	0.72	1.52 +/- 0.04	0.52
	Brain	Females	8	296.3 +/- 10.8	0.98	4.01 +/- 0.10	0.34
		Males	13	292.8 +/- 5.2	0.51	4.31 +/- 0.20	0.30

Table 2.A2 Organ and tissue weights (Part 1). A random effects model was used to compare each exposure group with the control group, using litter as a random effect and exposure as a fixed effect in order to account for similarities between mice from the same litter. **p ≤ 0.01 vs. controls, *p ≤ 0.05 vs. controls, ^p ≤ 0.10 vs. controls.

Exposure Group	Organ/Tissue	Sex	N	Mean Organ/Tissue Weight (mg) +/- SE	p-value (vs. controls)	Mean Relative Weight (% Body Weight) +/- SE	p-value (vs. controls)
DINP	Liver	Females	15	**328.4 +/- 18.5**	**0.02***	**4.17 +/- 0.15**	**0.02***
		Males	13	329.5 +/- 20.0	0.25	4.18 +/- 0.14	0.38
	Pancreas	Females	15	33.8 +/- 2.5	0.18	0.43 +/- 0.03	0.37
		Males	13	32.0 +/- 3.2	0.39	0.40 +/- 0.03	0.91
	Gonadal Fat	Females	15	9.9 +/- 0.9	0.28	0.13 +/- 0.01	0.29
		Males	13	22.1 +/- 3.7	0.44	0.26 +/- 0.04	0.58
	Spleen	Females	15	56.0 +/- 5.6	0.11	0.70 +/- 0.06	0.12
		Males	13	59.5 +/- 8.5	0.15	0.72 +/- 0.08	0.16
	Kidneys	Females	15	117.0 +/- 6.5	0.56	1.50 +/- 0.07	0.12
		Males	13	124.2 +/- 7.4	0.12	1.57 +/- 0.02	0.63
	Brain	Females	15	303.2 +/- 4.5	0.34	3.91 +/- 0.10	0.11
		Males	13	288.7 +/- 4.6	0.27	**3.76 +/- 0.17**	**0.07^**
DEHP+DINP	Liver	Females	14	**321.1 +/- 22.6**	**0.05***	**4.25 +/- 0.16**	**0.006****
		Males	10	311.3 +/- 8.2	0.36	4.08 +/- 0.07	0.56
	Pancreas	Females	14	31.1 +/- 3.8	0.33	0.40 +/- 0.04	0.55
		Males	10	33.2 +/- 3.9	0.30	0.43 +/- 0.04	0.69
	Gonadal Fat	Females	14	10.0 +/- 1.6	0.34	0.12 +/- 0.02	0.33
		Males	10	18.2 +/- 4.5	0.58	0.23 +/- 0.05	0.67
	Spleen	Females	14	54.9 +/- 6.8	0.34	0.71 +/- 0.08	0.31
		Males	10	52.4 +/- 5.9	0.30	0.67 +/- 0.06	0.26
	Kidneys	Females	14	120.2 +/- 5.6	0.62	1.59 +/- 0.04	0.54
		Males	10	118.9 +/- 5.2	0.23	1.55 +/- 0.04	0.88
	Brain	Females	14	289.0 +/- 5.9	0.46	3.89 +/- 0.15	0.13
		Males	10	300.3 +/- 4.4	0.73	3.94 +/- 0.08	0.14
DEHP+DINP +DBP	Liver	Females	13	**311.3 +/- 19.1**	**0.05***	**3.99 +/- 0.14**	**0.08^**
		Males	9	327.0 +/- 29.6	0.17	4.16 +/- 0.20	0.28
	Pancreas	Females	13	**35.0 +/- 3.9**	**0.07^**	0.45 +/- 0.04	0.18
		Males	9	**38.6 +/- 3.6**	**0.05***	0.49 +/- 0.04	0.14
	Gonadal Fat	Females	13	**12.6 +/- 2.1**	**0.03***	**0.15 +/- 0.02**	**0.02***
		Males	9	19.6 +/- 7.3	0.56	0.22 +/- 0.06	0.87
	Spleen	Females	13	**55.8 +/- 9.0**	**0.08^**	0.68 +/- 0.09	0.11
		Males	9	56.7 +/- 13.1	0.20	0.67 +/- 0.11	0.25
	Kidneys	Females	13	123.0 +/- 7.1	0.20	1.58 +/- 0.04	0.63
		Males	9	119.3 +/- 9.3	0.24	1.52 +/- 0.04	0.59
	Brain	Females	13	291.8 +/- 4.0	0.72	**3.83 +/- 0.14**	**0.04***
		Males	9	295.2 +/- 6.0	0.76	**3.87 +/- 0.21**	**0.07^**

Table 2.A3 Organ and tissue weights (Part 2). A random effects model was used to compare each exposure group with the control group, using litter as a random effect and exposure as a fixed effect in order to account for similarities between mice from the same litter. **p ≤ 0.01 vs. controls, *p ≤ 0.05 vs. controls, ^p ≤ 0.10 vs. controls.

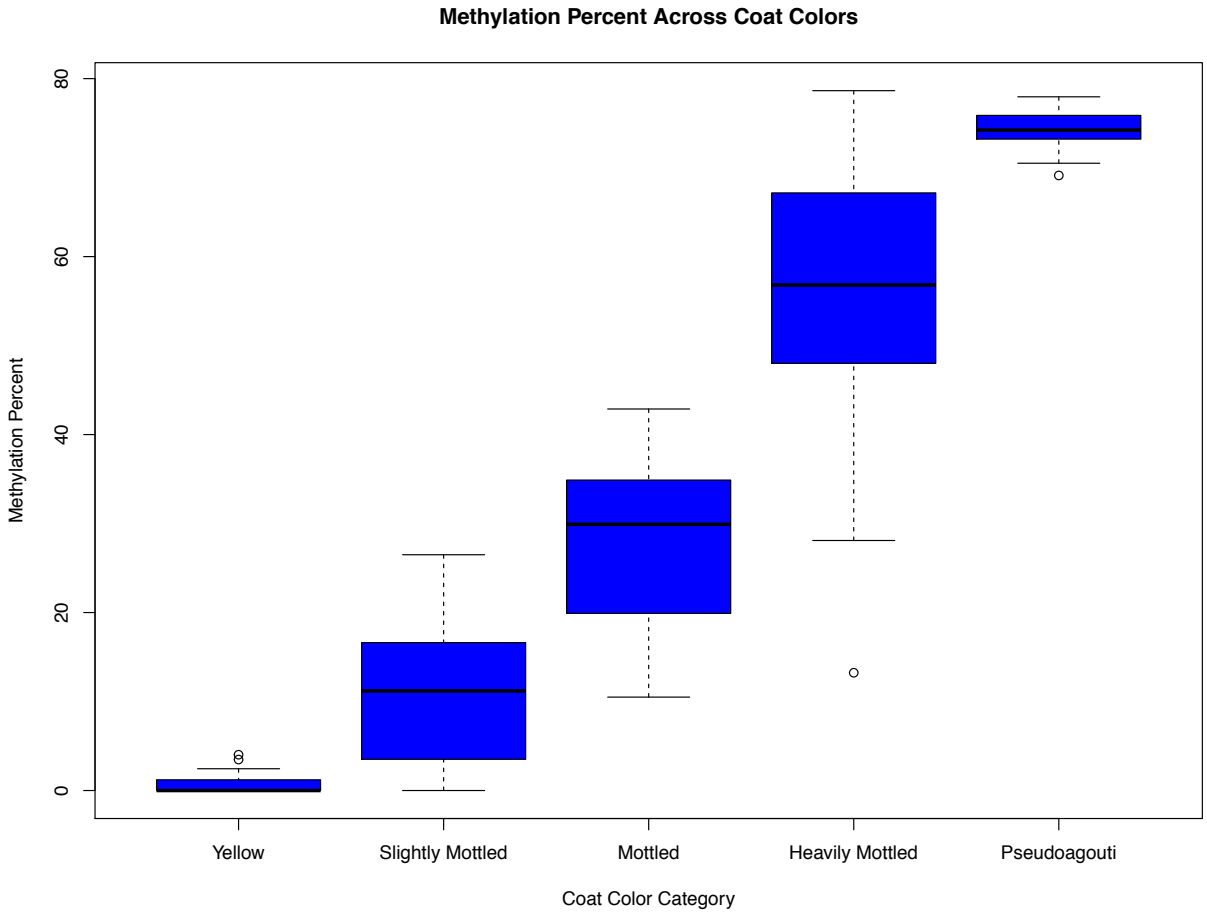

Figure 2.A1 Boxplots for tail Avy methylation percent across coat colors (n=299). Boxes represent the interquartile range (IQR), lines represent the median, whiskers represent maximum or minimum observation if within 1.5*IQR (interquartile range), circles represent values outside 1.5*IQR.

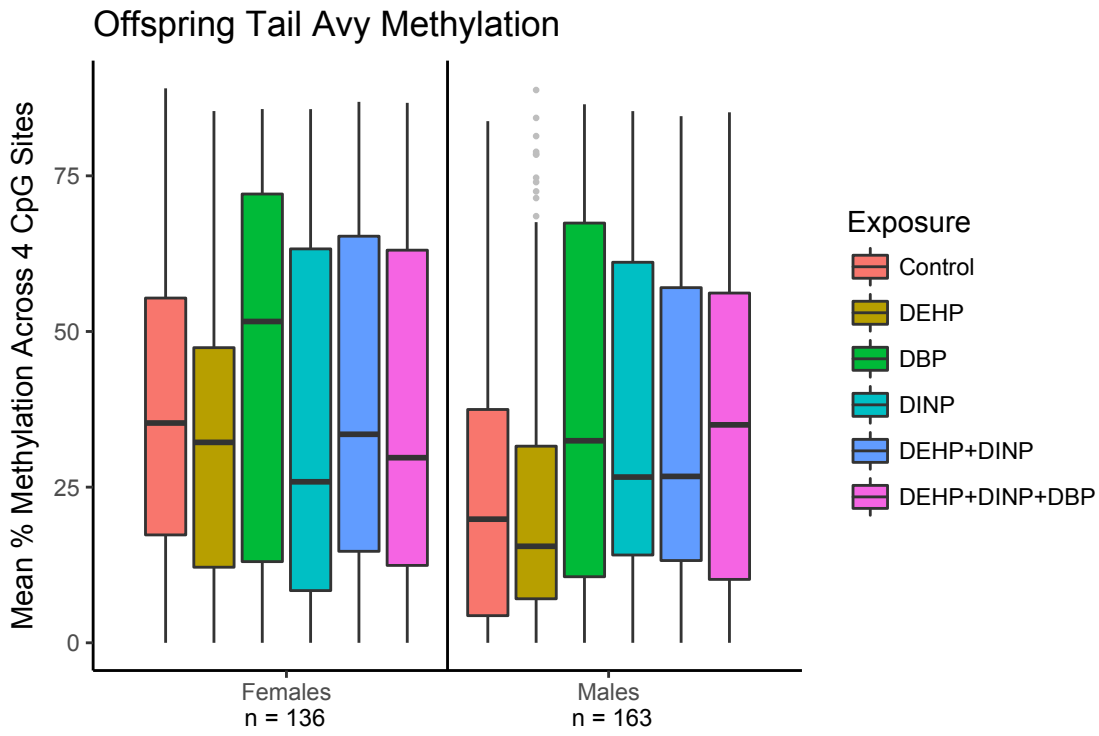

Figure 2.A2 Tail DNA methylation at the A^{vy} locus across exposure groups in A^{vy}/a offspring. Methylation was averaged across four CpG sites at the A^{vy} locus for each mouse. Because A^{vy} methylation was bimodally distributed in several exposure groups, the variation appears to be very large in box plot form. This box plot is most informative when comparing between A^{vy} methylation and global IAP methylation. Lines within boxes represent medians and whiskers represent 1.5*IQR. Gray dots are values outside of 1.5*IQR.

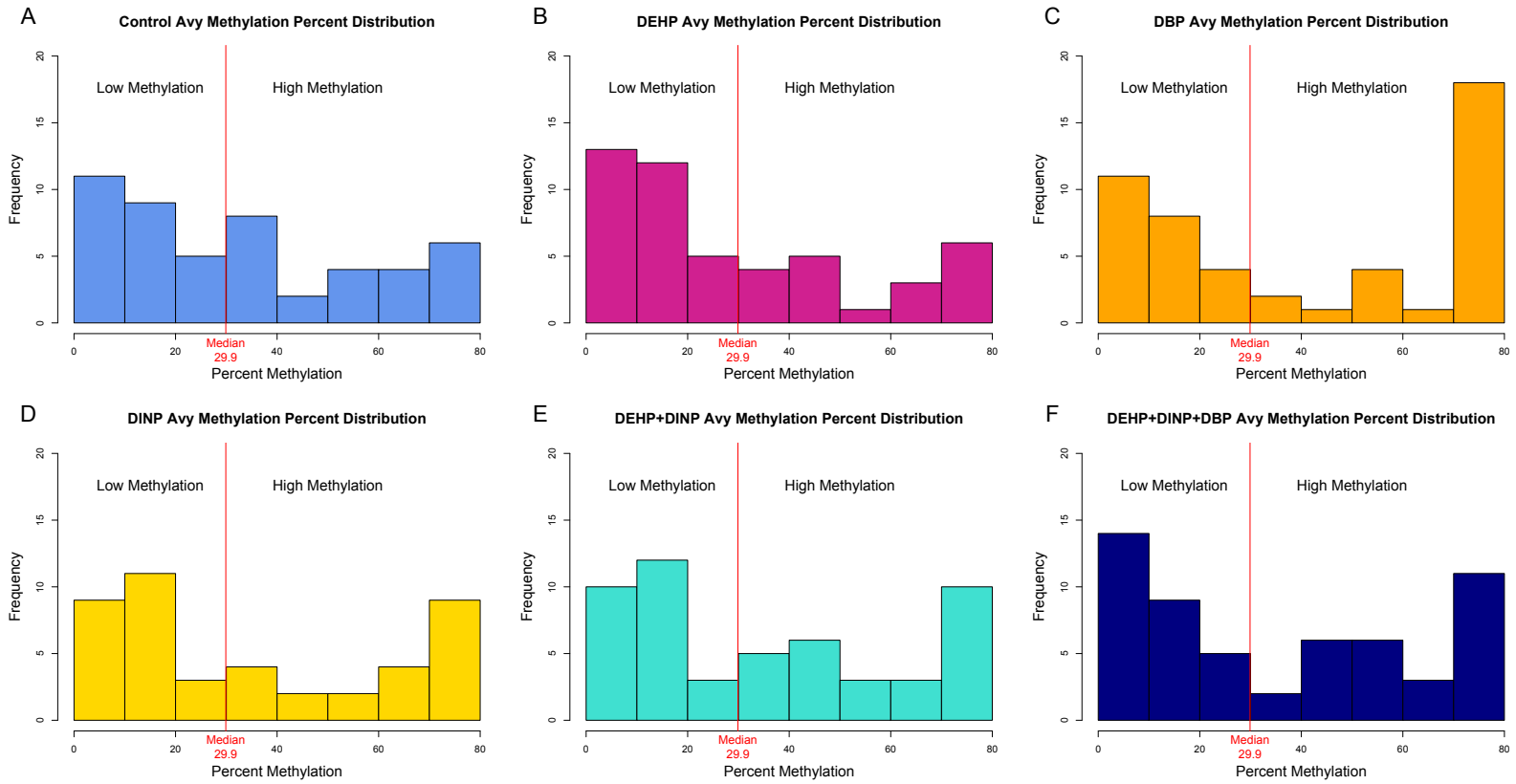

Figure 2.A3 Histograms of mean tail DNA methylation at the *A^vy* locus in each exposure group. A) Controls (n=49), B) DEHP (n=49), C) DBP (n=49), D) DINP (n=44), E) DEHP+DINP (n=52), F) DEHP+DINP+DBP (n=56).

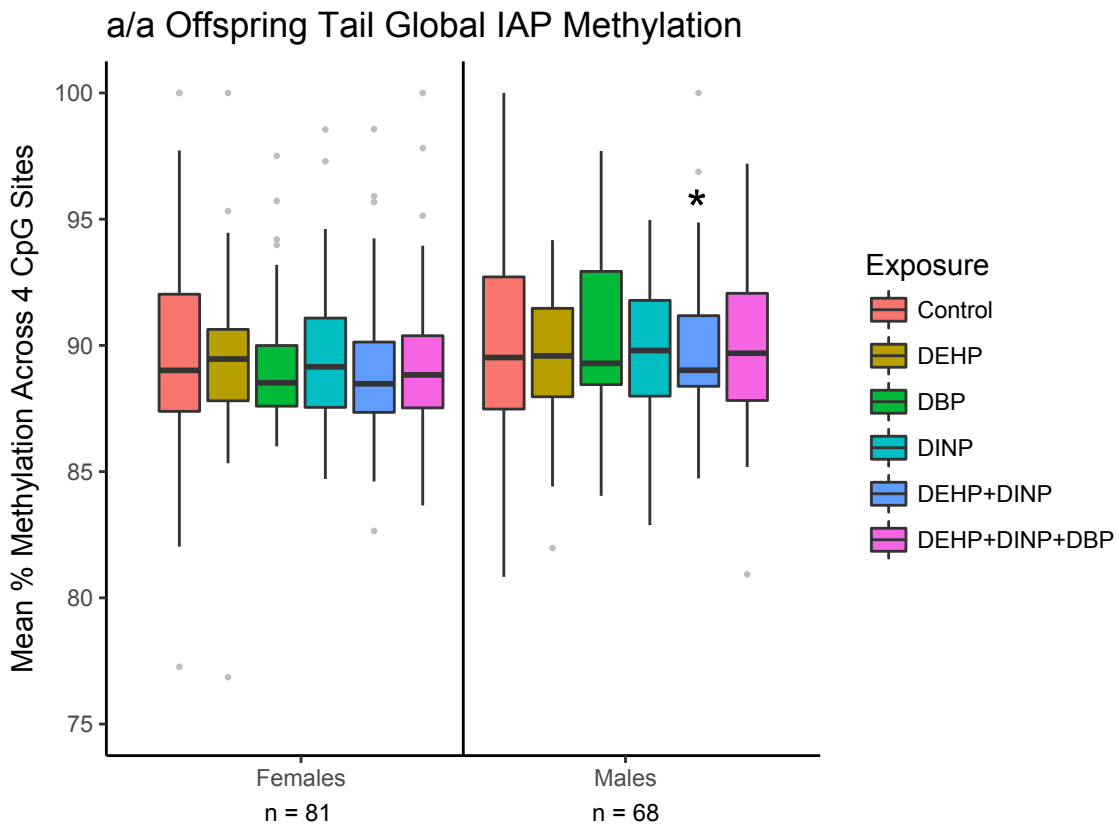

Figure 2.A4 Tail global IAP DNA methylation across exposure groups in *a/a* mice. Mean global IAP methylation across four CpG sites in female (n=81) and male (n=68) *a/a* mice. Linear mixed models were used to compare each exposure group to controls. Lines within boxes represent medians and whiskers represent 1.5*IQR. Gray dots are values outside of 1.5*IQR. ^p < 0.10, *p < 0.05.

Chapter 3

Aim 2: Longitudinal Metabolic Impacts of Perinatal Exposure to Phthalates and Phthalate Mixtures in Mice

Abstract

Developmental exposures to phthalates are suspected to contribute to risk of metabolic syndrome. However, findings from human studies are inconsistent and long-term metabolic impacts of early-life phthalate and phthalate mixture exposures are not fully understood. Furthermore, most animal studies investigating metabolic impacts of developmental phthalate exposures have focused on diethylhexyl phthalate (DEHP), while newer phthalates, such as diisononyl phthalate (DINP), are understudied. We utilized a longitudinal mouse model to evaluate long-term metabolic impacts of perinatal exposures to three individual phthalates, DEHP, DINP, and dibutyl phthalate (DBP), as well as two mixtures (DEHP+DINP and DEHP+DINP+DBP). Phthalates were administered to pregnant and lactating females through phytoestrogen-free chow at the following exposure levels: 25mg DEHP/kg chow, 25mg DBP/kg chow, and 75mg DINP/kg chow. One male and female per litter (n=9-13/sex/group) were weaned onto control chow and followed until 10 months of age. They underwent metabolic phenotyping at 2 and 8 months and adipokines were measured in plasma collected at 10 months. Longitudinally, females perinatally exposed to DEHP-only had increased body fat percent (BF%) and decreased lean mass percent, while females perinatally exposed to DINP-only had impaired glucose tolerance. Perinatal phthalate exposures

also modified the relationship between BF% and plasma adipokine levels at 10 months in females. Phthalate-exposed males did not exhibit statistically significant differences in the measured longitudinal metabolic outcomes. Surprisingly, perinatal phthalate mixture exposures were statistically significantly associated with few metabolic effects and were not associated with larger effects than single exposures, revealing complexities in metabolic effects of developmental phthalate mixture exposures.

Introduction

Obesity prevalence has been on the rise in recent decades, with over one third of US adults being affected.[1] The high prevalence of obesity is a threat to public health, due in part to its numerous comorbidities, including but not limited to metabolic syndrome, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, and some cancers.[2] Poor nutrition, sedentary lifestyle, and genetic polymorphisms are well established risk factors for obesity, but a growing body of evidence implicates a role for exposure to endocrine disrupting chemicals (EDCs) in the obesity epidemic [3–5]. EDCs that have been suggested to play a role in the development of obesity have been termed "obesogens."[5,6] Development is considered to be a particularly sensitive period of exposure to obesogens.[3,4] The developmental origins of health and disease (DOHaD) hypothesis postulates that environmental perturbations during critical periods of development can result in reprogramming of cells and tissues to influence susceptibility to chronic disease.[7] Exposures to obesogens during development have been linked to altered basal metabolic rate, glucose metabolism, energy storage, and food intake, thereby influencing susceptibility to

obesity, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes (T2D), and dyslipidemia in adulthood.[8,9]

Phthalates are classified as obesogens and are found in a wide variety of consumer products, including plastics and personal care products. High molecular weight (HMW) phthalates, such as diethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP), are used primarily as plasticizers and are found in items such as children's toys, medical, and vinyl flooring, whereas low molecular weight (LMW) phthalates, such as dibutyl phthalate (DBP), are used primarily as solvents in items such as shampoo and nail polish.[10] Human exposure to phthalates is nearly unavoidable. Biomonitoring samples collected from women of reproductive age as part of the National Human and Nutrition Examination Survey (NHANES) identified metabolites from over 13 different phthalates and detected at least one phthalate metabolite in all available samples.[11] Developmental exposures to one of these phthalates, DEHP, has been associated with increased body fat and impaired glucose tolerance in rodents.[12–17] Despite indications that human exposures to DEHP are decreasing, while exposure to other phthalates, such as DINP, are increasing[11], there is a lack of *in vivo* data regarding metabolic impacts of developmental exposures to phthalates other than DEHP. Animal studies examining metabolic health outcomes resulting from exposures to phthalates other than DEHP are needed to understand whether other phthalates are also capable of interfering with metabolic processes.

Human birth cohort studies have found inconsistent relationships between *in utero* phthalate exposures and obesity-related outcomes. Some studies have found positive associations between *in utero* phthalate exposures and body mass index (BMI)

and body fat in childhood[18,19], whereas other studies have found no association or negative associations.[20] One challenge in human studies that may contribute to disparate findings is that humans are co-exposed to a mixture of phthalates. Previous animal studies have indicated that exposure to multiple phthalates has a dose-additive or synergistic effect on the reproductive tract.[21,22] However, metabolic impacts following developmental exposures to phthalate mixtures are not well understood. Furthermore, existing human and animal studies have only measured metabolic outcomes during infancy, childhood, or early adulthood, despite strong trends of increased metabolic syndrome risk with increased age.[23,24] Thus, more studies are needed to better understand the extent to which developmental phthalate and phthalate mixture exposures impact metabolism across the lifecourse.

In this study, we utilized a longitudinal mouse model of perinatal exposure to characterize long-term metabolic impacts of perinatal exposures to phthalates and phthalate mixtures in order to inform human birth cohort studies and to complement the current animal literature. We included three exposure groups with individual phthalates and two exposure groups with phthalate mixtures. DEHP was chosen since it is the most widely studied phthalate and still presents high risk of exposure despite recent bans in certain products. Diisononyl phthalate (DINP), another HMW phthalate, was chosen because exposures to DINP have been increasing in recent years[11], and it is often used as a replacement for DEHP due to its structural similarity.[25] We also examined dibutyl phthalate (DBP) as a widely studied phthalate that represents one of the highest levels of human exposures to a LMW phthalate.[11] For mixtures, we examined a mixture of the two HMW phthalates (DEHP+DINP) and a mixture of all three

phthalates together (DEHP+DINP+DBP). Mice were exposed from preconception to weaning and followed out to 10 months of age, far longer than other similar rodent studies.[12,13,15] Multiple metabolic phenotyping measures, including body composition, glucose tolerance, energy expenditure, and food intake, were taken at two time points, early adulthood (2 months) and later adulthood (8 months), so effects could be assessed in a longitudinal manner. In addition, plasma adipokines were measured at 10 months of age. From this study, we aimed to test the following hypotheses: 1) perinatal exposures to DEHP, DINP, and DBP have long-lasting impacts on metabolism, and 2) perinatal exposures to phthalate mixtures have exaggerated effects on metabolism compared to individual exposures.

Materials and Methods

Experimental Design

To evaluate metabolic effects of developmental exposures to phthalates and phthalate mixtures, we utilized a longitudinal mouse model (**Figure 3.1**). Exposure to phthalates and phthalate mixtures was carried out through adding phthalates to chow, and the exposure duration spanned from periconception until weaning. Virgin females aged six to eight weeks were randomized onto one of six diets two weeks prior to mating: 1) phytoestrogen-free 7% corn oil control (Teklad Diet TD95092; Envigo), 2) 25 mg DEHP/kg chow, 3) 25 mg DBP/kg chow, 4) 75 mg DINP/kg chow, 5) 25 mg DEHP + 75 mg DINP/kg chow, and 6) 25 mg DEHP + 75 mg DINP + 25 mg DBP/kg chow. Details regarding exposure level selection are provided below, as well as in.[26] F_0 females remained on their assigned chow throughout gestation, birth, and lactation. At postnatal day 21 (PND21), one male and one female F_1 offspring per litter were weaned

onto control chow and followed to 10 months of age (n=9-13/sex/group). F_1 offspring underwent metabolic phenotyping at two time points across the lifecourse: 2 months and 8 months. At 10 months, F_1 offspring were euthanized; tissues were harvested and blood and plasma were collected via cardiac puncture.

Animals

For this study, we utilized *a/a* mice from a genetically invariant colony of viable yellow agouti (A^{vy}) mice maintained by sibling mating and forced heterozygosity through the male line for over 220 generations resulting in an isogenic background 93% identical to the C57BL/6J strain.[27,28] A^{vy}/*a* mice display a spectrum of metabolic phenotypes based on epigenetic marks at the A^{vy} locus and expression of the A^{vy} allele and have an increased susceptibility to obesity and tumorigenesis,[27,29–31] thus only *a/a* offspring underwent metabolic phenotyping.

A total of 108 mate pairs were set up from which a total of 98 litters were generated. Mean litter size was 6.57 pups/litter and was not significantly impacted by exposure.[26] The largest *a/a* male and largest *a/a* female from each litter were selected for follow-up systematically in attempt to ensure high survival rates and to reduce chances of complications during metabolic phenotyping at 2 months. We selected one mouse per sex per litter for follow-up to avoid controlling for within-litter effects in statistical analyses. Litters were generated until a minimum of 10 male *a/a* offspring and 10 female *a/a* offspring were available for follow-up; however, the DBP exposure group had only 9 females due to difficulty in generating *a/a* females, and one DINP male died after oral gavage during oral glucose tolerance testing (OGTT). A sample size of 10 per sex per exposure group was selected for this study based on previous work carried out

successfully using similar longitudinal mouse models examining perinatal exposures to EDCs.[32] At weaning (postnatal day 21, or PND21), the number of offspring in each group was as follows for females and males, respectively: Control n=13, n=12; DEHP n=11, n=11; DBP n=9, n=11; DINP n=12, n=10; DEHP+DINP n=13, n=12; DEHP+DINP+DBP n=12, n=11; total n=70, n=67. Experimental group was kept blinded to all lab personnel for the duration of the study.

All animal procedures were carried out in an AAALAC accredited facility under approval by the University of Michigan Institutional Animal Care & Use Committee (IACUC) and in compliance with the Guide for the Care and Use of Laboratory Animals. Mice were housed in two different facilities throughout the course of the study: their primarily housing location was at the University of Michigan School of Public Health (SPH) and their secondary location was at the University of Michigan Animal Phenotyping Core (APC). Mice were transported to the APC at 2 months and at 8 months of age and remained at the APC for approximately four weeks each time. They were given one week of adjustment period immediately after transport prior to any metabolic testing and were given one week after metabolic testing before transport back to SPH. Mice were housed in polycarbonate-free static cages with corn cob bedding. All mice were housed with one non-study cagemate throughout the duration of the study, with the exception of 4 days at 2 months of age and 4 days at 8 months of age when animals were undergoing CLAMS measurements. Males were given extra enrichment (Envirodry) to prevent fighting. All mice were provided food and water *ad libitum* throughout the duration of the study and were kept on a 12 hour light/dark cycle at

21°C. Lab personnel and husbandry staff monitored animals on a daily basis and reported health conditions to veterinary staff to ensure high quality of life.

Exposures

Virgin *a/a* females aged six to eight weeks were randomly assigned to one of six experimental groups: 1) phytoestrogen-free 7% corn oil control (Teklad Diet TD95092; Envigo), 2) 25 mg DEHP/kg chow, 3) 25 mg DBP/kg chow, 4) 75 mg DINP/kg chow, 5) 25 mg DEHP + 75 mg DINP/kg chow, and 6) 25 mg DEHP + 75 mg DINP + 25 mg DBP/kg chow. To generate phthalate-containing chow, phthalates were mixed into the corn oil that was used to make the chow. Phthalate-containing chows were also comprised of 7% corn oil and were the same as the control chow in nutritional values. Phytoestrogen-free chow was chosen as the control and background diet to minimize interference of phytoestrogens with molecular pathways that may be impacted by phthalate exposures. Ingestion through chow was chosen as a means of exposure since ingestion is a major route of phthalate exposures,[10,33] to mimic the gradual exposures that humans experience throughout the day as opposed to one bolus daily dose, and to minimize stress to pregnant mice that can incur during other exposure methods (e.g., oral gavage).[34] DEHP and DBP were added to chow at 25 mg phthalate/kg chow and DINP was added at 75 mg phthalate/kg chow, resulting in an estimated dose of 5 mg/kg-day and 15 mg/kg-day, respectively.[34] These estimated doses were based on assumptions used in our previous exposure studies[35] that average consumption rates were 5 grams of chow per day for a 25 gram pregnant female mouse. These perinatal exposure levels were chosen based on levels anticipated to result in effects indicative of metabolic syndrome in offspring observed in

previous rodent studies of DEHP and DBP[13,15] and based on published literature indicating that DINP is approximately ~3 fold less potent than DEHP and DBP with respect to antiandrogenic effects.[22] Furthermore, the exposure levels selected are human-relevant based on extrapolations between rodent and human data of phthalate exposures.[26] Previous rodent studies found mean levels of DEHP metabolites of 68 ng/mL in amniotic fluid of pregnant rats that were exposed to 11 mg/kg-day of DEHP,[36] a dose similar to the phthalate doses utilized in this study. A variety of human studies that measured DEHP metabolites in amniotic fluid found that the median levels ranged from 1.6 to 22.1 ng/mL, with a maximum level of 100 ng/mL.[37–41] Thus, the exposure levels used in this study likely result in human-relevant fetal exposure levels, albeit in the higher range.

Body Weight and Composition

Body weights were measured in F_1 offspring on a weekly basis between weaning (PND21) and 10 months of age. Body composition was measured at ages 2 months, 8 months, and 10 months. At 2 months and 8 months, body fat, lean mass, and free fluid were measured at the University of Michigan APC via a nuclear magnetic resonance (NMR) based analyzer (Minispec LF9011, Bruker Optics). For one subset of mice at 2 months of age (n=22, spread across all exposure groups), the instrument malfunctioned and body composition measurements were not recorded, but data was recorded for all remaining mice (n=114, spread across all exposure groups). Body fat mass and lean mass were measured at 10 months via magnetic resonance imaging (MRI) in the morning prior to sacrifice (EchoMRI). Body fat, lean mass, and free fluid measured via NMR at 2 months and 8 months were used for longitudinal phenotypic analyses,

whereas the EchoMRI measurements taken at 10 months were used primarily as covariates for analyzing plasma adipokine levels. Body fat, lean mass, and free fluid measures were reported as percent of body weight, which was recorded immediately prior to body composition analysis.

Comprehensive Lab Animal Monitoring System (CLAMS)

Respiratory exchange rate (RER), energy expenditure (EE), glucose oxidation, fat oxidation, spontaneous activity, and food intake were evaluated at 2 months and 8 months of age with Comprehensive Lab Animal Monitoring Systems (CLAMS, Columbus Instruments) at the APC. Mice were placed into a CLAMS chamber alone for four days at each time point (2 months and 8 months). The first 24 hours was designated as an acclimation period and therefore the first day was removed from subsequent analyses comparing experimental groups. While in the CLAMS chamber, physical activity was measured as number of times the mouse moves through laser fields, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured using indirect calorimetry, and food intake was measured by weight. VCO_2 and VO_2 were measured once every 20 minutes. RER was calculated as VCO_2/VO_2, and EE, fat oxidation, and glucose oxidation were calculated from VCO2 and VO2 based on previously published methods.[42] Resting metabolic rate (RMR) was calculated for each mouse at 2 months and 8 months of age using a penalized spline model incorporating both EE and ambulatory physical activity as described previously.[43,44]

Glucose Tolerance Testing

Oral glucose tolerance testing (OGTT) was carried out at 2 months and 8 months to evaluate glucose tolerance in F_1 offspring. Mice were fasted for 5 hours prior to

testing, and then administered an oral dose of glucose (2.0 g glucose/kg body weight) via oral gavage. Blood glucose was measured at T_0 (prior to glucose administration), 15, 30, 60, and 120 minutes post-glucose challenge via blood collected from the tail vein. Glucose was measured with an AccuChek (Roche) glucometer.

Plasma Adipokines

Adipokines were measured in plasma collected from F1 offspring at 10 months. Blood was collected via cardiac puncture post CO_2 euthanasia and immediately placed in EDTA-containing microcentrifuge tubes. Blood was centrifuged at 2,000 xg for 10 minutes to separate the plasma; plasma was then stored at -80°C. Leptin, MCP-1, PAI-1, Resistin, IL-6 and TNF-α were measured in plasma using the MILLIPLEX Map Mouse Adipokine Magnetic Bead Panel (Sigma-Aldrich),[45] a multiplex immunoassay that was carried out on a Luminex xMap. Each plasma sample was run in duplicate, per the manufacturer's instructions. Plasma Adiponectin was measured separately via enzyme-linked immunosorbent assay (ELISA) (Sigma-Aldrich),[46] with each sample run in duplicate. Three samples did not have enough sample volume left to analyze plasma Adiponectin levels.

Statistical Analysis

All statistical analyses were carried out using R version 3.5.0 (www.R-project.org). As EDCs, phthalates are expected to have sex-specific effects, so all analyses were stratified by sex. We first carried out cross-sectional analyses on data obtained at 2, 8, and 10 months via multiple linear regression (MLR) comparing each exposure group to controls. However, the primary goal of this study was to assess longitudinal metabolic impacts of perinatal phthalate exposures, and many of the metabolic measures were

repeated at two time points (2 and 8 months). Thus, we utilized linear mixed effects (LME) models to assess longitudinal effects and to account for temporal dependence. To determine whether exposure significantly modified the effect of age, we carried out an additional LME model with an interaction term for exposure*age. To test the hypothesis that mice perinatally exposed to phthalate mixtures exhibit exaggerated effects longitudinally compared to mice exposed to individual phthalates, we used LME models with a simple order constraint on the exposure combinations, the so-called constrained linear mixed effects implemented in the R package CLME. We employed 3 models to make the following sets of comparisons, with each model containing 2 comparisons: 1) Control versus DEHP and DEHP versus DEHP+DINP, 2) Control versus DINP and DINP versus DEHP+DINP, and 3) Control versus DBP and DBP versus DEHP+DINP+DBP. In addition, inter-mouse variability in certain measures was compared at 2 months of age versus 8 months of age via F-test.

A portion of F_1 offspring had gross liver masses at dissection.[47] Models that included data obtained at 8 or 10 months included a dichotomous variable to control for gross liver masses (0=no mass present, 1=mass(es) present), since the presence of gross liver masses significantly impacted several different outcomes. In evaluation of EE, fat and lean mass were also controlled for in each model, since fat and lean mass are both known to significantly impact EE. For glucose and fat oxidation, we examined models both with and without controlling for fat and lean mass. With respect to plasma adipokine levels, interaction terms were added to MLR models to test whether perinatal exposures to phthalates and phthalate mixtures modify the relationship between plasma adipokine levels and body fat percent. Plasma IL-6 and TNF-α had a large portion of

non-detects (17% and 44.3%, respectively). Thus, the Kolmogorov-Smirnov test for comparing empirical distribution functions of IL-6 and TNF-α for each group to controls was carried out.

In discussion of our findings, we considered differences with $p \leq 0.10$ to be marginally statistically significant, and differences with $p \leq 0.05$ to be statistically significant. Because this study included multiple groups, we also set more stringent p-value cut-offs based on the means of familywise type I error control to account for multiple comparisons. For cross-sectional MLR and longitudinal LME analyses which compared each of the 5 groups to controls, differences with $p \leq 0.02$ were considered marginally significant and differences with $p \leq 0.01$ were considered significant when taking multiple comparisons into account. For LME models with ordered constraints we made two comparisons per model and thus differences with $p \leq 0.05$ were considered marginally significant and differences with $p \leq 0.025$ were considered significant.

Results

Litter Parameters and Life Course Morbidity and Mortality

A total of 108 mate pairs were set up to generate mice for this study, of which 98 produced litters (90.7%; **Appendix Table 3.A1**).[47] The number of litters generated per exposure group ranged from 15 to 19. The DINP exposure group had a significantly lower birth rate, with 15 of 21 mating pairings resulting in pups (71.4%) compared to 17 of 17 mate pairings resulting in pups (100%) for Controls ($p=0.02$). Of the 137 total F_1 offspring that were followed until 10 months of age, seven died due to complications with metabolic testing or fight wounds, and one died from unknown causes at two months of age. Upon necropsy at 10 months of age, 22 offspring had observable gross

liver masses (**Appendix Table 3.A1**).[47] One female and one male control offspring had liver masses, representing a background liver mass rate of 8.3% and 9.1%, respectively. Males perinatally exposed to DINP and DEHP+DINP had the highest rate of liver masses (33.3% for both), although this was not significantly higher than the background rate ($p>0.10$).

Body Weight and Body Composition

Longitudinally, there were no significant differences in body weight across phthalate exposure groups in females or males across the life course ($p>0.10$; **Figure 3.2**). Despite a lack of statistical significance, females exposed to individual phthalates exhibited trends towards increased body weight longitudinally. Furthermore, perinatal exposure to phthalates modified the relationship between age and body weight in females. Females perinatally exposed to DEHP-only and DBP-only gained more weight as they aged than controls (interaction $p=0.006$ and $p<0.0001$, respectively; **Figure 3.2**). Interestingly, there was increased variability in body weights of control and exposed females as they aged. F-tests comparing the variance in body weights at 2 months versus variances in body weights at 8 months were statistically significant for control females ($p=0.0001$), DEHP females ($p=0.0004$), DINP females ($p=0.0002$), DBP females ($p=0.0001$), DEHP+DINP females ($p<0.00001$), and DEHP+DINP+DBP females ($p<0.00001$).

Females, but not males, perinatally exposed to individual phthalates had altered body composition in adulthood (**Table 3.1**). Longitudinal analyses indicated that females perinatally exposed to DEHP-only had increased body fat percent ($p=0.01$) and decreased lean mass ($p=0.01$) compared to controls. Females perinatally exposed to

DINP-only had increased body fat percent (p=0.05) and decreased lean mass percent (p=0.03) in comparison to controls, but these differences were not significant after accounting for multiple comparisons (p≤0.01 for significance and p≤0.02 for modest significance) (**Table 3.1).** On average, females in the DEHP exposure group displayed an increase of 3.63% body fat and a decrease of 2.98% lean mass relative to controls, and females in the DINP exposure group had an increase of 2.82% body fat and a decrease of 2.47% lean mass relative to controls. Cross-sectional analyses indicated similar trends in females perinatally exposed to DEHP-only and DINP-only at 2 and 8 months, but these trends were not statistically significant when accounting for multiple comparisons (p>0.02; **Appendix Table 3.A2**).[47] Notably, females perinatally exposed to mixtures of phthalates did not exhibit altered body composition in adulthood (p>0.10).

Food Intake and Physical Activity

Mice perinatally exposed to phthalates and phthalate mixtures did not exhibit notable differences in physical activity or food intake compared to controls longitudinally or cross-sectionally (p>0.05; **Appendix Table 3.A3**).[47] Thus, differences in body composition observed in females exposed to phthalates were more likely due to alterations in intrinsic metabolic processes and not due to differences in energy intake or energy expenditure due to physical activity.

Energy Expenditure and Resting Metabolic Rate

Longitudinally, there was no indication of statistically significant differences in energy expenditure (EE) rates in females or males perinatally exposed to phthalates and/or phthalate mixtures (p>0.05). Cross-sectionally at 2 months of age, females exposed to DEHP-only had average EE rates of 0.378 kcal/hr and controls had average

EE rates of 0.359 kcal/hr (p=0.03), but this difference was not significant when accounting for multiple comparisons. There were no significant differences in EE rates across exposure groups in females cross-sectionally at 8 months of age. Males perinatally exposed to DEHP+DINP had a mean EE rate of 0.431 kcal/hr during the light cycle at 2 months of age compared to control males who had a mean EE rate of 0.390 kcal/hr at 2 months of age; however, this difference was not statistically significant when accounting for multiple comparisons (p=0.05). Cross-sectional analyses at 8 months of age indicated a similar, non-significant trend in light cycle EE rates of DEHP+DINP males (p=0.06).

Females perinatally exposed to phthalates did not exhibit significant differences in resting metabolic rate (RMR) longitudinally. However, at 2 months of age, the mean RMR in DEHP females was 0.318 kcal/hr, which was higher than the mean RMR of 0.295 kcal/hr observed in control females to a modest degree of statistical significance (p=0.02; **Figure 3.3A, 3.3B**). On the other hand, males perinatally exposed to DEHP+DINP trended toward having a higher mean RMR than control males longitudinally (p=0.03; **Figure 3.3D**) and cross-sectionally at 2 months (p=0.04; **Figure 3.3C**), but these differences were not statistically significant after adjustment for multiple comparisons.

Respiratory Exchange Rate, Fat Oxidation Rate, and Glucose Oxidation Rate

Mice perinatally exposed to phthalates did not exhibit a statistically significant altered respiratory exchange rate (RER) or fat oxidation rate when accounting for multiple comparisons. However, females, but not males, perinatally exposed to DEHP-only, DINP-only, and DEHP+DINP trended towards decreased mean RER compared to

controls longitudinally across 2 and 8 months of age (p=0.07, p=0.08, p=0.05, respectively; **Appendix Table 3.A4**).[47] Cross-sectional analyses also indicated that there were trends towards lower RERs at 2 months in females perinatally exposed to DEHP+DINP (dark cycle p=0.03) and at 8 months in females perinatally exposed to DEHP (light cycle p=0.03, average of light and dark cycle p=0.05).

In concordance with decreased RERs, females perinatally exposed to only DEHP, only DINP, and a mixture of DEHP+DINP demonstrated trends towards increased fat oxidation rates longitudinally across 2 and 8 months of age by 5.17, 4.34, and 4.72 mg/hr compared to controls, respectively (p=0.05, p=0.09, p=0.05, respectively; **Appendix Table 3.A4**),[47] but these differences were not statistically significant after adjusting for multiple comparisons (p≤0.01). Similar trends were also evident in cross-sectional analyses, but none of the differences reached statistical significance at p≤0.01. Lastly, males did not exhibit significant differences in fat oxidation rates either longitudinally or cross-sectionally.

Perinatal phthalate exposure was not associated with longitudinal changes in glucose oxidation rates in females (**Appendix Table 3.A4**).[47] Males perinatally exposed to DEHP+DINP exhibited a trend towards increased glucose oxidation rates longitudinally, with an average increase of 14.87 mg/hr compared to controls (p=0.03), but this difference was not significant when accounting for multiple comparisons, and cross-sectional analyses demonstrated only non-significant trends (**Appendix Table 3.A4**).[47]

Glucose Tolerance

Females perinatally exposed to DINP-only exhibited modestly impaired glucose tolerance longitudinally. Across 2 and 8 months of age, females exposed to DINP-only had a mean increase in glucose area under the curve (AUC) by 1404.3 compared to controls (p=0.02; **Figure 3.4C**). Cross-sectional analyses revealed that differences in blood glucose levels during OGTT in females exposed to DINP-only were more pronounced at 2 months of age than at 8 months of age. At 2 months, females exposed to DINP had modestly increased blood glucose levels at 60 minutes post-gavage, and had increased glucose AUC, compared to controls (p=0.02, for both; **Figure 3.4A**). At 8 months, on the other hand, there were no significant differences in blood glucose levels following OGTT across groups in females (**Figure 3.4B**). Of note, DEHP+DINP+DBP females also had a trend towards higher blood glucose levels at 60 minutes post-gavage in comparison to controls at 2 months (p=0.03; **Figure 3.4A**), but this difference was not statistically significant after adjustment for multiple comparisons. Surprisingly, there was a trend of decreased glucose AUC with age in females perinatally exposed to DEHP+DINP compared to controls (p = 0.04; **Figure 3.4C**), suggesting that their glucose tolerance improved with age. Furthermore, at 8 months, females perinatally exposed to DEHP+DINP had a trend towards decreased blood glucose levels at 120 minutes post-gavage compared to controls (p=0.04; **Figure 3.4B**). However, these improvements in glucose tolerance with age were not statistically significant when accounting for multiple comparisons.

Although males perinatally exposed to phthalates and phthalate mixtures did not exhibit significant differences in blood glucose measures longitudinally, males

perinatally exposed to DINP-only had a modest decline in glucose AUC with age in comparison with controls (exposure*age interaction p=0.02; **Figure 3.4F**). Similar to females, males perinatally exposed to DEHP+DINP also exhibited a trend towards decreased glucose AUC with age (exposure*age interaction p=0.04), but this difference was not statistically significant after adjustment for multiple comparisons. Cross-sectionally, there were no differences across exposure groups in glucose levels at any time in 2-month-old males (**Figure 3.4D**), but males perinatally exposed to DBP-only exhibited a modestly decreased blood glucose levels at 120 minutes post-gavage when compared to controls at 8 months of age (p=0.02; **Figure 3.4E**).

Plasma Adipokines

Plasma adipokines did not significantly differ across exposure groups in males or females (**Appendix Table 3.A5-7**).[47] TNF-α and IL-6 plasma levels were too low to detect in many of the samples analyzed for this study; 46.1% of the samples did not have detectable TNF-α levels and 22% did not have detectable IL-6 samples (**Appendix Table 3.A7**).[47] Although there were no statistically significant differences by exposure group for TNF-α plasma concentrations, it was notable that none of the plasma samples from DINP males had detectable levels of TNF-α.

Since adipokines are produced by adipocytes, we next examined whether perinatal phthalate exposures modified the relationship between body fat mass and adipokine levels by examining interactions between exposures and body fat percentage via MLR, controlling for the presence of gross liver masses. Our findings indicated that perinatal exposures modified the relationship between body fat percent and 10-month plasma Resistin and MCP-1 levels in females (**Figure 3.5**). MLR analyses indicated that

perinatal exposure to DEHP-only modified the relationship between body fat percent and plasma Resistin levels in females at 10 months in a positive manner to a modest degree of statistical significance after adjusting for multiple comparisons (p=0.02; **Figure 3.5A**). Females perinatally exposed to DEHP+DINP and DEHP+DINP+DBP also trended toward positively modifying the relationship between body fat percent and plasma Resistin levels (p=0.08, p=0.04, respectively; **Figure 3.5A**). Perinatal phthalate exposure more strongly modified the relationship between 10 month plasma MCP-1 levels and body fat percent in females; DBP-only and DEHP+DINP had significant negative interactions between body fat percent and plasma MCP-1 levels (p=0.006, p=0.0009, respectively; **Figure 3.5B**). Perinatal exposure to DEHP-only also modified the relationship between body fat percent and plasma MCP-1 levels in females to a modest degree of statistical significance in the negative direction (p=0.02), and perinatal exposure to DEHP+DINP+DBP trended towards a negative modification of body fat percentage and plasma MCP-1 levels, though the effect modification was not statistically significant after accounting for multiple comparisons (p=0.03; **Figure 3.5B**). Thus, plasma MCP-1 levels in females perinatally exposed to phthalates increased less with increasing body fat percentage than in controls, potentially indicating that their adipocytes produced less MCP-1.

Longitudinal Mixture Effects

One of the primary objectives of this study was to characterize long-term metabolic impacts of perinatal exposure to phthalate mixtures and to understand whether perinatal exposures to mixtures of phthalates had exaggerated effects compared to perinatal exposures to individual phthalates. Thus, we utilized a

longitudinal mixed effects (LME) model with a simple order constraint to model longitudinal metabolic parameters and test whether perinatal exposure to phthalate mixtures had an exaggerated metabolic response than individual phthalates. LME models demonstrated longitudinal impacts of perinatal exposure to either individual phthalates or phthalate mixtures on body fat percentage, lean mass percentage, and glucose AUC in females (**Table 3.1, Figure 3.4**). There were also trends toward exposure-related effects on RER and fat oxidation in females ($0.02<p<0.05$) and RMR and glucose oxidation in males ($0.02<p<0.05$) that were further explored with simple order restraint models. These models were used to test the following comparisons to test the overall hypothesis that perinatal exposures to phthalate mixtures would have exaggerated effects compared to perinatal exposures to individual phthalates: 1) Control versus DEHP and DEHP versus DEHP+DINP, 2) Control versus DINP and DINP versus DEHP+DINP, and 3) Control versus DBP and DBP versus DEHP+DINP+DBP.

Perinatal exposures to a mixture of DEHP+DINP and DEHP+DINP+DBP did not exert exaggerated effects on body fat percent, lean mass percent, RER, fat oxidation, or glucose tolerance. Overall p-values calculated via the bootstrap likelihood ratio test (LRT) were statistically significant for body fat percent ($p=0.028$, $p=0.020$) and lean mass percent ($p=0.018$, $p=0.017$), for comparisons of control females to DEHP females to DEHP+DINP females and for comparisons of control females to DINP females to DEHP+DINP females (**Table 3.2**). Overall p-values were also significant for RER ($p=0.005$) and fat oxidation ($p=0.003$) when comparing control females to DEHP females to DEHP+DINP females, and they trended toward significance for control

females versus DINP females versus DEHP+DINP females (p=0.055 and p=0.057, respectively). However, these significant overall p-values were driven by the differences between control females and females that were exposed to individual phthalates, namely DEHP and DINP. Individual comparisons indicated that females perinatally exposed to a mixture of DEHP+DINP did not have exaggerated effects compared to females exposed to DEHP and DINP alone (p>0.35 in all instances**; Table 3.2**). Notably, the longitudinal models did not reveal any significant differences in glucose tolerance between exposure groups in females (**Table 3.2**). In addition, overall model p-values and individual comparisons between control females, DBP females, and DEHP+DINP+DBP females were not statistically significant for any of the metabolic outcomes evaluated, which was consistent with previous longitudinal LME models (**Table 3.3**).

Males perinatally exposed to a mixture of DEHP+DINP had significantly increased RMR and glucose oxidation in comparison to those exposed to DEHP or DINP alone (**Table 3.2**). Across 2 and 8 months of age, DEHP+DINP males had an increased RMR by 0.054 kcal/hr versus DEHP-only males (p=0.004) and had an increased RMR of 0.035 kcal/hr versus DINP-only males (p=0.016). Similarly, males perinatally exposed to DEHP+DINP had increased glucose oxidation rates of 17 mg/hr compared to those exposed to DEHP-only (p=0.003) and increased glucose oxidation rates of 10.71 compared to those exposed to DINP-only (p=0.035). However, males exposed to DEHP or DINP individually did not display significant differences in longitudinal metabolic outcomes compared to controls. Comparisons between male controls, males perinatally exposed to DBP-only, and males perinatally exposed to

DEHP+DINP+DBP did not exhibit statistically significant differences via LME models with simple constraints, which was consistent with findings from LME models without constraints and similar to our findings in females (**Table 3.3; Appendix Table 3.A3-A4**).[47]

Discussion

Mice perinatally exposed to phthalates exhibited alterations in metabolism throughout their life course. Metabolic impacts of perinatal phthalate exposures were sex-specific with females showing more sensitivity to phthalate exposures than males. Females exposed to phthalates had increased body fat percent, decreased lean mass percent, and impaired glucose tolerance. One previously published study utilized indirect calorimetry to evaluate metabolic outcomes in mice following direct exposure to phthalates during adulthood,[48] but this is the first study to use indirect calorimetry to characterize life course metabolic effects following indirect developmental exposure to phthalates. This is also the first study to follow mice perinatally exposed to phthalates out until middle age, with longitudinal measurements taken at multiple points throughout the life course. Furthermore, this is the first metabolic study on perinatal phthalate exposures to examine phthalates other than DEHP, as well as mixture effects.

In previous work, we identified a relationship between perinatal exposure to phthalates and phthalate mixtures and increased body weight in offspring at PND21.[26] However, the present study did not find significant persistent differences ($p>0.10$) in body weight longitudinally across the life course in phthalate-exposed offspring compared to controls. It is possible that the differences were too subtle to detect, since the sample size of animals that we followed longitudinally was approximately half of the

number of animals that we recorded body weights for at PND21 (n=~10 vs. n=~20 per group per sex, respectively). The fact that female body weights became more variable as they aged also may have contributed to the lack of statistically significant differences in body weight longitudinally. Despite the lack of a statistically significant direct exposure effect on body weight, we observed non-significant trends towards increased body weight and found that exposure to DEHP-only and DBP-only modified the effect of age on body weight in females, indicating that females exposed to phthalates gained more weight as they aged compared to controls. Other researchers have reported both increased and decreased body weights in adult rats and mice following perinatal exposure to phthalates.[12,13,49] These studies spanned a range of differing mouse strains, including inbred (C3H/N, C57BL6/J) and outbred (CD-1) strains, and also spanned a variety of ages at body weight measurement cross-sectionally (PND21, 8 weeks, 12 weeks), likely contributing to the inconsistency of findings. Our study, on the other hand, utilized weekly weights to analyze body weight longitudinally from PND21 to 10 months of age.

The association between perinatal phthalate exposures and increased body fat percent and impaired glucose tolerance observed in the present study are consistent with previously published studies examining perinatal exposure to DEHP in rodents. Multiple mouse studies demonstrated increased body fat in 2-month-old and 3-month-old mice perinatally exposed to DEHP,[12–14] while other rodent studies have reported associations between developmental DEHP exposure and impaired glucose tolerance in adults.[15–17] One distinctive aspect of our study design was the systematic selection of the largest male and female offspring in each litter. This may have resulted in selectively

measuring animals that were already more prone towards increased body fat accumulation. However, we selected the largest offspring in both control and exposure groups, and as mentioned above, our findings were consistent with other studies that examined similar doses of DEHP. Interestingly, our findings also indicated that perinatal exposure to DINP in males resulted in improvement in glucose tolerance with age when compared to controls. Furthermore, the impaired glucose tolerance observed in females perinatally exposed to DINP was more apparent at 2 months of age than 8 months. Although we did not find significant alterations in absolute levels of circulating adipokines following perinatal phthalate exposures, other studies have reported impacts of perinatal exposure to DEHP on TNF-α and Leptin.[14,50] To date, no other studies have examined circulating plasma adipokine levels in adult rodents perinatally exposed to phthalates, and therefore our findings that perinatal phthalate exposures modified the relationship between body fat percent and plasma MCP-1 and Resistin levels in females are novel. Additional studies examining the relationship between developmental phthalate exposure and adipokine levels are needed to corroborate these findings.

HMW phthalates appeared to more consistently impact the metabolic phenotypes measured in this study than the LMW phthalate we tested. The only statistically significant alterations observed in DBP-exposed females were the positive modification on the effect of age on body weight and a negative interaction between plasma MCP-1 levels and body fat percent at 10 months. The only statistically significant alteration observed in males exposed to DBP was decreased blood glucose at 120 minutes after glucose administration during OGTT at 8 months. In addition, mice exposed to phthalate mixtures containing DBP were not significantly different from controls in any of the

metabolic parameters measured, although there were trends towards a modification of the effect of body fat percent on plasma adipokine levels at 10 months in females. These findings are consistent with *in vitro* studies that demonstrated increased potency in HMW phthalates versus LMW phthalates with respect to activation of peroxisome proliferator-activated receptors (PPARs), which is considered to be an important mechanism for phthalates' obesogenic effects.[51,52] Since we only investigated one LMW phthalate and two HMW phthalates in this study, additional studies examining a larger variety of phthalates are needed to confirm that developmental exposures to HMW have greater impacts on metabolism than LMW phthalates.

This was the first animal study to examine metabolic impacts of perinatal DINP exposure. Previous animal studies have indicated that DINP is capable of endocrine disruption.[22,53] Human and rodent studies have indicated that developmental exposures to DINP is associated with adverse reproductive outcomes,[54,55] and a cross-sectional study on adolescents and teens indicated that direct exposure to DINP was associated with increased insulin resistance.[25] The findings in this study indicate an association between perinatal exposure to DINP and impaired glucose tolerance in females. Females perinatally exposed to DINP also had trends, albeit non-statistically significant after adjusting for multiple comparisons, towards increased body fat percentage and decreased lean mass percentage. Additional studies are warranted to further explore relationships between developmental exposures to DINP and metabolic outcomes, particularly since exposure trends in humans indicate that DINP exposures have been increasing in recent years.[11]

Our findings demonstrated striking sex-specific effects of perinatal phthalate exposure on adult metabolic phenotypes. Previously published rodent studies have also demonstrated sex-specific differences in metabolic outcomes following perinatal phthalate exposures. In concordance with our findings that females, but not males, perinatally exposed to phthalates had increased body fat percent compared to controls, at least one other study also suggested that females are more sensitive to fat accumulation following developmental DEHP exposure.[12] Some studies, however, reported no or minimal sex-specific effects with respect to fat accumulation.[13,14] Investigators who examined glucose tolerance following developmental phthalate exposures found conflicting sex-specific effects. One study exposed mice perinatally to DEHP with a subsequent high fat diet (HFD) challenge, and found that males, but not females, exposed to DEHP and challenged with a HFD had impaired glucose tolerance.[17] Conversely, another study found that female rats, but not male rats, had impaired glucose tolerance following developmental DEHP exposure.[15] Our findings were in concordance with the latter and demonstrated impaired glucose tolerance in females perinatally exposed to phthalates, but not males. Notably, the first study used a higher dose of DEHP (300 mg/kg-day) compared to the present study (estimated dose of 5 mg/kg-day), while doses used by the second study were 1.25 and 6.25 mg/kg-day, which are more comparable to the study presented here. Thus, the sex-specific effects on body fat percent and glucose tolerance that were evident in this study were also generally in concordance with other similar rodent studies that have reported on these metabolic outcomes and may be dose-dependent.

During lactation, it is possible that phthalate-exposed mice experienced “catch-up” growth, which may have mediated the long-term effects of perinatal phthalate exposures on metabolism. Catch-up growth can occur when infants experience growth restriction *in utero*, and accelerated catch-up growth is linked with an increased risk of metabolic disease in adulthood.[56] Perinatal phthalate exposures have been associated with premature birth[57] and low birth weight[58] in epidemiological studies. Thus, *in utero* phthalate exposures may result in in utero growth restriction and consequential catch-up growth to influence later-life metabolism. To avoid disturbing the nests, we did not measure birth weights and thus cannot determine whether catch-up growth during weaning mediated the relationships observed between perinatal phthalate exposures and long-term metabolic outcomes in this study. It is worth noting that other mouse studies did not find statistically significant changes in birth weights of pups exposed to phthalates *in utero*,[59,60] but a potential role for catch-up growth cannot be ruled out.

In females, perinatal phthalate mixture exposures did not result in exaggerated effects compared to perinatal exposures to individual phthalates. This was demonstrated by a lack of significant differences in females exposed to DEHP+DINP and DEHP+DINP+DBP in longitudinal models, and particularly in the simple order constrained models. On the other hand, the only exposure group that exhibited any differences that trended towards statistical significance in males was the DEHP+DINP exposure group. One possible explanation for the lack of effect observed in mixture groups in females is that the lack of mixture effect is really a non-monotonic dose effect. Lower total doses of phthalates perinatally, such as those used in the individual exposure groups, may have larger effects on body composition and glucose tolerance

than higher doses, such as those used in the mixture groups, or in other words, they exhibit a non-monotonic dose effect. Non-monotonic dose responses have been linked with EDCs, including phthalates, in several previous studies.[61,62] Future studies examining multiple doses of each phthalate individually and in mixture form are required to fully understand metabolic effects of perinatal exposures to phthalate mixtures.

Epigenetic reprogramming is considered to be a potential molecular mechanism linking developmental exposures with later-life health outcomes, and has also been explored as a mediating factor driving sex-specific effects of developmental EDC exposures.[63] Previously, we published a study that demonstrated sex-specific alterations in tail DNA methylation at repetitive intracisternal A-particles (IAPs) in weanling mice perinatally exposed to phthalates and phthalate mixtures.[26] Together with the longitudinal metabolic phenotypes presented in the current study, our data suggests that altered DNA methylation at IAPs may be a link between perinatal phthalate exposures and metabolic outcomes later in life. Specifically, females in the DEHP-only and DINP-only mixture exposure groups exhibited increased tail DNA IAP methylation at PND21, and females in these same exposure groups had increased body fat percent and impaired glucose tolerance, respectively, in adulthood. Females perinatally exposed to a mixture of DEHP and DINP also had increased tail DNA IAP methylation at PND21, but metabolic effects observed in DEHP+DINP females were not statistically significant after adjusting for multiple comparisons. In contrast to females, males perinatally exposed to a mixture of DEHP and DINP had decreased tail DNA IAP methylation at PND21 and had trends towards increased RMR and glucose oxidation rates longitudinally compared to controls. Thus, DNA methylation at repetitive elements

in surrogate tissues, such as tail, is a potential biomarker linking perinatal phthalate exposure and metabolic health outcomes.

Although we followed a relatively large number of animals per sex per group (N=9-12), we utilized six experimental groups which required adjustment for multiple comparisons when comparing each exposure group to controls, resulting in relatively stringent p-value cut-offs for statistical significance. Thus, we consider the metabolic phenotypes that we observed in mice perinatally exposed to phthalates to be robust. Additional metabolic outcomes that had p-values less than 0.05 and did not reach statistical significance after adjustment for multiple comparisons included RER, glucose oxidation, and fat oxidation.[47] Future studies evaluating metabolic impacts of developmental phthalate exposures should still consider these as potential outcomes of interest, but fewer experimental groups or a larger sample size may be required to achieve enough power to detect statistically significant effects.

The in-depth metabolic phenotyping measures and longitudinal nature of the study allowed us to characterize long-lasting metabolic changes in mice that were exposed to phthalates and phthalate mixtures perinatally. Females were particularly susceptible to long-term obesogenic effects and impaired glucose tolerance. It is also important to note that there were additional effects in females and males perinatally exposed to phthalates that could be considered beneficial, although many of these effects were not statistically significant after accounting for multiple comparisons. For example, males perinatally exposed to DINP-only had improved glucose tolerance with age compared to controls, and there were trends towards increased fat oxidation in females perinatally exposed to DEHP-only and DEHP+DINP. Furthermore, perinatal

exposures to phthalate mixtures did not result in larger effect sizes compared to perinatal exposures to individual phthalates, but it was not possible to delineate whether this was due to a non-monotonic dose effect or antagonistic mixture effects. A multi-dose mixture study focusing on one or a few of the outcomes identified here would help to disentangle these two possibilities.

Conclusion

Perinatal exposures to phthalates resulted in altered metabolism across the life-course long after exposure had ceased. These effects were sex-specific, phthalate-specific, and had a larger magnitude in mice exposed to individual phthalates compared to those exposed to phthalate mixtures. Compared to controls, females perinatally exposed to DEHP-only exhibited increased body fat percent and decreased lean mass percent longitudinally across 2 and 8 months, while females perinatally exposed to DINP-only had impaired glucose tolerance longitudinally. Females perinatally exposed to DEHP-only and DBP-only had larger increases in body weight with age compared to controls, and males perinatally exposed to DINP-only exhibited improved glucose tolerance with age compared to controls. In addition, at 10 months of age, females perinatally exposed to phthalates had increased plasma Resistin and decreased MCP-1 with increasing body fat percent. The LMW phthalate examined, DBP, appeared to have fewer effects, many of which were distinctive from the effects observed in the mice perinatally exposed to the HMW phthalates. Moreover, mixture effects were difficult to interpret; multi-dose studies are needed to better characterize these effects. Current human birth cohort studies have yielded conflicting results regarding in utero phthalate exposures and obesogenic effects; the findings from this study suggest some

possibilities for these conflicting results, including complex mixture effects. Additional animal and human studies are needed to fully understand mechanisms linking perinatal phthalate exposures and metabolic health outcomes, and to better understand the contribution of developmental phthalate exposures to the high incidence of metabolic syndrome.

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Tables and Figures

		Body Fat %						Lean Mass %					
		2 Months		8 Months		Longitudinal Analysis		2 Months		8 Months		Longitudinal Analysis	
	Exposure Group	**N**	**mean ± SE**	**N**	**mean ± SE**	**β**	p-value	**N**	**mean ± SE**	**N**	**mean ± SE**	**β**	p-value
Females	Control	11	13.05 ± 0.62	13	32.94 ± 1.92	Ref	Ref	11	67.98 ±0.48	13	52.76 ± 1.67	Ref	Ref
	DEHP	9	**15.14 ± 0.81**	11	**36.52 ± 1.24**	**3.63**	δ **0.01****	9	**66.53 ±0.47**	11	**49.66 ± 1.06**	**-2.98**	δ **0.01****
	DBP	8	14.58 ± 0.95	9	37.09 ± 1.70	2.62	0.08^	8	67.17 ±0.63	9	49.26 ± 1.37	-1.96	0.09^
	DINP	10	**15.10 ± 0.80**	12	**34.87 ± 1.57**	**2.82**	**0.05***	10	**66.20 ± 0.78**	12	**51.06 ± 1.34**	**-2.47**	**0.03***
	DEHP+DINP	11	13.61 ± 0.61	13	34.99 ± 1.93	1.68	0.21	11	67.77 ± 0.59	13	50.95 ± 1.58	-1.36	0.20
	DEHP+DINP+DBP	10	14.43 ± 0.55	12	32.00 ± 1.90	0.76	0.58	10	67.55 ± 0.49	12	53.41 ± 1.55	-0.04	0.74
Males	Control	10	12.18 ± 0.61	12	27.39 ± 0.95	Ref	Ref	10	69.37 ± 0.48	12	57.10 ± 0.81	Ref	Ref
	DEHP	8	13.51 ± 1.40	10	29.39 ± 1.07	1.62	0.12	8	69.15 ± 1.14	10	55.28 ± 0.87	-1.06	0.23
	DBP	9	12.53 ± 0.71	10	27.34 ± 1.00	-0.19	0.86	9	69.70 ± 0.65	10	56.81 ± 0.88	0.26	0.77
	DINP	9	13.68 ± 0.95	9	26.77 ± 0.85	0.51	0.63	9	68.19 ± 0.90	9	57.58 ± 0.78	-0.47	0.60
	DEHP+DINP	10	12.82 ± 0.57	12	26.56 ±1.26	0.006	1.00	10	69.29 ± 0.52	12	57.72 ± 1.15	0.1	0.91
	DEHP+DINP+DBP	9	12.56 ± 0.54	11	27.79 ± 0.80	0.17	0.87	9	69.26 ± 0.66	11	56.59 ± 0.70	-0.11	0.91

Table 3.1 Longitudinal Impacts of Perinatal Phthalate Exposures on Body Composition. **β** coefficients and p-values are from linear mixed effects (LME) models of longitudinal metabolic parameters, comparing each exposure group to controls. Body composition data was obtained at 2 and 8 months of age. Models controlled for the presence of gross liver masses at necropsy. Body Fat % and Lean Mass % are expressed as % of body weight. Each exposure group was compared to controls, resulting in 5 comparisons per model; to account for multiple comparisons, p-value cut-offs of 0.05 for significant and 0.10 for borderline significant are adjusted to 0.01 and 0.02, respectively. Comparisons that remained statistically significant after accounting for multiple comparisons are denoted with δ. ^p≤0.10, *p≤0.05, **p≤0.01, ***p≤0.001 compared to controls.

		Model 1: Control vs. DEHP vs. DEHP+DINP					Model 2: Control vs. DINP vs. DEHP+DINP				
			Control vs. DEHP		DEHP vs. DEHP+DINP			Control vs. DINP		DINP vs. DEHP+DINP	
	Outcome	Overall p-value	**β**	p-value	**β**	p-value	Overall p-value	**β**	p-value	**β**	p-value
Females	Body Fat %	**0.028***	**2.53**	δ **0.014***	0	1	**0.020***	**2.33**	δ **0.014***	0	1
	Lean Mass %	**0.018***	**-2.04**	δ **0.018***	0	1	**0.017***	**-2**	δ **0.014***	0	1
	RER (VCO2/VO2)	**0.005****	**-0.03**	δ **0.001****	0	1	**0.055^**	**-0.026**	δ **0.024***	-0.002	0.37
	Fat Oxidation (mg/hr)	**0.003****	**5.24**	δ **0.001****	0	1	**0.057^**	**4.31**	δ **0.023***	0.15	0.35
	Glucose AUC (mg/dL)	0.27	422.2	0.16	0	1	0.15	697.2	0.07^	0	1
Males	RMR (kcal/hr)	**0.005****	0	1	**0.054**	δ **0.004****	**0.035***	0.01	0.22	**0.035**	δ **0.016***
	Glucose Oxidation (mg/hr)	**0.023***	0	1	**17**	δ **0.003****	0.057^	2.95	0.24	**10.71**	ψ **0.035***

Table 3.2 Linear Mixed Effects Models with Simple Order Constraints Evaluating Effects of Individual Phthalates Compared to Phthalate Mixtures on Longitudinal Metabolic Outcomes (Part 1). Linear mixed effects (LME) models with simple order restraints were used to assess whether mice perinatally exposed to phthalate mixtures had greater effects compared to those exposed to individual phthalates. Overall p-value is the global p-value of the model calculated via bootstrap likelihood ratio test (LRT) based on 1,000 bootstraps. Individual p-values are Williams' type tests for each individual comparison. Individual tests had two comparisons for each model, and thus a p-value of 0.025 (0.05/2) was used as a cut-off for statistical significance after accounting for multiple comparisons and a p-value of 0.05 (0.10/2) was used as a cut-off for borderline statistical significance. Comparisons that remained statistically significant after accounting for multiple comparisons are denoted with δ, and those that are still marginally statistically significant are denoted with ψ. (^p≤0.10, *p≤0.05, **p≤0.01)

		Model 3: Control vs. DBP vs. DEHP+DINP+DBP				
		Overall p-value	Control vs. DBP		DBP vs. DEHP+DINP+DBP	
	Outcome		β	p-value	β	p-value
Females	Body Fat %	0.21	1.61	0.09^	0	1
	Lean Mass %	0.23	-1.08	0.11	0	1
	RER (VCO2/VO2)	0.33	-0.011	0.18	0	1
	Fat Oxidation (mg/hr)	0.37	1.6	0.2	0	1
	Glucose AUC (mg/dL)	0.2	582.7	0.09^	0	1
Males	RMR (kcal/hr)	1	0	1	0	1
	Glucose Oxidation (mg/hr)	0.65	0.15	0.39	0	1

Table 3.3 Linear Mixed Effects Models with Simple Order Constraints Evaluating Effects of Individual Phthalates Compared to Phthalate Mixtures on Longitudinal Metabolic Outcomes (Part 2). Linear mixed effects (LME) models with simple order restraints were used to assess whether mice perinatally exposed to phthalate mixtures had greater effects compared to those exposed to individual phthalates. Overall p-value is the global p-value of the model calculated via bootstrap likelihood ratio test (LRT) based on 1,000 bootstraps. Individual p-values are Williams' type tests for each individual comparison. Individual tests had two comparisons for each model, and thus a p-value of 0.025 (0.05/2) was used as a cut-off for statistical significance after accounting for multiple comparisons and a p-value of 0.05 (0.10/2) was used as a cut-off for borderline statistical significance. Comparisons that remained statistically significant after accounting for multiple comparisons are denoted with δ, and those that are still marginally statistically significant are denoted with ψ. (^p≤0.10, *p≤0.05, **p≤0.01).

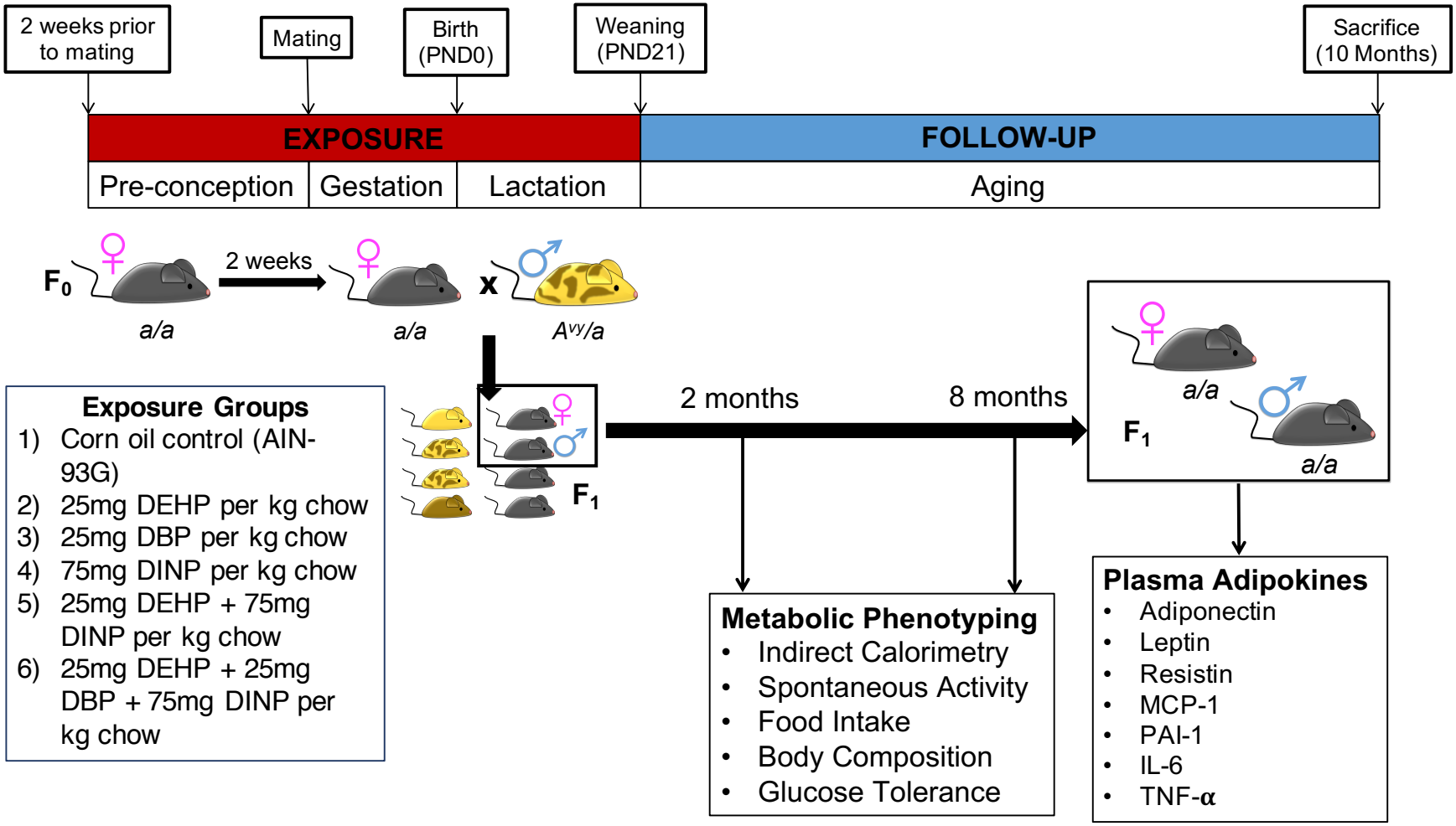

Figure 3.1 Experimental Design. Two weeks prior to mating, virgin a/a female mice (F_0) were randomly assigned to one of six exposure groups containing different combinations of phthalates. Phthalates were administered through chow, on a background diet of 7% corn oil (AIN-93G, phytoestrogen-free). Exposure spanned preconception, gestation, and lactation, and at postnatal day 21 (PND21), on male and one female F_1 offspring per litter were weaned onto control chow and followed until 10 months of age (N=9-13/sex/exposure group). Metabolic phenotyping was carried out at 2 and 8 months, and plasma adipokines were measured at 10 months.

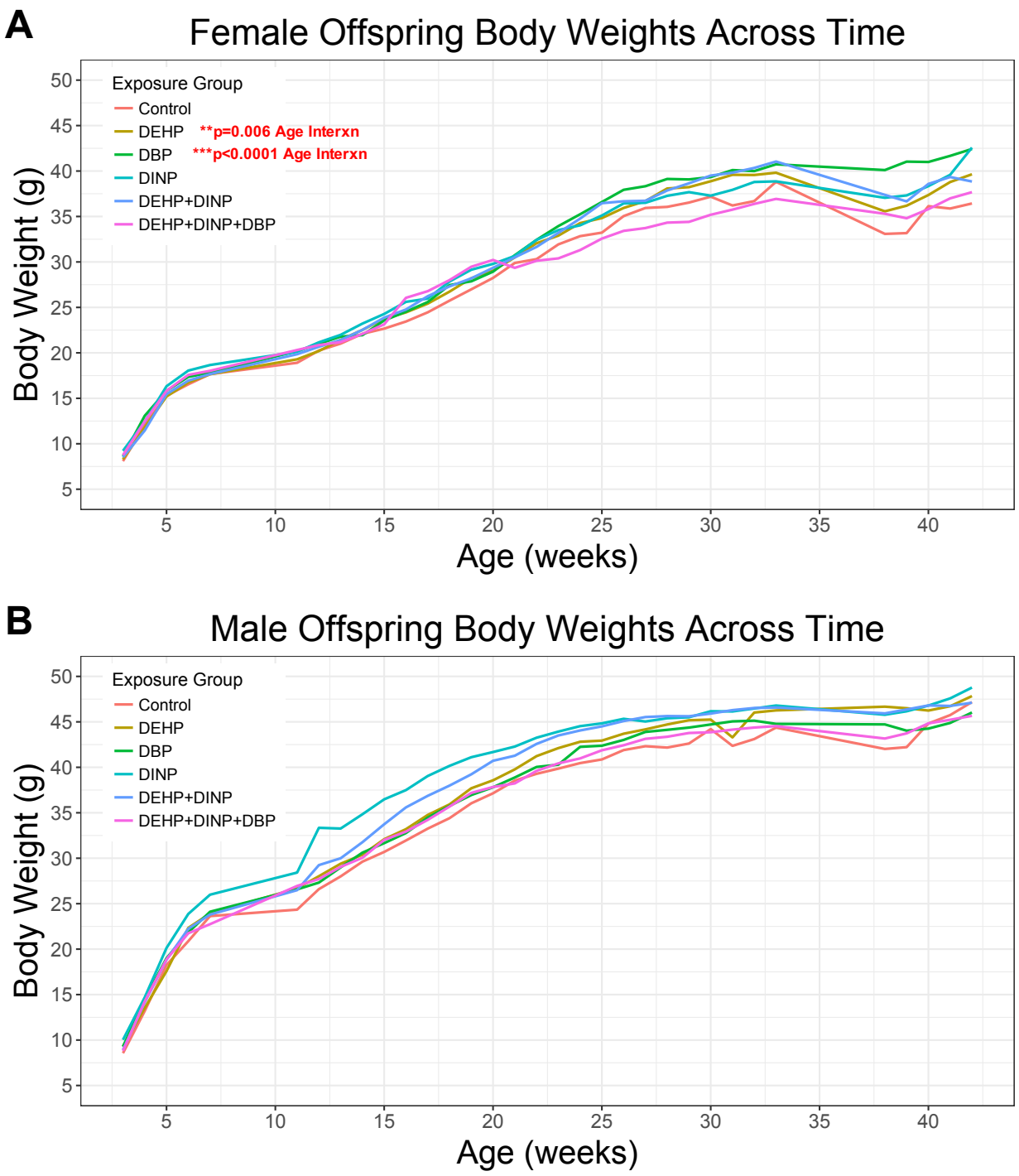

Figure 3.2 Body Weights Across Time. Weekly body weights were recorded in female (A) and male (B) offspring (N=9-13/sex/exposure group). No significant differences in body were detected via longitudinal linear mixed effects (LME) models. However, females perinatally exposed to DEHP-only, DBP-only, and DINP-only gained more weight with age than controls (p=0.006, p<0.0001, p=0.09, respectively).

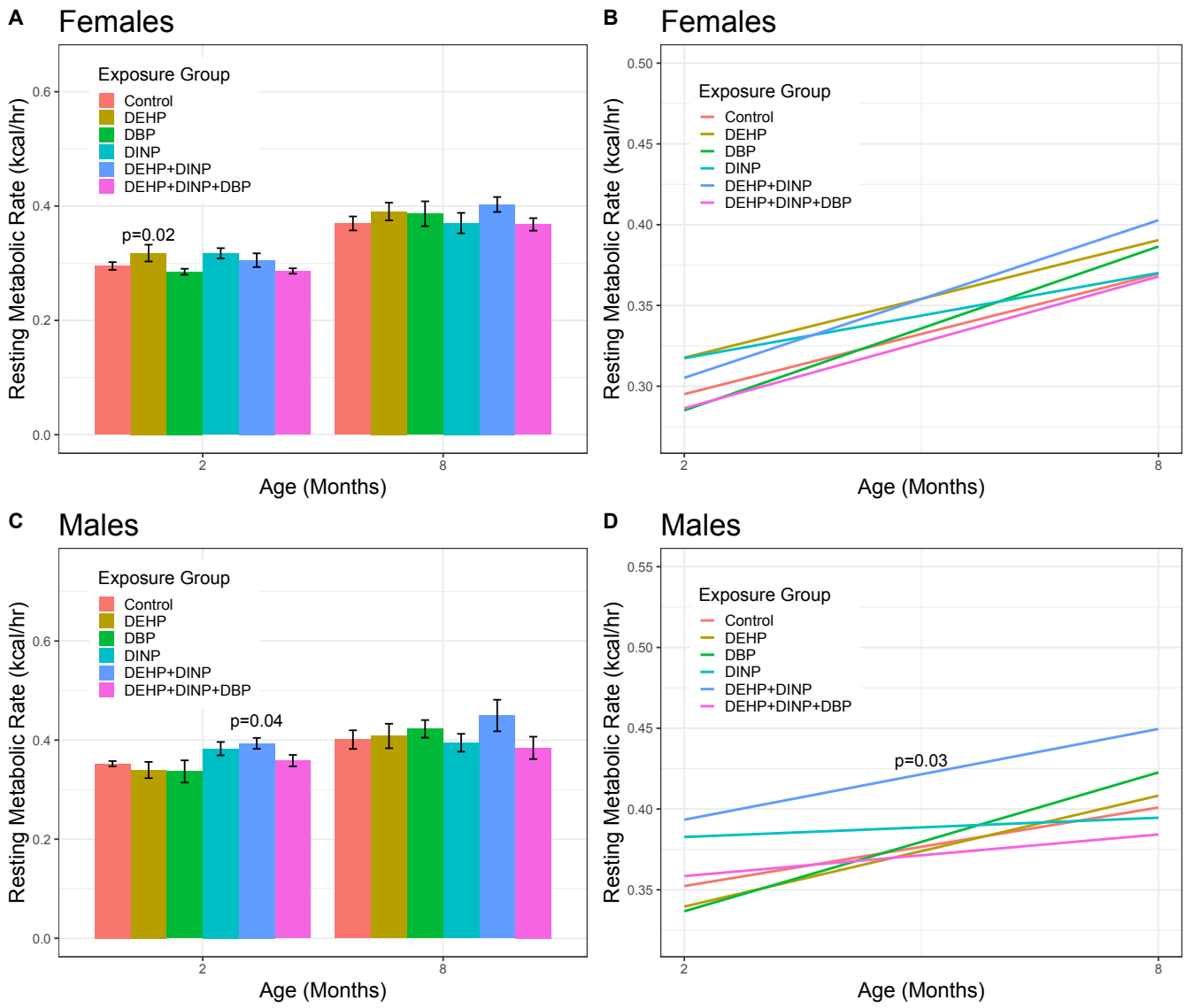

Figure 3.3 Resting metabolic rate (RMR) in mice perinatally exposed to phthalates. Cross-sectional analyses of females (A) and males (C) comparing RMRs across exposure groups at 2 and 8 months of age were carried out via multiple linear regression (MLR) (N=8-13/sex/exposure group). Models for 8 month data included a variable to control for the presence of gross liver abnormalities. Longitudinal analyses of females (B) and males (D) to examine the effects of perinatal phthalate exposures on RMR were carried out via linear mixed effects (LME) models. RMR was calculated based on spontaneous activity and indirect calorimetry. Bars represent mean RMR for each group and error bars represent +/- standard error (SE). Lines track from mean RMR at 2 months to the mean RMR at 8 months for each group.

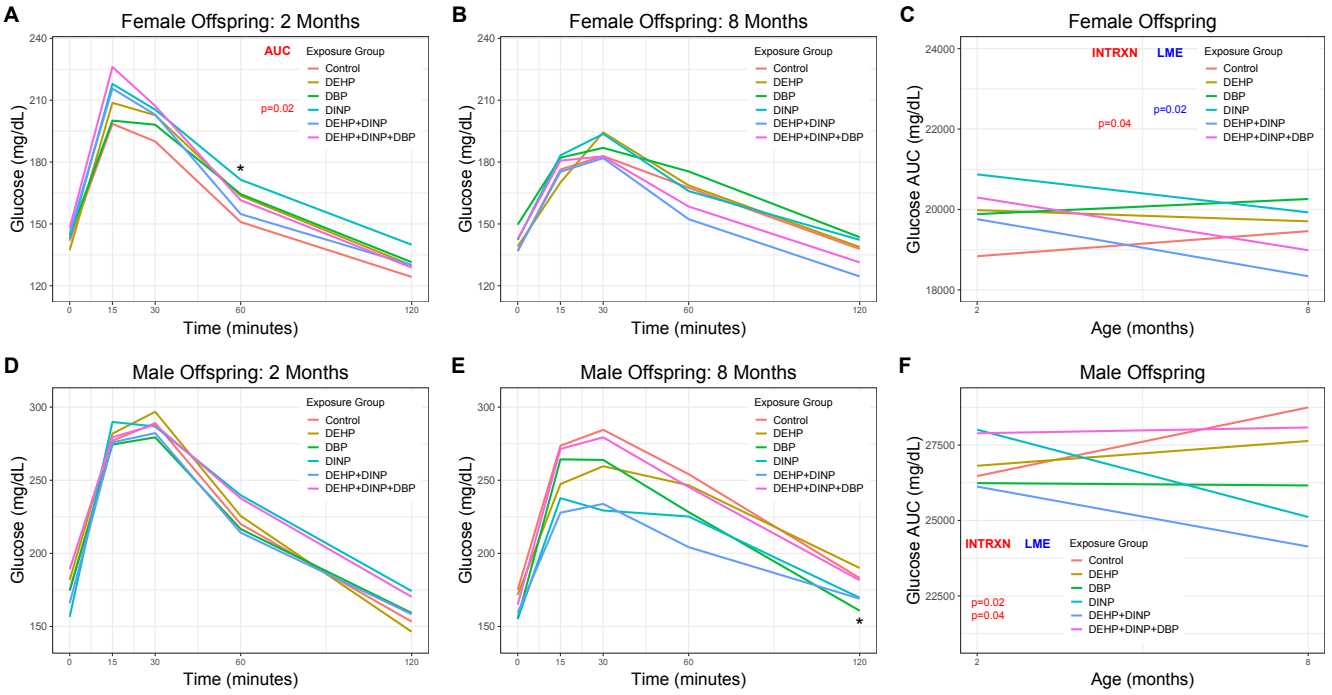

Figure 3.4 Oral Glucose Tolerance Testing. Oral glucose tolerance tests (OGTT) were carried out on females (A-C) and males (D-F) at 2 and 8 months (N=8-13/sex/exposure group). Lines in A, B, D, and E represent group means of blood glucose levels during the testing period (0-120 minutes after glucose challenge). Cross-sectional analyses were carried out via multiple linear regression (MLR) for each time point (0, 15, 30, 60, and 120 minutes) after glucose challenge, and were also carried out for area under the curve (AUC), a measurement of glucose tolerance. Mean AUC was plotted across the 2 and 8 month ages for females (C) and males (D) to examine longitudinal effects of exposure via linear mixed effects (LME) models, and also whether phthalate exposures modified the relationship with glucose tolerance and age (INTERXN). (*p≤0.02)

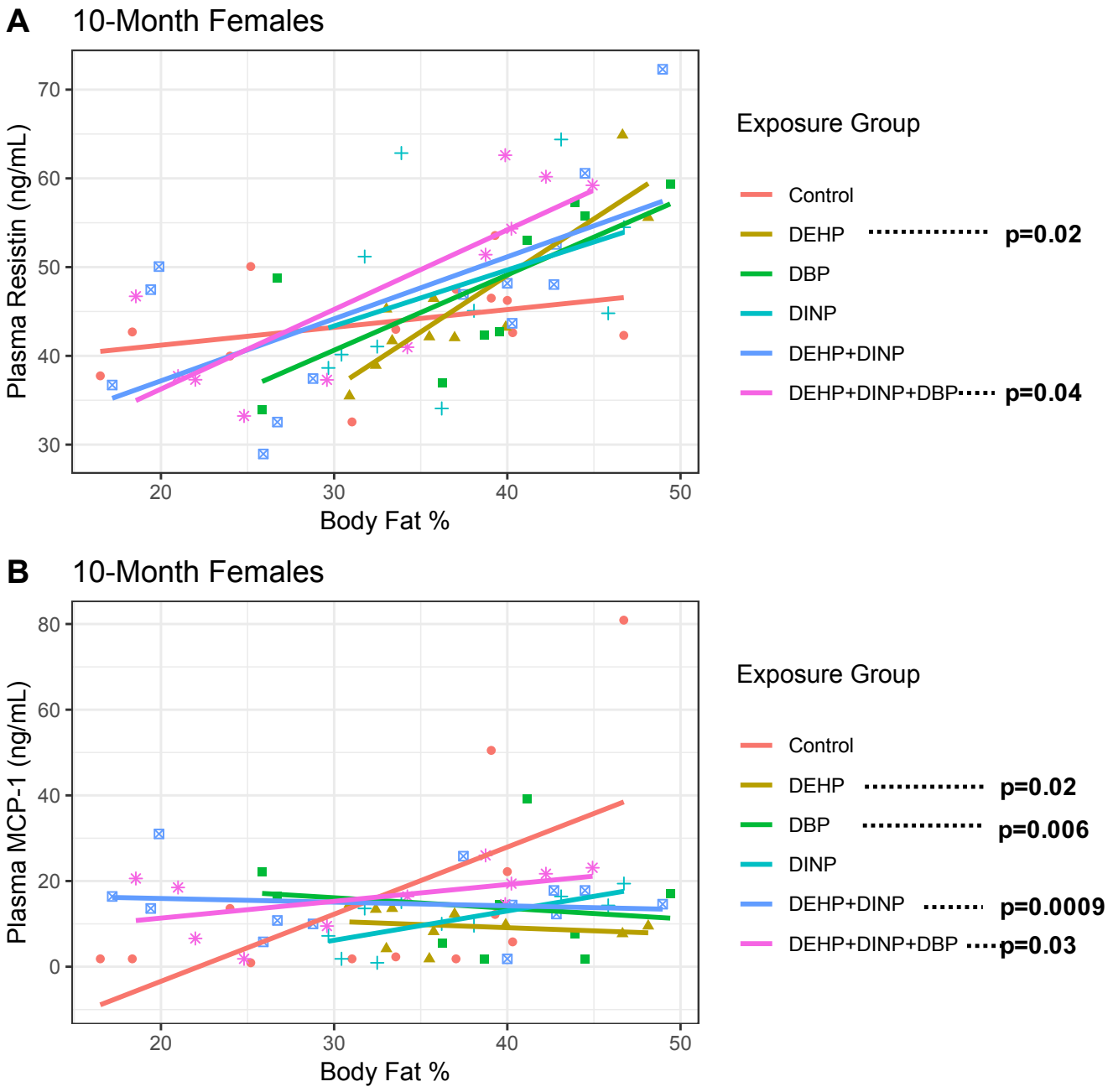

Figure 3.5 Modification of Relationship Between Plasma Adipokine Levels and Body Fat % at 10 Months. The relationship between body fat percent and plasma levels of Resistin (**A**) and MCP-1 (**B**) in females (N=9-13/exposure group). Dots represent individual female mice, lines represent a least squares regression curve of the relationship between body fat percent and plasma adipokine levels for each exposure group. Multiple linear regression (MLR) models with interaction terms for exposure*body fat percent were carried out to examine the effect modification of perinatal phthalate exposure. Analyses controlled for the presence of gross liver masses at necropsy.

Appendix

	Litter Parameters			# Follow-Up Offspring: PND21 (weaning)		# Follow-Up Offspring: 10 months		Mortality Rate (%)		# With Gross Liver Masses at Necropsy		Liver Mass Rate (%)	
Exposure Group	# Mate Pairs	# Litters	Birth Rate (%)	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males
Control	17	17	100	13	12	12	11	7.7	8.3	1	1	8.3	9.1
DEHP	17	16	94.1	11	11	10	10	9.1	9.1	2	2	20.0	20.0
DBP	17	15	88.2	9	11	9	10	0.0	9.1	2	1	22.2	10.0
DINP	21	15	**71.4***	12	10	11	9	8.3	10.0	2	3	18.2	33.3
DEHP+DINP	17	16	94.1	13	12	13	12	0.0	0.0	2	4	15.4	33.3
DEHP+DINP+DBP	19	19	100	12	11	11	11	8.3	0.0	1	1	9.1	9.1
Total	**108**	**98**	**90.7**	**70**	**67**	**66**	**63**	**5.7**	**6.0**	**10**	**12**	**15.2**	**19.0**

Table 3.A1 Litter Parameters and Offspring Morbidity and Mortality. A total of eight mice died during the follow-up period. One was euthanized for fight wounds, one was euthanized for a tail infection resulting from poor tail vein blood draws, one died during body composition measurement at 10 months, four died due to a poor oral gavage during oral glucose tolerance testing (OGTT), and one died at 2 months of age from unknown causes. Birth rate (%) and liver mass rate (%) for each exposure group was compared to controls using Fisher's exact test. (*p = 0.02 vs. Control)

FEMALES: 2 MONTHS	Body Fat %					Lean Mass %				
Exposure	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value
Control	11	13.05	0.62	Ref	Ref	11	67.98	0.48	Ref	Ref
DEHP	9	**15.14**	**0.81**	**2.09**	**0.04***	9	66.53	0.47	-1.45	0.08^
DBP	8	14.58	0.95	1.53	0.15	8	67.17	0.63	-0.81	0.35
DINP	10	**15.10**	**0.80**	**2.05**	**0.04***	10	**66.20**	**0.78**	**-1.78**	**0.03***
DEHP+DINP	11	13.61	0.61	0.56	0.56	11	67.77	0.59	-0.21	0.79
DEHP+DINP+DBP	10	14.43	0.55	1.38	0.17	10	67.55	0.49	-0.43	0.59
Overall p-value					0.23					0.20
FEMALES: 8 MONTHS	**Body Fat %**					**Lean Mass %**				
Exposure	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value
Control	13	32.94	1.92	Ref	Ref	13	52.76	1.67	Ref	Ref
DEHP	11	**36.52**	**1.24**	**4.56**	**0.05***	11	**49.66**	**1.06**	**-3.95**	**0.04***
DBP	9	37.09	1.70	3.61	0.14	9	49.26	1.37	-3.00	0.13
DINP	12	34.87	1.57	3.47	0.13	12	51.06	1.34	-3.13	0.09^
DEHP+DINP	13	34.99	1.93	2.60	0.23	13	50.95	1.58	-2.31	0.20
DEHP+DINP+DBP	12	32.00	1.90	0.07	0.97	12	53.41	1.55	-0.20	0.91
Overall p-value					0.25					0.21
MALES: 2 MONTHS	**Body Fat %**					**Lean Mass %**				
Exposure	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value
Control	10	12.18	0.61	Ref	Ref	10	69.37	0.48	Ref	Ref
DEHP	8	13.51	1.40	1.33	0.26	8	69.15	1.14	-0.22	0.83
DBP	9	12.53	0.71	0.35	0.76	9	69.70	0.65	0.32	0.75
DINP	9	13.68	0.95	1.50	0.19	9	68.19	0.90	-1.18	0.25
DEHP+DINP	10	12.82	0.57	0.64	0.56	10	69.29	0.52	-0.08	0.93
DEHP+DINP+DBP	9	12.56	0.54	0.38	0.74	9	69.26	0.66	-0.12	0.91
Overall p-value					0.78					0.79
MALES: 8 MONTHS	**Body Fat %**					**Lean Mass %**				
Exposure	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value
Control	12	27.39	0.95	Ref	Ref	12	57.10	0.81	Ref	Ref
DEHP	10	29.39	1.07	1.80	0.16	10	55.28	0.87	-1.65	0.13
DBP	10	27.34	1.00	-0.63	0.62	10	56.81	0.88	0.24	0.83
DINP	9	26.77	0.85	-0.32	0.81	9	57.58	0.83	0.17	0.88
DEHP+DINP	12	26.56	1.26	-0.53	0.66	12	57.72	1.15	0.30	0.78
DEHP+DINP+DBP	11	27.79	0.80	-0.21	0.86	11	56.59	0.70	0.05	0.96
Overall p-value					0.43					0.48

Table 3.A2 Cross-Sectional Analyses of Metabolic Measures at 2 and 8 months. Cross-sectional analyses were carried out via multiple linear regression (MLR) comparing each exposure group to the control group, while stratifying by sex. For data collected at 8 months, models were adjusted for the presence of gross liver masses at necropsy. Values in blue were still statistically significant to a modest degree following adjustment for multiple comparisons (p≤0.02). Values in bold had p≤0.05. (^p≤0.10, *p≤0.05). Remaining cross-sectional analyses are available at figshare.com DOI: 10.6084/m9.figshare.8141684.v1[47]

		Body Weight		Body Fat %		Lean Mass %		Free Fluid %		Food Intake		Physical Activity	
	Exposure Group	**β**	p-value	**β**	p-value	**β**	p-value	**β**	p-value	**β**	p-value	**β**	p-value
Females	DEHP	1.67	0.31	**3.63**	**0.01****	**-2.98**	**0.01****	0.05	0.71	-0.23	0.20	94.7	0.63
	DBP	2.17	0.19	2.62	0.08^	-1.96	0.09^	0.17	0.25	0.02	0.92	-30.3	0.88
	DINP	1.37	0.39	**2.82**	**0.05***	**-2.47**	**0.03***	0.10	0.47	-0.28	0.11	-53.1	0.78
	DEHP+DINP	1.63	0.29	1.68	0.21	-1.36	0.20	-0.15	0.27	-0.32	0.06^	41.9	0.82
	DEHP+DINP+DBP	0.03	0.98	0.76	0.58	-0.04	0.74	0.09	0.53	-0.008	0.96	60.3	0.75
	DEHP*Age	**0.012**	**0.006****	0.195	0.67	-0.22	0.57	0.04	0.44	-0.019	0.75	-8.01	0.86
	DBP*Age	**0.021**	**<0.0001*****	0.443	0.34	-0.46	0.25	0.06	0.22	0.007	0.90	-21.87	0.64
	DINP*Age	0.007	0.09^	0.114	0.8	-0.12	0.75	-0.06	0.22	-0.051	0.37	26.71	0.54
	DEHP+DINP*Age	0.002	0.63	0.244	0.56	-0.26	0.47	0.04	0.31	-0.03	0.58	2.89	0.95
	DEHP+DINP+DBP*Age	-0.006	0.16	-0.274	0.54	0.08	0.83	0.06	0.24	0.046	0.42	29.65	0.51
Males	DEHP	1.33	0.33	1.62	0.12	-1.06	0.23	0.15	0.30	-0.20	0.31	-53.3	0.59
	DBP	-0.08	0.95	-0.19	0.86	0.26	0.77	0.22	0.14	-0.24	0.24	75.8	0.45
	DINP	2.11	0.14	0.51	0.63	-0.47	0.60	0.12	0.44	-0.17	0.43	17.1	0.87
	DEHP+DINP	2.04	0.14	0.006	1.00	0.1	0.91	0.15	0.28	-0.11	0.57	55.6	0.57
	DEHP+DINP+DBP	0.82	0.54	0.17	0.87	-0.11	0.91	-0.07	0.65	0.10	0.62	33.2	0.73
	DEHP*Age	0.001	0.84	-0.009	0.97	-0.15	0.56	-0.15	0.56	0.01	0.86	-46.31	0.11
	DBP*Age	-0.002	0.68	-0.17	0.53	-0.002	0.99	-0	0.99	-0.09	0.20	-45.59	0.11
	DINP*Age	-0.009	0.07^	-0.48	0.09^	0.39	0.13	0.39	0.13	-0.05	0.44	-5.435	0.85
	DEHP+DINP*Age	-0.003	0.51	-0.38	0.15	0.24	0.32	0.24	0.32	-0.03	0.61	-37.09	0.18
	DEHP+DINP+DBP*Age	-0.005	0.29	-0.14	0.59	0.06	0.81	0.06	0.81	0.05	0.45	-50.76	0.07^

Table 3.A3 Longitudinal Metabolic Impacts in Phthalate Exposure Groups Compared to Controls (Part 1). **β** coefficients and p-values are from linear mixed effects (LME) models of longitudinal metabolic parameters, comparing each exposure group to controls. Body weight analysis was carried out for weights that were taken on a weekly basis. All other parameters include data obtained at 2 and 8 months of age. All models controlled for the presence of gross liver masses at necropsy. Models for EE (energy expenditure) and RMR (resting metabolic rate) included covariates to adjust for body fat and lean mass. Body Fat %, Lean Mass %, and Free Fluid % are expressed as percent of body weight. Interaction LME models were carried out separately from the primary LME models to determine whether exposure modified the relationship between age and each metabolic outcome. Number of animals per sex per group ranged from 8-13. Comparisons that remained statistically significant after accounting for multiple comparisons are colored red (p≤0.01) and those that remained modestly statistically significant after accounting for multiple comparisons are colored blue (p≤0.02). ^p≤0.10, *p≤0.05, **p≤0.01, ***p≤0.001.

		Energy Expenditure		RMR		RER		Glucose Oxidation		Fat Oxidation		Glucose AUC	
	Exposure Group	**β**	p-value	**β**	p-value	**β**	p-value	**β**	p-value	**β**	p-value	**β**	p-value
Females	DEHP	0.007	0.68	0.0145	0.32	-0.028	0.07^	-9.02	0.16	**5.17**	**0.05***	1028.3	0.09^
	DBP	-0.003	0.88	0.0002	0.99	-0.019	0.23	-5.71	0.38	2.99	0.26	769.3	0.21
	DINP	-0.004	0.80	0.0093	0.51	-0.026	0.08^	-8.68	0.16	4.34	0.09^	**1404.3**	**0.02***
	DEHP+DINP	0.016	0.30	0.0194	0.15	**-0.028**	**0.05***	-5.23	0.38	**4.72**	**0.05***	54.3	0.92
	DEHP+DINP+DBP	-0.014	0.38	-0.0042	0.76	-0.005	0.72	-3.84	0.53	0.47	0.85	377.8	0.52
	DEHP*Age	0.0026	0.54	0.00037	0.92	-0.0062	0.16	-2.31	0.20	1.19	0.11	-179.3	0.3
	DBP*Age	0.0059	0.18	0.0045	0.24	-0.0014	0.76	0.49	0.79	0.43	0.57	-41.4	0.82
	DINP*Age	-0.0014	0.73	-0.002	0.57	-0.0015	0.73	-1.59	0.36	0.48	0.51	-242.8	0.15
	DEHP+DINP*Age	0.0037	0.36	0.0038	0.26	-0.0013	0.74	-0.21	0.90	0.47	0.49	**-340.7**	**0.04***
	DEHP+DINP+DBP*Age	-0.0022	0.59	0.0018	0.62	0.0023	0.59	0.37	0.83	-0.38	0.60	-274.8	0.11
Males	DEHP	-0.014	0.58	-0.0061	0.80	-0.005	0.68	-3.22	0.64	0.64	0.73	-86.8	0.95
	DBP	-0.002	0.94	0.0014	0.95	0.005	0.68	3.21	0.64	-0.43	0.82	-2113.6	0.12
	DINP	0.005	0.84	0.0110	0.65	0.004	0.76	4.16	0.56	-0.23	0.91	328.8	0.82
	DEHP+DINP	0.044	0.08^	**0.0492**	**0.03***	0.012	0.31	**14.87**	**0.03***	-0.83	0.65	-1105.3	0.40
	DEHP+DINP+DBP	-0.013	0.60	-0.0130	0.55	-0.002	0.85	-2.62	0.69	0.28	0.88	-204.9	0.88
	DEHP*Age	0.0074	0.22	0.0026	0.66	0.0015	0.69	1.77	0.29	0.078	0.90	-428.1	0.34
	DBP*Age	0.0061	0.31	0.0050	0.40	-0.0023	0.53	-0.50	0.76	0.46	0.44	-513.2	0.25
	DINP*Age	-0.0044	0.47	-0.0063	0.31	-9E-05	0.98	-0.97	0.57	-0.078	0.90	**-1048**	**0.02***
	DEHP+DINP*Age	0.0027	0.64	0.0005	0.93	-0.0012	0.74	0.03	0.99	0.27	0.64	**-896.7**	**0.04***
	DEHP+DINP+DBP*Age	-0.0041	0.48	-0.0046	0.43	0.00011	0.97	-0.90	0.58	-0.076	0.90	-532.9	0.22

Table 3.A4 Longitudinal Metabolic Impacts in Phthalate Exposure Groups Compared to Controls (Part 2). **β** coefficients and p-values are from linear mixed effects (LME) models of longitudinal metabolic parameters, comparing each exposure group to controls. Body weight analysis was carried out for weights that were taken on a weekly basis. All other parameters include data obtained at 2 and 8 months of age. All models

controlled for the presence of gross liver masses at necropsy. Models for EE (energy expenditure) and RMR (resting metabolic rate) included covariates to adjust for body fat and lean mass. Body Fat %, Lean Mass %, and Free Fluid % are expressed as percent of body weight. Interaction LME models were carried out separately from the primary LME models to determine whether exposure modified the relationship between age and each metabolic outcome. Number of animals per sex per group ranged from 8-13. Comparisons that remained statistically significant after accounting for multiple comparisons are colored red (p≤0.01) and those that remained modestly statistically significant after accounting for multiple comparisons are colored blue (p≤0.02). ^p≤0.10, *p≤0.05, **p≤0.01, ***p≤0.001

	Leptin					Adiponectin				
Females: 10 Months	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value
Control	12	12156.08	2281.161	Ref	Ref	12	30667.75	3601.386	Ref	Ref
DEHP	10	12210	1423.359	536.72	0.85	10	31002.9	3881.81	-751.9	0.87
DBP	9	15027.22	1547.595	2526.28	0.39	9	29179.44	3426.897	-711.9	0.88
DINP	10	14910.5	1616.131	3237.22	0.26	8	28178.25	4223.792	-4042.4	0.41
DEHP+DINP	13	11827.38	2170.392	-36.89	0.99	12	28055.42	2592.176	-3388.8	0.44
DEHP+DINP+DBP	11	12273.82	2446.062	149.09	0.96	11	23038.46	2892.76	-7699.9	0.087^
Males: 10 Months	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value
Control	11	15998.27	2227.886	Ref	Ref	11	17130.36	1417.569	Ref	Ref
DEHP	10	14431.9	1365.559	-566.2	0.8	10	17360.1	1894.568	-189.4	0.93
DBP	10	14985.6	2024.178	-929.3	0.67	10	16390.9	2240.042	-774.4	0.72
DINP	9	17847.33	2485.835	4071.7	0.08^	9	15894.56	1440.338	-2167.2	0.34
DEHP+DINP	12	11440.17	1561.134	-2335.5	0.28	12	15598.42	1290.16	-2463.3	0.24
DEHP+DINP+DBP	11	16997.64	1678.523	999.4	0.64	11	15101.36	1035.669	-2029	0.34

Table 3.A5 Plasma Adipokine Levels in Females and Males at 10 Months (Part 1). Cross-sectional analyses evaluating Leptin, Adiponectin, Resistin, MCP-1, and PAI-1 were carried out via multiple linear regression (MLR) comparing each exposure group to the control group, while stratifying by sex. Models were adjusted for the presence of gross liver masses at necropsy. IL-6 and TNF-α levels were below the limit of

detection for many samples, and therefore the Kolmogorov-Smirnov test comparing cumulative distribution functions for each group to controls was carried out. Means and standard errors for IL-6 and TNF-αwere calculated by replacing samples that were below the limit of detection (LOD) with LOD/sqrt(2). None of the exposure groups were significantly different from controls at p≤0.01 or modestly significantly different at p≤0.02, which were the p-values cut-offs used for statistical significance after adjusting for multiple comparisons. (^p≤0.10)

	Resistin					MCP-1					PAI-1				
Females: 10 Months	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value
Control	12	1941.167	140.0843	Ref	Ref	12	16.315	7.167	Ref	Ref	12	4433.5	820.73	Ref	Ref
DEHP	10	2143.6	276.1673	278.8	0.48	10	9.514	1.324	-6.22	0.263	10	4188.5	610.69	-191.17	0.92
DBP	9	2361.111	289.4531	365.4	0.37	9	14.031	3.953	-2.70	0.635	9	5372.67	1352.47	900.72	0.64
DINP	10	2367.4	326.4093	502.6	0.21	10	10.836	1.935	-4.90	0.378	10	4335.6	593.47	-44.07	0.98
DEHP+DINP	13	2292.462	322.7357	397.5	0.29	13	14.78	2.118	-1.18	0.818	13	4844.92	672.17	443.96	0.8
DEHP+DINP+DBP	11	2346.636	310.3609	410.4	0.29	11	16.204	2.245	-0.074	0.989	11	7609.09	2536.56	3179.09	0.08^
Males: 10 Months	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value	N	Mean	SE	β	p-value
Control	11	1554.455	119.2083	Ref	Ref	11	11.713	2.016	Ref	Ref	11	4424.36	1029.23	Ref	Ref
DEHP	10	1767.5	180.4255	293.9	0.07^	10	14.62	1.624	2.91	0.35	10	5587	892.74	491.03	0.69
DBP	10	1697.7	141.9138	150	0.34	10	10.716	2.885	-1.00	0.75	10	4484.6	1179.90	4.27	1
DINP	9	1549.778	156.9087	175	0.29	9	12.722	1.604	1.01	0.76	9	6188.44	1715.99	271.62	0.83
DEHP+DINP	12	1490.667	137.2961	115.9	0.45	12	12.995	2.062	1.28	0.67	12	6318.5	1273.49	401.68	-0.73
DEHP+DINP+DBP	11	1699.455	116.2027	145	0.34	11	15.454	2.337	3.74	0.22	11	4310.46	510.04	-113.91	0.92

Table 3.A6 Plasma Adipokine Levels in Females and Males at 10 Months (Part 2). Cross-sectional analyses evaluating Leptin, Adiponectin, Resistin, MCP-1, and PAI-1 were carried out via multiple linear regression (MLR) comparing each exposure group to the control group, while stratifying by sex. Models were adjusted for the presence of gross liver masses at necropsy. IL-6 and TNF-α levels were below the limit of detection for many samples, and therefore the Kolmogorov-Smirnov test comparing cumulative distribution functions for each group to controls was carried out. Means and standard errors for IL-6 and TNF-αwere calculated by replacing samples that were below the limit of detection (LOD) with LOD/sqrt(2). None of the exposure groups were significantly different from controls at p≤0.01 or modestly significantly different at p≤0.02, which were the p-values cut-offs used for statistical significance after adjusting for multiple comparisons. (^p≤0.10

	IL-6					TNF-α				
Females: 10 Months	N total	N non-detects	Mean	SE	p-value	N total	N non-detects	Mean	SE	p-value
Control	11	0	13	6.46	Ref	10	4	3.03	2.15	Ref
DEHP	8	3	3.72	0.98	0.11	8	6	0.9	0.19	0.25
DBP	9	3	5.72	2.2	0.51	9	5	1.6	0.56	0.91
DINP	8	1	7.16	1.9	0.98	8	3	1.48	0.25	0.49
DEHP+DINP	11	1	5.35	0.77	0.83	11	4	2.27	0.58	0.68
DEHP+DINP+DBP	10	2	4.78	0.74	0.87	10	5	1.58	0.34	0.99
Males: 10 Months	N total	N non-detects	Mean	SE	p-value	N total	N non-detects	Mean	SE	p-value
Control	10	1	8.96	1.57	Ref	10	2	2.1	0.42	Ref
DEHP	8	3	5.85	1.4	0.56	8	4	1.46	0.16	0.81
DBP	9	1	7.45	1.74	0.66	9	2	1.45	0.28	0.59
DINP	6	0	29.72	20.71	0.49	6	6	1.24	0.19	0.26
DEHP+DINP	8	0	5.72	0.72	0.15	8	3	1.99	0.47	0.96
DEHP+DINP+DBP	10	3	7.31	2.57	0.46	10	5	1.75	0.37	0.81

Table 3.A7 Plasma Adipokine Levels in Females and Males at 10 Months (Part 3). Cross-sectional analyses evaluating Leptin, Adiponectin, Resistin, MCP-1, and PAI-1 were carried out via multiple linear regression (MLR) comparing each exposure group to the control group, while stratifying by sex. Models were adjusted for the presence of gross liver masses at necropsy. IL-6 and TNF-α levels were below the limit of detection for many samples, and therefore the Kolmogorov-Smirnov test comparing cumulative distribution functions for each group to controls was carried out. Means and standard errors for IL-6 and TNF-αwere calculated by replacing samples that were below the limit of detection (LOD) with LOD/sqrt(2). None of the exposure groups were significantly different from controls at p≤0.01 or modestly significantly different at p≤0.02, which were the p-values cut-offs used for statistical significance after adjusting for multiple comparisons. (^p≤0.10)

Chapter 4

Aim 3: Persistent Alterations in Gene Expression and Promoter DNA Methylation in Mice Perinatally Exposed to Phthalates

Abstract

Developmental exposures to environmental chemicals, including phthalates, have been implicated in the obesity epidemic. Phthalates have been demonstrated to influence metabolism through interacting with peroxisome proliferator-activated receptors (PPARs). However, mechanisms linking developmental phthalate exposures to long-term metabolic effects have not yet been elucidated. Here, we investigated the hypothesis that developmental exposure to phthalates is capable of long-lasting impacts on PPAR target gene expression and DNA methylation in the liver to influence metabolism across the life course. We utilized an established longitudinal mouse model of perinatal exposures to two phthalates, diethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP), as well as a mixture of DEHP+DINP. Exposure was through the diet and spanned from two weeks prior to mating until weaning at postnatal day 21 (PND21). Liver tissue was analyzed from mice at PND21 at the end of the exposure period and in a second cohort of mice at 10 months of age, long after exposure had ceased. RNA-seq was used to screen for PPAR target genes that were altered in liver from phthalate-exposed mice at both ages, followed by pathway enrichment analysis. The top enriched pathway in DINP females across PND21 and 10 months was acetyl-CoA metabolic process, of which 10/13 significant genes were PPAR target genes, 9 of which were up-

regulated. DNA methylation was increased in the promoter region of *Fasn* in DINP females at PND21 (p=0.007) and marginally increased at 10 months (p=0.08). Together, our data are indicative of persistently altered expression and promoter DNA methylation of PPAR target genes as a potential mechanism linking developmental phthalate exposures to lifecourse metabolic effects.

Introduction

Obesity, as defined by a body mass index (BMI) greater than 30, affects over 93 million adults and over 13 million children in the US.[1] Obesity is also associated with some of the leading causes of preventable death, making it a major concern for public health. Recently, exposures to environmental chemicals have been implicated as risk factors for obesity, and have thus been given the term "obesogens."[2,3] Many obesogens are also endocrine disrupting chemicals (EDCs) that interfere with hormone signaling to influence metabolism and feeding behaviors.[4] Phthalates are one subset of EDCs that have been classified as obesogens. Phthalates are chemicals found in a variety of consumer products, including plastics, furniture, and food packaging, resulting in ubiquitous exposure.[5] Diethylhexyl phthalate (DEHP), is a widely used phthalate and has been heavily investigated via *in vitro*, animal, and human studies. However, due to negative health risks associated with exposure to DEHP, other phthalates have been used to replace it, including diisononyl phthalate (DINP).

Several studies have indicated that phthalates interfere with metabolism by interacting with human and mouse peroxisome proliferator activated receptors (PPARs).[6–10] PPARs are nuclear receptors that activate transcription of target genes regulating a wide variety of metabolic processes.[11–14] PPARs are present in rodents and

humans in three main isoforms: PPARα, PPARγ, and PPARδ/β. PPARα expression is highest in the liver, PPARγ expression is highest in adipose tissue, and PPARδ/β is ubiquitously expressed across all tissues at low levels.[15,16] Phthalates have been demonstrated to interact with all three isoforms.[6,7,9] A critical window of exposure for phthalates and other obesogens is during development, a time period when PPAR signaling is critical for programming of metabolic organs and tissues.[17,18] However, few studies have directly examined PPAR activation following developmental phthalate exposures.[19,20]

PPARs recruit ten-eleven translocation (TET) enzymes to the promoter regions of target genes to locally de-methylate DNA and facilitate transcription [21]. DNA methylation is a well-established epigenetic modification that influences gene transcription and is heritable through cell division. DNA methylation consists of a methyl group to the 5' carbon of a cytosine (5mC), usually adjacent to a guanine (CpG). In general, higher levels of 5mC in the promoter region are generally associated with repression while lower levels of promoter 5mC are associated with activation.[22] TET enzymes catalyze the oxidation of 5mC to 5'-hydroxymethyl cytosine (5hmC), and then subsequently 5'-formyl cytosine (5fC) and 5'-carboxyl cytosine (5caC), which is removed by base excision repair machinery (BER) and replaced with an unmethylated cytosine.[23] During development, DNA methylation is particularly sensitive to environmental cues and undergoes reprogramming [24,25]. Because DNA methylation is relatively stable and heritable through cell division, if environmental chemicals, such as phthalates, interfere with DNA methylation during development when it is plastic, then this could result in altered DNA methylation that persists into adulthood.

We previously found that perinatal exposure to DEHP alone, DINP alone, and a combination of DEHP+DINP resulted in increased relative liver weights in weanling female mice at postnatal day 21 (PND21),[26] which may be an indication of PPARα activation.[27,28] Longitudinally, female mice perinatally exposed to DEHP-only had increased body fat percentage and those perinatally exposed to DINP-only had impaired glucose tolerance.[29] Building upon these findings, we hypothesized that early life exposures to phthalates resulted in long-lasting impacts on PPAR target gene expression in the liver by decreasing promoter region DNA methylation to influence metabolism across the life course. To investigate this hypothesis, we utilized liver tissue collected from a previously established mouse model of perinatal exposures to DEHP-only, DINP-only, and DEHP+DINP. We used transcriptomics (RNA-seq) in liver collected in early postnatal life at the end of the exposure period (PND21) and at 10 months of age, long after the exposure had ceased, to screen for PPAR target genes that were persistently activated by developmental phthalate exposures. We then measured promoter region DNA methylation levels for candidate PPAR target genes to elucidate the role of DNA methylation.

Materials and Methods

Animals and Exposures

The overall experimental design is laid out in **Figure 4** and is described in detail in previous studies.[26,29] Animals were obtained from a colony of viable yellow agouti (A^{vy}) mice maintained for over 220 generations with sibling mating and forced heterozygosity for the A^{vy} allele through the male line.[30] For this study, we utilized

tissues from only the “wild-type” *a/a* offspring, which are isogenic and 93% similar to C57BL/6.[31]

The exposure window captured the entire perinatal period spanning from preconception (two weeks prior to mating) through gestation and lactation until weaning at postnatal day 21 (PND21). Two weeks prior to mating, virgin *a/a* dams aged six to eight weeks were randomly assigned to one of four exposure groups: 1) Control, 2) DEHP-only, 3) DINP-only, and 4) DEHP+DINP. Phthalates (Sigma) were administered through the chow on a background 7% corn oil phytoestrogen-free diet (Teklad diet TD-95092; ENVIGO, Madison, WI). Controls were given 7% corn oil chow without phthalates added. Exposure levels for the three exposure groups were as follows: DEHP-only = 25mg DEHP/kg chow; DINP-only = 75mg DINP/kg-chow; DEHP+DINP = 25mg DEHP + 75mg DINP/kg chow. These exposure levels were selected based on a target maternal dose of 5mg/kg-day for DEHP and 15mg/kg-day for DINP, assuming that pregnant and nursing female mice weigh approximately 25g and eat approximately 5g of chow per day. These target doses were selected based on literature demonstrating obesity-related phenotypes in offspring that were developmentally exposed to 5mg/kg-day of DEHP.[32,33] A higher exposure level of DINP was chosen based on previous studies that have indicated it is three times less potent than DEHP with respect to antiandrogenic effects.[34] These exposure levels are estimated to fall within the range of exposures experienced by humans, although they are on the higher end.[26]

At PND21, one male and one female *a/a* offspring per litter were weaned onto control chow and followed to 10 months of age while the rest of the *a/a* mice were

euthanized for tissue collection. Study mice that were followed to 10 months were co-housed with one same-sex, same-age, non-study littermate that was used as a companion only. Full litter parameters and outcomes are reported in previous work.[26] There were no significant differences in number of pups per litter across exposure groups. Throughout the duration of the study, animals were given food and water ad libitum and remained on a 12-hour light/dark cycle. Health checks were carried out daily by lab personnel and the University of Michigan Unity for Laboratory Animal Medicine (ULAM). The guidelines for the use and care of laboratory animals were followed and mice were treated humanely. The University of Michigan Institutional Animal Care and Use Committee (IACUC) approved all animal procedures used for this project.

Tissue Collection and Nucleic Acid Isolation

At PND21, mice were fasted for four hours prior to euthanasia and at 10 months, mice were fasted for six hours prior to euthanasia. Estrus testing was performed on females at 10 months of age and synchronized so that all females were sacrificed during estrus. Euthanasia was carried out via inhalation of CO_2 followed by cardiac puncture and whole-body perfusion with cell culture grade 0.9% sodium chloride saline (Sigma-Aldrich). Liver and WAT tissue were collected and flash frozen in liquid nitrogen and then stored at -80°C.

RNA and DNA were extracted from liver and WAT collected from offspring at PND21 and 10 months using Universal All-Prep kits (Qiagen Cat# 80224). Approximately 10-15 mg of tissue from each liver was homogenized using a TissueLyser II (Qiagen). Kit protocols were followed exactly with one exception during isolation from WAT: the chloroform lipid extraction step was carried out prior to

dispensing tissue lysate onto the DNA spin column. This allowed us to use a large mass of tissue without overloading the column, enabling us to maximize recovery of nucleic acids from lipid-rich tissue. RNA isolated from WAT underwent an additional clean-up step with Qiagen RNeasy MinElute Cleanup kits. DNA and RNA quantity were measured using a NanoDrop spectrophotometer. Isolated RNA and DNA were stored at -80°C.

RNA-seq library preparation and sequencing

RNA-seq library preparation and sequencing were carried out at the University of Michigan DNA Sequencing Core in Ann Arbor, Michigan. RNA libraries from liver of PND21 and 10-month mice were sequenced (N=5-6/sex/age/tissue). We excluded samples from mice that had gross liver tumors and then selected 5-6 samples for each sex and each exposure group based on the highest RIN scores. Prior to library preparation, RNA quality was assessed using the Agilent 2200 TapeStation. Electropherograms indicated that the RINs ranged from 6.0-9.4 for RNA isolated from liver tissue. RNA isolated from liver was used to generate libraries with the Illumina TruSeq stranded mRNA Library Prep Kit and RNA isolated from WAT was used to generate libraries with the KAPA mRNA hyper prep kit following manufacturer instructions. Quantity and quality of the prepared libraries were confirmed with the Agilent 2200 TapeStation. Sequencing was carried out on the Illumina HiSeq 4000. Liver libraries were multiplexed across 16 sequencing lanes and were sequenced on one full flow cell to eliminate batch effects. Paired-end 50 base-pair reads were sequenced.

Bioinformatics Pipeline and Differential Expression

Sequenced reads were trimmed via Cutadapt,[35] quality assessment with Fastqc,[36] STAR alignment,[37] and expression quantification using RSEM.[38] The following quality parameters were assessed for each library: 1) Number of unique reads, 2) ratio of unique reads to duplicates, 3) number of reads covering gene bodies, and 4) area under the curve (AUC) for gene body reads. One liver sample had substantially lower reads (2.4 million unique reads and 2.1 million unique reads in gene bodies) than the others and was removed from downstream analyses. After removal of this sample, number of unique reads ranged from 15.2 million to 74.4 million, ratio of unique reads to duplicates ranged from 74.9 percent to 87.8 percent, number of unique reads in gene bodies ranged from 12.5 million to 62.8 million, and AUC for gene body reads ranged from 56.9 percent to 79.4 percent. Differential expression was analyzed using the quasi-likelihood function (QLF) of edgeR[39,40] in R (www.r-project.org) between each exposure group compared to controls, stratified by tissue, age and sex. Another package in R, DESeq2,[41] was used to obtain normalized read counts for each gene for plotting purposes.

Pathway Analysis

Pathway enrichment analyses were carried out via RNA-Enrich through LRpath [42]. To perform a pathway analysis on differential gene expression from both PND21 and 10 month time points together, we applied Fisher's meta-analysis method to combine p-values from PND21 and 10 months. We utilized the mean reads of controls in RNA-Enrich to adjust for read counts per gene to minimize potential bias introduced by differing read counts, and to improve type I error rates.

RT-qPCR

Quantitative reverse transcription PCR (RT-qPCR) was carried out for three candidate genes in liver collected from PND21 (N=7-10/group/sex) and 10-month mice (N=8-9/group/sex). RNA was converted to cDNA using iScript cDNA Synthesis Kits (Bio-Rad cat# 1708891) following manufacturers' instructions. We utilized a reference gene panel (PrimePCR H384 Reference Gene Panels, Bio-Rad) to test for reference genes that were stable in liver tissue across exposure groups and sex using pooled samples with eight mice per pool. This was carried out for RNA collected from livers at PND21 and 30 reference genes in triplicate were analyzed using geNorm.[43] The most stable genes based on geNorm M values were Hmbs, Actb, and Psmc4 (**Appendix Figure 4.A1A**). We also used geNorm to calculate V values, which is defined as systematic variation for repeated RT-qPCR experiments on the same gene which reflects the variation in the machine, enzymes, and pipetting. Our analyses indicated that even using just one reference gene would would provide reliable results, with a geNorm V value of <0.15, but elected to use two reference genes: Hmbs and Psmc4 (**Appendix Figure 4.A1B**). We excluded samples from the analysis if melt curve data indicated poor sample integrity or if Cq values were larger than 38, which is indicative of very low copies of the mRNA being amplified, and therefore would be unreliable.

Quantitative PCR reactions were set up using PrimePCR assays for *Acly*, *Cs*, and *Fasn* (Bio-Rad) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) via the manufacturer's instructions. PrimePCR assays have been designed and experimentally validated to meet MIQE guidelines. Reactions were set up in 384-well plates so that all samples from PND21 mice were on one plate and all samples from 10-

month mice were on a second plate. Samples were run in triplicate, and controls included a no template control (NTC), positive PCR control, and genomic DNA control. We analyzed one gene per plate using the CFX384 Real-Time PCR Detection System (Bio-Rad). The cycling protocol was as follows: 1) 2 mins at 95°C x 1 cycle, 2) 5 seconds at 95°C and 30 seconds at 60°C x 40 cycles, and 3) melt curve with 0.5°C 5 second increments from 65°C – 95°C. Relative expression was carried out via calculating delta Cq, comparing Cq values of each target gene to Cq values of the reference genes. The 2^{-ddCq} method was used to estimate fold-change.

DNA methylation

We measured DNA methylation using bisulfite conversion of DNA and subsequent pyrosequencing assays on liver from PND21 (N=7-12/group/sex) and 10-month mice (N=8-10/group/sex). We bisulfite converted DNA isolated from liver tissue using Zymo EZ-96 DNA Methylation kits (Zymo Cat# D5004) following manufacturer instructions, and then used pyrosequencing to measure DNA methylation in the promoter regions of three target genes: citrate synthase (*Cs*), ATP-citrate lyase (*Acly*), and fatty acid synthetase (*Fasn*). **Appendix Table 4.A1** describes the pyrosequencing assay parameters, including chromosomal location, primer sequences, annealing temperatures, sequence to analyze, and amplicon length. Primers were designed using PyroMark Assay Design software 2.0 and mm10 mouse genome. Pyrosequencing assays were designed to capture CpGs in the promoter regions of PPAR target genes, and publicly available data from Cistrome (cistrome.org)[44] was used to select regions that were adjacent to PPAR binding sites (**Appendix Figure 4.A2**). DNA methylation levels were measured using PyroMark Q96 ID instrument (Qiagen). All bisulfite-

converted DNA from PND21 mice were run on one plate and samples from 10-month mice were run on another plate to reduce plate-to-plate batch effects. Oxidative bisulfite-converted DNA was only run for females and therefore all PND21 and 10-month samples were run on the same plate. A subset of samples were run in duplicate to ensure that the coefficient of variation was <10% for each assay. Each plate included 0%, 25%, 50%, 75%, and 100% bisulfite converted methylation controls and a no template control to ensure that the assay was functioning properly.

Targeted Metabolomics

Targeted metabolomics assays were carried out on ~50mg frozen liver tissue at the Michigan Regional Comprehensive Metabolomics Resource Core (MRC^2) at the University of Michigan in Ann Arbor, MI. Two targeted assays were carried out: 1) a central metabolism profile[45] and 2) an acylcarnitine profile.[46] The central metabolism assay included analytes involved in multiple metabolic pathways, including the citric acid cycle (TCA cycle), glycolysis, pentose-phosphate shunt, and also includes measurement of select fatty acids. Samples underwent solvent extraction and was subsequently separated on a 1mm x150mm hydrophilic interaction liquid chromatography (HILIC) specific column using a 35-minute cycle. Analytes were measured on a quadrupole time of flight (Q-TOF) mass spectrophotometer. The acylcarnitines assay was carried out to measure 30 acylcarnitine species subsequent to solvent extraction. The samples were separated via 20-minute RPLC cycle and measured on a liquid chromatography triple quadrupole (LC-QQQ) mass spectrometer with multiple reaction monitoring (MRM) methods. Both assays included internal standards. The majority of analyte quantities were standardized to wet tissue weight,

but a subset of 40 analytes included in the central metabolism profile were reported as relative abundance.

Statistical Analyses

All statistical analyses were carried out using R version 3.5.2 (www.r-project.org). All analyses were stratified by age and sex. Differential expression was carried out via edgeR as described above, and genes with false discovery rates (FDRs) of < 0.10 were identified as signals. Comparisons of relative expression as measured via RT-qPCR and DNA methylation levels were carried out via linear mixed effects models for PND21 mice and linear regression for 10-month mice comparing each exposure group to controls. Since a large portion of PND21 mice had littermates included in the study, we used linear mixed effects models with litter-specific random effects to account for within-litter correlation . This was not an issue for 10-month mice because only one male and one female per litter were used and all analyses were stratified by sex. For the bisulfite-converted DNA methylation assay in the CpG island in the promoter of *Cs*, there were several values of 0% methylation and therefore distributions were not normal. Therefore, we used generalized linear models with a zero inflation compound Poisson distribution available via the cplm package in R[47] to analyze differences between exposure groups and controls for data generated from this assay. A Bonferroni correction factor was applied to RT-qPCR and DNA methylation analyses to account for multiple comparisons; three comparisons were made per analysis since there were three exposure groups and each were compared to the control group. For these analyses, we considered comparisons with adjusted p-values of < 0.05 as significantly different, and those < 0.10 to be marginally significant.

Results

RNA-seq

To identify PPAR target genes that were persistently altered by perinatal phthalate exposures, we utilized transcriptomics via RNA-seq to screen for PPAR target genes and/or biologically-relevant pathways that were altered at an early-life time point when offspring were still directly exposed (PND21) and a later-life time point when offspring had not been exposed for several months (10 months; >9 months after exposure had ceased). Differential expression analyses comparing hepatic gene expression in exposed groups versus controls, stratified by age and sex, revealed that PND21 females perinatally exposed to DINP-only had the most differentially expressed genes with FDR<0.10, with 61 (**Table 4.1**). PND21 females perinatally exposed to a combination of DEHP+DINP had one differentially expressed gene with FDR<0.10, while PND21 females perinatally exposed to DEHP-alone did not have any with FDR<0.10. Females at 10 months of age did not exhibit any differentially expressed genes at FDR<0.10 by exposure group, nor did males at PND21 or males at 10 months.

The top 10 differentially expressed genes in DINP female livers at PND21 are presented in **Table 4.2**, and the full list can be found in **Appendix Table 4.A2**. The top ten differentially expressed genes were *Atp2a1*, *Myh1*, *Fabp3*, *Tnni2*, *Acta1*, *Dsg1c*, *Pgam2*, *Ryr1*, *Clip4*, and *Tpm2*. A majority of the 61 differentially expressed genes were up-regulated while very few were down-regulated; out of 61 differentially expressed genes (FDR<0.10), 56 were up-regulated and only five were down-regulated.

WAT differential gene expression analyses were also stratified by age and sex, and indicated that 10-month old females perinatally exposed to DINP-only and

DEHP+DINP had three (*Rn7sk*, ENSMUSG00000106106, and ENSMUSG00000089417) and two genes (*Rn7sk,* ENSMUSG00000106106), respectively, that were differentially expressed compared to 10-month control females at FDR<0.10 (**Table 4.3**). No other exposure groups had differentially expressed genes in WAT tissue in either sex and at either age at FDR<0.10. *Rn7sk* and ENSMUSG00000106106 are both RNA genes as opposed to protein coding genes. *Rn7sk* is a small nuclear RNA and ENSMUSG00000106106 is part of 18s RNA. Both *Rn7sk* and ENSMUSG00000106106 showed decreased expression in WAT from DINP-only females (log fold change, or LFC=-2.86, FDR=0.002; LFC=-3.10, FDR=0.012, respectively) and DEHP+DINP females (LFC=-2.77, FDR=0.008; LFC=-2.85, FDR=0.09, respectively) at 10 months compared to controls.

Pathway Enrichment Analysis

Since a primary objective of this study was to identify long-lasting alterations in gene expression in metabolic pathways that were influenced by perinatal phthalate exposures, we utilized pathway enrichment analyses to determine whether there were biologically relevant gene pathways that may have been impacted at both PND21 and 10 months. Although we did not observe differential gene expression by exposure at 10 months with FDR<0.10, we considered the possibility that sets of biologically relevant genes may have been differentially expressed at relatively small effect sizes that were not detected via differential gene expression and FDR<0.10. We utilized the RNA-Enrich function in LRpath to carry out pathway enrichment analysis for Gene Ontology (GO) Biological Pathways (BP). To identify pathways that were enriched at both PND21 and 10 months, we used Fisher's method to combine p-values from differential

expression analyses at PND21 and at 10 months, and those p-values were used as input for LRpath.

Pathway analyses revealed several metabolic pathways in the liver that were potentially reprogrammed by perinatal phthalate exposures. The top 10 pathways for DEHP females, DINP females, and DEHP+DINP females are presented in **Table 4.4 and 4.5**; a list of the top ten enriched pathways in males is located in **Appendix Table 4.A3 and 4.A4**. Females and males perinatally exposed to DINP had the largest number of enriched pathways at FDR<0.05, with 12 and 15 pathways, respectively. Notable pathways that were in enriched in PND21 and 10 month females perinatally exposed to DINP included acetyl-CoA metabolic process (OR=2.42, FDR=0.0018), acyl-CoA metabolic process (OR=2.10, FDR=0.0018), and thioester metabolic process (OR=2.10, FDR=0.0018), which are processes regulated by PPARs (CITE). DINP males also had several enriched metabolic pathways, including alpha-amino acid metabolic process (OR=2.49, FDR=0.0006), organic acid metabolic process (OR=1.69, FDR=0.003), dicarboxylic acid metabolic process (OR=2.50, FDR=0.012), and small molecule metabolic process (OR=1.50, FDR=0.019) (**Appendix Table 4.A3**).

Within the most significantly enriched hepatic pathway for DINP females, acetyl-CoA metabolic process, there were 13 significant genes and 10 of those are PPAR target genes (**Table 4.6**), as identified via PPARgene.org with confirmed experimental data (GEO accession numbers are referenced within **Table 4.6**). Of these 10 PPAR target genes, nine were up-regulated in DINP females compared to control females (unadjusted p-value≤0.05) at either PND21 or 10 months: *Fasn, Pdk4, Pdha1, Acacb, Acly, Acss2, Pdk2, Cs*, and *Dlat*. *Mlycd* was the only PPAR target gene that was not up-

regulated at either time point; it showed a non-statistically significant trend towards down-regulation in PND21 DINP females and was down-regulated in 10-month DINP females (unadjusted p=0.0085). Of the nine up-regulated genes, four were up-regulated at both PND21 and 10 months (unadjusted $p \leq 0.05$ vs. controls): *Fasn*, *Pdk4, Acacb,* and *Cs* (**Table 4.6**). Two were up-regulated at PND21 to a modest degree of statistical significance (unadjusted $p < 0.10$) and were also up-regulated at 10 months (unadjusted $p \leq 0.05$): *Acss2* and *Dlat* (**Table 4.6**). Out of the 10 PPAR target genes driving the acetyl-CoA metabolic process pathway, five were PPARα targets, 5 were PPARδ/β targets, and seven were PPARγ targets. Notably, females perinatally exposed to DEHP-only and DEHP+DINP also exhibited differences in PPAR target gene expression, although these differences were less consistent than those observed in DINP-only females (**Figure 4.2A-L**).

The most enriched pathway in males perinatally exposed to DINP across the PND21 and 10 month time points was alpha-amino acid metabolic process. Within this pathway, two of 15 significant genes were identified as potential PPAR target genes with experimental evidence, and another two were identified as potential PPAR target genes only due to putative PPRE binding sites located flanking the transcription start site (TSS) (**Appendix Table 4.A5**). However, in the second most enriched pathway in DINP males, organic acid metabolic process, 13 out of 33 significant genes in the pathway were identified as PPAR target genes with experimental evidence and two genes had PPREs flanking the TSS (**Appendix Table 4.A6**). Despite the relatively large number of PPAR target genes that were significant in this pathway, there was only one gene that had the same directional change to a degree of at least modest statistical

significance (unadjusted p<0.10) at both PND21 and 10 months: *Cyp2e1*. Furthermore, there was a mixture of up- and down-regulated genes at both PND21 and 10 months.

Carrying out parallel pathway analyses in WAT tissue indicated that many GO Biological Process pathways were potentially reprogrammed in WAT by perinatal phthalate exposures in both males and females (**Tables 4.7-4.10**). In females, those perinatally exposed to DEHP-only had 166 enriched pathways (FDR<0.05), those perinatally exposed to DINP-only had 62 enriched pathways (FDR<0.05), and those perinatally exposed to DEHP+DINP had 13 enriched pathways (FDR<0.05). DEHP males had 123 enriched pathways (FDR<0.05), DINP males had 83 enriched pathways (FDR<0.05), and DEHP+DINP males had 79 enriched pathways (FDR<0.05). Although there was a relatively large number of potentially reprogrammed pathways identified in WAT from females perinatally exposed to phthalates, PPAR target genes made up a relatively small proportion of genes driving these pathways (**Table 4.11**). WAT from females perinatally exposed to DEHP-only was enriched for several pathways involving lipid, cholesterol, and steroid processing and esterification (**Table 4.7-4.8**). These pathways were driven primarily by just two genes: *Apoe* and *Apoa1*. *Apoe* is a target of PPARγ, and it showed very modest down-regulation in DEHP females at PND21 (LFC = -0.62, unadjusted p=0.053) and very modest up-regulation at 10 months (LFC = 0.49, unadjusted p=0.08). WAT from females perinatally exposed to DINP-only and DEHP+DINP was enriched for multiple pathways involving RNA processing and metabolism, including RNA processing, RNA metabolic process, and RNA splicing, but not for metabolic pathways associated with PPAR activation. Furthermore, the few PPAR target genes that were significant in these pathways were not uni-directionally

differentially expressed at both PND21 and 10 months, except for one gene in DINP females (**Table 4.11**). *Smarca2* was up-regulated in WAT at PND21 (LFC=1.11, unadjusted p=0.039) and exhibited modest up-regulation at 10 months (LFC=0.33, unadjusted p=0.089).

The vast majority of enriched pathways in WAT from males perinatally exposed to phthalates across PND21 and 10 months of age were related to muscle development and function (**Table 4.9-4.10**). The most significant enriched pathway in WAT of males perinatally exposed to DEHP-only, DINP-only, and DEHP+DINP across PND21 and 10 months was muscle contraction (FDR<0.00001 for all three groups). Within the muscle contraction pathway, there were a handful of PPAR target genes that were at least modestly down-regulated at both PND21 and 10 months (unadjusted p<0.10; **Table 4.12**). One PPAR target gene exhibited decreased expression at both time points in all three exposure groups compared to controls: *Tpm2* (**Figure 4.3A-B**). Expression of both *Atp2a2* and *Mybpc1* were decreased at both PND21 and 10 months in DEHP-only males and DINP-only males relative to controls with at least a modest degree of statistical significance when p-values are not corrected for multiple comparisons (unadjusted p<0.10; **Figure 4.3C-F**).

In addition to pathways involved in muscle function and development, WAT from males perinatally exposed to DINP-only was also enriched for several metabolic pathways, including pathways that were enriched in DINP-only female livers. These pathways included generation of precursor metabolites and energy (FDR=0.0000034), acyl-CoA metabolic process (FDR=0.00013), thioester metabolic process (FDR=0.00013), and acetyl-CoA metabolic process (FDR=0.00053). Within these

pathways, *Pdk4* and *Mlycd* were at least modestly differentially expressed (unadjusted p<0.10) relative to controls at both PND21 and 10 months, although expression was decreased for both at PND21 (LFC=-3.53, unadjusted p=0.0024; LFC=-0.89, unadjusted p=0.095, respectively) and increased for both at 10 months (LFC=0.47, unadjusted p=0.094; LFC=0.43, unadjusted p=0.034, respectively).

RT-qPCR

We next carried out RT-qPCR to examine relative expression for three genes that are involved in acetyl-CoA metabolic processes and were identified as being altered in the liver by perinatal exposure to phthalates in females: *Acly*, *Cs*, and *Fasn*. Analyses of RT-qPCR for relative expression of these three genes were confirmatory of the RNA-seq expression patterns at PND21. Females perinatally exposed to DINP-only and DEHP+DINP had increased relative expression of *Cs* (adjusted p=0.033 and 0.040, respectively) and *Fasn* (adjusted p=0.081 and 0.007, respectively) in the liver compared to controls (**Figure 4.4A, 4.4E**). Females perinatally exposed to DEHP+DINP also had increased relative expression of *Acly* in the liver at PND21 (p=0.015) (**Figure 4.4C**). Although there was not a statistically significant difference in hepatic expression of *Acly* at PND21 via RNA-seq reads, the trends in *Acly* expression at PND21 across exposure groups was similar between RNA-seq (**Figure 4.2C**) and RT-qPCR data (**Figure 4.4B**).

In livers collected from females at 10 months, RT-qPCR relative expression data were less consistent with RNA-seq expression data than it was for PND21 livers. In contrast to RNA-seq read count data, there were no statistically significant differences in relative expression of *Cs* or *Fasn* at 10 months via RT-qPCR (adjusted p>0.10; **Figure 4.4B, 4.4F**). Relative hepatic *Acly* expression, however, was increased in 10-month

females perinatally exposed to DINP and DEHP+DINP compared to controls (adjusted p=0.012 and 0.097, respectively) (**Figure 4.4D**). Differential expression analysis of RNA-seq data indicated similar trends in hepatic *Acly* expression across exposure groups (**Figure 4.2D**).

RT-qPCR assays for *Acly*, *Fasn*, and *Cs* were also carried out in livers collected from males at PND21 and 10 months. Males perinatally exposed to DINP had decreased relative expression of *Cs* in the liver at 10 months (adjusted p=0.028). However, there were no other statistically significant relationships between relative hepatic expression of *Fasn*, *Acly*, or *Cs* and perinatal exposures to phthalates at PND21 or 10 months of age in males.

DNA methylation

Since PPARs can recruit TET enzymes to de-methylate promoter region DNA of PPAR target genes,[21] we measured CpG methylation levels in the promoter regions of *Acly*, *Fasn*, and *Cs* in the livers collected from mice at PND21 and 10 months of age. To do this, we utilized bisulfite conversion of DNA coupled with pyrosequencing. Pyrosequencing assays were designed to capture CpG-rich regions adjacent to PPAR binding sites in the promoter regions of the three genes (**Appendix Figure 4A.2**). PPAR Chromatin Immunoprecipitation Sequencing (ChIP-seq) peaks from Cistrome (cistrome.org)[44] were used to visually identify potential PPARa and PPARy binding sites on the genome.

Females perinatally exposed to phthalates had altered DNA methylation levels in the promoter regions of *Cs*, *Acly*, and *Fasn* (**Figure 4.5**). At PND21, there were no statistically significant differences in *Cs* promoter region DNA methylation levels in

females between control and exposure groups, although non-statistically significant trends were reflective of mRNA expression level patterns across exposure groups (**Figure 4.5A, Figure 4.2E, Figure 4.4A**). At 10 months, females perinatally exposed to DEHP+DINP had significantly decreased hepatic DNA methylation percentage in the *Cs* promoter by 1.06 compared to controls (adjusted p=0.04) (**Figure 4.5B**). As noted above, hepatic *Cs* expression was increased in females perinatally exposed to DEHP+DINP at PND21, but not at 10 months of age.

In PND21 females, promoter *Acly* DNA methylation percentages in the liver reflect *Acly* expression levels at PND21 (**Figure 4.5C, Figure 4.2C, Figure 4.4C**). Compared to control females, females perinatally exposed to DEHP+DINP had a 1.39 decrease in percent DNA methylation in the *Acly* promoter region at PND21 (adjusted p=0.048). This was in concordance with RT-qPCR expression data which indicated that DEHP+DINP females had increased hepatic *Acly* expression at PND21. Despite our observations that there were changes in hepatic mRNA expression of *Acly* in females perinatally exposed to DINP-only and DEHP+DINP relative to controls at 10 months of age, there were no statistically significant exposure-related changes in *Acly* promoter region DNA methylation at 10 months (**Figure 4.5D**).

Fasn promoter region DNA methylation levels were unexpectedly increased in livers from female mice perinatally exposed to phthalates at PND21 and 10 months of age when compared to controls (**Figure 4.5E, 4.5F,** respectively). At PND21, females perinatally exposed to DEHP-only and DINP-only had higher percent methylation than control females by 4.88 and 3.75, respectively (adjusted p=0.002 and 0.007, respectively). This was the largest effect size observed for DNA methylation in this

study. Females perinatally exposed to DINP-only also had modestly increased hepatic DNA methylation in the *Fasn* promoter compared to controls (effect size = 0.78%; adjusted p=0.084). Interestingly, the observed increase in *Fasn* promoter region DNA methylation in DINP-only females corresponded to increased *Fasn* expression at both time points, which was unanticipated based on typical relationships between promoter region DNA methylation and gene expression.

Males perinatally exposed to phthalates exhibited minimal effects on hepatic DNA methylation in the promoter regions of *Cs*, *Acly*, and *Fasn*. The only statistically significant difference was in males perinatally exposed to DEHP+DINP at PND21, who had increased DNA methylation in the CpG island of the *Cs* promoter compared to controls (effect size = 0.91%, adjusted p=0.013). However, there was no complementary significant alteration in *Cs* expression in DEHP+DINP males at PND21.

Discussion

Liver

The findings presented here are consistent with the numerous studies that have demonstrated phthalates' abilities to activate PPARα, PPARγ and PPARδ/β,[6,7,9] but this is the first study providing evidence that developmental exposures to phthalates may have long-lasting impacts on metabolic pathways that are regulated by PPARs in the liver. We found that perinatal exposures to phthalates were associated with altered hepatic gene expression and promoter region DNA methylation in PPAR target genes in female mice at both PND21 and 10 months. Pathway enrichment analyses of RNA-seq data indicated that pathways regulated by PPARs were altered in DINP-only female livers at PND21 and 10 months of age, indicating that these pathways were potentially

reprogrammed by perinatal exposure to DINP. The 10 PPAR target genes that were significant in the top enriched pathway, acetyl-CoA metabolic process, indicated a shift in utilization of acetyl-CoA to fatty acid metabolism (**Figure 4.6**). RT-qPCR analyses for relative expression of three PPAR target genes involved in these pathways, *Acly, Cs,* and *Fasn*, confirmed differential expression analyses of RNA-seq data for PND21 livers, but less consistently confirmed findings for 10-month livers. Targeted analyses in the liver of females at PND21 and 10 months revealed that DNA methylation in the promoter regions of *Cs*, *Acly*, and *Fasn* was altered by perinatal phthalate exposures.

To the best of our knowledge, this was the first study to utilize transcriptomics to evaluate hepatic gene expression of PPAR target genes at two different ages of mice following perinatal phthalate exposure. Despite the relatively few previous studies that have examined hepatic gene expression changes following developmental phthalate exposures, our findings are generally consistent with studies that have examined developmental exposures to other obesogens and in studies that evaluated direct exposures to DEHP and other PPAR agonists. In concordance with our data, mice that were directly exposed to 200 or 1150 mg/kg-day of DEHP in adulthood exhibited increased *Acacb, Acss2,* and *Pdk4* hepatic mRNA expression.[48] Furthermore, Ren et al. 2010 found that the effects of DEHP on hepatic gene expression of *Acacb, Acss2,* and *Pdk4* were PPARα-dependent. Although we could not identify any studies that examined expression of *Cs* or *Dlat* in the liver following phthalate exposures, one study found that *Cs* and *Dlat* were up-regulated in the hearts of mice exposed to DEHP in adulthood,[49] which is consistent with our data indicating *Cs* and *Dlat* were upregulated in livers of mice perinatally exposed to phthalates. *Fasn* mRNA expression was

upregulated in the livers of mice perinatally exposed to another environmental obesogen, tributyltin, as well as the PPAR-y agonist rosiglitazone,[50] which was similar to our findings with respect to phthalates in the present study. However, direct exposure to DEHP in adulthood has been associated with decreased hepatic *Fasn* gene expression.[51] Also in contrast to our findings, previous studies indicated that direct treatment of PPARα and PPARγ agonists to adult mice has resulted in increased, not decreased, *Mlycd* expression in the liver in previous studies.[52,53]

We found evidence of altered DNA methylation in the promoter regions of PPAR target genes that were differentially expressed in the liver of females perinatally exposed to phthalates. Specifically, we measured DNA methylation in the promoter region of *Cs, Acly*, and *Fasn*. In our previous work, we found sex-specific differences in DNA methylation at repetitive elements (intercisternal-a particles, or IAPs) in tails of mice perinatally exposed to phthalates at PND21; specifically, females perinatally exposed to DEHP-only, DINP-only, and DEHP+DINP had increased DNA methylation at IAPs relative to controls [26]. In the present study, we found that females perinatally exposed to DEHP+DINP had decreased DNA methylation in the promoter region of *Cs* in 10-month livers; however, hepatic *Cs* expression was increased in DEHP+DINP females only at PND21 and not at 10 months of age. Additionally, females perinatally exposed to DINP-only did not exhibit statistically significantly altered DNA methylation at the CpGs we analyzed in the *Cs* promoter region despite having increased hepatic *Cs* expression at both PND21 and 10 months of age. There were, however, non-statistically significant trends towards decreased DNA methylation in DINP-only females at both time points.

Females perinatally exposed to DEHP+DINP had decreased DNA methylation in the promoter region of *Acly* in the liver at PND21, which was accompanied by increased *Acly* mRNA expression at PND21. Trends in *Acly* DNA methylation and expression across exposure groups in females at PND21 were suggestive of an inverse relationship. Interestingly, DNA methylation in the *Acly* promoter decreased with age; mean *Acly* methylation in control females was 9.86% at PND21 and 4.20% at 10 months (t-test $p<0.00001$). With age, significant and non-significant differences in *Acly* methylation across exposure groups were also diminished. Promoter *Fasn* DNA methylation was increased in DINP females compared to controls at both PND21 and 10 months. Increased promoter region DNA methylation is typically associated with decreased mRNA transcription; however, sequencing bisulfite conversion of DNA cannot distinguish between 5'-methyl cytosine (5mC) and 5'-hydroxymethyl cytosine (5hmC). Thus, this increase in DNA methylation may be due to an increase in 5hmC. 5hmC is an intermediate of active DNA de-methylation catalyzed by TET enzymes, which can be recruited to target regions by PPARs, and is also increasingly being recognized as a stable epigenetic mark that may have distinctive relationships with mRNA expression.[54,55] In addition, previous work in our lab found that developmental exposure to another EDC, bisphenol-A (BPA), influenced 5hmC levels longitudinally across the genome in mouse blood, demonstrating that developmental EDC exposures are capable of altering 5hmC.[56] Future studies to analyze 5hmC levels in response to developmental phthalate exposures are needed. It is worth noting that our pyrosequencing assays only covered between 3 and 10 CpG sites per assay, so there may be other CpGs within the promoter region that have regulatory effects on gene

transcription that we did not measure. Furthermore, DNA methylation at other regulatory regions (e.g., enhancers), as well as other epigenetic factors (e.g., histone modifications) likely also play a role in regulating expression of these PPAR target genes.

Our data indicated that females exhibited more convincing evidence of reprogrammed PPAR pathways in the liver than males. For example, PPAR target genes driving the top pathways enriched in the livers of females perinatally exposed to DINP-only were more consistently up-regulated at both PND21 and 10 months than the PPAR target genes driving pathways enriched in males perinatally exposed to DINP. The sex-specific effects observed in females in this study are consistent with our previous work that demonstrated increased susceptibility to longitudinal metabolic phenotypes in females.[29] PPARs exhibit cross-talk with estrogen signaling,[57] and pharmaceutical PPARα and PPARγ agonists have sexually dimorphic side effects.[58] Since phthalates have been implicated in interfering with sex hormones[59] as well as activating PPARs, sexually dimorphic effects following phthalate exposures were expected. A previously published study examining liver reprogramming following developmental DEHP exposures found sex-specific reprogramming in males but not females; however, this study examined glycogen storage/depletion as the main outcome of interest, utilized higher doses of DEHP, analyzed younger mice, and only evaluated hepatic expression of one gene.[60] Other researchers who examined hepatic gene expression in mice perinatally exposed to a mixture of food contaminants, including DEHP, observed increased gene expression of cholesterol-related genes in males only.[61] However, this chemical mixture included other chemicals with diverse

modes of action, including bisphenol-A, polychlorinated biphenyl 153, and 2,3,7,8-tetrachlorodibenzo-p-dioxin. Additional studies are needed to fully elucidate the underlying mechanisms driving sex-specific effects of developmental phthalate exposures on PPAR target genes.

A majority of previous studies that have examined metabolic impacts of developmental phthalate exposures have focused on investigating DEHP. The inclusion of DINP and a mixture of DEHP+DINP in the present study is unique and also of critical importance in the context of public health due to trends indicating that exposure to DEHP is declining while exposure to DINP is increasing in women of reproductive age in the US population.[62] Furthermore, humans are exposed to mixtures of phthalates, and it is therefore important to understand metabolic impacts of developmental phthalate mixture exposures. Our findings with respect to DINP in the present study combined with whole-body metabolic phenotyping data published in previous studies highlight the need for continued examination of this phthalate. Results from this study and previous studies also have interesting implications with respect to metabolic impacts of phthalate mixtures. A combination of RNA-seq and RT-qPCR data indicated that females perinatally exposed to DEHP+DINP had increased hepatic expression of some PPAR target genes at PND21, but in most cases, these effects did not persist to 10 months of age. This was consistent with our previous phenotyping studies that indicated females perinatally exposed to DEHP+DINP had increased body weight and relative liver weight at PND21, but did not exhibit metabolic phenotypes longitudinally. Further studies are needed to better understand the observed effects on molecular

metabolic pathways and whole-body metabolism in mice perinatally exposed to phthalate mixtures.

Up-regulation of the genes in the acetyl-CoA metabolic process pathway diagrammed in **Figure 4.6** could result in a predisposition towards increased fatty acid biosynthesis in the liver. If those fatty acids are used for triglyceride synthesis and subsequent storage in the liver, or are exported to circulation as free fatty acids, then this could increase the susceptibility of developing insulin resistance, NAFLD, or coronary heart disease.[63,64] Previous studies have demonstrated that direct exposure to high doses of DEHP induced fat accumulation in the liver cells *in vitro*.[65] In our previously published work,[29] we found that female mice perinatally exposed to DINP had impaired glucose tolerance across the life course, and it is possible that altered hepatic gene expression of the PPAR target genes identified in this study could have influenced glucose tolerance in these mice. Additional studies are needed to better understand the role that these persistently altered PPAR target genes play in mediating the relationship between developmental phthalate exposures and susceptibility to metabolic disorders.

The data presented in this study provide evidence for altered mRNA expression and promoter DNA methylation of PPAR target genes in the liver following perinatal phthalate exposures. However, our analyses were on bulk liver tissue, and we did not carry out analyses to determine whether cellular composition of the liver was altered in phthalate-exposed mice. Therefore, we were unable to determine whether perinatal phthalate exposures reprogrammed the cells of the liver, or if they reprogrammed the cellular composition of the liver. It should also be noted that phthalate-related changes

in PPAR target gene expression were relatively subtle. None of the PPAR target genes that we examined had an FDR<0.10 when analyzing the entire transcriptome, and log fold changes in genes that had un-adjusted p-values of < 0.05 ranged from -0.45 to 2.49. DNA methylation changes also had relatively small magnitudes, with magnitudes ranging from 1.06 to 4.88 percent. These relatively small magnitude changes in gene expression and DNA methylation are consistent with the subtle metabolic phenotypes observed in these same mice.[26,29] In addition, small effect sizes are common for DNA methylation changes associated with developmental exposures to environmental chemicals.[66] In general, RT-qPCR data replicated the RNA-seq data, but for *Cs* and *Fasn* expression in 10-month livers, RT-qPCR data did not agree with RNA-seq data. One possible reason for this discrepancy is that RT-qPCR measures relative expression whereas RNA-seq measures read counts. We selected two reference genes based on a reference gene panel that included cDNA from PND21 livers across control and exposure groups, but it is possible that these two genes, Psmc4 and Hmbs, were less suitable for reference genes in 10-month livers.

WAT

Overall, differential expression and pathway analyses in WAT did not indicate that there were PPAR target genes reprogrammed in females perinatally exposed to phthalates. Interestingly, RNA processing and metabolism pathways appeared to be impacted by developmental phthalate exposures in females. *Rn7sk* and ENSMUSG00000106106 were both significantly down-regulated in WAT from 10-month females that were perinatally exposed to DINP-only and DEHP+DINP. Although these genes are unlikely to be directly regulated by PPARs, they may be downstream of direct

PPAR effects. Interestingly, *Rn7sk* has been implicated in playing a role in cellular senescence in adipose tissue,[67] though relatively little is known about the function of this small nuclear RNA. *Smarca2* is a potential PPARα target gene within the nucleic acid metabolic process pathway that demonstrated increased WAT expression in DINP-only females at PND21 and modestly increased WAT expression at 10 months compared to controls. This gene is involved in chromatin remodeling and is highly expressed in the ovary, brain, thyroid, and fat tissue.[68] Further investigation to understand how developmental phthalates impact RNA processing and nucleic acid metabolism are needed to understand mechanisms and implications for metabolism.

WAT from males perinatally exposed to phthalates exhibited enrichment for muscle function and development pathways. This was an unexpected pathway for WAT, but there were several PPAR target genes involved in driving enrichment of these pathways. Notably, the PPAR target genes that were driving these pathways were all down-regulated in WAT from phthalate-exposed males, and all were PPARα or PPARδ/β target genes as opposed to PPARγ, which is the predominant isoform in WAT. Three genes were up-regulated in both PND21 and 10-month WAT from males perinatally exposed to DEHP-only and DINP-only: *Tpm2*, *Atp2a2*, and *Mybpc1*. Although muscle contraction is a primary function of these genes, they are also all expressed in adipose tissue according to data made publicly available in the Genotype-Tissue Expression (GTEx) portal (www.gtexportal.org). *Tpm2* and *Mybpc1* bind actin and stabilize the cytoskeleton of non-muscle cells,[69] and *Atp2a2* regulates calcium homeostasis.[70] However, there is very little in the published literature with respect to adipose-specific functions of these genes.

Conclusion

Liver

Overall, our data suggest that perinatal exposures to phthalates have persistent effects on mRNA expression and promoter DNA methylation of PPAR target genes in the liver. Long-lasting alterations in PPAR target gene expression and DNA methylation were more evident in females than in males, providing evidence for sex-specific effects of developmental phthalate exposures on the liver. Metabolic pathways were most enriched in females perinatally exposed to DINP-only, demonstrating the need for increased animal and human studies evaluating metabolic effects of DINP. Perinatal exposure to a mixture of DEHP+DINP was associated with increased gene expression of several PPAR target genes at PND21, but only one gene at 10 months, indicating a potential adaptive response. Additional studies are needed in other metabolic tissues such as skeletal muscle, cardiac muscle, and adipose tissue to determine whether similar PPAR target genes are impacted across multiple tissue types and to provide more context for how molecular mechanisms influence whole-body metabolic effects.

WAT

We did not find clear evidence of persistent PPAR target gene activation in WAT from females perinatally exposed to phthalates or phthalate mixtures. In contrast, RNA-seq and pathway analysis data indicated that perinatal phthalate exposures were associated with dysregulation of RNA metabolism and processing pathways in female WAT. In males, PPAR target genes involved in muscle contraction and cytoskeleton stabilization had decreased mRNA expression in WAT from phthalate-exposed mice compared to controls. The specific roles that these genes play in WAT are unclear and

additional work is needed to determine how down-regulation of these genes may impact metabolism.

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Tables and Figures

	# Differentially expressed genes (FDR<0.10)			
	PND21 Females	10-Month Females	PND21 Males	10-month Males
DEHP v. Control	0	0	0	0
DINP v. Control	**61**	0	0	0
DEHP+DINP v. Control	1	0	0	0

Table 4.1 Differentially expressed genes in the livers of mice perinatally exposed to phthalates. Differential gene expression was determined by the quasi-likelihood function (QLF) in edgeR comparing each exposure group to controls. The number of differentially expressed genes in this table was determined by FDR<0.10.

Top 10 Differentially Expressed Genes in PND21 DINP Female Livers vs. Controls

Gene Symbol	Log Fold Change	p-value	FDR
Atp2a1	11.77168701	7.49E-06	0.038647
Myh1	11.30810269	8.60E-06	0.038647
Fabp3	11.58658065	1.68E-05	0.038647
Tnni2	10.72416927	1.69E-05	0.038647
Acta1	11.22848784	1.87E-05	0.038647
Dsg1c	-1.989642518	2.04E-05	0.038647
Pgam2	9.268813642	2.22E-05	0.038647
Ryr1	8.62230757	2.24E-05	0.038647
Clip4	7.161814544	2.50E-05	0.038647
Tpm2	4.626968688	2.67E-05	0.038647

Table 4.2 Top 10 Differentially Expressed Genes in PND21 DINP Female Livers vs. Controls. Diffrential gene expression was determined by the quasi-likelihood function (QLF) in edgeR comparing each exposure group to controls.

	# Differentially expressed genes (FDR<0.10)			
	PND21 Females	10-Month Females	PND21 Males	10-month Males
DEHP v. Control	0	0	0	0
DINP v. Control	0	3	0	0
DEHP+DINP v. Control	0	2	0	0

Table 4.3 Differentially expressed genes in WAT of mice perinatally exposed to phthalates. Differential gene expression was determined by the quasi-likelihood function (QLF) in edgeR comparing each exposure group to controls. The number of differentially expressed genes in this table was determined by FDR<0.10.

DEHP					DINP				
Pathway	# Genes	Odds Ratio	p-value	FDR	Pathway	#Genes	Odds Ratio	p-value	FDR
epithelial cell apoptotic process	27	3.4447791	3.46E-05	0.2097744	acetyl-CoA metabolic process	35	2.41708568	6.02E-07	0.0017998
vascular endothelial growth factor signaling pathway	7	6.741962	6.81E-05	0.2097744	acyl-CoA metabolic process	60	2.10335551	8.76E-07	0.0017998
positive regulation of phosphoprotein phosphatase activity	4	8.2277563	1.65E-04	0.2541489	thioester metabolic process	60	2.10335551	8.76E-07	0.0017998
protein K63-linked deubiquitination	5	6.8486845	2.10E-04	0.2541489	skeletal muscle contraction	5	5.25817636	3.79E-06	0.005839
hepatocyte apoptotic process	6	6.03992	2.47E-04	0.2541489	myofibril assembly	10	2.92499363	7.19E-06	0.008302
positive regulation of protein dephosphorylation	10	4.6290056	2.47E-04	0.2541489	muscle contraction	46	2.04154321	8.08E-06	0.008302
purine nucleobase metabolic process	12	5.7938222	3.94E-04	0.3245004	multicellular organismal movement	8	3.1928107	1.96E-05	0.0151104
response to organic substance	737	1.4750605	4.21E-04	0.3245004	musculoskeletal movement	8	3.1928107	1.96E-05	0.0151104
response to chemical	1000	1.4239517	5.09E-04	0.348267	monocarboxylic acid biosynthetic process	106	1.71963759	4.11E-05	0.0281485
positive regulation of phosphatase activity	5	6.1364769	6.45E-04	0.3783425	thioester biosynthetic process	22	2.37262318	6.77E-05	0.0368805

Table 4.4 Top 10 Enriched GO Biological Process Pathways Across PND21 and 10-Month Female Livers (Part 1). Pathway analyses were carried out via the RNA-Enrich function in LRpath for Gene Ontology Biological Processes. A combination of p-values from differential expression analysis at PND21 and 10 months calculated via Fisher's method was used for input into LRpath. Pathways above are enriched and do not include depleted pathways. Pathways were considered significantly enriched with FDR<0.05.

	DEHP+DINP			
Pathway	#Genes	Odds Ratio	p-value	FDR
acute-phase response	17	6.50315493	4.05E-06	0.024986
B cell homeostasis	3	26.1226456	3.87E-05	0.091743
acute inflammatory response	33	4.20303942	4.79E-05	0.091743
lipid biosynthetic process	263	2.16912803	7.70E-05	0.091743
brown fat cell differentiation	13	6.14357306	9.20E-05	0.091743
cytokine-mediated signaling pathway	78	3.20446311	9.34E-05	0.091743
negative regulation of lymphocyte apoptotic process	5	11.4268032	1.09E-04	0.091743
fat cell differentiation	51	3.4369137	1.23E-04	0.091743
innate immune response	136	2.49015132	1.36E-04	0.091743
steroid metabolic process	188	2.33796569	1.74E-04	0.091743

Table 4.5 Top 10 Enriched GO Biological Process Pathways Across PND21 and 10-Month Female Livers (Part 2). Pathway analyses were carried out via the RNA-Enrich function in LRpath for Gene Ontology Biological Processes. A combination of p-values from differential expression analysis at PND21 and 10 months calculated via Fisher's method was used for input into LRpath. Pathways above are enriched and do not include depleted pathways. Pathways were considered significantly enriched with FDR<0.05.

Gene	PPAR target gene	Confidence level	GEO Accession #	# PPREs in TSS-flanking region	PND21 LFC	PND21 p-value	10M LFC	10M p-value
Fasn	**PPAR*α*, PPAR*δ*/*β***	**High**	**GSE32706, GSE11803, GSE11804, GSE29055**	**5**	**1.62**	**0.011***	**1.66**	**0.0074****
Acaca	NA	NA	NA	NA	0.76	0.06^	1.53	0.0041**
Pdk4	**PPAR*α*, PPAR*δ*/*β*, PPAR*γ***	**High**	**GSE17250, GSE30553, GSE32706, GSE5777, GSE11803, GSE11804, GSE20371, GSE29055, GSE32498, GSE1458, GSE35011**	**2**	**2.49**	**0.0011****	**1**	**0.053***
Pdha1	PPAR*γ*	Medium	GSE1458	0	0.54	0.015*	0.16	0.32
Acacb	**PPAR*α*, PPAR*γ***	**High**	**GSE17250, GSE35011**	**6**	**1.49**	**0.014***	**1.39**	**0.0015****
Acly	PPAR*δ*/*β*	High	GSE11803, GSE29055	6	0.76	0.17	1.44	0.0091**
Mpc1	NA	NA	NA	NA	-0.48	0.015*	-0.47	0.13
Acss2	PPAR*δ*/*β*, PPAR*γ*	High	GSE29055, GSE30116	8	1.24	0.08^	1.31	0.027*
Mlycd	**PPAR*α*, PPAR*δ*/*β*, PPAR*γ***	High	GSE17250, GSE5777, GSE11803, GSE11804, GSE35011	2	-0.25	0.13	-0.45	0.0085**
Pdk2	PPAR*γ*	Medium	GSE35011	9	0.11	0.55	0.75	0.005**
Cs	PPAR*γ*	**High**	**GSE1458, GSE35011**	**3**	**0.76**	**0.0016****	**0.58**	**0.0099****
Mpc2	NA	NA	NA	NA	-0.26	0.29	-0.9	0.01**
Dlat	**PPAR*α*, PPAR*γ***	Medium	GSE32706, GSE35011	3	0.3	0.099^	0.55	0.0054**

Table 4.6 Significant genes driving pathway enrichment for acetyl-CoA metabolic process in DINP females. Genes were identified as PPAR target genes based on PPARgene.org, which utilizes a combination of evidence from published datasets and PPAR response elements (PPREs) in regions flanking th TSS. PPAR isoform was assigned based on experimental gene expression data in the Gene Expression Omnibus (GEO). Confidence levels are from PPARgene.org. PND21 and 10-month (10M) log fold change (LFC) and p-values (unadjusted) are from differential gene expression of RNA-seq data analyzed via edgeR quasi likelihood function (QLF) comparing DINP females to controls. Bolded genes were differentially expressed with an unadjusted p-value of <0.10 at both PND21 and 10 months.

DEHP					DINP				
Pathway	# Genes	Odds Ratio	p-value	FDR	Pathway	#Genes	Odds Ratio	p-value	FDR
high-density lipoprotein particle remodeling	4	6.23863066	2.49E-07	5.95E-04	nucleic acid metabolic process	2577	1.506462	1.87E-10	1.19E-06
positive regulation of cholesterol esterification	6	6.1129021	3.60E-07	5.95E-04	RNA processing	454	1.88064366	1.37E-09	4.36E-06
high-density lipoprotein particle assembly	6	5.88911531	7.29E-07	5.95E-04	mRNA processing	300	2.04434081	4.22E-09	8.93E-06
DNA alkylation	27	3.48046067	8.18E-07	5.95E-04	mRNA metabolic process	354	1.91125516	1.61E-08	2.56E-05
DNA methylation	27	3.48046067	8.18E-07	5.95E-04	RNA splicing	255	2.03338333	5.83E-08	7.42E-05
regulation of cholesterol esterification	7	5.32698386	8.42E-07	5.95E-04	RNA metabolic process	2370	1.42123991	8.84E-08	9.36E-05
steroid esterification	8	5.32698386	8.42E-07	5.95E-04	nucleobase-containing compound metabolic process	2895	1.38778681	1.69E-07	1.53E-04
sterol esterification	8	5.32698386	8.42E-07	5.95E-04	heterocycle metabolic process	2939	1.37536559	3.33E-07	2.62E-04
cholesterol esterification	8	5.32698386	8.42E-07	5.95E-04	chromatin organization	445	1.76343719	3.75E-07	2.62E-04
very-low-density lipoprotein particle remodeling	5	6.59124429	1.47E-06	8.26E-04	protein-DNA complex subunit organization	67	2.59506007	4.12E-07	2.62E-04

Table 4.7 Top 10 Enriched GO Biological Process Pathways Across PND21 and 10-Month Female WAT (Part 1). Pathway analyses were carried out via the RNA-Enrich function in LRpath for Gene Ontology Biological Processes. A combination of p-values from differential expression analysis at PND21 and 10 months calculated via Fisher's method was used for input into LRpath. Pathways above are enriched and do not include depleted pathways. Pathways were considered significantly enriched with FDR<0.05.

DEHP+DINP				
Pathway	#Genes	Odds Ratio	p-value	FDR
regulation of RNA splicing	75	2.5950739	1.21E-06	0.007706
regulation of RNA metabolic process	1832	1.4479118	6.58E-06	0.020708
RNA metabolic process	2370	1.41516142	9.76E-06	0.020708
gene expression	2954	1.38566804	1.34E-05	0.021245
regulation of gene expression	2199	1.40098005	1.84E-05	0.023385
cartilage morphogenesis	3	6.15637469	2.69E-05	0.028525
nucleic acid metabolic process	2577	1.37728097	3.41E-05	0.031032
negative regulation of regulated secretory pathway	6	4.45041753	5.38E-05	0.033799
regulation of nucleobase-containing compound metabolic process	1962	1.38045229	6.11E-05	0.033799
phospholipid homeostasis	4	5.10232447	6.38E-05	0.033799

Table 4.8 Top 10 Enriched GO Biological Process Pathways Across PND21 and 10-Month Female WAT (Part 2). Pathway analyses were carried out via the RNA-Enrich function in LRpath for Gene Ontology Biological Processes. A combination of p-values from differential expression analysis at PND21 and 10 months calculated via Fisher's method was used for input into LRpath. Pathways above are enriched and do not include depleted pathways. Pathways were considered significantly enriched with FDR<0.05.

DEHP					DINP				
Pathway	# Genes	Odds Ratio	p-value	FDR	Pathway	#Genes	Odds Ratio	p-value	FDR
muscle contraction	106	4.16850222	4.52E-32	2.90E-28	muscle contraction	106	3.45285068	1.78E-12	1.15E-08
muscle system process	160	3.375519322	4.14E-29	1.33E-25	muscle system process	160	2.71710505	1.09E-10	3.49E-07
striated muscle contraction	52	3.575642964	2.60E-18	5.58E-15	generation of precursor metabolites and energy	224	2.36748853	1.60E-09	3.43E-06
muscle cell development	93	2.917312928	3.08E-17	4.94E-14	energy derivation by oxidation of organic compounds	165	2.45133753	1.37E-08	2.19E-05
skeletal muscle contraction	14	6.702399412	4.26E-17	5.47E-14	coenzyme metabolic process	193	2.40050689	1.70E-08	2.19E-05
multicellular organismal movement	20	4.795771331	8.19E-17	7.51E-14	acyl-CoA metabolic process	55	3.58711512	1.47E-07	1.34E-04
musculoskeletal movement	20	4.795771331	8.19E-17	7.51E-14	thioester metabolic process	55	3.58711512	1.47E-07	1.34E-04
striated muscle cell development	86	2.938585081	9.91E-17	7.96E-14	acetyl-CoA metabolic process	29	4.59776522	7.36E-07	5.29E-04
muscle structure development	328	2.090722124	1.72E-14	1.23E-11	thioester biosynthetic process	22	5.32267265	8.24E-07	5.29E-04
myofibril assembly	36	3.579062337	2.23E-14	1.43E-11	acyl-CoA biosynthetic process	22	5.32267265	8.24E-07	5.29E-04

Table 4.9 Top 10 Enriched GO Biological Process Pathways Across PND21 and 10-Month Male WAT (Part 1). Pathway analyses were carried out via the RNA-Enrich function in LRpath for Gene Ontology Biological Processes. A combination of p-values from differential expression analysis at PND21 and 10 months calculated via Fisher's method was used for input into LRpath. Pathways above are enriched and do not include depleted pathways. Pathways were considered significantly enriched with FDR<0.05.

	DEHP+DINP			
Pathway	#Genes	Odds Ratio	p-value	FDR
muscle contraction	106	4.83733293	1.32E-27	8.47E-24
muscle system process	160	3.74542672	1.33E-24	4.28E-21
striated muscle cell development	86	3.41626135	3.68E-14	6.02E-11
muscle cell development	93	3.32170378	3.75E-14	6.02E-11
striated muscle contraction	52	4.18240908	6.21E-14	7.97E-11
skeletal muscle contraction	14	9.14792498	5.42E-13	5.8E-10
muscle fiber development	30	4.73320026	1.83E-12	1.68E-09
multicellular organismal movement	20	5.86081863	1.07E-11	7.67E-09
musculoskeletal movement	20	5.86081863	1.07E-11	7.67E-09
sarcomere organization	25	5.21094165	1.21E-11	7.76E-09

Table 4.10 Top 10 Enriched GO Biological Process Pathways Across PND21 and 10-Month Male WAT (Part 2). Pathway analyses were carried out via the RNA-Enrich function in LRpath for Gene Ontology Biological Processes. A combination of p-values from differential expression analysis at PND21 and 10 months calculated via Fisher's method was used for input into LRpath. Pathways above are enriched and do not include depleted pathways. Pathways were considered significantly enriched with FDR<0.05.

Exposure	Pathway(s)	# Significant genes	# Significant PPAR target genes			# PPAR targets up-regulated at both PND21 and 10 Months	# PPAR targets down-regulated at both PND21 and 10 Months
			High Confidence	Medium Confidence	Low Confidence		
DEHP	High-density lipoprotein particle remodeling, positive regulation of cholesterol esterification, high-density lipoprotein particle assembly, regulation of cholesterol esterification, steroid esterification, sterol esterification, cholesterol esterification, very-low-density lipoprotein remodeling	2	-	1	-	0	0
DEHP	DNA alkylation, DNA methylation	4	-	-	-	0	0
DEHP	Humoral immune response	1	-	-	-	0	0
DEHP	Positive regulation of steroid metabolic process	3	-	1	-	0	0
DINP	Nucleic acid metabolic process	153	-	5	6	1	1
DINP	mRNA metabolism	36	-	-	1	0	0
DEHP+DINP	Regulation of RNA metabolic process	59	1	4	2	0	0
DEHP+DINP	Phospholipid homeostasis	3	1	1	-	0	0

Table 4.11 PPAR target genes driving enriched pathways in female WAT. Significant genes were identified as genes with combined p-value using Fisher's method from PND21 and 10-month analyses as < 0.05. PPAR target genes and their confidence intervals were identified using PPARgene.org. A gene was defined as being up- or down-regulated if differential expression analyses via edgeR quasi-likelihood function (QLF) tests yielded an unadjusted p-value of <0.10 when comparing each exposure group to the control.

Exposure	Pathway	# Significant genes	# Significant PPAR target genes			# PPAR targets up-regulated at both PND21 and 10 Months	# PPAR targets down-regulated at both PND21 and 10 Months
			High Confidence	Medium Confidence	Low Confidence		
DEHP	Muscle contraction	32	1	6	10	0	3
DEHP	Muscle system process	36	1	7	11	0	4
DINP	Muscle contraction	18	-	2	3	0	3
DINP	Generation of precursor metabolites and energy	17	3	3	2	0	0
DINP	Acyl-CoA metabolic process	153	-	5	6	0	0
DINP	Glucose metabolic process	36	-	-	1	0	0
DEHP+DINP	Muscle contraction	30	1	3	5	0	1

Table 4.12 PPAR target genes driving enriched pathways in male WAT. Significant genes were identified as genes with combined p-value using Fisher's method from PND21 and 10-month analyses as < 0.05. PPAR target genes and their confidence intervals were identified using PPARgene.org. A gene was defined as being up- or down-regulated if differential expression analyses via edgeR quasi-likelihood function (QLF) tests yielded an unadjusted p-value of <0.10 when comparing each exposure group to the control.

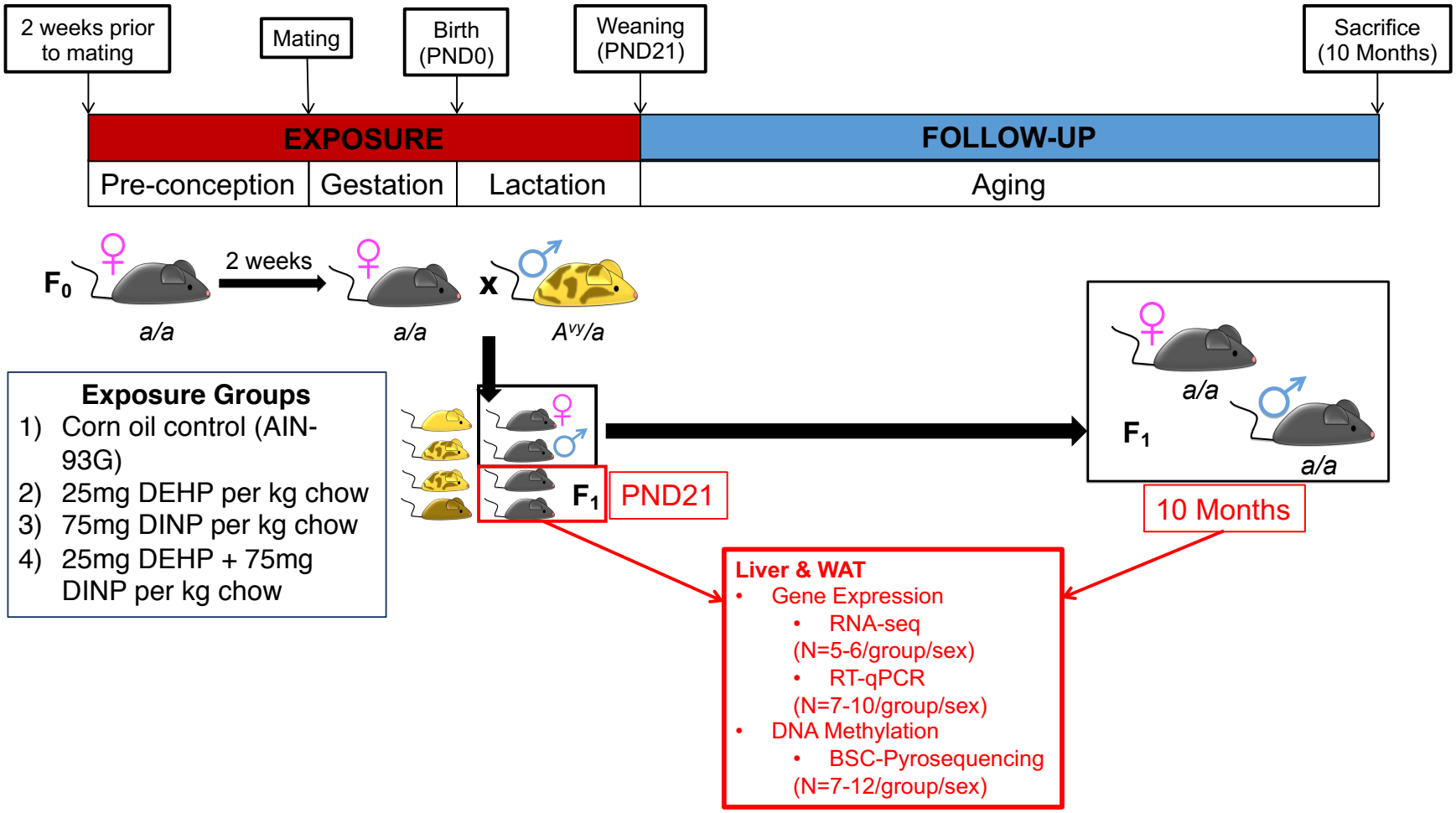

Figure 4.1 Experimental Design. Two weeks prior to mating, virgin *a/a* female mice (F_0) were randomly assigned to one of four exposure groups containing different combinations of phthalates. Phthalates were administered through chow, on a background diet of 7% corn oil (phytoestrogen-free). Exposure spanned preconception, gestation, and lactation, and at weaning on postnatal day 21 (PND21), one male and one female F_1 offspring per litter were weaned onto control chow and followed until 10 months of age. At PND21 and 10 months of age, mice were euthanized and livers were collected for analysis via RNA-seq, RT-qPCR, and pyrosequencing of bisulfite converted (BSC) DNA.

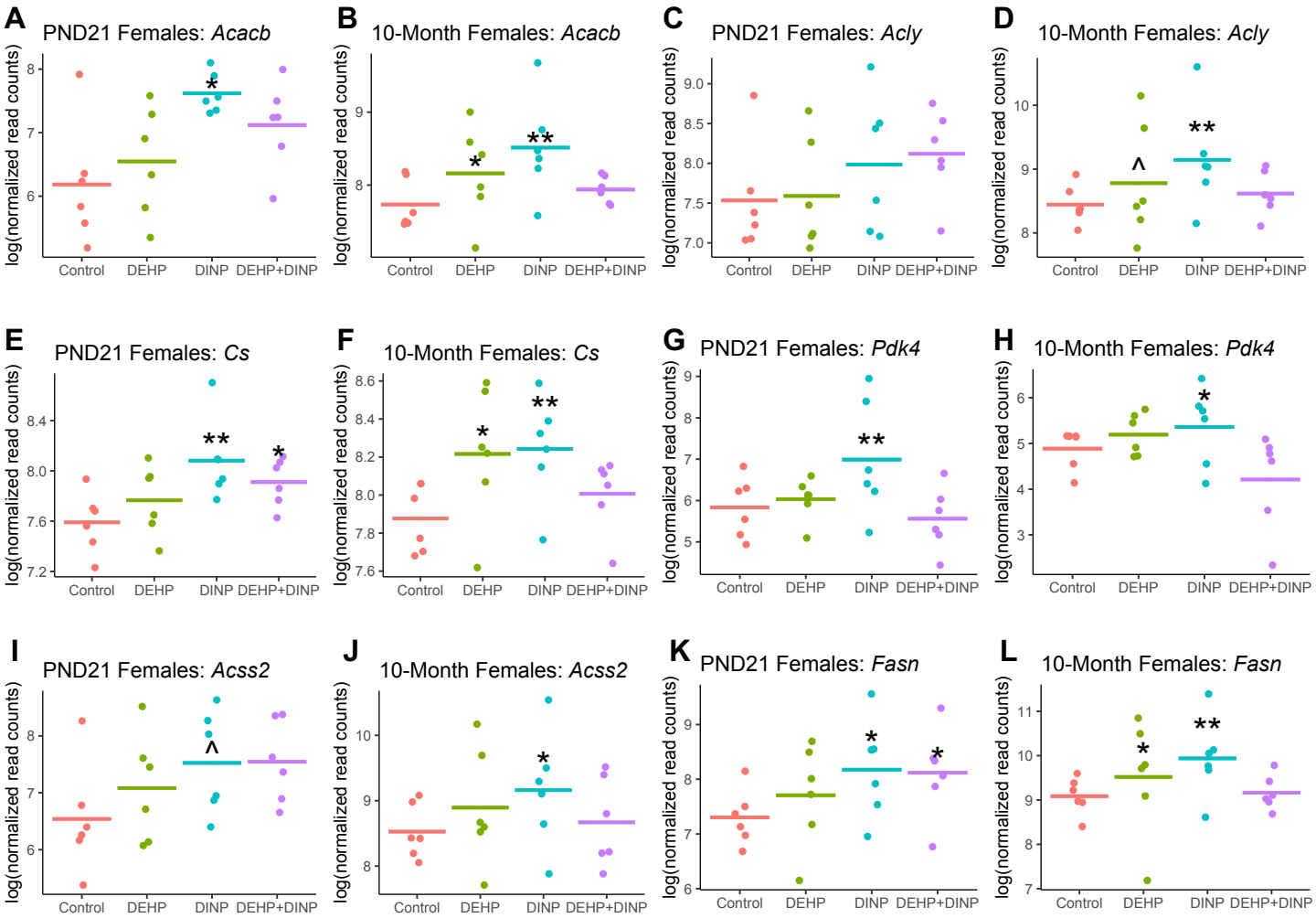

Figure 4.2 RNA-seq read counts for select PPAR target genes in the acetyl-CoA metabolic process pathway. Read counts for each gene are normalized to library size and log transformed for data visualization. **A)** PND21 females: *Acacb*, **B)** 10-month females: *Acacb*, **C)** PND21 females: *Acly*, **D)** 10-month females: *Acly*, **E)** PND21 females: *Cs*, **F)** 10-month females: *Cs*, **G)** PND21 females: *Pdk4*, **H)** 10-month females: *Pdk4*, **I)** PND21 females: *Acss2*, **J)** 10-month females: *Acss2*, **K)** PND21 females: *Fasn*, **L)** 10-month females: *Fasn*. All symbols represent unadjusted p-values from differential expression analyses in edgeR comparing each exposure group to controls. ^p<0.10, *p<0.05, **p<0.01. N=5-6/group/age.

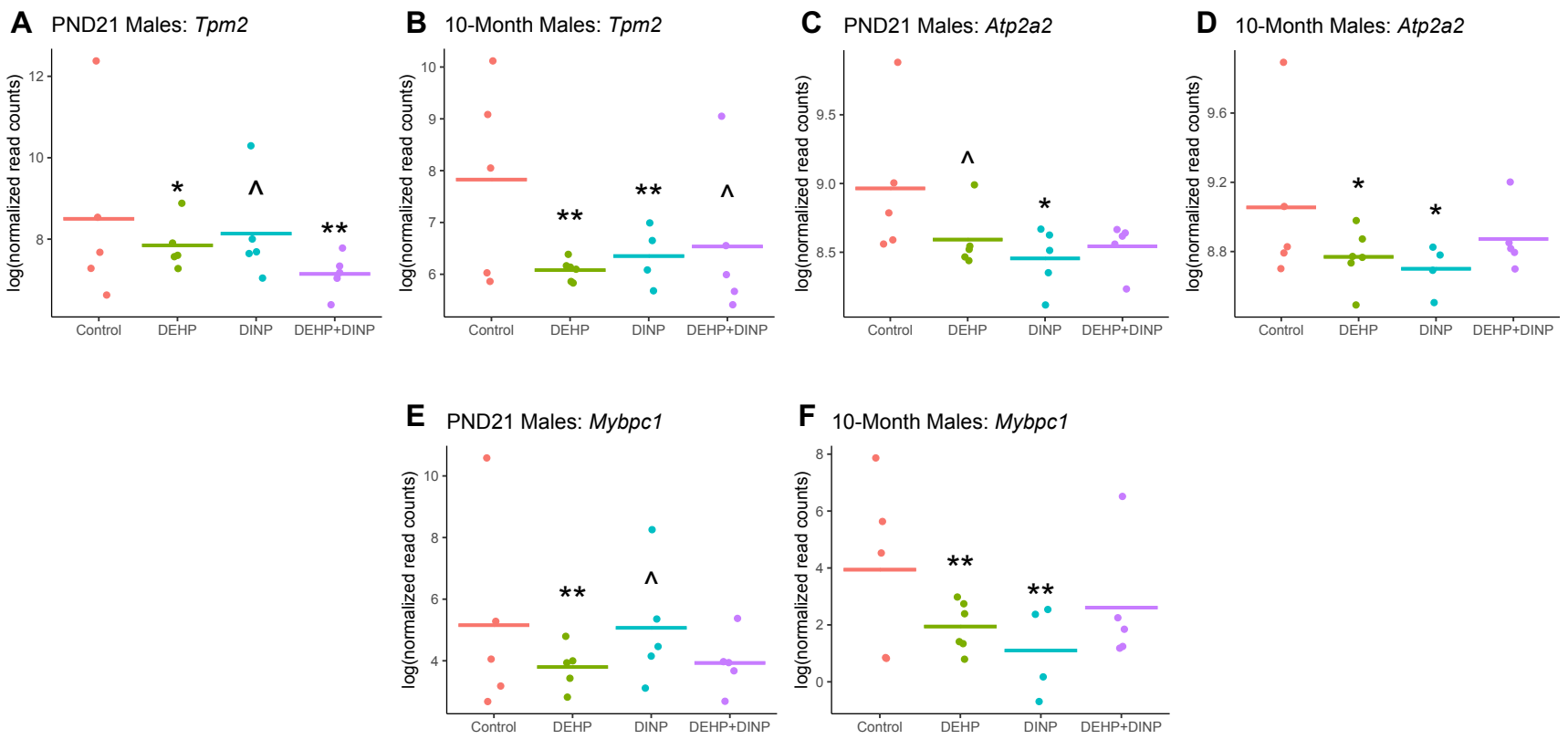

Figure 4.3 RNA-seq read counts in WAT for PPAR target genes in the muscle contraction pathway. Read counts for each gene are normalized to library size and log transformed for data visualization. **A)** PND21 males: *Tpm2*, **B)** 10-month males: *Tpm2*, **C)** PND21 males: *Atp2a2*, **D)** 10-month males: *Atp2a2*, **E)** PND21 males: *Mybpc1*, **F)** 10-month males: *Mybpc1.* All symbols represent unadjusted p-values from differential expression analyses in edgeR comparing each exposure group to controls. ^p<0.10, *p<0.05, **p<0.01. N=4-6/group/age.

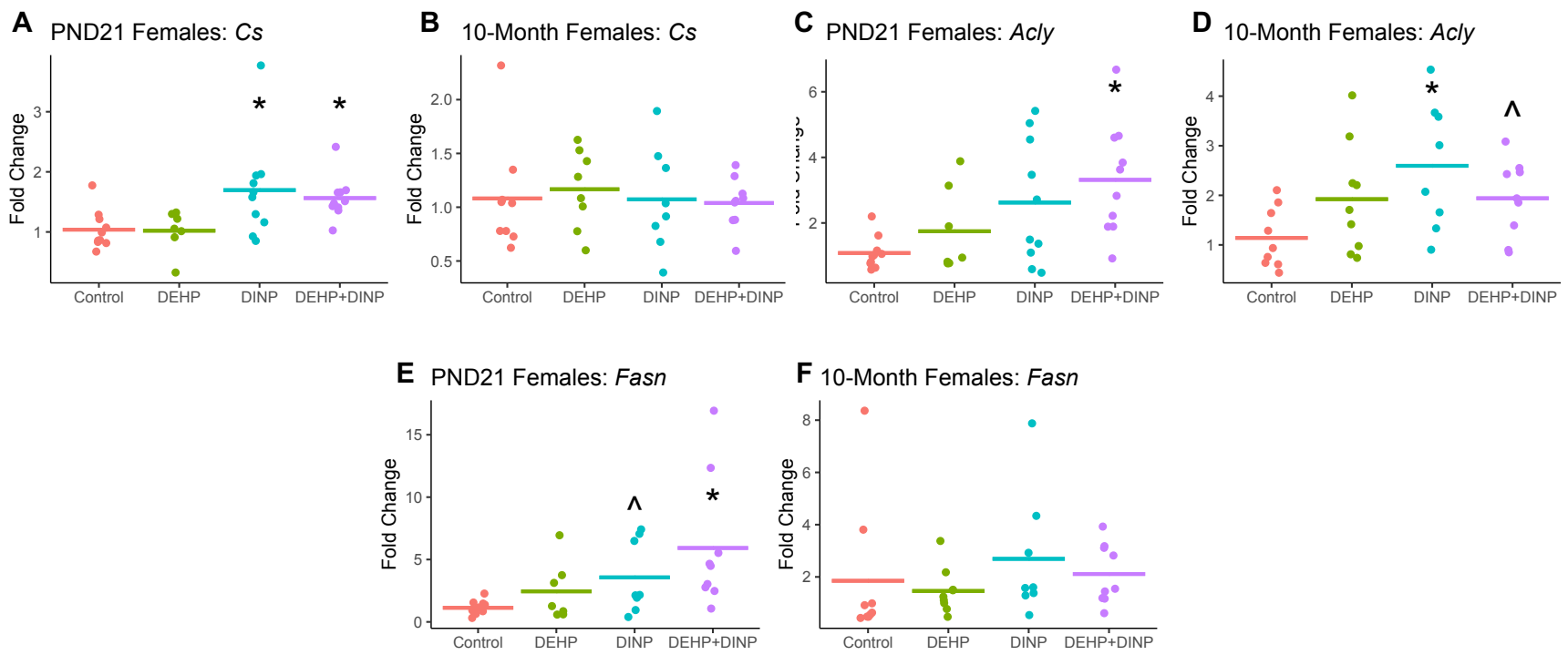

Figure 4.4 RT-qPCR for candidate PPAR target genes in the liver. Fold change was calculated via the 2^{-ddCq} method and plotted for data visualization. **A)** PND21 females: *Cs*, **B)** 10-month females: *Cs*, **C)** PND21 females: *Acly*, **D)** 10-month females: *Acly*, **E)** PND21 females: *Fasn*, **F)** 10-month females: *Fasn*. Relative expression (Cq_{target} – mean($Cq_{reference}$)) was compared for each exposure group to controls using 1) linear mixed effects models with litter as the random effect in analyses on PND21 mice, 2) and linear regression in analyses on 10-month mice. P-values were Bonferroni corrected for multiple comparisons. ^p<0.10, *p<0.05. N=7-10/group/age.

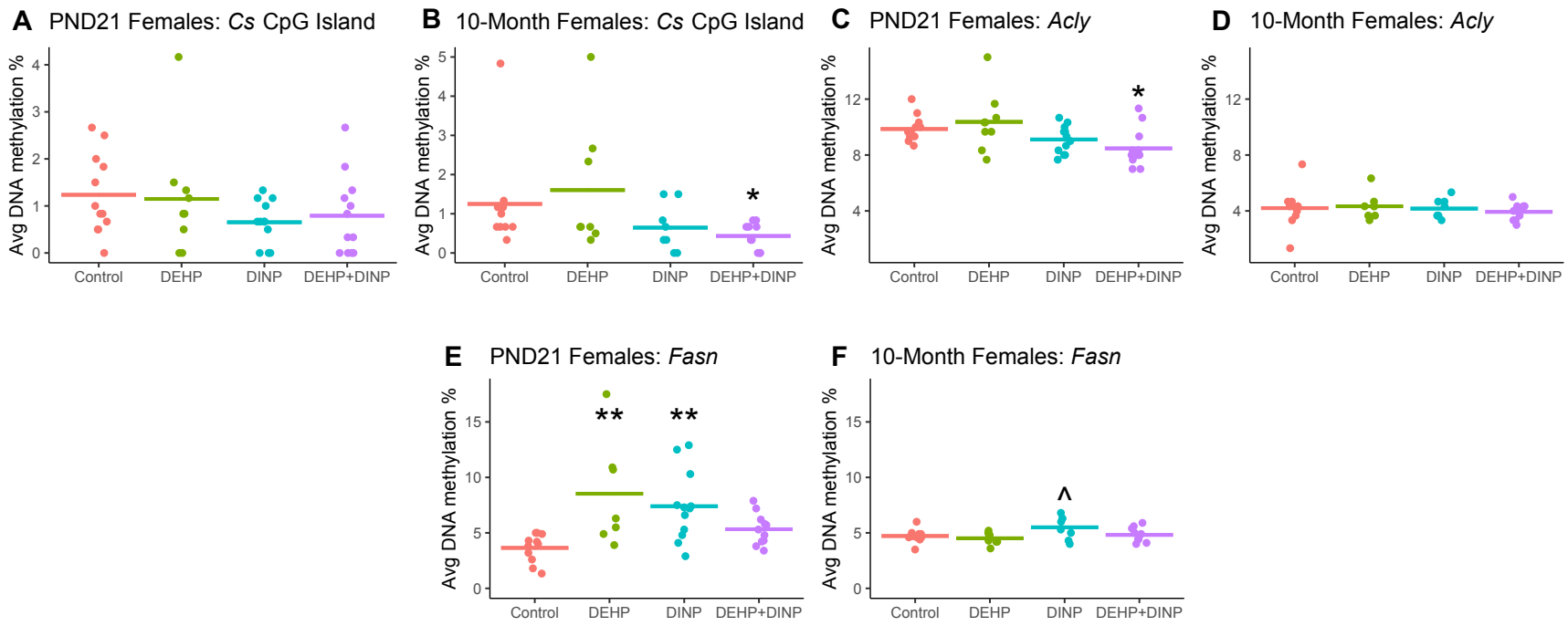

Figure 4.5 Promoter DNA methylation in candidate PPAR target genes. **A)** PND21 females: *Cs*, **B)** 10-month females: *Cs*, **C)** PND21 females: *Acly*, **D)** 10-month females: *Acly*, **E)** PND21 females: *Fasn*, **F)** 10-month females: *Fasn*. Mean DNA methylation across CpG sites was compared for each exposure group to controls using 1) linear mixed effects models with litter as the random effect in analyses on PND21 mice, 2) linear regression in analyses on 10-month mice, and 3) compound poisson regression with zero inflation in analyses of Cs DNA methylation. P-values were Bonferroni corrected for multiple comparisons. ^p<0.10, *p<0.05. N=7-12/group/age

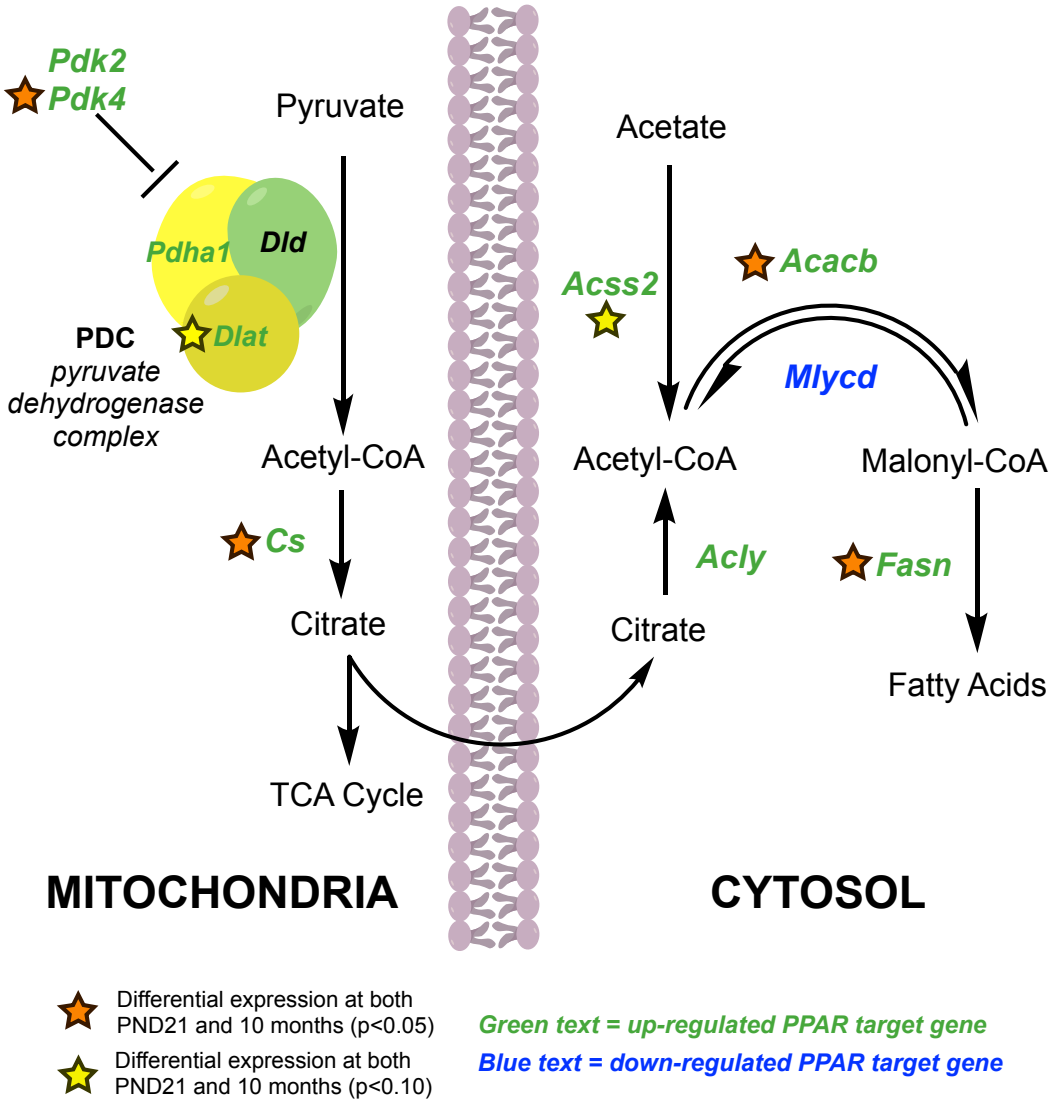

Figure 4.6 Metabolic pathway connecting 10 PPAR target genes identified via RNA-seq and pathway analysis. Arrows represent conversion of one metabolite to another and --| indicates negative regulation. Italicized genes encode enzymes responsible for these enzymatic conversions. Enzymes/genes in green and blue text are PPAR target genes identified in pathway analyses via RNA-Enrich function in LRpath as drivers of the top enriched acetyl-CoA metabolic process GO BP pathway. Genes in green text were up-regulated in DINP females relative to controls via edgeR QLF differential expression analyses at either PND21 or 10 months with unadjusted p-values <0.10. Genes in blue text were up-regulated in DINP females relative to controls via edgeR QLF differential expression analyses at either PND21 or 10 months with unadjusted p-values <0.10.

Appendix

Primer/Sequence to Analyze	**Cs**	**Acly**	**Fasn**
Location (mm10)	chr10:128337792-128337832	chr11:100528430-100528470	chr11:120824870-120824920
Forward PCR Primer (5' → 3')	AGGGGGGAGGGTTTAAGT	GGTTGTTGGTTTAAAAGGTTATAGTATT	GGGAAGGGAGTAGTAGGTAGGATAG
Reverse PCR Primer (5' → 3')	biotin-CCTCCTTTAAACTCACCTTAACTC	biotin-ACTAATTTACCCCTTCCTCCA	biotin-TCATCCCCCCCCCCCAAATT
Sequencing Primer (5' → 3')	GTTTGTGTAGGTTTGTTTAT	TTTGGAATTTTTTTTAGGGAA	GTAGGTAGGATAGGGA
Sequence to Analyze	T TTTTTTTTTAGTYGTTTTTTTTT AATTTTGTTYGTYGGTTGGYGYG GTTTTTATTATAGYGG	TTTATTAGAGTTAGTTTATAYGA TTAYGTTATATAATTAGATTGTT YGATTTAGGATATAGATTGGAGG AAGGGGTAA	ATATTGAYGGGYGYGTGT GGYGGTTTTYGGAGTTYG TTTAGTGTTTAGGYGYGG TTATTTGGYGAGYGGGATA TTATTTTTTTTTYGTTTGGT GGGAATAA
Amplicon Length (bp)	274	287	229
Annealing Temperature (C)	53	53	53
Number of Cycles	50	50	50
Number of CpG Sites	5	3	10

Table 4.A1 Pyrosequencing Assay Details

Differentially Expressed Genes (FDR<0.10) in the Liver in DINP females versus Controls

ENSEMBL ID	SYMBOL	Log Fold Change	p-value	FDR	ENSEMBL ID	SYMBOL	Log Fold Change	p-value	FDR
ENSMUSG00000030730	*Atp2a1*	11.77169	7.49E-06	0.0386	ENSMUSG00000064179	*Tnnt1*	4.043706	9.66E-05	0.05157
ENSMUSG00000056328	*Myh1*	11.3081	8.60E-06	0.0386	ENSMUSG00000018566	*Slc2a4*	3.148917	9.77E-05	0.05157
ENSMUSG00000028773	*Fabp3*	11.58658	1.68E-05	0.0386	ENSMUSG00000060600	*Eno3*	4.519588	0.00011	0.05387
ENSMUSG00000031097	*Tnni2*	10.72417	1.69E-05	0.0386	ENSMUSG00000030972	*Acsm5*	-0.7079	0.00011	0.05409
ENSMUSG00000031972	*Acta1*	11.22849	1.87E-05	0.0386	ENSMUSG00000025488	*Cox8b*	10.8484	0.00011	0.05409
ENSMUSG00000034774	*Dsg1c*	-1.98964	2.04E-05	0.0386	ENSMUSG00000022594	*Lynx1*	4.104466	0.00011	0.05409
ENSMUSG00000020475	*Pgam2*	9.268814	2.22E-05	0.0386	ENSMUSG00000026950	*Neb*	5.392665	0.00012	0.05713
ENSMUSG00000030592	*Ryr1*	8.622308	2.24E-05	0.0386	ENSMUSG00000034377	*Tulp4*	1.163161	0.00013	0.05805
ENSMUSG00000024059	*Clip4*	7.161815	2.50E-05	0.0386	ENSMUSG00000008658	*Rbfox1*	6.729889	0.00014	0.0608
ENSMUSG00000028464	*Tpm2*	4.626969	2.67E-05	0.0386	ENSMUSG00000051748	*Wfdc21*	1.740077	0.00016	0.06389
ENSMUSG00000030672	*Mylpf*	7.777575	2.74E-05	0.0386	ENSMUSG00000021929	*Kpna3*	0.584696	0.00016	0.06389
ENSMUSG00000006221	*Hspb7*	7.489467	2.80E-05	0.0386	ENSMUSG00000075307	*Klhl41*	7.317099	0.00016	0.06389
ENSMUSG00000001334	*Fndc5*	4.618679	2.92E-05	0.0386	ENSMUSG00000007097	*Atp1a2*	4.710633	0.00016	0.06389
ENSMUSG00000006457	*Actn3*	12.09428	3.47E-05	0.0386	ENSMUSG00000033065	*Pfkm*	2.553511	0.00018	0.07003
ENSMUSG00000032366	*Tpm1*	3.461934	3.52E-05	0.0386	ENSMUSG00000031791	*Tmem38a*	3.525896	0.00021	0.07973
ENSMUSG00000026817	*Ak1*	6.681673	3.60E-05	0.0386	ENSMUSG00000069456	*Rdh16*	-1.14711	0.00022	0.08335
ENSMUSG00000047419	*Cmya5*	8.142225	3.77E-05	0.0386	ENSMUSG00000042828	*Trim72*	6.406327	0.00024	0.08533
ENSMUSG00000026208	*Des*	4.524383	4.01E-05	0.0388	ENSMUSG00000028207	*Asph*	1.380966	0.00025	0.08669
ENSMUSG00000085834	*NA*	3.368851	5.21E-05	0.0457	ENSMUSG00000061540	*Orm2*	5.12618	0.00025	0.08669
ENSMUSG00000061462	*Obscn*	6.924677	5.25E-05	0.0457	ENSMUSG00000023092	*Fhl1*	2.528667	0.00026	0.08685
ENSMUSG00000106040	*NA*	4.96844	5.70E-05	0.0473	ENSMUSG00000017817	*Jph2*	5.57789	0.00026	0.08685
ENSMUSG00000022519	*Srl*	5.116667	6.02E-05	0.0476	ENSMUSG00000034842	*Art3*	2.078756	0.00026	0.08691
ENSMUSG00000030401	*Rtn2*	6.111908	7.05E-05	0.0508	ENSMUSG00000049134	*Nrap*	6.249872	0.00028	0.08837
ENSMUSG00000068699	*Flnc*	6.074038	7.46E-05	0.0508	ENSMUSG00000001403	*Ube2c*	2.31211	0.00028	0.08837
ENSMUSG00000007122	*Casq1*	6.906864	7.55E-05	0.0508	ENSMUSG00000021811	*Dnajc9*	0.919009	0.0003	0.09459
ENSMUSG00000057465	*Saa2*	5.748397	7.78E-05	0.0508	ENSMUSG00000067225	*Cyp2c54*	-1.82065	0.00032	0.09566
ENSMUSG00000007877	*Tcap*	10.37799	8.20E-05	0.0508	ENSMUSG00000036854	*Hspb6*	1.879356	0.00032	0.09566
ENSMUSG00000024049	*Myom1*	4.246464	8.37E-05	0.0508	ENSMUSG00000025197	*Cyp2c23*	-0.89597	0.00033	0.09654
ENSMUSG00000047746	*Fbxo40*	7.261901	8.46E-05	0.0508	ENSMUSG00000065037	*Rn7sk*	2.130189	0.00034	0.0982
ENSMUSG00000074115	*Saa1*	4.79833	9.38E-05	0.0516	ENSMUSG00000062929	*Cfl2*	0.668002	0.00034	0.09824
ENSMUSG00000029683	*Lmod2*	6.138273	9.56E-05	0.0516					

Table 4.A2 Differentially expressed genes (FDR<0.10) in the liver in DINP females versus controls. Differential expression was carried out using the quasi-likelihood function (QLF) in edgeR.

DEHP					DINP				
Pathway	# Genes	Odds Ratio	p-value	FDR	Pathway	#Genes	Odds Ratio	p-value	FDR
organic hydroxy compound biosynthetic process	66	5.488428	2.75E-05	0.0974	alpha-amino acid metabolic process	122	2.4886873	4.85E-07	5.95E-04
polyol biosynthetic process	9	23.259336	3.17E-05	0.0974	organic acid metabolic process	636	1.6882503	4.58E-06	0.003125
mitotic spindle assembly	12	12.576798	1.50E-04	0.1495	alpha-amino acid catabolic process	48	2.8309594	2.54E-05	0.012479
microtubule cytoskeleton organization involved in mitosis	12	12.576798	1.50E-04	0.1495	dicarboxylic acid metabolic process	61	2.5047567	2.67E-05	0.012479
response to wounding	123	3.3636381	1.64E-04	0.1495	cellular amino acid catabolic process	44	2.751145	2.71E-05	0.012479
platelet aggregation	12	10.981142	2.64E-04	0.1495	carboxylic acid metabolic process	574	1.6197961	4.84E-05	0.01903
alcohol biosynthetic process	47	5.2110501	2.68E-04	0.1495	small molecule metabolic process	1065	1.4969044	5.63E-05	0.01903
organic hydroxy compound metabolic process	181	2.9324252	3.13E-04	0.1495	oxoacid metabolic process	577	1.6096433	5.89E-05	0.01903
negative regulation of sister chromatid segregation	6	30.347262	3.63E-04	0.1495	aspartate family amino acid metabolic process	24	3.4034176	7.38E-05	0.020604
negative regulation of chromosome segregation	6	30.347262	3.63E-04	0.1495	glutamate metabolic process	17	3.3344269	7.39E-05	0.020604

Table 4.A3 Top 10 Enriched GO Biological Process Pathways Across PND21 and 10-Month Male Livers (Part 1). Pathway analyses were carried out via the RNA-Enrich function in LRpath for Gene Ontology Biological Processes. A combination of p-values from differential expression analysis at PND21 and 10 months calculated via Fisher's method was used for input into LRpath. Pathways above are enriched and do not include depleted pathways. Pathways were considered significantly enriched with FDR<0.05.

DEHP+DINP				
Pathway	#Genes	Odds Ratio	p-value	FDR
regulation of systemic arterial blood pressure	18	4.85909661	2.03E-04	0.73796
positive regulation of heterotypic cell-cell adhesion	3	11.4405805	3.02E-04	0.73796
platelet aggregation	12	4.76146293	6.66E-04	0.73796
fibrinolysis	21	4.12371225	8.27E-04	0.73796
plasminogen activation	11	4.75480722	0.001081	0.73796
regulation of body fluid levels	114	2.31443179	0.001117	0.73796
monocyte differentiation	3	9.26942302	0.001368	0.73796
mononuclear cell differentiation	3	9.26942302	0.001368	0.73796
zymogen activation	18	3.85105232	0.001416	0.73796
positive regulation of vasoconstriction	7	5.63035665	0.001716	0.73796

Table 4.A4 Top 10 Enriched GO Biological Process Pathways Across PND21 and 10-Month Male Livers (Part 2). Pathway analyses were carried out via the RNA-Enrich function in LRpath for Gene Ontology Biological Processes. A combination of p-values from differential expression analysis at PND21 and 10 months calculated via Fisher's method was used for input into LRpath. Pathways above are enriched and do not include depleted pathways. Pathways were considered significantly enriched with FDR<0.05.

Pathway	Gene	PPAR target gene	Confidence level	GEO Accession #	# PPREs in TSS-flanking region	PND21 LFC	PND21 p-value	10M LFC	10M p-value
Alpha-amino acid metabolic process	*Comt*	No							
	Gclc	PPARα	Low	GSE30553	0	0.5	0.046	-0.41	0.018
	Ass1	Unspecified	Low	-	8	-0.45	0.038	0.31	0.12
	Hpd	Unspecified	Low	-	6	-0.62	0.011	0.077	0.64
	Bhmt	NA							
	Got1	NA							
	Asl	NA							
	Aadat	NA							
	Gldc	PPARδ	Low	GSE32498	2	-0.49	0.017	-0.18	0.29
	Aass	NA							
	Gls2	NA							
	Sds	NA							
	Gpt2	NA							
	Acmsd	NA							
	Dmgdh	NA							

Table 4.A5 Significant genes driving pathway enrichment for top two enriched pathways in DINP males livers (Part 1). Genes were identified as PPAR target genes based on PPARgene.org, which utilizes a combination of evidence from published datasets and PPAR response elements (PPREs) in regions flanking th TSS. PPAR isoform was assigned based on experimental gene expression data in the Gene Expression Omnibus (GEO). Confidence levels are from PPARgene.org. PND21 and 10-month (10M) log fold change (LFC) and p-values (unadjusted) are from differential gene expression of RNA-seq data analyzed via edgeR quasi likelihood function (QLF) comparing DINP females to controls. Bolded genes were differentially expressed with an unadjusted p-value of <0.10 at both PND21 and 10 months.

Pathway	Gene	PPAR target gene	Confidence level	GEO Accession #	# PPREs in TSS-flanking region	PND21 LFC	PND21 p-value	10M LFC	10M p-value
Organic acid metabolic process	***Cyp2e1***	**PPARδ**	**High**	**GSE11803, GSE11804, GSE29055**	**4**	**-0.42**	**0.06**	**-0.43**	**0.04**
	Fabp1	PPARα	High	GSE17250, GSE30553	6	0.94	0.05	-0.34	0.13
	G6pc	PPARα	Low	GSE17250, GSE30553	4	-1.19	0.005	-0.09	0.76
	Comt	NA							
	Por	PPARα	High	GSE5777	3	-0.51	0.1	0.47	0.018
	Ddit4	PPARδ, PPARγ	High	GSE11804, GSE1458	8	-1.79	0.0069	0.35	0.25
	Gclc	PPARα	Low	GSE30553	0	0.5	0.046	-0.41	0.018
	Abdhd5	NA							
	Gpd1	PPARγ	High	GSE1458, GSE35011	7	0.18	0.35	-0.61	0.0015
	Ass1	Unspecified	Low	-	8	-0.45	0.038	0.31	0.12
	Hpd	Unspecified	Low	-	6	-0.62	0.011	0.077	0.64
	Abcd2	NA							
	Elovl6	PPARδ	Low	GSE11803	1	1.8	0.0098	-0.12	0.74
	Hal	NA							
	Fh1	PPARγ	Medium	GSE1458, GSE35011	4	-0.6	0.028	0.52	0.06
	Bhmt	NA							
	Got1	NA							
	Asl	NA							
	Aadat	NA							
	Gpat4	NA							
	Gldc	PPARδ	Low	GSE32498	2	-0.49	0.017	-0.18	0.29
	Sc5d	PPARγ	Low	GSE35011	3	0.98	0.017	-0.22	0.33
	Acat2	NA							
	Aass	NA							
	Stard4	PPARα	Medium	GSE5777	0	0.67	0.0074	-0.19	0.39
	Gls2	NA							
	Acsl5	PPARα	Medium	GSE17251	1	0.62	0.031	-0.26	0.09
	Sds	NA							
	Gpt2	NA							
	Acmsd	NA							
	Cyp39a1	NA							
	Dmgdh	NA							
	Acox2	NA							

Table 4.A6 Significant genes driving pathway enrichment for top two enriched pathways in DINP males livers (Part 2). See caption for Table 4.A5.

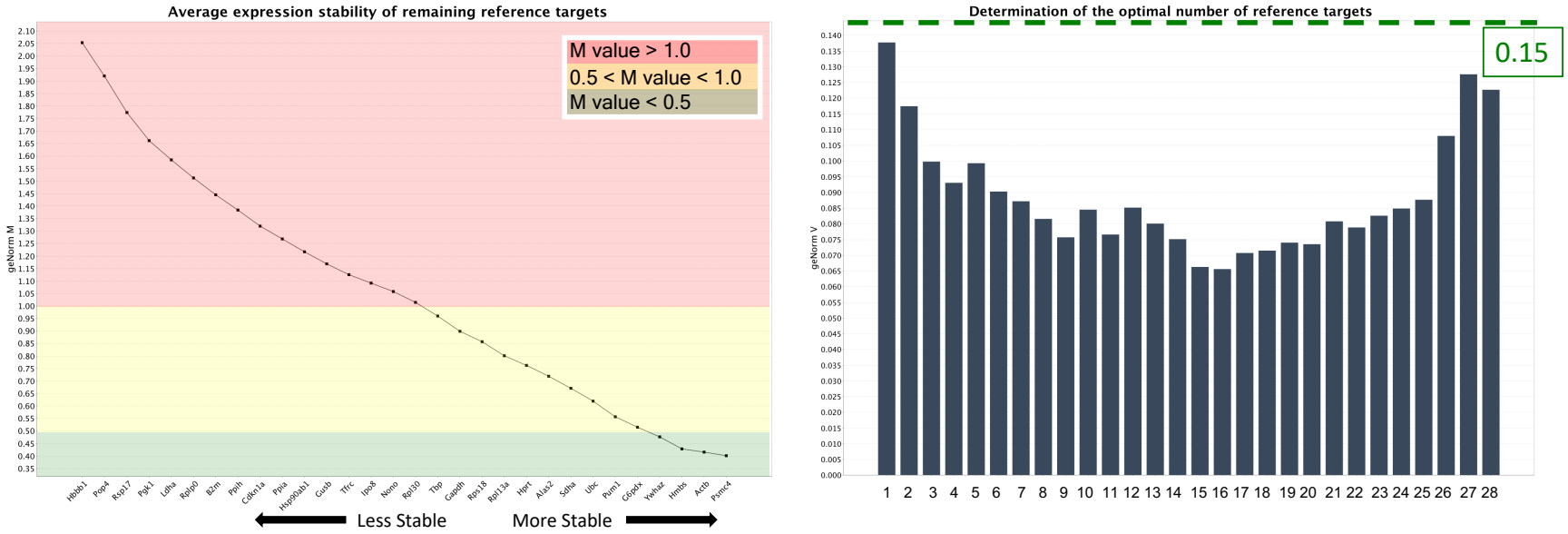

Figure 4.A1 Reference gene analysis. 30 reference genes were analyzed using geNorm across pooled samples (n=~8 mice/pool) representing PND21 males and females across 6 different perinatal phthalate exposure groups. There were two additional exposure groups represented in the reference gene plate that were not included in the RT-qPCR study: DBP-only and a mixture of DEHP+DINP+DBP. **A)** The internal control gene-stability measure M is defined as the average pairwise variation of a particular gene with all other control genes. Vandesompele et al., Genome Biology, 2002. **B)** The measure V is defined as the systematic variation calculated as the pairwise variation for repeated RT-qPCR experiments on the same gene which reflects the variation in machine, enzymes and pipetting Vandesompele et al., Genome Biology, 2002. V-values are plotted under the assumption of using 1 – 28 reference genes. M values lower than 0.5 and V values <0.15 are ideal.

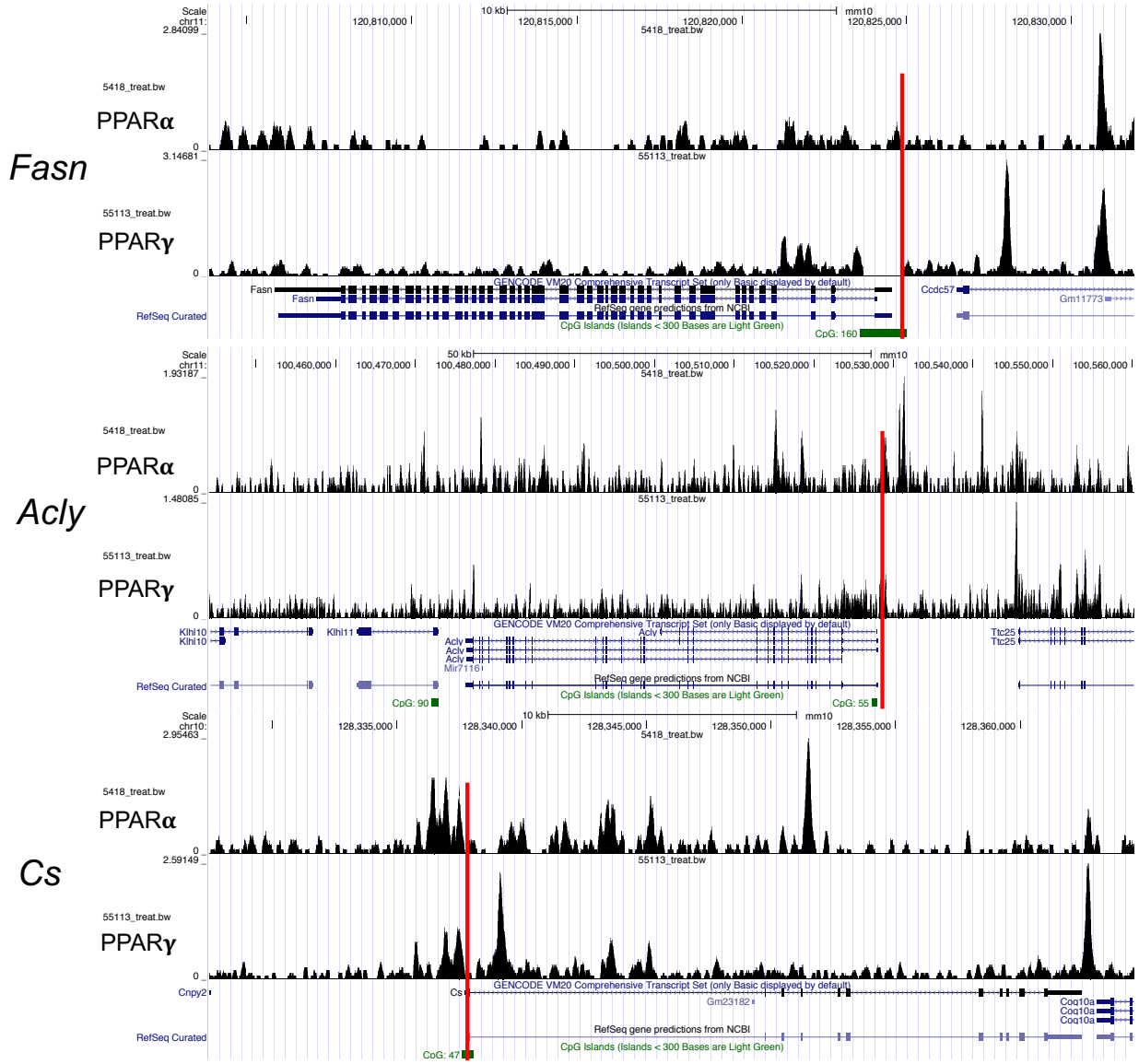

Figure 4.A2 Pyrosequencing Assay CpG Genomic Locations. ChIP-seq tracks for PPARa in liver (top tracks) and PPARy in adipose (mid tracks) were loaded to the UCSC genome browser from Cistrome (cistrome.org). RefSeq genes are listed on the lower tracks. The red lines represent the location of the CpGs analyzed in each pyrosequencing assay.

CHAPTER 5

DISCUSSION

Summary of research findings

In this dissertation research, we utilized a longitudinal mouse model of perinatal exposures to phthalates and phthalate mixtures to provide evidence supporting the theory that developmental exposures to phthalates have long-term negative impacts on metabolic health outcomes, and to present a novel molecular mechanism underlying this relationship. The findings from this work also provide insights into metabolic responses following developmental exposures to phthalate mixtures, indicating unexpected complexities that should be taken into account in interpreting human studies.

In Aim 1, we found that perinatal exposures to individual phthalates and phthalate mixtures were associated with altered metabolic phenotypes and an altered epigenome in early postnatal life. Males and females perinatally exposed to DINP alone and to phthalate mixtures had increased body weights at PND21. With the exception of the DINP-only exposure group, coat color distributions of A^{vy}/a offspring were significantly different in exposure groups compared to controls, which suggested that early-life phthalate exposures altered the epigenome. We also found early evidence of sex-specific effects. Females, but not males, perinatally exposed to DINP-only and a mixture of DEHP+DINP also had increased relative liver weights at PND21. In addition, females exposed to phthalates and phthalate mixtures during development had

increased tail repetitive element DNA methylation whereas males perinatally exposed to phthalates had decreased tail repetitive element DNA methylation.

In the second aim, we found evidence for sex-specific and phthalate-specific longitudinal metabolic disruptions in mice perinatally exposed to phthalates, and observed a relative lack of longitudinal effects in mice perinatally exposed to phthalate mixtures. Life course body weights were not significantly different in exposed mice compared to controls; however, females perinatally exposed to DBP-only and DEHP-only gained more weight with age than control females. Females perinatally exposed to DEHP-only also exhibited increased body fat percentage and decreased lean mass percentage at two and eight months of age, whereas females perinatally exposed to DINP-only had impaired glucose tolerance longitudinally in comparison to control females. On the other hand, males perinatally exposed to DINP-only and a mixture of DEHP+DINP showed improved glucose tolerance with age relative to controls. We utilized linear mixed effects models with a simple order constraint to specifically test the hypothesis that perinatal exposures to phthalate mixtures exert a larger magnitude of effect on longitudinal metabolic health outcomes and did not find evidence to support this hypothesis. Lastly, we found that perinatal phthalate and phthalate mixture exposures altered the relationship between body fat percentage and plasma adipokine levels in females at 10 months of age, which suggested that developmental exposures to phthalates may have modified adipokine secretion from adipose tissue.

In Aim 3, we elucidated a potential mechanism linking developmental phthalate exposures to long-term metabolic health outcomes. We utilized transcriptomics and subsequent pathway enrichment analyses to identify PPAR target genes that were

persistently up-regulated in liver and WAT from mice perinatally exposed to phthalates. The acetyl-CoA metabolic process pathway was enriched in the livers from females perinatally exposed to DINP across PND21 and 10 months relative to controls. Of the 13 genes driving this pathway, 10 were confirmed PPAR target genes, and 9 of those 10 exhibited up-regulation, which is consistent with PPAR activation. Interestingly, females perinatally exposed to DINP-only had increased hepatic DNA methylation in the promoter region of one of these PPAR target genes, *Fasn*, at both PND21 and 10 months. Increased DNA methylation is generally associated with decreased mRNA expression,[1] and yet *Fasn* expression was increased in the liver of DINP females. Although this presented an unexpected relationship between DNA methylation and gene expression, it nonetheless indicated persistent effects on hepatic gene expression and promoter DNA methylation of *Fasn* in females perinatally exposed to DINP. Furthermore, the observed increase in DNA methylation may have been due to increased hydroxymethylation (5hmC), a DNA modification that may be associated with increased transcription.[2,3] Males perinatally exposed to DINP-only also exhibited enriched metabolic pathways in the liver across PND21 and 10 months relative to controls; however, the directionality of differential gene expression was not consistent in PPAR target genes across both PND21 and 10 months. In contrast to the liver, there was less convincing evidence of persistent changes in PPAR target gene expression in WAT. In females, we found that perinatal phthalate exposures may impact pathways involved with RNA metabolism and processing in WAT. In males, we identified three PPAR target genes involved in muscle contraction that were down-regulated in WAT from phthalate-exposed mice, but the implications that these gene expression changes

have for metabolism are unknown. Additional studies are needed to better understand persistent effects of developmental phthalate exposures on WAT tissue.

This dissertation research corroborates previous animal literature demonstrating associations between perinatal exposures to DEHP and metabolic health effects,[4–6] and builds on it by incorporating exposures to additional phthalates and phthalate mixtures, as well as longitudinal metabolic phenotyping. The longitudinal aspect of our study allowed us to characterize patterns in metabolic effects across time, which revealed striking findings with respect to developmental exposures to phthalate mixtures compared to individual phthalates. Perinatal exposures to both phthalates and phthalate mixtures were associated with metabolic effects in early postnatal life at PND21. Although metabolic effects were also observed longitudinally in mice perinatally exposed to individual phthalates, longitudinal metabolic effects were far less evident in mice perinatally exposed to phthalate mixtures. This presents the possibility that perinatal phthalate mixture exposures elicit an initial metabolic response followed by an adaptive response when the exposure is removed. Furthermore, the relatively diminished long-term metabolic effects in mixture exposure groups may be evidence of a non-monotonic dose-response. Non-monotonic dose-responses and low-dose effects have been widely observed for developmental exposures to EDCs, including phthalates.[7,8] Since the phthalate mixture groups were exposed to higher total phthalate levels, the metabolic response patterns observed here may be a manifestation of non-monotonic dose-response. Additional studies are needed to characterize the underlying biology to explain these observations.

The overall findings in this dissertation indicate that females were more sensitive to perinatal phthalate exposures than males. Specifically, females perinatally exposed to phthalates exhibited altered metabolic phenotypes longitudinally and also exhibited more striking alterations in hepatic gene expression and DNA methylation than males. Exposures to EDCs, including phthalates, are frequently associated with sex-specific effects in animals and in humans.[9–13] Furthermore, phthalates have well-documented effects on sex hormones,[14,15] and PPARs exhibit cross-talk with estrogen signaling.[16] Thus, it comes as no surprise that developmental phthalate exposures resulted in sex-specific metabolic effects. However, the previous animal literature is inconsistent; some studies have reported metabolic effects specific to females[4,5] while others have reported metabolic effects specific to males.[17] These divergent findings may be attributable to differences in species, mouse/rat strain, exposure level, and exposure timing.

Synthesis of evidence for proposed mechanism

The work in this dissertation provided evidence of a potential molecular mechanism linking developmental exposure to DINP and impaired glucose tolerance. In Aim 2, we found that female mice perinatally exposed to DINP-only had impaired glucose tolerance across the life course, and in Aim 3, we found that females perinatally exposed to DINP had up-regulated PPAR target genes in the liver across PND21 and 10 months of age that could contribute to increased fatty acid biosynthesis. These PPAR target genes were involved in acetyl-CoA metabolic processes, and taken together, indicated a shift towards utilization of acetyl-CoA for fatty acid biosynthesis (Figure 4.6). Nine PPAR target genes involved in acetyl-CoA metabolic processes were up-regulated in liver tissue from DINP-only females at either PND21 and 10 months:

Pdk2, *Pdk4*, *Dlat, Pdha1*, *Cs*, *Acly*, *Acss2*, *Acacb*, and *Fasn*. Pyruvate dehydrogenase E1 alpha 1 (*Pdha1*) and dihydrolipoamide S-acetyltransferase (*Dlat*) are part of the pyruvate dehydrogenase complex (PDC) that converts pyruvate to acetyl-CoA in the mitochondria. Pyruvate dehydrogenase kinase 2 and 4 (*Pdk2* and *Pdk4*) inhibit the activity of the PDC, and their up-regulation may represent a feedback compensatory mechanism. Citrate synthase (*Cs*) converts acetyl-CoA to citrate, which can then either enter the citric acid (TCA) cycle or can be transported from the mitochondria to the cytosol to enter fatty acid biosynthesis pathways. ATP-citrate lyase (*Acly*) converts citrate to acetyl-CoA in the cytosol, and acetyl-CoA synthetase 2 (*Acss2)* converts acetate to acetyl-CoA in the cytosol. Thus, upregulation of these two genes, in combination with up-regulation of *Cs*, could result in increased acetyl-CoA in the cytosol. Acetyl-CoA carboxylase beta (*Acacb*) converts acetyl-CoA to malonyl-CoA, which is the base substrate for fatty acid biosynthesis. The gene responsible for catalyzing the reverse reaction, malonyl-CoA decarboxylase (*Mlycd*), was down-regulated, further suggesting an increase in production of malonyl-CoA. Importantly, females perinatally exposed to DINP had persistently up-regulated hepatic mRNA levels and promoter DNA methylation levels of *Fasn* (fatty acid synthase), which is the gene directly responsible for synthesizing fatty acids. Taken together, persistent up-regulation of these PPAR target genes and persistently altered DNA methylation at the *Fasn* promoter suggests that perinatal DINP exposure reprograms the liver to have increased fatty acid biosynthesis.

In theory, increased fatty acid synthesis could lead to increased hepatic triglyceride (TG) synthesis and storage in the liver, which could in turn lead to glucose

intolerance.[18,19] However, we did not observe a significant relationship between hepatic TG levels and perinatal phthalate exposures at PND21 (Aim 1). Alternatively, fatty acids synthesized in the liver could be exported into the circulation. Increased circulating free fatty acids (FFAs) can cause decreased uptake of glucose in skeletal muscle,[20,21] which may ultimately result in impaired whole-body glucose tolerance. This proposed mechanism linking developmental exposure to DINP and glucose intolerance is outlined in **Figure 5.1**.

Evidence from this dissertation in combination with evidence from previous studies is consistent with this proposed mechanism. It has been well established that phthalate metabolites activate PPARs, and the findings from Aim 3 are also supportive of phthalate-induced PPAR activation in the liver. In Aim 3, we found that developmental exposures to phthalates may have persistent effects on promoter DNA methylation and gene expression of PPAR target genes that were involved in fatty acid biosynthesis. This evidence suggests the possibility that activation of PPARs via DINP exposure during liver organogenesis may have resulted in epigenetic reprogramming of PPAR target genes to have an increased propensity for fatty acid synthesis. However, we did not measure gene expression or DNA methylation during liver organogenesis and the resulting functional outcomes in the liver are still unclear. Targeted metabolomics analyses are planned to determine whether the observed up-regulation of PPAR target genes in the liver of females perinatally exposed to phthalates were accompanied by an altered metabolic profile, including whether acetyl-CoA and malonyl-CoA levels were altered. Although we did not directly measure FFAs in circulation, a previous study demonstrated that mice perinatally exposed to DEHP had increased plasma FFA levels

compared to controls.[22] Additionally, another study found that developmental exposure to DEHP in rats was associated with down-regulation of GLUT4 (*Slc2a4*) expression and activity in gastrocnemius muscle accompanied by impaired glucose tolerance.[23] Further investigations to determine whether perinatal DINP exposures can also influence circulating FFAs and GLUT4 activity in the muscle are needed to confirm the proposed mechanism in **Figure 5.1**.

The role that WAT plays in mediating the relationship between developmental phthalate exposures and later-life metabolic health outcomes was less clear based on the evidence from this dissertation. Differential expression and pathway analyses indicated that PPAR target genes involved in muscle contraction were altered in WAT from males exposed to phthalates at both PND21 and 10 months. However, the connection between muscle contraction pathways in WAT and whole-body metabolism is uncertain. In addition, only three PPAR target genes were consistently differentially expressed in WAT across both time points, and they were down-regulated, which was unexpected based on the theory that phthalates activate PPARs. Further studies are needed to elucidate the role of WAT mediating the relationship between developmental phthalate exposures and long-term metabolic impacts.

Relevance to human health

One of the overall objectives of this dissertation was to utilize a mouse model to inform human studies, and therefore we designed the exposure paradigm to be human relevant. Exposures included human-relevant levels of both LMW and HMW phthalates, as well as newer phthalates and phthalate mixtures. Furthermore, exposures were through the diet, which is the most relevant route for human exposure to phthalates, and

also resulted in a gradual exposure over time as opposed to a bolus dose each day. Of particular relevance to human studies, we found that perinatal exposures to DINP resulted in long-term metabolic impacts. Thus, characterization of *in utero* DINP exposures should be implemented in birth cohort studies that examine metabolic health outcomes. Some human epidemiological studies have begun to develop and use statistical modeling techniques to better understand health effects of mixture exposures.[24] However, use of an animal model allowed us to isolate individual phthalate exposures and to evaluate two different mixture combinations. We found that perinatal exposures to individual phthalates were associated with adverse metabolic health effects both cross-sectionally in early postnatal life and longitudinally across the life course, but that perinatal exposures to phthalate mixtures were only associated with effects in early postnatal life. This is an important finding that will aid in the interpretation of human studies examining developmental exposures to phthalates.

The longitudinal aspect of this dissertation was also implemented as a means for informing epidemiological studies. We found that longitudinally, there were no significant differences in body weight in exposed versus control groups, but that female mice perinatally exposed to DEHP-only and DBP-only gained more weight with age than control females. Thus, human studies may want to consider examining associations between developmental phthalate exposures and body weight trajectories as opposed to examining body weight cross-sectionally. Our findings also indicated that males perinatally exposed to a mixture of DEHP+DINP and DINP-alone and females perinatally exposed to DEHP+DINP had improved glucose tolerance with age compared

with controls. This further demonstrated the importance of examining patterns over time when examining metabolic effects following developmental phthalate exposures.

Our findings from Aim 1 that developmental phthalate exposures impacted tail DNA methylation at repetitive elements are similar to birth cohort studies that found associations between DNA methylation at repetitive elements in cord blood and developmental phthalate exposures. Multiple studies have demonstrated a negative correlation between developmental phthalate exposures and Alu DNA methylation in cord blood,[9,25] and another has suggested a negative association between developmental phthalate exposures and LINE1 methylation in cord blood.[26] We also found that perinatal phthalate exposure was associated with DNA methylation at repetitive elements, but we found that females perinatally exposed to phthalates had increased DNA methylation at IAPs globally and males had decreased DNA methylation at IAPs. Furthermore, we did not find an association between perinatal phthalate exposures and LINE1 methylation. Thus, the data from this dissertation are consistent with some, but not all, human data pointing to associations between developmental phthalate exposures and DNA methylation at repetitive elements.

Impact and Innovation

This is the first animal study to investigate metabolic outcomes following developmental exposures to DINP. Historically, the highest phthalate exposures in humans were to DEHP. However, in recent years, exposure to DINP has been increasing while exposure to DEHP has been declining.[27] Although DINP is considered to be less potent than DEHP, these potency estimates are based off of reproductive health effects,[28] and little is known with respect to metabolic health effects. Our findings

suggest that developmental exposures to DINP are associated with adverse metabolic impacts that last into adulthood. Therefore, this work suggests that more research investigating health impacts of developmental DINP is needed to better understand the risk to human health.

This study is also the first to examine metabolic impacts of developmental exposures to individual phthalates as well as phthalate mixtures. Humans are co-exposed to mixtures of phthalates, but in utilizing an animal model, we can isolate effects that are due to individual phthalates and compare them to effects from phthalate mixtures. Our findings demonstrate that perinatal exposures to phthalate mixtures have distinctive metabolic effects from perinatal exposures to individual phthalates, especially when examining metabolic parameters longitudinally. This work is informative for future human epidemiological studies.

In this dissertation, we took a novel approach to investigate potential mechanisms linking developmental phthalate exposures and long-term metabolic effects. To our knowledge, this is the first study to use transcriptomics to examine PPAR activation following developmental phthalate exposures. Furthermore, we measured gene expression and DNA methylation at two different time points: 1) early postnatal life when mice were still directly exposed and 2) adulthood long after exposure had ceased. Combining data from these two time points allowed us to delineate between genes and pathways that were only impacted by direct exposure, pathways that may have been impacted only by phenotype, and pathways that were potentially reprogrammed by developmental phthalate exposures. The resulting proposed mechanism (**Figure 5.1**) is

a novel mechanism for developmental exposures to obesogens and paves the way for future mechanistic studies.

Limitations

Despite the strengths and innovative aspects of this study, there are also aspects of this research that limit the ability to definitively prove underlying molecular mechanisms. The approaches used in this study allowed us to identify potential molecular mechanisms and generate new hypotheses, but were associative in nature as opposed to causative. Specifically, we did not utilize positive controls for PPAR activation (e.g., experimental groups exposed to pharmaceutical PPAR agonists such as rosiglitazone and fibrates), and we did not utilize genetic tools such as knock out models to prove the necessity of PPARs linking developmental phthalate exposures and metabolic health outcomes. Furthermore, the study design did not incorporate measurements during gestation, and we were therefore unable to identify molecular events that may have taken place during crucial periods of development, such as early post-fertilization epigenetic reprogramming and organogenesis.

An additional limitation to this study was the relatively low number of samples utilized for transcriptomic analyses given the subtle metabolic phenotypes observed. In this study, we used between five and six samples per sex, per group, per age. Only female PND21 DINP-only-exposed livers exhibited a large number of differentially expressed genes. We were able to utilize pathway analyses to dig deeper into the data to uncover significantly impacted metabolic pathways, but there may be other significant pathways that were missed. Therefore, we recommend that future studies with subtle

phenotypes incorporate larger sample sizes to more robustly identify key genes and molecular pathways.

Recommendations for future research

This dissertation work indicated that DINP is a chemical of concern with respect to early-life exposures and later life metabolic health outcomes. However, replication of these findings in an animal model with multiple doses of DINP and/or in human cohorts are needed to fully understand the risks of developmental DINP exposures. In addition, multi-dosing studies with phthalate mixtures are needed to determine whether the mixture effects observed here were due to a non-monotonic dose-response.

Measurement of circulating FFAs and GLUT4 activity in muscle tissue are obvious next steps to confirm our proposed mechanism. A future study incorporating measurements of FFAs in plasma at PND21 and 10 months of age would provide evidence of whether developmental phthalate exposures result in persistently increased release of FFAs into circulation. Analysis of GLUT4 expression and activity in skeletal muscle tissue at PND21 and 10 months would then help to elucidate whether glucose uptake in skeletal muscle is persistently hindered by developmental phthalate exposures. Additional studies incorporating tissue-specific knock-out animal models would more definitively provide proof of mechanism. Finally, single-cell RNA-seq in the liver would help determine whether altered gene expression in the liver via developmental phthalate exposures was due to reprogramming of individual liver cells, or reprogramming the cellular composition of the liver.

The DNA methylation analyses in Aim 3 only covered very small regions of the genome. DNA methylation analyses in the promoter region of additional PPAR target

genes identified as being potentially reprogrammed in the liver by developmental phthalate exposures would be beneficial. Examples include *Acacb*, *Pdk4*, and *Acss2*, which were all up-regulated in PND21 and 10-month livers of females perinatally exposed to DINP. Higher throughput techniques for measuring DNA methylation such as bisulfite capture or enhanced reduced representation bisulfite sequencing (eRRBS) would provide even more insights. Another informative approach would be to measure 5hmC. Bisulfite conversion of DNA, the method used in this work, does not distinguish between 5mC and 5hmC. We found increased *Fasn* promoter DNA methylation in the liver concurrent with increased gene expression, but the relationship between promoter 5mC and gene expression is usually inverse.[1] Furthermore, PPARs recruit TET enzymes, which convert 5mC to 5hmC, to the promoter region of target genes.[29] Thus, measurement of 5hmC using oxidative bisulfite conversion, antibody pull-down with chromatin immunoprecipitation, or other methods that distinguish between 5mC or 5hmC would provide valuable insights.

In addition to collecting gonadal WAT and liver tissue, we also biobanked several additional tissues that could be analyzed to provide additional mechanistic insights, and could give more context from a systems biology perspective. Biobanked tissues of interest include mesenteric WAT, brain, and kidneys. Mesenteric WAT is more vascularized and has more metabolic activity than gonadal WAT,[30,31] and would likely exhibit different phthalate-related effects than gonadal WAT. PPARs are also known to play important roles in the brain, which plays a central role in choreographing metabolic processes throughout the body.[32,33] Thus, reprogramming of PPAR target genes in the brain via developmental phthalate exposures could have important impacts on whole-

body metabolism. PPARs are also important in regulating renal metabolism and the renin-angiotensin-aldosterone system,[34] and exposure to phthalates has been associated with kidney toxicity.[35] Thus, the kidney would also be an important target organ for developmental exposures to phthalates.

Overall conclusions

The work in this dissertation characterized short- and long-term metabolic effects of developmental exposures to phthalate mixtures. This is the first animal study to investigate metabolic effects of developmental exposure to DINP, and we uncovered associations with increased body weight and relative liver weight in early postnatal life and impaired glucose across the life course in females. Incorporating both individual phthalates and phthalate mixtures was another unique aspect of this project. We found that developmental exposures to phthalate mixtures resulted in increased body weight and relative liver weights in females at PND21, but did not have adverse metabolic effects longitudinally. The contrast in metabolic effects we observed between individual phthalates and phthalate mixtures may be one explanation for the discrepancies in metabolic effects reported in human studies.

This dissertation also provides evidence for a novel molecular mechanism linking developmental phthalate exposures and long-term metabolic impacts. Females perinatally exposed to DINP exhibited up-regulation of PPAR target genes in the acetyl-CoA metabolic process pathway in the liver. Up-regulation of the genes in this pathway were suggestive of increased fatty acid synthesis, which if transported to the circulation, could impact skeletal muscle glucose uptake and result in impaired glucose tolerance.[20,21] Furthermore, we found that promoter DNA methylation of *Fasn* in the liver

was altered in DINP females at both PND21 and 10 months, suggesting that epigenetic reprogramming may play a role. Because human exposures to phthalates are ubiquitous, the findings from this study demonstrate a need for continued efforts to characterize metabolic health impacts of developmental exposures to phthalates, including DINP and other newer phthalates, as well as phthalate mixtures.

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Figures

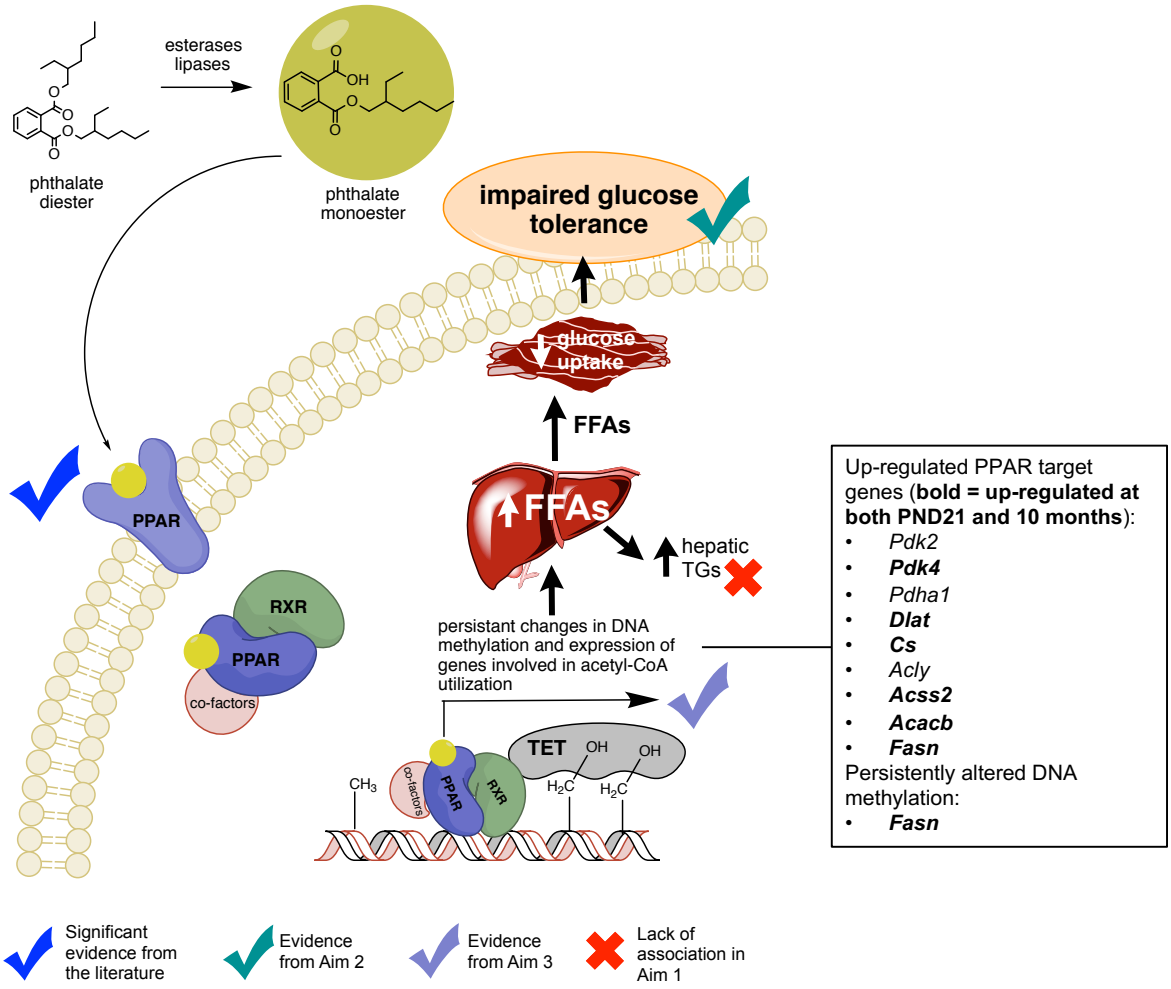

Figure 5.1 Proposed mechanism linking developmental DINP exposure and impaired glucose tolerance. The primary phthalate metabolites, phthalate monoesters, have been demonstrated to activate all three PPAR isoforms. Upon ligand binding, PPARs heterodimerize with RXR-α and other co-factors and then bind to PPAR response elements (PPREs) in the promoter regions of target genes. PPARs have been demonstrated to recruit ten-eleven translocation (TET) enzymes, which can locally alter DNA methylation to facilitate transcription. In Aim 3, we found that *Fasn* promoter DNA methylation was increased in the liver of females perinatally exposed to DINP-only at both PND21 and 10 months, indicating that developmental phthalate exposures had impacts on DNA that lasted long after exposure had ceased. We also found that hepatic *Fasn* expression was up-regulated in these mice, along with a host of other genes involved in transporting and converting acetyl-CoA into fatty acid biosynthesis intermediates. In Aim 1, we did not observe a significant difference in hepatic triglyceride levels by phthalate exposure. Thus, instead of being utilized for triglyceride (TG) biosynthesis, free fatty acids (FFAs) may have instead been transported to the circulation.