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In Utero Growth Restriction and Catch-up Adipogenesis After Developmental Di (2-ethylhexyl) Phthalate (DEHP) Exposure Cause Glucose Intolerance in Adult Male Rats Following a High-fat Dietary Challenge

Rita S. Strakovsky[a], **Stéphane Lezmi**[b], **Ielyzaveta Shkoda**[a], **Jodi A. Flaws**[c], **William G. Helferich**[a], and **Yuan-Xiang Pan**[a,d,*]

[a]Department of Food Science and Human Nutrition

[b]Department of Pathobiology

[c]Department of Comparative Biosciences

[d]Division of Nutritional Sciences

Abstract

Phthalates impact adipocyte morphology *in vitro*, but the sex-specific adipogenic signature immediately after perinatal di(2-ethylhexyl) phthalate (DEHP) exposure and adulthood physiology following a high-fat (HF) dietary challenge are unknown. In the current study, pregnant and lactating dams received DEHP (300 mg/kg body weight) or oil. At weaning (postnatal day (PND) 21), adipose tissue was sampled for real-time PCR. The remaining offspring consumed a control or HF diet. DEHP decreased % fat in males at birth from 13.9%±0.2 to 11.8%±0.6 (mean±SEM), representing a 15.1% decrease in fat by DEHP, and these males caught up in adiposity to controls by PND21. Adult DEHP-exposed males had a 27.5% increase in fat (12.5%±0.9% in controls vs. 15.9%±1.5% in the DEHP group); adipocyte perimeter was increased as well, with fewer small/medium-sized adipocytes, and decreased cell number compared to oil controls. HF diet intake in DEHP-exposed males further increased male energy intake and body weight and led to glucose intolerance. In PND21 males, DEHP increased the expression of adipogenic markers (*Pparg1, Cebpa, Adipoq, Ppard, Fabp4, Fasn, Igf1*), decreased Lep, and decreased markers of mesenchymal stem cell commitment to the adipogenic lineage (*Bmp2, Bmp4, Stat1, Stat5a*) compared to oil controls. These data suggest that DEHP may decrease the adipocyte pool at birth, which initially increases adaptive adipocyte maturation and lipid accumulation, but leads to adipose tissue dysfunction in adulthood, decreasing the capacity to adapt to a HF diet, and leading to systemic glucose intolerance.

*Corresponding author: Yuan-Xiang Pan, PhD, 461 Bevier Hall, MC-182, 905 South Goodwin Avenue, Urbana, IL, 61801, Tel 217-333-3466, Fax 217-265-0925, yxpan@illinois.edu.

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Keywords

adipose; phthalates; diabetes; Pparg; obesity; programming

1. INTRODUCTION

Despite efforts to curb obesity rates, the obesity epidemic continues to contribute to an increase in metabolic syndrome in developed as well as developing countries. Obesity and metabolic syndrome have been associated with various lifestyle and genetic factors. Importantly, although the role of environmental chemicals in the development of metabolic syndrome is not clearly understood, a growing body of evidence is beginning to suggest that exposures to several endocrine disrupting environmental chemicals (EDCs), including phthalates [1], are associated with metabolism-related diseases in humans, including obesity and type 2 diabetes.

Of the EDCs being investigated, the plasticizer di (2-ethylhexyl) phthalate (DEHP) is one of the most ubiquitous environmental contaminants, with measurable concentrations of its metabolites detected in the urine of nearly 100% of the humans sampled [2–4]. While DEHP exposure has been primarily associated with reproductive problems [5, 6], studies in humans have shown that phthalate exposure is also associated with increased waist circumference as well as diabetes [7, 8]. In animals, adulthood exposure to DEHP has been shown to influence insulin sensitivity, liver metabolism, and energy balance [9–12], and studies specifically focusing on prenatal exposure to numerous EDCs, including phthalates, suggest that prenatal exposure to EDCs may disrupt metabolism (reviewed [13]). However, the mechanisms underlying these effects remain to be elucidated, especially in males versus females.

White adipose tissue (WAT) in adult animals is primarily comprised of mature adipocytes that contain a single lipid vacuole and are responsible for both buffering circulating lipids and contributing to systemic glucose homeostasis. The process of adipocyte maturation occurs in several regulated steps. Immature preadipocytes originate from mesenchymal stem cells in the process that regulates adipocyte cell number (hypo- or hyper-plasia), and preadipocytes further mature to adipocytes, which can then adapt in size (hypo-or hyper-trophy) in response to metabolic cues [14]. Due to this multi-step and ontogenic regulation, the process of white adipose tissue expansion is complicated and involves both prenatal and postnatal cues. Studies in animals suggest that increased adipocyte size and number can both be programmed *in utero* by numerous factors [15–17]. This prenatal shift in adipocyte morphology likely increases an organism's propensity for increased fat mass accumulation in response to certain postnatal signals, including the intake of a high-fat diet. Perinatal exposure to DEHP can induce obesity and adipose tissue inflammation in adult offspring [18]. Furthermore, while mono-(2-ethylhexyl) phthalate (MEHP), the active metabolite of DEHP, has previously been shown to induce pre-adipocyte [19] and adipocyte [20] differentiation in human cell lines, and DEHP was shown to induce adipogenesis in a murine mesenchymal stem cell line [21], the molecular mechanisms behind the observed consequences of developmental DEHP exposure *in vivo* are largely unknown.

Due to the critical role of both *in utero* and postnatal events in adipose tissue metabolism, the proposed role of DEHP in inducing adipogenesis, and the known sex differences in adipose tissue metabolism, the current study investigated the effect of a postnatal high-fat diet on adipose morphology in male and female rats perinatally exposed to DEHP. Furthermore, unlike previous studies investigating the effects of DEHP on adipogenesis in adults, we specifically assessed sex-specific molecular markers in adipose tissue immediately following developmental exposure, and then investigated physiology and adipose tissue morphology in adult animals. We observed that male, but not female offspring perinatally exposed to DEHP had increased adiposity in adulthood. Furthermore, exposure of these males to a post-weaning HF diet increased energy intake and body weight, decreased the number of small and medium-sized adipocytes, and led to glucose intolerance. At birth, DEHP-exposed pups had the same body weight, but lower % body fat than their oil controls, and these males caught up in adiposity to controls by the end of the weaning period (PND21). Adipose tissue of PND21 male, but not female pups had increased expression of markers of adipogenesis and *de novo* fatty acid synthesis, but decreased expression of markers of mesenchymal stem cell to pre-adipocyte commitment. These data suggest that in the current model, gestational DEHP exposure may decrease the adipocyte pool at birth, which initially increases adaptive catch-up adipocyte maturation and lipid accumulation, but decreases the capacity of adipose tissue to appropriately respond to a HF diet in adulthood, leading to glucose intolerance.

2. METHODS

2.1. DEHP Dosing and Animal Experimental Design during Gestation and Weaning

Timed-pregnant female Sprague-Dawley rats (n=6 per treatment group) were obtained from Charles River Laboratories (Wilmington, MA, USA) on gestational day 2. Pregnant dams were individually housed in ventilated cages in a temperature-controlled environment, fed a modified AIN-93G diet (Table 1), and received DEHP-free (reverse osmosis) water *ad libitum*. Beginning on gestational day 6 until postnatal day (PND) 21, pregnant and lactating dams were orally dosed with either vehicle control (Oil; tocopherol-stripped corn oil) or DEHP (DEHP; 300 mg/kg BW; Cat# 36735, Sigma-Aldrich, St. Louis, MO) using a 1000 μL Eppendorf pipette and sterilized tips. Pregnant dams were weighed every 3 days, and the dose of DEHP was adjusted based on body weight. To account for the additional calories being consumed from oil by oil vehicle controls, the liquid DEHP solution for the DEHP treatment group was first mixed with the same volume of oil as was given to controls.

While most humans have regular daily exposure to DEHP, exact exposures have yet to be characterized. Although the U.S. Environmental Protection Agency reference dose for DEHP is 20 μg/kg/day and the lowest-observed-adverse-effect level (LOAEL) of DEHP is 140 mg/kg/day, it is likely that humans are routinely exposed to much higher doses of phthalates because Becker *et al*. have shown that levels of DEHP in household dust range from 400–700 mg/kg [22]. Multiple doses were not used in the current study because the addition of postnatal diet as a factor limited our ability to include additional dosing groups. However, the dose (300 mg/kg BW) was selected to mimic other rodent studies that have reported adverse reproductive outcomes at this dose, which allowed for the evaluation of

metabolic disruptions by DEHP at a dose where male reproductive dysregulations were previously clearly documented [23, 24].

Immediately after birth, litter sex distributions and offspring weights were recorded, and 4 male and 4 female pups were randomly placed back with each dam in respective oil or DEHP-treated groups.

2.2. Animal Experimental Design after Weaning Using a High-fat Dietary Challenge

To determine the effects of a HF dietary challenge in male and female pups developmentally exposed to DEHP, PND21 pups were mixed (within sex and treatment group), 5 male and 5 female removed from each treatment group for sampling, and the remainder of the pups were randomized and placed into new cages, with 2 or 3 offspring per cage, separated by sex and maternal dosing group. At this point, offspring were separated into 4 groups (n=5 males and n=5 females), such that half of the oil-treated offspring consumed a C diet (AIN93G rodent diet for growth Oil-C), while half consumed a high-fat diet (Oil-HF) to model the intake of a western-style diet alone without DEHP exposure, and half of the DEHP-exposed offspring consumed a C diet (DEHP-C), while half were further challenged with a HF diet (DEHP-HF), to model the intake of a HF diet in animals prenatally exposed to DEHP. The HF diet has a higher amount of kcal coming from fat (45%) when compared to the AIN93G diet (16%). Recent NHANES data show that the average intake of fat in the U.S. is 33.7% of total kcals, which is at the higher range of recommended intake for adults of 20–35% kcals from fat [25] and the intake of an energy-dense western-style or high-fat (HF) diet is unquestionably linked to the development of metabolic syndrome [26]. Since many individuals likely consume more fat than the population mean, the HF diet utilized in the current study models a relevant dietary pattern in humans.

At the onset of puberty, all animals were single-housed, but remained on these different diets until the end of the study (PND110) when they were euthanized with CO_2. At both collection points (weaning and adulthood), all tissues were either snap-frozen in liquid nitrogen and stored at −80°C or fixed in 10% formalin for future analyses.

2.3. Serum Testosterone Measurement

As DEHP has been suggested to be anti-androgenic in previous rodent models, total testosterone was measured at weaning on PND21 and in adult males on PND110. Serum samples were thawed on ice and analyzed in triplicate per manufacturer's instruction using the Testosterone enzyme-linked immuno assay (ELISA) kit from DRG (Cat #EIA-1559). The kit is reported to have 100.0 cross-reactivity with testosterone, 3.3 cross reactivity with 11β-Hydroxytestosterone and 19-Nortestosterone, <1.0 with 5α-Dihydrotestosterone and Androstenedione, and <0.01 with other steroid hormones. The intra-assay variation (as the mean of %CV values) was 1.9%.

2.3. Oral Glucose Tolerance Test (OGTT)

Because DEHP exposure has previously been associated with altered glucose homeostasis in humans and animal models, we assessed glucose tolerance on PND22 and PND100, when offspring were fasted overnight, and fasted blood glucose was measured as the baseline the

following morning. Immediately following the fasted measurement, animals were orally gavaged with a 2g/kg BW bolus of D-glucose dissolved in water (50% w/v). Plasma glucose measurements were taken after 30, 60, and 120 minutes of the bolus using the Accu-Chek Glucometer and Comfort Curve strips (Roche, Indianapolis, IN) from a tail vein nick.

2.4. Body Composition

To determine whether DEHP exposure affected fat mass throughout the study, and whether a HF diet had an additional impact, body composition was measured on PND1, at weaning on PND21, after the onset of puberty on PND60, and on PND90 using the EchoMRI-700 Body Composition Analyzer (Echo Medical Systems, Houston, TX), which allows for the measurement of fat and lean mass in conscious and unrestrained animals. On PND1 and PND21, male and female pups were scanned as a litter but separated by sex, and on PND60 and PND90, offspring were scanned individually.

2.5. Adipose Histopathology

To assess the effect of DEHP exposure, with and without a HF diet, on adipose tissue morphology in adult animals, gWAT samples were fixed in formalin, embedded in paraffin using a routine protocol and 3 μm thick sections were stained with H&E. Images were taken under 20X magnification and 2 independent fields were chosen for analysis from 5 male or female offspring. Approximately 80–150 independent adipocytes were manually circled and counted per image in order to calculate the perimeter and cell number (ImageJ Software, NIH, Bethesda, MD). Cell number density calculation was performed using a geometric approach as previously reported [27]. Briefly, “adipose tissue cell density” was calculated by first determining cell area and the number of these cells that are expected to fit into each independent window view without overlap. Where cellular borders were not clearly obvious, circularity was predicted by finding the center of cells where at least half of the perimeter was visible, and using the circle feature within the software to predict the full cell perimeter (and area). The actual number of cells within each view was then divided by the calculated expected cell number, such that a lower ratio indicates a decrease in adipose tissue cell density.

2.6. Adipose RNA Isolation and RT-PCR Analysis in PND21 Pups and PND110 Adults

To investigate the adipogenic expression signature in PND21 pups immediately following developmental DEHP exposure, and the mRNA expression of *Ar* in adult animals, RNA isolation and RT-PCR were performed as previously described [27]. Briefly, frozen WAT (100 mg) from 5 male and 5 female PND21 pups from each treatment group was ground in liquid nitrogen with mortar and pestle. Because pre-pubertal PND21 pups had very little gWAT, adipose sampling included gonadal and renal adipose tissue to assure that sufficient sample was available for analysis. Total RNA isolation, DNase I treatment (to eliminate any DNA contamination), cDNA synthesis and real-time PCR were performed as previously described [28] with an additional spin down step during RNA isolation to remove the lipid layer in adipose. *Pparg1* and *Pparg2* transcription rate was determined by designing primers that cover both an exon and intron of the gene to measure unspliced pre-mRNA, as previously described [29]. This procedure has been reported to be a valid technique for

analyzing transcription rate [30], and was adapted from a method originally described by Lipson & Baserga [31], except that pre-mRNA amounts were quantified by real-time PCR. For all realtime PCR reactions, a serial dilution was used to create a standard curve for quantification and a dissociation curve was analyzed following each reaction. All primers for real-time PCR analysis (Table 2) were designed using the VectorNTI® software (Life Technologies™, Grand Island, NY), analyzed using BLAST, and synthesized by IDT (Coralville, IA). All mRNA data were normalized to the housekeeping gene encoding ribosomal protein L7a (*rpl7a*).

2.7. Statistical Analysis

Maternal weight gain, litter size, sex distribution, maternal %fat mass at delivery and weaning, % fat or lean mass at PND1 and PND21, glucose values and the area under the curve, and all mRNA data were analyzed using one-way ANOVA and body weight from PND1 to PND21 was analyzed using repeated measures one-way ANOVA to determine the overall effect of DEHP. Body weight from PND1 to PND21 was analyzed using repeated measures. All data in adult animals were analyzed in n=5 offspring (except for the OGTT) by first using one-factor ANOVA to confirm the significance of the model, followed by independent comparisons of all groups. gWAT adipocyte perimeter, adipocyte density, and adipocyte perimeter distribution (small+medium adipocytes) at adulthood were calculated in n=5 male or female offspring from each treatment group with 2 fields counted per animal. Values for the OGTT glucose area under the curve (AUC) at adulthood were analyzed in n=4 male or female offspring and on PND22 in n=3 male or female offspring from each treatment group using the following equation: AUC = (0.25*fasted) + (0.5*30min) + (0.75*60min) + (0.5*120min). For all one-way ANOVA, normality was checked using the Shapiro-Wilk test (with all data being normal); for the repeated measures one-way ANOVA, normality was checked using the Kolmogorov-Smirnov test and confirmed using a Q-Q plot of the residuals. All ANOVA and regression analyses (with Pearson's correlation being shown to demonstrate relationships between variables) were performed in SAS (Chicago, IL) and differences were considered significant at $P<0.05$.

3. RESULTS

3.1. DEHP exposure had no observable effect on pregnancy outcomes, liver weights and testosterone levels after weaning or in adulthood

DEHP had no effect on litter size or sex distribution (%male). DEHP exposure also had no effect on maternal gestational weight gain, or % fat mass either at delivery or at weaning on PND21 (Table 3).

As a marker of toxicity, DEHP had no significant effect on liver weights, either at weaning on PND21 (1.89±0.07 g in the oil group vs. 1.97±0.89 g in DEHP males, and 1.49±0.07 g in the oil group vs. 1.63+0.06 g in DEHP females), or in adults on PND110 (22.28±0.98 g in the oil group vs. 25.91±1.54 g in DEHP males, and 12.08±0.25 g in the oil group vs. 10.85±1.16g in DEHP females).

3.2. Perinatal DEHP exposure decreased % fat mass at birth, but induced catch-up adipogenesis during weaning in male, but not female pups, with no effect on circulating testosterone levels

DEHP had no effect on body weight from birth until PND21 in either male (Figure 1A) or female (Figure 1D) pups. However, % fat mass was significantly lower ($P<0.05$) and lean mass was significantly higher ($P<0.05$) in males at birth when compared to controls (representing a 15.1% decrease in fat mass or increase in lean mass by DEHP), and adiposity in males caught up to controls by PND 21 (Figure 1B). DEHP trended to decrease adiposity in female pups at birth and on PND21, but this was not significant during either period (Figure 1E). DEHP had no effect on PND21 OGTT glucose levels or the glucose AUC in males (Figure 1C). In females, DEHP decreased OGTT glucose levels at 60 ($P<0.005$) and 120 ($P<0.05$) minutes, without a significant effect on the glucose AUC (Figure 1F). DEHP had no significant effect on circulating total testosterone levels in males on PND21 (0.53±0.11 ng/mL in the oil group vs. 0.51±0.12 ng/mL in the DEHP group).

3.3. Perinatal DEHP exposure increased %fat, and a post-weaning HF dietary challenge in DEHP-exposed males increased body weight, energy intake, %fat, and glucose intolerance in adults, with no effect on circulating testosterone levels

In adult males, developmental DEHP exposure alone had no effect on body weight (Figure 2A) or energy intake (Figure 2B), but DEHP-exposed males also consuming a post-weaning HF diet had significantly higher ($P<0.05$) body weight (Figure 2A) and energy intake (Figure 2B) when compared to controls and either the DEHP or HF groups alone. DEHP-exposed males had increased ($P<0.05$) % fat mass from the post-puberty until adulthood period (representing a 27.5% increase in fat mass by DEHP), and while the DEHP-HF group appeared to have higher % fat mass when compared to all groups, the difference was only significant ($P<0.05$) when compared to the control group (Figure 2C). Also in males, developmental DEHP exposure alone had no effect on OGTT glucose levels or the glucose AUC, but DEHP-exposed males also consuming a post-weaning HF diet had significantly higher ($P<0.05$) glucose levels at 60min, as well as a higher ($P<0.05$) glucose AUC when compared to controls and either the DEHP or HF groups alone (Figure 2D). In females, neither DEHP exposure alone nor in combination with a post-weaning HF diet affected adulthood body weight (Figure 2E), energy intake (Figure 2F), % fat mass (Figure 2G), or the OGTT glucose levels and the glucose AUC (Figure 2H). DEHP had no significant effect on circulating total testosterone levels in adult males (1.12±0.31 ng/mL in the oil group vs. 1.31±0.21 ng/mL in the DEHP group).

3.4. Perinatal DEHP exposure increased adipocyte perimeter, adipocyte density, and the number of small/medium adipocytes in males, and a post-weaning HF dietary challenge in DEHP-exposed males further decreased the number of small/medium adipocytes

Adipocyte size has been associated with altered insulin sensitivity and glucose uptake [32], and in the current study, glucose AUC was significantly correlated to adipocyte perimeter in males ($r = 0.73$, $P<0.001$), where increased adipocyte perimeter was associated with a higher glucose AUC, and no relationship was observed in females. In males, the postnatal HF diet group (Oil-HF) and both DEHP groups (DEHP-C and DEHP-HF) had increased

(P<0.05) adipocyte perimeter when compared to the control (Oil-C), and DEHP-HF males also had larger (P<0.05) adipocyte perimeter than DEHP-C males (Figure 3A). Furthermore, male adipocyte density, as a marker of adipocyte number in the sampled depot, was decreased (P<0.05) in both DEHP groups (DEHP-C and DEHP-HF) when compared to unexposed groups (Oil-C and Oil-HF) (Figure 3B). To further characterize the morphology of the adipose tissue, adipocytes were subcategorized by perimeter into 4 size groups: small (<200 μm), medium (200–300 μm), large (300–450 μm), and extra-large (450+ μm). The Oil-HF, DEHP-C, and DEHP-HF had significantly decreased (P<0.05) percentage of small/medium-sized adipocytes in males, and the percentage was lower in DEHP-HF males when compared to DEHP-C and Oil-HF males (Figure 3C, representative images in Figure 3D). In females, the Oil-HF, and DEHP-HF groups had increased (P<0.05) adipocyte perimeter when compared to Oil-C (Figure 3E), whereas there were no group differences in female adipocyte density (Figure 3F) or perimeter distribution (Figure 3G, representative images in Figure 3H).

3.5. Perinatal exposure to DEHP increased the PND21 expression of adipogenic genes in the adipose tissue of male offspring

Because DEHP-exposed males had increased adiposity when compared to Oil-C males, and because a post-weaning HF diet increased glucose intolerance in perinatally DEHP-exposed males (DEHP-HF), we assessed the adipose tissue expression of genes associated with the developmental adipogenic cascade immediately after weaning, including the expression of *Pparg*, the master regulator of adipogenesis (diagram of its 2 major transcripts is shown in Figure 4A). DEHP-exposed male pups had increased expression (P<0.005) and transcription rate (P<0.05) of *Pparg1* (Figure 4B), but not *Pparg2* (Figure 4C). DEHP-exposed males also had increased (P<0.05) expression of *Cebpa, Fasn, Igf1, Adipoq, Ppard, and Fabp4*, and decreased (P<0.05) *Lep* expression, without any change in *Cebpb, Irs1, Lpl, or Ppara* (Figure 4D). In females, DEHP exposure had no effect on the expression or transcription rate of either *Pparg1* (Figure 4E) or *Pparg2* (Figure 4F), but DEHP decreased (P<0.05) the expression of *Cebpb* (P<0.005), *Fasn* (P<0.05), *Lpl* (P<0.005), increased *Irs1* (P<0.05), and had no effect on *Adipoq, Cebpa, Fabp4, Igf1, Lep, Ppara, or Ppard* (Figure 4G).

3.6. Perinatal exposure to DEHP decreased the PND21 expression of genes associated with the mesenchymal stem cell commitment to the adipogenic lineage in the adipose tissue of male offspring

Because adipocyte density, a marker of adipocyte cell number, was decreased in DEHP-exposed males, we also assessed the PND21 expression of genes associated with the mesenchymal stem cell transition to the adipocyte lineage. In males, DEHP significantly decreased (P<0.05) the expression of *Bmp2* and *Bmp4*, as well as of *Stat1* and *Stat5a*, without a significant effect on *Stat5b* (Figure 5A). DEHP had no effect on the expression of these genes in females (Figure 5B).

4. DISCUSSION

The Developmental Origins of Health and Disease (DOHaD) hypothesis first suggested that maternal nutrition is critical for the fetal programming of adult cardiac and metabolic

disorders, and subsequent studies have also suggested that the adulthood adipocyte "set point" can be programmed by numerous *in utero* factors [33], which may increase the propensity of adipocytes to accumulate lipids and impact the ability of adipose tissue to contribute to systemic glucose handling in response to postnatal cues (including diet). While numerous epidemiological and animal studies have focused on the effects of nutrition and stress during pregnancy on metabolic outcomes, prenatal exposure to environmental endocrine disrupting chemicals has also been proposed to have a role in programming adult-onset metabolic diseases [34]. In the current study, prenatal DEHP exposure resulted in metabolic dysregulations in males, which is consistent with previous studies in rodents [35, 36]. Furthermore, in the current model, glucose intolerance was only observed when developmentally DEHP-exposed males were further challenged with an adulthood HF diet, which could potentially be related to the ability of developmental DEHP exposure to affect adipose tissue structure and function and subsequently alter the response to adulthood fat intake (Figure 6).

4.1. Developmental DEHP exposure had sex-specific effects on early-life adipose tissue adipogenic signaling, adulthood morphology, as well as the response to a post-weaning HF diet

In the current study, metabolism-related dysregulations were observed in males, but not females in response to developmental DEHP exposure and a post-weaning HF diet. Adipose metabolism and glucose homeostasis are intrinsically different between males and females, with females being more insulin sensitive, as well as having differently localized adipose depots and increased lipogenesis when compared with postmenopausal females and males [37–39]. A study in mice also showed that in response to a high-fat diet, males and ovariectomized females, but not intact females, were more susceptible to developing obesity and glucose intolerance than controls [40], which agrees with our finding that females did not respond to a HF diet, and that adipocyte size was associated with glucose AUC in males, but not in females. Because animals in the current study were still relatively young adults at the end of the study, it is possible that HF females would have increased adiposity and metabolic disorders with age.

The sex-specific response to DEHP treatment might be due to intrinsic baseline morphological differences between adipose depots, likely driven by the differential expression levels observed in PND21 pups. For example, in the current study, adult females had a higher average % fat mass at baseline compared to males (19.7% and 12.5%, respectively) and also presented with larger adipocytes at baseline than males (320 μm and 262 μm, respectively). These morphological differences were accompanied by obvious sex differences in the baseline expression of several genes at PND21 (*Pparg1*, *Fasn*, and *Lep*), and a drastically different adipogenic signature in response to DEHP. For example, numerous adipogenic genes were affected in males but not females (*Pparg1*, *Cebpa*, *Igf1*, *Adipoq*, *Lep*, *Ppard*, *Fabp4*), while several other genes affected in females appeared to suggest an anti-adipogenic signature in females in response to DEHP (decreased *Cebpb*, *Fasn*, *Lpl* and increased *Irs1*). The exact cause for this sex-specific response is unknown, but may be linked to endocrine signals within the adipose tissue itself. DEHP has been proposed to be anti-androgenic, and in men, testosterone has been shown to be inversely correlated

with % body fat [41] and to also decrease adipogenesis *in vitro* [42]. Previous studies in male rodents utilizing doses of DEHP that are similar to the dose used in the current study have shown decreased testosterone levels in adult offspring [23, 24]. While we did not observe differences in circulating total testosterone in adult male offspring in the current study, we assessed the mRNA expression of Androgen Receptor (*Ar*) in gWAT of adult animals on PND110 (data not shown), and found that in males (but not females), DEHP significantly decreased *Ar* mRNA expression (1.00±0.04 Oil-C vs. 0.79±0.05 DEHP-C, $P<0.05$). HF diet also decreased *Ar* expression (1.00±0.04 Oil-C vs. 0.68±0.60 Oil-HF, $P=0.01$), and the DEHP-HF group had the lowest *Ar* expression (0.43±0.03, $P<0.0001$ to Oil-C; $P<0.05$ to Oil-HF; and $P<0.001$ to DEHP-C). Furthermore, there was a negative correlation between adipocyte size in all adult males and *Ar* expression ($r=-0.73$, $P<0.01$). *Ar* knockout mice have been shown to develop late-onset obesity, but have increased adiponectin release and are not insulin resistant or diabetic [43], which agrees with the observed increased adiposity (but not disrupted glucose homeostasis) in DEHP-exposed males on a control diet in the current study. Based on these observations, as well as the differences in baseline adipocyte measures between the sexes and the effect of DEHP on male adipocyte parameter, it is possible that DEHP directly disrupted hormonal signaling within adipose tissue, and future studies that focus on developmental endocrine disruption within gWAT itself will be needed to determine the precise endocrine mechanisms for the metabolic dysregulation observed in males but not females in response to prenatal DEHP exposure and a postnatal HF diet.

4.2. Developmental DEHP exposure initially decreased adipose tissue expansion, but this was followed by catch-up adipogenesis and increased adulthood adiposity in males

Fetal growth restriction has long been associated with increased adiposity in adulthood, both in humans and animal models [44]. David Barker's "fetal programming" hypothesis in humans led to numerous studies in animals that utilized developmental nutrition deficits to established intrauterine growth restriction (IUGR) that was followed by an increase in adulthood chronic diseases. Furthermore, the adaptive process of rapid postnatal catch-up growth has been shown to be strongly associated with adulthood chronic diseases, including diabetes and insulin resistance in animal models and human [45, 46]. In the current study, PND1 DEHP-treated males had significantly lower adiposity than oil-treated controls (but not lower body weight), and caught up to the controls by PND21, reflecting a more rapid gain in adiposity during the weaning period. These males also had increased adiposity in adulthood when compared to the oil controls. The mechanisms involved in this adaptive process have not been fully elucidated, but in the case of gestational nutrient restriction, it has been suggested that IUGR establishes a "thrifty phenotype" that is well adapted to its predicted nutrient-deficient postnatal environment. Whether this is also true in the case of environmental chemical exposure is unclear. As previously discussed, DEHP exposure has been associated with metabolic disturbances in humans, and several studies have also shown that DEHP exposure is associated with altered birth outcomes. A population study in China showed that prenatal DEHP exposure was associated with decreased birth length and exposure to another phthalate, dibutyl phthalate, was associated with low birth weight [47]. Furthermore, a study in rats showed that DEHP-exposed rat pups experienced intrauterine growth restriction [48], suggesting that DEHP exposure can interfere with certain aspects of

fetal growth, which likely includes metabolic maturation. Additional studies in humans and mechanistic studies in animals are needed to investigate whether growth restriction mediates the proposed relationship between DEHP exposure and increased metabolic syndrome.

4.3. Catch-up adipogenesis in DEHP-exposed males was associated with the increased expression of adipogenic markers but decreased expression of genes associated with the commitment of stem cells to the adipogenic lineage

The commitment of mesenchymal pluripotent stem cells to the adipogenic lineage involves numerous factors. Bmp2 and Bmp4 have been shown to drive stem cell commitment, giving rise to preadipocytes [49], and members of the STAT family of proteins have also been implicated in adipogenic commitment [50, 51]. In the current study, males developmentally exposed to DEHP had decreased *Bmp2, Bmp4, Stat1*, and *Stat5a* expression at weaning, which was also associated with a significant decrease in adipocyte density (as a marker of cell number) in adulthood. At birth, these males had decreased adiposity and increased lean mass when compared to controls. These data suggest that decreased adiposity at birth in response to DEHP exposure may be due to a decrease in the number of adipocyte precursors.

We also observed rapid catch-up adipogenesis in these male pups after birth, which was likely due to an increase in adipocyte size and *de novo* fatty acid synthesis, rather than adipocyte hyperplasia. The differentiation of preadipocytes to mature adipocytes that are capable of storing excess lipids and synthesizing lipids as well as contributing to systemic glucose homeostasis is primarily regulated by peroxisome proliferator-activated receptor gamma (PPARg)-mediated signaling [52], which is accompanied by the increased expression of CCAAT/enhancer binding proteins (*Cebpa* and *Cebpb*) [53], as well as other genes associated with lipid accumulation and *de novo* fatty acid synthesis, including *Fasn* [54] and *Fabp4* [55]. In the current study, PND21 DEHP-exposed males had increased expression of *Pparg1, Cebpa, Fasn*, *Fabp4*, *Ppard*, and *Igf1*. These mRNA expression data suggest that PND21 male pups had increased adipogenesis at weaning, potentially as an adaptive mechanism in response to restricted adipose tissue development *in utero*. Interestingly, *Adipoq* mRNA expression was increased in PND21 DEHP-exposed males. Insulin resistance and obesity are typically associated with a decrease in circulating adiponectin. However, It has been shown that testosterone could actually decrease adiponectin secretion from 3T3-L1 adipocytes and mice (high molecular weight form) [56, 57], and body fat and adiponectin are elevated in androgen receptor knockout mice [58]. As discussed above, we did not observe an overall decrease in circulating testosterone in adult males, but did find that DEHP decreased androgen receptor expression (together with adiponectin expression), which agrees with the data from androgen receptor knockout mice. It is possible that increased *Adipoq* mRNA expression on PND21 acts as an adaptive mechanism to compensate for the decrease in adipogenic precursors at birth, but additional studies will be needed to determine whether this has a direct impact on the observed consequence of DEHP exposure on adulthood glucose homeostasis in response to a HF diet.

4.4. Developmental DEHP exposure decreased the number of small and medium adipocytes in males, which was associated with glucose intolerance in adults consuming a HF diet

Adipose tissue expansion and large adipocytes, in particular, are thought to contribute to the insulin resistant phenotype [32, 59]. This is due to the fact that in addition to acting as the body's energy storage depot (in the form of fatty acids and triglycerides), mature adipocytes are also critical for regulating whole-body glucose homeostasis by clearing circulating glucose in response to insulin signals. In the current study, DEHP-exposed males that were further challenged by a post-weaning HF diet had glucose intolerance, which was associated with increased adipocyte perimeter, decreased adipocyte number density, and decreased % of small/medium-sized adipocytes. As previously discussed, PND21 males experienced catch-up adipose tissue expansion, which was potentially associated with an increased rate of adipogenesis but a decrease in the number of adipogenic precursors. Leptin is released from mature adipocytes [60], and PND21 males had decreased adipose *Lep* expression, which has been shown to positively correlate to preadipocyte number [61], preadipogenic induction [62], and adipogenic differentiation [63]. These data suggest that developmental DEHP exposure and subsequent catch-up adipogenic expansion during weaning increased the tendency for adipocytes from DEHP-exposed males to efficiently store lipids. Increased cell size is known to cause inflammation that leads to insulin resistance [32], so while adipocyte size (and subsequent glucose intolerance) were further increased in DEHP males in response to a HF diet, this potentially occurred due to the apparent decrease in adipocyte number, which may have put additional stress on the adipose tissue "organ" by decreasing individual storage depots (adipocytes).

Interestingly, these physiological adaptations in males in response to DEHP were associated with a substantial increase in the expression and transcription rate of *Pparg1* on PND21, without a concurrent increase in *Pparg2* expression. The nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARg) is a critical transcription factor during adipogenesis. In addition to regulating adipose tissue expansion, PPARg has been shown to directly participate in adipose tissue-related insulin sensitivity. Pparg adipose-specific knockout mice have lipoatrophy and uncontrolled diabetes [64], and individuals with the Pro12Ala polymorphism within the Pparg gene are at an increased risk of having type 2 diabetes [65], highlighting the critical role of this transcription factor in regulating glucose homeostasis. While Pparg has widespread tissue distribution, mature human adipocytes have been shown to have different levels of Pparg1 and Pparg2, which have identical sequences, except for additional nucleotides at the 5-prime end of Pparg2. Mutations in Pparg2, but not Pparg1, have been associated with obesity and diabetes in humans, and Pparg2 has been designated as the "nutrition-responsive" transcript. However, Pparg1 protein appears to be activated earlier during pre-adipocyte differentiation and is expressed at a higher level than that of Pparg2 [66], and both Pparg1 and Pparg2 have been shown to have the ability to independently induce adipogenic differentiation and adipogenesis in 3T3 fibroblasts, but through different transcription factor mechanisms [67]. These observations suggest that the two isoforms have unique upstream regulation as well as differential downstream consequences. Numerous reproductive toxicity and carcinogenicity studies in animals have established that DEHP can act through PPPARg-mediated signaling

[68], and cell culture modeling studies suggest that phthalates can efficiently bind to the human Pparg [69], as well as to induce Pparg expression and induce adipogenesis [70]. However, this is the first report, to our knowledge, to suggest that DEHP exposure has transcript-selective effects during development, which appears to be associated with increased lipid accumulation but decreased adipogenic precursor formation. These data suggest that in the current model of developmental DEHP exposure, *Pparg1*, rather than *Pparg2* may have a critical role in driving DEHP-induced adipocyte morphology during development, thus selectively activating signals responsible for lipid accumulation and *de novo* fatty acid synthesis but decreasing stem cell commitment and decreasing the capacity of adipose tissue to contribute to systemic glucose homeostasis.

In conclusion, the present study is the first to demonstrate that the early-life adipogenic signature in males in response to DEHP-induced growth restriction and catch-up adipogenesis is predictive of adulthood adipocyte morphology and glucose intolerance following a high-fat dietary challenge. Our data suggest that DEHP may decrease the adipocyte pool but hasten early-life adipogenesis, thus disrupting adulthood glucose homeostasis, which is a marker of the metabolic syndrome. The sex-specific deleterious effects of prenatal DEHP exposure and a postnatal HF diet on whole-body energy homeostasis is especially relevant in the current obesogenic environment, and data from the current study characterizing these energy-balance sex differences *in vivo* contributes to unraveling the roles of EDCs in metabolism-related chronic diseases.

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Abbreviations Used

Adipoq	adiponectin
AUC	area under the curve
Bmp2 or 4	bone morphogenic protein 2 or 4
Cebpa or b	CCAAT/enhancer binding protein alpha or beta
DEHP	di (2-ethylhexyl) phthalate
EDC	endocrine disrupting chemical
Fabp4	fatty acid binding protein 4
Fasn	fatty acid synthase
gWAT	gonadal white adipose tissue
HF	high-fat
Igf1	insulin-like growth factor 1

Irs1	insulin receptor substrate 1
L7a	ribosomal protein L7a
Lep	leptin
Lpl	lipoprotein lipase
MRI	magnetic resonance imaging
OGTT	oral glucose tolerance test
Ppara, Ppard, Pparg 1 or 2	peroxisome proliferator-activated receptor alpha, delta, or gamma 1 or 2
PND	postnatal day
Stat1 or 5a or 5b	Signal Transducers and Activators of Transcription

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HIGHLIGHTS

- Adult DEHP-exposed males had increased % fat and adipocyte perimeter
- HF diet in DEHP-exposed adult males led to glucose intolerance
- At birth, DEHP decreased % fat mass in males
- At weaning, DEHP increased adipogenic markers in males
- At weaning, DEHP decreased markers of commitment to the adipogenic lineage in males

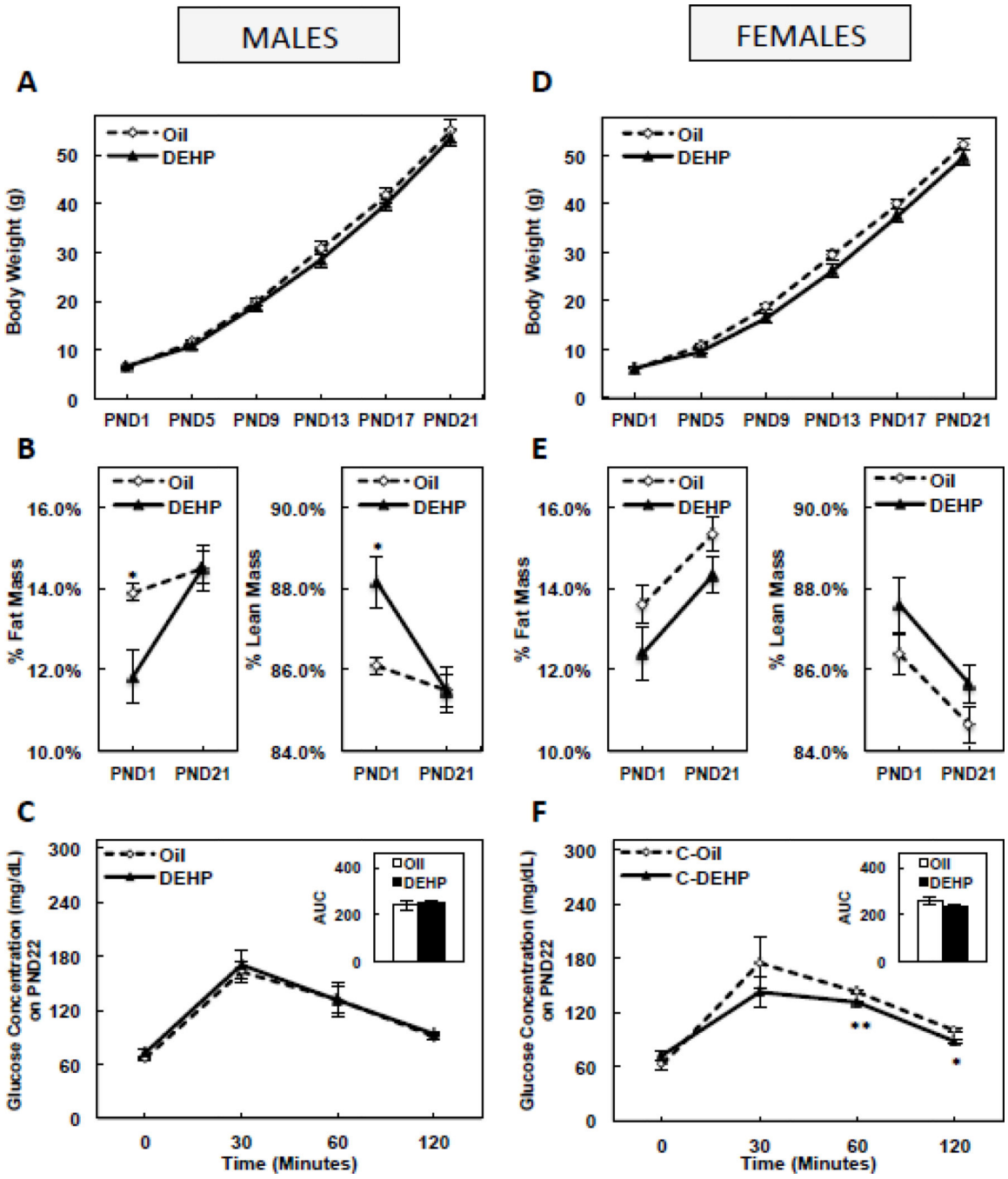

FIGURE 1. Body weight from postnatal day (PND) 1 until PND21 in male (A) and female (D) offspring, fat mass or lean mass on PND1 and PND21 in male (B) and female (E) offspring, and oral glucose tolerance and glucose area under the curve (AUC) on PND 22 in male (C) and female (F) offspring

Offspring were exposed to maternal DEHP or vehicle (oil). Values are means ± SEM. n=5 male or female offspring. *P<0.05 and **P<0.005.

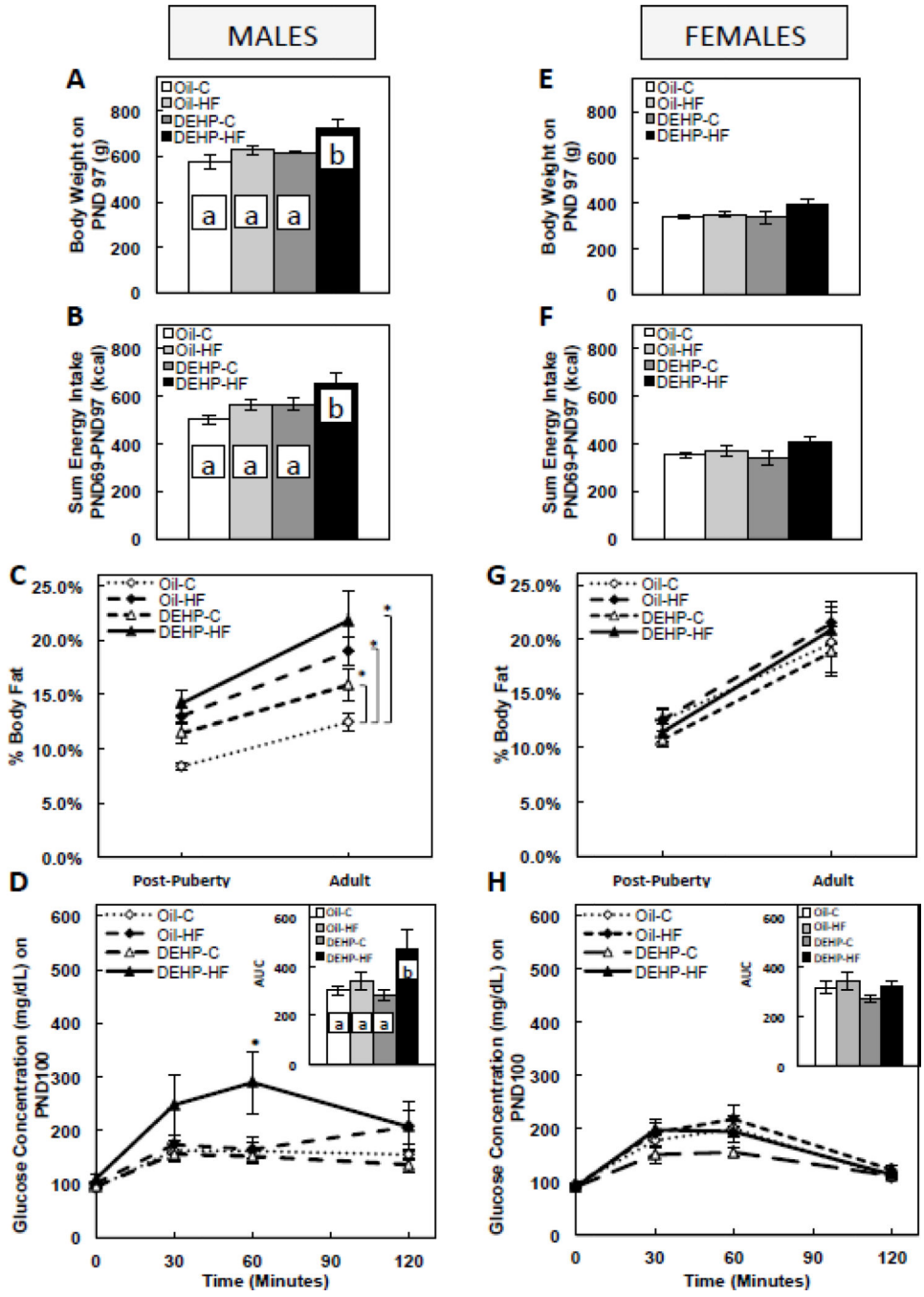

FIGURE 2. Body weight on postnatal day (PND) 97 in male (A) and female (E) offspring, sum of energy intake from PND 69–97 in male (B) and female (F) offspring, % fat mass post-puberty (PND 60) and in adulthood (PND 90) in male (C) and female (G) offspring, and oral glucose tolerance and glucose area under the curve (AUC) in adult male (D) and female (H) offspring (PND 100)

Offspring were exposed to maternal DEHP or vehicle (oil) and then consumed a postnatal control (C) or high-fat (HF) diet. Values are means ± SEM. n=5 male or female offspring for all figures, except n=4 male or female offspring for Figs. D & H. Bars with different letters

differ at $P<0.05$. *$P<0.05$ when compared to Oil-C (Fig. C) and to Oil-C, Oil-HF, and DEHP-C (Fig. D).

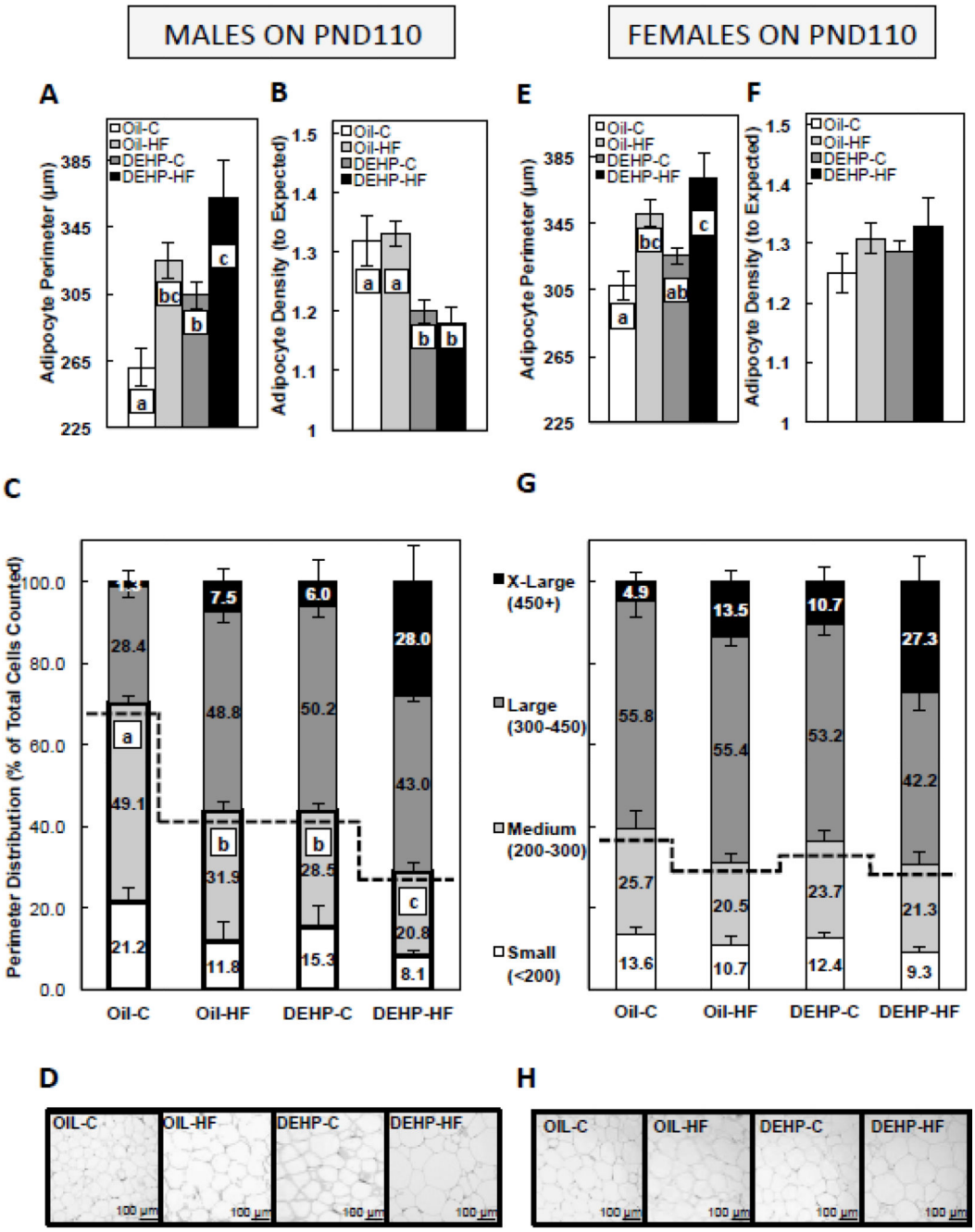

FIGURE 3. Gonadal white adipose adipocyte perimeter in adult (PND 110) male (A) and female (E) offspring, gonadal white adipose adipocyte density in adult male (B) and female (F) offspring, adipocyte perimeter distribution in male (C) and female (G) offspring, and representative images of gonadal fat depots in male (D) and female (H) offspring

Offspring were exposed to maternal DEHP or vehicle (oil) and then consumed a postnatal control (C) or high-fat (HF) diet. Values are means ± SEM. n=5 male or female offspring with 2 fields counted per animal. Bars with different letters differ at $P<0.05$. For perimeter distribution, data are shown as amount of small, medium, large or extra-large adipocytes as percent of total adipocytes counted, and statistical analysis represents group differences of

small+medium-sized adipocytes combined. Representative images are 3–4μm hematoxylin & eosin-stained sections under 20X magnification.

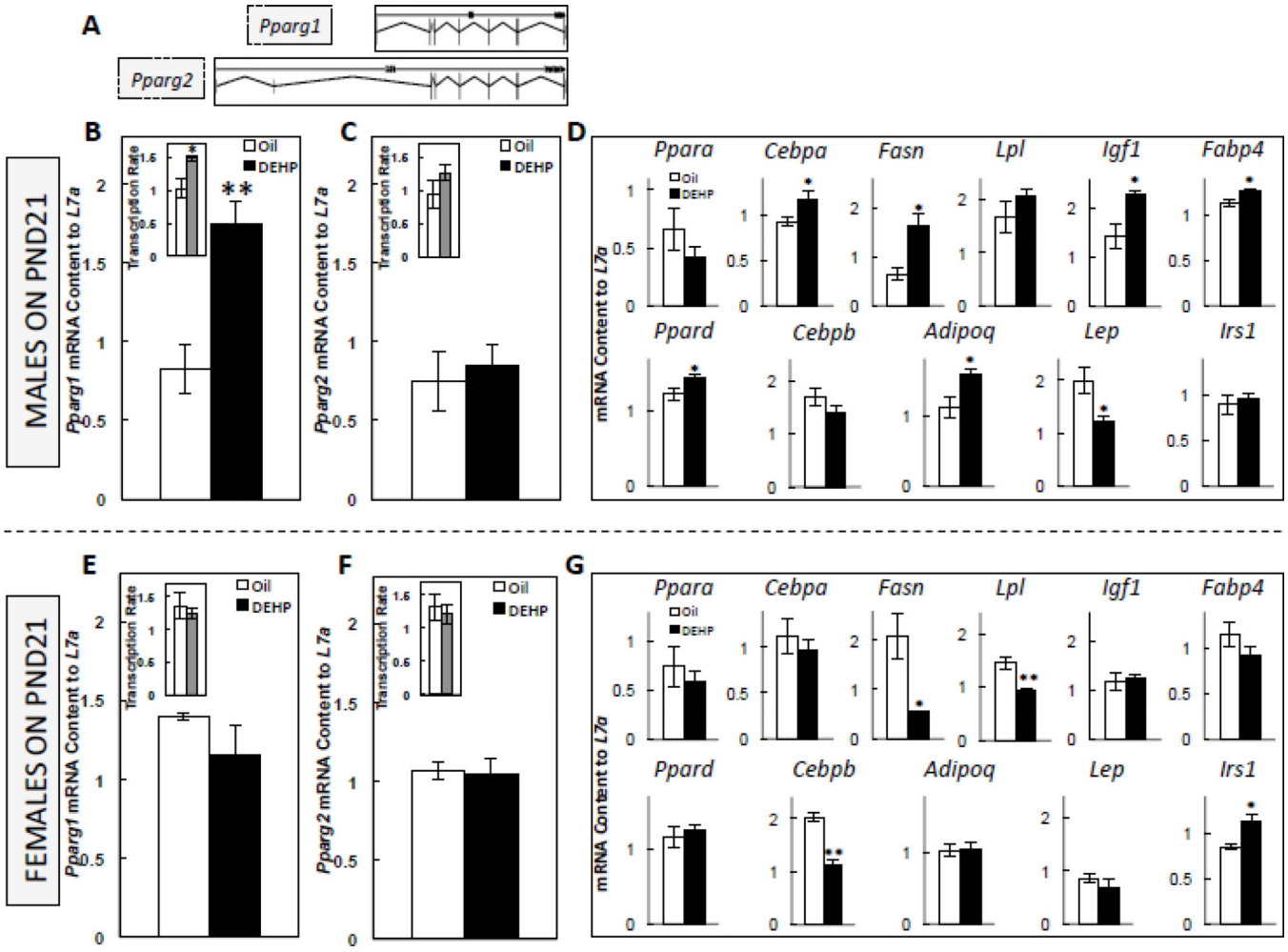

FIGURE 4. Diagram of two major transcripts of the *Pparg* gene (A), the mRNA expression of Pparg1 in postnatal (PND) 21 male (B) and female offspring (E), the mRNA expression of Pparg2 in PND21 male (C) and female (F) offspring, and the mRNA expression of adipogenic genes in PND 21 male (D) and female (E) offspring

Offspring were exposed to maternal DEHP or vehicle (oil). Values are means ± SEM. n=5 male or female offspring. *P<0.05 and **P<0.005.

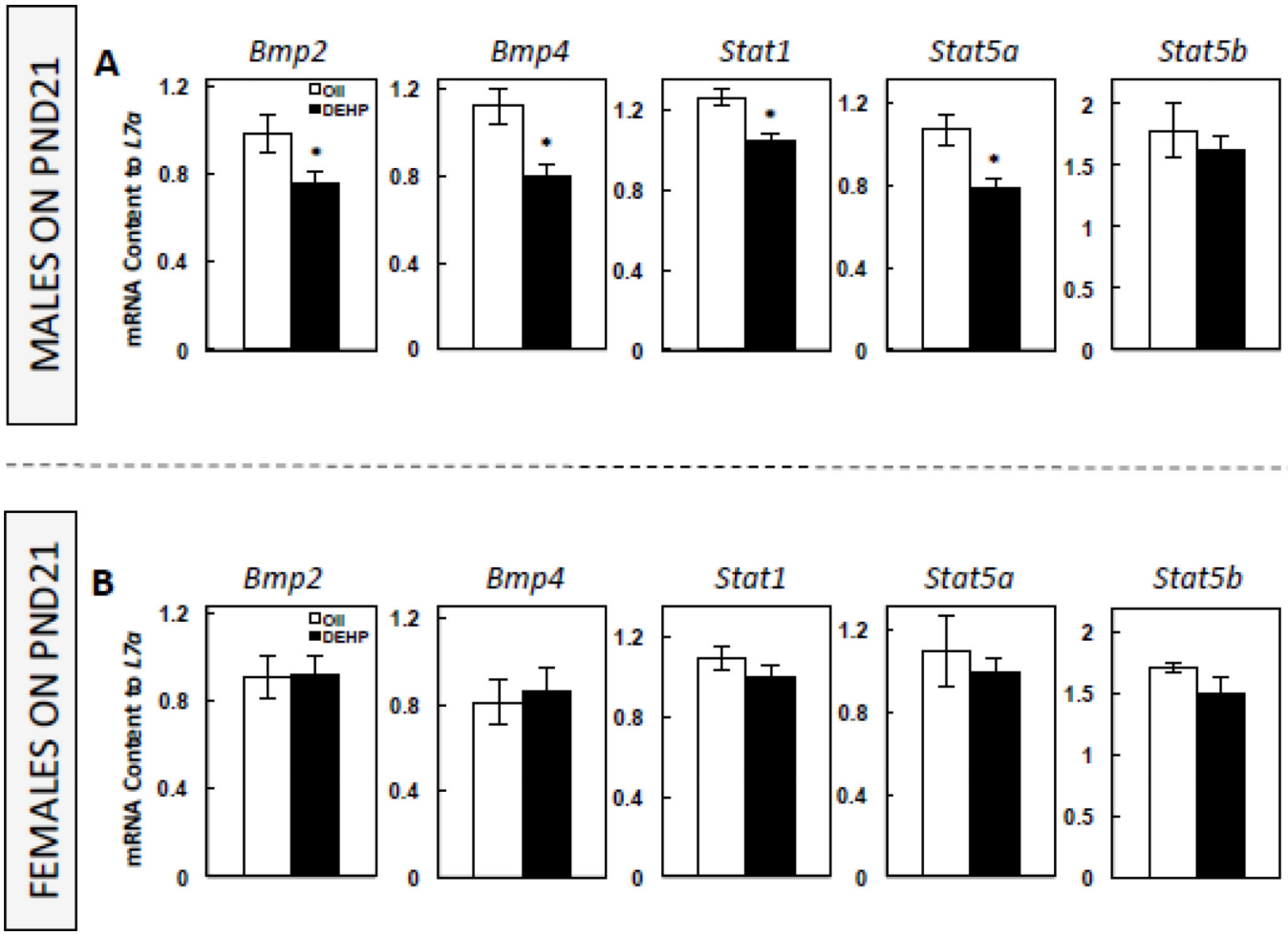

FIGURE 5. The mRNA expression of genes associated with the mesenchymal stem cell commitment to the adipogenic lineage in postnatal (PND) 21 male (A) and female offspring (B)

Offspring were exposed to maternal DEHP or vehicle (oil). Values are means ± SEM. n=5 male or female offspring. *P<0.05.

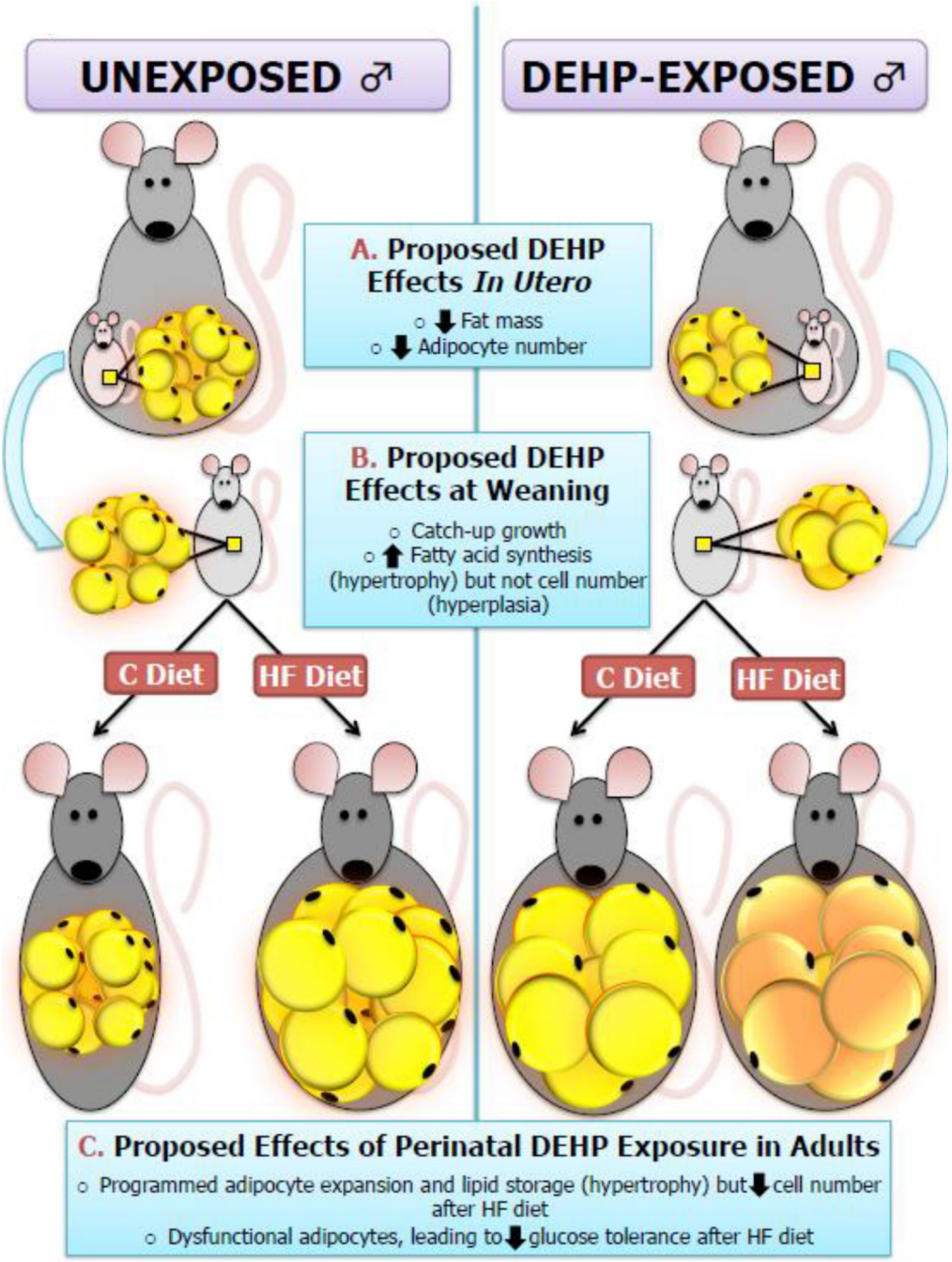

FIGURE 6. The proposed action of developmental DEHP exposure on adipose tissue in male offspring in the current model

Our data suggest that *in utero* DEHP exposure in males was associated with decreased fat mass, potentially due to a decrease in adipocyte number at birth (A). DEHP-exposed male offspring experienced catch-up adipogenesis at weaning, potentially due to an increase in lipid accumulation and *de novo* fatty acid synthesis (B). Adult males developmentally exposed to DEHP had increased adiposity and cell perimeter, but fewer small/medium-sized

adipocytes and decreased overall cell number. Furthermore, these adult males had glucose intolerance when they also consumed a high-fat diet after weaning.

TABLE 1

Diet Composition*

	Control (C)		High-Fat (HF)	
%	**Gram**	**kcal**	**Gram**	**kcal**
Protein	**20**	**20**	**24**	**20**
Carbohydrate	**64**	**64**	**41**	**35**
Fat	**7**	**16**	**24**	**45**
Ingredients				
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	397.5	1590	105	420
Maltodextrin	132	528	132	528
Sucrose	100	400	100	400
Cellulose	50	0	50	0
Soybean Oil	70	630	70	630
Lard	0	0	130	1170
Mineral Mix	35	0	35	0
Vitamin Mix	10	40	10	40
Choline Bitartrate	2.5	0	2.5	0

*Research Diets, Inc, New Brunswick, NJ

TABLE 2

Primer Information

Gene	Forward Sequence and Location	Reverse Sequence and Location
Adipoq	TTCAAGAAGGACAAGGCCGT (+540)	CCAGATGGAGGAGCATGGA(+630)
Ar	GAAATGGGACCTTGGATGGAGA (+2508)	TAAAACGTGGTCCCTGGTACTGTC (+2585)
Bmp2	TGATGCGATGGACAGCACAGG (+688)	ACCTGGCTTCTCCTCTAAGTGGGC (+764)
Bmp4	CAGCAGCATCCCAGAGAATGAGG (+573)	GTCCACCTGCTCCCGAAATAGC (+639)
Cebpa	AGTCGGTGGATAAGAACAGCAACG (+821)	GCTGTTTGGCTTTATCTCGGCTC(+910)
Cebpb	AGAACGAGCGGCTGCAGAAGA (+1220)	GAACAAGTTCCGCAGCGTGC(+1287)
Fabp4	AAGAAGTGGGAGTTGGCTTCGC (+106)	ACCAAGTCCCCTTCTACGCTGATG (+190)
Fasn	CTTTGTGAGCCTCACCGCCAT (+1747)	ATGCCATCAGGTTTCAGCCCC (+1811)
Igf1	GGTGGACGCTCTTCAGTTCGTG (+454)	TCTGTGGTGCCCTCCGAATG (+545)
Irs1	AGGACTGGGGGAGACTTAGTC (+307)	TCATGCCCAGAGGGAAAG (+384)
Rpl7a	GAGGCCAAAAAGGTGGTCAATCC (+64)	CCTGCCCAATGCCGAAGTTCT(+127)
Lep	CTCACCAGCTTGCCTTCCCA (+112)	AGGTCTCGCAGGTTCTCCAGGT (+176)
Lpl	GAGAACATTCCCTTCACCCTGCC (+1296)	CCGATGTCCACCTCCGTGTAAA (+1375)
Ppara	ATGGCTGAGAAGACGCTTGTGG (+1122)	TCGGACCTCTGCCTCCTTGTT (+1190)
Ppard	GCTCACCGAGTTCGCCAAGAAC (+1024)	CCTCATGCACGCCGTACTTGAG (+1112)
Pparg1		
mRNA	CGCTGATGCACTGCCTATGAGC (+51)	GGTCCACAGAGCTGATTCCGAAGT (+148)
Transcription Rate	CGCTGATGCACTGCCTATGAGC (+51)	CCCGAAGCATCCCTTACAGCAA (+123)
Pparg2		
mRNA	TAGCCTGGGCTGCTTTTATA (+15)	GGGAGTTAAGATGAATTTAGCG (+117)
Transcription Rate	ACTCCCATTGAGTAGGTG (+112)	ACCCTCTAACAAAGTGCC (+164)
Stat1	CAGTGGCTAGAAAAGCAAGACTGGG (+396)	AGAGCAGGTCATGGAAGCGGAT (+474)
Stat5a	GTCTCAGTTCAGCGTTGGCAGC (+1385)	ACAATGACGACCACAGGGAGGG (+1459)
Stat5b	TCAACGCCACCATCACAGACATC (+962)	GGAGGCTGCTTCTCGATGATGAA (+1031)

TABLE 3

Maternal Characteristics

Variable	Oil Control	DEHP
Maternal Weight Gain (g)	171.4 ± 6.4	179.9 ± 11.4
Litter Size	12.8 ± 0.7	12.7 ± 0.4
Sex Distribution (% Male)	41.5 ± 5.7	51.8 ± 4.6
Maternal % Fat Mass at Delivery (PND1)	14.7 ± 0.4	15.9 ± 0.2
Maternal % Fat Mass at Weaning (PND21)	10.3 ± 0.9	11.3 ± 0.3

* $P<0.05$ comparing Oil Control vs. DEHP within each gender

PND: Postnatal day