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## Prenatal and ancestral exposure to di(2-ethylhexyl) phthalate alters gene expression and DNA methylation in mouse ovaries

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### Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a commonly used plasticizer and known endocrine disrupting chemical, which causes transgenerational reproductive toxicity in female rodents. However, the mechanisms of action underlying the transgenerational toxicity of DEHP are not understood. Therefore, this study determined the effects of prenatal and ancestral DEHP exposure on various ovarian pathways in the F1, F2, and F3 generations of mice. Pregnant CD-1 dams were orally exposed to corn oil (vehicle control) or DEHP (20 µg/kg/day-750 mg/kg/day) from gestation day 10.5 until birth. At postnatal day 21 for all generations, ovaries were removed for gene expression analysis of various ovarian pathways and for 5-methyl cytosine (5-mC) quantification. In the F1 generation, prenatal DEHP exposure disrupted the expression of cell cycle regulators, the expression of peroxisome-proliferator activating receptors, and the percentage of 5-mC compared to control. In the F2 generation, exposure to DEHP decreased the expression of steroidogenic enzymes, apoptosis factors, and ten-eleven translocation compared to controls. It also dysregulated the expression of phosphoinositide 3-kinase (PI3K) factors. In the F3 generation, ancestral DEHP exposure decreased the expression of steroidogenic enzymes, PI3K factors, cell cycle regulators, apoptosis factors, *Esr2*, DNA methylation mediators, and the percentage of 5-mC compared to controls. Overall, the data show that prenatal and ancestral DEHP greatly suppresses gene expression of pathways required for folliculogenesis and steroidogenesis in the ovary in a transgenerational manner and that gene expression may be influenced by DNA methylation. These results provide insight into some of the mechanisms of DEHP-mediated toxicity in the ovary across generations.

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Competing interest statement

The authors have no conflicts of interest to disclose.

## Keywords

Ovary; DEHP; endocrine disruptor; transgenerational; methylation

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## Introduction

Phthalates are a family of synthetic chemicals that act as plasticizers to confer flexibility and reduce breakage [1]. Phthalates are critical for the production of consumer goods. Many types of phthalates exist, but di(2-ethylhexyl) phthalate (DEHP) is a common plasticizer found in polyvinyl chloride products. DEHP is incorporated into a multitude of products including personal care products, medical equipment (i.e., blood and I.V. bags), car upholstery, food and beverage packaging, and building materials, particularly vinyl products [1–3]. DEHP is non-covalently bound to the polymer chains within these products; therefore, DEHP may leach from the products and into the environment after repeated use, heating, and cleaning [4]. Humans are exposed to DEHP by oral ingestion, inhalation, and dermal contact. However, the most common route of exposure to DEHP and phthalates in general is by ingestion. The estimated range of human exposure to DEHP is between 3 – 30 µg/kg/day [2, 5–7]. Human urine samples persistently test positive for DEHP and its metabolites, indicating that humans are repeatedly and continuously exposed to DEHP [8]. This is further supported by the detection of DEHP in human tissues such as blood, amniotic fluid, umbilical cord blood, breast milk, and ovarian follicular fluids in humans [2, 9–12].

DEHP is an endocrine disrupting chemical (EDC) [2, 13, 14], and reproductive tissues such as the gonads are particularly susceptible to EDCs. In humans, *in utero* exposure to DEHP is associated with decreased free testosterone and free testosterone:estradiol ratio the in cord blood of both male and female newborns [15, 16]. DEHP exposure also interferes with obstetrical outcomes, puberty, and gonadal function [17]. DEHP exposure is also associated with an early age of pubic hair development in young girls, an indicator of precocious puberty [18]. Further, *in utero* exposure to DEHP metabolites is associated with an earlier age of menarche in young girls [19]. Finally, urinary concentrations of DEHP metabolites are negatively associated with total oocytes, mature oocytes, fertilized oocytes, and top quality embryos, indicating that DEHP impairs oocyte parameters [20].

The ability of DEHP to cause adverse reproductive outcomes is a major concern for the F1 and subsequent generations. DEHP-induced alterations in the ovary may be passed to the subsequent generations through the female germ cells [21]. Transmission of disease due to direct prenatal DEHP exposure may cause multigenerational effects and ancestral exposure to DEHP may cause transgenerational effects in the F3 generation. This is because during a developmental exposure window, the pregnant mouse (F0 generation) is exposed to DEHP via ingestion. Therefore, the F1 generation is directly exposed to DEHP as a fetus, and the F2 generation is directly exposed to DEHP as the developing germ cells in the F1 fetus, causing multigenerational effects in the F1 and F2 generations [22]. Effects observed in the F3 generation are not due to direct exposure, but instead are due to ancestral exposure, therefore demonstrating transgenerational inheritance [22].

Experimental studies show that DEHP exposure causes numerous multigenerational and transgenerational phenotypes in female reproduction. DEHP exposure during prenatal development dysregulates folliculogenesis, alters sex steroid hormone levels, and increases the presence of ovarian cysts in a multigenerational manner [23, 24]. Further, prenatal DEHP exposure decreases the percentage of dams that give birth in the F2 generation of mice [25]. Ancestral DEHP exposure accelerates ovarian follicle formation, onset of puberty, and reproductive senescence in the F3 generation of female mice [23, 25]. In addition, ancestral exposure to DEHP accelerates early folliculogenesis in a transgenerational manner [24]. Although studies demonstrate that phthalate exposure causes transgenerational effects on the ovary, the mechanisms underlying these effects are not well understood.

Epigenetic modification is thought to be the mechanism by which transgenerational effects are inherited [26]. Epigenetics are mitotically and meiotically heritable changes in gene function, without changing DNA sequences [27, 28]. These heritable changes in the epigenome define and control cell and tissue development by controlling gene expression [29]. Multiple molecular mechanisms alter the epigenome, however, DNA methylation is the most commonly studied epigenetic mechanism [30]. DNA methylation patterns are mediated by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) enzymes [31–33]. DNMTs are a family of enzymes that methylate CpG dinucleotides in DNA. DNMT1 is the maintenance DNMT that maintains original DNA methylation patterns in a cell lineage; it methylates CpG sites during DNA replication so that both daughter cells have the same DNA methylation patterns [34–36]. DNMT3A and DNMT3B methylate CpG sites on naked DNA outside of DNA replication and are required for genome-wide de novo methylation [31]. TETs are enzymes that oxidize 5-methyl cytosine (5-mC) as a demethylation mechanism [32, 37]. TET1 is primarily responsible for oxidizing 5-mC into 5-hydroxymethyl cytosine (5-hmC), whereas TET2 and TET3 primarily oxidize 5-hmC into further oxidized cytosines that are replaced with an unmethylated, unmodified cytosine [32, 38].

Studies have demonstrated that DEHP exposure modulates DNA methylation. Specifically, prenatal DEHP exposure induces long-lasting and robust promoter methylation-related silencing of fundamental genes in sperm physiology [39]. *In utero* DEHP exposure is associated with an enrichment of DNA methylation of genes involved in the androgen response, estrogen response, and spermatogenesis [40]. Prenatal exposure to DEHP differentially expressed 406 genes related to reproductive processes in rat ovaries [41]. Although these studies indicate that DEHP acts through methylation, studies do not indicate if DEHP modifies methylation statuses throughout generations. Therefore, the current study was designed to evaluate the pathways and mechanisms by which prenatal and ancestral exposure to DEHP influence key ovarian functions in the F1, F2, and F3 generations of mice. Specifically, this study tested the hypothesis that prenatal and ancestral DEHP exposure disrupt ovarian functions by altering gene expression of several ovarian pathways critical for cell growth, proliferation, and function (i.e., the sex steroid hormone synthesis pathway, phosphoinositide 3-kinase pathway (PI3K), cell cycle regulators, apoptosis and oxidative stress factors, steroid hormone receptors, and insulin-like growth factors) [24, 42–49], DNA methylation, and DNA methylation effectors such as DNMTs and TET enzymes.

## Materials and Methods

### Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of DEHP (0.022, 0.224, 560, and 840 mg/mL) were prepared by diluting DEHP in tocopherol-stripped corn oil (MP Biomedicals, Solon, OH). These stock solutions were diluted to create doses of 20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day of DEHP. DEHP concentrations were chosen based on previous studies and their environmental relevance [45, 46, 50–53]. Specifically, the 20 µg/kg/day dose of DEHP was selected because the U.S. Environmental Protection Agency established the chronic oral reference dose as 20 µg/kg/day of DEHP. The reference dose is an estimate of the daily oral exposure of DEHP in the general population that has a low risk of adverse effects during the lifetime [54]. In addition, 20 µg/kg/day of DEHP falls within the estimated human exposure range based on urinary metabolite levels [5]. The 200 µg/kg/day dose of DEHP was used because it falls within the estimated occupational range of exposure [2]. In addition, adult exposure to 200 µg/kg/day of DEHP has been shown to cause abnormal estrous cyclicity and accelerate primordial follicle recruitment in female CD-1 mice [45]. The 500 mg/kg/day dose of DEHP was selected because it has been shown to cause abnormalities in spermatogonial stem cells across multiple generations in male CD-1 mice [52]. The 750 mg/kg/day dose of DEHP was selected because adult exposure has been shown to cause abnormal estrous cyclicity and accelerate primordial follicle recruitment in adult female CD-1 mice [45].

### Animals and dosing paradigm

Adult female and male CD-1 mice (Charles River, USA) were housed at 25°C in conventional polysulfone, ventilated cages on 12L:12D cycles. The mice were fed Teklad Rodent Diet 8604 (Harlan) and provided highly purified water (reverse osmosis filtered water) in polysulfone water bottles *ad libitum*. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and abide by the guidelines set forth by the National Institute of Health for the Care and Use of Laboratory Animals.

At 8 weeks of age, female mice (F0) were mated with control male mice of the same age. The female mice were monitored twice a day for the presence of a copulatory vaginal sperm plug to confirm mating. Once a copulatory vaginal sperm plug was confirmed, the presence of which was considered gestational day (GD) 0.5, the females were removed, weighed, and individually housed. Subsequently, the mice were weighed twice a week to confirm successful pregnancy. From GD 10.5 until birth of the pups, pregnant dams (F0) were orally dosed once a day with the vehicle control (tocopherol-stripped corn oil) or with DEHP (20 µg/kg/day, 200 µg/kg/day, 500 mg/kg/day, or 750 mg/kg/day) by placing a pipette tip with the dosing solution into the cheek pouch of the mouse. This dosing regimen was selected to mimic oral exposure to DEHP in humans [1, 45, 51]. The doses were calculated and adjusted based on daily body weights, and delivered in 25 – 33 µL of tocopherol-stripped corn oil. The treatment window was chosen because it is a critical time period of ovarian development. Specifically, this is when primordial germ cells arrive at the gonad [55, 56],

sex determination occurs [57], and global demethylation and imprint erasure of primordial germ cells occur [58].

Pregnant mice were allowed to deliver naturally and the day of birth was considered postnatal day (PND) 0. Mice born from the F0 generation were labeled the F1 generation. Female mice from the F1 generation were mated with non-treated male CD-1 mice to produce the F2 generation. Females from the F2 generation were mated with non-treated male CD-1 mice to produce the F3 generation. No mice were mated with family members. At PND 21, mice ( $n = 3 - 15$  dams/treatment group) were euthanized by CO<sub>2</sub> affiliation followed by cervical dislocation. PND 21 was selected because mice are juvenile, not sexually mature, and no corpora lutea are present. Whole ovaries were collected from each mouse. One ovary was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for RNA and DNA extraction.

### RNA sequencing analysis

Frozen whole ovaries collected at PND 21 from control and 20  $\mu\text{g}/\text{kg}/\text{day}$  ( $n = 3$  ovaries/treatment group) from the F3 generation were used for RNA sequencing. Raw reads were checked for quality using FASTQC (v 0.11.5) then trimmed and filtered using Trimmomatic (v 0.36) to remove residual adapter content, low quality bases (Phred quality score  $< 28$ ), and resulting reads shorter than 30 nt. Trimmed/filtered reads were aligned to NCBI's *Mus musculus* GRCm38.p6 genome and gene model annotation release 106 using STAR (v 2.5.3a). Post-alignment gene counts were then determined for each NCBI EntrezGene ID using featureCounts from Subread (v 1.5.2-pl) with multi-mapping reads excluded.

The raw read counts were input into R [59] (v 3.4.3) for pre-processing and analysis together using Bioconductor [60] packages as listed below. Approximately  $\sim 23$  million reads aligned uniquely within the 41,595 *M. musculus* genes. We used TMM method [61] in the edgeR package [62] (v 3.20.5) to normalize the counts to log<sub>2</sub>-transformed counts per million (logCPM), using the cpm() function with prior.count = 3. Specifically, 25,141 genes did not have logCPM  $> \log_2(0.5)$  in at least three samples and were filtered out, leaving 16,454 genes to be analyzed for differential expression. TMM-values and logCPM normalized values were re-calculated with prior.count = 3 after gene filtering. Principle components analysis clustering of the samples (data not shown) indicated one of the treatment replicates was more variable than the other two. Rather than remove this sample completely, we did a surrogate variables analysis [63, 64] on the logCPM values, which estimated one surrogate variable that corrected for the difference in this replicate (data not shown). This surrogate variable was added to edgeR's quasi-likelihood negative binomial generalized log-linear model [65], which was fit on the original read counts + TMM values to find differential expression between the treated and control groups. Multiple hypothesis test correction was done using the False Discovery Rate method [66].

Data obtained from RNA sequencing were functionally analyzed using The Database of Annotation, Visualization, and Integrated Discovery Bioinformatics (DAVID) 6.8 following the previously published protocol [67, 68]. Genes with a false discovery rate  $< 0.62$  and  $p < 0.007$  were entered into DAVID for functional annotation analysis for a total of 177 genes. "Gene\_Ontology" and "Pathways" and the denoted DAVID defined defaults were selected

for functional annotation clustering. To determine if functional gene groups were valuable, annotation clusters with a significant enrichment score  $\geq 1$  were further explored [67].

### Gene expression analysis

Frozen whole ovaries collected at PND 21 were used for quantitative real-time polymerase chain reaction (qPCR) analysis ( $n = 3 - 6$  ovaries/treatment group). Total RNA ( $>100$  ng) was extracted from the whole ovaries using the AllPrep DNA/RNA Mini Kit (Qiagen, Austin, TX, USA) according to the manufacturer's protocol, including DNase digestion. Total RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) using the iScript RT Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Each cDNA sample was diluted 1:8 using nuclease-free water prior to qPCR analysis. Analysis of qPCR was performed using the CFX96 C1000 Real-Time PCR Detection System and CFX Manager Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Each qPCR reaction was done in duplicate using 2  $\mu$ L of cDNA, forward and reverse primers (5 pmol) for select genes, nuclease-free water, and SsoFastEvaGreen Supermix for a final reaction volume of 10  $\mu$ L. Target genes were analyzed in reference to the housekeeping gene, beta-actin (*Bactin*). A list of gene primers (Integrated DNA Technologies, Coralville, IA, USA) and the housekeeping gene, beta-actin, are included in the supplementary files (Table S1).

The CFX96 C1000 Real-Time PCR Detection machine quantifies the amount of PCR product generated by measuring SsoFastEvaGreen dye (Bio-Rad Laboratories, Inc., Hercules, CA) that fluoresces when bound to double-stranded DNA. The qPCR program consisted of an enzyme activation step (95 °C for 1 min), an amplification and quantification program (40 cycles of 95 °C for 10 s, 60 °C for 10 s, single fluorescence reading), a 72 °C for 5 min, a melt curve (65 °C-95 °C heating 0.5 °C/s with continuous fluorescence readings), and a final step at 72 °C for 5 min per the manufacturer's protocol. All gene expression data were normalized to the housekeeping gene. Relative fold changes were calculated and analyzed using a mathematical model for relative quantification of real-time PCR data developed by Pfaffl [69] and then normalized as a ratio to control group.

### DNA methylation analysis

DNA was extracted from frozen whole PND 21 ( $n = 3 - 7$  ovaries/treatment group) ovaries using the AllPrep DNA/RNA Mini Kit (Qiagen, Austin, TX, USA) per the manufacturer's protocol. DNA was extracted, eluted in 100  $\mu$ L of EB buffer, and stored in  $-80$  °C until further DNA methylation testing. To measure global DNA methylation status, the enzyme-linked immunosorbent assay MethylFlash Methylated DNA 5-mC Quantification Kit (Colorimetric assay, Epigentek Group Inc., Farmingdale, NY, USA) was used according to the manufacturer's protocol. Briefly, ovarian DNA (100 ng) was added to high affinity strip wells. Methylated DNA was detected using capture and detection antibodies for 5-methyl cytosine (5-mC) and quantified by reading absorbance at 450 nm using a 354 Multiskan Ascent Microplate Reader (Thermo Electron Corp., Shanghai, China). The absolute amount and percentage of methylated DNA were calculated using the absolute quantification method per the manufacturer's protocol. Briefly, a standard curve was calculated from five known concentrations of methylated DNA (0.5, 1, 2, 5, 10 ng). The slope of the standard curve was

quantified and used in the provided formulas per the manufacturer's protocol to calculate the amount and percentage of methylated DNA (5-mC) in each sample.

### Statistical analyses

Data were expressed as the mean  $\pm$  standard error of the mean (SEM). In all generations, data from multiple female pups originating from the same litter were averaged and combined as  $n = 1$ , and data from at least 3 separate litters were used in the analyses. Data were analyzed by comparing treatment groups to control using IBM SPSS version 24 software (SPSS Inc., Chicago, IL, USA). Outliers were removed by the Grubb's test using GraphPad outlier calculator software (GraphPad Software Inc., La Jolla, CA, USA). Data that were continuous were assessed for normal distribution by Shapiro-Wilk analysis. If data met assumptions of normal distribution and homogeneity of variance, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey HSD or Dunnett 2-sided *post-hoc* comparisons. However, if data met assumptions of normal distributions, but not homogeneity of variance, data were analyzed by ANOVA followed by Games-Howell or Dunnett's T3 *post-hoc* comparisons. If data were presented as percentages or were not normally distributed, the independent sample Kruskal-Wallis H followed by Mann-Whitney U non-parametric tests were performed. For all comparisons, statistical significance was determined by p-value  $\leq 0.05$ . In instances in which p-values were greater than 0.05, but less than 0.10, data were considered to exhibit a trend towards significance.

## Results

### The effects of ancestral exposure to DEHP on gene expression in the F3 generation as determined by RNA sequencing and The Database of Annotation, Visualization, and Integrated Discovery Bioinformatics

Functional annotation gene clustering analysis via DAVID provided 5 annotation clusters from the "Gene\_Ontology" selection (Figure 1A). Within the annotation cluster containing the highest enrichment score was insulin-like growth factor binding, regulation of cell growth, and growth factor binding (Figure 1A). Functional annotation gene clustering from the "Pathway" selection provided 1 annotation cluster (Figure 1B). Within the annotation cluster, extra cellular matrix-receptor interaction, amoebiasis, focal adhesion, and the PI3K-Akt signaling pathway were listed (Figure 1B). Based on these results, subsequent qPCR was conducted to assess the effects of DEHP exposure on ovarian gene expression.

### The effects of DEHP exposure on hormone receptors and insulin-like growth factor gene expression in ovaries from the F1 – F3 generations

Several studies suggest that DEHP and its many metabolites act through steroid hormone receptors and peroxisome proliferator-activated receptors (PPAR) [70, 71]. Therefore, the current study examined the effects of prenatal and ancestral exposure to DEHP on the expression of hormone receptors and PPARs. Further, based on the RNA sequencing results, the insulin-like growth factor (IGF) family was examined. In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Ppara*, *Fshr*, or *Igfbp4* compared to controls (Figures 2A, 2E, and 2G). However prenatal exposure to DEHP decreased the expression of *Pparg* in the 20  $\mu\text{g}/\text{kg}/\text{day}$  and 750  $\text{mg}/\text{kg}/\text{day}$  groups, increased *Esr2*

expression in the 750 mg/kg/day group, decreased the expression of *Ar* in the 20 µg/kg/day group, and increased the expression of *Igf1* in the 500 mg/kg/day group compared to controls (Figures 2B, 2C, 2D, and 2F, n = 3 – 5 ovaries/treatment group, p < 0.05). In the F2 generation, exposure to DEHP did not affect the expression of *Ppara*, *Pparg*, *Ar*, *Fshr*, or *Igf1* compared to controls (Figures 2A, 2B, 2D, 2E, and 2F). In contrast, exposure to DEHP decreased the expression of *Esr2* in the 20 µg/kg/day group and decreased *Igfbp4* expression in the 500 mg/kg/day group compared to controls (Figures 2C and 2G, n = 3 ovaries/treatment group, p < 0.05). In the F3 generation, ancestral exposure to DEHP did not affect the expression of *Ppara*, *Pparg*, *Fshr*, *Igf1*, or *Igfbp4* compared to controls (Figures 2A, 2B, 2E, 2F, and 2G). However ancestral exposure to DEHP decreased the expression of *Esr2* in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups, and decreased the expression of *Ar* in the 200 µg/kg/day group compared to controls (Figures 2C and 2D, n = 3 – 6 ovaries/treatment group, p < 0.05 but p = 0.071 for *Ar* in 200 µg/kg/day).

### **The effects of DEHP on steroidogenic enzyme gene expression in ovaries from the F1 – F3 generations**

Our previous work showed that prenatal DEHP exposure dysregulated steroid hormone levels in F2 generations, but not the F1 and F3 generations of mice at PND 21 [24]. The current work was performed to examine estrogen synthesis and expand our knowledge of DEHP dysregulation of steroid hormones by determining if it is due to prenatal or ancestral DEHP effects on expression of steroidogenic enzymes. In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Star*, *Hsd17b1*, *Cyp11a1*, *Cyp17a1*, *Cyp19a1*, *Cyp11a1*, or *Cyp11b1* (Figures 3A, 3C, 3D, 3E, 3F, 3G, and 3H), but DEHP at 750 mg/kg/day increased the expression of *Hsd3b1* compared to controls (Figure 3B, n = 3 – 5 ovaries/treatment group, p < 0.05). In the F2 generation, exposure to DEHP decreased the expression of *Star* in the 20 µg/kg/day group, decreased *Hsd3b1* expression in the 20 µg/kg/day group, decreased *Hsd17b1* expression in the 20 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day groups, and decreased *Cyp19a1* expression in the 20 µg/kg/day and 500 mg/kg/day groups compared to controls (Figures 3A, 3B, 3C, and 3F, n = 3 ovaries/treatment group, p < 0.05, but p = 0.081 for *Star* in 20 µg/kg/day). In the F3 generation, ancestral exposure to DEHP did not affect the expression of *Star*, *Hsd3b1*, *Cyp11a1*, *Cyp17a1*, *Cyp19a1*, or *Cyp11a1* compared to controls (Figures 3A, 3B, 3D, 3E, 3F, and 3G), but ancestral exposure decreased the expression of *Hsd17b1* in the 20 µg/kg/day and 750 mg/kg/day groups and decreased *Cyp11b1* expression in the 200 µg/kg/day, 500 mg/kg/day, and 750 mg/kg/day treatment groups compared to controls (Figures 3C and 3H, n = 3 – 6 ovaries/treatment group, p < 0.05, but p = 0.068 and 0.069 for *Cyp11b1* in 500 mg and 750 mg/kg/day, respectively).

### **The effects of DEHP exposure on phosphoinositide 3–kinase pathway gene expression ovaries from the F1 – F3 generations**

Our previous work showed that ancestral exposure to DEHP accelerated primordial follicle recruitment in the F3 generation, but not in the F1 and F2 generations of mice [24]. Previous studies also indicate that adult exposure to DEHP dysregulates the PI3K pathway, a critical pathway for primordial follicle recruitment [45]. Further, the RNAseq data indicate that ancestral DEHP exposure alter the PI3K pathway (Figure 1). Thus, we examined the effects



of prenatal and ancestral exposure to DEHP on the PI3K factors in the F1 – F3 generations. In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Foxl2*, *Kitl*, *Mtorc1*, *Foxo3a*, *Pdk1*, *Kit*, *Tsc1*, or *Rps6* compared to controls (Figures 4A, 4B, 4C, 4D, 4F, 4G, 4H, and 4J). However, prenatal exposure to 20 µg/kg/day of DEHP increased the expression of *Pten* compared to controls (Figure 4E, n = 3 – 5 ovaries/treatment group, p 0.05). In the F2 generation, exposure to DEHP did not affect the expression of *Foxl2*, *Kitl*, *Pdk1*, *Kit*, *Tsc1*, or *Rps6* compared to controls (Figures 4A, 4B, 4F, 4G, 4H, and 4J), but DEHP exposure increased the expression of *Mtorc1* at 20 µg/kg/day and 750 mg/kg/day and decreased *Mtorc1* expression at 200 µg/kg/day, decreased the expression of *Foxo3a* in the 20 µg/kg/day group and increased *Foxo3a* expression in the 750 mg/kg/day group, and decreased the expression of *Pten* in the 20 µg/kg/day group compared to controls (Figures 4C, 4D, and 4E, n = 3 ovaries/treatment group, p 0.05). In the F3 generation, ancestral exposure to DEHP did not affect the expression of *Foxl2*, *Kitl*, *Mtorc1*, *Foxo3a*, *Kit*, or *Tsc1* compared to controls (Figures 4A, 4B, 4C, 4D, 4G, and 4H). Ancestral exposure to DEHP decreased the expression of *Pten* in all treatment groups, decreased *Pdk1* expression in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups, and decreased *Rps6* expression in the 20 µg/kg/day group compared to controls (Figures 4E, 4F, and 4J, n = 3 – 6 ovaries/treatment group, p 0.05).

### The effects of DEHP exposure on cell cycle regulator gene expression in ovaries from the F1 – F3 generations

Our previous work showed the prenatal exposure to DEHP dysregulated folliculogenesis at PND 21 in all three generations of mice [24]. Folliculogenesis is regulated by many factors, but the cell cycle regulators are heavily involved in cell proliferation and follicle growth [49, 72]. The RNAseq data suggested that ancestral exposure to DEHP affected regulators of growth (Figure 1). Therefore, we measured the mRNA expression levels of cyclins, cyclin dependent kinases, and cyclin dependent kinase inhibitors. In the F1 generation, prenatal exposure to DEHP increased the expression of *Ccna2* in the 500 mg/kg/day group, decreased the expression of *Ccnd2* in the 750 mg/kg/day group, decreased the expression of *Cdkn1a* in the 750 mg/kg/day group, increased the expression of *Cdkn1c* in the 200 µg/kg/day group, and increased the expression of *Cdkn2a* expression in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups compared to controls (Figure 5A, 5C, 5F, 5G, and 5H n = 3 – 6 ovaries/treatment group, p 0.05, but p = 0.101 for *Ccnd2* in 20 µg/kg/day, p = 0.086 for *Cdkn1c* in 200 µg/kg/day, and p = 0.086 for *Cdkn2a* for 200 µg/kg/day). Prenatal exposure to DEHP did not affect the expression of *Ccnb1*, *Ccne1*, or *Cdk4* compared to controls (Figures 5B, 5E, and 5D). In the F2 generation, exposure to DEHP did not affect the expression of *Ccna2*, *Ccnd2*, *Ccne1*, *Cdk4*, *Cdkn1a*, *Cdkn1c*, or *Cdkn2a* compared to controls (Figures 5A, 5C, 5D, 5E, 5F, 5G, and 5H). However, exposure to DEHP at 500 mg/kg/day decreased the expression of *Ccnb1* compared to controls, but it was borderline statistically significant (Figure 5B, n = 3 ovaries/treatment group, p = 0.057). In the F3 generation, ancestral exposure to DEHP decreased the expression of *Ccna2* in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups, decreased *Ccnb1* expression in the 750 mg/kg/day group, decreased *Ccnd2* expression in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups, decreased *Cdk4* expression in the 750 mg/kg/day group, and decreased the expression of *Cdkn2a* in the 20 µg/kg/day and 200 µg/kg/day groups compared to

controls (Figures 5A, 5B, 5C, 5E, and 5H n = 3 – 6 ovaries/treatment group, p = 0.05, but p = 0.084 and 0.060 for *Ccnd2* in 200 µg/kg/day and 750 mg/kg/day, respectively). Further, in the F3 generation, ancestral exposure to DEHP did not affect the expression of *Ccne1*, *Dckn1a*, or *Cdkn1c* compared to controls (Figures 5D, 5F, and 5G).

### **The effects of DEHP exposure on apoptosis and oxidative stress pathway gene expression in ovaries from the F1 – F3 generations**

Our previous study showed the prenatal exposure to DEHP decreased the percentage of atretic follicles in the F1 generation [24]. The B-cell lymphomas/leukemia-2 (Bcl-2) family includes inhibitors and promoters of apoptosis in the ovary [47]. The balance of promoters and inhibitors of apoptosis is critical for the healthy development and maintenance follicular cells. The Bcl-2 family has been shown to directly regulate apoptosis in the ovary [47, 73]. In addition, oxidative stress is an imbalance of pro-oxidant molecules and anti-oxidant defenses and the balance of these factors are critical for adequate growth and development of follicles [48]. Thus, we examined the effects of prenatal and ancestral exposure to DEHP on the expression of the Bcl-2 family and oxidative stress factors in the ovary of the F1 – F3 generations.

In the F1 generation, prenatal exposure to DEHP did not affect the expression of *Bcl2*, *Bax*, *Bad*, *Casp3*, *Casp8*, *Catalase*, *Gpx*, or *Gsr* compared to controls (Figures S1A, S1B, S1C, S1E, S1F, S1H, S1J, and S1K). In contrast, prenatal exposure to DEHP at 750 mg/kg/day decreased the ratio of *Bax/Bcl2* and DEHP at 500 mg/kg/day increased the expression of *Bok* compared to controls, but it was borderline statistically significant (Figures S1D and S1G, n = 3 – 5 ovaries/treatment group, p = 0.05 and p = 0.076 for *Bok*). In the F2 generation, exposure to DEHP did not affect the expression of *Bcl2*, *Bax*, *Bax/Bcl2* ratio, *Bok*, *Catalase*, or *Gsr* compared to controls (Figures S1A, S1B, S1D, S1E, S1H, and S1K). However, exposure to 20 µg/kg/day of DEHP decreased the expression of *Bad*, *Casp3*, and *Casp8*, and exposure to 500 mg/kg/day of DEHP increased the expression of *Gpx* compared to controls (Figures S1C, S1E, S1F, and S1J, n = 3 ovaries/treatment group, p = 0.05). In the F3 generation, ancestral exposure to DEHP decreased the expression of *Bcl2* in all treatment groups, increased the expression of *Bax/Bcl2* ratio in the 20µg/kg/day, 200 µg/kg/day, and 500 mg/kg/day groups, decreased the expression of *Casp3* in the 20 µg/kg/day, 200 µg/kg/day, and 500 mg/kg/day groups, decreased the expression of *Casp8* in all treatment groups, decreased the expression of *Bok* in the 20 µg/kg/day group, decreased the expression of *Gpx* in the 750 mg/kg/day group, and decreased the expression of *Gsr* in the 20 µg/kg/day, 200 µg/kg/day, and 750 mg/kg/day groups compared to controls (Figures S1A, S1D, S1E, S1F, S1G, S1J, and S1K, n = 3 – 6 ovaries/treatment group, p < 0.05, but p = 0.071 for *Casp8* in 750 mg/kg/day, p = 0.088 for *Bok* in 20 µg/kg/day, and p = 0.067 for *Gsr* in 200 µg/kg/day).

### **The effects of DEHP exposure on DNA methyltransferases and ten-eleven translocation enzyme gene expression in ovaries from the F1 – F3 generations**

Although previous studies demonstrate that DEHP exposure causes transgenerational inheritance of ovarian dysfunction [24, 74, 75], the DNA methylation mediators underlying these changes have not been well studied. Therefore, we examined the expression levels *Dnmt* and *Tet* in the F1 – F3 generations. In the F1 generation, prenatal exposure to DEHP

did not affect the expression of *Dnmt3a*, *Dnmt3b*, *Tet1*, *Tet2*, and *Tet3* compared to controls (Figures 6B, 6C, 6D, 6E, and 6F). However, prenatal exposure to DEHP at 750 mg/kg/day increased the expression of *Dnmt1* compared to controls, but it was borderline statistically significant (Figure 6A, n = 3 – 5 ovaries/treatment group, p = 0.068). In the F2 generation, exposure to DEHP did not affect the expression of *Dnmt1*, *Dnmt3a*, or *Dnmt3b* compared to controls (Figures 6A, 6B, and 6C). In contrast, DEHP exposure decreased the expression of *Tet1* in all groups, decreased the expression of *Tet2* in the 20 µg/kg/day group, and decreased the expression of *Tet3* in the 500 mg/kg/day group compared to control, but it was borderline statistically significant (Figures 6D, 6E, and 6F, n = 3 ovaries/treatment group, p = 0.05, but p = 0.085 for *Tet3* in 500 mg/kg/day). In the F3 generation, ancestral exposure to DEHP decreased the expression of *Dnmt1* in all groups compared to control, but some of the decreases were borderline statistically significant (Figure 6A, n = 3 – 6 ovaries/treatment group, p = 0.05, but p = 0.097 for 200 µg/kg/day, p = 0.095 for 500 mg/kg/day, and p = 0.059 for 750 mg/kg/day). Further, ancestral exposure to DEHP decreased the expression of *Dnmt3a* in the 750 mg/kg/day group, decreased the expression of *Dnmt3b* in all groups, decreased *Tet2* expression in the 200 µg/kg/day and 750 mg/kg/day groups, and decreased *Tet3* expression in the 200 µg/kg/day and 750 mg/kg/day groups compared to controls (Figures 6B, 6C, 6E, and 6F, n = 3 – 6 ovaries/treatment group, p = 0.05, but p = 0.064 for *Tet2* in 750 mg/kg/day and p = 0.063 for *Tet3* in 200 µg/kg/day). In the F3 generation, ancestral exposure to DEHP did not affect the expression of *Tet1* compared to controls (Figure 6D).

### **The effects of DEHP exposure on DNA methylation percentage in whole ovaries from the F1 – F3 generations**

Previous studies determined that prenatal exposure to DEHP causes both multigenerational and transgenerational inheritance in ovarian dysfunction [23–25]. Transgenerational inheritance is thought to be mediated by epigenetic mechanisms, and DNA methylation is a commonly studied epigenetic mechanism. Therefore, the current study measured the percentage of 5-mC in the whole ovary in the F1 – F3 generations. In the F1 generation, prenatal exposure to 20 µg/kg/day of DEHP increased the percentage of 5-mC compared to controls and in the F3 generation, ancestral exposure to 500 mg/kg/day and 750 mg/kg/day of DEHP decreased the percentage of 5-mC in the whole ovary compared to controls (Figure 7, n = 3 – 7 ovaries/treatment group, p = 0.05).

### **Discussion**

Our previous study showed that prenatal and ancestral exposure to DEHP disrupted sex steroid hormone levels in the F1 and F2 generations, disrupted ovarian follicle counts in the F1 – F3 generations, and altered select reproductive outcomes in the F1 – F3 generations [23, 24]. Our current study provides additional information on the multigenerational and transgenerational effects of DEHP exposure on the ovary. We show that prenatal exposure to DEHP disrupts the expression of the sex steroid hormone synthesis pathway, factors in the PI3K pathway, steroid hormone receptors, DNA demethylation processes, and DNA methylation in the F1 and the F2 generations of the ovary. Further, we show that ancestral exposure to DEHP disrupts the expression of estrogen metabolism, the PI3K pathway, cell

cycle regulators, apoptosis and oxidative stress factors, estrogen receptor beta, DNA methylation and demethylation factors, and DNA methylation in the F3 ovary. This study provides potential mechanisms and pathways explaining how both prenatal and ancestral exposure to DEHP disrupt ovarian functions in the F1–F3 generations of mice.

In this study, mice were orally exposed daily to DEHP starting at embryonic day 10.5 and ending at birth. During this exposure window, primordial germ cells in the fetus (F2 generation) migrate to the genital ridge and undergo mitosis, meiosis, methylation, and demethylation processes [76–79]. We anticipate that this window of exposure targets epigenetic inheritance and likely causes transgenerational inheritance in the F3 generation [79]. This window of exposure is critical because the F1, F2, and F3 generations receive exposure at different developmental windows. The F1 generation is exposed as a developing pup, and therefore, the hypothalamus-pituitary-gonadal axis is directly exposed to DEHP. The F2 generation is exposed as the developing germ cells in the gonad. The F3 generation is not directly exposed to DEHP and thus, it is the first generation to experience transgenerational inheritance. Each generation is exposed to DEHP at different developmental time points; therefore, we anticipated and observed that the effects of DEHP on ovarian functions would be different in each generation.

Endocrine disruptors, such as DEHP, cause nonmonotonic dose responses such as sigmoid, U-shaped, or inverted-U-shaped curves [80, 81]. Although the mechanisms behind such nonmonotonic effects are not fully understood, they may be due to receptor type and abundance in specific cells or tissues [82], receptor down-regulation and desensitization [83, 84], and endocrine feedback loops [85, 86]. Therefore, it is not surprising that the majority of the results indicate that DEHP exposure did not have a linear dose response effect on gene expression in the ovary. Further, in each generation, baseline differences in control groups were present. Baseline differences in control groups between generations is a phenomenon that has been observed in numerous transgenerational studies [23–25, 75, 87]. Although it is not clear what causes baseline changes between control groups, it may be due to differences in the timing of exposure and/or age of mice [87].

Data from RNA sequencing were analyzed using DAVID. Of the 177 genes, themes and pathways were identified. Genes were selected for further qPCR analysis as a mean to verify sequencing data. Interestingly, some themes including regulation of cell growth, insulin-like growth factor binding, and PI3K-Akt signaling pathway were identified. Previous work in the Flaws' laboratory has determined that exposures to toxicants such as DEHP and bisphenol A disrupts genes associated with cell growth, insulin-like growth factor, and the PI3K-Akt signaling pathway in the ovary [45, 88, 89]. Thus, numerous signaling pathways were analyzed.

Our results indicate that DEHP exposure affected the expression of *Pparg*, *Ar*, and *Esr2* in the F1 generation. These findings are in contrast to a previous study that showed that DEHP exposure repressed *Esr1* gene expression via PPAR $\alpha$ -dependent pathways in a multigenerational manner [90]. We were surprised that ancestral DEHP exposure did not change *Ppar* expression in the F3 generation in our study because it is thought that the endocrine disrupting effects of DEHP are mediated through PPAR action [90–93]. However,

most notably in the F3 generation, ancestral DEHP exposure caused significant decreases in *Esr2* in our study. Given that *Esr2* is important for regulatory effects of estrogens on granulosa proliferation, it is possible that DEHP-induced decreases in *Esr2* may lead to many of the observed gene expression changes in the F3 generation [94–96].

Our current results show that DEHP exposure disrupted expression of steroidogenic enzymes in the F2 and F3 generations, but not in the F1 generation. According to our previous study, prenatal DEHP exposure did not affect serum 17 $\beta$ -estradiol levels in the F1 generation at PND 21 [24]. Therefore, it is not surprising that prenatal DEHP exposure did not significantly affect the expression of sex steroid hormone synthesis enzymes in the F1 generation. However, in the F2 generation, our previous study showed that prenatal exposure to DEHP borderline decreased serum 17 $\beta$ -estradiol levels and increased serum progesterone levels in the 20  $\mu$ g/kg/day treatment group compared to control [24]. In our current study, DEHP exposure at 20  $\mu$ g/kg/day decreased the expression of *Star*, *Hsd3b1*, *Hsd17b1*, and *Cyp19a1* in the F2 generation and this decrease in enzymes correlates well with the previously observed serum sex steroid hormone levels. Likely, the increase in serum progesterone level is due to the decrease of enzymes necessary to further biotransform it to androgens and estrogens. Further, the decrease of *Hsd17b1* and *Cyp19a1* likely leads to a decrease in serum 17 $\beta$ -estradiol levels because these two enzymes biotransform estrone and testosterone into 17 $\beta$ -estradiol, respectfully [97]. Finally, in the F3 generation, we observed a decrease in *Hsd17b1* expression with DEHP exposure, which is important for biotransforming androstenedione into testosterone and estrone into 17 $\beta$ -estradiol [97]. However, in the previous study, we did not observe a serum sex steroid hormone change in response to DEHP exposure [24].

Interestingly, our steroidogenic enzyme gene expression results are in contrast with another study that exposed mice to DEHP during an early developmental time period. Specifically, Pocar *et al.* perinatally dosed mice throughout gestation and lactation with low doses of DEHP and observed decreases in steroidogenic enzyme expression in the F1 generation and not the F2 or F3 generations [75]. The reason why our results and Pocar *et al.* vary may be due to the many differences between the experiments. In our study, we dosed animals only during the second half of gestation and our doses included 20  $\mu$ g/kg/day – 750 mg/kg/day, whereas Pocar *et al.* dosed animals throughout lactation and gestation with 50  $\mu$ g/kg/day and 5 mg/kg/day [75]. Additional studies that examine the direct effects of DEHP exposure on steroidogenesis show mixed results, demonstrating that the route, timing, and dose of DEHP greatly contribute to DEHP-induced effects [23, 24, 51, 98].

Results from our study indicate that prenatal and ancestral DEHP exposure disrupted the PI3K pathway in the F1, F2, and F3 generations. In the F1 generation, prenatal DEHP exposure increased *Pten* expression in the PI3K pathway, but it did not affect other factors in the pathway. *Pten* is a gene that encodes the PI3K negative regulator; if deleted, the entire pool of primordial follicles activates [42]. Therefore, an increase in *Pten* expression suggests primordial follicle quiescence. Interestingly, in our previous study, we observed data supporting primordial follicle quiescence in the F1 generation [24]. Our previous data also showed that at PND 21, ancestral DEHP exposure decreased primordial follicle numbers [24]. A correlated decrease in *Pten*, *Pdk1*, and *Rps6* expression correlates well with

decreased primordial follicle numbers because decreased *Pten* expression suggests that primordial follicles activate and continue folliculogenesis [42]. Further, decreased *Pdk1* and *Rps6* expression decreases primordial follicle survival [99]. Therefore, decreased expression of these factors supports previously published follicle count numbers in the F3 generation [24] and provides a potential mechanism for follicle count disruption observed at PND 21.

Prenatal and ancestral exposure to DEHP significantly decreased gene expression of cell cycle regulators in the F1 and F3 generations. In somatic cells, the cell cycle is made of four phases, with different cyclin-dependent kinases and cyclins to control the cell cycle [100]. Cyclin A2 is expressed during the S phase and is critical for DNA replication [49]. Cyclin B1 is necessary for cell cycle progression through mitosis [49]. Cyclin D2 binding to CDK4 is a critical positive regulator for ovarian granulosa cell proliferation in response to follicle-stimulating hormone (FSH) [72, 101]. In the F1 generation, it is likely that prenatal DEHP exposure inhibits cell cycle progression by decreasing promoters of the cell cycle such as *Ccnd2* and increasing the expression of inhibitors of cell cycle such as *Cdkn2a* and *Cdkn1c* [102, 103]. Although the expression of *Cdkn1a*, another cell cycle inhibitor, was decreased in the F1 generation, it is likely that it was not biologically significant enough to counteract the expression of the other inhibitors. Further, the effects of prenatal DEHP exposure on cell cycle regulators is somewhat similar to studies that directly exposed the ovary to DEHP. Direct exposure to DEHP *in vitro* increased the expression of *Ccna2*, *Ccnb1*, *Ccnd2*, *Cdk4*, and *Ccne1* after 72 hours of exposure [50]. In the F3 generation, DEHP-induced decreases in cyclins and cyclin-dependent kinase suggest that ancestral DEHP exposure reduces cell cycle progression and proliferation, likely causes cells to undergo cell cycle arrest.

DEHP exposure disrupted DNA methylation in the ovaries in each generation. In the F1 generation, prenatal DEHP exposure increased *Dnmt* expression and increased the percentage of 5-mC. Increased *Dnmt1* expression supports increased 5-mC in the ovary because *Dnmt1* is important for translating DNMT1, the maintenance DNA methyltransferase. Prenatal DEHP exposure increased *Dnmt* expression in testicular Leydig cells, increased methylation in promoter regions of steroidogenic transcription factors, and decreased gene expression of steroidogenic enzymes in the F1 generation of rats [104]. In the F2 generation, DEHP exposure did not affect DNA methylation percentage, but it significantly decreased *Tet* expression. These data suggest that prenatal DEHP exposure modulates DNA demethylation pathways, but not to a large enough degree for the decrease to significantly affect 5-mC percentage in the ovary. In the F3 generation, ancestral DEHP exposure decreased *Dnmt*, *Tet*, and 5-mC expression. It is likely that ancestral DEHP exposure decreases *Dnmt*, subsequently decreasing 5-mC in the ovary, and that DEHP-induced changes in 5-mC percentage in the F1 and F3 generations may contribute to some of the DEHP-induced changes in expression [40, 105–107]. However, additional studies are necessary to determine if global 5-mC translates to altered methylation in promoters of transcripts for critical ovarian functions.

In summary, our observations indicate that prenatal and ancestral DEHP exposure causes differential gene expression in multiple pathways necessary for healthy ovarian function in the F1, F2, and F3 generations. Further, our study suggests that DEHP-induced DNA methylation may underly some of the transgenerational effects of DEHP. However, our

studies focus on the expression of mRNA and not protein levels in the ovary and therefore, caution should be taken when interpreting these results and comparing them to studies with protein expression. Therefore, future studies should measure protein expression of the genes and examine the specific epigenetic mechanisms underlying the transgenerational effects of DEHP exposure. Finally, the metabolic and pharmacokinetic differences between mice and humans are not clear for DEHP [108, 109], therefore, the unknowns in species differences in metabolism may contribute to uncertainty in the species specific effects of DEHP.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Highlights

- Prenatal DEHP exposure disrupts gene expression in the F1, F2, and F3 ovary.
- Prenatal DEHP exposure disrupts *Dnmt* in the F1 and F3 generations.
- Prenatal DEHP exposure decreases *Tet* in F2 and F3 generation.
- DEHP increases DNA methylation in the F1, but decreases it in the F3 generation.

**A**

Functional Annotation  
Clustering  
Background: Mus musculus  
177 DAVID IDs  
Classification Stringency: Medium

12 Clusters

Annotation Cluster	Enrichment Score	Count	P_Value Benjamini
Annotation Cluster 1	2.62		
GOTERM_MF_DIRECT	insulin-like growth factor binding	4	5.00E-04 1.30E-01
GOTERM_BP_DIRECT	regulation of cell growth	5	8.20E-04 5.80E-01
GOTERM_MF_DIRECT	growth factor binding	3	3.30E-02 9.50E-01
Annotation Cluster 2	2.11		
GOTERM_CC_DIRECT	proteinaceous extracellular matrix	11	6.30E-05 5.80E-03
GOTERM_BP_DIRECT	extracellular matrix organization	6	1.40E-03 5.30E-01
GOTERM_CC_DIRECT	basement membrane	3	1.40E-01 8.90E-01
GOTERM_BP_DIRECT	cell adhesion	6	2.80E-01 1.00E+00
Annotation Cluster 3	1.56		
GOTERM_MF_DIRECT	serine-type endopeptidase inhibitor activity	5	1.00E-02 7.50E-01
GOTERM_BP_DIRECT	response to cytokine	4	2.10E-02 8.50E-01
GOTERM_BP_DIRECT	acute-phase response	3	2.70E-02 8.70E-01
GOTERM_MF_DIRECT	peptidase inhibitor activity	4	5.20E-02 9.70E-01
GOTERM_BP_DIRECT	negative regulation of peptidase activity	4	5.30E-02 9.20E-01
Annotation Cluster 4	1.2		
GOTERM_BP_DIRECT	blood vessel development	4	1.50E-02 8.60E-01
GOTERM_BP_DIRECT	positive regulation of gene expression	7	7.00E-02 9.30E-01
GOTERM_BP_DIRECT	kidney development	3	2.40E-01 9.90E-01
Annotation Cluster 5	1.06		
GOTERM_BP_DIRECT	negative regulation of apoptotic process	11	8.20E-03 9.40E-01
GOTERM_BP_DIRECT	male gonad development	5	9.00E-03 9.10E-01
GOTERM_BP_DIRECT	heart looping	4	1.00E-02 8.90E-01
GOTERM_BP_DIRECT	positive regulation of transcription, DNA-templated	7	2.40E-01 9.90E-01
GOTERM_BP_DIRECT	negative regulation of transcription, DNA-templated	7	2.40E-01 9.90E-01
GOTERM_MF_DIRECT	transcription factor activity, sequence-specific DNA binding	4	9.40E-01 1.00E+00
GOTERM_BP_DIRECT	positive regulation of transcription from RNA polymerase II promoter	4	9.80E-01 1.00E+00

**B**

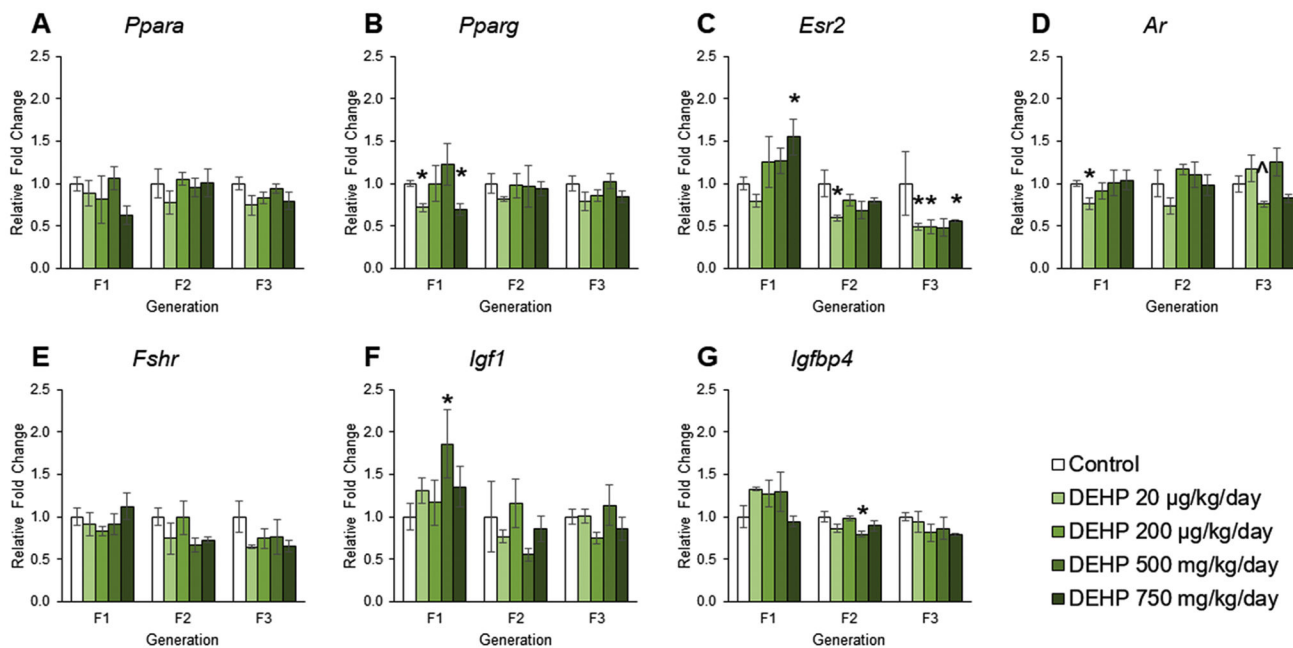
Functional Annotation  
Clustering  
Background: Mus musculus  
177 DAVID IDs  
Classification Stringency: Medium

12 Clusters

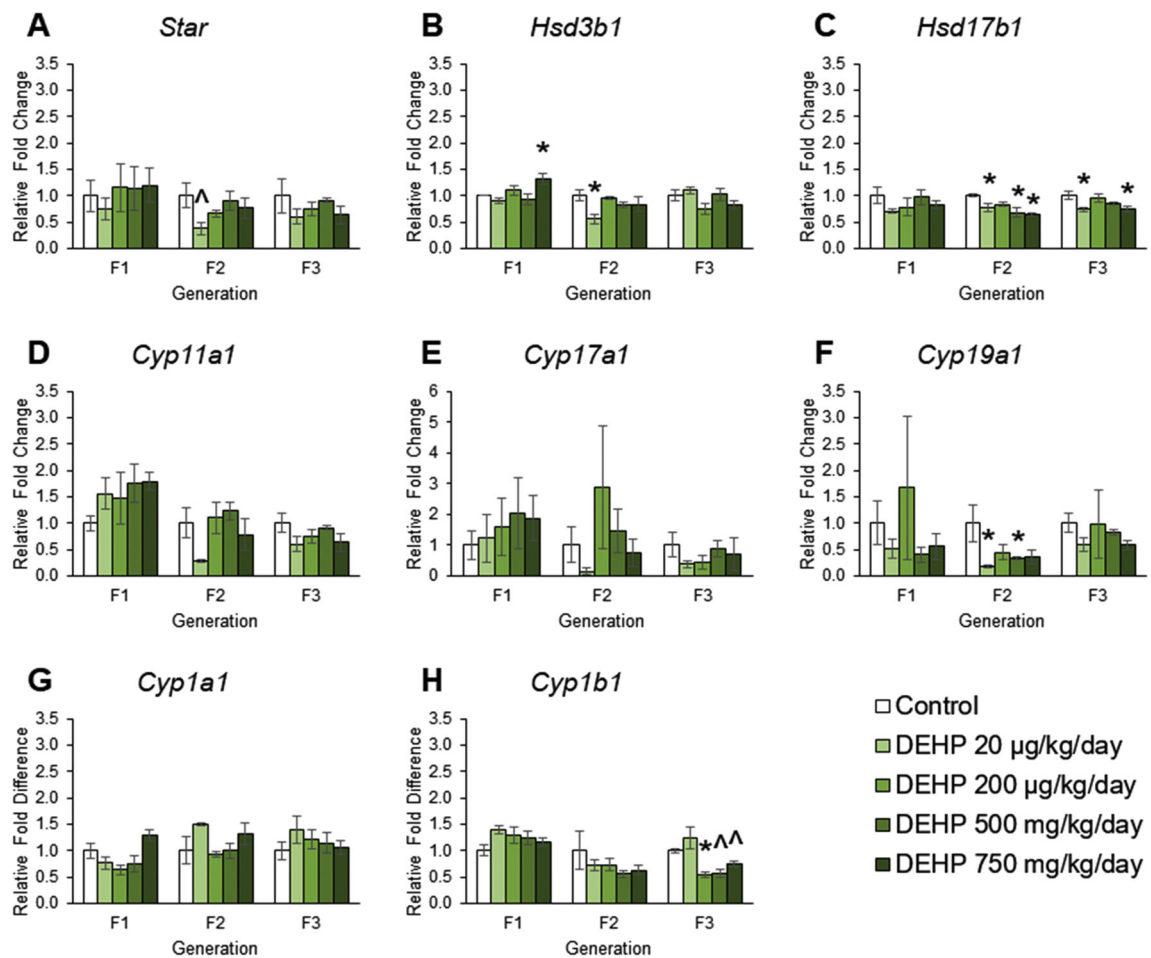
Annotation Cluster	Enrichment Score	Count	P_Value Benjamini
Annotation Cluster 1	1.18		
KEGG_PATHWAY	ECM-receptor interaction	4	2.50E-02 9.30E-01
KEGG_PATHWAY	Amoebiasis	4	5.10E-02 9.40E-01
KEGG_PATHWAY	Focal adhesion	5	6.00E-02 8.90E-01
KEGG_PATHWAY	PI2K-Akt signaling pathway	5	2.40E-01 9.70E-01

**Figure 1.**

Data obtained from the RNA sequencing were functionally analyzed using The Database of Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics version 6.8. A total of 177 genes were entered into DAVID (false discovery rate < 0.62 and  $p < 0.007$ ) for functional annotation analysis. “Gene\_Ontology” results yield 5 annotation clusters (A) and “Pathways” results yield 1 annotation cluster (B) with a significant enrichment score = 1.



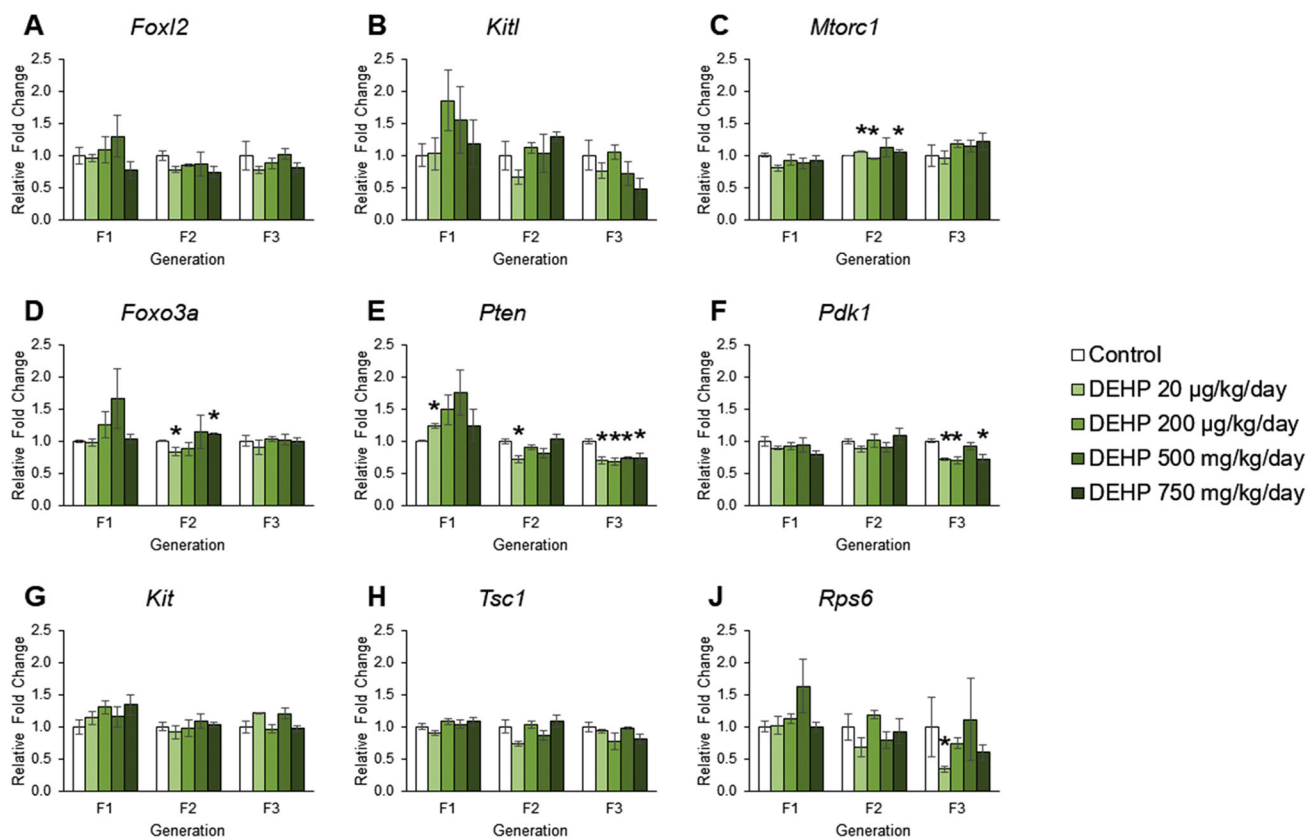
**Figure 2.** The effects of prenatal and ancestral DEHP exposure on steroid hormone receptors and insulin-like growth factors in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin*, and the relative fold change is normalized to 1 for control. Graphs represent mean  $\pm$  SEM from 3 – 6 ovaries per treatment group. \*  $p < 0.05$  (significant difference compared to control with generation),  $0.05 < p < 0.10$ .



**Figure 3.**

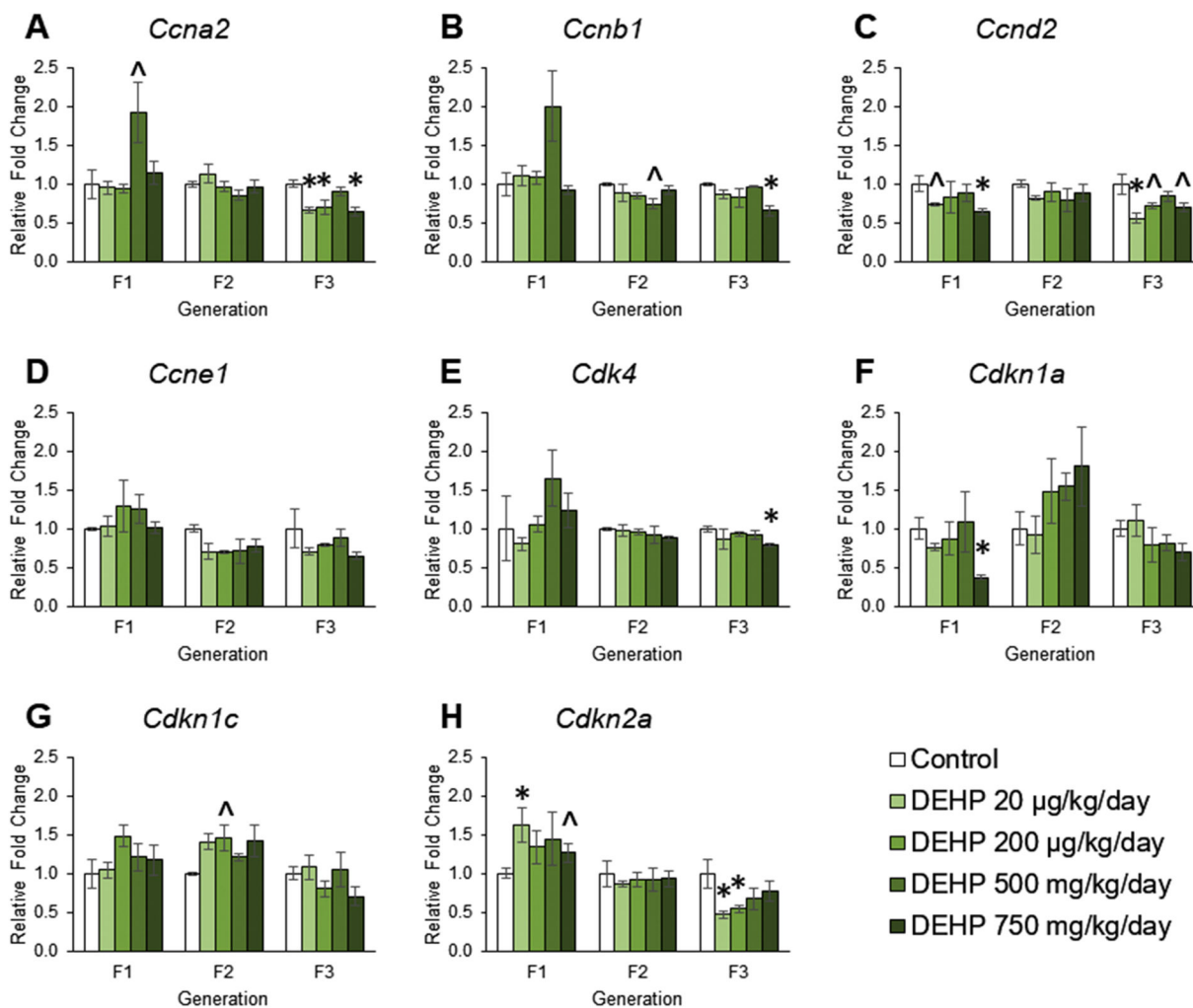
The effects of prenatal and ancestral DEHP exposure on steroidogenesis and estradiol metabolism in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin*, and the relative fold change is normalized to 1 for control. Graphs represent mean  $\pm$  SEM from 3 – 6 ovaries per treatment group. \*  $p < 0.05$  (significant difference compared to control with generation),  $0.05 < p < 0.10$ .





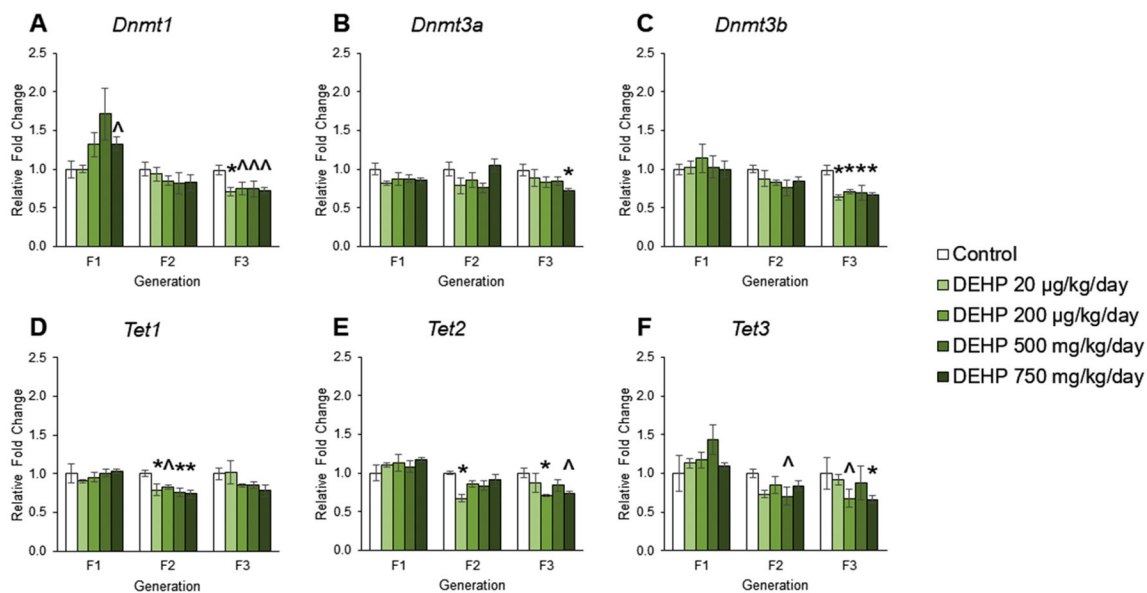
**Figure 4.**

The effects of prenatal and ancestral DEHP exposure on the phosphoinositide 3-kinase pathway in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin*, and the relative fold change is normalized to 1 for control. Graphs represent mean  $\pm$  SEM from 3 – 6 ovaries per treatment group. \*  $p < 0.05$  (significant difference compared to control with generation),  $0.05 < p < 0.10$ .



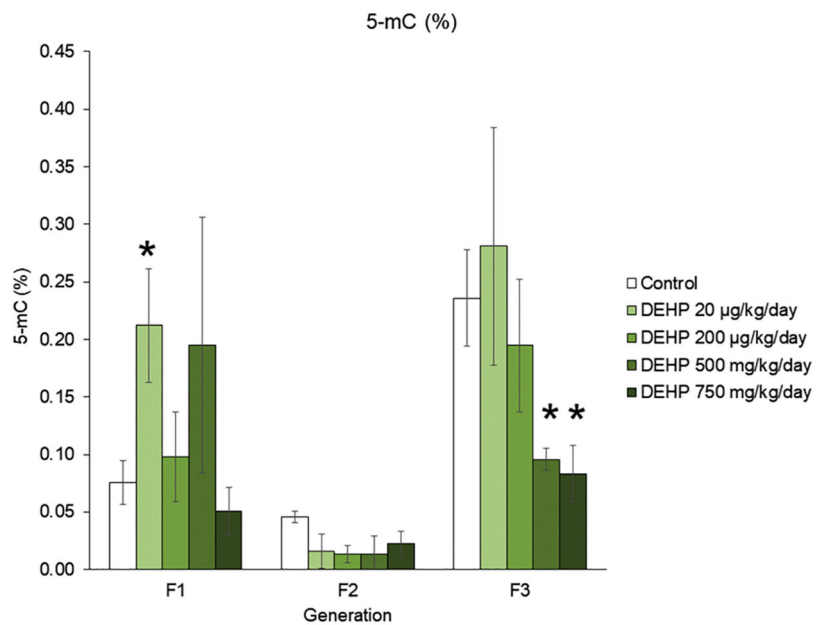
**Figure 5.**

The effects of prenatal and ancestral DEHP exposure on cell cycle regulators in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin*, and the relative fold change is normalized to 1 for control. Graphs represent mean  $\pm$  SEM from 3 – 6 ovaries per treatment group. \*  $p < 0.05$  (significant difference compared to control with generation),  $0.05 < \Lambda < 0.10$ .



**Figure 6.**

The effects of prenatal and ancestral DEHP exposure on DNA methyltransferases and ten-eleven translocation enzymes in PND 21 ovaries in the F1 – F3 generations. All gene expression is relative to the housekeeping gene, *Bactin*, and the relative fold change is normalized to 1 for control. Graphs represent mean  $\pm$  SEM from 3 – 6 ovaries per treatment group. \* p < 0.05 (significant difference compared to control with generation), 0.05 <  $\lambda$  p < 0.10.



**Figure 7.** The effects of prenatal and ancestral DEHP exposure on the percentage of 5-mC in whole ovaries at PND 21 in the F1 – F3 generations. Graphs represent mean  $\pm$  SEM from 3 – 7 ovaries per treatment group. \*  $p < 0.05$  (significant difference compared to control with generation).