Published in final edited form as:

Reprod Toxicol. 2015 June ; 53: 23–32. doi:10.1016/j.reprotox.2015.02.013.

Prenatal exposure to di-(2-ethylhexyl) phthalate (DEHP) affects reproductive outcomes in female mice

Sarah Niermann, Saniya Rattan, Emily Brehm, and Jodi A. Flaws¹

Department of Comparative Biosciences, University of Illinois at Urbana-Champaign, 2001 S. Lincoln Ave., Urbana, Illinois, 61802, USA

Sarah Niermann: steinma1@illinois.edu; Saniya Rattan: rattan2@illinois.edu; Emily Brehm: esbrehm@illinois.edu

Abstract

This study tested the hypothesis that prenatal DEHP exposure affects female reproduction. To test this hypothesis, pregnant female CD-1 mice were orally dosed daily with tocopherol-stripped corn oil (vehicle control) or DEHP (20µg/kg/day-750mg/kg/day) from gestation day 11-birth. Pups were counted, weighed, and sexed at birth, ovaries were subjected to evaluations of follicle numbers on postnatal days (PNDs) 8 and 21, and fertility was evaluated at 3-9 months. The results indicate that prenatal DEHP exposure increased male-to-female ratio compared to controls. Prenatal DEHP exposure also increased preantral follicle numbers at PND 21 compared to controls. Further, 22.2% of the 20 µg/kg/day treated animals took longer than 5 days to get pregnant at 3 months and 28.6% of the 750 mg/kg/day treated animals lost some of their pups at 6 months. Thus, prenatal DEHP exposure alters F1 sex ratio, increases preantral follicle numbers, and causes some breeding abnormalities

Introduction

Phthalates are a class of chemicals found in wide range of consumer, medical, and building materials and products [1-4]. Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizing agent commonly found in polyvinyl chloride (PVC) products such as shower curtain liners, car upholstery, baby toys, wire sheathing, synthetic wall coverings, medical tubing, and IV and blood transfusion bags [1-4]. DEHP is not covalently bonded to the polymers of these products and, as a result, readily leaches from products into the environment [5-7].

Because of the quantity and variety of products containing DEHP, exposure can occur via oral ingestion, inhalation, dermal contact, and intravenously through medical procedures [8-12]. DEHP and its metabolites have been identified in a number of human tissues and fluids, including urine, serum, cord blood, breast milk, amniotic fluid, and ovarian follicular

© 2015 Published by Elsevier Inc.

¹Corresponding author: Dr. Jodi A. Flaws, Department of Comparative Biosciences, University of Illinois at Urbana-Champaign, 2001 S. Lincoln Ave., Room 3223, Urbana, Illinois, 61802, Phone: (217) 333-7933 Fax: (217) 244-1652, jflaws@illinois.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

fluid [13-14]. The presence of DEHP in follicular fluid indicates its ability to reach the ovary, and its presence in amniotic fluid and cord blood implies it is able to reach the fetus. The Agency for Toxic Substances and Disease Registry estimates that humans are exposed daily to DEHP between 3-30 µg/kg/day [15].

Human exposure to DEHP is of concern because it is a known endocrine disrupting chemical and a reproductive toxicant [16-19]. Epidemiological studies have associated *in utero* exposure to DEHP with reduced anogenital distance (AGD) and testosterone levels in males [16], and experiments in rodent models have corroborated these findings [17]. Additionally, epidemiological studies have reported correlations between prenatal exposure to DEHP and endometriosis, precocious puberty, early pregnancy loss, preterm birth, and low birth weight, though these findings have been inconsistent [18-19].

The mechanism by which phthalate exposure is associated with adverse reproductive outcomes in humans is unknown. Recent studies, however, have shown that DEHP, through its bioactive metabolite mono-(2-ethylhexyl) phthalate (MEHP), may alter peroxisome proliferator-activated receptor (PPAR) downstream activity in a number of tissues in animal models. PPARs exist in three different isoforms: α , β , and γ [20]. The receptors are located in adipose tissue [21], liver cells [22], uterine endometrial cells [23], and throughout the ovary [24]. Changes in activity of PPARs in these tissues have been linked with reproductive problems in animal models [19] and thus, may play a role in the association between phthalate exposure and reproductive outcomes in humans.

Although few studies have examined phthalates in humans, the effects caused by exposure to DEHP have been extensively researched in rodent models using a variety of exposure windows and concentrations [17, 19, 25-28]. However, most previous studies have focused on males [17, 25-27] and, thus, not much is known about the effects of prenatal exposure to DEHP on female reproduction. Further, few studies have been conducted using a wide range of doses of DEHP during a critical period of ovarian development and few studies have focused on the impact of prenatal DEHP exposure on ovarian development and future fertility of the female offspring.

Thus, this study was designed to test the hypothesis that prenatal DEHP exposure adversely affects ovarian development and reproductive outcomes in the female offspring of mice.

Materials and Methods

Chemicals

DEHP (99% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of DEHP were prepared by diluting the chemical in tocopherol-stripped corn oil (MP Biomedicals, Solon, OH) to achieve the desired concentrations.

Animals

Adult cycling female outbred CD-1 mice (8 weeks old) or CD-1 males (8 weeks) were housed at 25°C in conventional polystyrene cages on 12L:12D cycles. The mice were provided Teklad Rodent Diet 8604 (Harlan) and high purity water (reverse osmosis filtered)

ad libitum. Male CD-1 mice (8 weeks old) were housed with females during breeding (2 females and 1 male per cage). All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Breeding Protocol

At 8 weeks of age, 50 female mice (F0) were mated with control males of the same age. Mating was confirmed by the presence of vaginal plug. The day the vaginal plug was detected was defined as gestation day (GD) 1. Once the vaginal plug was observed, females were individually housed.

Dosing Regimen

Previous studies have shown that DEHP appears to have a non-monotonic dose-response curve [25] so we used a wide range of doses in these studies. Each pregnant female was randomly assigned to one of 6 treatment groups: control, 20 µg/kg/day, 200 µg/kg/day, 200 mg/kg/day, 500 mg/kg/day, or 750 mg/kg/day. The lowest dose, 20 µg/kg/day, was selected because it is the EPA reference dose for humans [15]. The 200 µg/kg/day, 200 mg/kg/day, and 750 mg/kg/day doses were selected because exposure to them during adulthood has been shown to affect primordial follicle recruitment in adult mice [28]. The 500 mg/kg/day dose was selected because it has been shown to have adverse effects in male mice, such as abnormal seminiferous tubule formation, decreased sperm count and motility, impaired stem cell function, decreased nipple retention, decreased anogenital distance, and delayed pubertal onset following prenatal exposure [17, 26].

From GD 11 until birth of the pups, pregnant dams were orally dosed once a day with tocopherol-stripped corn oil (vehicle control) or the assigned dose of DEHP by placing a pipette tip containing the dosing solution into the mouth. This dosing regimen was selected to mimic oral exposure in humans. GD 11 until birth was selected as the exposure window because this is a critical time period of ovarian development [29].

Tissue collection (F1)

On post-natal day (PND) 0, the numbers of pups, numbers of males and females, and numbers of dead pups (if any) were counted. The average birth weights of the pups were determined by measuring weights of all live pups in the litter combined, and dividing the total weight by the total number of live pups in the litter. On PND 1 – 60 (5-16 females per treatment group), tissues (ovaries, uteri, and livers) were collected and weighed as described below. Further, one ovary per pup was fixed in Dietrich fixative for histological evaluation as described below in the section called *Histological Evaluation of Ovaries*.

Body and Organ Weight Analysis (F1)

At each of the tissue collections, the pups were weighed prior to euthanasia and organs were aseptically removed, cleaned of interstitial tissue, and weighed. Organ weights were recorded as whole organ weights in grams (g). Average body weights for each treatment group at PND 1, 8, 21, 60, three months, and six months were recorded and compared to control weights at each age.

Analysis of Estrous Cyclicity (F1)

After weaning at PND 21, at least four F1 females from each treatment group were selected for examination of puberty onset and estrous cyclicity. These mice were weighed and checked for vaginal opening daily. Once vaginal opening occurred, estrous cyclicity was evaluated by examining vaginal smears daily for 30 days. The first observed estrus and the percentage of time that F1 females remained in each stage of the estrous cycle were calculated and reported. A mouse was considered to be in proestrus if a majority of cells in the vaginal smear were nucleated epithelial cells. A mouse was considered to be in estrus if an overwhelming majority of the cells observed in the vaginal smear were cornified epithelial cells. A mouse was considered to be in metestrus if the cells present were a mixture of cornified epithelial cells and a large number of leukocytes. A mouse was considered to be in diestrus when all cells present were leukocytes, or mostly leukocytes with a small number of nucleated epithelial cells. Metestrus and diestrus were combined during analysis because these two stages are very similar in cytology and hormone profile.

Histological evaluation of ovaries (F1)

The ovaries from F1 females on PND 8 and 21 were collected and fixed in Dietrich fixative. The fixed tissues were embedded in paraffin, serially sectioned (8 μ m), mounted on glass slides, and stained with Weigert's hematoxylin and picric acid–methyl blue. Every 10th section of the ovary was used to count the numbers of primordial follicles, primary follicles, preantral follicles, and antral follicles. All sections were evaluated without knowledge of the treatment group. Follicles were classified based on the following criteria: primordial follicles contained an oocyte surrounded by a single layer of squamous granulosa cells, primary follicles contained an oocyte surrounded by a single layer of cuboidal granulosa cells, preantral follicles contained an oocyte surrounded by at least two layers of cuboidal granulosa cells and theca cell layers, and antral follicles contained an oocyte surrounded by multiple layers of cuboidal granulosa cells with a fluid-filled antral space and theca cell layers [29-31]. All primordial and primary follicles with oocytes, regardless of nuclear material in the oocytes, were counted, whereas only preantral and antral follicles with a clear nucleus in the oocyte were counted to avoid redundant counting of follicles large enough to span multiple sections [30-31].

Analysis of Fertility (F1)

At least three F1 females from each treatment group (all F1 females were from different F0 dams) were selected to examine fertility at the ages of three, six, and nine months. By placing the mice in breeding studies at the selected time points, we were able to test breeding capacity over time and in turn, determine whether the mice prenatally exposed to DEHP experience premature reproductive senescence (evidenced by losing the ability to become pregnant and produce normal sizes of healthy litters earlier than controls). In each fertility test, the selected F1 females were first subjected to daily vaginal smears for two weeks to evaluate estrous cyclicity and then they were mated with fertility confirmed untreated males for two weeks or until a vaginal plug was observed. During mating, all the females were weighed twice weekly to monitor pregnancy weight gain. Once a vaginal plug was observed, females were weighed and individually caged. Litter sizes, average pup

weights, sex ratios, and percent of dead pups were recorded on PND 0. After the two-week mating period, females who did not become pregnant were singly housed.

Various breeding anomalies and complications were also recorded (i.e. number of dead pups, no pups born and no weight gain following observation of a vaginal plug, lack of pregnancy after more than five days with a male). The cut-off of 5 days was selected because the average estrous cycle for mice is about 4-5 days, and mice are proficient breeders so most should become pregnant during the first cycle [32].

Statistical analyses

All data were analyzed using the F0 dam as the experimental unit (n). Data from multiple F1 females from the same litter were averaged and combined as $n = 1$. For all analyses except those for data on breeding complications, data were expressed as means $\pm$ standard error of the means (SEM) from at least three different F0 litters. One-way ANOVA followed by Dunnett (2-sided) *post hoc* comparisons was used to make multiple comparisons between treatment groups. If data had unequal variances according to Levene, Brown-Forsythe, or Welch tests, Games-Howell and Dunnett T3 *post hoc* comparisons were performed to compare treatment groups. If the data did not have a normal distribution according to Shapiro-Wilk analysis, the data from each treatment group were compared using Mann-Whitney and Kruskal-Wallis tests. For data on breeding complications, comparisons were made using Chi-square and Fisher Exact tests. Statistical significance was assigned at $p = 0.05$ for all comparisons.

Results

Effect of In Utero DEHP Exposure on F0 Fertility Outcomes

To determine if there were any effects on F0 fertility outcomes from exposure to DEHP from GD 11 until birth, F1 litters were analyzed for number of live pups and birth weight (Figure 1), and sex ratio (Figure 2). DEHP exposure did not significantly affect the number of live pups or birth weight when compared to controls ($n = 5-18$ dams/treatment group). However, DEHP exposure (200 $\mu\text{g/kg/day}$) significantly increased the ratio of males to females compared to controls ($n = 5-15$ dams/treatment group; $p = 0.05$).

Effect of In Utero DEHP Exposure on F1 Body and Organ Weights

Prenatal exposure to 200 mg/kg/day DEHP decreased body weight at PND 8 and prenatal exposure to 750 mg/kg/day DEHP decreased body weight at PND 60 (Table 1, $n = 3-15$ dams/treatment group except $n = 2$ dams for 20 $\mu\text{g/kg/day}$ DEHP at PND 60 only; $p = 0.05$). Prenatal DEHP exposure, however, did not significantly affect F1 body weight at any other doses or time-points. At PND 8, DEHP at 200 mg/kg/day significantly reduced liver weight compared to controls (Table 1, $n = 4-15$ dams/treatment group; $p = 0.05$). DEHP exposure did not significantly affect liver weight at any other doses or time-points. At PND 8 and 60, DEHP did not significantly affect uterine weight, but at PND 21, DEHP exposure significantly increased uterine weight compared to controls at the 200 $\mu\text{g/kg/day}$ dose compared to controls ($n = 3-16$ dams/treatment group except $n = 2$ dams for 20 $\mu\text{g/kg/day}$ DEHP at PND 60 only; $p = 0.05$). At PND 21, DEHP exposure significantly decreased

ovarian weight in mice exposed to 20 µg/kg/day compared to controls (Table 1, n = 3-14 dams/treatment group; p = 0.05). In contrast, DEHP did not significantly affect ovarian weight compared to controls at any other doses or time-points.

Effect of In Utero DEHP Exposure on F1 Pubertal Outcomes

The age at vaginal opening (Figure 3A), weight at vaginal opening (Figure 3B), and age at first estrus (Figure 3C) were recorded as indicators of pubertal onset. DEHP exposure did not significantly affect any of these markers of puberty compared to controls (n = 4-15 dams/treatment group). Further, estrous cyclicity for 30 days after vaginal opening as well as average cycle length (Figures 4A and 4B, respectively) were not significantly different for any DEHP treatment group compared to control animals (n = 4-14 dams/treatment group).

Effect of In Utero DEHP Exposure on F1 Ovarian Morphology

On PND 8, DEHP exposure did not significantly affect numbers of primordial, primary, preantral, or antral follicles (Figure 5A, n = 4-14 dams/treatment group). In contrast, on PND 21, prenatal exposure to DEHP at the 200 µg/kg/day and 500 mg/kg/day doses significantly increased the number of preantral follicles compared to controls (Figure 5B; n = 3-10 dams/treatment group; p = 0.05).

Effect of In Utero DEHP Exposure on Fertility Outcomes of F1 offspring

Prenatal DEHP exposure did not significantly affect the days between mating and presence of vaginal plug (Figure 6A), number of F2 pups per litter (Figure 6B), or F2 birth weight (Figure 6C) compared to controls (n = 4-15 dams/treatment group). Unlike what we observed in the F1 litters (Figure 2), DEHP exposure did not significantly affect male to female ratio in the F2 litters (Figure 6D, n = 4-13 dams/treatment group).

Interestingly, prenatal DEHP exposure increased the percentage of F1 dams with some breeding complications at 3 months compared to controls (Table 2). For example, none of the control dams took longer than 5 days to get pregnant, whereas 22.2% of the dams exposed to 20 µg/kg/day DEHP took longer than 5 days to get pregnant (n=9-19 dams per treatment group; p<0.05).

Prenatal DEHP exposure also increased the percentage of F1 dams with some breeding complications at 6 months (Table 3). For example, none of the control dams lost pups, but 25% of the dams exposed to 20 µg/kg/day (n=4-10; p <0.06) and 28.6% of the dams exposed to 750 mg/kg/day lost some pups (n=4-10 dams/treatment group; p = 0.05). Interestingly, 25% of the control dams took longer than 5 days to become pregnant, but all of the dams treated with 200 µg/kg/day DEHP became pregnant in less than 5 days. In contrast, prenatal DEHP did not significantly affect the ability of the dams to produce a litter at 6 months (Table 3). It also did not significantly affect the ability of dams to get pregnant, the time to pregnancy, and the loss of pups at 9 months (Table 4).

Discussion

The purpose of this study was to evaluate the effects of prenatal exposure to the plasticizer DEHP on female reproductive function. Previous studies examining the effects of prenatal exposure to DEHP on reproductive outcomes have been limited and primarily focused on males [16-17, 25-27]. These studies have observed effects of DEHP exposure such as reduced anogenital distance, reduced testes weights, reduced testosterone production [17, 25], delayed pubertal onset, and abnormal seminiferous tubule formation [26]. A few studies have examined the effects of exposure to DEHP on female reproduction [28, 33-35]. These studies have shown that DEHP exposure causes a decline in pregnancy rates and irregular estrous cyclicities at very high doses [33], decreased overall fertility [34], atresia of tertiary follicles in adult rats, and delayed onset of puberty [35]. These previous studies on females, however, did not examine the effects of prenatal exposure to DEHP on female reproduction in the F1 offspring. Thus, limited information was available on the effects of only *in utero* DEHP exposure and female reproductive outcomes.

Our data indicate that prenatal exposure to DEHP (200 µg/kg/day) increases the ratio of males to females in F1 litters. Since dosing began after determination of chromosomal sex, the mechanism by which DEHP affects sex ratio is unclear. It is possible that DEHP was toxic to recently implanted female embryos, causing more females to die *in utero*, and resulting in fewer live female pups at birth than male pups in each litter. One would expect that if this were the case, there would be an effect of DEHP exposure on overall litter size as well, and this was not observed in the current study. It is possible that prenatal exposure to 200 µg/kg/day DEHP resulted in more pups per litter *in utero*, but the litter sizes normalized due to some post-implantation losses from DEHP exposure. Thus, future experiments should examine the uteri from F0 dams for implantation sites throughout pregnancy to determine whether DEHP causes implantation loss. Interestingly, DEHP exposure did not alter the sex ratio in F2 litters, suggesting that the effect of DEHP on sex ratio in F1 litters is not multigenerational. Our data differ from a study by Xi *et al.*, which examined the effects of prenatal DEHP exposure on sex ratio in mice [36]. Specifically, Xi *et al.* noted a dose-dependent decrease in male-to-female ratio in F1 litters, which the investigators attributed to a possible interaction between DEHP and SRY activity [36]. The reasons for the differences between our results and those of Xi *et al.* [36] could be that we used a smaller dosing window, which included only prenatal exposure, whereas Xi *et al.* [36] used a larger dosing window, which included perinatal and lactational exposure and spanned the time of sex determination. Additionally, Xi *et al.* [36] used a mixture of DEHP and bisphenol-A (BPA), which could also explain the different effect on sex ratio observed in their study compared to our study. Our data, however, are similar to other studies showing that endocrine disrupting chemicals alter sex ratio [37-38]. For example, Mocarelli *et al.* have shown that prenatal exposure to dioxin is associated with a lowered male/female sex ratio in humans, which persists for years after exposure [37-38].

Our results also indicate that DEHP exposure (200 mg/kg/day and 750 mg/kg/day) significantly decreased pup weights at PND 8 and PND 60. A difference in body weight caused by factors that occur prenatally is possibly due to a change in gene expression during development. Thus, expression of genes involved in metabolism and body fat deposition,

such as PPARs, should be analyzed as a possible explanation for the observed DEHP-induced decrease in body weight. It is also possible that the type of chemical exposure, dose, and timing of exposure play a role in determining which specific chemicals affect body weight. A study by Hao *et al.* observed an increase in body weight in adult life following perinatal exposure to DEHP, and proposed that this occurred through PPAR-mediated pathways [39]. Our data showed a contrasting effect, but this could be explained by the difference in timing of exposure, as well as doses used in our study versus those used by Hao *et al.* [39].

Our data also showed a significant reduction in mean liver weight at PND 8 for animals in the 200 mg/kg/day group, and a trend towards reduction in liver weight for the two higher doses (500 and 750 mg/kg/day) compared to controls. This is interesting because exposure to DEHP is usually associated with an increase in liver weight due to activation of PPARs in the liver, resulting in liver cell hypertrophy and hyperplasia [22]. Like our unexpected results in overall body weight, the reduced liver weight could be a result of interaction with the PPARs differently than expected because of the selected doses and specific exposure window. This possibility is supported by David *et al.*, who showed a possible threshold dose for PPAR-induced liver effects in mice [40].

Our study shows that prenatal exposure to 200 µg/kg/day DEHP significantly increases uterine weight at PND 21 compared to controls. Uterine weight increases in response to estrogen or estrogen-mimicking compounds [41-42], and this may occur through cross-talk between estrogen and PPARs [23]. An increase in uterine weight could be explained by an increase in PPAR activation related to an increase in estrogen synthesis by preantral and antral follicles present in the ovaries of mice at these two time-points. The increase in follicle numbers observed in PND 21 ovaries from DEHP exposed animals versus controls supports this explanation, but future studies should compare estrogen levels in control and DEHP-treated animals.

Our data indicate that prenatal exposure to 20 µg/kg/day DEHP significantly decreases ovarian weight at PND 21. This decrease in ovarian weight could be explained by a significant decrease in preantral and antral follicle numbers at the same dose. However, this was not observed in our study. In contrast, prenatal exposure to 200 µg/kg/day and 500 mg/kg/day DEHP significantly increased preantral follicle numbers compared to controls. A decrease in ovarian weight, but no change or an increase in preantral follicle numbers could be explained by the follicles being smaller on average than follicles in control ovaries, with fewer granulosa cells per follicle. This possibility is supported by previous studies indicating that DEHP and its metabolite MEHP inhibit follicle growth [43-45].

The reason that prenatal exposure to DEHP increases pre-antral follicle numbers is unclear. It could be that the prenatal exposure is somehow inhibiting the natural atretic process that occurs in preantral follicles, allowing more than a normal number of preantral follicles to survive. It is also possible that prenatal exposure to DEHP accelerated growth of primordial and primary follicles to the prenatal stage, resulting in an increased number of preantral follicles. If this were the case, we would have expected to see a reduced number of primordial and primary follicles in the DEHP treatment group compared to controls, but we

did not observe this to be the case. It is possible, however, that we missed the time-point in which primordial and primary follicle numbers declined because we only examined the ovaries at PND 8 and PND 21. The decline could have occurred between PND 8 and PND 21.

A few studies have shown that exposure to DEHP prior to and during pregnancy affects overall female fertility based on whether a litter was produced following pairing with an untreated male [33-34]. However, the effects were only observed at very high doses that also caused general toxicity. Our data shows that prenatal exposure to DEHP (at doses that do not cause overt toxicity) does not significantly affect days to pregnancy, F2 birth weight, F2 pups per litter, and F2 male-to-female sex ratio in the F1 females in comparison to control animals. However, DEHP exposure resulted in a few breeding anomalies in F1 females. Approximately 22.2% of the F1 females in the 20 µg/kg/day group took more than 5 days to become pregnant and 28.6% of the females in the 750 mg/kg/day group lost some pups at 6 months. While the reasons for the pup losses are unknown, it could be that DEHP exposed dams had difficulty nursing their entire litter or that some pups were born with abnormalities that resulted in their death.

Interestingly, our data also indicate that prenatal DEHP exposure does not have clear typical dose-response effects on the female reproductive system. The dose of DEHP that alters some outcomes in one tissue does not affect outcomes in other tissues. Further, sometimes the low doses of DEHP cause a much more profound effect than the high doses of DEHP. It is likely that DEHP, like other endocrine disrupting chemicals, exhibits non-monotonic dose response curves. Specifically, previous studies have indicated that DEHP has a non-monotonic dose response curve specific to gene expression in cultured mouse antral follicles [44-45]. Further, studies in males have shown non-monotonic responses in terms of testicular and serum testosterone levels and anogenital distance and brain aromatase activity [25, 46]. The reasons for the non-monotonic responses to DEHP are unknown, but could stem from different tissues having different capacities to respond to DEHP or to metabolize DEHP to less toxic compounds. Alternatively, although we estimate that we had enough power to detect significant differences between treatment groups, it is always possible that this was not the case. Thus, future studies should further examine the effects of DEHP on female reproduction using larger sample sizes.

In conclusion, the present study provides evidence that prenatal exposure to 200 µg/kg/day DEHP affects the ratio of males to females in F1 litters, and that prenatal exposure to 200 mg/kg/day and 750 mg/kg/day DEHP reduces overall body weight at PND 8 and PND 60, though these shifts are not dramatic. The present study also shows that prenatal exposure to 200 mg/kg/day DEHP reduces liver weight at PND 8, and that prenatal exposure to 200 µg/kg/day of DEHP significantly increases uterine weight at PND 21. Further, this study shows that prenatal exposure to 20 µg/kg/day DEHP significantly reduces ovarian weight at PND 21 and that prenatal exposure to 200 µg/kg/day and 500 mg/kg/day DEHP increases the number of preantral follicles per ovary at PND 21. Finally, this study shows that prenatal DEHP exposure causes a few breeding complications in the F1 offspring at 3 and 6 months. Future studies should examine the mechanisms by which prenatal DEHP exposure causes these female reproductive outcomes.

Acknowledgments

We thank members of Dr. Flaws' Laboratory for their help with dosing and tissue collection. This work was supported by NIH P01 ES022848 and EPA RD-83459301.

References

1. US Dept of Health and Human Services, Public Health Service, National Toxicology. Program Di(2-ethylhexyl) phthalate. Report on carcinogens : carcinogen profiles. 2011; 12:156–9.
2. NTP. Report on Carcinogens. Eight. Research Triangle Park, NC: National Toxicology Program; 1998.
3. Kelley KE, et al. Identification of phthalates in medications and dietary supplement formulations in the United States and Canada. *Environ Health Perspect.* 2012; 120(3):379–84. [PubMed: 22169271]
4. Hernandez-Diaz S, et al. Medications as a potential source of exposure to phthalates in the U.S. population. *Environ Health Perspect.* 2009; 117(2):185–9. [PubMed: 19270786]
5. Berge A, et al. Meta-analysis of environmental contamination by phthalates. *Environmental Science and Pollution Research International.* 2013; 20(11):8057–76. [PubMed: 23917738]
6. Hongjun Y, et al. Distribution of phthalate esters in topsoil: a case study in the Yellow River Delta, China. *Environmental Monitoring and Assessment.* 2013; 185(10):8489–500. [PubMed: 23609921]
7. Martine B, Marie-Jeanne T, Cendrine D, Fabrice A, Marc C. Assessment of adult human exposure to phthalate esters in the urban centre of Paris (France). *Bulletin of Environmental Contamination and Toxicology.* 2013; 90(1):91–6. [PubMed: 23090363]
8. Wittassek M, et al. Assessing exposure to phthalates - the human biomonitoring approach. *Molecular Nutrition & Food Research.* 2011; 55(1):7–31.
9. Becker K, et al. DEHP metabolites in urine of children and DEHP in house dust. *International Journal of Hygiene and Environmental Health.* 2004; 207(5):409–17. [PubMed: 15575555]
10. Silva MJ, et al. Urinary levels of seven phthalate metabolites in the U.S. population from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. *Environ Health Perspect.* 2004; 112(3):331–8. [PubMed: 14998749]
11. Kato K, et al. Mono(2-ethyl-5-hydroxyhexyl) phthalate and mono-(2-ethyl-5-oxohexyl) phthalate as biomarkers for human exposure assessment to di-(2-ethylhexyl) phthalate. *Environ Health Perspect.* 2004; 112(3):327–30. [PubMed: 14998748]
12. Hogberg J, et al. Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. *Environ Health Perspect.* 2008; 116(3):334–9. [PubMed: 18335100]
13. Latini G, et al. Exposure to Di(2-ethylhexyl)phthalate in humans during pregnancy. A preliminary report. *Biol Neonate.* 2003; 83(1):22–4. [PubMed: 12566679]
14. Koch HM, Drexler H, Angerer J. An estimation of the daily intake of di(2-ethylhexyl)phthalate (DEHP) and other phthalates in the general population. *Int J Hyg Environ Health.* 2003; 206(2):77–83. [PubMed: 12708228]
15. Agency for Toxic Substances and Disease Registry (ATSDR) Toxicological profile for Di(2-ethylhexyl)phthalate (DEHP). P.H.S.A. Department of Health and Human Services; GA: U.S.: 2002. p. 11Editor
16. Swan SH, et al. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect.* 2005; 113(8):1056–61. [PubMed: 16079079]
17. Gray LE Jr, et al. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci.* 2000; 58(2):350–65. [PubMed: 11099647]
18. Cobellis L, et al. High plasma concentrations of di-(2-ethylhexyl)-phthalate in women with endometriosis. *Hum Reprod.* 2003; 18(7):1512–5. [PubMed: 12832380]
19. Kay VR, Chambers C, Foster WG. Reproductive and developmental effects of phthalate diesters in females. *Critical Reviews in Toxicology.* 2013; 43(3):200–219. [PubMed: 23405971]

20. Corton JC, et al. Mode of action framework analysis for receptor-mediated toxicity: The peroxisome proliferator-activated receptor alpha (PPARalpha) as a case study. *Crit Rev Toxicol*. 2014; 44(1):1–49. [PubMed: 24180432]
21. Desvergne B, Feige JN, Casals-Casas C. PPAR-mediated activity of phthalates: A link to the obesity epidemic? *Molecular and Cellular Endocrinology*. 2009; 304(1–2):43–48. [PubMed: 19433246]
22. Rusyn I, Peters JM, Cunningham ML. Modes of action and species-specific effects of di-(2-ethylhexyl)phthalate in the liver. *Critical Reviews in Toxicology*. 2006; 36(5):459–479. [PubMed: 16954067]
23. Gallo D, et al. Comparative effects of 17beta-estradiol and phytoestrogens in the regulation of endometrial functions in the rodent uterus. *J Endocrinol Invest*. 2008; 31(1):48–56. [PubMed: 18296905]
24. Komar CM. Peroxisome proliferator-activated receptors (PPARs) and ovarian function--implications for regulating steroidogenesis, differentiation, and tissue remodeling. *Reprod Biol Endocrinol*. 2005; 3:41. [PubMed: 16131403]
25. Do RP, et al. Non-monotonic dose effects of in utero exposure to di(2-ethylhexyl) phthalate (DEHP) on testicular and serum testosterone and anogenital distance in male mouse fetuses. *Reproductive Toxicology*. 2012; 34(4):614–621. [PubMed: 23041310]
26. Doyle TJ, et al. Transgenerational effects of di-(2-ethylhexyl) phthalate on testicular germ cell associations and spermatogonial stem cells in mice. *Biol Reprod*. 2013; 88(5):112. [PubMed: 23536373]
27. Parks LG, et al. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol Sci*. 2000; 58(2):339–49. [PubMed: 11099646]
28. Hannon PR, Peretz J, Flaws JA. Daily exposure to Di(2-ethylhexyl) phthalate alters estrous cyclicity and accelerates primordial follicle recruitment potentially via dysregulation of the phosphatidylinositol 3-kinase signaling pathway in adult mice. *Biol Reprod*. 2014; 90(6):136. [PubMed: 24804967]
29. Hirshfield, AN. Development of Follicles in the Mammalian Ovary. In: Jeon, KW.; Friedlander, M., editors. *International Review of Cytology*. Academic Press; 1991. p. 43–101.
30. Benedict JC, et al. Aryl hydrocarbon receptor regulates growth, but not atresia, of mouse preantral and antral follicles. *Biol Reprod*. 2003; 68(5):1511–7. [PubMed: 12606443]
31. Borgeest C, et al. Methoxychlor may cause ovarian follicular atresia and proliferation of the ovarian epithelium in the mouse. *Toxicol Sci*. 2002; 68(2):473–8. [PubMed: 12151644]
32. Allen E, Allen. The oestrous cycle in the mouse. *American journal of anatomy*. 1922; 30(3):297–371.
33. Takai R, et al. Collaborative work on evaluation of ovarian toxicity. 10) Two- or four-week repeated dose studies and fertility study of di-(2-ethylhexyl) phthalate (DEHP) in female rats. *J Toxicol Sci*. 2009; 34 Suppl 1:Sp111–9. [PubMed: 19265277]
34. Lamb, JCT, et al. Reproductive effects of four phthalic acid esters in the mouse. *Toxicol Appl Pharmacol*. 1987; 88(2):255–69. [PubMed: 3564043]
35. Grande SW, et al. A dose–response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): Reproductive effects on adult female offspring rats. *Toxicology*. 2007; 229(1–2):114–122. [PubMed: 17098345]
36. Xi W, et al. Effects of perinatal exposure to bisphenol A and di(2-ethylhexyl)-phthalate on gonadal development of male mice. *Environmental Science and Pollution Research*. 2012; 19(7):2515–2527. [PubMed: 22828881]
37. Mocarelli P, et al. Change in sex ratio with exposure to dioxin. *Lancet*. 1996; 10:409. [PubMed: 8709758]
38. Mocarelli P, et al. Paternal concentrations of dioxin and sex ratio of offspring. *Lancet*. 2000; 355:1858–63. [PubMed: 10866441]
39. Hao C, et al. Perinatal exposure to diethyl-hexyl-phthalate induces obesity in mice. *Front Biosci (Elite Ed)*. 2013; 5:725–33. [PubMed: 23277027]

40. David RM, et al. Chronic Toxicity of Di(2-ethylhexyl)phthalate in Mice. *Toxicological Sciences*. 2000; 58(2):377–385. [PubMed: 11099649]
41. Papaconstantinou AD, et al. Increases in mouse uterine heat shock protein levels are a sensitive and specific response to uterotrophic agents. *Environ Health Perspect*. 2002; 110(12):1207–12. [PubMed: 12460799]
42. Anderson JN, et al. Estrogen-induced uterine responses and growth: relationship to receptor estrogen binding by uterine nuclei. *Endocrinology*. 1975; 96(1):160–167. [PubMed: 162876]
43. Gupta RK, et al. Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate inhibit growth and reduce estradiol levels of antral follicles in vitro. *Toxicol Appl Pharmacol*. 2010; 242(2):224–30. [PubMed: 19874833]
44. Wang W, et al. Di (2-ethylhexyl) phthalate inhibits growth of ovarian antral follicles through an oxidative stress pathway. *Toxicology and Applied Pharmacology*. 2012; 258(2):288–295. [PubMed: 22155089]
45. Wang W, et al. Mono-(2-ethylhexyl) phthalate induces oxidative stress and inhibits growth of mouse ovarian antral follicles. *Biol Reprod*. 2012; 87(6):152. [PubMed: 23077170]
46. Andrade AJ, et al. A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose-response and low dose effects on rat brain aromatase activity. *Toxicology*. 2006; 227(3):185–92. [PubMed: 16949715]

Highlights

- Prenatal exposure to DEHP increases male-to-female sex ratio in the F1 generation
- Prenatal exposure to DEHP increases preantral follicle numbers on postnatal day 21
- Prenatal exposure to DEHP (20 µg/kg/day) increases time to pregnancy
- Prenatal exposure to DEHP (750 mg/kg/day) causes loss of some pups

Figure 1. Effect of DEHP on F1 Litter Outcomes

At birth, the number of live pups in each litter (A) was counted and compared in each treatment group. Graph represents means $\pm$ SEM (n=5-17 dams/treatment group). Average weight of live pups (B) was also calculated as described in methods and compared in each treatment group. Graph represents means $\pm$ SEM (n=5-18 dams/treatment group).

Figure 2. Effect of DEHP on F1 Litter Sex Ratio

At birth, male to female ratio was calculated as described in methods and compared in each treatment group. Graph represents means $\pm$ SEM (n=5-15 dams/treatment group). Asterisk (*) represents significant difference from vehicle control ($p \leq 0.05$).

Figure 3. Effect of DEHP on Pubertal Outcomes

Age at vaginal opening (A), weight at vaginal opening (B), and age at first estrus (C) were determined and analyzed as described in methods. The means were compared in each treatment group. Graph represents means $\pm$ SEM (n=4-15 dams/treatment group).

Figure 4. Effect of DEHP on Estrous Cyclicity

After vaginal opening, estrous cyclicity was monitored for 30 days and the average cycle length for this time frame was determined as described in the methods. The percent of time out of 30 days spent in each stage was calculated (A), as well as the average cycle length (B). The means were compared in each treatment group. Graph represents means $\pm$ SEM (n=4-14 dams/treatment group).

Figure 5. Effect of DEHP on Ovarian Morphology

Ovaries collected from mice on PND 8 (A) and PND 21 (B) were subjected to histological evaluation of follicle numbers based on criteria described in the methods. The graphs represent the mean total number of each follicle type present $\pm$ SEM (n=3-14 dams/treatment group). Asterisks (*) represent significant difference from vehicle control (p 0.05).

Figure 6. Effect of DEHP on F1 Fertility

At three months of age, F1 females were paired with proven breeder males to evaluate the fertility of the females. Fertility was based on time to pregnancy as determined by the number of days between pairing with a male and observing a plug (A), litter size (B), average pup birth weight (C), and sex ratio (D). These data were compared in each treatment group. Graphs represent means $\pm$ SEM (n=4-15 dams/treatment group).

Table 1

F1 Body and Tissue Weights

		Age of Pups (F1)				
		PND1	PND8	PND21	PND60	3 months
Body Weight (g)						
Control		1.80 ± 0.04	6.30 ± 0.22	15.48 ± 0.35	30.63 ± 0.66	33.49 ± 1.09
DEHP 20 µg/kg/day		1.85 ± 0.09	6.20 ± 0.32	15.39 ± 0.98	30.51 ± 1.15	40.19 ± 1.56
DEHP 200 µg/kg/day		1.93 ± 0.10	6.19 ± 0.29	15.61 ± 1.26	31.28 ± 2.96	43.50 ± 2.21
DEHP 200 mg/kg/day		1.80 ± 0.06	5.58 ± 0.27*	14.83 ± 0.82	30.30 ± 0.58	40.27 ± 2.17
DEHP 500 mg/kg/day		1.87 ± 0.05	5.71 ± 0.18	13.86 ± 0.57	29.61 ± 2.19	-
DEHP 750 mg/kg/day		1.77 ± 0.05	5.74 ± 0.23	14.63 ± 0.55	28.14 ± 0.19*	38.05 ± 1.58
Liver Weight (g)						
Control		0.071 ± 0.003	0.195 ± 0.008	0.815 ± 0.026	1.694 ± 0.056	-
DEHP 20 µg/kg/day		0.071 ± 0.003	0.198 ± 0.014	0.761 ± 0.060	1.704 ± 0.110	-
DEHP 200 µg/kg/day		0.075 ± 0.004	0.192 ± 0.013	0.822 ± 0.050	1.536 ± 0.274	-
DEHP 200 mg/kg/day		0.071 ± 0.004	0.156 ± 0.008*	0.746 ± 0.039	1.611 ± 0.031	-
DEHP 500 mg/kg/day		0.084 ± 0.002	0.163 ± 0.008	0.757 ± 0.021	1.749 ± 0.122	-
DEHP 750 mg/kg/day		0.076 ± 0.003	0.168 ± 0.012	0.853 ± 0.051	1.536 ± 0.076	-
Uteri Weight (g)						
Control		-	0.0037 ± 0.0002	0.0206 ± 0.0018	0.0974 ± 0.0087	-
DEHP 20 µg/kg/day		-	0.0030 ± 0.0003	0.0153 ± 0.0025	0.0966 ± 0.0076	-
DEHP 200 µg/kg/day		-	0.0033 ± 0.0002	0.0303 ± 0.0037*	0.1466 ± 0.0445	-
DEHP 200 mg/kg/day		-	0.0037 ± 0.0002	0.0212 ± 0.0060	0.1297 ± 0.0138	-
DEHP 500 mg/kg/day		-	0.0038 ± 0.0002	0.0187 ± 0.0024	0.0828 ± 0.0072	-
DEHP 750 mg/kg/day		-	0.0031 ± 0.0002	0.0233 ± 0.0025	0.1929 ± 0.0215	-
Ovary Weight (g)						
Control		-	-	0.0062 ± 0.0004	0.0153 ± 0.0012	-
DEHP 20 µg/kg/day		-	-	0.0039 ± 0.0003*	0.0165 ± 0.0006	-
DEHP 200 µg/kg/day		-	-	0.0056 ± 0.0004	0.0126 ± 0.0016	-
DEHP 200 mg/kg/day		-	-	0.0070 ± 0.0007	0.0145 ± 0.0010	-

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Age of Pups (F1)					
9 months	3 months	PND60	PND21	PND8	PND1
-	-	0.0141 ± 0.0008	0.0061 ± 0.0010	-	-
-	-	0.0145 ± 0.0004	0.0051 ± 0.0002	-	-
DEHP 500 mg/kg/day					
DEHP 750 mg/kg/day					
* 0.05 d					

Table 2
F1 Breeding Complications Following DEHP Exposure (3 months)

Treatment	no litter produced	>5 days to get pregnant	Lost some pups (2 or less)	Lost all pups
control	5.3 (n=19)	0.0 (n=19)	11.1 (n=18)	11.1 (n=18)
20 µg/kg/day	11.1 (n=9)	22.2 (n=9)*	0.0 (n=8)	0.0 (n=8)
200 µg/kg/day	18.2 (n=11)	9.1 (n=11)	11.1 (n=9)	11.1 (n=9)
200 mg/kg/day	11.1 (n=9)	11.1 (n=9)	12.5 (n=8)	0.0 (n=8)
500 mg/kg/day	25.0 (n=4)	0.0 (n=3)	0.0 (n=3)	0.0 (n=3)
750 mg/kg/day	10.0 (n=10)	0.0 (n=10)	11.1 (n=9)	22.2 (n=9)

*
p < 0.05

Table 3
F1 Breeding Complications Following DEHP Exposure (6 months)

Treatment	No litter produced	>5 days to get pregnant	Lost some pups (2 or less)	Lost all pups
control	16.7 (n=12)	25.0 (n=12)	0.0 (n=10)	10.0 (n=10)
20 µg/kg/day	11.1 (n=9)	25.0 (n=8)	25.0 (n=8) [^]	25.0 (n=8)
200 µg/kg/day	27.3 (n=11)	0.0 (n=11) [*]	0.0 (n=8)	12.5 (n=8)
500 mg/kg/day	0.0 (n=4)	0.0 (n=4)	0.0 (n=4)	25.0 (n=4)
750 mg/kg/day	30.0 (n=10)	20.0 (n=10)	28.6 (n=7) [*]	0.0 (n=7)

^{*}
p < 0.05

[^]
p < 0.06

Table 4
F1 Breeding Complications Following DEHP Exposure (9 months)

Treatment	No litter produced	>5 days to get pregnant	Lost some pups (2 or less)	Lost all pups
control	45.5 (n=11)	12.5 (n=8)	50.0 (n=5)	33.3 (n=6)
20 µg/kg/day	55.6 (n=9)	25.0 (n=8)	25.0 (n=4)	25.0 (n=4)
200 µg/kg/day	27.3 (n=11)	22.2 (n=9)	37.5 (n=7)	37.5 (n=8)
500 mg/kg/day	16.7 (n=6)	20.0 (n=5)	20.0 (n=5)	20.0 (n=5)
750 mg/kg/day	22.2 (n=9)	0.0 (n=10)	42.9 (n=6)	42.9 (n=7)