Independent and combined effects of Bisphenol A and Diethylhexyl Phthalate on gestational outcomes and offspring development in Sprague-Dawley rats

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HIGHLIGHTS

- Prenatal exposure to combinations of BPA and DEHP increased abortion rates in rats. Exposure to individual chemicals did not induce abortions.
- Relative heart weight increased in male offspring prenatally exposed to the combination of BPA and high dose-DEHP.
- Prenatal exposure to BPA or HD-DEHP reduced the relative thymus weight in males; while exposure to HD-DEHP increased the apoptotic index in both sexes.

ARTICLE INFO

ABSTRACT

Bisphenol A (BPA) and Diethylhexyl Phthalate (DEHP) are well-studied endocrine disrupting chemicals (EDCs), however, the effects of mixtures of these EDCs are not. To assess the consequences of prenatal exposure to a mixture of these EDCs, dams were orally administered either saline (control), BPA (5 μg/kg BW/day), high dose DEHP (HD-D; 7.5 mg/kg BW/day), or a combination of BPA with HD-D in experiment 1; saline, BPA (5 μg/kg BW/day), low-dose DEHP (LD-D; 5 μg/kg BW/day) or a combination of BPA with LD-D in experiment 2. Gestational weights, number of abortions, litter size and weights, number of live births and stillbirths were recorded. Morphometric measures were obtained at birth and body weight, food and water intake were monitored weekly from postnatal weeks 3—12. Offspring were sacrificed at 16—24 weeks of age and organ weights were measured. The abortion rate of dams exposed to HD-D and the mixtures, BPA + LD-D and BPA + HD-D were higher at 9, 14 and 27% respectively. Prenatal exposure to BPA or HD-D significantly decreased relative thymus weights in male but not female offspring. Apoptotic cells were detected in thymus sections of both male and female offspring prenatally exposed to DEHP. Relative heart weights increased in BPA + HD-D exposed male offspring compared to the other groups. The results indicate that a mixture of BPA and DEHP, produced a pronounced effect on pregnancy outcomes. Male offspring appear to be more susceptible to the programming effects of these EDCs or their mixture suggesting a need to reconsider the possible additive, antagonistic or synergistic effects of EDC mixtures.

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1. Introduction

Increased prevalence of human health risks has been correlated with environmental contaminants, including the plastic-derived compounds: Bisphenol-A (BPA) and Di-2-ethylhexyl phthalate (DEHP). BPA is a weak estrogen while DEHP has anti-androgenic properties. They interfere with the endocrine system by activating a broad range of signaling pathways (Gould et al., 1998; Grun and Blumberg, 2006; Latini et al., 2004). BPA and DEHP are ubiquitously present in the environment. They are extensively used in a variety of consumer products including plastic bottles, dental fillings and sealants, eyeglass lenses, sports equipment, household electronics and food packaging (Hashimoto and Nakamura, 2000; Yoshida et al., 2001). Exposure to these chemicals occurs through ingestion of contaminated food or water, inhalation of contaminated air and dust, and dermal absorption (Hernandez-Diaz et al., 2009; Erythropel et al., 2014; Diamanti-Kandarakis et al., 2009; Morgan et al., 2018). BPA and DEHP can leach into the environment from plastic products as they degrade over time and increase human exposures (Erythropel et al., 2014; Yoshida et al., 2001; Yamamoto and Yasuhara, 1999).

The prevalence of BPA and DEHP in the environment has raised concerns, in particular because they have been detected in the serum (Schonfelder et al., 2002), amniotic fluid (Jensen et al., 2012; Calafat et al., 2006; Edlow et al., 2012; Ikezuki et al., 2002), placental tissue (Schonfelder et al., 2002; Jimenez-Diaz et al., 2010) and fetal serum (Talsness et al., 2009; Troisi et al., 2014; Schonfelder et al., 2002; Ikezuki et al., 2002). This implies that BPA and DEHP can cross the placenta during the critical periods of development and program the fetus for possible adult-onset disorders (Liu et al., 2013).

Animal and human studies have demonstrated some of the permanent and irreversible *in utero* effects of low-dose BPA and DEHP on different organ systems (Colborn, vom Saal, and Soto, 1993) (vom Saal et al., 1998; Hatch et al., 2010; Naville et al., 2013). Early life exposure to DEHP is associated with a decrease in pregnancy weight gain (Gray et al., 2000), fetal growth (Ferguson et al., 2016), pup weight at birth (Gray et al., 2000) and anogenital distance in male infants (Swan et al., 2005; Gray et al., 2000) (Parks et al., 2000). *In utero* exposure to BPA is shown to produce sex-specific reductions in offspring birth weight (Huo et al., 2015), increase in gestational length (Veiga-Lopez et al., 2015), and shortened anogenital distance in male offspring (Sun et al., 2018). Besides, exposure to environmentally relevant low doses BPA and DEHP have been linked to a number of chronic diseases including metabolic, reproductive, cardiovascular, and neurological disorders (De Coster and van Larebeke, 2012; Rochester, 2013; Richter et al., 2007; Vandenberg et al., 2009; Rubin and Soto, 2009; Rubin, 2011; Lyche et al., 2009). However, the effects of prenatal exposures to a mixture of low doses of these chemicals have not been studied.

This study aimed to investigate the effects of prenatal exposure to a mixture of low dose BPA and DEHP on Sprague Dawley rat development, as well as the sex differences in their biological effects. We used a single low dose of BPA (5 μg/kg BW) in combination with either a low dose (5 μg/kg BW) of DEHP to match the BPA dose or a higher dose (7.5 mg/kg BW) based on earlier studies (Rattan et al., 2018; Tinwell et al., 2002). Exposures were limited to the prenatal period (gestational days 6–21). We hypothesized that prenatal exposure to EDCs will "program" the offspring and affect their postnatal morphometric parameters, body weights, and organ weights in a sex-specific manner with the EDC mixture showing a more pronounced effect than individual EDCs.

2. Materials and methods

2.1. Chemicals

BPA (Lot # MKBH2096V) and DEHP (Lot # BCBR8079V) were purchased from Sigma Aldrich (St. Louis, MO). Stock solutions of BPA and DEHP were made in DMSO and diluted with saline prior to oral administration. Pregnant dams were administered BPA (5 μg/kg BW), low dose (LD) DEHP (5 μg/kg BW), high dose (HD) DEHP (7.5 mg/kg BW) or a combination of BPA with either dose of DEHP (As summarized in Fig. 1). Animals were dosed from gestational days (GD) 6–21. The dosing volume was adjusted based on individual maternal weight changes. The dose of BPA used in this study (5 μg/kg BW) is well below the established reproductive/developmental NOAEL values of 50 mg/kg BW and systemic NOAEL value of 5 mg/kg BW (Tyl et al. 2002, 2008). It is also in the range of doses that simulate human exposures: 0.4–5 μg/kg BW (Lakind and Naiman, 2008). The high dose of DEHP (7.5 mg/kg BW) was selected based on a published study in mice (Rattan et al., 2018) and is slightly higher than the established NOAEL values for this chemical (4.8 mg/kg BW) (Blystone et al., 2010) but lower than the NOAEL for occupational exposures (28.9 g/kg) (Kim, 2016). However, the low dose of DEHP used (5 μg/kg BW) is much lower than the established NOAEL values and lies within the range of human DEHP exposures of 0.5–25 μg/kg BW/day (Wittassek et al., 2011).

2.2. Animals

Harlan Sprague-Dawley (SD) rats were procured from Envigo (Indianapolis, IN) and maintained at the Animal Resource Facility. SD dams and their offspring were housed in polycarbonate cages in a temperature- and humidity-controlled facility under standard conditions (12 h:12 h light-dark cycle). Food and water were provided *ad libitum*. All animal procedures were in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University and the University of Georgia, Athens.

2.3. Experimental design

A graphic representation of the study design is presented in Fig. 1. Vaginal cytology was used to identify female rats in proestrus. They were co-housed with a randomly assigned male rat for one day. The day of copulation was marked as gestational day (GD) 0. In experiment 1, the SD dams were randomly assigned to one of 4 different treatment groups: control (10 μl Phosphate Buffered Saline or PBS; n = 7), BPA (5 μg/kg BW/day; n = 7), high dose DEHP (HD-D; 7.5 mg/kg BW/day; n = 11), or a combination of BPA and HD-D (n = 11). In experiment 2, the dams received either PBS (control; n = 6), BPA (5 μg/kg BW/day; n = 6), low dose DEHP (LD-D; 5 μg/kg BW/day; n = 6), or a combination of BPA and LD-D (n = 7).

2.4. Gestational and reproductive outcomes

Gestational weights were tracked and those dams that lost weight drastically and returned to pre-pregnancy weight were marked as aborted. Immediately following parturition, number of live births and stillbirths were counted. Litter size and weights
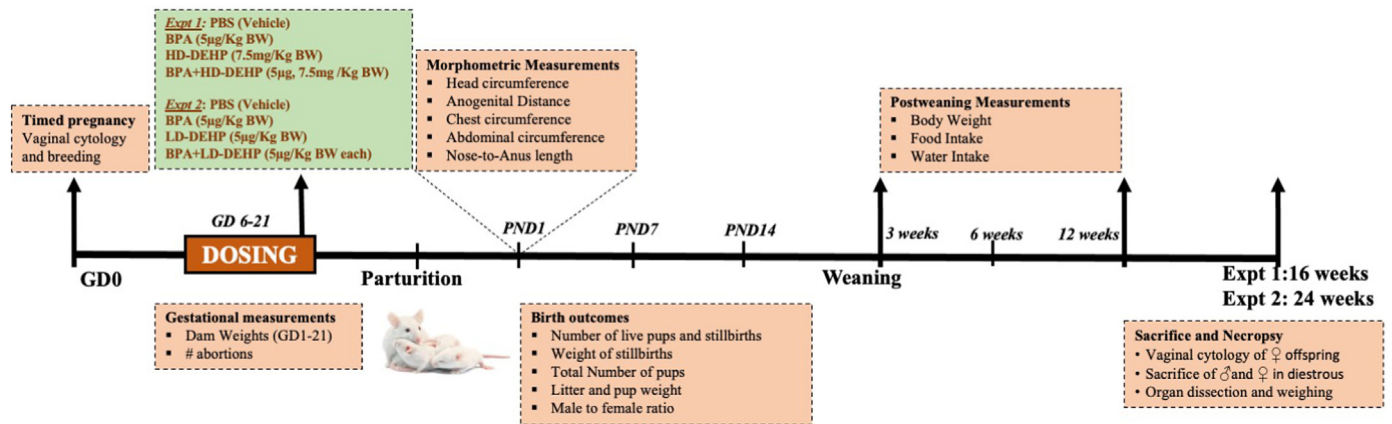
Fig. 1. Study design summary. Female Sprague Dawley rats in proestrus were mated with males (GD 0). Pregnant dams were used in two experiments: In experiment 1, they were orally dosed from GD 6−21 daily with saline (n = 7), BPA (5 μg/kg BW; n = 7), high dose (HD) DEHP (7.5 mg/kg BW; n = 11) or BPA + HD DEHP (5 μg, 7.5 mg/kg BW; n = 11). In experiment 2, they were dosed with saline (n = 6); BPA (5 μg/kg BW; n = 6), low dose (LD) DEHP (5 μg/kg BW; n = 6), or BPA + LD DEHP (5 μg, 5 μg/kg BW; n = 7). Gestational and postnatal measurements were performed to examine the effect of *in utero* EDC exposure on pregnancy outcomes and offspring development on postnatal day (PND) 1, 7, 14 and 21 as well as postnatal weeks 3, 6, and 12. Male and female offspring (in diestrous) were sacrificed at 16 weeks of age (Experiment 1) or 24 weeks of age (experiment 2) and organs were harvested and weighed.

were monitored on postnatal days (PND) 1, 7, 14, and 21. The gestational index was calculated as % of pregnancies yielding live litters. The live birth and stillbirth index was calculated as the % of live and stillborn pups to total number of pups based on published formulae (Tyl et al., 2008), with minor modifications.

2.5. Sex differentiation of genital morphology

In rodents, anogenital distance (AGD) is a marker of abnormalities in genital differentiation and is most often studied as a marker of sex differentiation after prenatal EDC exposure (Welsh et al., 2008). AGD was measured as the distance between the anus and the genital papilla (penis or clitoris) on postnatal day 1. Sex and female-to-male ratios were determined at weaning on PND 21.

2.6. Postnatal growth

Immediately following parturition, birth morphometric parameters were measured individually for the live and stillborn pups. These measurements included head circumference, chest circumference, and crown to rump length. Pups were weaned on PND21 and housed by sex and litter, three to four in a cage. Litter weights were measured on postnatal days 1, 7, 14 and 21. Body weights, food and water intakes were recorded every week from weaning through 12 weeks of age.

2.7. Gross necropsy and organ weights

Males and females in diestrus (as confirmed by vaginal smears) were sacrificed by rapid decapitation at 16 (experiment 1) or 24 weeks of age (experiment 2). Organs and tissues were dissected according to established methods (Fiette and Slaoui, 2011), weighed, and stored for further processing. Organs collected included the pituitary gland, thymus, heart, lungs, spleen, adrenal glands, kidneys, visceral adipose tissues and reproductive organs (ovaries and uterus in females and testes and epididymis in males).

2.8. Thymus histology

A histopathological evaluation of the thymus was performed to detect apoptotic DNA fragmentation in control versus prenatally treated female and male offspring. Formalin-fixed, paraffin embedded (FFPE) rat thymus samples were sectioned. Terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) staining of nicked DNA with Alexa Fluor 594 dye was utilized to detect the fragmented DNA using a Click-iT™ Plus TUNEL Assay (Invitrogen) according to the manufacturer's instructions. Tissues were counterstained with Hoechst 33342. TUNEL + cells were visualized and imaged using Zeiss LSM 710 confocal microscope at the UGA Biomedical Microscopy Core.

2.9. TUNEL quantitative analysis

Images of random and non-overlapping fields were examined at an objective magnification of 20× and 80×. FIJI® free software (https://fiji.sc/) was used to quantify the TUNEL labeled nuclei in thymus tissues. A threshold of fluorescence from negative control images was set and applied to all images. TUNEL-positive cells were systematically counted in defined cortical and medullary regions. A cell was considered apoptotic only if its nucleus was labeled with both TUNEL (red) and Hoechst (blue). Apoptotic index (% of apoptotic cells) was calculated. Mean apoptotic index of 5−7 rats/ group was represented.

2.10. Statistical analysis

Statistical analysis was performed using Prism 8.0.2 (GraphPad, Inc) and R statistical software. Chi-Square test of homogeneity was used to analyze the proportion of dams that aborted, the number of stillbirths, the proportion of dams with stillbirths, and male to female ratios. Gestational data, birth and pre-weaning measurements from experiments 1 and 2 were pooled for ease of presentation. Gestational weight gain, stillborn weights, and litter size at every time point were analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons post hoc test. Preweaning litter sizes at PND 1, 7, 14, and 21 were analyzed using ANCOVA, with prenatal treatment and time as covariates. Birth morphometric measures (AGD, chest circumference, abdominal circumference, and crown-to-rump length) were analyzed using a linear mixed effect model, with programming as a fixed effect and dam as a random effect. Weaning BWs, food intakes, and water intakes were analyzed using a linear mixed effect model, with programming and sex as fixed effects and dam as a random effect. Organ weights were analyzed by experiment. Organ weights and

apoptotic index were analyzed using two-way ANOVA (sex x programming), followed by Tukey's multiple comparisons post hoc test. R software was used to analyze linear mixed effect models and ANCOVA tests. Prism software was used to generate graphs and perform one-way/two-way ANOVA and Chi Square analysis. A p-value < 0.05 was considered to be statistically significant. Data were expressed as mean ± standard error of mean (SEM).

3. Results

3.1. Effect of in utero BPA and DEHP exposure on gestational parameters

Pregnancy outcomes are outlined in Table 1 and Fig. 2 (A). There were no significant differences in percent gestational weight gain between pregnant dams from different treatment groups. However, the reproductive outcome was impaired in dams exposed to DEHP and BPA + DEHP. Dams exposed to BPA or LD-D had no abortion and were comparable to control rats. In contrast, exposure to HD-D induced abortions in 9% of the dams. Exposure to BPA + LD-D increased the abortion rate to 14%, and exposure to BPA + HD-D increased it even further (27%).

3.2. Effect of in utero BPA and DEHP exposure on birth outcomes

Birth outcomes are summarized in Table 1 and Fig. 2B. The gestational index was reduced in HD-D (73%), BPA + LD-D (86%), and BPA + HD-D (73%) compared to control (100%). The HD-D group had the lowest live birth (90.75%) and highest stillbirth index (9.3%) among all treatment groups. At PND1, the number of live and stillborn pups were counted and weighed. Surviving pups were monitored on PND 7, 14, and 21 (Fig. 2C, D). Exposure to BPA or DEHP or their combinations did not affect litter size (Fig. 2C), average pup weight (Fig. 2D), and male-to-female ratio on PND21 (Table 1).

3.3. Effect of in utero BPA and DEHP exposure on morphometric parameters and postnatal growth

Changes in morphometric measures of neonatal rats are depicted in Fig. 3. A linear mixed effect model revealed significant differences in head circumference ($p = 0.008$), chest circumference ($p < 0.0001$), and crown-to-rump length ($p = 0.0004$) among the different treatment groups (Fig. 3A, B, C respectively). While exposure to LD-D increased chest circumference, the higher dose of DEHP decreased it. Similarly, there was an increase in chest circumference (Fig. 3B) with BPA + LD-D exposure ($p = 0.027$) but a reduction in chest circumference with BPA + HD-D exposure ($p = 0.037$). When compared with control group, offspring prenatally exposed to BPA ($p = 0.047$) and BPA + LD-D ($p = 0.006$) had a higher crown-to-rump length (Fig. 3C). All these parameters indicate that offspring growth in utero is being altered by BPA and DEHP, alone and in combination. Prenatal exposure to BPA/DEHP had no overt impact on head circumference (Fig. 3A) or anogenital distance ($p = 0.687$; Fig. 3D).

Post-weaning body weights, food intake, and water intake of female and male offspring are shown in Fig. 4. Prenatal exposure to BPA and DEHP had no significant impact on post-weaning female and male body weights at weeks 3, 6, and 12. Food and water intake were variable and showed no clear treatment-related effects.

3.4. Effect of in utero BPA and DEHP exposure on relative organ weights

Relative organ weights (organ weight to BW ratios) were used to avoid body weight-associated increases in organ weights that are apparent in males and to examine true programming effects induced by BPA and DEHP exposure. Organ weights from offspring prenatally treated with LD-D, alone and in combination with BPA, are depicted in Fig. 5. Relative organ weights from offspring that were exposed to HD-D alone or in combination with BPA are depicted in Fig. 6. Two-way ANOVA revealed sex effects in the thymus, heart, spleen, lungs, pituitary, and adrenals, with females having higher relative organ weights compared to males (Figs. 5 and 6). There was no programming or sex-related effects on relative kidney weights (Tables 2 and 3). In contrast to LD-D, exposure to HD-D produced a marked effect on relative thymus weight in male offspring (Fig. 6B; $p = 0.0071$) and interaction effect on relative heart weight (Fig. 6A; $p < 0.025$). These effects were apparent only in males and not in females. Prenatal BPA exposure caused a 40% decrease in relative thymus weight compared to controls ($p = 0.0006$; Fig. 6B). HD-D exposure on the other hand, produced only a 27% decrease in relative thymus weight ($p = 0.0369$; Fig. 6B). This effect was completely reversed in the group treated with both

Table 1
Pregnancy and birth outcomes of control and treated dams.

	Control	BPA	LD DEHP	HD DEHP	B + LD-D	B + HD-D
Pregnant dams (n)	13	13	6	11	7	11
Dam weight Gain (GD6-21)	38.11 ± 2.28	41.59 ± 2.42	40.04 ± 4.35	39.09 ± 2.75	42.25 ± 2.22	35.8 ± 3.37
Dams that delivered (n)	13	13	6	10	6	8
Total number of offspring (n)	177	166	80	114	79	107
Abortion						
Number of abortions	0	0	0	1	1	3
% of dams that aborted [a]	0	0	0	9	14	27
Indices						
Gestational Index (%)[b]	100	100	100	73	86	73
Stillbirth Index/dam (%)[c]	1.9 ± 1.0	2.4 ± 1.9	3.45 ± 1.56	9.3 ± 5.3	_	7.9 ± 6.1
Live birth Index/dam (%)[d]	98.77 ± 1.4	97.62 ± 1.9	96.55 ± 1.6	90.75 ± 5.3	100 ± 0	92.12 ± 6.1
Male to Female Ratio on PND21						
F1 Total Females (n)	73	75	42	58	35	47
F1 Total Males (n)	95	81	35	45	41	54
Male: Female Ratio	1.3	1.08	0.83	0.78	1.17	1.15

Data are presented as mean ± SEM or as stated.
[a] % of Abortion = (# of abortions/# of pregnancies) x 100.
[b] Gestational Index = (# of dams with live litters/# of pregnant dams) x 100.
[c] Stillbirth Index = (# of pups dead on PND 0/# of total pups on PND0) x 100.
[d] Livebirth Index = (# of live pups on PND0/# of total pups on PND0) x 100.
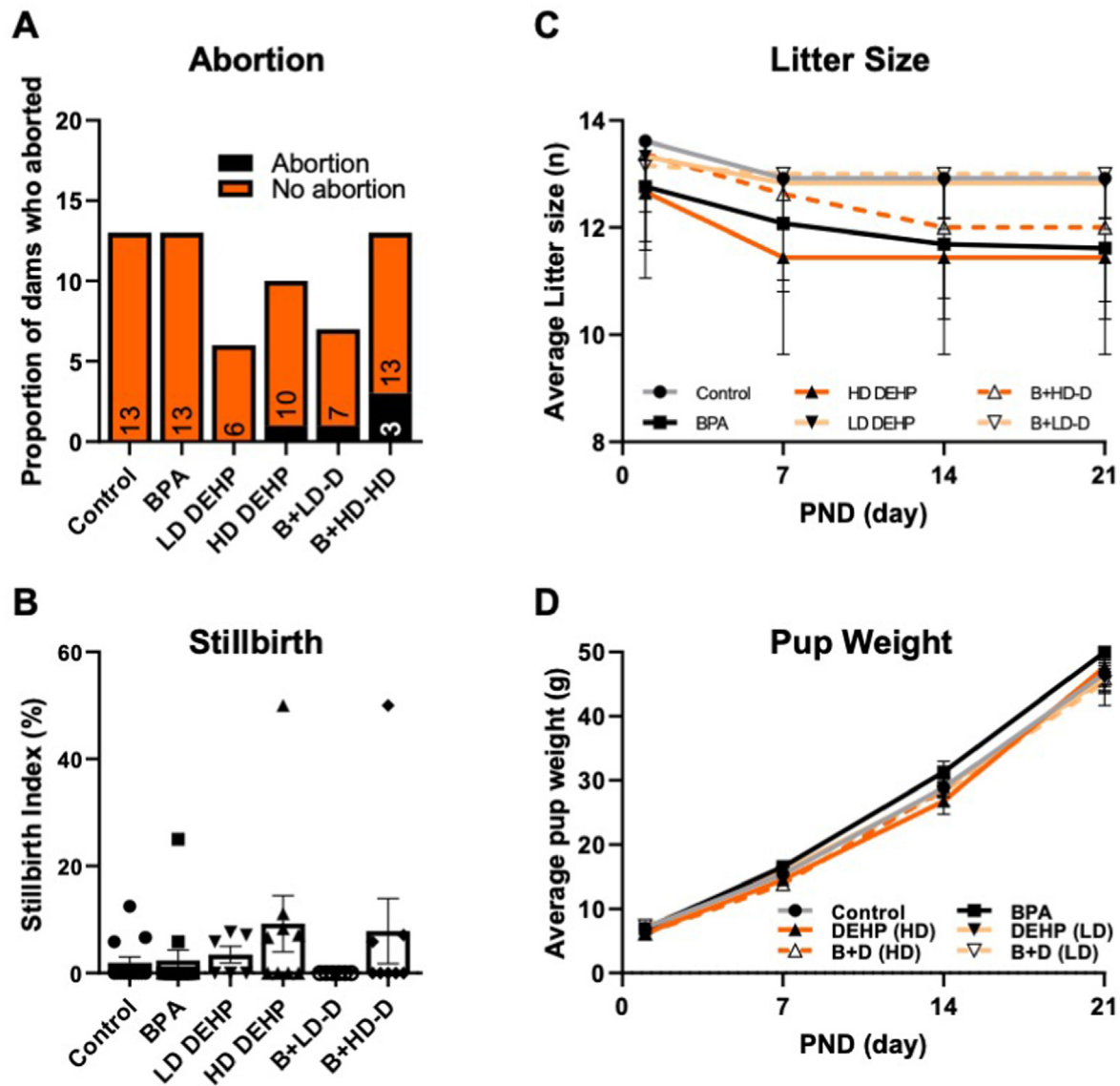
Fig. 2. Pregnancy and birth outcomes of control and treated dams. Data from experiments 1 and 2 are merged here. Body weights of dams were recorded every day during pregnancy. Dams that returned to pre-breeding weights were marked as aborted. Chi square analysis did not show significant difference in proportion of dams who aborted (Fig. 2A). At PND1, number of stillbirths were counted. One-way ANOVA did not show any significant difference in stillbirth index/dam (Fig. 2B). However, a clear trend is seen across treatment groups with the DEHP (HD) and B + HD-D showing the highest still birth index. Number of pups per dam were counted and weighed on PND1,7,14, and 21 (Fig. 2C). Pup weight was measured once a week prior to weaning (Fig. 2D). No significant prenatal programming effect was observed.

BPA and HD-D ($p > 0.999$). There was a 16% increase in relative heart weight in the group treated with BPA and HD-D compared to the groups treated with BPA or HD-D alone ($p = 0.0112$; Fig. 6D).

3.5. Effect of in utero EDC exposure on apoptosis in the thymus

To understand if the reduction in thymus weight was caused by early thymus involution or atrophy through apoptosis, we performed TUNEL staining of nicked DNA on thymic sections, followed by Hoechst 33342 counterstain. TUNEL + apoptotic cells were hardly seen in the thymus sections from control rats, but numerous apoptotic cells were apparent in the thymus sections of male and female offspring prenatally treated with BPA or HD-D suggesting apoptotic changes. Apoptosis was most pronounced in HD-D offspring (Fig. 7). Exposure to HD-D produced a marked increase in apoptosis in the thymic cortex of both male and female offspring (Fig. 7Y) while it increased apoptosis in the medullary region only

in females (Fig. 7Z). Exposure to a combination of BPA + HD-D completely blocked the apoptosis caused by BPA and HD-D (Fig. 7Y and Z).

4. Discussion

Previous studies have identified BPA and DEHP independently as reproductive (Shi et al., 2019, 2019b), developmental, and systemic toxicants (Naule et al., 2014) (Tyl et al., 2008; Shi et al., 2019). However, studies investigating the effects of BPA and DEHP mixtures are few. Exposure to EDC mixtures is probably more common rather than exposure to any individual EDC due to their ubiquitous presence in the environment. Moreover, both BPA and DEHP metabolites have been found in the urine of pregnant women (Ye et al., 2008; Braun et al., 2012; Martinez-Ibarra et al., 2019) indicating that pregnant women are also exposed to a mixture of these chemicals. There are several reports to indicate adverse pregnancy
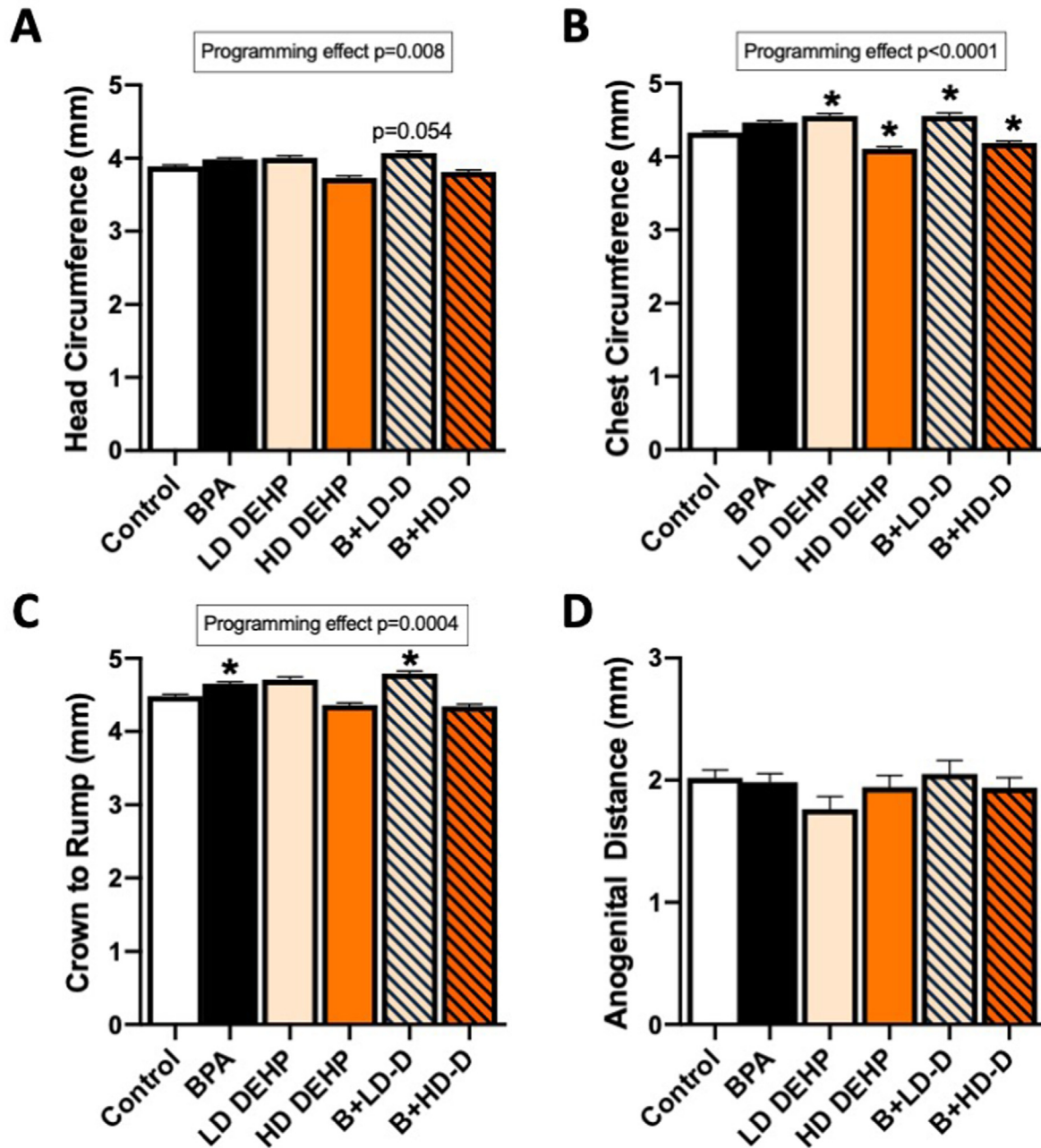
Fig. 3. Birth morphometric parameters of rats exposed to BPA, DEHP or the mixtures *in utero*. Morphometric measurements (mean ± SEM) were performed on PND1. Linear mixed effect model revealed a significant prenatal programming effect with respect to head circumference (Fig. 3A; $p = 0.008$), chest circumference (Fig. 4B; $p < 0.0001$), and crown to rump length (Fig. 3C; $p = 0.0004$). No programming effect was seen on anogenital distance. Graphs represent mean ± SEM from 6 to 15 dams per treatment group. Asterisk (*) represents a significant difference from the control group ($p \leq 0.05$).

outcomes with prenatal exposures to BPA or DEHP alone (reviewed in (Strakovsky and Schantz, 2018; Zhou et al., 2019; Pergialiotis et al., 2018)). However, there are no experimental studies examining the effects of exposure to low dose BPA-DEHP mixtures on gestational outcomes.

In the present study, we observed an increase in the rate of abortions in dams that were treated with the HD-D alone and a dose-dependent increase in the abortion rate with BPA-HD-D mixtures. The abortions occurred within the first few days of treatment and since treatment was started on the 6th day of gestation when the blastocyst is implanted in the endometrium, it appears that the developing embryo is highly susceptible to these

mixtures. These findings are supported by a recent study in humans that correlated BPA and DEHP metabolites in the urine of pregnant women with lower rates of implantation, clinical pregnancy and live birth (Minguez-Alarcon et al., 2019). Other studies have found correlations between urinary phthalate concentrations in women and higher incidence of preterm birth (Ferguson et al., 2014; Chin et al., 2019a). More than BPA, it appears that DEHP and its metabolites may affect the survival of the developing embryo. In fact, increased risk of clinical pregnancy loss was associated with higher levels of DEHP metabolites in the urine of pregnant mothers (Mu et al., 2015). High doses of DEHP (500 mg/kg BW) caused abortions in 100% of pregnant mice (Schmidt et al., 2012) and we have
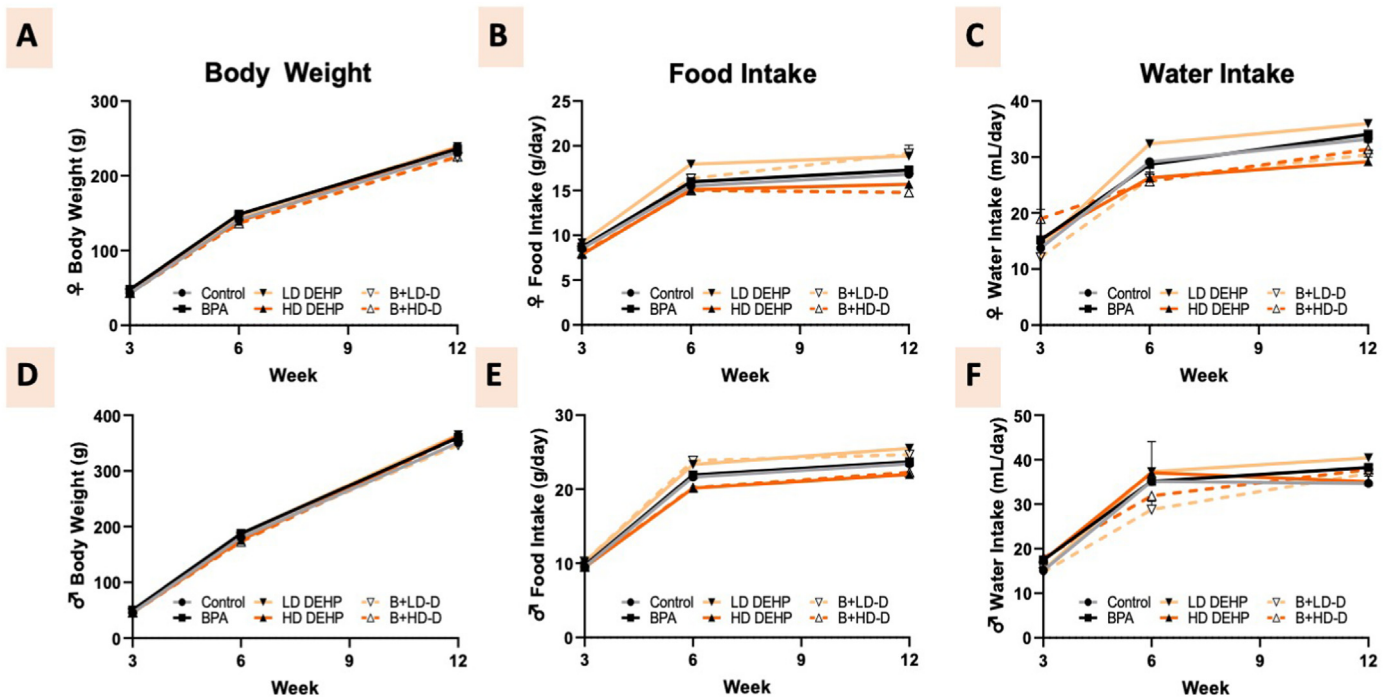
Fig. 4. Post-weaning changes in body weight, food intake and water intake of offspring exposed to BPA, DEHP or the mixture *in utero*. Top panels depict changes in female offspring and bottom panels depict changes in male offspring. Data are presented as mean ± SEM for group averages of 6–13 dams per treatment group. There were no significant programming effects on absolute body weight (grams; Fig. 4 A, D), food intake (g/day; Fig. 4B, E), and water intake (mL/day; Fig. 4C, F) at 3, 6, and 12 weeks of age.

observed a similar phenomenon with a 750 mg/kg BW dose of DEHP in pregnant rats (unpublished observations). There is some evidence to suggest that DEHP could disrupt MAPK and NK-kB signaling pathways leading to possible embryo detachment and abortion (Li et al., 2012). Another study has found that a DEHP metabolite can inhibit the activity of matrix metalloproteinase-9 which is essential for extravillous trophoblast invasion during the early stages of development, thus contributing to early pregnancy loss (Gao et al., 2017). Whether a similar mechanism could play a role after exposure to BPA-DEHP mixtures needs to be investigated.

Exposure to HD-D also increased the risk for stillbirths. This could be associated with placental insufficiency or birth defects which need to be explored further. In fact, recent evidence suggests that BPA and phthalate exposures can change micro-RNA expression in the human placenta, induce DNA methylation and genomic imprinting leading to adverse placental outcomes (Strakovsky and Schantz, 2018). However, there was no evidence of intrauterine growth retardation in the present study that would suggest placental dysfunction after exposure to HD-DEHP or the mixture. Nor were there any effects in terms of gestational length, litter size, and sex ratio. Modest changes in birth morphometric measures were apparent after prenatal exposure to BPA-DEHP mixtures, but it did not significantly affect postweaning body weight, food intake, and water intake. The reason for this discrepancy is not clear. There is major controversy in the literature about the effects of prenatal exposure to BPA and DEHP on birth outcomes and postnatal life. While one study has observed no changes in postnatal development (Arcadi et al., 1998) many studies have reported intrauterine growth retardation with exposure to BPA or DEHP(Muller et al., 2018; Snijder et al., 2013; Shen et al., 2017; Yu et al., 2018). Several factors including animal model used, dose and duration of exposure, route of administration and stage of gestation at the time of exposure all contribute to the inconsistent findings (Melnick et al., 2002).

Exposure to BPA and DEHP did not affect the weights of most organs when the offspring were sacrificed after reaching adulthood. Treatment specific effects were observed only in male offspring and they were restricted to the thymus and the heart. BPA or DEHP alone did not produce any change in heart weight, however the mixture of BPA and HD-D produced a significant increase in heart weight that could suggest possible cardiac hypertrophy. We have previously reported that prenatal BPA exposure in a sheep model increased a marker of cardiac hypertrophy, atrial natriuretic peptide, in the ventricles, and increased collagen expression in the left ventricle (MohanKumar et al., 2017). Prenatal BPA exposure also increased fibrosis in the heart of a mouse model (Rasdi et al., 2020). This could translate into increased risk for cardiovascular dysfunction. In fact, there is some evidence to suggest that prenatal BPA exposure could increase cardiovascular risk in children (Ouyang et al., 2020). In contrast to BPA, prenatal DEHP exposure has been shown to decrease blood pressure in rats (Martinez-Arguelles et al., 2013) and children (Vafeiadi et al., 2018). The increase in heart weight observed in the present study after exposure to the mixture could be caused by an increase in hypertrophy of cardiac myocytes or increased collagen deposition as mentioned above. Although the underlying mechanisms are unclear, it is possible that signaling pathways of these two chemicals likely intersect resulting in a variety of responses ranging from complete negation (as seen in birth morphometric measures) to a more synergistic effect as seen in the heart.

Apart from the heart, the most marked change in organ weights was apparent in the thymus of male offspring in the first experiment where we used BPA in combination with HD-D. It is important to note that animals from experiment 1 (with HD-D) were sacrificed at 16 weeks, while animals from experiment 2 (with LD-D) were sacrificed 8 weeks later. The natural involution of the thymus with age could account for the lower thymus weights in the LD-D groups. However, the remarkable treatment effects of
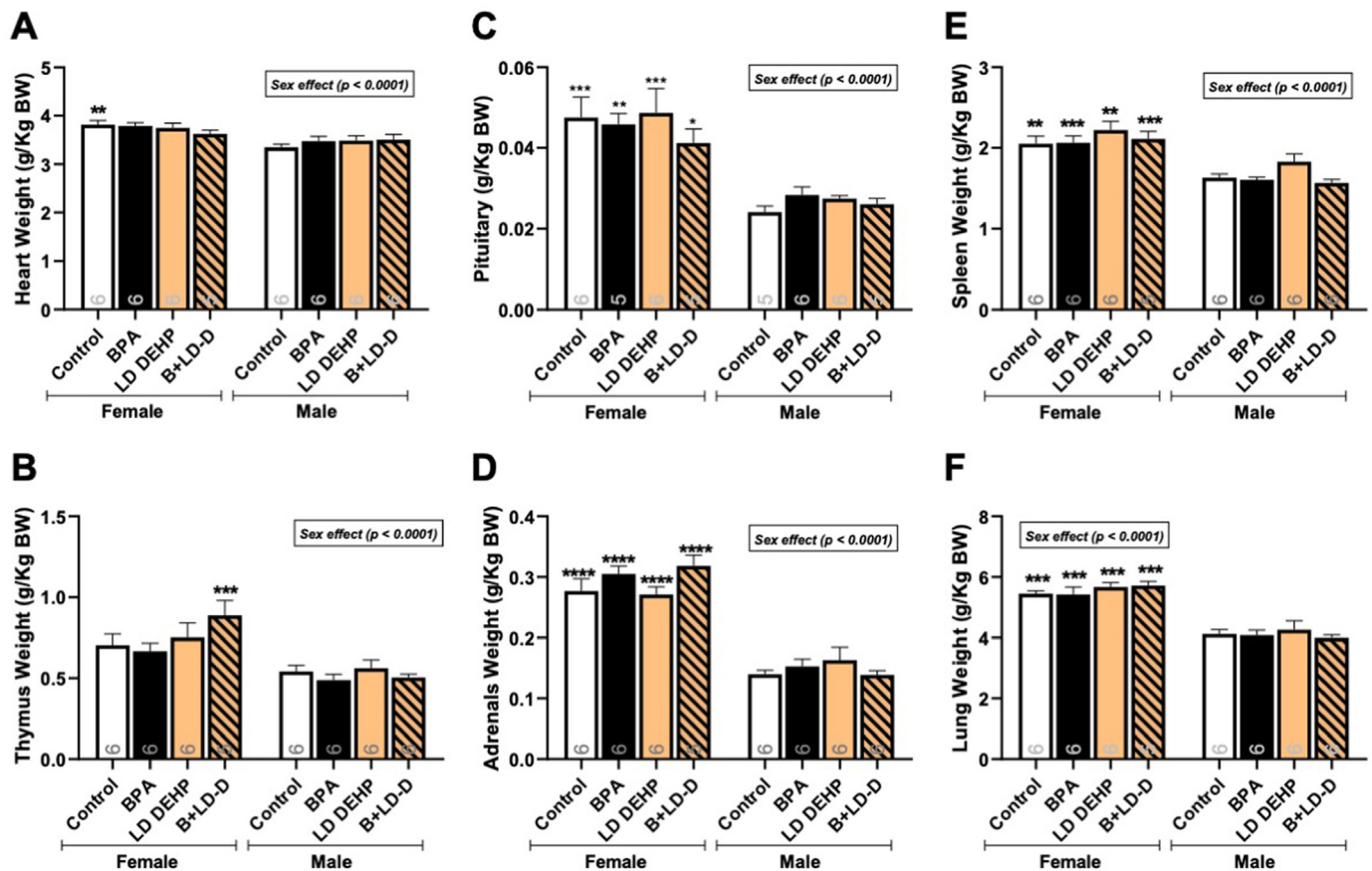
Fig. 5. Sex-specific effect of prenatal exposure to BPA, LD-DEHP or their mixtures on relative organ weights in male and female offspring. Top panels reflect changes in the heart, pituitary and spleen. Bottom panels reflect changes in the relative weights of the thymus, adrenals and lungs. Animals were sacrificed at 24 weeks of age. Sample sizes are provided inside the bars. * represents sex differences (*p* < 0.05; **p* < 0.01; ***p* < 0.001).

prenatal exposures were apparent in experiment 1 when compared to corresponding controls. Although males that were prenatally exposed to BPA or HD-D had lower thymus weights compared to control, apoptosis was more pronounced only in the HD-D group. This could indicate that there could be other underlying processes contributing to reduced thymus weights in the BPA-exposed animals. One such process could be changes in thymic stromal cells that cause contraction of complex cell projections (Venables et al., 2019) leading to reduction in thymus weight. Another possibility is that DEHP could reduce the levels of progenitor T cells leading to reduced seeding of the thymus and reduced thymus weights possibly by acting through the aryl hydrocarbon receptor. A similar phenomenon has been observed after prenatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Besteman et al., 2007).

Interestingly in females, there was a marked increase in apoptosis without any effect on organ weight in the HD-D group. This could be attributed to a possible quicker replenishment of apoptotic cells in females. In fact, female mice are known to have higher number of thymocytes than males even in middle age (Hun et al., 2020) and this disparity between the sexes could be responsible for the present observations. Moreover, apoptosis was apparent both in the cortex and the medulla in females but only in the cortex in males. Developing T cells in the cortex undergo apoptosis when they fail to interact with class I or class II major histocompatibility complex molecules. In the medulla, they undergo apoptosis if they interact too strongly with self-antigens presented by antigen presenting cells (Shah and Zuniga-Pflucker, 2014). Prenatal exposure to DEHP possibly influences both these

phenomena in females and only the former in males. These are complex processes involving multiple cytokines, chemokines, and ligands produced by the thymic epithelium (Shah and Zuniga-Pflucker, 2014). Nevertheless, the fact that prenatal exposure to BPA and HD-D by themselves could potentially influence these processes is interesting and needs further investigation. Further, exposure to the mixture of BPA and HD-D reversed the apoptotic change induced by either of these chemicals alone. This suggests that the dynamics involved in the combined effects of BPA and DEHP were able to cancel some of the deleterious effects as reported previously (Chin et al., 2019b).

In conclusion, the overall developmental and reproductive toxicities attributed to prenatal BPA and DEHP exposures are complex and controversial. To our knowledge, this is the first study to examine the effects of environmentally relevant doses of mixtures of DEHP and BPA. While individual EDCs produced dramatic effects, the combination appeared to neutralize or augment the effects depending on the gestational or postnatal parameter examined. Our study highlights the necessity to consider the possible additive, antagonistic, or synergistic activities of EDC mixtures, to which pregnant individuals are likely exposed. More experiments are underway to better characterize the effects of the prenatal exposure to low dose EDC mixtures and to define whether they represent adverse or adaptive events.

Credit author statement

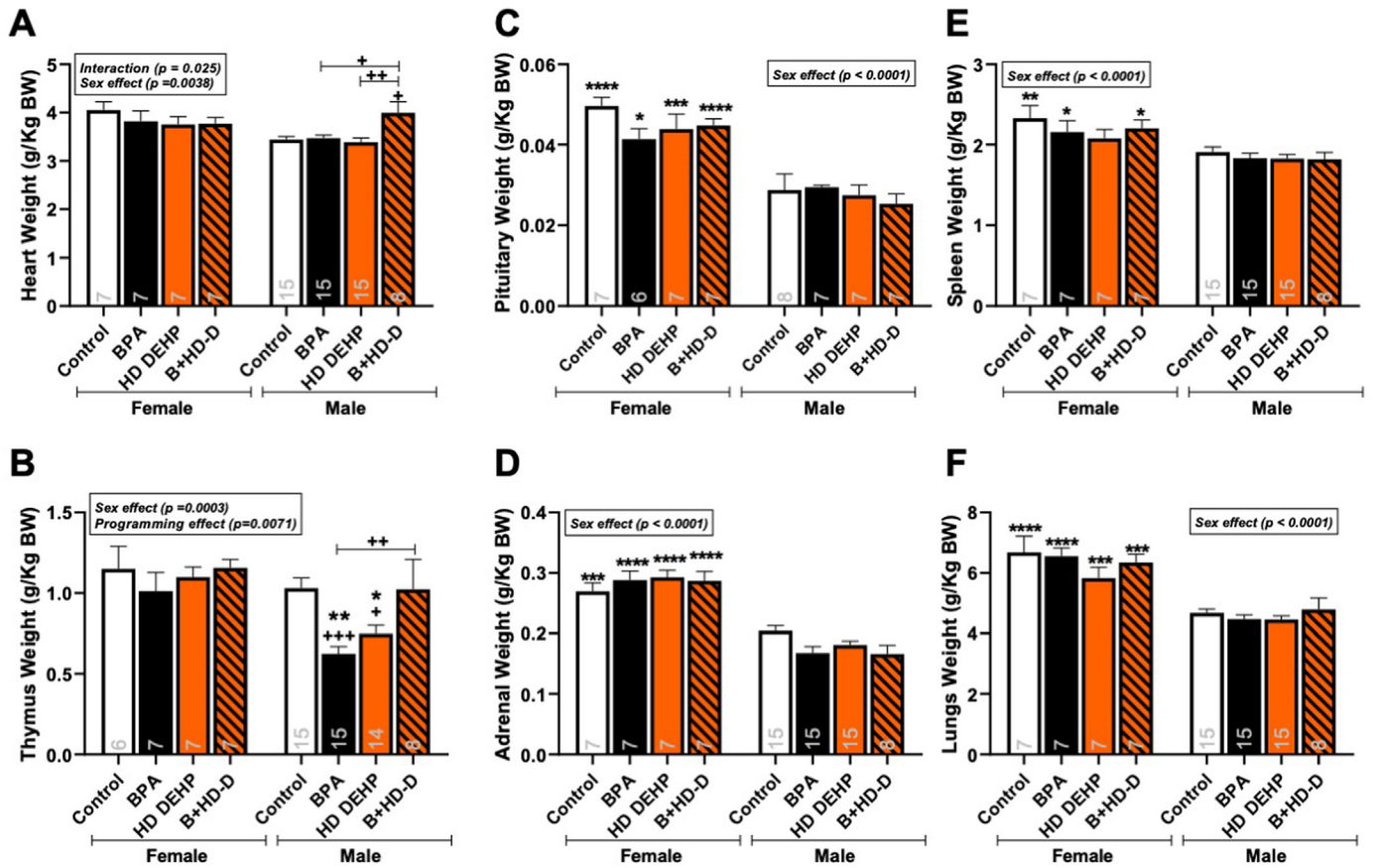Josephine Bou Dagher: Investigation, Formal analysis,

Fig. 6. Programming effect of prenatal exposure to BPA, HD-DEHP, or their mixture on relative organ weights. Top panels reflect changes in the heart, pituitary and spleen. Bottom panels reflect changes in the relative weights of the thymus, adrenals and lungs. Animals were sacrificed at 16 weeks of age. Sample sizes are provided inside the bars. * represents sex differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). + represents treatment differences within the same sex (+$p < 0.05$; ++ $p < 0.01$; +++$p < 0.001$).

Table 2
Relative kidney, visceral adipose tissue and reproductive organ weights of control and prenatally-treated offspring with BPA and/or low-dose DEHP.

Organ Weight	Control	BPA	DEHP	B + D	Sex Effect p-value
Kidneys					NS
Female	6.72 ± 0.22	6.63 ± 0.09	6.51 ± 0.17	6.62 ± 0.09	
Male	6.77 ± 0.16	6.70 ± 0.12	6.87 ± 0.36	6.51 ± 0.32	
Visceral AT					<0.01
Female	11.62 ± 2.31	10.41 ± 0.98	$9.91 \pm 1.27*$	13.06 ± 0.98	
Male	14.02 ± 0.69	13.35 ± 0.66	15.67 ± 1.78	15.04 ± 0.44	
Uterus + Ovaries	2.435 ± 0.2	2.52 ± 0.19	2.409 ± 0.13	2.686 ± 0.44	–
Testes + Epididymus	13.5 ± 0.33	12.47 ± 0.37	14.14 ± 0.6	13.32 ± 0.31	–

Data are presented as mean $\pm$ SEM for group averages. *p < 0.05, significantly different from the male group of similar treatment.

Table 3
Relative kidney, visceral adipose tissue and reproductive organ weights of control and prenatally-treated offspring with BPA and/or high-dose DEHP.

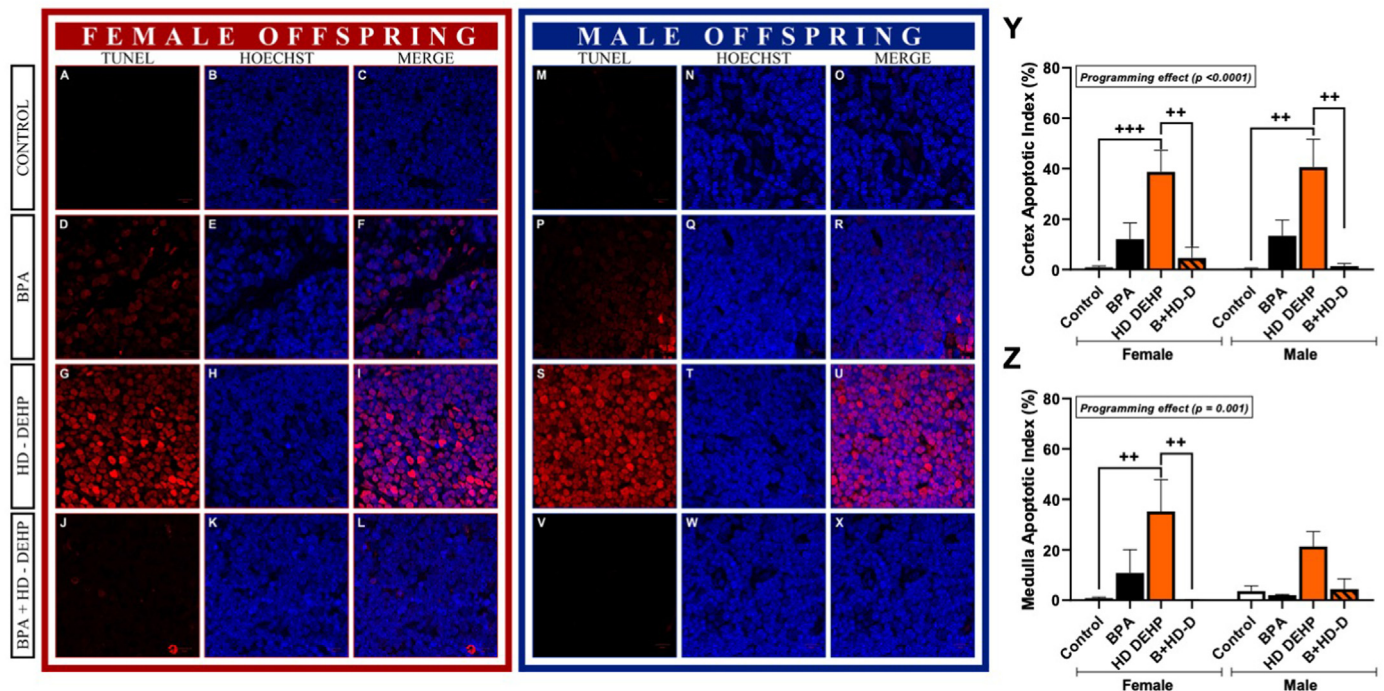| Organ Weight	Control	BPA	DEHP	B + D	Sex Effect p-value
Kidneys					<0.01
Female	6.51 ± 0.1	6.53 ± 0.19	6.29 ± 0.27	6.30 ± 0.17	
Male	6.79 ± 0.1	7.04 ± 0.18	6.82 ± 0.23	6.67 ± 0.24	
Visceral AT					NS
Female	10.86 ± 1.27	13.25 ± 1.86	10.51 ± 1.22	10.82 ± 1.98	
Male	14.20 ± 1.06	11.96 ± 1.38	11.54 ± 1.17	11.47 ± 0.80	
Uterus + Ovaries	2.755 ± 0.2	2.877 ± 0.21	3.469 ± 0.43	3.378 ± 0.34	–
Testes + Epididymus	14.81 ± 0.3	14.37 ± 0.4	15.27 ± 0.3	14.77 ± 0.45	–

Fig. 7. Effect of prenatal exposure to BPA, HD-DEHP, or the combination on apoptosis in the thymus. Representative samples of immunofluorescent TUNEL (red) assays of thymus sections from control and prenatally EDC-exposed female (Fig. 7. A,D,G,J) and male rats (Fig. 7. M,P,S,V). Hoechst (blue) was used for nuclear staining (Fig. 7. B,E,H,K,N,Q,T,W). *(Magnification: 40x oil, Zoom 2.0; Scale bar:*10 μm). Colocalization of Hoechst and TUNEL signals are represented in Figures C,F,I,L,O,R,U,X. Quantification of TUNEL-positive nuclei in cortical (Fig. 7 Y) and medullary (Fig. 7 Z) regions of the thymus from control and prenatally treated rats. 2-way ANOVA shows a significant increase in apoptotic nuclei in both cortex and medullary regions of the thymus in female offspring. These changes are apparent only in the cortex in male offspring prenatally exposed to HD-DEHP. + represents treatment differences within the same sex ($^{++}p < 0.01$; $^{+++}p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Caleb J. Bacak: Investigation, data curation. Hannah E. Buechter: Investigation, data curation. Celexis Cambric: Investigation, data curation. Jared Spivey: Investigation, data curation. Yen-Jun Chuang: Investigation, data curation, Supervision. Elyssa J. Campbell: Project administration, Investigation. Abhyuday Mandal: Formal analysis, resources. Puliyur S. Mohankumar: Conceptualization, funding acquisition, writing-review and editing. Sheba M.J. MohanKumar: Conceptualization, methodology, resources, Supervision, Funding acquisition, writing-review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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