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Prenatal exposure to bisphenols affects pregnancy outcomes and offspring development in rats

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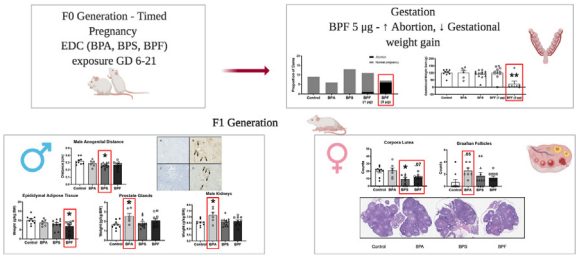
HIGHLIGHTS

- BPF increases spontaneous abortions in pregnant dams in a dose-dependent manner.
- Prenatal exposure to BPS and BPF in females decreases corpora lutea in the ovary.
- Prenatal exposure to BPS reduces anogenital distance in males.
- Prenatal exposure to BPA and BPS induces oxidative stress in weanling testes.
- Prenatal exposure to BPA increases kidney and prostate gland weights in males.

GRAPHICAL ABSTRACT



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ABSTRACT

The objective of this study was to evaluate the effects of gestational exposure to low doses of bisphenol A (BPA), bisphenol S (BPS), and bisphenol F (BPF) on pregnancy outcomes and offspring development. Pregnant Sprague-Dawley rats were orally dosed with vehicle, 5 µg/kg body weight (BW)/day of BPA, BPS and BPF, or 1 µg/kg BW/day of BPF on gestational days 6–21. Pregnancy and gestational outcomes, including number of abortions and stillbirths, were monitored. Male and female offspring were subjected to morphometry at birth, followed by pre- and post-weaning body weights, post-weaning food and water intakes, and adult organ weights. Ovarian follicular counts were also obtained from adult female offspring. We observed spontaneous abortions in over 80% of dams exposed to 5 µg/kg of BPF. BPA exposure increased Graafian follicles in female offspring, while BPS and BPF exposure decreased the number of corpora lutea, suggesting reduced ovulation rates. Moreover, BPA exposure increased male kidney and prostate gland weights, BPF decreased epididymal adipose tissue weights, and BPS had

Abbreviations: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F; EDC, endocrine disrupting chemical; PBS, Phosphate Buffered Saline; DMSO, dimethylsulfoxide; NOAEL, no-observed-adverse-effect-level; AGD, anogenital distance; AAT, abdominal adipose tissue; EAT, epididymal adipose tissue; OAT, ovarian adipose tissue; PAT, perirenal adipose tissue.

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Ovulation
Oxidative stress

modest effects on male abdominal adipose tissue weights. Prenatal BPS exposure reduced anogenital distance (AGD) in male offspring, suggesting possible feminization, whereas both BPS and BPA induced oxidative stress in the testes. These results indicate that prenatal exposure to BPF affects pregnancy outcomes, BPS alters male AGD, and all three bisphenols alter certain organ weights in male offspring and ovarian function in female offspring. Altogether, it appears that prenatal exposure to BPA or its analogues can induce reproductive toxicity even at low doses.

1. Introduction

The adverse health effects of BPA, a ubiquitous endocrine disrupting chemical (EDC) in the environment, have been extensively investigated. There is abundant evidence associating BPA exposure with repercussions on development, reproduction, metabolism, and neurobehavior (Rochester, 2013; Mileva et al., 2014; Gioiosa et al., 2015; Ma et al., 2019). Therefore, in an effort to phase out the use of BPA in consumer products, manufacturers have turned to the use of chemicals that are structurally similar to BPA in BPA-free products. Among the most commonly used substitutes of BPA are its structural analogues BPS and BPF (Rochester and Bolden, 2015).

BPS is used in industrial applications including certain agents found in cleaning products, and in thermal paper products such as cashier's receipts (Siracusa et al., 2018). BPF is found in epoxy resins (Liao and Kannan, 2013) and is a contaminant in a variety of fresh and canned foods including vegetables, meats, and dairy products (Cabaton et al., 2009; Audebert et al., 2011). Both BPF and BPS are also used in a variety of applications such as structural adhesives, dental materials, electrical varnishes, industrial applications such as grouts, coatings, flooring, tank and pipe linings, and road and bridge deck sealants (Rochester and Bolden, 2015; Siracusa et al., 2018). They have additionally been detected in a variety of consumer products including food packaging and plastics, and in personal care items such as hair care products, lotions, and toothpaste (Liao and Kannan, 2014). In the environment, BPS and BPF are particularly prevalent in indoor dust, water, sediment, and sewage (Fromme et al., 2002; Liao et al., 2012; Song et al., 2014; Yang et al., 2014). Exposure to these chemicals occurs through the dermal, oral, and inhalation routes. Consequently, these chemicals have been found in human urine samples in concentrations comparable to BPA (Zhou et al., 2014). Research into the health consequences of BPS and BPF is expanding, with increasing evidence identifying similarities in the adverse effects of these analogues with those of BPA itself (Rochester and Bolden, 2015).

Rodent studies examining the effects of perinatal exposure to BPS report dose-dependent changes in offspring body weight (BW) and organ weights. Male mice offspring with low-dose BPS treatment (100 ng/g BW) show increases in BW and specific organ weights (Meng et al., 2019b). Interestingly, male and female rats with perinatal exposure to very low doses of BPS (10 and 50 μg/kg BW) have lower food intake (da Silva et al., 2019). BPS can cross the placental barrier (Gingrich et al., 2018), but its ability to do so is ten times less than that of BPA (Grandin et al., 2018). The current Environmental Protection Agency (EPA) recommended no-observed-adverse-effect-level (NOAEL) for BPS is 10 mg/kg/day (EPA, 2014), which is relatively higher than the doses used in the aforementioned studies.

Studies investigating the effects of perinatal BPF exposure in rodent models to date have primarily focused on neuroendocrine, metabolic, oxidative stress, and behavioral endpoints in the offspring (Castro et al., 2015; Ohtani et al., 2017; Meng et al., 2019a), but none have studied the gestational effects or offspring development. Nevertheless, studies examining direct exposure to high-dose BPF (20–750 mg/kg BW) report lower BWs in mature male and female rats coupled with increased organ weights (Higashihara et al., 2007b; Igarashi et al., 2018), and studies in male mice have used BPF at doses of 0.044 or 4.4 mg/kg/day and report less weight gain (Drobna et al., 2019). Although a NOAEL for BPF has not been published yet, the EPA has unofficially classified it as a strong developmental hazard (Catron et al., 2019) and a moderate reproductive hazard (den Braver-Sewradj et al., 2020). Moreover, BPF is known to cross the placental barrier and reach the fetus (Cabaton et al., 2006).

The prenatal period is a critical window of development (Selevan et al., 2000) during which exposure to exogenous compounds – including EDCs – can impact fetal development. The fetus is particularly vulnerable during this sensitive period with limited capacity to metabolize and process these chemicals (Unüvar and Büyükgebiz, 2012). This may result in long-lasting tissue level changes that contribute to adverse health outcomes in adulthood (Fenton, 2006; Dietert, 2012; Tucker et al., 2018). In this study, we aimed to uncover pregnancy and developmental outcomes that result from prenatal exposures to BPA, BPS, and BPF at environmentally relevant doses that are significantly lower than the established NOAEL doses and those used in prior studies.

2. Materials and methods

2.1. Animal husbandry

Adult female and male Sprague-Dawley (SD) rats (3 months old) were obtained from Envigo (Indianapolis, IN). They were housed in light- (12:12 light-dark cycle) and temperature-controlled rooms (23 ± 2 °C, 50 ± 20% relative humidity) within accredited animal facilities. Animals were housed in polycarbonate cages and food (LabDiet 5053) and water were provided *ad libitum*. We did not control for bisphenol exposures from the environment (cages, water bottles etc.) since all animals were maintained in the same environment. All animal procedures were compliant 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 Committees (IACUC) at the University of Georgia.

2.2. EDC exposure paradigm

The experimental design is depicted in Fig. 1. Prior to mating, vaginal cytology was performed on the female breeders for 10 consecutive days to document the regularity of estrous cycles. Following this, a female in proestrus and a randomly assigned male were co-housed for 1 day. Mating was confirmed by the presence of a vaginal plug. The day of copulation was marked as GD 0. Each dam was randomly assigned to one of 4 different treatment groups: control (10 μl Phosphate Buffered Saline or PBS; $n = 9$), BPA (5 μg/kg BW/day; $n = 6$), BPF (5 or 1 μg/kg BW/day; $n = 10$), and BPS (5 μg/kg BW/day; $n = 13$). The samples sizes varied between different

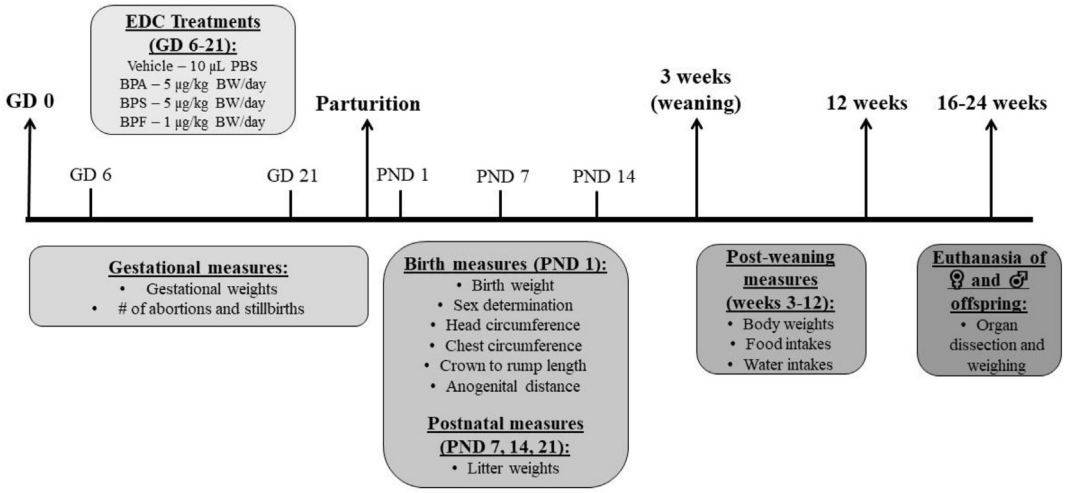

Fig. 1. Schematic depicting the experimental design. Pregnant Sprague Dawley dams were orally dosed daily from gestational days (GD) 6–21 with vehicle (control) (10 µL PBS; $n = 9$), BPA (5 µg/kg/day; $n = 6$), BPS (5 µg/kg/day; $n = 13$), or BPF (5 or 1 µg/kg/day; $n = 7$ and 10 respectively). Several measurements were obtained during gestation and on postnatal days (PND) 1, 7, and 14 as well as following weaning (week 3) until euthanasia (weeks 16–24). Note: EDC, endocrine disrupting chemical; PBS, Phosphate Buffered Saline; BPA, Bisphenol A; BPS, Bisphenol S; BPF, Bisphenol F; BW, body weight.

treatment groups because the experiment was repeated twice, and animals from both studies are reported here. The dam was considered the experimental unit. At GD 6, females were orally exposed to environmentally relevant doses of EDC or vehicle. The dams remained group-housed with others of the same treatment group for the duration of the exposure and were separated into individual cages on GD 22. The dams remained with their litters until weaning.

BPA (Catalog No. 239658; Lot MKBH2096V; ≥99% purity), BPF (Catalog No. 51453; Lot BCBQ5566V; >98% purity), and BPS (Catalog No. 43034; Lot BCBV2462; ≥98% purity) were obtained from Sigma Aldrich (St. Louis, MO). Stock solutions were made in dimethylsulfoxide (DMSO) to obtain complete dissolution. Doses were calculated daily based on BW and small aliquots were mixed with 10 µl PBS for oral dosing. Daily oral dosing occurred from GD 6–21, during which the vehicle or EDC solution (~15 µl) was discharged into the oral cavity with a micropipettor to avoid causing irritation to the gastrointestinal tract and potential stress to the pregnant dam. This procedure was relatively quick and induced minimal stress. All dams received vehicle or EDC treatments daily. Dams were group-housed (3 to a cage) based on treatment and it is likely that they were exposed to chemicals that were excreted in the feces and urine of their cage-mates. The BPS and BPA doses were selected because they are well below the EPA recommended NOAEL doses of 10 mg/kg/day for BPS and 5 mg/kg/day for BPA (EPA, 2014). The BPA dose we used is also 10-fold lower than the current daily reference dose of 50 µg/kg/day (Almeida et al., 2018). No NOAEL is currently set for BPF. However, the lowest-observed-adverse-effect level (LOAEL) for BPF is 20 mg/kg/day based on a sub-acute oral toxicity study (Higashihara et al., 2007a), and a proposed tolerable daily intake value is 11 µg/kg/day for BPF (Zoller et al., 2016). The BPF dose we used for this study was significantly lower than both of these doses. The dams were separated into individual cages on GD22 and they remained in separate cages with their litter until weaning.

2.3. Gestational and offspring measurements

The number of pregnant dams and number of abortions per dam were tracked. Body weights of dams were obtained daily from GD 6. Any sudden reduction in body weight and return to pre-breeding weight was considered an abortion. Each dam needed to gain an average of 35 g to confirm pregnancy. Sudden weight loss below this body weight was considered an abortion. Dams that aborted typically stopped gaining weight by gestational days 15 or 16. The number of stillbirths and live births were recorded following parturition. Gestational index was defined as the ratio of the number of dams with live litters to the number of pregnant dams, and was calculated using the equation: (# of dams with live litters/# of pregnant dams) x 100. Stillbirth index was defined as the ratio of the number of stillbirths to the total number of pups on PND1, and was calculated using the equation: (# of stillborn pups/# of total pups born) x 100.

Pups were typically counted within 24 h of initiation of the delivery process. Sex was determined on PND 1 and morphometric measurements were collected individually for the live pups. These measurements included head circumference, chest circumference, crown to rump length (measured from the midpoint on the top of the head to the base of the tail), and anogenital distance (AGD). Since it was not possible to identify the pups individually prior to weaning, weekly litter weights were collected on postnatal days (PND) 1, 7, 14, and 21 until weaning. Litter weights were divided by the litter sizes to obtain average pre-weaning BW for male and female pups, which is reported in this paper. After weaning, animals were identified by ear punches and individual BWs were obtained until they were 12 weeks old. Pups were housed by sex and litter, with three or four pups from the same litter per cage. BWs, food intakes, and water intakes were recorded at 3, 6, and 12 weeks of age. Post-weaning BWs were collected to determine if there was any catch-up growth in the event of intrauterine growth restriction.

2.4. Tissue collection and preparation

Adult male and female offspring in diestrus (as confirmed by vaginal cytology) were euthanized by rapid decapitation in adulthood (at 16–24 weeks of age). Blood was collected following euthanasia, and organs and tissues were dissected, weighed, and stored for further processing. Organs collected included the pituitary gland, thymus, heart, lungs, liver, spleen, adrenal glands, kidneys, abdominal adipose tissue (AAT), epididymal adipose tissue (EAT) from males or ovarian adipose tissue (OAT) from females,

perirenal adipose tissue (PAT), and reproductive organs (ovaries and uterus in females, paired testes, prostate glands, and seminal vesicles in males). All organ weights that were measured at the time of sacrifice were normalized to the body weight of the animals to address the difference in the age of the animals.

2.5. Oxidative stress in the testis of weanlings

At the time of weaning, some pups from each treatment group were culled. Testes from male pups were fixed in formalin and subjected to immunohistochemistry (IHC) for 8-hydroxy deoxy guanosine (8-OHdG), a DNA oxidation product and a marker of oxidative stress (Lih-Brody et al., 1996). Four μm sections were deparaffinized in xylene and rehydrated in graded alcohol and PBS. They were subject to permeabilization in 0.25% Triton in PBS for 10 min. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide followed by blocking using PBS-Caesin for 1 h. After rinsing in PBS-tween, sections were incubated with primary antibody (8-hydroxy deoxy guanosine antibody tagged with HRP; Santa Cruz biotechnology, Dallas, TX; Cat. No.SC393871; 1:100) overnight at room temperature. They were rinsed in PBS-tween followed by PBS before adding DAB substrate (Vector labs, Burlingame, CA). The sections were counter stained with methylene blue before dehydration and coverslipping. The slides were scanned at 40x using an Aperio AT2 digital whole slide scanner (Leica Microsystems, Buffalo Grove, IL) and the images were obtained using the Aperio eSlide viewer software.

2.6. Testosterone measurement

Following euthanasia, trunk blood was collected from adult male rats, and the serum was separated and stored at −80 °C for hormone assays. Serum testosterone levels were measured by a double antibody radioimmunoassay (MP Biomedicals, Santa Ana, CA; SKU:0718910-CF) according to the manufacturer's protocol. 50 μl serum volume was used in duplicates. Values were expressed as ng/ml.

2.7. Morphometric analysis of ovaries

Four sections from the ovaries (4 μm thick, 20 mm apart) were collected and stained with hematoxylin and eosin using standard protocols. The slides were scanned at 40x using an Aperio AT2 digital whole slide scanner (Leica Microsystems, Buffalo Grove, IL) and the images were analyzed using the Aperio eSlide Manager and viewer software. The follicles were characterized as primordial, primary, secondary, tertiary, Graafian, corpus luteum, or atretic, according to Myers et al. (2004). A primordial follicle was defined as an oocyte surrounded by a single layer of squamous cells. A primary follicle was defined as an oocyte surrounded by a single layer of cuboidal cells. A secondary follicle was defined as an oocyte surrounded by multiple layers of cuboidal granulosa cells with or without antral space development. A tertiary follicle was any follicle with a confluent antral space. A Graafian follicle was an oocyte located on a cumulus oophorus containing multiple layers of granulosa cells and a single, confluent, large antral space (size of the follicle is greater than 300 μm). A corpus luteum is a dense body, composed of luteal cells. Finally, atretic follicles were degenerating follicles with inflammatory cells and macrophages. The entire section was evaluated and the different follicles and corpora lutea were counted for statistical analysis.

2.8. Statistical analysis

Prism 8.0 (GraphPad, Inc.) and R statistical software were used to perform statistical analyses. Chi-square tests of homogeneity were applied to the number of normal pregnancies, abortions, stillbirths and sex ratios. We used Tukey's post hoc analysis for multiple comparisons. In addition, in exploratory data analysis, standard checks for heterogeneity of variances were performed and necessary transformations were applied, if needed. Gestational weight gain and stillborn weights were analyzed using one-way ANOVA. Gestational weight gain by day was analyzed using repeated measures two-way ANOVA, with treatment and time as variables. Differences in stillbirth index were analyzed using the Kruskal-Wallis test. Pre-weaning litter sizes and body weights were analyzed using analysis of covariance (ANCOVA), with treatment and time as covariates.

Morphometric measures, as well as post-weaning measures including BWs, food intakes, and water intakes were analyzed using a linear mixed effect model, with treatment as a fixed effect and dam as a random effect, followed by Tukey's multiple comparisons post hoc analyses to identify differences between the control and EDC groups. Serum testosterone levels, relative organ weights (organ weights normalized to body weight), and absolute organ weights were analyzed by one-way ANOVA, with treatment as a variable. Tukey's multiple comparisons post hoc test was used to identify differences between the control and EDC groups. Finally, the number of ovarian follicles per treatment group was analyzed by one-way ANOVA, followed by Tukey's multiple comparisons post hoc test. Prism software was used for ANOVA and Chi-Square tests, and R software was used to analyze linear mixed effect models and ANCOVA tests. P-value < 0.05 was considered to indicate a statistically significant difference. Data was expressed as mean ± standard error of mean (SEM).

3. Results

3.1. Gestational and birth measurements

Fig. 2 and Table 1 provide information regarding pregnancy outcomes. In the BPF group, dams were initially treated with a dose of 5 μg/kg/day; however, this dose produced spontaneous abortions in a majority (86%) of the dams ($X^2 = 32.3$, $p < 0.0001$) (Fig. 2A). We were able to detect the abortions by closely monitoring the body weight of the dam throughout gestation. Dams treated with 5 μg/kg BPF stopped gaining weight within one week of treatment (around day 15 or 16 of pregnancy), suggesting that this dose of BPF was lethal to the developing fetus (Fig. 2B). Therefore, the BPF dose was lowered to 1 μg/kg/day for a new set of dams, and the abortion rate was reduced from 86% to 9.1% (Table 1). As a result, the gestational index increased from 14% in the high dose BPF group to 90% in the low dose BPF group (Table 1). In comparison, the gestational index was 100% in the other treatment groups.

Furthermore, dams treated with BPA, BPS, and 1 μg of BPF gained weight at rates comparable to control dams throughout the gestational period (Fig. 2C). In contrast, dams exposed to 5 μg of BPF showed a drastic decrease in gestational weight gain ($F = 6.6$; $p = 0.0003$), providing further confirmation that a majority of the pregnancies in this group resulted in abortions. Finally, BPS- and BPF (1 μg)-treated dams had stillborn pups, while the control and BPA groups had none. Stillborn pups were observed in 2 out of 13 BPS dams and 2 out of 11 low dose BPF dams (Table 1).

In terms of pre-weaning growth parameters, there were no significant effects of treatment on litter size or body weight (Table 2). There were also no differences in the sex ratio between the treatment groups (Table 2).

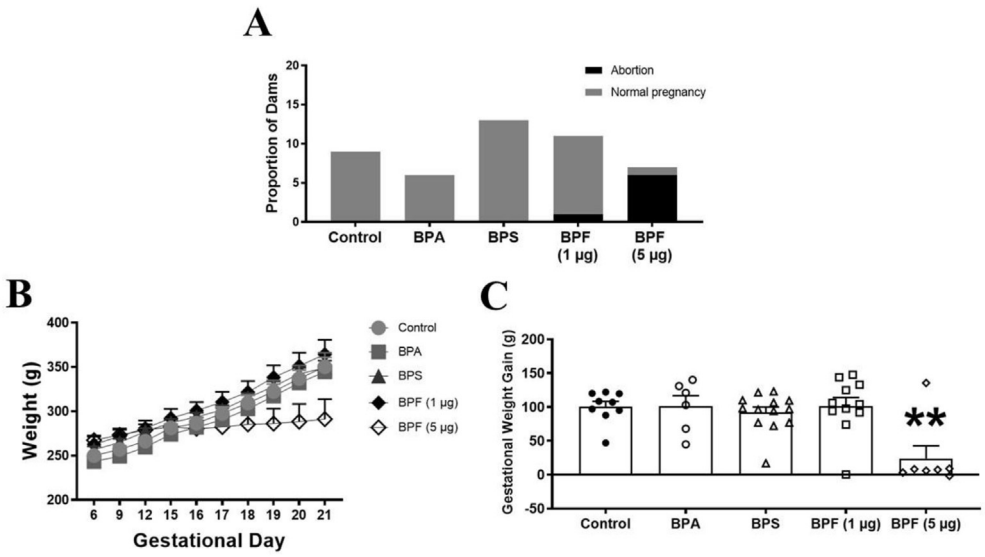

Fig. 2. Pregnancy outcomes and weight gain during gestation in dams following exposure to vehicle or bisphenols during gestational days 6–21. (*A*) Proportion of dams with normal pregnancies or abortions (complete litter loss), (*B*) gestational weight gain per day, and (*C*) overall gestational weight gain in dams treated with vehicle (control) ($n = 9$), BPA (5 µg/kg BW; $n = 6$), BPS (5 µg/kg BW; $n = 13$), high dose BPF (5 µg/kg BW; $n = 7$) or low dose BPF (1 µg/kg BW; $n = 11$) during pregnancy. **p < 0.01, one-way ANOVA, followed by Tukey's multiple comparisons between bisphenol groups and the control group.

Table 1
Pregnancy outcomes of control and treated dams.

Parameter	Control	BPA (5 µg)	BPS (5 µg)	BPF (5 µg)	BPF (1 µg)
Pregnant dams (n)	9	6	13	7	11
Normal Deliveries (n)	9	6	13	1	10
Total number of offspring (n)	83	85	160	17	133
Abortions (% of dams)	0	0	0	86.71	9.09
Stillbirths and live births					
Total live offspring (n)	83	85	158	17	130
Live birth index/dam (%)[a]	100 ± 0	100 ± 0	98.75 ± 0.88	100 ± 0	97.74 ± 1.29
% of dams with stillbirths	0	0	15.38	0	18.18
Total number of stillborn pups (n)	0	0	2	0	3
Stillbirth index/dam (%)[b]	0	0	1.25 ± 0.88	0	2.26 ± 1.29
Average stillborn weight (g)	–	–	0.95 ± 0.64	–	1.04 ± 0.70
Gestational index (%)[c]	100	100	100	14.29	90.91

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F. Measures were obtained from offspring with prenatal exposure to Vehicle (control), BPA (5 µg/kg BW), BPS (5 µg/kg BW), high dose BPF (5 µg/kg BW), or low dose BPF (1 µg/kg BW). Data are presented as mean ± SEM.
[a] Livebirth Index = (# of live pups/# of total pups born) x 100.
[b] Stillbirth Index = (# of stillborn pups/# of total pups born) x 100.
[c] Gestational Index = (# of dams with live litters/# of pregnant dams) x 100.

3.2. Morphometric measurements

Table 2 and Fig. 3A list the measurements obtained at birth from offspring of each group including head circumference, chest circumference, crown to rump length, and anogenital distance (AGD). A significant treatment effect was observed in male AGD ($p = 0.02$) (Fig. 3A). Male offspring exposed to BPS had lower AGD (0.26 ± 0.01) than control males (0.32 ± 0.02; $p = 0.02$). No significant differences were observed in female AGD or any of the other morphometric measures (Table 2).

3.3. Oxidative stress in the testis of Weanlings and adult serum testosterone

Since BPS male offspring showed a reduction in AGD, oxidative stress in the testes and serum testosterone levels were next examined. Fig. 3B depicts representative IHC images of the testis from weanlings and Fig. 3C shows serum testosterone levels from adult male rats. There was an increase in the production of 8-OHdG, a marker of oxidative stress, in BPA- and BPS-exposed testes. From their location in the germinal epithelium, it appears that the cells with marked levels of 8-OHdG are primary spermatocytes. On the contrary, control and BPF males did not show oxidative stress in the testes. No significant differences were observed in testosterone levels at postnatal weeks 16–24.

3.4. Post-weaning measurements

Table 3 displays post-weaning BWs, food and water intakes of male and female offspring at 3, 6, and 12 weeks of age. No significant differences were observed in any of the post-weaning measures.

3.5. Organ weights

Table 3 provides the relative organ weights (g/kg BW, mean ± SEM) as well as absolute organ weights (g, mean ± SEM) obtained from adult male and female offspring. Modest treatment

Table 2
Pre-weaning measures of control and prenatally-exposed offspring.

Parameter	Control		BPA		BPS		BPF (low dose)	
Litter size								
PND 1	10.38 ± 1.85		14.17 ± 1.35		12.31 ± 0.96		13.44 ± 1.22	
PND 7	9.88 ± 1.68		14.00 ± 1.39		12.08 ± 0.98		12.89 ± 1.17	
PND 14	9.75 ± 1.65		13.50 ± 0.99		12.00 ± 0.97		12.78 ± 1.13	
PND 21	9.63 ± 1.60		13.50 ± 0.99		12.00 ± 0.97		12.78 ± 1.13	
	Males	Females	Males	Females	Males	Females	Males	Females
Body weight (g)								
PND 1	16.58 ± 1.88	14.05 ± 1.81	13.48 ± 1.03	15.44 ± 1.44	17.55 ± 4.93	14.47 ± 1.31	13.49 ± 0.86	14.60 ± 1.33
PND 7	34.04 ± 3.66	31.11 ± 3.85	29.79 ± 2.93	32.98 ± 3.64	45.47 ± 13.42	32.89 ± 2.72	30.43 ± 1.95	33.87 ± 3.27
PND 14	68.24 ± 7.95	62.36 ± 6.42	53.76 ± 5.62	58.74 ± 5.96	80.95 ± 21.14	61.26 ± 4.85	55.96 ± 2.83	62.21 ± 6.29
PND 21	108.13 ± 13.22	99.66 ± 13.00	88.10 ± 9.19	96.09 ± 9.42	124.10 ± 37.25	88.88 ± 8.03	84.10 ± 7.58	94.54 ± 12.37
Sex ratio	40	43	45	39	82	77	62	59
Head circumference (cm)	3.72 ± 0.47	3.52 ± 0.45	4.08 ± 0.04	4.06 ± 0.05	3.85 ± 0.06	3.84 ± 0.07	3.87 ± 0.12	3.86 ± 0.10
Chest circumference (cm)	4.53 ± 0.10	4.40 ± 0.07	4.52 ± 0.13	4.44 ± 0.13	4.35 ± 0.06	4.30 ± 0.07	4.33 ± 0.15	4.26 ± 0.12
Crown to rump length (cm)	4.26 ± 0.55	4.00 ± 0.53	4.81 ± 0.10	4.68 ± 0.10	4.62 ± 0.08	4.53 ± 0.08	4.51 ± 0.11	4.47 ± 0.12
Anogenital Distance (cm)		0.13 ± 0.02		0.11 ± 0.00		0.11 ± 0.01		0.11 ± 0.00

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F; PND, postnatal day. Measures were obtained from offspring with prenatal exposure to Vehicle (control), BPA (5 μg/kg BW), BPS (5 μg/kg BW), or low dose BPF (1 μg/kg BW). Sex ratio, head and chest circumferences, and crown to rump lengths were determined on PND 1. Data are presented as mean ± SEM.

effects were observed in the relative weights of kidneys ($F = 3.8$; $p = 0.02$) (Fig. 4A) and epididymal adipose tissue (EAT) ($F = 3.1$; $p = 0.04$) (Fig. 4C) in male offspring. Male offspring exposed to BPA (7.15 ± 0.24) had slightly increased kidney relative weights compared to control males (6.53 ± 0.09; $p = 0.02$). Relative EAT weights were markedly reduced in male offspring exposed to BPF (6.83 ± 0.96) compared to controls (10.19 ± 0.75; $p = 0.03$).

Male offspring exposed to BPF had lower EAT absolute weights (2.88 ± 0.45; $p = 0.04$) (Table 3). On the other hand, there were no apparent effects of EDCs on female kidneys (Fig. 4B) or ovarian adipose tissue (OAT) (Fig. 4D). While there were no effects of EDC exposure on the testes, significant treatment effects were apparent in the absolute weights ($F = 3.0$; $p = 0.04$) (Fig. 5C) as well as relative weights ($F = 3.4$; $p = 0.03$) (Fig. 5D) of the prostate gland in male offspring. Male offspring prenatally exposed to BPA had significantly higher prostate gland absolute weights (1.11 ± 0.11; $p = 0.04$) and relative weights (2.54 ± 0.29; $p = 0.03$). Finally, significant treatment effects were apparent in the absolute weights ($F = 3.3$; $p = 0.04$) and relative weights ($F = 3.1$; $p = 0.04$) of the seminal vesicles, but no differences between any of the treatment groups were found following post hoc analyses (Table 3). No significant differences were observed in the other organ weights as

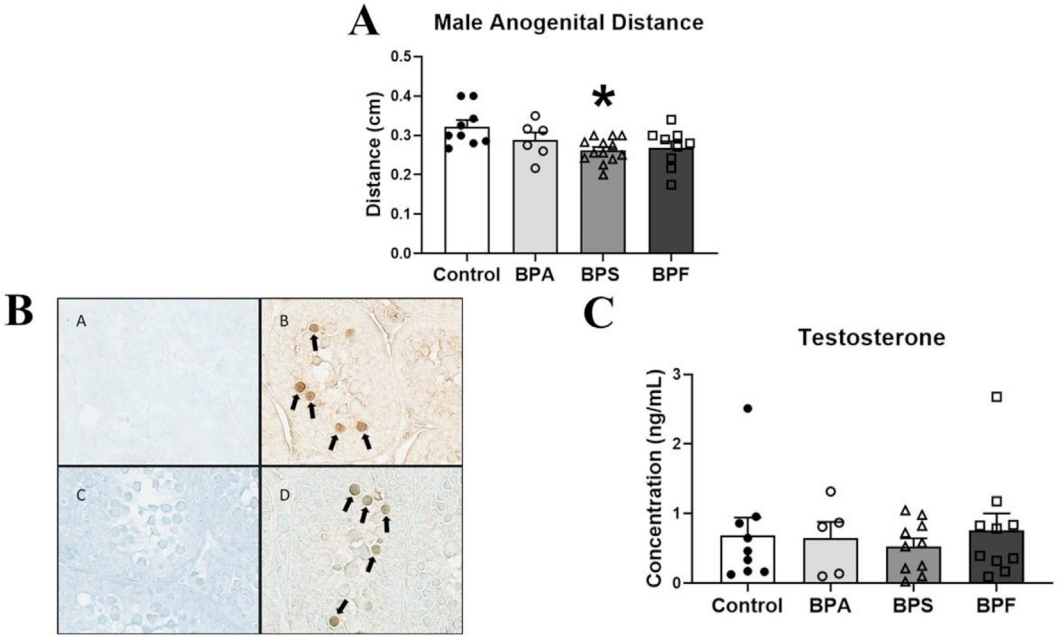

Fig. 3. Reproductive measures from male offspring with prenatal exposure to vehicle or bisphenols. (*A*) BPS exposure reduced anogenital distance in male offspring at PND 1 (Control: $n = 9$, BPA 5 μg/kg BW: $n = 6$, BPS 5 μg/kg BW: $n = 13$, BPF 1 μg/kg BW: $n = 9$). (*B*) Representative IHC images of testes sections show accumulation of 8-OHdG in the seminiferous tubules of male offspring with BPA (panel B) and BPS (panel D) exposure. (*C*) Serum testosterone levels were not significantly different between treatment groups in adulthood (Control: $n = 9$, BPA 5 μg/kg BW: $n = 5$, BPS 5 μg/kg BW: $n = 10$, BPF 1 μg/kg BW: $n = 10$). *$p < 0.05$, linear mixed effect model, followed by Tukey's multiple comparisons between control and EDC groups. Error bars represent the standard error of the mean (SEM).

Table 3
Post-weaning parameters and organ weights in male and female offspring after prenatal bisphenol exposure.

Parameter	Control		BPA		BPS		BPF (low dose)	
	Males	Females	Males	Females	Males	Females	Males	Females
Post-Weaning								
Body weights (g)								
Week 3	51.50 ± 1.01	48.09 ± 0.78	43.70 ± 0.86	42.04 ± 0.93	45.02 ± 0.92	43.86 ± 0.99	47.05 ± 1.12	44.01 ± 1.01
Week 6	189.80 ± 1.57	148.98 ± 1.46	178.36 ± 1.98	139.51 ± 1.40	178.25 ± 2.09	139.13 ± 1.45	180.45 ± 2.24	142.61 ± 1.47
Week 12	359.74 ± 4.25	235.97 ± 4.95	347.97 ± 3.10	215.57 ± 3.67	357.85 ± 3.10	232.63 ± 2.64	345.32 ± 4.02	235.87 ± 3.49
Food intakes (g/day)								
Week 3	9.80 ± 0.22	8.93 ± 0.13	9.07 ± 0.30	8.51 ± 0.23	9.62 ± 0.16	8.74 ± 0.16	9.54 ± 0.13	8.66 ± 0.12
Week 6	22.34 ± 0.31	17.02 ± 0.45	23.18 ± 0.25	16.44 ± 0.20	21.64 ± 0.41	15.60 ± 0.19	21.53 ± 0.31	15.96 ± 0.22
Week 12	23.58 ± 0.20	18.25 ± 0.68	24.31 ± 0.24	17.37 ± 0.20	23.42 ± 0.23	16.13 ± 0.25	22.19 ± 0.29	17.02 ± 0.24
Water intakes (mL/day)								
Week 3	14.77 ± 0.75	14.03 ± 0.68	13.40 ± 0.49	14.82 ± 0.49	14.74 ± 0.30	14.04 ± 0.26	15.51 ± 0.31	13.68 ± 0.22
Week 6	35.69 ± 0.80	28.70 ± 0.48	30.80 ± 2.12	29.49 ± 0.57	33.31 ± 0.68	27.17 ± 0.42	33.96 ± 0.40	28.07 ± 0.45
Week 12	37.17 ± 0.33	32.35 ± 0.41	37.70 ± 1.12	33.03 ± 0.46	37.10 ± 0.53	32.74 ± 0.60	36.31 ± 0.53	33.23 ± 0.82
Relative Organ Weights (g/kg BW)								
Pituitary gland	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
Thymus	0.72 ± 0.10	0.95 ± 0.13	0.52 ± 0.03	0.58 ± 0.03	0.61 ± 0.07	0.82 ± 0.08	0.76 ± 0.10	0.94 ± 0.12
Heart	3.48 ± 0.11	3.92 ± 0.14	3.54 ± 0.12	4.33 ± 0.38	3.70 ± 0.18	4.21 ± 0.31	3.38 ± 0.11	3.80 ± 0.13
Lungs	4.41 ± 0.16	6.03 ± 0.48	4.73 ± 0.30	5.76 ± 0.32	4.46 ± 0.11	4.65 ± 0.27	4.61 ± 0.24	5.65 ± 0.28
Spleen	1.68 ± 0.06	2.18 ± 0.12	1.68 ± 0.12	1.93 ± 0.10	1.66 ± 0.04	2.16 ± 0.07	1.71 ± 0.03	2.19 ± 0.07
Liver	28.57 ± 1.46	27.09 ± 0.95	30.89 ± 1.40	28.16 ± 1.14	29.19 ± 1.52	28.26 ± 1.39	31.10 ± 1.72	26.37 ± 0.92
Adrenal glands	0.16 ± 0.01	0.28 ± 0.01	0.19 ± 0.02	0.28 ± 0.02	0.16 ± 0.01	0.27 ± 0.02	0.17 ± 0.01	0.31 ± 0.02
Testes and epididymis	14.42 ± 0.43	–	14.26 ± 0.22	–	14.04 ± 0.40	–	14.56 ± 0.24	–
Seminal vesicles	4.04 ± 0.34	–	4.15 ± 0.22	–	3.14 ± 0.23	–	3.30 ± 0.32	–
Uterus + ovaries	–	2.52 ± 0.19	–	2.64 ± 0.11	–	2.72 ± 0.19	–	2.36 ± 0.15
Abdominal adipose tissue	4.89 ± 0.54	2.37 ± 0.30	3.31 ± 0.84	2.28 ± 0.30	3.32 ± 0.3[a]	2.85 ± 0.41	3.50 ± 0.33	2.51 ± 0.29
Perirenal adipose tissue	1.81 ± 0.17	1.89 ± 0.60	1.52 ± 0.24	1.41 ± 0.28	1.39 ± 0.14	1.89 ± 0.17	1.32 ± 0.11	1.64 ± 0.31
Absolute Organ Weights								
Pituitary gland (mg)	11.74 ± 0.62	12.16 ± 0.44	11.90 ± 0.67	11.58 ± 0.50	11.37 ± 0.62	11.30 ± 0.58	11.12 ± 0.42	11.06 ± 0.74
Thymus (g)	0.30 ± 0.04	0.25 ± 0.03	0.23 ± 0.01	0.17 ± 0.01	0.26 ± 0.03	0.20 ± 0.02	0.30 ± 0.04	0.24 ± 0.03
Heart (g)	1.50 ± 0.06	1.05 ± 0.04	1.56 ± 0.07	1.09 ± 0.04	1.58 ± 0.08	1.04 ± 0.06	1.39 ± 0.07	0.99 ± 0.03
Lungs (g)	1.90 ± 0.07	1.60 ± 0.13	2.09 ± 0.16	1.62 ± 0.10	1.90 ± 0.04	1.51 ± 0.08	1.88 ± 0.08	1.49 ± 0.09
Spleen (g)	0.73 ± 0.04	0.57 ± 0.04	0.74 ± 0.06	0.54 ± 0.03	0.71 ± 0.02	0.54 ± 0.01	0.70 ± 0.02	0.57 ± 0.01
Kidneys (g)	2.83 ± 0.12	1.69 ± 0.05	3.16 ± 0.15	1.85 ± 0.05	2.83 ± 0.12	1.63 ± 0.06	2.74 ± 0.12	1.65 ± 0.06
Liver (g)	12.52 ± 1.02	7.23 ± 0.33	13.66 ± 0.86	7.95 ± 0.40	12.68 ± 1.02	7.08 ± 0.37	12.88 ± 1.01	6.35 ± 0.58
Adrenal glands (mg)	69.41 ± 4.12	74.41 ± 3.51	82.42 ± 7.68	79.92 ± 4.92	66.37 ± 4.40	67.66 ± 5.01	70.18 ± 3.98	81.51 ± 5.17
Testes and epididymis (g)	6.21 ± 0.15	–	6.28 ± 0.14	–	5.96 ± 0.11	–	5.96 ± 0.18	–
Seminal vesicles (g)	1.68 ± 0.17	–	1.81 ± 0.11	–	1.33 ± 0.11	–	1.36 ± 0.11	–
Uterus + ovaries (g)		0.66 ± 0.04		0.75 ± 0.05		0.68 ± 0.04		0.62 ± 0.04
Abdominal adipose tissue (g)	2.13 ± 0.25	0.64 ± 0.08	1.43 ± 0.36	0.65 ± 0.09	1.45 ± 0.16	0.72 ± 0.11	1.46 ± 0.17	0.67 ± 0.09
Epididymal/ovarian adipose tissue (g)	4.43 ± 0.37	1.77 ± 0.20	3.87 ± 0.30	1.47 ± 0.26	3.53 ± 0.37	1.70 ± 0.16	2.88 ± 0.45*	1.61 ± 0.16
Perirenal adipose tissue (g)	0.80 ± 0.10	0.51 ± 0.16	0.67 ± 0.10	0.40 ± 0.09	0.61 ± 0.08	0.48 ± 0.05	0.55 ± 0.06	0.43 ± 0.08

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F; BW, body weight. Measures were obtained from offspring with prenatal exposure to Vehicle (control), BPA (5 μg/kg BW), BPS (5 μg/kg BW), or low dose BPF (1 μg/kg BW). Data are presented as mean ± SEM.
*$p < 0.05$, one-way ANOVA, followed by Tukey's multiple comparisons between control and BPF males.
[a] $p = 0.07$, one-way ANOVA, followed by Tukey's multiple comparisons between control and BPS males.

well (Table 3).

3.6. Ovarian morphology

Figs. 6 and 7 depict changes in ovarian structures observed in each treatment group. A non-significant trend for a treatment effect was apparent in the number of Graafian follicles ($F = 2.5$; $p = 0.08$) (Fig. 6A). A trend for increased number of Graafian follicles was apparent in the BPA group compared to control (2.6 ± 0.43 vs. 0.7 ± 0.47; $p = 0.05$). Additionally, a significant treatment effect was found in the number of corpora lutea (CL) ($F = 6.1$; $p = 0.002$) (Fig. 6C). Prenatal exposure to BPS (9.5 ± 2.41; $p = 0.01$) significantly reduced the number of CL when compared to control offspring (20.1 ± 2.26), and BPF (11.9 ± 1.35) had the same effect, but it did not reach statistical significance ($p = 0.07$). BPA offspring did not show any differences in CL compared to controls.

Finally, there was a significant treatment effect ($F = 3.4$; $p = 0.03$) in the number of primary follicles (Table 4); however, post hoc analyses revealed no significant differences between treatment groups. No significant differences were observed in the number of atretic follicles (Fig. 6B), old CL (Fig. 6D), primordial, secondary, or tertiary follicles (Table 4).

4. Discussion

The results of this study indicate for the first time that BPS and BPF even at low doses were capable of producing significant effects on the reproductive system. While BPF at doses comparable to BPA and BPS (5 μg/kg BW) induced spontaneous abortions in pregnant dams, lower doses of BPF (1 μg/kg BW) still induced abortions, but at a significantly lower rate. Both BPF and BPS decreased the number of CL in the ovaries suggesting a possible reduction in ovulation. BPA on the other hand, produced a modest increase in Graafian follicles. In male offspring, BPS decreased the anogenital distance in male offspring which could indicate possible feminization or compromised testicular function while BPF reduced epididymal adipose tissue weights. Moreover, exposure to BPA increased kidney and prostate gland weights. Taken together, it appears that prenatal exposure to low doses of BPA or its analogues is capable of inducing reproductive toxicity both in male and female offspring.

Our findings regarding the gestational outcomes are among the

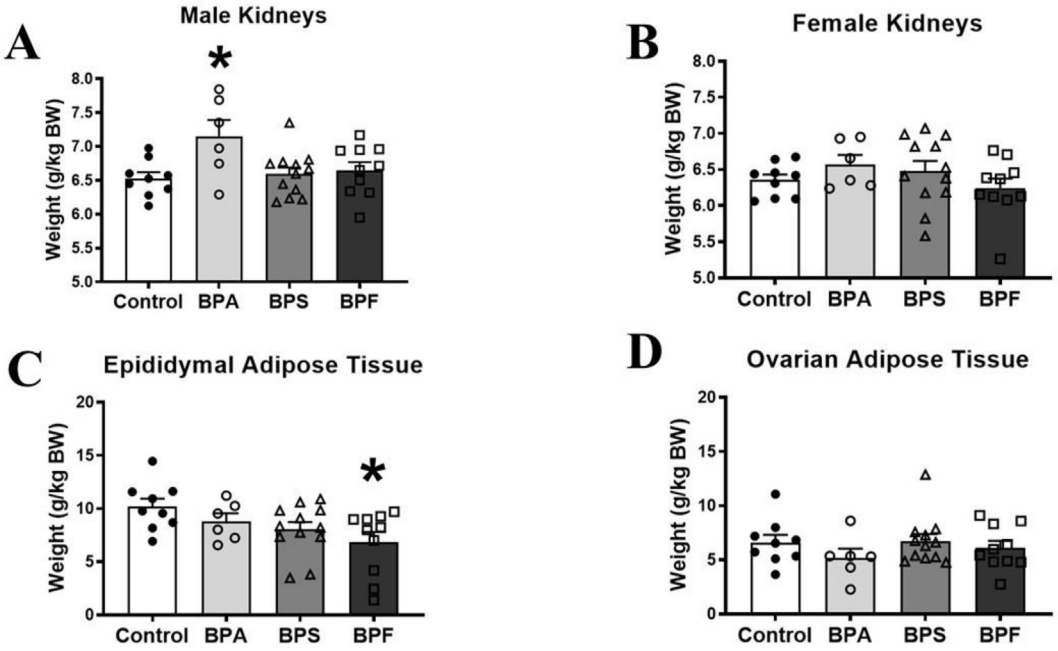

Fig. 4. Kidney and gonadal adipose tissue weights of adult offspring exposed to vehicle (control) ($n = 9$), BPA (5 μg/kg BW; $n = 6$), BPS (5 μg/kg BW; $n = 12$), or low dose BPF (1 μg/kg BW; $n = 10$) *in utero*. We used data from only 12 offspring in the BPS group because 1 BPS-treated mom had only 4 offspring which were used in related studies and were not included here. BPA exposure increased relative weights of the kidneys (*A*) and BPF exposure decreased relative weights of the epididymal adipose tissue (*C*) in male offspring. In contrast, no changes were observed in female organ weights, including kidneys (*B*) or ovarian adipose tissue (*D*). *$p < 0.05$, one-way ANOVA, followed by Tukey's multiple comparisons between control and EDC groups. Error bars represent the standard error of the mean (SEM).

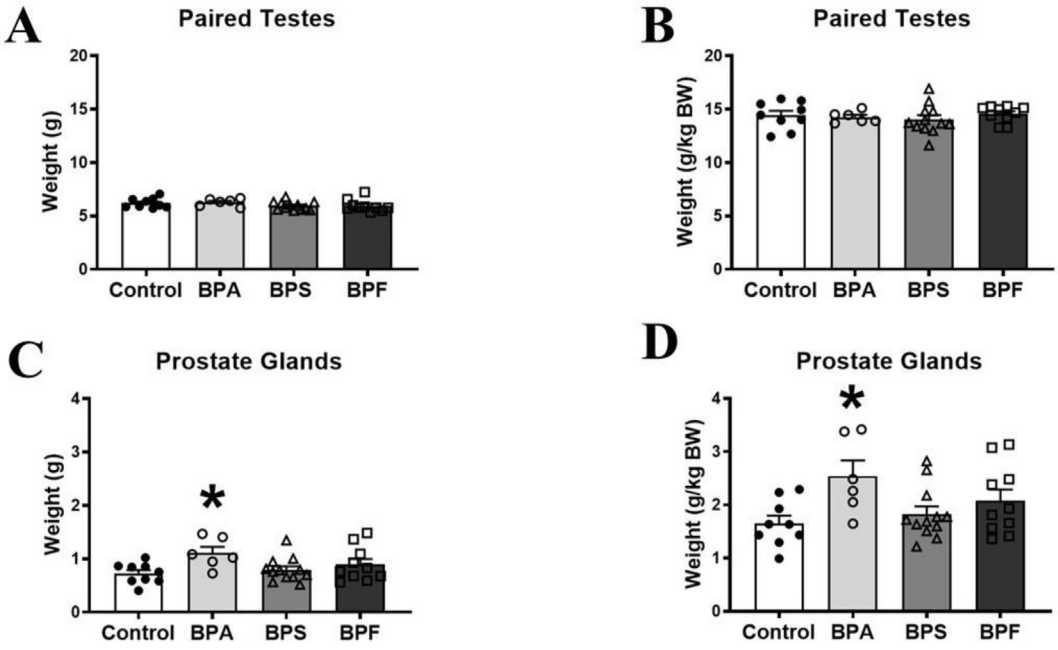

Fig. 5. Paired testes and prostate gland weights of adult male offspring exposed to EDCs *in utero*. Prenatal EDC exposure did not alter absolute weight (*A*) or relative weight (*B*) of the testes. However, prenatal exposure to BPA increased prostate gland absolute weights (*C*) and prostate gland relative weights (*D*). Organs were harvested from male offspring prenatally exposed to vehicle (control) ($n = 9$), BPA (5 μg/kg BW; $n = 6$), BPS (5 μg/kg BW; $n = 12$), or BPF (1 μg/kg BW; $n = 10$) when they were 16–24 weeks of age. *$p < 0.05$, one-way ANOVA, followed by Tukey's multiple comparisons between control and EDC groups. Error bars represent the standard error of the mean (SEM).

most insightful results of this study. We discovered that even a dose as low as 5 μg/kg/day of BPF could lead to abortions in 86% of the dams, indicating its high potential for reproductive toxicity. Lowering the dose to 1 μg/kg/day produced a dose-dependent reduction in the abortion rate, indicating that this is a very real effect. Since the dams were group housed, it is likely that they were exposed to additional BPF that was excreted in the urine and feces of their cage-mates. Also, the estrogenic potential of BPF is similar to or slightly greater than that of BPA (Rochester and Bolden, 2015), probably contributing to its ability to induce abortions. Human

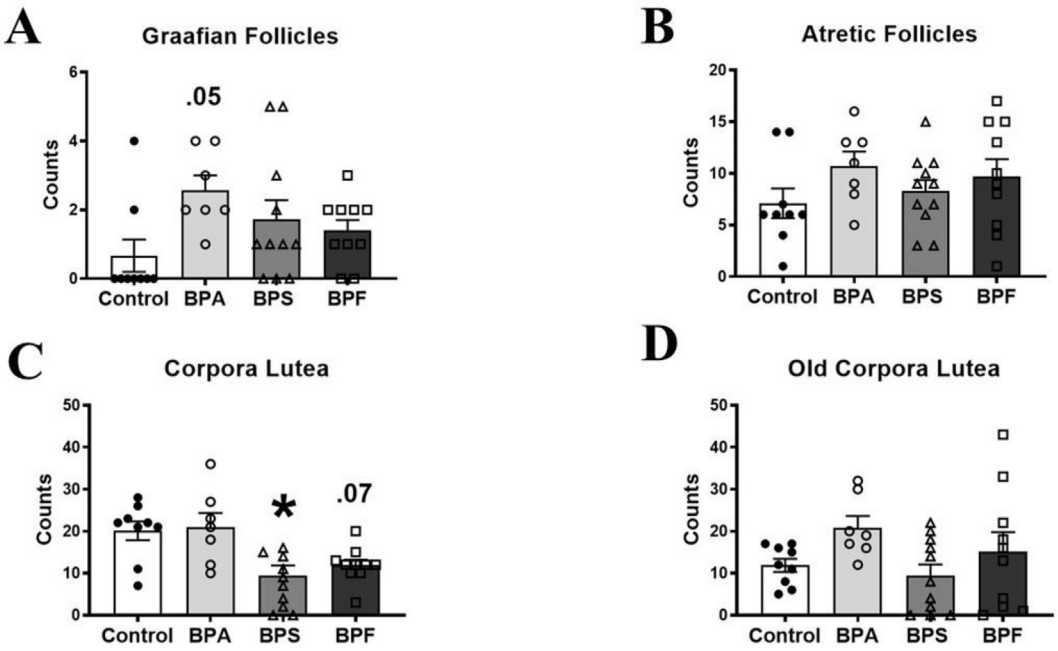

Fig. 6. Ovarian follicle counts from adult female offspring exposed to BPA, BPS or BPF *in utero*. Numbers of Graafian follicles (*A*) atretic follicles (*B*), CL (*C*) and old CL (D) from female offspring after prenatal exposure to vehicle (control) ($n = 9$), BPA (5 μg/kg BW; $n = 7$), BPS (5 μg/kg BW; $n = 11$), or low dose BPF (1 μg/kg BW; $n = 10$). Data were obtained from ovaries of adult female offspring sacrificed when they were in diestrus. *$p < 0.05$, one-way ANOVA, followed by Tukey's multiple comparisons between BPS and the control group. Error bars represent the standard error of the mean (SEM).

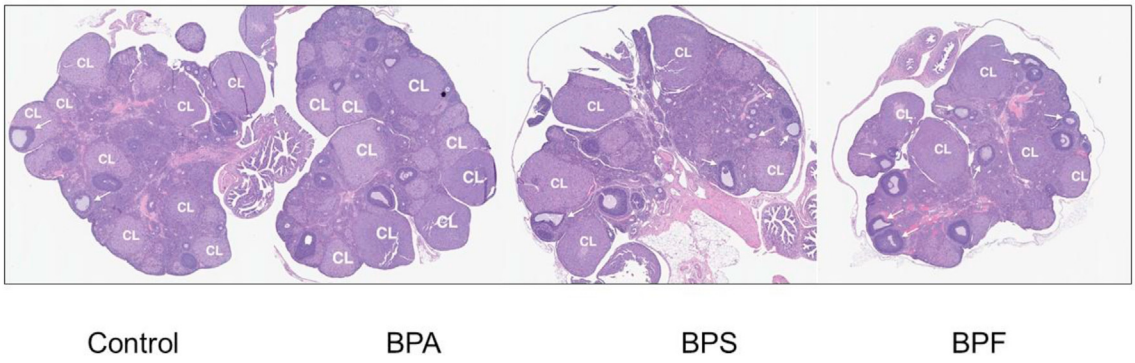

Fig. 7. Representative sections of the ovary from the different treatment groups. CL: corpus luteum; arrows indicate atretic follicles. Ovaries were obtained from adult female offspring sacrificed when they were in the state of diestrus.

Table 4
Ovarian follicle counts from adult female offspring with prenatal EDC exposure.

Ovarian Follicle	Control	BPA (5 μg)	BPS (5 μg)	BPF (1 μg)
Primordial follicles	23.56 ± 3.72	33.43 ± 4.27	31.00 ± 2.91	25.80 ± 5.36
Primary follicles	8.89 ± 1.95	12.00 ± 2.26	5.18 ± 1.15	6.70 ± 0.99
Secondary follicles	15.44 ± 3.75	20.29 ± 3.36	14.18 ± 2.34	12.80 ± 2.40
Tertiary follicles	4.78 ± 1.15	5.00 ± 1.09	7.91 ± 1.07	4.90 ± 0.84

Note: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F. Data are presented as mean ± SEM. Ovarian follicle counts were obtained from female offspring with prenatal exposure to vehicle, 5 μg/kg BPA, 5 μg/kg BPS, or 1 μg/kg BPF. Data were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons post hoc analyses for differences between control and EDC groups.

studies have associated BPA exposure with recurrent spontaneous abortions (Lathi et al., 2014), and rodent studies further show that gestational BPA exposure at doses of 5–40 mg/kg/day are correlated with increased rates of abortion in mice (Tachibana et al., 2007; Wei et al., 2019). Recent studies investigating the mechanisms by which BPA produces abortions indicate that BPA may disrupt blastocyst formation and increase generation of reactive oxygen species that contribute to mitochondrial and DNA damage in the developing embryo (Guo et al., 2017). In rats, the first heart beat is evident on day 11 of gestation and certain organs such as the lung and liver are detectable with ultrasound from day 16 (Kirberger et al., 2019). Since the dams began to lose weight by GD15, it is safe to say that BPF produces an acute toxic effect on the developing vital organs in the embryo leading to abortions. Interestingly, BPA and BPS did not cause abortions at the same dose indicating that BPF is a more potent developmental toxicant. Further studies are needed to determine the possible underlying mechanisms.

Besides inducing spontaneous abortions, BPF along with BPS also decreased the number of corpora lutea (CL). This is the first report to indicate a reduction in CL number with prenatal BPS and BPF exposure which would suggest inhibition of ovulation (Sirivelu et al., 2009). Ovulation is a complex process that involves a number

of hypothalamic neurotransmitters, releasing hormones and pituitary hormones. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) play an important role in ovulation and it is possible that prenatal exposure to BPF and BPS inhibits their secretion. Another reason for the reduction of these hormones could be the fact that BPF and BPS have higher progestogenic activity than BPA (Rosenmai et al., 2014). Progesterone is known to suppress both FSH and LH secretion (Messinis, 2006). Since the exposure to these EDCs occurred *in utero*, it is likely that they altered the expression of hormone receptors that manifest in adulthood possibly as reduced secretion of LH and FSH and lower rates of ovulation. In contrast to BPS and BPF, prenatal exposure to BPA increases the number of Graafian follicles. It is possible that BPA could have stimulated the hypothalamic-pituitary-gonadal axis due to its weak estrogenic action (Matuszczak et al., 2019). It will be useful to assess the levels of gonadotrophic and gonadal hormones in these offspring.

Even though EDC-induced changes did not affect other morphometric parameters, we observed a modest reduction in the AGD of male offspring prenatally exposed to BPS. No prior studies to our knowledge have explored the relationship between prenatal BPS exposure and AGD in the offspring. Perinatal treatment with BPA at low (50 μg/kg) (El Henafy et al., 2020) and high doses (0.25–50 mg/kg) (Christiansen et al., 2014) have been shown to decrease AGD in male offspring. This effect has been observed in humans as well (Miao et al., 2011; Sun et al., 2018), causing further concern about BPA exposure during gestation. AGD is a sensitive biomarker of fetal androgen exposure and could predict testicular development and function in later life (Thankamony et al., 2016; Freire et al., 2018). A reduction in AGD suggests feminization of the male reproductive tract (Welsh et al., 2008). However, we did not observe any significant changes in serum testosterone levels in adult animals. Interestingly, we observed an increase in the accumulation of 8-OHdG in the BPA and BPS-exposed groups, indicative of oxidative stress (Valavanidis et al., 2009) possibly in primary spermatocytes within the seminiferous tubules. These changes were apparent in male offspring at the time of weaning. Further studies are needed to determine if prenatal exposure to BPA and BPS do alter the male reproductive system/function in adult animals.

Male offspring exposed to BPA had higher relative kidney weights compared to control males. This is supported by another study that used much higher doses of BPA and found increases in the weight of the liver, adrenal, spleen, pituitary and brain besides the kidney (Tyl et al., 2002). The reason for the increase in kidney weight is not clear, however, it could suggest altered tissue function as seen in diabetes or obesity (Maric-Bilkan, 2013). Other than the kidney, prenatal exposure to BPA also increased the weight of the prostate gland. This is supported by studies in mice where gestational exposure to BPA at low doses of 2–50 μg/kg/day (Nagel et al., 1997; Gupta, 2000; Timms et al., 2005) increased prostate weights. This is especially concerning because prenatal BPA exposure was found to increase the risk for prostate cancer in rats (Prins et al., 2017) and pre-cancerous lesions of the prostate have been observed in male rats after prenatal exposure to BPA (Ho et al., 2006).

While prenatal exposure to BPA increased kidney and prostate gland weights, exposure to BPS significantly reduced epididymal fat weight without affecting other fat depots. This is in contrast to another study that found lower visceral adipose tissue mass in male offspring only due to reduced food intake (da Silva et al., 2019). We did not observe any change in food intake in male or female offspring after prenatal BPS exposure. Further analysis of the metabolic parameters are essential to determine why other fat depots were not affected with BPS exposure.

Finally, differences in the rodent models used, doses of EDCs, and duration of exposure (prenatal vs. perinatal) could have all contributed to the differences in observations in terms of organ weights compared to previous studies (Ullah et al., 2019) (Tyl et al., 2002). In addition, a major limitation of our study was that the pregnant dams were group housed while receiving EDC treatment. We did not control for any potential contamination of the housed animals with bisphenol metabolites that may have been released through urine or feces. However, all animals used in this experiment were housed in similar cages and were provided water in glass bottles. Therefore, any bisphenol exposure from the environment would have been similar across treatments. The only way to examine this is to measure the bisphenol levels in the animals and determine any differences between EDC-exposed animals and control animals.

Regardless, results from this study provide robust associations between prenatal programming with low doses of BPF or BPS and adverse effects on gestational outcomes, offspring morphometry and changes in organ weights that are apparent in adulthood. These results are concerning because BPF and BPS appear to exert actions different from those of BPA, and require further investigation. To our knowledge, this is the first study to address the sex-specific differences in developmental parameters of male and female offspring with prenatal low-dose BPS and BPF exposures. These studies underline the need to revisit current regulatory practices on EDCs with the hope that they are appropriately modified to protect public health.

Credit author statement

Amrita Kaimal: Writing – original draft, Investigation, Formal analysis, Visualization. Maryam H. Al Mansi: Investigation, Data curation, Validation. Josephine Bou Dagher: Investigation, Formal analysis. Catherine Pope: Investigation, Data curation. Marissa G. Varghese: Investigation, Data curation. Thomas B. Rudi: Investigation, Data curation. Ansley E. Almond: Investigation, Data curation. Loren A. Cagle: Investigation, Data curation. Hermela K. Beyene: Investigation, Data curation. William T. Bradford: Investigation, Data curation. Benjamin B. Whisnant: Investigation, Data curation. Baobsom D.K. Bougouma: Investigation, Data curation. Karim J. Rifai: 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 & editing. Sheba M.J. MohanKumar: Conceptualization, Methodology, Resources, Supervision, Funding acquisition, Writing – review & 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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