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Title: Developmental Programming: Sex specific programming of growth upon prenatal bisphenol A exposure

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Abstract:

In both human and animals, in utero exposure to bisphenol A (BPA), an endocrine disrupting chemical used in the production of plastics and epoxy resins, has been shown to affect offspring reproductive and metabolic health during adult life. We hypothesized that the effect of prenatal exposure to environmentally relevant doses of BPA will be evident during fetal organogenesis and fetal / postnatal growth trajectory. Pregnant ewes were administered BPA subcutaneously from days 30-90 days of gestation (term 147 days). Fetal organ weight, anthropometric measures, maternal / fetal hormones and postnatal growth trajectory were measured in both sexes. Gestational BPA administration resulted in higher accumulation in male than female fetuses only at fetal day 65, with minimal impact on fetal / maternal steroid milieu in both sexes at both time points. BPA-treated male fetuses were heavier than BPA-treated female fetuses at fetal day 90 whereas this sex difference was not evident in the control group. At the organ level, liver weight was reduced in prenatal BPA-treated female fetuses, while heart and thyroid gland weights were increased in BPA-treated male fetuses relative to their sex-matched control groups. Prenatal BPA treatment also altered postnatal growth trajectory in a sex-specific manner. Males grew slower during the early postnatal period, caught up later. Females, in contrast, demonstrated the opposite growth trend. Prenatal BPA-induced changes in fetal organ differentiation and early life growth strongly implicate translational relevance of in utero contributions to reproductive and metabolic defects previously reported in adult female offspring.

Short abstract (80 words)

Prenatal exposure to bisphenol A (BPA) from early to mid-gestation leads to sex specific changes in fetal organ weight and postnatal growth.

Introduction

Intrauterine growth restriction (IUGR) is defined as birth weight and / or length below the 10th percentile for gestational age secondary to pathological restriction of fetal growth *in utero* (Kanaka-Gantenbein et al., 2003; Romo et al., 2009). Incidence of IUGR in newborns of term pregnancies ranges from 3 to 7% (Romo et al., 2009). IUGR followed by catch up growth has been associated with chronic disease later in life, including

development of hypertension, impaired glucose tolerance, type 2 diabetes and lipid abnormalities (Barker, 2004; Valsamakis et al., 2006). Genetic factors account for one third of human IUGR births, and two thirds are thought to be secondary to adverse *in utero* environment (Valsamakis et al., 2006), including infection, disease states, nutritional deficit/excess, maternal stress, endocrine abnormalities, smoking or alcohol consumption, and exposure to environmental chemicals (Hakim et al., 2017).

Programming of IUGR can be mediated by alteration of the feto-maternal hormonal milieu (Fowden et al., 2006). Since steroid hormones orchestrate the dialogue between the uterine environment and the fetus, inappropriate exposure to sex steroids, native or environmental, can reprogram fetal development (Padmanabhan & Veiga-Lopez, 2011). Additionally, alterations in the gestational hormonal milieu can affect postnatal growth of the IUGR fetus leading to catch up growth and permanent defects throughout the lifespan of the individual. Such reprogramming of pre- and postnatal growth has been linked to cardio-metabolic and reproductive dysfunction (Manikkam et al., 2004; Ong et al., 2000; Ong & Dunger, 2002).

In recent years, endocrine disrupting chemicals (EDC) that mimic both estrogenic and androgenic actions have received considerable attention relative to pregnancy outcomes and origin of human disease (Veiga-Lopez et al., 2015; Veiga-Lopez et al., 2018; Vom Saal et al., 2012). One such ubiquitous EDC with estrogenic and anti-androgenic actions is bisphenol A (BPA), used in the manufacture of plastics and epoxy resins used in consumer products (vom Saal & Hughes, 2005). Worldwide, >7 billion pounds of BPA are produced annually, generating 1 million dollars per day in revenue for corporations (Erler & Novak, 2010). Human exposure to BPA occurs through dust, air and water (Vandenberg et al., 2013). Regulatory agencies, such as the Environmental Protection Agency, the Federal Drug Administration, and the National Toxicology Program have set different threshold doses of BPA (50, 5, and 0.5 mg/kg/day, respectively (Beronius et al., 2010; Erler & Novak, 2010) below which adverse effects are not present. BPA has been found in maternal circulation, amniotic fluid, adipose tissue, liver, placenta, and breast milk (Calafat et al., 2009; Ikezuki et al., 2002; Sun et al., 2004). There are, however, only a handful of human prospective studies that have assessed birth outcomes following prenatal BPA exposure (Snijder et al., 2013). Our recent study found that elevated first trimester BPA concentrations are associated with increased oxidative stress (Veiga-Lopez, Pennathur, et al., 2015) leading to low birth weight in female, but not male offspring (Veiga-Lopez et al., 2015). The fact that higher maternal BPA concentrations at

term were not associated with low birth weight (Padmanabhan et al., 2008; Veiga-Lopez et al., 2015) in these human studies emphasizes the importance of windows of susceptibility, particularly during pregnancy. Other epidemiological studies have found prenatal BPA exposure to be positively associated with offspring BMI in early to mid-childhood (Vafeiadi et al., 2016; Yang et al., 2017), also a risk factor for adult origins of disease.

Animal models have been used to address BPA's effect on pregnancy and offspring outcomes to establish causality. Studies with precocial models, exhibiting developmental trajectories similar to humans, are essential for establishing causality of developmentally programmed pathologies that can be translated into novel human therapies. Sheep models, in particular, have been extensively used to assess pregnancy outcomes and developmental origin of diseases stemming from inappropriate exposure to native steroids and environmental steroid mimics (Padmanabhan et al., 2010). Specifically, prenatal exposure to 5 mg/kg/day BPA from day 30-90 days of gestation (term: ~147 days), resulting in maternal BPA concentrations 2-fold higher than the highest values observed in pregnant U.S. women (Padmanabhan et al., 2008; Savabieasfahani et al., 2006), deliver low birth weight female offspring exhibiting reproductive cycle and metabolic dysfunction, including insulin resistance, adipocyte hypertrophy, and elevated pro-inflammatory markers in adipose tissue (Abi Salloum et al., 2013; Savabieasfahani et al., 2006; Veiga-Lopez et al., 2014).

Considering the non-monotonic dose responses that have been observed relative to the impact of BPA (Vandenberg et al., 2014), as well as the limited number of reports addressing the early impact of BPA in precocial animals, the primary objectives of the present study were to 1) determine BPA concentrations achieved in both mother and fetus following gestational exposure to BPA at doses spanning human exposure levels, and 2) assess the effects of prenatal BPA exposure on birth and organ weights, and postnatal growth trajectory, key biomarkers for assessing risk of adult onset disease. Since steroid hormones are major regulators of growth trajectory, and BPA alters steroidogenesis (Bloom et al., 2016; Peretz et al., 2014), our third objective was to determine the effects of prenatal BPA exposure on maternal/fetal steroid hormone milieu and its association with fetal organ weights.

Material and Methods

The study was conducted at the University of Michigan Sheep Research Facility (Ann Arbor, MI; 42º 18´N). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Michigan and are consistent with the National Institutes of Health Guide for Use and Care of Animals. Healthy adult Suffolk sheep between the ages of 3 and 5 years were purchased from local farmers and used for generating control and prenatal BPA-treated animals. Animals were blocked by maternal weight and body score and distributed between control and BPA-treated groups. Details regarding animal maintenance, breeding, and lambing have been described previously (Veiga-Lopez et al., 2016). Briefly, ewes were group-fed daily with 0.5 kg shelled corn and 1.0-1.5 kg alfalfa hay/ewe and mated to raddled Suffolk rams. Pregnant ewes maintained good health throughout pregnancy. They were weighed weekly and mean weight gain during pregnancies were determined. After weaning at ~8 weeks of age, lambs were maintained outdoors and fed a pelleted diet (Shur-Gain, Nutreco Canada Inc., Guelph, Canada) comprised of 3.6 MCal/kg digestible energy and 18% crude protein.

Two studies were conducted. Study 1 addressed the long-term consequences of prenatal BPA treatment and therefore offspring were maintained from birth to adulthood. Study 2 focused on determining maternal and fetal milieu and fetal organ weights. For this study only the mid dose (0.5 mg/kg/day) was selected as this was more environmentally relevant.

Study 1: Effects of prenatal BPA on birth weight and postnatal growth trajectory

Gestational BPA treatment consisted of daily subcutaneous (s.c.) BPA injections into dams of 0.05 (low), 0.5 (medium), or 5 (high) mg/kg/day of BPA (purity ≥99%, cat# 239658; Aldrich Chemical Co., Milwaukee, WI) in corn oil from days 30 through 90 of gestation (term: ~ 147 days). Control (C) dams received corn oil injections, alone. Umbilical arterial BPA concentrations using the medium dose are already published (Veiga-Lopez et al., 2013) and approximate maternal BPA blood levels reported for U.S. women (Padmanabhan et al., 2008). Offspring were delivered by 14, 21, 26 and 22 dams, respectively, in control, low, medium and high BPA treatment groups. Final

experimental group sizes for control, low, medium and high BPA treatments were 8, 12, 13 and 7, respectively for female offspring, and 6, 9, 13 and 15 for male offspring.

Newborn samples were collected by jugular venipuncture in sodium fluoride/potassium oxalate tubes (BD Vacutainer, NJ, USA) and plasma frozen at -20 °C until assayed for glucose and insulin. From day of birth to 11 weeks of age, females and male offspring were weighed weekly, after which males were not maintained to avoid unwanted pregnancies in the flock. After 11 weeks, female weights were taken weekly until ~6 months of age, and monthly thereafter until they were ~20 months old.

Study 2: Effect of prenatal BPA on maternal and fetal steroid, hormones, and organ weights

To determine the early effects of gestational BPA treatment, dams received daily s.c. injections of either the medium BPA dose (0.5 mg/kg/day), or corn oil (controls, C). This medium BPA dose achieved circulating levels of free BPA of ~2.6 ng/mL in umbilical artery of female fetus, approximating median values found in urine of pregnant women and cord blood in human studies (Veiga-Lopez et al., 2013). Dams from C and BPA groups were anesthetized on gestational day 65 (GD65) or 90 (GD90) with ketamine hydrochloride (Fort Dodge, Iowa; 3-4 mg/kg, i.v.) and xylazine (Anased Injection, Shenandoah, Iowa, USA; 0.4 mg/kg/ewe, i.m.), and subsequently maintained under general anesthesia with isoflurane (RxElite Holdings Inc., Meridian, Indiana, USA). Umbilical arterial and venous samples were collected after accessing the gravid uterus via the midline and uterine wall incisions, as previously described (Veiga-Lopez et al., 2011). Samples were frozen until assayed for free and total BPA, androstenedione, cortisol, dehydroepiandrosterone (DHEA), deoxycorticosterone (DOC), 11-deoxycortisol, estradiol, estrone, glucose, insulin, pregnenolone, 17-hydroxypregnenolone, 17-hydroxyprogesterone and testosterone (T).

Fetuses were then removed and euthanized by intra-cardiac barbiturate administration. Fetal body measures were undertaken and tissues procured. After removal of fetuses, mothers were also euthanized with a barbiturate overdose (15 ml, i.v. Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). Group sizes for gestational day 65 comprised: BPA, 9 female and 11 male fetuses delivered by 10 dams; C, 9 female and 6 male fetuses delivered by 8 dams. Gestational day 90 groups comprised: BPA, 9 female and

4 male fetuses delivered from 7 dams, C, 8 female and 10 male fetuses delivered from 9 dams. Fetal measurements included anogenital distance, crown to rump length (from the highest midpoint on the top of the head to the base of the tail), head circumference, fetal weight, as well as the weights of adrenal, brain, heart, kidney liver, lung, ovaries, testes, pancreas, pituitary, spleen, thymus, thyroid, and uterus. The ratios of organ weights to body weight were determined.

Hormonal and metabolic measures

Plasma concentrations of androstenedione, cortisol, DHEA, DOC, 11-deoxycortisol, estradiol, estrone, pregnenolone, 17-hydroxypregnenolone, 17-hydroxyprogesterone and T were assayed in a subset of maternal and umbilical cord samples collected at 65 and 90 days of gestation. The steroid hormone determinations were undertaken at the Optimized Analytical Solutions Laboratories, LLC (Durham, North Carolina, USA) using high performance liquid chromatography / tandem mass spectrometry (LC/MS-MS) Agilent MassHunter Workstation Data Acquisition for Triple Quad B.03.01 (B2065) and Agilent MassHunter Quantitative Analysis for QQQ (B.04.00/Build 4.0.225.0) (Santa Clara, California, USA) with electrospray ionization. Samples were subjected to a liquid / liquid extraction method followed by derivatization with dansyl chloride to enable the detection of estrone and estradiol. Calibration curves of steroid hormone standard / internal standard peak area ratio versus steroid concentration were constructed and a weighted 1/x2 linear regression applied to the data. Concentrations of steroid hormones in samples were defined based on the appropriate calibration curve. Commercially obtained female monkey plasma was used as quality controls (for cortisol, estradiol, and T: Sigma Aldrich, St Louis, Missouri, USA and for androstenedione, DHEA, DOC, 11-deoxycortisol, estrone, pregnenolone, 17-hydroxypregnenolone, 17-hydroxyprogesterone: Steraloids Inc, Newport, RI). Lower detection limits were: androstenedione 0.1 ng/ml, cortisol 0.5 ng/ml, DHEA 0.5 ng/ml, 11-deoxycortisol 0.05 ng/ml, DOC 0.05 ng/ml, estradiol 5 pg/ml, estrone 5 pg/ml, pregnenolone 1 ng/ml, 17-hyrdoxypregnenolone 1 ng/ml, 17-hydroxyprogesterone 0.2 ng/ml and T 0.02 ng/ml. The respective coefficients of variation were: androstenedione 3%, cortisol 11%, DHEA 8%, DOC 19%, 11-deoxcortisol 5%, estradiol 2%, estrone 4%, pregnenolone 16%, 17-hyrdoxypregnenolone 16%, 17-hydroxyprogesterone 2%, and T 8%.

Plasma insulin and glucose were measured in all maternal / umbilical cord blood samples collected at gestational days 65 and 90 (Study 2) and during the neonatal

period (Study 1). Insulin concentrations were measured using a radioimmunoassay kit (MP Biomedicals, Solon, OH). Samples from each study were each measured within a separate assay and assay sensitivity averaged 0.61 and 0.39 µU/ml for the 2 studies. Mean intra-assay CVs based on two quality-control pools were 3.4 and 7.3%, and 2.7 and 0.4%, respectively. Inter assay CVs were 3.4 and 5.3%, respectively. Glucose concentrations for the two studies (each measured within a separate assay) were determined using the Glucose Oxidase Method (Pointe Scientific, Inc., Canton, MI) as described previously[40]. Assay sensitivity was 5.9 and 4.5 mg/dl, respectively. Intra-assay CVs were 1.2 and 0.5% (25 mg/dl QC pool), 1.2 and 0.5% (75 mg/dl QC pool), and 1.6 and 1.3% (150 mg/dl QC pool), and respective inter assay CVs were 4.0, 0.6, and 0.9%, respectively.

Bisphenol-A measurements

Free BPA (unconjugated) and total BPA were measured using a validated protocol published from the NIEHS-funded Round Robin study in one of the laboratories that participated in the study (Vandenberg et al., 2014). In brief, after thawing samples at room temperature, plasma or amniotic fluid (0.5 ml) was transferred to a 15 ml glass tube, and internal standards (d6-BPA and 13C12-BPA-G), ammonium acetate buffer, formic acid and milli-Q water were added to a total volume of 3 ml. An Oasis MCX cartridge (60 mg/3cc; Waters, Milford, MA) was used for extraction and clean-up procedure. The cartridge was pre-conditioned with methanol and water. After loading the sample, the cartridge was washed with 15% methanol in water and eluted with methanol. The eluate was concentrated to 0.5 ml. BPA levels in samples were quantified using a high-performance liquid chromatography (HPLC) coupled with API 5500 electrospray triple-quadrupole mass spectrometer (ESI-MS/MS). Ten µl of the extract was injected onto an analytical column (Betasil C18, 100 x 2.1 mm column; Thermo Electron Corporation, Waltham, MA), which was connected to a Javelin guard column (Betasil C18, 20 x 2.1 mm). The mobile phase comprised methanol and 10 mM ammonium acetate in water. The ESI-MS/MS was operated in the electrospray negative ion mode. Data were acquired using multiple reaction monitoring (MRM) for the transitions of 227 > 212 for BPA and 233 > 215 for d6-BPA.

Quality assurance and quality control parameters include validation of the method by spiking internal standards into the sample matrices and passing through the entire analytical procedure to calculate recoveries of target analytes through the analytical

method. A procedural blank was analyzed with the samples to check for interferences or laboratory contamination. The limit of detection was 0.02 ng/ml for BPA. The recovery of d6-BPA spiked into samples was 95 ± 29% (mean ± SD). Reported concentrations were corrected for the recoveries of surrogate standard (isotope dilution method). The native standards spiked into procedural blank and selected sample matrices and passed through the entire analytical procedure yielded recoveries of 105 ± 5% and 102 ± 7% for BPA, respectively. An external calibration curve was prepared by injecting 10 µl of 0.01-100 ng/ml standards and calibration coefficient was > 0.99.

Validation study

To determine if the sample collection procedure routinely used in the laboratory leaches BPA, three females were administered a single BPA dose (0.5 mg/kg BW; s.c.). Two simultaneous plasma samples were collected before, 6 and 24 hours after BPA administration using both a routine collection procedure (involving plastic instruments) and a plastic-free collection procedure. For the routine procedure, plastic syringes (BD, Franklin Lakes, NJ) for blood extraction and plastic Pasteur pipets (Fisher, Pittsburgh, PA) for plasma transfer to storage plastic tube (DOT, Burton, MI) sealed with a plastic cap (DOT, Burton, MI) were used. For the plastic-free procedure, a vacutainer blood extraction system (BD, Franklin Lakes, NJ) was used and blood was collected in a vacutainer glass tube (BD, Franklin Lakes, NJ). Plasma was then transferred to a glass tube (Fisher, Pittsburg, PA) with a glass Pasteur pipet (Fisher, Pittsburg, PA). All samples were immediately frozen and stored at -20 °C.

Statistical analysis

Postnatal growth data were collected for 11 weeks in male offspring and 86 weeks (~600 days) in female offspring. In the first analysis, birth weight and growth curves from birth to 11 weeks of age using all offspring (including both female and male animals) among treatment groups were analyzed. A linear random effect mixed model was used for both analyses to account for correlation between lambs born to the same mother and repeated measurements from the same offspring. Quadratic function was found to fit the growth curve well from birth to 11 weeks of age. A second analysis was then conducted to study long-term postnatal weight trajectories in female offspring, alone. Quadratic splines with knots at 3 and 6 months of age were used to fit the growth curve. For all the

above analyses, all BPA dose groups were combined before comparing them to the controls.

For all analyses: To assess and adjust for sex differences, sex and treatment interaction was tested in each model. To adjust for litter size, numbers of female and males siblings in the same litter were included in the model. In addition, comparisons of fetal organ weights, fetal steroids and fetal hormonal measures were made using a regression model. Interactions between data collection time and gender (data collection and treatment) were assessed, within BPA treated and C (male and female) groups, respectively.

Appropriate transformations were applied, as needed, to account for normality of data. Comparisons of fetal BPA concentrations (free and total) were analyzed using a mixed model with Tukey post-hoc tests with number of fetuses as a covariate.

For analysis of maternal steroidal measures and insulin to glucose ratio in Study 2, two-tailed t test was performed. Comparisions for fetal insulin to glucose ratio were made between BPA *vs* C in each sex using Mann-Whitney Test. In addition, same sex fetuses born to the same dam were averaged within each group to account for dam effects. A univariate analysis of variance was used for comparing newborn insulin to glucose ratio (Study 1) and maternal weight gain.

Spearman's correlation analysis was performed to relate significant outcomes in Study 2 with maternal / fetal BPA (free and total) levels. All statistics were run using PASW Statistics for Windows release 18.0.1 or SAS (Windows version 9.4) and all plots were generated using R software. Differences were considered significant at $P<0.05$. All data are presented as mean ± SEM unless otherwise stated.

Results

Mean weight gain during pregnancy were 1.20, 1.36, 1.54 and 1.96 kg for the control, $BPA_{low,}$ BPA_{medium}, and BPA_{high} respectively and did not differ significantly between control and BPA-treated groups (p=1.00 for all 3 comparisons).

Study 1: Effects of prenatal BPA on birth weight and early postnatal growth trajectory (0 to 11 weeks)

Table 1a shows the mean birth weight for each treatment group by sex. As expected, birthweight of female offspring were significantly lower compared to males, (p = 0.0023). There was no treatment effect on birth weight for either sex when the three BPA treated groups were analyzed separately or combined. Growth trajectories from birth to 11 weeks of age are shown in Figure 1. Growth curves differed significantly between treatment groups and sex (p < 0.0001), as described below. Despite the similarity in birth weight, the growth rate of BPA-treated males after birth (Figure 1, *left panels*) differed from controls. While control males grew ~2.7 kg/week during week 1, prenatal BPA-treated males grew more slowly (~2.0 kg/week; p = 0.0004). In C males, growth rates remained approximately the same during the subsequent 11 weeks. In contrast, growth rate of BPA males increased over time (p<0.0001). By ~6 weeks of age, BPA male growth rate approximated that of C males. By 10 weeks of age, BPA males grew faster than control males (p=0.0052 Figure 1, *left panels*). Immediately after birth, C females grew ~1.7 kg/week, while BPA females grew faster (~2.1 kg/week; p=0.030; Figure 1, *right panels*). Growth rate increased with increasing age in both C and BPA groups, but was less pronounced in BPA-treated females (p < 0.001). By 10 weeks of age, growth rates in C and BPA females were similar.

Table 1b shows the insulin to glucose ratio (I:G ratio) in newborn lambs. There was no difference in the I:G ratio between BPA and C females (p= 0.22, 0.41, and 0.61 respectively, for $BPA_{low,}$ $BPA_{medium,}$ and BPA_{high}) or males (p= 0.91, 0.63, and 0.99 respectively).

Effects of prenatal BPA on later growth trajectory (up to 86 weeks of age) in female offspring

Weights of control and prenatal BPA-treated females from birth to 86 weeks of age are shown in Figure 2. As opposed to increased growth rate of BPA-treated females during the first few weeks, C females showed a higher growth rate compared with the BPA-treated group at 13 weeks of age. Growth rate was 2.98 kg/week in C vs. 2.76 kg/week in BPA-treated females ($p = 0.0029$) at 13 weeks of age. At 26 weeks, growth rates had slowed in both groups (C: 0.77 *vs*. BPA: 0.61 kg/week; $p = 0.0074$). By 80 weeks, growth rates for both groups were minimal and did not differ (C: 0.00 *vs*. BPA: 0.1 kg/week; $p = 0.28$).

Study 2: Effect of prenatal BPA on maternal fetal BPA measures

Mean unconjugated and total BPA concentrations in sheep cord blood, amniotic fluid, and maternal circulation is shown in Figure 3. The majority of BPA in maternal and cord blood, as well as amniotic fluid was in conjugated form (Panel A *vs.* B) at both gestational time points. Concentrations of free and total BPA were significantly higher in BPA-treated groups compared to C groups in both maternal and fetal circulation (white asterisks). In general, free BPA concentrations were 10-fold lower in fetal circulation compared to maternal circulation at GD65 in both male and female fetuses (Figure 3, Panel A; a *vs*. b), but were comparable at GD 90. Total BPA concentrations achieved were similar in maternal and fetal circulation at GD65 (Figure 3, Panel B). In contrast, total BPA concentrations achieved were 2-fold higher in both male and female fetal circulation and amniotic fluid compared to maternal circulation on GD90. Total BPA concentrations in both male and female fetuses at GD90 were elevated compared to concentrations on GD65.

Focusing on sex differences, there were no differences in free BPA concentrations between male and female fetuses on GD65 or GD90 in the BPA-treated group, although in controls, higher concentrations of BPA were evident in males compared to females on GD65 (Figure 3, Panel A, grey diamond). In contrast, higher concentrations of total BPA were observed in BPA-treated males compared to females at GD65, but not GD90.

Validation studies in sheep provided no evidence of BPA contamination originating from any of the collection system used (Figure 4).

Effects of prenatal BPA on hormones and metabolic measures

No difference was observed between BPA vs. C group in maternal I:G ratio at GD65 (BPA: 0.20 ± 0.04 *vs*. C: 0.18± 0.05 units) or at GD90 (BPA: 0.20 ± 0.05 *vs*. C: 0.16 ± 0.06). Similarly, there was no difference between BPA vs. C groups in fetal I:G ratio at GD65 (females: 1.48 ±0.33 *vs*. 9.10 ±14.69; males: 1.57±0.72 vs.5.61±3.94, respectively). At GD90, BPA-treated females tended to have lower I:G ratio compared to controls (BPA: 1.86 ± 0.97 *vs*. C: 4.76 ± 2.44 p value 0.052). No such difference was evident in males (BPA: 1.8±0.54 *vs*. C: 2.1±1.29).

Maternal steroidal measures are summarized in Supplemental Table 1. Measures of DHEA, androstenedione, 17-hydroxypregnenolone, 17-hydroxyprogesterone and T were under the limit of detection for the assay used in both maternal and fetal samples. BPA treatment increased maternal corticosterone concentrations at day 65 (p=0.036) and pregnenolone level at GD90 (p=0.004). DOC in maternal circulation tended to be higher at day 65 with BPA treatment. Fetal steroidal profiles are summarized in Supplemental Table 2. There was no significant interaction between sex and treatment at GD65 or GD90 (Supplemental Table 2a). When comparing within treatment groups progesterone and pregnenolone concentrations in females were significantly higher in the BPA compared to C females at GD65. Interestingly, when comparing ratios of adrenal steroid precursors we noted increased ratio of progesterone to DOC in the BPA-treated females compared to C females at GD65 (Supplemental Table 2b). Corticosterone and DOC were significantly lower in BPA females compared to control females at GD90, but similar differences were not seen between BPA and control male fetuses and there were no differences in adrenal steroid precursor ratios (Supplemental Table 2b). Deoxycortisol and cortisol were also significantly lower in control females compared with control males, at GD90. At GD90, BPA-males had significantly higher deoxycortisol than BPA-females (Supplemental Table 2b). Except from pregnenolone that correlated with total BPA at day 65 in female fetus (R=0.683 p=0.029) none of the other adrenal steroids that were significantly altered correlated with either free BPA or BPA.

Effects of prenatal BPA on fetal weight, size and organ weights

Fetal body weight and anthropometric measurements are shown in Table 2. In the control group, no differences were observed in fetal body weight, head circumference or crown to rump length between females and males at GD65 or GD90. In BPA fetuses,

males had larger body weights than females at GD90 (p = 0.008), but not at GD65 (Table 2). As expected, anogenital distance (AGD) was greater in males compared to females in both the control and BPA groups at both GD65 and GD90 (Table 2). Within sex comparison of control and BPA groups found BPA males tended to have higher bodyweight compared to control males (p=0.061).

Organ to body weight ratios for heart, liver, thyroid and gonads are shown in Table 3 (for other organs weights are summarized in Supplemental Table 3). No significant differences were noted between sex when compared within each treatment group (Table 3) however, heart:bodyweight ratio tended to be lower in C males compared to C females at GD65 (p = 0.091), the BPA males tended to have an increased heart:bodyweight ratio at GD90 (p = 0.061) compared to BPA females.

Focusing on BPA treatment effect within each sex, BPA females had a lower liver:bodyweight ratio relative to the control group (p = 0.023) at GD90 (Table 3). Similar differences were not observed in BPA males at both gestational ages. In contrast, BPA males had a higher heart:bodyweight ratio compared to controls (p =0.030) at GD90 (Table 3). No differences in gonadal (ovary or testis):bodyweight ratios were evident between control and BPA groups at both gestational time points.

A significant increase in thyroid:bodyweight ratio was evident in BPA males compared to controls at GD65 (p=0.028) (Table 3). There were no other differences in organ:bodyweight ratio with lungs, pancreas, kidney, adrenal, and pituitary (Supplemental Table 3). Spearman correlation analysis revealed no significant correlation between organ weight:body weight ratio for heart, liver and with either free or total BPA.

Discussion

This study, to our knowledge the first to assess the sex-specific effects of gestational BPA exposure on fetal anthropometrics and postnatal growth trajectory in a precocial mammalian model, reveals significant sex-by-treatment interactions. Gestational BPA

led to sexually dimorphic effects on fetal organ weights, with liver weight affected in females in contrast to heart and thyroid weights in males. Sex differences were also evident in early postnatal growth trajectories with BPA males growing more slowly than controls during the early postnatal period followed by catch up growth, while BPA females showing increased growth early on followed by growth slowing. Although the contribution of sexually dimorphic differences in fetal concentrations of total BPA achieved on gestational day 65 following gestational BPA exposure remains unclear, the relevance of the sex specific impact of BPA on fetal organ weights, steroidal profiles and growth trajectories are discussed below.

Maternal and fetal concentrations of BPA

Consistent with transplacental transfer of BPA seen in earlier studies (Balakrishnan et al., 2010; Gingrich et al., 2019; Ikezuki et al., 2002; Schonfelder et al., 2002), we observed trans-placental transfer of BPA to the fetus with measurable concentrations of free BPA detectable in umbilical cord blood. In this assessment of chronic BPA exposure at two time points during pregnancy (GD65 and GD90 of a 147 day gestation pregnancy), concentrations of free and total BPA in maternal circulation were comparable between GD65 and GD90, indicative of the short half life and fast clearance of BPA reported in earlier studies (Collet et al., 2010; Gingrich et al., 2019; Viguie et al., 2013) and supportive of a lack of an accummulation of BPA in the maternal compartment after chronic BPA exposure. Maternal and umbilical cord concentrations of BPA achieved following daily subcutaneous administration of environmentally relevant dose of BPA have not previously been studied in detail. One study investigating pharmacokinetics of BPA in fetal plasma following single maternal intravenous infusion of 1 mg/kg of BPA in sheep found concentrations approaching ~90 ng/ml 1 hour post injection (Gauderat et al., 2017). Maternal concentrations of free BPA achieved in the present study, averaging 12.2 and 26.5 ng/ml respectively at GD65 and GD90, are within the the range reported in a Korean study (range 0.44 to 47.1 ng/ml) (Lee et al., 2018) and our Michigan-based study at first trimester (<LOD to 96.43 ng/ml) and term (<LOD to 89.60 ng/ml) (Veiga-Lopez et al., 2015). Umbilical cord blood concentrations of ~2 ng/ml of free BPA, achieved in the present study at both GD65 and GD90 following daily s.c. administration of 0.5 mg/kg/day BPA, are also within the range of free BPA found in mid-gestation umbilical cord blood concentrations (<LOD - 52.26 ng/ml) in a California based study (Gerona et al., 2013), term cord blood concentrations (<LOD - 51.5 ng/ml) in the Korean study (Lee et al., 2018) and our Michigan based study (<LOD - 41.83 ng/ml) (Veiga-Lopez et al., 2015).

Comparative estimates of free and total BPA in the cord blood of human are limited. The umbilical cord concentrations of BPA glucuronide in the California study (<LOD – 3.05 ng/ml) and our human Michigan study (<LOD – 4.85 ng/ml) (Gerona et al., 2013; Veiga-Lopez et al., 2015) are much lower than the free BPA concentrations. In contrast, total BPA concentrations are higher than free BPA in our sheep study. In addition, in the present study, the ratio of free to total BPA is much lower in umbilical and aminiotic fluid compared to that seen in maternal circulation. The high presence of total BPA over free BPA in the sheep study indicates that the fetal-placental unit is efficient in metabolizing BPA into its conjugated form, and that conjugated metabolites appear to accumulate in the fetal placental compartment. High concentrations of accumulated conjugated BPA in the sheep fetus have been reported following a single intravenous infusion of BPA (Corbel et al., 2013). Since BPA has been shown to be de-conjugated in the liver, these findings suggest that enzymes required for de-conjugation and elimination of BPA may not be fully functional in the fetal liver (Coughtrie et al., 1988). The sex differences in levels of BPA achieved at gestational day 90 may reflect differences in metabolism / clearance of BPA between males and females. Accumulation of conjugated BPA in the fetal compartment may have physiological relevance, as BPA glucuronide has also been reported to be biologically active (Boucher et al., 2015). Finally, the validation study we have conducted negates the concern that some of the measured BPA reflects processing contamination.

Prenatal BPA programming of organogenesis and fetal growth

There are limited epidemiological studies addressing the association of prenatal BPA exposure on organogenesis and/or fetal health. Most human studies have focused on birth outcomes as the endpoint of prenatal exposure to BPA. A recent study noted that elevated maternal concentrations of BPA in mid-late gestation were correlated with impaired fetal growth and head circumference (Snijder et al., 2013). Interestingly, sex differences in birth outcomes of maternal BPA exposure have been reported in a recent study, with elevated risk for large for gestational age, as well as small for gestational age male neonates depending on exposure dose of BPA. Similar effects on birth weight were not evident in female offspring (Chou et al., 2011). Interestingly, BPA-treated male fetuses were found to be also heavier than BPA-treated females at day 90 of gestation in the present study, possibly reflecting the increased concentrations of total BPA found in gestation day 65 male.

Lack of effect of prenatal BPA treatment on birth weight at all doses in Study 1 differs from that of our previous study where we found that prenatal BPA (5 mg/kg/day) resulted in decreased body weight of female fetuses (Savabieasfahani et al., 2006). These differences may reflect changes in environmental conditions. Epidemiological studies investigating the association of BPA with birth weight are also inconsistent, with some studies reporting a reduction (Burstyn et al., 2013; Miao, Yuan, Zhu, et al., 2011; Snijder et al., 2013), no change (Padmanabhan et al., 2008), while others report an increase (Lee et al., 2008) or an U-shaped response curve (Philippat et al., 2012). These discrepancies in birth weight variability could be explained by both the exposure and timing of BPA exposure during pregnancy and interaction with other environmental exposures, diet, and/or lifestyle factors.

In contrast to the lack of effects on birth weight, significant sex- and time-specific effects of prenatal BPA treatment,although not directly correlated, were evident in the organs involved in cardiometabolic function. In females, this is manifested by decreased fetal liver weight at GD90. The lower levels of total BPA in female GD 65 fetuses compared to males in the face of reduced liver weight suggest female fetuses may metabolize BPA quicker than males. Available evidence indicates BPA accumulates in human fetal liver and elevated BPA is associated with adult liver dysfunction (Lee et al., 2014; Nahar et al., 2014; Nahar et al., 2015). While studies addressing causal relationships between prenatal exposure to BPA on postnatal programming of human liver are not feasible, animal studies show that developmental exposure to BPA induces alterations in hepatic gene expression and diseases such as hepatic steatosis, liver tumors and metabolic syndrome (DeBenedictis et al., 2016; Moustafa & Ahmed, 2016; Wei et al., 2014). For instance, prenatal BPA from embryonic day 7.5 to 18.5 in mice affects liver maturation in a sex-specific fashion with effects seen only in females (DeBenedictis et al., 2016). Another study also found exposure to BPA 25 µg/kg/day from GD8 through postnatal day 16, increased hepatic lipid content in 5-week and 39-week old female, but not male mice (Shimpi et al., 2017). Other animal studies exploring the impact of BPA on liver focused on either of the sexes. For instance, in a study focusing on male offspring noted that at exposure to 50 mg/kg/day BPA from GD0 to the end of lactation at postnatal day 21 resulted in abnormalities of liver function and hepatic damage in male rats (Xia et al., 2014). Similarly, prenatal BPA treatment was found to program fatty liver disease in male offspring when subjected postnatally to high fat diet (Wei et al., 2014). In contrast, a study in mice focusing on female offspring showed exposure to 50 and 200 mg/kg BW

BPA from beginning of gestation through 3 months post weaning increased vacuolation of hepatic cells, proliferation of Kupffer cells and altered liver enzymes in females (Moustafa & Ahmed, 2016). The decrease in fetal female liver weight seen in our study reflecting early programming effects may contribute to later metabolic dysfunctions.

As opposed to lack of effect of prenatal BPA on fetal male liver, prenatal BPA treatment increased heart to body weight ratio at GD90 in males, alone. Consistent with lack of effect on the female heart during fetal life, there were no effects of prenatal BPA treatment on blood pressure or morphometric measures in adult BPA-treated offspring (Koneva et al., 2017; MohanKumar et al., 2017). Prenatal BPA treatment, however, up-regulated several genes involved in regulation of myocardium growth, heart development and remodeling, and down-regulation of genes involved in cellular respiration and inflammation in adult prenatal BPA-treated females (Koneva et al., 2017). Prenatal BPA treatment was also found to reduce collagen expression in the right ventricle (MohanKumar et al., 2017). These changes have the potential to lead to cardiac compromise with adult aging. The changes in fetal heart weight, while not correlating with measured BPA levels, may serve as proxy for later functional defects that may develop in the male. Cardiac-specific studies have not been carried out in adult, prenatal BPA-treated rams. In our studies, males were not maintained in parallel with females.

The male-specific effect of prenatal BPA on the thyroid, an important regulator of metabolic functions including hepatic steatosis (Mullur et al., 2014), is of interest. Our findings of increased thyroid weight in prenatally treated BPA male fetuses, coupled with recent findings in sheep that prenatal BPA treatment induces disruption of fetal thyroid function (Guignard et al., 2017), raises the possibility that prenatal exposure to BPA may compromise thyroid function in adulthood. This does not hold true with females, since thyroid gland size was not affected (this study). Endocrine disrupting chemicals have been shown to affect thyroid function (Miller et al., 2009). BPA in particular has been shown to affect thyroid volume (BPA concentrations inversely associated with thyroid volume) and also affect thyroid function in human studies (Meeker & Ferguson, 2011; Wang et al., 2015; Zoeller, 2007). Prenatal exposure to BPA around the third trimester of pregnancy has been shown to affect offspring thyroid axis in a sex-specific pattern, with conflicting data in one study showing elevated BPA correlated with low TSH in male neonates and other showing the same pattern in female neonates (Chevrier et al., 2013; Romano et al., 2015). Similarly in rodent studies, there are conflicting data on sex

specific effects of BPA on thyroid gland with effects seen in both sexes vs male alone vs no effect on either sex (Kobayashi et al., 2005; Xu et al., 2007; Zoeller et al., 2005). Prenatal BPA exposure is shown to alter thyroid hormone in sheep, however, this effect was not sex specific and there was no mention on fetal thyroid organ weight (Viguie et al., 2013). Since the thyroid has been shown to have a critical role in fetal and postnatal growth, (Shields et al., 2011; Smallridge & Ladenson, 2001) further studies to assess the functional effects of this sex-specific pattern of change in size of thyroid gland are warranted.

Interestingly, while we saw sex specific effects of BPA on fetal organ weights we did not see sex differences in fetal organ weights. To what extent these sex-specific impact of BPA on organ systems are key to cardiometabolic function are the result of masculinization or defeminization of systems is an important line of research. Our studies found no effect of BPA on anogenital distance (AGD), a biomarker for masculinization at GD65 and GD90 in both sexes, although the expected increase in AGD was evident in the males at GD65 and GD90 compared to females in both control and BPA-treated groups. In contrast, an earlier study found prenatal BPA exposure reduced AGD in male offspring (Mammadov et al., 2018; Miao, Yuan, He, et al., 2011).

Prenatal BPA and maternal/fetal steroidogenesis and metabolic measures

Because steroid hormones can program fetal growth and adult phenotype, and BPA has been shown to influence steroidogenesis (Peretz et al., 2014), we investigated the impact of prenatal BPA treatment on maternal and fetal steroidogenesis. Lack of effect of prenatal BPA treatment on maternal and fetal estrogens may suggest the programming of adult reproductive and metabolic dysfunction by prenatal BPA-treatment (Alonso-Magdalena et al., 2015) are not mediated via changes in maternal and fetal estrogens. We did, however, note a significant increase in corticosterone in the BPA treated mothers at GD65, but not at GD90. In general, maternal stress raises corticosterone concentrations during pregnancy with negative effects on feto-placental unit (Jafari et al., 2017; Vaughan et al., 2012). Elevations in corticosterone rather than cortisol concentrations strongly correlate with fetal stress in human studies (Wynne-Edwards et al., 2013). Despite elevated corticosterone in maternal circulation, corticosterone and DOC, both downstream products in the adrenal steroidal pathway leading to aldosterone synthesis, were lower in the D90 BPA exposed female fetuses. While similar changes in corticosterone and DOC levels were not seen In the GD65

BPA-exposed female fetuses higher levels of pregnenolone and progesterone, upstream products of the aldosterone synthetic pathway and a higher progesterone/DOC ratio were evident. These findings could be suggestive of a fetal compensatory response to overcome inhibition of adrenal enzyme activity upstream (e.g., CYP21) of the aldosterone synthesis pathway. Due to multiple precursors being below the level of detection, similar assessment of cortisol and androgen pathways were not possible. A recent *in vitro* study with a human cell line found BPA altered adrenal steroidogenesis by inhibiting CYP17 (Zhang et al., 2011) and similar to our study noted an increase in progesterone and a decrease in downstream steroidal hormone levels. One possibility is prenatal BPA blocked CYP17 activity leading to unmeasurable downstream steroidal products.

In epidemiological studies, BPA exposure has been shown to be associated with type 2 diabetes (Lang et al., 2008). In rodent models prenatal BPA has been shown to lead to insulin resistance in the male offspring (Alonso-Magdalena et al., 2015). Our group has previously shown that female offspring exposed to prenatal BPA also develop reduced insulin sensitivity in adulthood (Veiga-Lopez et al., 2016). To our knowledge this is the first study to assess insulin sensitivity in fetal circulation at early and later gestation in both sexes. A trend for increased insulin sensitivity only in the BPA-treated female fetus at GD90 in the absence of such differences in the maternal or newborn circulation may be a reflection of early programming effects of BPA on insulin homeostasis (Veiga-Lopez et al.,2016). Lack of effect of BPA on maternal insulin sensitivity suggests that effects of BPA on adult offspring's insulin sensitivity are programmed indirectly via other maternal mechanisms or through direct effects at the fetal level.

Prenatal BPA programming of sexually-dimorphic postnatal growth trajectory

A significant sex difference was evident in early postatal growth trajectory of prenatal BPA treated offspring, independent of the BPA dose. Prenatal BPA-treated female lambs grew faster immediately after birth with slowing of growth velocity overtime compared to their control counterparts. In contrast, prenatal BPA treated males grew slower in the immediate postnatal period, but had increased growth rate over time compared to the control group. Such sex differences in growth trajectories may be related to higher concentrations of total BPA found in male fetuses during early gestation (GD65). While the relative biological potency of conjugated BPA is unknown, there is

some evidence for bioactivity of the glucuronide form of BPA (Boucher et al., 2015). Native steroids such as estrogen, when conjugated with bovine serum albumin have the capability to stimulate surface receptors leading to rapid signaling (Chen et al., 2017; Watson et al., 2014). Considering the estrogenic properties of BPA, it is conceivable the same holds true for conjugated form of BPA.

Low birth weight followed by rapid catch up weight gain have been shown to lead to adverse cardio-metabolic phenotype in adulthood (Barker, 2004). Although we did not see low birth weight in our cohort, the female offspring did show a brief period of increase weight gain soon after delivery. One epidemiological study reported larger increases in BMI slope from 2 to 5 years associated with higher BPA with association being stronger in girls, while another found girls exposed to higher concentrations of prenatal BPA have lower body mass index in childhood (Braun et al., 2014; Harley et al., 2013). The increased growth rate evidenced in our study may contribute to the insulin resistance and increase in adipocyte size and inflammatory markers evidenced in adult prenatal BPA-treated sheep (Veiga-Lopez et al., 2016). The implication of the initial slower trajectory of postnatal weight gain followed by increased weight gain velocity later in males needs to be explored further, since several other factors such environmental exposure to other ubiquitously present environmental chemicals and their interaction with diet could also be playing a role. Considering that low birth weight followed by catch up growth has been shown to lead to cardio-metabolic diseases later in life (Barker, 2004; Valsamakis et al., 2006), the enhanced growth rate in the absence of change in birth weight is also a potential risk factor.

Finally, the major strengths of our current study assessing the impact of prenatal BPA on fetal and postnatal health of the offspring are: 1) use of a precocial species, sheep, with similar developmental trajectory as human; 2) use of three different doses of BPA spanning environmental to occupational exposure concentrations; 3) measurement of both free and total BPA concentrations using a well validated assay (Vandenberg et al., 2014; Veiga-Lopez et al., 2015) at two different time points in fetal and maternal serum allowing exploration of the contribution of time specific impact of BPA; 4) use of LCMS for measures of steroid hormones allowing for improved accuracy of steroid measures; and 5) more importantly the first longitudinal study of assessment of the sex-specific impact of prenatal BPA on growth trajectory. Study limitations include lack of follow up of the male offspring beyond 11 weeks of age in parallel with the female offspring, a

necessity to avoid unwanted pregnancy in our cohort, and absence of information on adult male phenotype to relate these early findings.

In conclusion, this study documents internal concentrations of maternal and fetal BPA achieved at two time points in gestation following treatment of pregnant sheep with environmentally relevant doses of BPA. Importantly, the study demonstrates sexually dimorphic effects of prenatal BPA on organogenesis and early postnatal growth trajectory - aspects of relevance to programming of adult reproductive and metabolic dysfunctions - in the absence of major disruptions in the maternal/fetal steroid hormone milieu.

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Figure legends

Figure 1. Growth trajectory and growth rate from birth to 11 weeks in males (*left panels*) and females (*right panels*) from control and BPA-treated groups. *Top panels*: means and standard errors of observed weight over time by treatment groups. *Middle panels*: estimated growth trajectories of the control and pooled BPA-treated groups based on the linear mixed effects model. *Bottom panels:* estimated weekly growth rate of the control and pooled BPA-treated groups, based on the linear mixed effect model. *p<0.05 for comparing growth rate between control and pooled BPA-treated groups.

Figure 2. Weights from birth to 86 weeks for females in the control (*solid black line*) *vs*. all BPA groups combined (dashed line). *: At birth, growth rate was 2.1kg per week for BPA and 1.6kg per week for C, p<0.0001; At 13 weeks, growth rate was 2.76kg per week for BPA and 2.98kg per week for C, p=0.0029; At 26 weeks, growth rate was 0.61kg per week for BPA and 0.77kg per week for C, p=0.0074.

Figure 3. Mean (±SEM) concentrations of free (Panel A) and total (Panel B) BPA in maternal blood (*left*) and umbilical artery blood and amniotic fluid (*right*) and samples at gestational day 65 (*top*) and 90 (*bottom*) in control (*open bars*) and BPA-treated (*closed bars*) groups. White Asterisks denote significant difference between control and BPA for each category. Diamond symbol (control: grey; black: BPA group) symbol indicate male vs female differences in BPA levels at each time point. a vs. b indicate differences between maternal vs fetal levels of BPA achieved (control: grey; black: BPA group). X vs. Y (control: grey; black: BPA group) indicate differences in BPA levels achieved between day 65 and 90 within each category for each sex.

Figure 4. BPA validation study. Three sheep females were administered a single BPA dose (0.5 mg/kg BW; s.c.) was administered to each female. Two simultaneous plasma samples were collected before, 6 and 24 hours after the BPA dose using both a routine collection procedure (involving use of plastic instruments) and a plastic-free collection procedure

Table 1a. Mean birth weight for each treatment group by sex. BPA doses: 0.05, 0.5, or 5 mg/kg/day (referred as: low, medium, and high, respectively).

		Female	Male
	Treatment Group	Mean ± SD ***N***	Mean ± SD ***N***
BW (Kg)	Control	6.22 ± 1.44 ***8***	6.60 ± 0.94 **6**
	Low	5.88 ± 1.02 **12**	6.22 ± 1.44 **9**
	Medium	5.42 ± 1.07 **13**	6.36 ± 1.66 **13**
	High	5.90 ± 1.42 **7**	5.66±1.27 **15**

BW: birth weight, N=number of lambs per group. Standard deviation expressed as SD

Table 1b. Insulin to Glucose ratio (I:G) for each treatment group by sex in newborn lambs. BPA doses: 0.05, 0.5, or 5 mg/kg/day (referred to as: low, medium, and high, respectively).

		Female	Male
	Treatment Group	Mean ± SD ***N***	Mean ± SD ***N***
I:G ratio	Control	0.20 ± 0.06 ***5***	0.16 ± 0.04 **5**

	Low	0.13± 0.04 **7**	0.18 ± 0.10 **6**
	Medium	0.15± 0.04 **10**	0.20± 0.09 **7**
	High	0.16± 0.11 **5**	0.16± 0.07 **9**

N=number of lambs per group. Standard deviation expressed as SD.

Table 2. Anthropometric measurements of fetuses: Comparing BPA (0.5 mg/kg dose) vs control within each sex and comparing male vs female within each treatment group at gestational days 65 and 90.

Female	Male

		Control Mean± SD ***N***	BPA Mean± SD ***N***	Control Mean ± SD ***N***	BPA Mean ± SD ***N***
FW (g)	GD 65	126.67± 18.7 ***9***	124.44± 21.3 ***9***	143.33± 36.7 **6**	140± 17.9 **11**
	GD 90	662.5± 97.1 **8**	**647.78± 32.3**[a*] **9**	653± 45.5 **10**	**715± 59.2**[b*] **4**
HC (cm)	GD 65	13.31± 1.1 **9**	13.3± 0.8 **9**	13.17± 0.6 **6**	13.76± 1.1 **11**
	GD 90	21.34± 0.8 **8**	21.27± 0.9 **9**	21.15± 0.7 **10**	21.58± 0.6 **4**
CR (cm)	GD 65	16.18± 1.6 **9**	16.14± 1.5 **9**	15.82± 1.4 **6**	16.38± 1.5 **11**
	GD 90	27.91±1.3 **8**	27.64±0.9 **9**	27.17±1.4 **10**	26.93±3.9 **4**
AGD (cm)	GD 65	**0.32± 0.1**[a*] **9**	**0.28± 0.1**[a*] **9**	**2.57± 0.16**[b*] **6**	**2.39± 0.3**[b*] **11**
	GD 90	**0.71± 0.2**[a*] **8**	**0.96± 1.1**[a*] **9**	**4.16± 0.4**[b*] **10**	**4.20± 0.4**[b*] **4**

GD: Gestational day, AGD: anogenital distance, CR: crown to rump, FW: fetal weight, HC: head circumference,g: grams,cm: centimeters. *Standard deviation expressed as SD. N= number of lambs per group*. a≠b denotes differences between sex within treatment groups p<0.05. A≠B denotes differences between treatment groups within sex p<0.05. * p value adjusted for #fetus per ewe. *FW Day 90 BPA female vs BPA male p=0.008, AGD for Control Male vs Female at GD 65 and 90 p=<0.001. AGD for BPA Male vs Female at GD 65 and 90 p=<0.001.*

Table 3. Fetal organ weights: Comparing BPA (0.5 mg/kg dose) vs control within each sex and comparing male vs female within each treatment group at gestational days 65 and 90.

Female	Male

		Control Mean ± SD ***N***	BPA Mean ± SD ***N***	Control Mean ± SD ***N***	BPA Mean ± SD ***N***
Heart: Body weight ratio #	GD 65	0.909± 0.228 ***9***	0.826± 0.129 ***9***	0.750± 0.063 **6**	0.782± 0.085 **11**
	GD 90	0.836± 0.089 **8**	0.824± 0.073 **9**	**0.852± 0.075**^{B*} **10**	**0.983± 0.126**^{A*} **4**
Liver: Body weight ratio #	GD 65	6.822± 0.828 **9**	6.501± 0.568 **9**	6.407± 0.785 **6**	6.220± 0.576 **11**
	GD 90	**5.129± 0.495**^{B*} **8**	**4.895±0.329**^{A*} **9**	4.993± 0.639 **10**	5.307± 0.783 **4**
Thyroid: Body weight ratio #	GD 65	0.051± 0.024 **9**	0.040± 0.013 **9**	0.045± 0.010 **6**	0.035± 0.012 **11**
	GD 90	0.038± 0.015 **8**	0.041± 0.009 **9**	**0.040± 0.008**^{B*} **10**	**0.052± 0.011**^{A*} **4**
Gonad$^{\$}$: Body weight ratio #	GD 65	0.021± 0.008 **9**	0.025± 0.008 **9**	0.051± 0.021 **6**	0.048± 0.008 **11**
	GD 90	0.006± 0.001 **8**	0.007± 0.002 **9**	0.043± 0.019 **10**	0.064± 0.035 **4**

Gonad$^{\$}$ = Ovaries in females and Testes in males. GD: Gestational day. # adjusted for body weight, then multiplied by 100. *Standard deviation expressed as SD.* a≠b denotes differences between sex within treatment groups p<0.05. A≠B denotes differences between treatment groups within sex p<0.05. * p value adjusted for #fetus per ewe. *Female liver BPA vs Control p=0.023, Male heart BPA vs Control p=0.03, Thyroid p=0.028*

Supplemental Table 1: Maternal steroid measures BPA (dose 0.5mg/kg/day) vs control at day 65 and 90 gestations.

		Control **Mean ± SD** *N*	**BPA** **Mean ± SD** *N*
Cortisol	GD 65	95.00± 15.7 **6**	120.40± 37.68 **6**
	GD 90	112.36± 23.15 **4**	128.12 ± 41.44 **5**
Corticosterone	GD 65	**1.82± 0.64** [B] **6**	**2.77 ± 0.77** [A] **6**
	GD 90	2.84 ± 1.36 **4**	2.42± 0.82 **5**
Deoxycortisol	GD 65	1.02 ± 0.49 **6**	1.27 ± 0.52 **6**
	GD 90	1.17± 0.54 **4**	1.49± 0.96 **5**
DOC	GD 65	0.07± 0.02 **6**	0.11± 0.04 **6**
	GD 90	0.10± 0.04 **4**	0.10± 0.03 **5**
Progesterone	GD 65	8.63± 2.17	7.86± 3.07

		6	**6**
	GD 90	12.1 ± 5.74 **4**	15.7 ± 2.59 **5**
Pregnenolone	GD 65	2.43 ± 0.81 **6**	2.54 ± 0.89 **6**
	GD 90	**2.39± 0.49** [B] **4**	**4.23 ± 0.65** [A] **5**
Estrone	GD 65	0.00 ± 0.00 **6**	0.01± 0.00 **6**
	GD 90	0.01 ± 0.00 **4**	0.01± 0.00 **5**
Estradiol	GD 65	0.18 ± 0.42 **6**	0.02± 0.03 **6**
	GD 90	0.02± 0.01 **4**	0.02± 0.00 **5**

GD: Gestational day, Deoxycorticosterone (DOC)**.** *Standard deviation expressed as SD*. A≠B denotes differences between treatment groups p<0.05. *GD65 BPA vs Control: Corticosterone p=0.036, GD90 BPA vs Control: Pregnenolone p=0.004.*

Supplemental Table 2a:Fetal steroid measure: Interaction effects between gender and treatment in fetal steroidal measures.

		P value*		
		Gender	Treatment	Interaction
Cortisol	GD 65	0.637	0.733	0.457
	GD 90	0.294	0.622	0.297
Corticosterone	GD 65	0.996	0.642	0.399
	GD 90	0.497	0.850	0.244
Deoxycortisol	GD 65	0.917	0.565	0.779
	GD 90	**0.009**	0.713	0.293
DOC	GD 65	0.856	0.415	0.383
	GD 90	0.476	0.688	0.637
Progesterone	GD 65	0.165	**0.005**	0.172
	GD 90	0.488	0.265	0.939
Pregnenolone	GD 65	0.088	**0.011**	0.144
	GD 90	0.391	0.510	0.448
Estrone	GD 65	0.374	0.338	0.892
	GD 90	0.062	0.849	0.220
Estradiol	GD 65	0.694	0.378	0.839
	GD 90	**0.042**	0.864	0.250

* Adjusted for # fet/Dam. Deoxycorticosterone (DOC).

Supplemental table 2b: Fetal steroid measure: Comparison BPA (dose 0.5mg/kg/day) vs control within each sex and comparing male vs female within each treatment group at gestational days 65 and 90.

		Female		Male	
		Control Mean ± SD ***N***	BPA Mean ± SD ***N***	Control Mean ± SD ***N***	BPA Mean ± SD ***N***
Cortisol	GD 65	9.56± 6.42 **7**	10.49± 7.82 **5**	11.61± 10.2 **4**	7.13± 1.22 **5**
	GD 90	**2.18± 1.21** [a*] **5**	1.66 ± 1.41 **6**	**3.43± 2.27** [b*] **8**	2.29± 1.56 **4**
Corticosterone	GD 65	1.63± 1.04 **7**	1.29± 1.02 **5**	2.6± 2.05 **4**	1.32± 0.58 **5**
	GD 90	**1 ± 1.08** [B*] **5**	**0.53 ± 0.07** [A*] **6**	1.90± 1.25 **8**	0.78± 0.56 **4**
Deoxycortisol	GD 65	0.34± 0.22 **7**	0.40± 0.16 **5**	0.37± 0.15 **4**	0.39± 0.16 **5**
	GD 90	**0.17± 0.07** [a*] **5**	**0.18± 0.08** [a*] **6**	**0.27± 0.05** [b*] **8**	**0.43± 0.31** [b*] **4**
DOC	GD 65	0.10± 0.05 **7**	0.07± 0.02 **5**	0.12± 0.04 **4**	0.08± 0.01 **5**
	GD 90	**0.062± 0.02** [B*] **5**	**0.057± 0.01** [A*] **6**	0.08± 0.02 **8**	0.06± 0.02 **4**

Progesterone	GD 65	**0.14± 0.04** [B*] **7**	**0.41± 0.36** [A*] 5	0.17± 0.03 **4**	0.21± 0.07 **5**
	GD 90	0.39± 0.10 **5**	0.50± 0.23 **6**	0.44± 0.21 **8**	0.57± 0.32 **4**
Pregnenolone	GD 65	**1.00± 0.00** [B*] **7**	**1.39± 0.60** [A*] **5**	1.02± 0.03 **4**	1.00± 0.00 **5**
	GD 90	1.17± 0.22 **5**	1.28± 0.59 **6**	1.14± 0.25 **8**	2.12± 2.21 **4**
Estrone	GD 65	0.02± 0.01 **7**	0.01± 0.01 **5**	0.02± 0.02 **4**	0.02± 0.01 **5**
	GD 90	0.04± 0.01 **5**	0.04± 0.01 **6**	0.05± 0.01 **8**	0.27± 0.46 **4**
Estradiol	GD 65	0.01± 0.01 **7**	0.01± 0.01 **5**	0.01± 0.00 **4**	0.01± 0.00 **5**
	GD 90	0.01± 0.01 **5**	0.01± 0.01 **6**	0.02± 0.01 **8**	0.04± 0.05 **4**
Preg/Prog ratio	GD 65	7.58± 2.27 **7**	5.61± 4.13 **5**	6.03± 1.17 **4**	5.37± 1.93 **5**
	GD 90	3.03± 0.44 **5**	2.79± 1.07 **6**	3.12± 1.36 **8**	3.22± 1.44 **4**
Prog/DOC ratio	GD 65	**1.93± 1.28** [B*] **7**	**5.38± 4.17** [A*] **5**	1.57± 0.52 **4**	2.59± 0.90 **5**
	GD 90	7.023± 3.07	9.05± 4.78	5.92± 3.21	9.88± 7.44

		5	**6**	**8**	**4**
DOC /Corticosterone ratio	GD 65	0.073± 0.02 **7**	0.08± 0.04 **5**	0.07± 0.04 **4**	0.07± 0.02 **5**
	GD 90	0.091± 0.04 **5**	0.11± 0.03 **6**	0.06± 0.04 **8**	0.10± 0.02 **4**

Pregnenolone (preg),Progesterone(prog),deoxycorticosterone(DOC). GD: Gestational day. *Standard deviation expressed as SD*. a≠b denotes differences between sex within treatment groups $p<0.05$. A≠B denotes differences between treatment groups within sex $p<0.05$. * p value adjusted for #fetus per ewe.

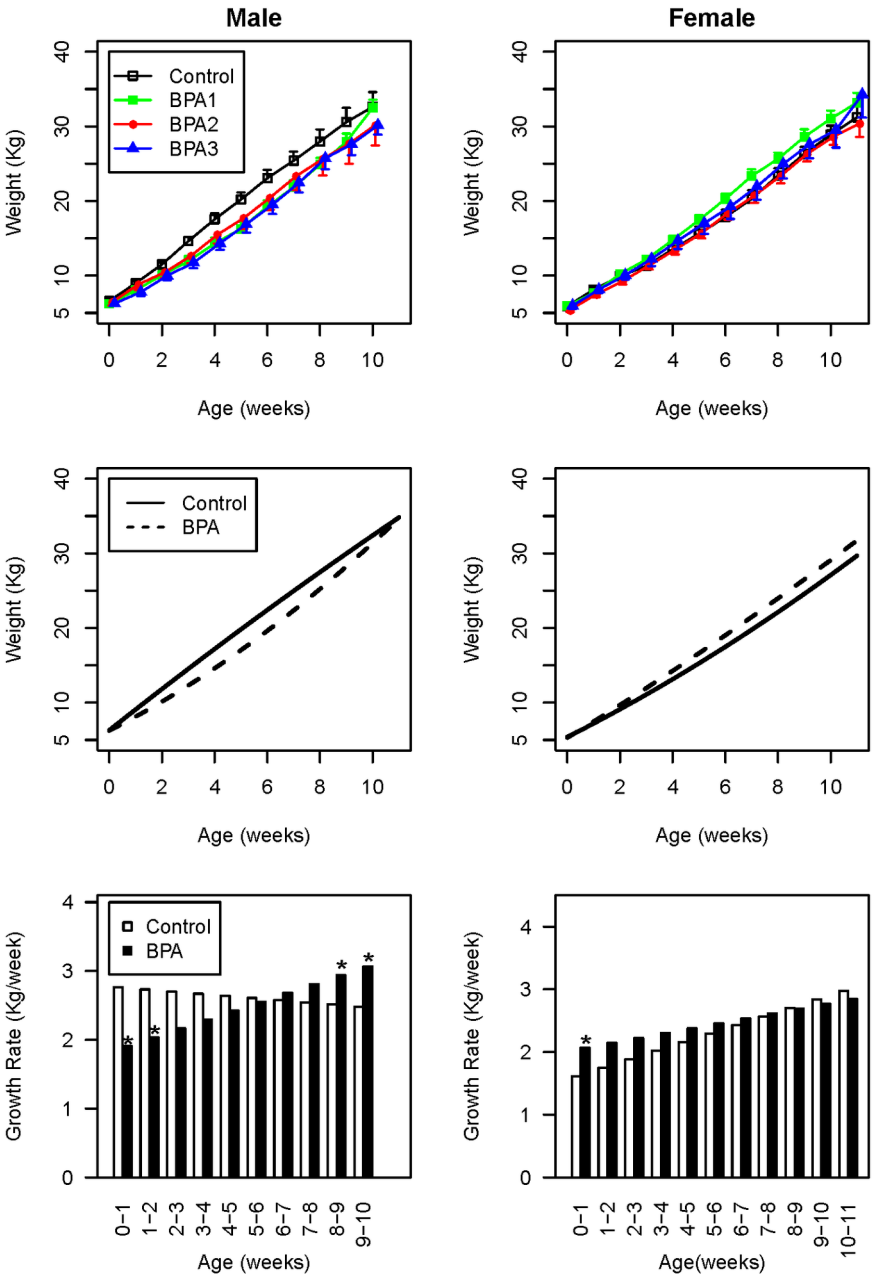
Female GD65 *BPA vs Control Progesterone p=0.002, Pregnenolone p=0.020, Prog:DOC ratio p=0.004. Female GD90 BPA vs Control Corticosterone p=0.045, DOC p=0.029. Control GD90 Female vs Male Cortisol p=0.032, Deoxycortisol p=0.008. BPA GD90 Female vs Male Deoxycortisol p=0.027.*

Supplemental table 3. Fetal organ weights: Difference in fetal organ weights between BPA (dose 0.5mg/kg/day) vs control within each sex and comparing male vs female within each treatment group at gestational days 65 and 90.

		Female		Male	
		Control Mean ± SD ***N***	BPA Mean ± SD ***N***	Control Mean ± SD ***N***	BPA Mean ± SD ***N***
Lung: Body weight ratio #	GD 65	56.11± 8.77 **9**	55.74± 3.57 **9**	49.49± 5.74 **6**	55.7± 5.29 **11**
	GD 90	52.9± 4.91 **8**	53.13± 9.73 **9**	53.06± 4.41 **10**	53.16± 3.87 **4**
Pancreas: Body weight ratio #	GD 65	1.74± 0.96 **9**	1.39± 1.07 **9**	1.33± 0.2 **6**	1.17± 0.11 **11**
	GD 90	1.08± 0.38 **8**	1.11± 0.51 **9**	1.21± 0.21 **10**	1.08± 0.17 **4**
Kidney: Body weight ratio #	GD 65	9.81± 1.12 **9**	9.49± 1.4 **9**	8.29± 0.65 **6**	8.67± 1.06 **11**
	GD 90	8.87± 1.59 **8**	9.55± 0.59 **9**	8.37± 1.05 **10**	8.46± 0.93 **4**
Adrenal: Body weight ratio #	GD 65	0.48± 0.15 **9**	0.48± 0.14 **9**	0.18± 0.03 **6**	0.18± 0.05 **11**
	GD 90	0.52± 0.17 **8**	0.51± 0.18 **9**	0.19± 0.04 **10**	0.19± 0.06 **4**

Pituitary: Body weight ratio #	GD 65	0.08± 0.02 **9**	0.08± 0.02 **9**	0.13± 0.22 **6**	0.05± 0.01 **11**
	GD 90	0.07± 0.02 **8**	0.07± 0.02 **9**	0.05± 0.01 **10**	0.06± 0.01 **4**

adjusted for body weight, then multiplied by 1000. *Standard deviation expressed as SD.* a≠b denotes differences between sex within treatment groups $p<0.05$. A≠B denotes differences between treatment groups within sex $p<0.05$. * p value adjusted for #fetus per ewe.



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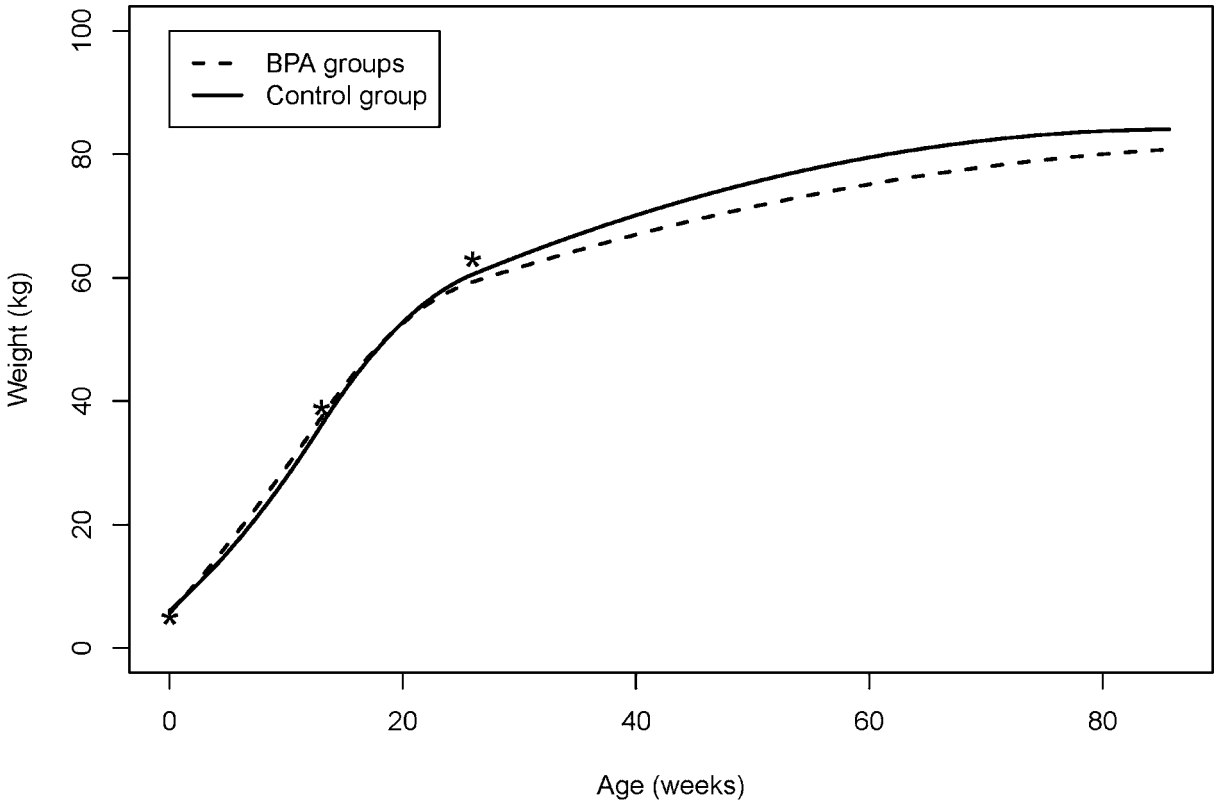

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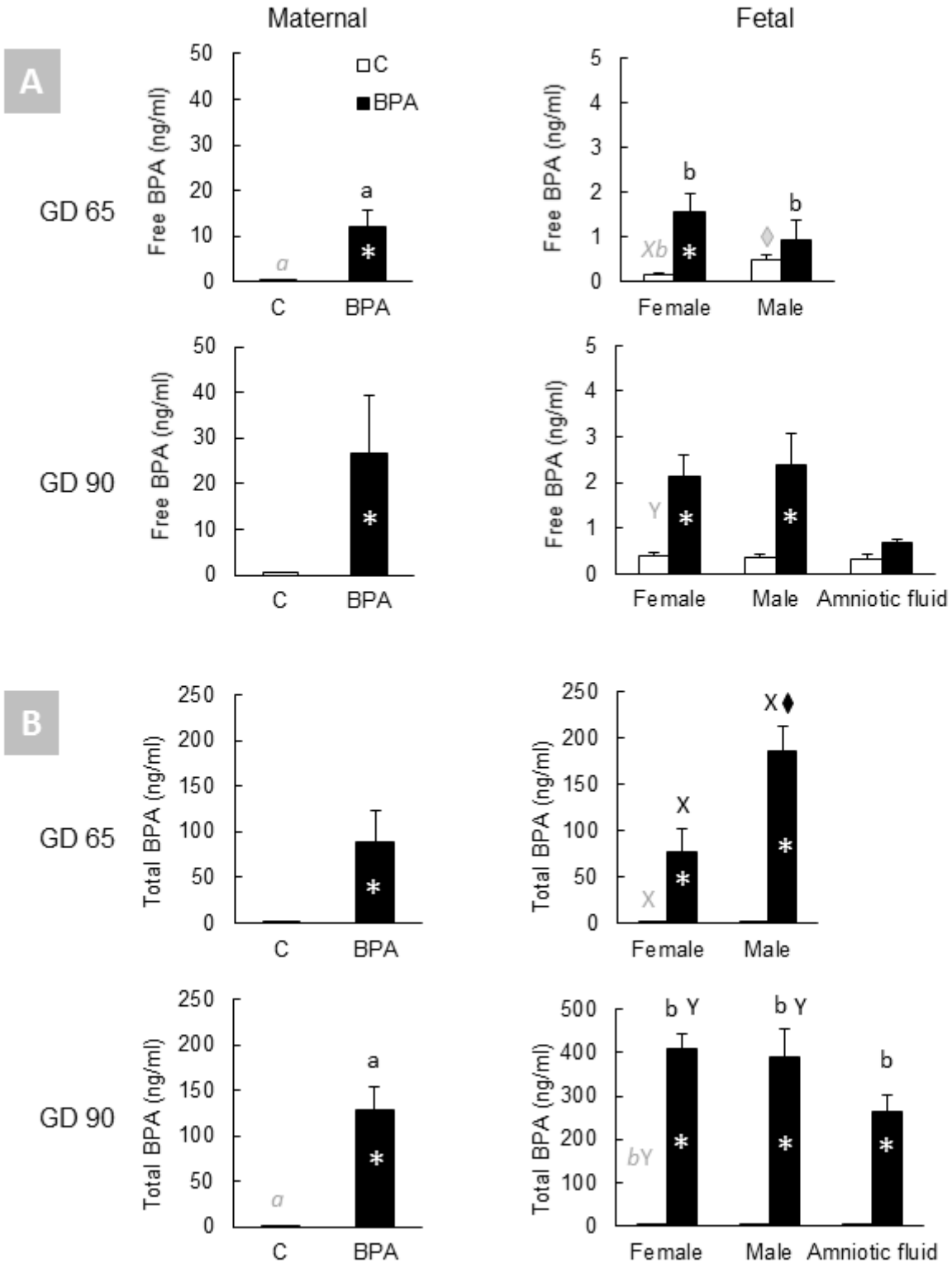

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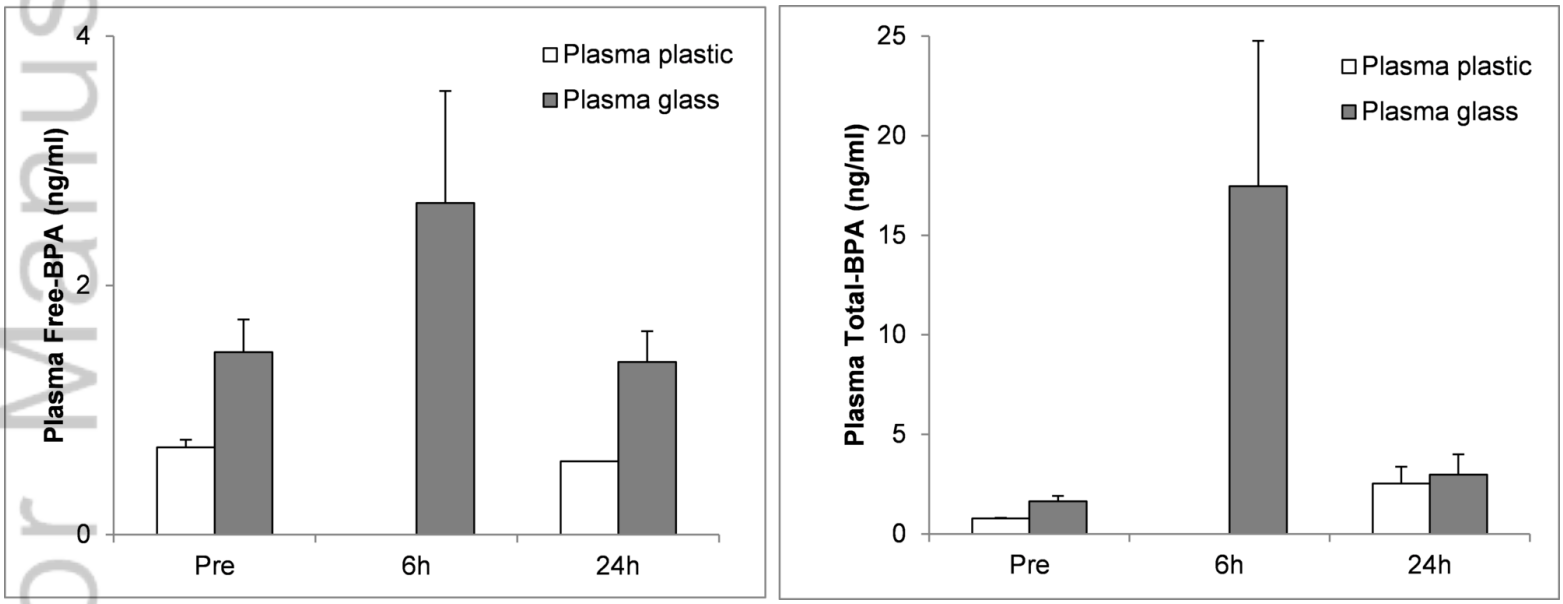

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