

Gestational and Lactational Exposure to Ethinyl Estradiol, but not Bisphenol A, Decreases Androgen-Dependent Reproductive Organ Weights and Epididymal Sperm Abundance in the Male Long Evans Hooded Rat

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Many chemicals released into the environment are capable of disrupting normal sex steroid balance, including the oral contraceptive ethinyl estradiol (EE) and the plastic monomer bisphenol A (BPA). EE and BPA are reported to impair reproductive organ development in laboratory animals; however, effects of lower doses of these chemicals have been debated. The goal of the current study was to determine whether relatively low oral doses of EE or BPA would alter male reproductive morphology and associated hormone levels of Long Evans hooded rat. Dams were gavaged with corn oil vehicle, EE (0.05–50 $\mu\text{g}/\text{kg}/\text{day}$) or BPA (2, 20, and 200 $\mu\text{g}/\text{kg}/\text{day}$) during pregnancy through lactation from gestational day 7 to postnatal day (PND) 18. Anogenital distance was measured at PND2 and nipple retention was measured at PND14 in male pups. Male offspring were euthanized beginning at PND150, and sera and organs were collected for analyses. Adult body weight was significantly decreased in males exposed to 50 μg EE/kg/day. Developmental EE exposure reduced androgen-dependent tissue weights in a dose-dependent fashion; for example, seminal vesicle and paired testes weights were reduced with ≥ 5 μg EE/kg/day. Epididymal sperm counts were also significantly decreased with 50 μg EE/kg/day. In contrast, treatment with 2, 20, or 200 μg BPA/kg/day or EE at 0.05–1.5 $\mu\text{g}/\text{kg}/\text{day}$ did not significantly affect any male endpoint in the current study. These results demonstrate that developmental exposure to oral micromolar doses of EE can permanently disrupt the reproductive tract of the male rat.

Key Words: ethinyl estradiol; bisphenol A; male reproduction; Long Evans rat; testes; epididymal sperm.

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Many chemicals with endocrine activity are released into the environment, including ethinyl estradiol (EE) and bisphenol A (BPA). EE is a potent synthetic estrogen used principally in the female oral contraceptive pill. Unintentional exposure of the developing human fetus to EE can occur when oral contraception is continued through the early months of undetected pregnancy (reviewed in Smithells, 1981). Due to its widespread use as an oral contraceptive, EE has been detected in river systems in the United States and Europe (Kolpin *et al.*, 2002; Zuo *et al.*, 2006), and is known to negatively impact fish reproduction (Jobling *et al.*, 1998, 2006). BPA is an estrogenic monomer used in the manufacture of polycarbonate products and is known to leach from them with use (see review in Howdeshell *et al.*, 2003). Concerns have been expressed about potential effects of BPA on human reproductive development because of its extensive use in consumer products, including baby bottles (vom Saal *et al.*, 2007).

The estrogenic effects of EE and BPA have been demonstrated in the laboratory in both *in vitro* and *in vivo* assays. A multilaboratory validation study found consistent, significant uterine stimulation with EE or BPA in intact prepubertal and ovariectomized adult female rats (Wistar and SD strains) administered over 3 days by oral (pubertal only) or subcutaneous injection (Kanno *et al.*, 2001). In Wistar male rats, subcutaneous injection of a high dose of BPA (50 mg/kg/day) to prepubertal males from 22 to 32 days of age significantly increased lateral prostate weight and inflammation at 120 days of age relative to control males (Stoker *et al.*, 1999). However, the reproducibility and nature of the effects of these chemicals at lower doses are currently the subject of intense debate in the scientific community and popular press (EWG, 2007; National Toxicology Program, 2007b; vom Saal *et al.*, 2007).

The objective of the current study was two-fold: (1) to determine if relatively low oral doses of EE (as a positive control estrogen) and BPA, administered to the dam during gestation and lactation, would alter reproductive morphology

of male Long Evans (LE) hooded rat offspring, and (2) to determine if developmental EE exposure would induce similar effects on the LE rat strain as were previously reported for the Sprague–Dawley (SD) rat (Sawaki *et al.*, 2003). Pregnant rats were gavaged daily from gestation day (GD) 7 through postnatal day (PND) 18 to expose their offspring during the period of sexual differentiation of the reproductive organs as well as the initial period of sexual differentiation of the brain. The oral route of exposure was selected for this rat study to simulate the most common route of exposure of humans to EE and BPA. We selected the LE rat strain because it had previously been used in our laboratory for the evaluation of estrogenic endocrine-disrupting chemicals, such as methoxychlor (Gray *et al.*, 1988, 1989), and antiandrogenic compounds on reproductive development (Gray *et al.*, 1999). Furthermore, the LE rat strain is commonly used for the study of reproductive behaviors, and the female offspring of the current study were observed for alterations in sexually dimorphic behaviors (Ryan and Gray, in preparation). The dosing period and the EE dose range, which spanned 4 log units (0.05–50 µg/kg/day), were modeled after a developmental study in CD(SD)IGS rats by Sawaki *et al.* (2003). The BPA dose range (2–200 µg/kg/day) was selected because it encompassed the lower doses previously studied in mice (Nagel *et al.*, 1997; Richter *et al.*, 2007; vom Saal *et al.*, 1998) and rats (Akingbemi *et al.*, 2004; Ema *et al.*, 2001; Tinwell *et al.*, 2002; National Toxicology Program CERHR Expert Panel Report on BPA-National Toxicology Program, 2007b). It was not the intention of this study to determine the sensitivity of the LE rat reproductive tract to developmental exposure to BPA over a range of doses.

MATERIAL AND METHODS

Animals. Adult female LE rats (Charles River Laboratories, Raleigh, NC) of approximately 90 days of age were mated by the supplier and shipped on GD2. Mating was confirmed by sperm presence in vaginal smears (day of sperm plug positive = GD1). All animals were housed in transparent, 20 × 25 × 47-cm polycarbonate cages with laboratory-grade, heat-treated pine shavings (Northeastern Products, Warrensburg, NY) with a 14L:10D photoperiod at 20°C–24°C and 40–50% relative humidity. Previous research has demonstrated that polycarbonate rodent caging can release increasing amounts of BPA as it becomes cloudy and worn with use (Howdeshell *et al.*, 2003); all caging used in the current experiment was clear and without evidence of significant wear. Pregnant and lactating dams were fed Purina Rat Chow 5008 *ad libitum*, and weanling and adult rats were fed Purina Rat Chow 5001 *ad libitum*. Animals were provided constant access to filtered (5-µm filter) municipal drinking water (Durham, NC) via an automatic watering system; the purpose of this noncharcoal filter was to remove potential bacterial growth. Water was tested monthly for *Pseudomonas* and every 4 months for a suite of chemicals, including pesticides and heavy metals. The current study was conducted under a protocol approved by the Environmental Protection Agency's National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care approved animal facility.

Doses and administration of chemicals. Pregnant rat dams were assigned to treatment groups on GD7 in a manner that provided similar mean body weight per treatment group within each block prior to dosing. Laboratory-grade

corn oil (CAS 8001-30-7; Lot 062K006), EE (CAS 57-63-6; Lot 103K1230; purity 98%), and BPA (CAS 80-05-7; Lot 03105ES; purity ≥ 99%) were purchased from Sigma-Aldrich (St Louis, MO). Pregnant dams were dosed via oral gavage from GD7 through PND18 with 0 (corn oil, vehicle control), EE at 0.05, 0.5, 1.5, 5, 15, or 50 µg/kg/day, or BPA (BPA) at 2, 20, or 200 µg/kg/day. The doses were delivered in 0.5 µl corn oil/g body weight, thus all dams in the study received the same amount of vehicle per body weight. The dams were weighed daily during the dosing period to administer the dose per kilogram body weight and to monitor their health.

This study was performed in two blocks. The first block involved 169 dams with 13–29 dams per treatment group: oil vehicle, EE (0.05, 0.5, 5 or 50 µg/kg/day), or BPA (2, 20, or 200 µg/kg/day). The majority of the males in the first block were necropsied beginning at 150 days of age (Block 1a), whereas a second set (Block 1b) was used in a breeding study with females from the first block prior to necropsy at PND229. The second block (Block 2) was performed to expand the range of EE doses tested and involved 82 dams with 6–14 dams per treatment group: oil vehicle, EE (0.05, 0.15, 0.5, 1.5, 5, 15, or 50 µg/kg/day), or BPA (20 or 200 µg/kg/day). A subset of control males from Block 2 were used in a breeding study with females from the same block, thus they were not included in the necropsy for the Block 2 males at PND150. Vehicle corn oil-treated dams and untreated control dams (one dam in the first block and five dams in the second block) were not significantly different from one another and thus were combined to calculate the control values for each block.

Maternal body weight gain during pregnancy was calculated from inception of dosing (GD7) to GD20 and the analysis of these data included only those dams that were pregnant and survived through GD20. Maternal body weight gain during lactation was calculated from PND2 through end of dosing (PND18) and included only those dams with pups surviving to weaning.

Neonatal and pubertal data. All dams were allowed to deliver naturally, and on PND2, the sex, body weight, and anogenital distance (AGD) of each pup were recorded; the observer measuring these endpoints was blinded as to the treatment group of the pup. AGD was measured using a dissecting microscope at 15× magnification with an ocular micrometer as per Hotchkiss *et al.* (2004). The AGD was defined as the distance between the anterior end of the anus and the posterior end of the genital papilla. On PND14, pups were reweighed, identified by sex, and the number of female-like areolae/nipples was counted. Male rat pups do not normally retain areolae because their higher levels of endogenous androgens, relative to females, cause regression of the nipple anlagen *in utero*.

Offspring were weaned at PND22 to PND25 and male rats were housed two per cage, with the exception of eight control males from each block that were used for lordosis behavior testing with females. Female offspring were evaluated by a series of measures of reproductive function and morphology as well as assays of sexually dimorphic behaviors; the female data will be published elsewhere (Ryan and Gray, in preparation). The rat dams were euthanized after their pups were weaned and the number of uterine implantation scars was counted and recorded. The number of live pups at PND2 was subtracted from the number of uterine scars to provide an indicator of fetal/neonatal mortality.

Necropsy. Male rats were necropsied when they reached at least 4 months old, following CO₂ anesthesia and decapitation. The total number of individual males and litters in Block 1a by treatment were as follows: vehicle control—20, 8; BPA (2 µg/kg/day—18, 7; 20 µg/kg/day—18, 7; 200 µg/kg/day—20, 9); and EE (0.05 µg/kg/day—16, 6; 0.5 µg/kg/day—20, 8; 5 µg/kg/day—20, 7; and 50 µg/kg/day—20, 7). The total number of individual males and litters, respectively, necropsied in Block 1b by treatment were as follows: vehicle control—11, 6; and EE (5 µg/kg/day—9, 7; and 50 µg/kg/day—3, 2). Finally, the total number of individual males and litters, respectively, necropsied in Block 2 by treatment were as follows: vehicle control—35, 10; BPA (20 µg/kg/day—16, 6; 200 µg/kg/day—34, 9; and EE (0.05 µg/kg/day—17, 6; 0.15 µg/kg/day—18, 6; 0.5 µg/kg/day—26, 7; 1.5 µg/kg/day—28, 9; 5 µg/kg/day—41, 8; 15 µg/kg/day—23, 6; and 50 µg/kg/day—1, 1).

The Block 1a and 2 males were necropsied beginning at 150 days of age, whereas the Block 1b males were necropsied beginning at 229 days of age after

use in a breeding experiment with females from Block 1 (Ryan and Gray, in preparation). These data were initially examined separately and then pooled to display overall treatment effects; the litter means of each treatment are reported per block and total (all blocks combined) in Supplementary Data. However, due to the incomplete block design of the study (not all EE or BPA doses were included in each block), the data from blocks 1a, 1b, and 2 were pooled and reanalyzed following conversion to percent of control using the control values for each respective block. The results of the statistical analysis for the percent of control and unadjusted litter mean values were essentially identical.

At necropsy, trunk blood was collected after decapitation and stored at -80°C until analysis of serum hormone levels. The ventral surface of each male was shaved and examined for abnormalities, including the number and location of retained nipples, hypospadias, cleft phallus, cleft prepuce, vaginal pouch, exposed os, and abnormal glans penis (e.g., incomplete preputial separation). The animals were examined for any internal reproductive malformations, including epididymal agenesis, testicular malformations (e.g., hypoplasia, atrophy, cryptorchid, or fluid-filled testes), as well as agenesis of the vas deferens, prostate, and seminal vesicles. The animals were also examined for hydronephrosis, bladder stones, and blood in the bladder.

Organ weights were recorded for the following: ventral and lateral prostate, seminal vesicles, testes, epididymides, glans penis, levator ani-bulbocavernosus (LABC) muscle, Cowper's gland, and liver. Sometimes, the seminal vesicles and Cowper's glands leaked during removal and prior to weighing; in these cases, the tissue weights were not included in the statistical analysis. The right epididymis was processed for the total number of sperm (see below). In addition, organ weights were recorded for pituitary, whole brain, kidneys, adrenals, spleen, heart, and lungs; these weights were only recorded for a subset of Block 1a males. These organs were not examined further, because there was no evidence of any treatment-related effects.

Tissues were subsequently preserved in Bouin's fixative for 24 h, then transferred into 70% ethanol for histological examination. The tissues selected for histopathology from Block 1a included the following: the right and left testes, left epididymis, glans penis, lateral and ventral prostate, seminal vesicles, Cowper's glands, and LABC. The tissues were paraffin embedded and a 6- μm section of each tissue was stained with hematoxylin and eosin. The tissue preparation and histopathological evaluation were conducted by Experimental Pathology Laboratories, Inc. (Research Triangle Park, NC). Of the tissues from Block 2 males, only the testes of control and BPA-treated males were examined for histopathology to confirm the findings of the first experiment. The tissues of the males from the breeding study (Block 1b) were not processed for histopathology.

Epididymal sperm counts. The abundance of epididymal sperm was measured as per Gray *et al.* (1997). At necropsy, the right epididymis was cut into two pieces (the cauda, distal-most 16 mm; and the corpus-caput, remainder of the epididymis) and weighed separately. The cauda and corpus-caput epididymal pieces were placed in separate 20-ml glass scintillation vials containing 2 ml of M199 media (Gibco through Invitrogen Corp., Carlsbad, CA), then were minced with dissecting scissors for approximately 30 s. After mincing, three additional milliliters of M199 media was added and the vials were incubated for 1 h in a water bath at 37°C to allow sperm to disperse into the medium. After incubation, 15 ml of 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ) was added to the vial to preserve the sperm and the sample was stored at room temperature. The sperm in the samples were counted electronically on a Coulter Counter Multisizer II (Beckman Coulter Inc., Fullerton, CA). The sperm were further diluted with 10% buffered formalin to produce counts within a range of 10,000–20,000 particles/0.5 ml of sample. The settings were as follows: sperm sample volume 0.5 ml, aperture size 70 μm ; aperture current 1600 μA ; gain 2, lower threshold 3.797 μm and upper threshold 6.797 μm , and channels 256. Three separate counts of each sample were taken and the mean value was used. All of Block 1 and one-half of Block 2 (Block 2a) samples were counted on the Coulter Counter Multisizer. We counted the remaining samples from Block 2 (Block 2b) on a newly purchased Coulter Counter Multisizer III (Beckman Coulter, Inc., Brea, CA) with the same settings with the exception of an aperture size of 100 μm and channels 512.

Serum steroid hormone radioimmunoassay. At necropsy, the blood collected from each animal was allowed to clot at 4°C for a minimum of 30 min in Vacutainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ), then centrifuged at $1000 \times g$ for 15 min at 4°C . The serum was then stored in Eppendorf tubes at -80°C until assayed by radioimmunoassay (RIA) for the levels of six hormones including estradiol, testosterone, corticosterone, total T4, luteinizing hormone (LH), and prolactin (PRL). Estradiol, total testosterone, corticosterone, and total thyroxine levels were measured using Coat-Count kits according to manufacturer's protocols (Diagnostic Products Corporation, Los Angeles, CA). Serum LH and PRL were measured by RIA as described in Goldman *et al.* (1986) and Stoker *et al.* (1999), respectively, using materials supplied by the National Hormone and Pituitary Program (Torrance, CA): iodination preparation I-6; reference preparation RP-3; and antisera S-11 for LH or S-9 for PRL. Iodination material for the LH and PRL assays was radiolabeled with ^{125}I (Dupont/New England Nuclear) by a modification of the chloramine-T method (Greenwood *et al.*, 1963). The level of detection of the RIAs were the following: estradiol, 1.5 pg/ml; testosterone, 0.2 ng/ml; LH, 0.115 ng/ml; PRL, 0.5 ng/ml; corticosterone, 20 ng/ml; and total thyroxine, 1 $\mu\text{g}/\text{dl}$. The sera samples were run as duplicates in the LH and PRL assays, and as single samples in the remainder of the hormone assays. Intra-assay coefficients of variation (CV) for the following assays were calculated based on the variability of the standard curve replicates: estradiol, 2.9%; testosterone, 3.3%; corticosterone, 1.6%; total thyroxine, 5.4%, and PRL, 2.6%. The intra-assay CV for the LH assay was 10.2% based upon the variability of the quality controls duplicates in the assay. There was less than 1% cross-reactivity between the RIA antibodies for the steroid hormones (estradiol, testosterone, and corticosterone) or the pituitary hormones (LH and PRL).

Statistical analyses. The maternal and F1 pup data adult body and organ weights, sperm counts, and hormone measurements were analyzed using PROC GLM by one-way analysis of variance (ANOVA) using PC SAS, version 8.2 (Cary, NC). The statistical analyses of F1 adult male rat organ weights at necropsy and epididymal sperm counts were initially analyzed separately for each age cohort (Block 1a, 1b, and 2), then the data were pooled on a percent of control values using the respective control values for each block to account for differences in size and age at necropsy. The percent of control analysis was performed because it was not possible to evaluate the data for block by treatment interactions using ANOVA because the different blocks did not have identical treatment groups. The influence of body weight on adult organ weights was subsequently determined by using body weight at necropsy as a covariate in an analysis of covariance (ANCOVA). Finally, the ANCOVA analyses were restricted to the dose range of EE or BPA, and controls, and differences between treatments were determined by *post hoc* least squared means analysis. The hormone measurements were log-transformed to normalize the data, then evaluated by ANOVA using PC SAS. As with the necropsy data, the hormone data were first analyzed using all treatment groups followed by an ANOVA of the EE or BPA dose range compared with controls and *post hoc* comparisons were made by least squared means. The statistical analyses presented in this paper for the F1 males are of the litter means; the litter means data were generated by computing an average litter mean for each endpoint with PROC MEANS. The frequency of dams not giving birth, although pregnant, was analyzed by Fisher Exact Test. The incidence of histopathological lesions was analyzed on an individual basis (not by litter) by chi-squared test. Statistical significance was considered $p \leq 0.05$.

RESULTS

Maternal and Pregnancy Data

There was a significant main effect of treatment on maternal body weight gain during gestation ($F = 16.37$, $p < 0.001$). Treatment with EE at 1.5 $\mu\text{g}/\text{kg}/\text{day}$ and higher significantly decreased maternal body weight gain during gestation relative

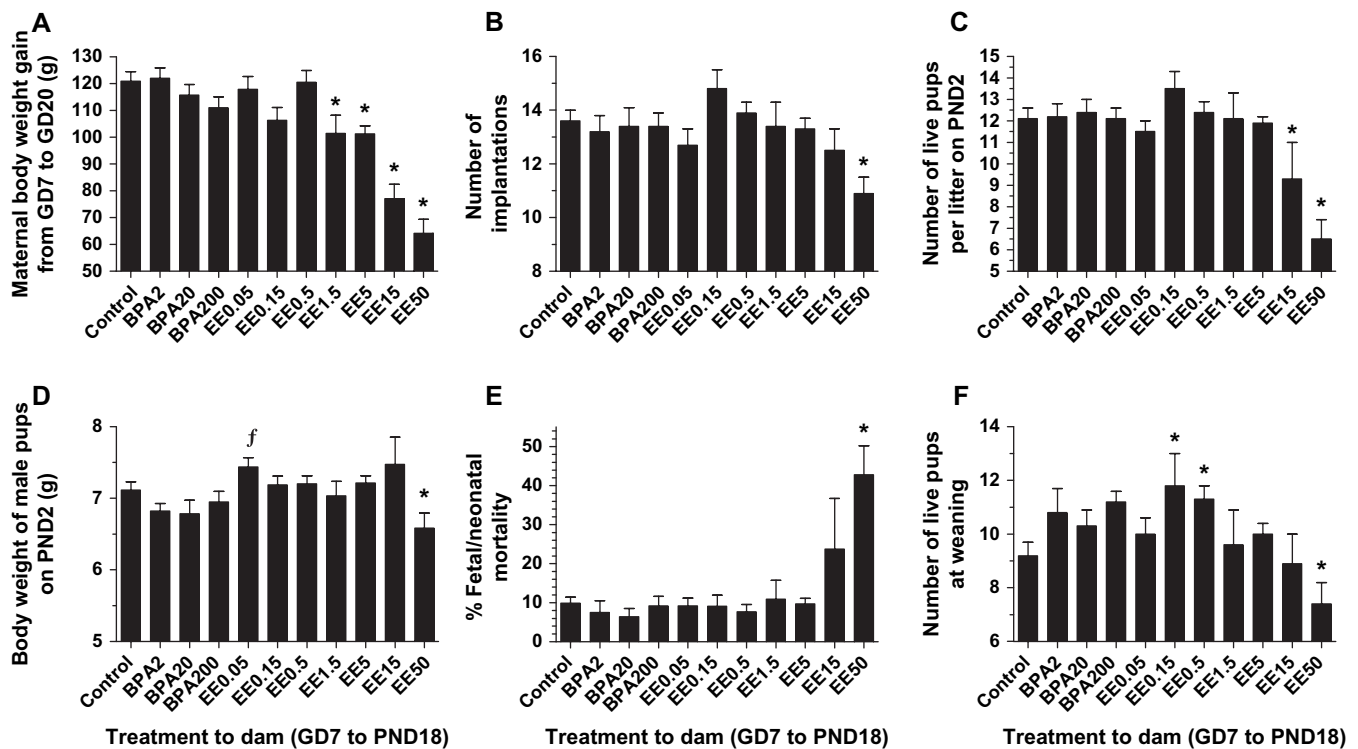


FIG. 1. Maternal body weight gain (A), number of implantations (B), number and body weight of live pups on PND2 (C, D), fetal/neonatal mortality (E), and number of live pups at weaning of LE rats dams administered EE or BPA via gavage on GD7 to PND18 (F). The doses of BPA and EE are expressed as $\mu\text{g}/\text{kg}/\text{day}$ (e.g., BPA2 = 2 $\mu\text{g}/\text{kg}/\text{day}$ BPA). The number of litters per treatment was as follows: vehicle control, 33–38; BPA 2, 7–13; BPA 20 13–20; BPA 200, 15–22; EE 0.05, 17–19; EE 0.15, 6; EE 0.5, 29–35; EE 1.5, 9–10; EE 5, 30–37; EE 15, 7–8; and EE 50, 14–24. Data are litter means \pm SE; * $p < 0.05$ versus controls.

to controls (Fig. 1A; Table 1). During lactation, maternal body weight gain was significantly increased in dams administered EE at 15 $\mu\text{g}/\text{kg}/\text{day}$ or higher, and tended to be higher at 5 $\mu\text{g}/\text{kg}/\text{day}$, relative to controls. However, dams administered EE at 0.05 $\mu\text{g}/\text{kg}/\text{day}$ had decreased maternal body weight gain during lactation relative to controls. There were no treatment effects of BPA on maternal body weight gain during pregnancy or lactation.

There was a significant main effect of treatment on the number of uterine implantations ($F = 2.76$, $p < 0.005$) with the 50 $\mu\text{g}/\text{kg}/\text{day}$ EE group having significantly fewer uterine implantation scars (Fig. 1B, Table 1). Dams dosed with EE at 50 $\mu\text{g}/\text{kg}/\text{day}$ had an increased incidence of not giving birth, although they were pregnant (4/24 dams; $p < 0.05$). In addition, one dam in each of the 0.5 and 15 $\mu\text{g}/\text{kg}/\text{day}$ EE groups was pregnant but did not give birth. One dam of the 50 $\mu\text{g}/\text{kg}/\text{day}$ EE group gave birth to only one pup and died on PND4. In a few litters, the pups died before weaning: one litter each of 20 and 200 $\mu\text{g}/\text{kg}/\text{day}$ BPA, 1.5 and 50 $\mu\text{g}/\text{kg}/\text{day}$ EE groups, and two litters in the 0.5 $\mu\text{g}/\text{kg}/\text{day}$ EE group. Data were accidentally not recorded on the number of implantations for three dams (one dam each in the control, 20 and 200 $\mu\text{g}/\text{kg}/\text{day}$ BPA treatment groups); the maternal body weight on PND2 was not recorded for one dam treated with 50 $\mu\text{g}/\text{kg}/\text{day}$

day. A total of five dams involved in this study were not pregnant: one dam each in the 5 and 50 $\mu\text{g}/\text{kg}/\text{day}$ EE groups and the 200 $\mu\text{g}/\text{kg}/\text{day}$ BPA group, and two dams in the control group. A total of 8 dams died of unknown causes, not due to treatment or dosing: two dams each in the control, 0.05, 0.5, and 50 $\mu\text{g}/\text{kg}/\text{day}$ EE groups. One dam in the 0.5 $\mu\text{g}/\text{kg}/\text{day}$ EE group and five dams in the 50 $\mu\text{g}/\text{kg}/\text{day}$ EE group were removed from the study due to dosing errors.

There was a significant main effect of treatment on the number and body weight of live pups on PND2 ($F = 8.48$, $p < 0.0001$) and postimplantation fetal/neonatal mortality ($F = 8.04$, $p < 0.0001$) at PND2. Treatment with EE at 15 and 50 $\mu\text{g}/\text{kg}/\text{day}$ decreased the number of live pups at PND2 and EE at 50 $\mu\text{g}/\text{kg}/\text{day}$ also decreased the body weight of live pups on PND2 relative to control values (Figs. 1C and 1D, Table 1). The percent of fetal/neonatal morbidity was significantly higher ($p < 0.0001$) in the 50 $\mu\text{g}/\text{kg}/\text{day}$ EE group relative to controls (Fig. 1E). *Post hoc* analysis detected no significant effect of BPA treatment on the number of implantations, number of live pups at PND2, or fetal/neonatal mortality. Treatment with 50 $\mu\text{g}/\text{kg}/\text{day}$ EE significantly decreased the number of pups at weaning relative to controls, although the number of weaned pups was significantly increased in the 0.15 and 0.5 $\mu\text{g}/\text{kg}/\text{day}$, and 200 $\mu\text{g}/\text{kg}/\text{day}$ BPA groups (Fig. 1F, Table 1).

TABLE 1
Maternal Body Weight, Number Implantations, Fetal and Pup Data (Litter Means ± SE) for Dams Administered BPA and EE during Pregnancy, GD7, through Lactation, PND18

	Vehicle control	BPA 2 (µg/kg/day)	BPA 20 (µg/kg/day)	BPA 200 (µg/kg/day)	EE 0.05 (µg/kg/day)	EE 0.15 (µg/kg/day)	EE 0.5 (µg/kg/day)	EE 1.5 (µg/kg/day)	EE 5 (µg/kg/day)	EE 15 (µg/kg/day)	EE 50 (µg/kg/day)
No. of dams pregnant on GD20	38	13	20	22	19	6	35	10	37	8	24
Maternal body wt. at GD7 (g)	236.9 ± 4.5	241.2 ± 5.2	249.6 ± 6.3 ^a	248.7 ± 4.3	240.8 ± 7.4	277.6 ± 5.5 ^b	239.1 ± 4.9	261.4 ± 8.1 ^b	236.8 ± 5.2	262.8 ± 9.5 ^b	244.7 ± 4.4
Maternal body wt. at GD20 (g)	357.8 ± 5.2	363.2 ± 7.2	365.3 ± 6.3	359.6 ± 5.4	358.7 ± 5.6	383.9 ± 7.7 ^b	359.7 ± 5.6	362.9 ± 7.7	338.1 ± 5.7 ^b	339.8 ± 4.4	309.0 ± 6.9 ^b
Maternal body wt. gain from GD7–GD20 (g)	120.9 ± 3.6	122.0 ± 3.9	115.7 ± 4.0	111.0 ± 4.1	117.9 ± 4.8	106.3 ± 4.8	120.6 ± 4.3	101.4 ± 6.8^{b,f}	101.3 ± 2.9^b	77.0 ± 5.4^b	64.2 ± 5.2^b
Maternal body wt. at PND2 ^c (g)	306.1 ± 4.5	310.8 ± 6.6	315.5 ± 5.2 (19) ^c	308.6 ± 4.8 (21)	310.5 ± 5.0	328.7 ± 5.2 ^b	308.1 ± 4.0 (32)	314.5 ± 8.0 (9)	288.0 ± 4.2 ^b	294.1 ± 7.4 (7)	264.4 ± 7.1 ^b (16)
Maternal body wt. at PND18 ^c (g)	318.8 ± 4.6	329.1 ± 6.8	324.2 ± 5.2 (19)	318.9 ± 3.6 (21)	307.5 ± 7.7	332.2 ± 2.9	315.8 ± 3.3 (32)	328.3 ± 5.4 (9)	307.9 ± 4.0 ^a	325.0 ± 7.3 (7)	292.5 ± 8.1 ^b (17)
Maternal body wt. gain from PND2–PND18 ^c (g)	12.2 ± 2.2	18.3 ± 3.6	8.7 ± 2.6 (19)	10.3 ± 3.7 (21)	−3.0 ± 6.3^b	3.5 ± 4.6	7.7 ± 2.4 (32)	13.8 ± 6.6 (9)	19.9 ± 2.6 ^a	30.9 ± 5.3^b (7)	26.5 ± 6.5^b (16)
No. of implantations	13.6 ± 0.4 (37)	13.2 ± 0.6	13.4 ± 0.7 (19)	13.4 ± 0.5 (20)	12.7 ± 0.6	14.8 ± 0.7	13.9 ± 0.4	13.4 ± 0.9	13.3 ± 0.4	12.5 ± 0.8	10.9 ± 0.6^b
No. of live pups per litter on PND2	12.1 ± 0.5	12.2 ± 0.6	12.4 ± 0.6	12.1 ± 0.5	11.5 ± 0.5	13.5 ± 0.8	12.4 ± 0.5	12.1 ± 1.2	11.9 ± 0.3	9.3 ± 1.7^b	6.5 ± 0.9^b
Fetal/neonatal mortality ^d (%)	9.9 ± 1.5 (37)	7.5 ± 3.0	6.4 ± 2.1 (19)	9.2 ± 2.4 (21)	9.2 ± 2.0	9.1 ± 2.8	7.7 ± 1.8 (32)	10.9 ± 4.8	9.7 ± 1.4	23.7 ± 13.0	42.8 ± 7.4^b
No. of live pups per litter at weaning ^e	9.2 ± 0.5	10.8 ± 0.9	10.3 ± 0.6 (19)	11.2 ± 0.4 (21)	10.0 ± 0.6	11.8 ± 1.2^b	11.3 ± 0.5^b (32)	9.6 ± 1.3 (9)	10.0 ± 0.4	8.9 ± 1.1 (7)	7.4 ± 0.8^b (17)
Male pup body wt. on PND2	7.12 ± 0.12 (186, 33) ^c	6.82 ± 0.10 (41, 7)	6.79 ± 0.19 (87, 13)	6.95 ± 0.15 (92, 15)	7.44 ± 0.13 ^a (105, 19)	7.19 ± 0.13 (44, 6)	7.20 ± 0.12 (186, 29)	7.04 ± 0.20 (46, 9)	7.21 ± 0.10 (183, 30)	7.47 ± 0.38 (34, 7)	6.58 ± 0.21^b (51, 14)
Male pup AGD on PND2	3.54 ± 0.07 (138, 24)	NA	3.38 ± 0.08 (40, 6)	3.30 ± 0.08 (56, 9)	3.59 ± 0.07 (93, 17)	3.45 ± 0.06 (44, 6)	3.55 ± 0.06 (119, 19)	3.39 ± 0.05 (46, 9)	3.56 ± 0.45 (131, 21)	3.50 ± 0.15 (34, 7)	3.34 ± 0.10 (23, 7)

^a*p* = 0.05–0.08 versus control.

^b*p* < 0.05 versus control.

^cData based on litters with live pups at weaning.

^dFetal/neonatal mortality = (no. of implantations – no. of pups on PND3)/no. of implantations.

^eFor male pup body weight and AGD, the parentheses contain the number of individuals and litters measured.

^fValues in bold were significantly different than controls due to treatment.

The number of dams was indicated in parenthesis when different from the total number of dams pregnant on GD20.

Neonatal and Pup Data

Table 2 displays a list of all the endpoints measured in the F1 male rats exposed EE and BPA from GD7 to GD18. Prenatal treatment with 50 µg EE/kg/day significantly ($p < 0.01$) decreased male pup body weight on PND2 with a 7.5% decrease relative to control values (Fig. 1D). Male pup body weight was not significantly impacted by developmental exposure to BPA. There was no effect of treatment on the AGD of male rats at PND2 (Table 1). One-half of the litters from Block 1 were excluded from the PND2 body weight and AGD analysis because the pups were measured on PND3, instead of PND2 (Fig. 1D). However, when the neonatal body weight measurements for PND2 and PND3 were analyzed together after they were converted to percent of controls, the combined neonatal body weights were the same as for the PND2 males alone (data not shown). There was no areolae/nipple retention, a female rat trait, among male rats developmentally exposed to EE or BPA.

Necropsy of Adult Offspring

Three of the males developmentally exposed to 50 µg EE/kg/day were lethargic at necropsy, and each of them displayed

large, externally visible epididymal granulomas ($p < 0.02$ higher incidence [3/26] vs. controls [0/66] by Fisher Exact Test). The data from these males were not included in the adult organ weight analyses. There were no other abnormalities, or reproductive malformations (external or internal) noted for any male in this study.

Maternal high-dose EE treatment significantly reduced body weight of the males in adulthood; the main effect of treatment in the ANOVA comparing EE doses versus control ANOVA was $F = 2.23$, $p < 0.05$. *Post hoc* analysis indicated that developmental exposure to 50 µg EE/kg/day significantly reduced body weight in adulthood ($p < 0.005$; Fig. 2A). In contrast, treatment with BPA did not significantly influence body weight at necropsy. Body weight at necropsy was retained as a covariate in the final analyses, because all of the reproductive organs measured with the exception of ventral prostate were significantly influenced by body weight at collection. Overall, androgen-dependent reproductive organs weights were decreased in a dose-dependent manner by EE. The weights of the seminal vesicles and paired testes were both significantly decreased in a dose-dependent manner by EE exposure at 5 µg/kg/day and higher (Figs. 2B and 2C). Ventral prostate was decreased 36.5% by 50 µg EE/kg/day ($p < 0.0001$) with nonsignificant decreases observed at 5 (3%) and 15 µg EE/kg/day (10.4%; Fig. 2D). The 50 µg EE/kg/day treatment also decreased the weights of the glans penis and the LABC (Figs. 2E and 2F). Although the weight of the paired epididymides was decreased by 50 µg EE/kg/day, this decrease was associated with the decrease in body weight at this dose (Fig. 3A).

Developmental exposure to EE did not affect the weight of the Cowper's glands. There were no BPA effects on any reproductive organ weight. Many of the nonreproductive organ weights were significantly correlated with body weight at necropsy, however, no significant treatment effects were found for either EE or BPA. All body weight, absolute organ weights (reproductive and nonreproductive), and percent of control values for these endpoints are included in Supplementary Data (Tables 1–2).

Histopathology of Adult Organs at Necropsy

Histopathological examination confirmed the adverse effects of developmental exposure to high doses of EE on F1 male reproductive organs. The histopathological evaluation of Block 1a males confirmed the presence of epididymal granulomas in three 50 µg EE/kg/day males; a granuloma is caused by inflammation and is not necessarily considered a malformation. It is possible that the lesion in these three males resulted from subtle malformations in the epididymal ducts, but no gross or histological malformations were noted in any of the other males. These three males also exhibited inflammation in all or a combination of the following organs: testes, seminal vesicles, LABC, ventral prostate, and glans penis. The incidence of bilateral seminal vesicle hypoplasia was significantly higher in rats exposed to 50 µg EE/kg/day (5/26 males; 19.2%) relative

TABLE 2
Endpoints Studied in Male LE Rats Exposed to EE and BPA from GD8 through PND18

Endpoint	Affected	Not affected
Body weight at PND2	X	
AGD at PND2		X
Areolae/nipple retention at PND13		X
Body weight in adulthood	X	
Nipple retention in adulthood		X
Glans penis weight	X	
Ventral prostate weight	X	
Seminal vesicle weight	X	
Paired testes weight	X	
Paired epididymides weight		X
LABC muscle weight	X	
Cowper's gland weight		X; supplementary data
Liver weight		X; supplementary data
Kidney weight		X; supplementary data
Paired adrenal gland weight		X; supplementary data
Spleen weight		X; supplementary data
Heart weight		X; supplementary data
Paired lung weight		X; supplementary data
Brain weight		X; supplementary data
Pituitary weight		X; supplementary data
Testosterone, serum levels		X; supplementary data
LH, serum levels		X; supplementary data
PRL, serum levels		X; supplementary data
Estradiol, serum levels		X; supplementary data
Corticosterone, serum levels		X; supplementary data
Thyroxine levels, serum levels		X; supplementary data
Sperm number per epididymis	X	
Reproductive tract malformations		X
Histopathology of reproductive tissues	X	

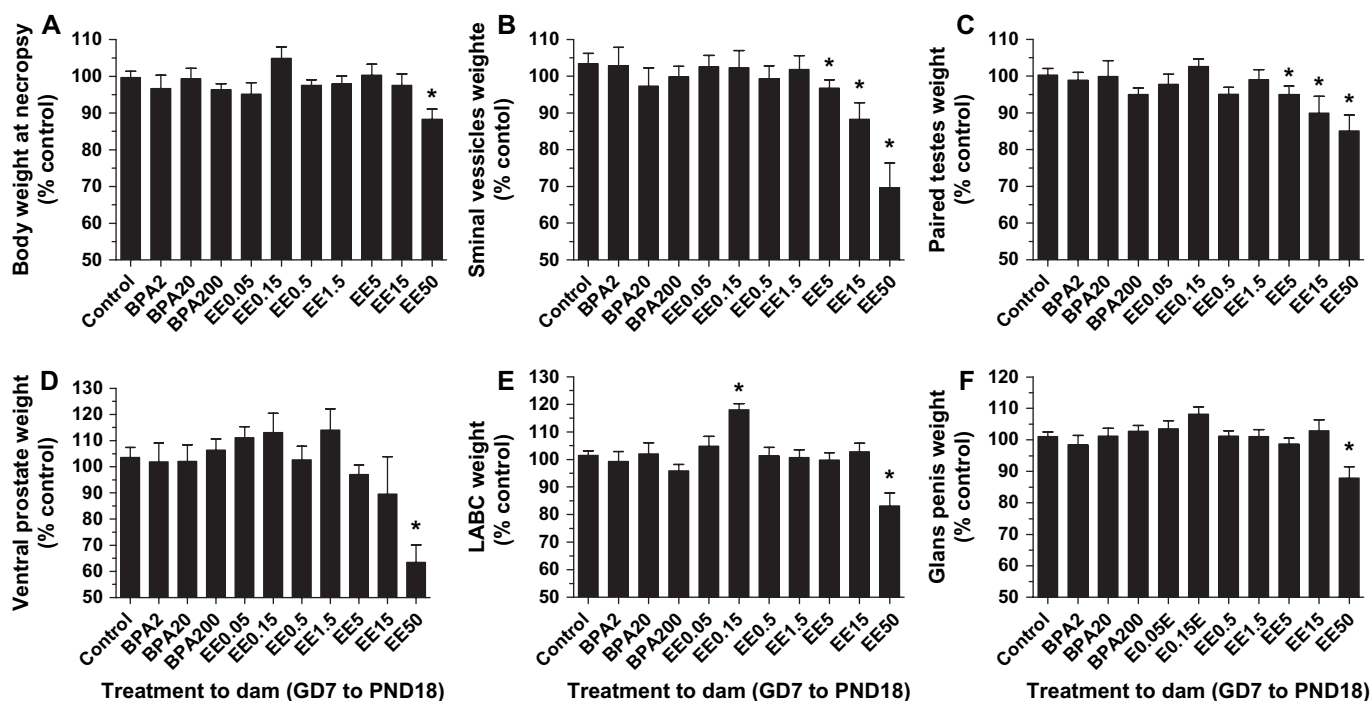


FIG. 2. Body weight at necropsy (A) and weights of the seminal vesicles (B), paired testes (C), ventral prostate (D), levator ani-bulbocavernosus muscle (LABC; E), and glans penis (F) were decreased by exposure to EE, administered via gavage to the dam on GD7 to PND18, in LE male adult rats. BPA and EE doses are $\mu\text{g}/\text{kg}/\text{day}$ (e.g., BPA2 = 2 $\mu\text{g}/\text{kg}/\text{day}$ BPA). The number of litters per treatment was as follows: vehicle control, 24; BPA 2, 7; BPA 20, 13; BPA 200, 18; EE 0.05, 12; EE 0.15, 6; EE 0.5, 15; EE 1.5, 9; EE 5, 21–22; EE 15, 6; and EE 50, 9. Data are litter means \pm SE expressed as percent of control; * $p < 0.05$ versus controls.

to controls (1/31 males; 3.2%); no seminal vesicle hypoplasia was noted for the lower doses of EE or any of the BPA treatments. The occurrence of ventral prostate hyperplasia in EE-treated males appeared to be dose-related with a 6.9% incidence at 5 μg EE/kg/day (2/29 males; nonsignificant [NS]), and a 45.8% incidence at 50 μg EE/kg/day (11/24 males; $p < 0.001$) relative to controls (0/31 males). Ventral prostate hyperplasia was noted in only one BPA-treated male (20 μg BPA/kg/day: 1/18 males, 5.6%) compared with 0% incidence in controls (0/31 males); this effect was not seen in males treated with the lower doses of EE.

The BPA-treated males of the first block (Block 1a) displayed an increased incidence of testicular degeneration. Testicular degeneration was characterized as minimal, mild, moderate, moderately severe, or severe. In Block 1a, minimal to moderately severe testicular degeneration occurred in 3/18 (16.7%, NS) of 20 μg BPA/kg/day treated males, and minimal to mild testicular degeneration occurred in 7/20 males (35%; $p < 0.005$) at 200 μg BPA/kg/day versus one control male with moderately severe testicular degeneration (1/31 males; 3.2%). Testicular degeneration was not seen with 2 μg BPA/kg/day (0/18 males) and did not reach significance with any EE treatment (0.05 $\mu\text{g}/\text{kg}/\text{day}$ = 0/16 males; 0.5 $\mu\text{g}/\text{kg}/\text{day}$ = 2/20 males, 10%; 5 $\mu\text{g}/\text{kg}/\text{day}$ = 1/29 males, 3.4%; and 50 $\mu\text{g}/\text{kg}/\text{day}$ = 4/26 males, 15.4%). The increase in germinal epithelium observed in the lumens of epididymal ducts of 20 μg BPA/

kg/day (1/18 males; 5.6%) and 200 μg BPA/kg/day males (3/20 males; 15%) was attributed to the testicular degeneration observed at these doses. However, the BPA-induced testicular histopathology occurred in the absence of effects on testes weight or epididymal sperm counts in Block 1a. In Block 2, we examined only the histopathology of the testes from control and BPA-treated males to determine if this effect was replicated in Block 2. Treatment with BPA did not increase the incidence of testis degeneration in the second block: control, 2/35 males (5.7%) with mild to severe testicular degeneration; 20 μg BPA/kg/day, 0/16 (0%); and 200 μg BPA/kg/day, 1/34 males (3%) with minimal degeneration. When the histopathology results from Blocks 1a and 2 were pooled, the total incidence of testicular degeneration induced by BPA was not statistically different from controls.

Epididymal Sperm Counts

The number of mature sperm per epididymis was decreased in a dose-dependent fashion by developmental exposure to EE (Fig. 3, Table 3). When expressed as a percentage of control, the number of mature sperm per epididymis was reduced 7.1% (NS) and 25.9% ($p < 0.0001$) by exposure to 15 and 50 μg EE/kg/day, respectively, as compared with control values. Treatment with BPA did not significantly lower mature sperm counts per epididymis at the doses used in this study.

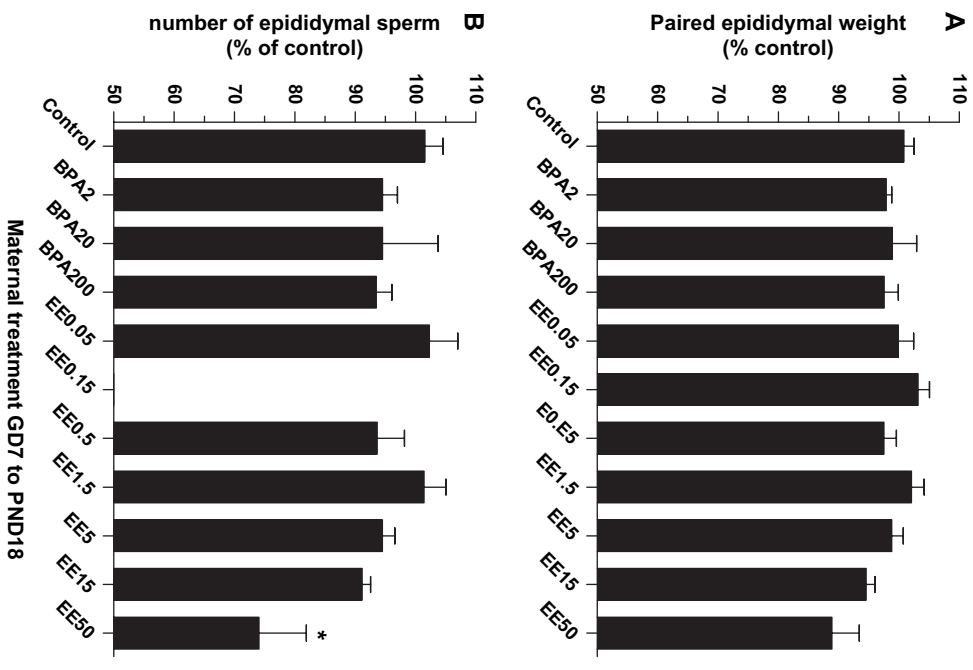


TABLE 3

Mature Sperm Counts (Mean \pm SE) from the Right Epididymis of Adult Male LE Rats Exposed to EE or BPA via their Rat Dams on GD7 through PND18

	Vehicle control	BPA 2 ($\mu\text{g/kg/day}$)	BPA 20 ($\mu\text{g/kg/day}$)	BPA 200 ($\mu\text{g/kg/day}$)	EE 0.05 ($\mu\text{g/kg/day}$)	EE 0.15 ($\mu\text{g/kg/day}$)	EE 0.5 ($\mu\text{g/kg/day}$)	EE 1.5 ($\mu\text{g/kg/day}$)	EE 5 ($\mu\text{g/kg/day}$)	EE 15 ($\mu\text{g/kg/day}$)	EE 50 ($\mu\text{g/kg/day}$)
Block 1a	112.8 \pm 6.5 (20, 8) ^a	104.0 \pm 2.6 (18, 7)	104.0 \pm 10.1 (18, 7)	98.7 \pm 3.1 (18, 7)	112.5 \pm 5.1 (16, 6)	NA	107.3 \pm 4.5 (20, 8)	NA	97.6 \pm 3.4 (20, 7)	NA	75.7 \pm 9.1 (20, 7)
Block 1b	122.2 \pm 5.4 (11, 6)	NA ^b	NA	NA	NA	NA	NA	NA	123.2 \pm 1.7 (9, 7)	NA	112.0 \pm 26.1 (3, 2)
Block 2a	118.0 \pm 5.9 (21, 5)	NA	NA	114.5 \pm 4.0 (22, 4)	NA	NA	95.2 \pm 13.7 (14, 3)	116.1 \pm 4.2 (17, 4)	108.9 \pm 6.4 (24, 5)	104.4 \pm 1.6 (13, 4)	95.0 (1, 1)
Block 2b	134.3 \pm 3.9 (8, 4)	NA	130.7 \pm 6.9 (12, 6)	138.0 \pm 2.9 (8, 4)	115.0 \pm 23.2 (12, 6)	148.4 \pm 9.3 (4, 6)	127.0 \pm 8.7 (8, 4)	130.5 \pm 5.3 (8, 4)	135.7 \pm 11.1 (12, 3)	130.4 \pm 7.2 (10, 2)	NA
Total	120.1 \pm 3.3 (60, 23)	104.0 \pm 2.6 (18, 7)	116.3 \pm 7.2 (30, 13)	113.4 \pm 4.7 (48, 15)	113.7 \pm 11.3 (28, 12)	148.4 \pm 9.3 (4, 6)	110.1 \pm 4.98 (42, 15)	123.3 \pm 4.1 (25, 8)	113.5 \pm 3.76 (65, 22)	113.10 \pm 5.98 (23, 6)	84.1 \pm 9.8 (24, 9)
Total (% of control)	101.1 \pm 2.5	94.6 \pm 2.4	95.4 \pm 5.3	95.7 \pm 2.2	102.3 \pm 4.7	109.5 \pm 6.9	93.7 \pm 3.6	98.8 \pm 2.7	95.2 \pm 2.1	92.9 \pm 1.9	74.1 \pm 7.8*

Note. Shaded values differ significantly from control. * $p < 0.05$ versus control. Block 1 animals were necropsied in 2005 at PND150 (1a) and PND229 (1b). All Block 2 animals were all necropsied in 2006 at PND150. Sperm samples for Block 2b were evaluated on a different Coulter counter than Block 1a, 1b, and 2a samples, thus are listed separately.

^aNumber of individuals and litters for each block are in parentheses.

^bNA indicates that data were not available.

Hormone RIAs

There were no significant main effects of treatment on sera levels of any of the six hormones measured. Developmental exposure to neither EE nor BPA resulted in sera levels of LH, testosterone, estradiol, PRL, corticosterone, or total thyroxine in adult male rats that were significantly different from control values. All RIA results are included in Supplementary Data (Table 3).

DISCUSSION

This study represents a comprehensive assessment of the effects of developmental EE exposure administered orally to the dam on reproductive organ development and endocrine parameters in male LE rat offspring, with an EE dose range

consisting of seven doses and spanning 4 log units (0.05 to 50 $\mu\text{g EE/kg/day}$). We report here that prenatal and lactational exposure to EE significantly decreased neonatal and adult body weight at 50 $\mu\text{g/kg/day}$, reproductive organs weights at $\geq 5 \mu\text{g/kg/day}$, and sperm production at 50 $\mu\text{g/kg/day}$ of male LE rats. We selected the EE dose range to observe whether LE rats would be affected at comparable doses as reported for CD(SD)IGS BR rats, who were exposed via oral gavage to the rat dam from GD6 to PND17 (Sawaki *et al.*, 2003). We observed that the LE male offspring of our study were more adversely affected by developmental exposure to EE and at lower dosage levels than reported for the CD(SD)IGS BR rats. The only effects observed for the male CD(SD)IGS rats were decreased body weights from PND4 through weaning for animals developmentally exposed to 50 $\mu\text{g EE/kg/day}$ versus controls (Sawaki *et al.*, 2003). The differences in the observed effects of developmental EE exposure on reproductive morphology and physiology between our laboratory and Sawaki *et al.* (2003) could be due to differences in sample size, rat strain sensitivities to EE, or laboratory techniques. Furthermore, prenatal and lactational EE exposure elicited more severe effects on the female LE rat siblings than the male rats of the current study with the induction of a reproductive malformation (cleft phallus), accelerated vaginal opening, and decreased fertility observed at EE doses of 5 $\mu\text{g/kg/day}$ and higher (Ryan and Gray, in preparation).

A recent panel convened by National Toxicology Program to review the chemical BPA reviewed the scientific literature on rat strain sensitivity to estrogenic chemicals (National Toxicology Program, 2007b). The panelists reported that several different traits have been shown to display differences in sensitivities to estrogenic chemicals among rat strains. The sensitivity to estrogens varies among the rat strains depending on the trait. There is not a consistent pattern of increased sensitivity to estrogenic chemicals among all traits with one strain being deemed more or less useful in the detection of estrogenic chemicals over another. For example, treatment with estradiol ($1 \times 10^{-5} \mu\text{g/kg}$) increased uterine epithelial cell height in F344 but not SD rats, demonstrating increased sensitivity of the F344 strain to estradiol. In contrast, treatment with $1 \times 10^{-3} \text{ mg/kg}$ 4-OH tamoxifen increased uterine weight and epithelial cell height in SD but not F344 rats, demonstrating that the SD strain is more sensitive to the antiestrogen (Bailey and Nephew, 2002). In some rat strains, administration of estrogens induces pyometritis, an inflammation of the uterus associated with infection. It has been reported that long-term diethylstilbestrol (DES) treatment induced pyometritis in 100% of Brown Norway rats, whereas 0% of similarly treated F344 displayed pyometritis (Pandey *et al.*, 2005). Rat strains also vary in their sensitivity to DES-induced thymic atrophy (Gould *et al.*, 2000) and pituitary tumors (Wiklund and Gorski, 1982), estrogen mediated pituitary PRL release (Lawson *et al.*, 1984). Because each trait reflects a tissue-specific response and these traits map to different genes and chromosomes, it is not

surprising that they also do not segregate together. To properly address the relative sensitivities of the LE versus other rat models, the rat strains under consideration would need to be included in the same experiment, administered the same doses over the same dosing period.

Developmental exposure to EE affects some of the same male reproductive organs affected by one- and multigenerational chronic exposure rat studies in which EE was administered via the diet. In a recent study by the National Toxicology Program, chronic dietary EE exposure of the male SD rats from GD7 through adulthood lead to decreased ventral prostate and testicular weights and induced seminal vesicle atrophy at 200 ppb (ingested dose of 13 $\mu\text{g EE/kg/day}$) (National Toxicology Program, 2007a). Interestingly, the lowest observed adverse effect level (LOAEL) for the NTP study was EE 25 ppb (ingested dose of 2 $\mu\text{g EE/kg/day}$), which induced ductal mammary gland hyperplasia in the male rats with dietary exposure to EE from gestation through adulthood. Our study observed a comparable LOAEL of 5 $\mu\text{g EE/kg/day}$, which decreased seminal vesicle and testes weights in LE males exposed to oral doses of EE via their dam during gestational and lactation. However, it is important to note that the developmental exposure to EE exerts permanent, organizational effects on these endpoints, although the chronic dietary exposures is likely a combination of both organizational effects as well as transient effects due to continuous exposure.

Exposure to EE during pregnancy decreased maternal body weight gain and compromised fetal development. Treatment with EE decreased body weight gain of the dam during pregnancy at doses of 1.5 $\mu\text{g/kg/day}$ and higher. In addition, the decrease in maternal body weight gain during pregnancy reflected the increase in fetal/neonatal mortality and decreased birth weight of the pups at the highest EE doses. The dose-dependent decrease in number of implantation scars suggests that EE treatment induced early postimplantation pregnancy loss, because dosing began after implantation; implantation occurs between GD5 and GD7 considering GD1 as day sperm positive (Garside *et al.*, 1996; Weitlauf, 1994). Early implantation does not result in implantation scars, thus decreases in early implantation would need to be verified by an alternate method not performed in the current study (such as subtracting the number of corpora lutea present in the ovary by the number of implantation scars or staining the uterus with ammonium sulfide). We considered the increase in number of pups at weaning seen with 0.15 and 0.5 $\mu\text{g EE/kg/day}$ as spurious deviations from the control group, which had slightly lower numbers of pups weaned than most of the treatment groups; our *a priori* hypothesis was that estrogenic treatments would reduce pup viability, as seen in the 50 $\mu\text{g EE/kg/day}$ dose group. Decreases in maternal body weight during pregnancy and live pup birth weight have been reported with dietary doses of 100 and 200 ppb EE (actual ingested doses of 7 and 13 $\mu\text{g EE/kg/day}$, respectively) in SD rats (Ferguson *et al.*, 2003; National Toxicology Program, 2007a). Previous studies

reported uterotrophic stimulation by EE at doses of 5 µg/kg/day administered directly to pubertal or ovariectomized female rats (Kanno *et al.*, 2001, 2003), which might suggest that the fetus is comparably as sensitive to EE as the mature animal. However, the effects of developmental exposure on the offspring reported in this study are permanent, whereas the effects of direct administration of EE reported in pubertal or adult animals are usually transient and largely disappear following cessation of treatment.

Prenatal and lactational exposure to 2–200 µg BPA/kg/day did not significantly affect male reproductive development, reproductive hormone levels or sperm production at the doses tested in the current study. Our BPA data are consistent with the findings of other prenatal exposure studies (Tinwell *et al.*, 2002) and large, transgenerational studies using the SD and Wistar rat strains (Ema *et al.*, 2001; Tyl *et al.*, 2002), which administered BPA orally to the rat dam within the 2–200 µg/kg/day range.

In the current study, adult serum sex steroids and LH levels were not significantly affected by developmental exposure to either EE or BPA in the current study, suggesting that the mature hypothalamic-pituitary-gonadal endocrine axis was functioning normally following developmental exposure to the dose ranges of EE and BPA tested. Although PRL levels were previously reported to increase at 29 days of age with daily subcutaneous injection of BPA at 50 mg/kg/day from 22 to 32 days of age to SD male rats (Stoker *et al.*, 1999), our data indicate that prenatal and lactational exposure to BPA at the dose range tested here does not lead to an increase in serum PRL levels in the F1 adult LE rat offspring. Thyroid hormone is critical for brain development, and these parameters were evaluated due to previous reports of *in vitro* binding of BPA to the thyroid hormone receptor (Moriyama *et al.*, 2002) and increased thyroxine levels in juvenile SD male rats exposed to BPA (1–50 mg/kg/day) from GD6 through PND15 (Zoeller *et al.*, 2005). In our current study, developmental exposure to BPA at lower doses did not lead to increased levels of thyroxine in the sera nor were there any treatment differences in pituitary or whole brain weights of the male offspring in adulthood; we did not evaluate sera levels of thyroxine in juvenile rats in our study.

Our study addressed some of the criticisms of previous low dose research on BPA (vom Saal and Hughes, 2005). We included a positive control (EE) over a wide dose range, which evaluated possible nonmonotonic dose responses to known potent synthetic estrogen. Treatment with the positive control EE demonstrated estrogenic action at doses of 5–50 µg/kg/day, as predicted by the studies referenced above, and an absence of effects at doses lower than 5 µg EE/kg/day resulting in a lack of nonmonotonic dose response for the endpoints evaluated in the male offspring reported here. Although there was not an equivalent EE dose for the lowest BPA dose, the lower EE doses did overlap the dose range of BPA exposure tested in this study. In order to determine the effective dose of BPA in the

LE rat (which was not the focus of the current study), a wider dose range of BPA would need to be employed that encompassed doses comparable with the 50 µg EE/kg/day. We used the LE rat, which had not previously been tested comprehensively for effects of EE or BPA following both gestational and lactational exposure via the dam. In addition, we administered the EE and BPA doses orally to the rat dam to best simulate the main route of exposure of humans to either chemical. Thus, we believe this was an accurate assessment of the effects of developmental exposure to EE (from 0.05 to 50 µg/kg/day) and BPA (from 2 to 200 µg/kg/day) in the LE rat strain.

In conclusion, the current study demonstrated that maternal exposure to 5–50 µg EE/kg/day during gestation and lactation produces permanent adverse effects on the male rat reproductive system, similar to those effects reported from developmental exposure other potent estrogens like DES and 17-beta estradiol (E2) (Behrens *et al.*, 2000; Biegel *et al.*, 1998; Fielden *et al.*, 2002). If the human fetus responds similarly to EE as did the rats in the current study, our findings suggest that EE could affect reproductive tract development in the human fetus. The lowest effective dose for F1 males in our rat study (5 µg/kg/day) is approximately fivefold higher than is used what is for oral contraceptives (assuming a 50 µg EE daily dose to a 110 lb woman). However in wildlife, EE at environmentally relevant concentrations is known to induce feminization and decrease reproductive potential of fish in the laboratory and similar effects are seen in natural environments (Kidd *et al.*, 2007; Lange *et al.*, 2001; Parrott *et al.*, 2004). Recent reports of feminized wild fish populations in the Potomac River around Washington, DC, have prompted the Office of Water at the U.S. Environmental Protection Agency to consider EE as a candidate for criteria derivation for its endocrine-disruptive effects on aquatic species (Fahrenthold, 2006; Beaman, personal communication). Furthermore, although the risk assessment community has traditionally focused on the effects of individual chemicals, a multitude of estrogenic contaminants can occur in the environment. It will be important to determine if mixtures of these chemicals act in a cumulative manner when exposures occur during critical developmental periods in human as well as in lower vertebrates.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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