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Developmental Treatment with Ethinyl Estradiol, but Not Bisphenol A, Causes Alterations in Sexually Dimorphic Behaviors in Male and Female Sprague Dawley Rats

Sherry A. Ferguson,[*,1] Charles Delbert Law,[*] and Grace E. Kissling[†]

[*]*Division of Neurotoxicology, National Center for Toxicological Research/Food and Drug Administration, Jefferson, Arkansas 72079; and* [†]*Biostatistics Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709*

[1]To whom correspondence should be addressed at HFT-132, Division of Neurotoxicology, National Center for Toxicological Research/FDA, 3900 NCTR Road, Jefferson, AR 72079. Fax: (870) 543-7745. E-mail: sherry.ferguson@fda.hhs.gov.

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The developing central nervous system may be particularly sensitive to bisphenol A (BPA)-induced alterations. Here, pregnant Sprague Dawley rats ($n = 11–12$/group) were gavaged daily with vehicle, 2.5 or 25.0 μg/kg BPA, or 5.0 or 10.0 μg/kg ethinyl estradiol (EE_2) on gestational days 6–21. The BPA doses were selected to be below the no-observed-adverse-effect level (NOAEL) of 5 mg/kg/day. On postnatal days 1–21, all offspring/litter were orally treated with the same dose. A naïve control group was not gavaged. Body weight, pubertal age, estrous cyclicity, and adult serum hormone levels were measured. Adolescent play, running wheel activity, flavored solution intake, female sex behavior, and manually elicited lordosis were assessed. No significant differences existed between the vehicle and naïve control groups. Vehicle controls exhibited significant sexual dimorphism for most behaviors, indicating these evaluations were sensitive to sex differences. However, only EE_2 treatment caused significant effects. Relative to female controls, EE_2-treated females were heavier, exhibited delayed vaginal opening, aberrant estrous cyclicity, increased play behavior, decreased running wheel activity, and increased aggression toward the stimulus male during sexual behavior assessments. Relative to male controls, EE_2-treated males were older at testes descent and preputial separation and had lower testosterone levels. These results suggest EE_2-induced masculinization/defeminization of females and are consistent with increased volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) at weaning in female siblings of these subjects (He, Z., Paule, M. G. and Ferguson, S. A. (2012) Low oral doses of bisphenol A increase volume of the sexually dimorphic nucleus of the preoptic area in male, but not female, rats at postnatal day 21. *Neurotoxicol. Teratol.* 34, 331–337). Although EE_2 treatment caused pubertal delays and decreased testosterone levels in males, their behaviors were within the range of control males. Conversely, BPA treatment did not alter any measured endpoint. Similar to our previous reports (Ferguson, S. A., Law, C. D. Jr and Abshire, J. S. (2011) Developmental treatment with bisphenol A or ethinyl estradiol causes few alterations on early preweaning measures. *Toxicol. Sci.* 124, 149–160; Ferguson, S. A., Law, C. D. and Abshire, J. S. (2012) Developmental treatment with bisphenol A causes few alterations on measures of postweaning activity and learning. *Neurotoxicol. Teratol.* 34, 598–606), the BPA doses and design used here produced few alterations.

Key words: **bisphenol A; ethinyl estradiol; developmental; behavior; estrous cycle; puberty.**

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Bisphenol A (BPA) is a high volume industrial chemical. More than 90% of the U.S. and Canadian populations exhibit detectable urinary levels of BPA (Bushnik *et al.*, 2010; Calafat *et al.*, 2008). Although there may be other routes of exposure (e.g., dermal), the most common exposure route appears to be oral (Geens *et al.*, 2012). Estimated daily BPA intake is higher for infants and children and highest for infants that are exclusively formula-fed. Formula-fed infants could receive 0.2–0.4 μg BPA/kg body weight/day (Food and Drug Administration, 2009).

Despite the many studies of BPA exposure in laboratory animals, there are widely differing views on the potential relevance of such studies to humans (Beronius *et al.*, 2010). The general consensus is that there may be some risks of BPA exposure during development, particularly central nervous system exposure (National Toxicology Program, 2008). Further, the behaviors potentially altered by developmental BPA exposure may be those that are not required for assessment as detailed in typical test guidelines (e.g., OECD guidelines). For example, in a detailed review of 44 developmental neurotoxicity studies of BPA treatment, Beronius *et al.* (2013) reported that a higher number of significant BPA effects were detected in anxiety, social, and sexual endpoints than in conventional motor activity endpoints,

suggesting the need to assess a wide range of endpoints in behavioral studies of BPA exposure.

Previously, we described few BPA effects on pre- and post-weaning behaviors that are not generally thought to be sexually dimorphic (Ferguson *et al.*, 2012, 2011). Our study design incorporated test methods and testing considerations that have been suggested for BPA research (recently reviewed in Shelnutt *et al.*, 2013 and see also Hunt *et al.*, 2009; Li *et al.*, 2008; Richter *et al.*, 2007). Specifically, BPA was orally administered, a reference estrogen-treated group was included, exogenous estrogens were carefully controlled, and potential litter effects were statistically controlled by assessing no more than 1 pup/sex/litter. Here, from that same study design, we describe the results of assessments of behaviors typically thought to be sexually dimorphic as well as pubertal landmark age, adult serum hormone levels, estrous cyclicity, and body weight. Because BPA is a weak estrogen and because androgens/estrogens are critical for sexual differentiation of the brain and behavior of rodents, we tested the hypothesis that BPA could influence sexually dimorphic behaviors when administered during the developmental period that is known to be affected by estrogens in the rat.

MATERIALS AND METHODS

Detailed descriptions of the animals, physical environment, diet, breeding, and treatment have been published (Ferguson *et al.*, 2011, 2012) and are only briefly described below.

Animals. The National Center for Toxicological Research (NCTR) Breeding Colony supplied female (total $n = 364$) and male (total $n = 180$) postnatal day (PND) 21 Sprague Dawley rats. At breeding age (see below), those animals became the dams and sires (i.e., F_0) of subsequent litters. *Ad libitum* food (see below for diet) and Millipore filtered water were provided throughout. Housing rooms were maintained on a 12/12-h light/dark cycle (on at 6 A.M.–off at 6 P.M.) at 22 ± 1°C (mean ± SE) and 45–55% humidity. All animal procedures followed the "Guide for the care and use of laboratory animals" (National Research Council, 1996) and were approved in advance by the NCTR Institutional Animal Care and Use Committee.

Housing environment. Rats were housed in polysulfone cages with polysulfone microfilter tops and glass water bottles. All chow was stored in metal containers. This environment began for the F_0 generation at PND 21 and continued throughout.

Diet. Upon arrival from the NCTR Breeding Colony and throughout the study, all rats (i.e., F_0 and F_1) were maintained on a low phytoestrogen chow (TestDiet 5K96 (irradiated pellets), Verified Casein Diet 10 IF, TestDiet, Richmond, IN). Levels of daidzein, genistein, and BPA in six lots of this diet were reported in Delclos *et al.* (2014) and averaged 0.249 ± 0.064 ppm, 0.374 ± 0.118 ppm, and 2.6 ± 0.8 ppb (mean ± standard deviation), respectively.

Breeding and treatment assignment. Each of the nine breeding rounds was separated by 2 weeks and consisted of a 24 h pairing of an F_0 PNDs 87–90 male and female. Following pairing, the female and the cage bottom were visually examined for a sperm plug. Breeding occurred prior to any treatment; thus, success or failure was not treatment-related. Females for which a sperm plug was detected were randomly assigned to one of the six treatment groups within their body weight stratum. This assignment yields approximately equal dam body weight distributions across treatments.

BPA and EE_2 treatment. Bisphenol A (2,2-bis(4-hydroxyphenyl)propane, Product no. B0494, TCI America, Portland, OR) and EE_2 (17α-ethinyl estradiol, Product no. E4876, Sigma, St Louis, MO) were each dissolved in a 0.3% (by weight) aqueous solution of carboxymethylcellulose sodium salt (CMC, high viscosity) (Product no. C5013, Sigma). CMC was selected as vehicle, rather than an oil, because certain oils may possess estrogenic activity (Ashby and Lefevre, 1997; Ryan, 2005).

Beginning on the morning of gestational day (GD) 6, females were gavaged with 5.0 ml 0.3% CMC/kg/day (vehicle control), 2.5 μg BPA/kg/day (2.5 BPA), 25.0 μg BPA/kg/day (25.0 BPA), 5.0 μg EE_2/kg/day (5.0 EE_2), or 10.0 μg EE_2/kg/day (10.0 EE_2) using a Hamilton Microlab 500 system (Hamilton Company, Reno, NV) interfaced with a weight scale and data collection software. An automated algorithm calculated the necessary volume based on each rat's daily body weight. Daily gavage continued through GD 21 (the day prior to parturition). Dams in the naïve control group were removed daily (GDs 6–21) from their home cage, weighed, restrained in the gavage position, but were not gavaged. Thus, there were six treatment groups: (1) naïve control, (2) vehicle control, (3) 2.5 BPA, (4) 25.0 BPA, (5) 5.0 EE_2, and (6) 10.0 EE_2.

On the day after parturition (PND 1), the eight offspring in each litter (after randomly culling to 4 pups/sex/litter) were orally treated with the same dose and volume (5 ml/kg) as their dams had received. Daily treatment of offspring continued through PND 21. Offspring in the naïve control group were removed daily from their home cage, weighed, and restrained in the gavage position, but were not gavaged.

The two doses of BPA (2.5 and 25.0 μg/kg/day) were specifically selected to be below the no-observed-adverse-effect level (NOAEL) of 5 mg/kg/day. Many of the developmental neurotoxicity effects of BPA have been reported at BPA doses less than the NOAEL; thus, it was essential to investigate doses within that range. The two doses of EE_2 (5.0 and 10 μg/kg/day) were selected based on our previous study of dietary EE_2 exposure (Ferguson *et al.*, 2003) in which male and female offspring of dams consuming chow containing 200 ppb EE_2 (≈10–13 and 18–38 μg/kg/day during pregnancy and lactation, respectively) exhibited reduced body weight but few behavioral alterations.

Postweaning environment. On PND 21, two offspring/sex/litter were weaned, tail tattooed for identification, and pair-housed in caging and with access to *ad libitum* water and food as described above. With the exception of residential running wheel assessments on PNDs 58–69 and a brief 24 h separation prior to play behavior assessments on PND 35, the rats remained pair-housed with their same-sex sibling throughout the study. Both siblings had been assessed for early preweaning behaviors (Ferguson *et al.*, 2011) and certain postweaning behaviors (Ferguson *et al.*, 2012). For each same-sex sibling pair, one rat was designated as the major behavioral assessment subject and is referred to here as male no. 1 or female no. 1. With the exception of flavored solution intake and female sex behavior, that subject underwent all behavioral assessments as well as measurement of serum hormone levels. The other subject underwent female sex behavior assessments (females only) and flavored solution intake tests (both sexes) and is referred to here as male no. 2 or female no. 2. For each endpoint, typical group subject numbers were 12 naïve controls (i.e., 12/sex), 12 vehicle controls, eleven 2.5 BPA, twelve 25.0 BPA, eleven 5.0 EE_2, and twelve 10.0 EE_2.

Body weight, food, and water intake. All rats were weighed once weekly beginning at PND 27, except during residential running wheel assessments (PNDs 58–69). Because rats were pair-housed with a same-sex sibling, both rats were weighed and the average of the two body weights was used for statistical analyses. If measured more than once weekly, food and water intakes were first averaged for each week and then divided by two in order to approximate the average intake/rat. Group sizes for these endpoints were: naïve control $n = 12$/sex; vehicle control $n = 12$/sex; 2.5 BPA $n = 11$/sex; 25.0 BPA $n = 12$/sex; 5.0 EE_2 $n = 11$/sex; 10.0 EE_2 $n = 12$/sex.

Pubertal landmarks. Beginning on PND 1, all offspring/litter were examined daily for developmental and pubertal landmarks. Preweaning landmarks (i.e., ear canal and eye opening) for the subjects of this study have been reported (Ferguson *et al.*, 2011). Postweaning, daily examinations continued in the remaining 2 pups/sex/litter for preputial separation and testes descent (males) and vaginal opening (females) until the landmark was present or until the end of the study. Group sizes for these endpoints were: naïve control $n = 12$/sex; vehicle control $n = 12$/sex; 2.5 BPA $n = 11$/sex; 25.0 BPA $n = 12$/sex; 5.0 EE_2 $n = 11$/sex; 10.0 EE_2 $n = 12$/sex.

During euthanasia procedures, it was noted that some females appeared to exhibit abnormal genitalia. These were photographed and evaluated by a veterinary pathologist. Sample photos are presented here; however, not all females were examined and the number affected in each treatment group is not known.

Estrous cyclicity. Female no. 1 underwent daily vaginal lavage on PNDs 82–109 for vaginal cytology after manual lordosis assessments (described below). Vaginal cytology slides were air-dried, immersion-fixed in methanol, stained with toluidine blue, coverslipped, and evaluated by light microscopy for determination of estrous phase by experienced personnel. Group sizes for these endpoints were: naïve control $n = 12$; vehicle control $n = 12$; 2.5 BPA $n = 11$; 25.0 BPA $n = 12$; 5.0 EE_2 $n = 5$; 10.0 EE_2 $n = 7$. EE_2 group sizes were smaller as some females in those two groups never experienced vaginal opening.

Serum hormone measures. Serum hormone levels were measured in male and female no. 1 (i.e., the subject that underwent the majority of behavioral assessments). Levels were measured in males of replicates 1–3 at PND 110 and the remaining replicates (i.e., 4–9) at PND 90. Levels were measured in all females at PND 110. Each rat was lightly anesthetized with CO_2, after which blood was collected via cardiac puncture. All samples were allowed to clot and then centrifuged. Serum was removed and stored at −80°C until analysis. Most samples were collected between 8:15 A.M. and 12:00 P.M. Through an inadvertent error, samples from females in replicates 6 ($n = 8$) and 9 ($n = 7$) were collected between 12:45 and 2:30 P.M. Several hormones measured here exhibit circadian rhythms (e.g., Kalra and Kalra, 1977; Mock *et al.*, 1978). Thus, hormone levels were statistically analyzed twice: once including all subjects and once without the 15 females for which samples were obtained in the afternoon. Further, several hormones measured here are associated with estrous phase. Although estrous phase was not measured on the day of euthanasia (i.e., PND 110), it was measured the day prior as part of the estrous cyclicity assessment. Group sizes for these endpoints were: naïve control $n = 12$/sex; vehicle control $n = 12$/sex; 2.5 BPA $n = 11$/sex; 25.0 BPA $n = 12$/sex; 5.0 EE_2 $n = 11$ males, 10 females; 10.0 EE_2 $n = 12$/sex.

Total thyroxine (T4), total triiodothyronine (T3), total estradiol (E_2), total testosterone, corticosterone, and luteinizing hormone (LH) were analyzed in duplicate with Siemens radioimmunoassay reagents (Los Angeles, CA) and counted on a Perkin Elmer Cobra 5005 gamma counter (Shelton, CT). Leptin (Millipore rat leptin ELISA, Billerica, MA) and total ghrelin (Linco rat/mouse ghrelin ELISA, St Charles, MO) were analyzed via ELISA and read on a BioTek EL808 microplate reader (Winooski, VT). ELISA results were calculated with the instrument's data reduction software using a sigmoidal 4-parameter logistic equation. The radioimmunoassay results were calculated with the instrument's data reduction software from a logit-log representation of the linear calibration curve. Two levels of assayed controls were included in each assay as internal controls.

Whole and regional brain weights. Whole and regional brain weights were measured in those males that were assessed for play behavior and flavored solution intake (i.e., male no. 2). Their paired female sibling (i.e., female no. 2) was not mea-

sured as this subject had been assessed for sex behavior and may have been pregnant. Each male was euthanized with CO_2 and the whole brain was removed and weighed. Frontal cortex and hippocampus were then dissected as previously described (Ferguson *et al.*, 1993) and weighed. Absolute and body weight adjusted (ratio) whole brain, frontal cortex, and hippocampal weights were subjected to analysis. Group sizes for these endpoints were: naïve control $n = 11$; vehicle control $n = 11$; 2.5 BPA $n = 10$; 25.0 BPA $n = 12$; 5.0 EE_2 $n = 9$; 10.0 EE_2 $n = 9$.

Play behavior. On PND 34, each same-sex sibling pair was individually housed in clean cages. After 24 h and during the lighted portion of the light cycle, the pair was reunited in their original home cage and behavior was videorecorded for 7 min. A single tester blind to treatment status scored all sessions after training to reliability coefficients of 0.94 or better on the two behaviors. Frequency of pins and dorsal contacts were measured during the last 5 min of the session only as initially there is increased environmental exploration and little play behavior. Group sizes for these endpoints were: naïve control $n =$ 12 pairs/sex; vehicle control $n = 12$ pairs/sex; 2.5 BPA $n =$ 11 pairs/sex; 25.0 BPA $n = 12$ pairs/sex; 5.0 EE_2 $n = 10$ male pairs, 11 female pairs; 10.0 EE_2 $n = 12$ pairs/sex.

Residential running wheel activity. On PNDs 58–69, one offspring/sex/litter (male and female no. 1) was housed in a residential running wheel cage (34.3 cm diameter, Mini-Mitter, Sunriver, OR) interfaced with a computer. Number of wheel revolutions was recorded by 10 min intervals and the lighting cycle in the testing room was identical to that of the housing room (12/12-h; 6 A.M.–6 P.M.). On PND 63, each rat was removed briefly for normal husbandry routines. Group sizes for these endpoints were: naïve control $n = 12$/sex; vehicle control $n = 12$/sex; 2.5 BPA $n = 11$/sex; 25.0 BPA $n = 12$/sex; 5.0 EE_2 $n = 11$/sex; 10.0 EE_2 $n = 12$/sex.

Intake of flavored solutions. On PNDs 59–62, two bottles were placed on the home cage of the remaining offspring/sex/litter (i.e., male and female no. 2). One bottle contained regular water and the other contained a 0.05% (0.0027M) saccharin solution. On PNDs 63–66, a similar procedure was conducted with one bottle of regular water and the other containing a 0.3% (0.0513M) sodium chloride solution. Bottles were weighed daily to determine intake (ml consumed). Prior to statistical analyses, each daily intake (ml) was divided by PND 59 (for saccharin intake data) or PND 63 body weight (for sodium intake data). We have previously shown this endpoint (ml intake/g body weight) to be sexually dimorphic in adult Sprague Dawley rats (Ferguson and Boctor, 2010; Ferguson *et al.*, 2007; Flynn *et al.*, 2005). In addition, saccharin and sodium preference for each of the 3 days was calculated as 100 × (flavored solution intake/total intake) and subjected to analysis as well. Group sizes for these endpoints were: naïve control $n = 12$/sex for saccharin intake and $n = 11$/sex for sodium intake; vehicle control $n = 12$/sex for saccharin intake and $n =$ 11/sex for sodium intake; 2.5 BPA $n = 11$/sex for saccharin intake and $n = 9$/sex for sodium intake; 25.0 BPA $n = 12$/sex for saccharin intake and $n = 10$/sex for sodium intake; 5.0 EE_2 $n =$ 11/sex for saccharin intake and $n = 10$/sex for sodium intake; 10.0 EE_2 $n = 12$/sex for saccharin and sodium intake.

Manual lordosis measures. On PNDs 96–100, female no. 1 was assessed for manual elicitation of the lordosis response similar to that previously described (Gans and McClintock, 1993; Gans *et al.*, 1995; Hoffman *et al.*, 2002). This assessment was conducted in a test room under dim red light, although during the light period of the light cycle. The home cage was gently opened and an experienced tester attempted to elicit lordosis by using their dominant hand and gently gripping the female on the dorsal side near the middle of the back while allowing the rat's feet to remain firmly on the cage floor. The tester then gently stroked their hand toward the rat's tail using very light pressure on the flanks while at the same time, using one finger to lightly touch the perineal area under the tail. Each female was palpated three times in succession and all palpations were done by the same tester who was blind to treatment status. Each of those three trials was scored on a 0–3 scale: 0 = no reflex at all (back was hunched and head parallel with the bottom of the cage or lower), 1 = marginal reflex (slight flex of spine, slightly raised head and hips with tail base elevated from cage floor), 2 = normal reflex (spine was flexed, head at approximate angle of 30° with horizontal, front paws placed slightly forward and hind legs straightened up stiffly), 3 = intense/exaggerated reflex (pronounced spinal flex, head at an angle of 45° or more with horizontal). Some 5.0 and 10.0 EE_2 females scheduled for manual lordosis assessments never experienced vaginal opening (see Results section). Manual lordosis assessments were conducted on those females and the dataset was analyzed twice: once including all females and once without those females that did not experience vaginal opening. Group sizes for these endpoints were: naïve control $n = 12$; vehicle control $n = 12$; 2.5 BPA $n = 11$; 25.0 BPA $n = 12$; 5.0 EE_2 $n = 11$; 10.0 EE_2 $n =$ 12.

Female sex behavior. Female no. 2 was assessed for male-elicited sex behavior on PNDs 96–100. Fifteen experienced Sprague Dawley breeders obtained from the NCTR Breeding Colony were used as stimulus males. Seven were used with females of replicates 1–4 and the remaining eight were used with females of replicates 5–9. Each female subject was placed with a different stimulus male for each of the five test days. Sessions were conducted under dim red lighting. Between 12:30 and 1:00 P.M., the stimulus male was placed into one of four Plexiglas chambers (38.5 cm × 22.0 cm × 30.0 cm) which contained a small amount of bedding. After 10 min, the female was placed into the chamber with the stimulus male and behavior was videorecorded for 20 min. Videorecordings were later scored by a single experienced tester blind to treatment status of the female.

For each session, frequency of lordosis postures and hopping or darting episodes exhibited by the female were recorded. In addition, each session was scored for the presence or absence of the following behaviors: female investigation of male anogenital area, male investigation of female anogenital area, female aggression directed at male, and male aggression directed at female. These aggressive behaviors were generally biting (by either sex), jumping on top of male by female (or *vice versa*), or the female being held down by the male. Some 5.0 and 10.0 EE_2 females that were scheduled for female sex behavior assessments never experienced vaginal opening (see Results section). Those females were not tested; thus, group numbers are somewhat smaller for the EE_2 groups. Group sizes for these endpoints were: naïve control $n = 12$; vehicle control $n = 12$; 2.5 BPA $n = 11$; 25.0 BPA $n = 12$; 5.0 EE_2 $n = 5$; 10.0 EE_2 $n = 7$.

Statistical analyses. All analyses were conducted using SAS (version 9.2, SAS Institute Inc., Cary, NC) or SigmaPlot (version 11.0, Systat Software, Inc., San Jose, CA). Significant effects (i.e., those at $p < 0.050$) were further detailed using appropriate comparisons to the vehicle control group. Litter was the experimental unit in all analyses. Data were analyzed separately by sex for those endpoints in which the variances for males and females were not similar as the analysis of variance (ANOVA) utilizes pooled estimates of variance. Thus, body weight, and food and water intake were analyzed separately by sex, each using a repeated measures (RM) ANOVA with factors of treatment, PND (or week), and the interaction.

Initially, the behavior of the vehicle control group was assessed for sexual dimorphism. For play behavior, *t*-tests determined the effect of sex in the vehicle control group for frequency of dorsal contacts and pins. For residential running wheel activity, a single datapoint was calculated for each vehicle control subject by averaging over days for each 12-h light period wheel activity and for each 12-h dark period wheel activity. Similarly, averages of the 3 days of saccharin-flavored solution intake/preference and sodium-flavored solution intake/preference were calculated. Each of those datapoints (i.e., light period wheel activity, dark period wheel activity, saccharin-flavored solution intake/preference, and sodium-flavored solution intake/preference) was subjected to a *t*-test. Where the data were not normally distributed, a Mann-Whitney/Wilcoxon test assessed sex differences.

Because 2/sex/litter were examined for pubertal landmarks (i.e., preputial separation, testes descent, and vaginal opening), litter correlation was accounted for by using a RM ANOVA with a compound symmetric variance structure. If one same-sex sibling did not achieve the landmark, the other sibling's datapoint was used. If both same-sex siblings did not achieve the landmark, that litter was not included in the analysis.

Proportion of days in each estrous phase (proestrus, estrus, and diestrus) for female no. 1 (measured daily on PNDs 82–109) was analyzed via a multivariate analysis of variance (MANOVA) on the arcsine-square root transformed estrous phase relative frequencies. Pairwise comparisons to the vehicle control group were adjusted for multiple comparisons using Dunnett's test. Analysis of aberrant estrous cycles was conducted using a method similar to that described by Girard and Sager (1987). This method uses a Markov chain to model transitions between estrous states. Here, aberrant states were defined as extended diestrus (4 or more consecutive days) or extended estrus (3 or more consecutive days). All other states were defined to be normal. A chi square analysis of transition matrices compared the relative frequencies of transitions involving aberrant states (any transition involving extended diestrus or estrus). Pairwise comparisons to the vehicle control group were adjusted using a Hochberg adjustment.

Serum hormone levels were analyzed via two-way ANOVA with factors of treatment, sex, and the interaction. For subjects in which a level was below the limit of detection, that value (i.e., the limit of detection) was used in the analysis.

Absolute and weight adjusted whole and regional brain weights of male no. 2 were analyzed via one-way ANOVAs with a factor of treatment. Play behaviors were analyzed via two-way ANOVAs with factors of treatment, sex, and the interaction.

Because the estrous cycle can affect residential running wheel activity (Dixon *et al.*, 2003; Eckel *et al.*, 2000; Steiner *et al.*, 1982) and EE_2 treatment had a substantial effect on estrous cyclicity (see Results section), running wheel revolutions were first summed for each 12-h light and dark period and then averaged for each subject, resulting in two datapoints for each subject: average light period revolutions and average dark period revolutions. Those data were analyzed separately, each using an ANOVA with treatment, sex, and the interaction as factors. In addition to those typical analyses, running wheel data were modeled using a sigmoidally transformed cosine curve described by Marler *et al.* (2006) in which it is assumed that activity is cyclical over a 24-h period. This running wheel model was previously shown to be sensitive to cerebellar development alterations (Kim *et al.*, 2010). The parameters of interest were: (1) initial amplitude (on day 1), (2) daily increase in amplitude (the rate at which amplitude increased each day), (3) t_{max} (the time each day at which maximum activity occurred), (4) beta or activity slope (how quickly activity increased or decreased from the inactive or active portion of the cycle), (5) $t_{1/2\ up}$ (the time each day at which increasing activity reached half the amplitude), (6) $t_{1/2\ down}$ (the time each day at which decreasing activity reached half the amplitude), (7) duration of maximum activity (the number of hours each day during which the rat had higher activity levels; calculated as $t_{1/2\ down} - t_{1/2\ up}$), and (8) duration of minimum activity (the number of hours each day during which the rat had lower activity levels; calculated as $t_{1/2\ up\ on\ the\ next\ day} - t_{1/2\ down}$). The model was modified to account for the increase in activity over days. A "slope" term was included in the amplitude parameter to allow for the probability that amplitude increases over days. Thus, amplitude was split into an intercept and a slope: $a0$ (baseline amplitude at day 0)

and $a1$ (rate of increase in amplitude/day). The modeled parameters were estimated for each subject and then subjected to exact Mann-Whitney tests. The "time" parameters of t_{max}, $t_{1/2\ up}$, and $t_{1/2\ down}$ are actual clock times on a 24-h clock. Each day, the dark cycle began at approximately 18:30 (6:30 P.M.) and ended at 06:30 A.M. For many rats, the time that maximum activity was achieved, t_{max}, occurred near midnight. To avoid averaging premidnight values near 24 with postmidnight values near 0, 24 was added to the postmidnight values of t_{max} prior to averaging; thus, t_{max} values could range above 24:00.

Intake of regular water and flavored solutions were analyzed using four separate RM ANOVAs: (1) intake of regular water during the time saccharin-flavored solution was available, (2) intake of saccharin-flavored solution, (3) intake of regular water during the time sodium-flavored solution was available, and (4) intake of sodium-flavored solution. Preference was analyzed using two separate RM ANOVAs: (1) saccharin preference, and (2) sodium preference. Each RM ANOVA contained factors of treatment, sex, day, and all interactions as factors.

Manual lordosis of female no. 1 was assessed for five consecutive days. Thus, it was expected that on at least one of those days, the rat would be in estrus and expected to exhibit a lordosis response. Therefore, the number of females with a nonzero response on at least one of the three daily trials was subjected to a two-way RM logistic regression analysis with factors of treatment and days.

During sex behavior assessments of female no. 2, there was a relatively low occurrence of lordosis postures and hops/darts; thus, these endpoints were analyzed as binary variables. Any observation with a lordosis posture or a hop/dart was treated as an occurrence; those with no such behaviors were treated as a nonoccurrence. Data were analyzed using a categorical ANOVA-type model of these binary variables with factors of treatment, test day, and the interaction.

RESULTS

Differences between the Naïve and Vehicle Control Groups

The naïve control group was included as a potential control for stress induced by gavage, because prenatal stress can alter later behaviors (reviewed in Buynitsky and Mostofsky, 2009; Maccari and Morley-Fletcher, 2007; Weinstock, 2001). This became potentially relevant because an earlier study of siblings of the subjects of the current study described differences in the naïve and vehicle control groups (Cao *et al.*, 2013). However, similar to the results of previous behavioral assessments in these subjects (Ferguson *et al.*, 2011, 2012), there were no statistically significant comparisons between the naïve and vehicle control groups in any of the significant main effects of treatment or interactions with treatment described below.

Sexual Dimorphisms in the Vehicle Control Group

Table 1 shows mean ± SE for these endpoints for the vehicle control group. Vehicle control males performed significantly more dorsal contacts during play behavior assessments than females ($df = 22, t = 2.36, p < 0.028$). Although a statistically significant effect was not noted for number of pins (Mann-Whitney $U = 44.5, p < 0.118$), vehicle control males performed an average 34% more pins than vehicle control females. Average light period running wheel activity of vehicle control males was significantly less than that of vehicle control females ($df = 22, t = 5.42, p < 0.001$). Similarly, vehicle control males were significantly less active in running wheels than vehicle control females during the dark period (Mann-Whitney $U = 3.0, p < 0.001$). Relative to vehicle control females, vehicle control males consumed significantly less of the saccharin-flavored solution ($df = 22, t = 4.12, p < 0.001$) and the sodium-flavored solution ($df = 20, t = 2.87, p < 0.010$) on a ml/kg basis. However, saccharin preference averaged over the 3-day period did not differ between vehicle control males and females ($df = 22, t = -0.81, p < 0.428$), nor did sodium preference ($df = 20, t = -1.35, p < 0.192$).

Body Weight, Food, and Water Intake

Analysis of male body weight indicated significant main effects of treatment ($F(5, 64) = 5.35, p < 0.001$) and PND ($F(10, 538) = 3638.48, p < 0.001$). However, pairwise comparisons did not indicate any significant differences between the vehicle control group and any other treatment group (see Fig. 1A). The main effect of PND indicated that all male groups gained weight with age. Analysis of female body weight indicated a significant interaction of treatment with PND ($F(50, 640) = 4.65, p < 0.001$). Pairwise comparisons indicated that, beginning at PND 41 and continuing through the end of the study, 5.0 EE_2 females weighed more than vehicle control females (all comparisons $p < 0.010$) (see Fig. 1B). Beginning at PND 55 and continuing through the end of the study, 10.0 EE_2 females weighed more than same-sex vehicle controls (all comparisons $p < 0.011$).

Analysis of food intake by females indicated a significant interaction of treatment with PND ($F(50, 622) = 1.58, p < 0.008$) as did the analysis of food intake by males ($F(50, 489) = 1.44, p < 0.030$) (data not shown). Pairwise comparisons indicated the 5.0 and 10.0 EE_2 females consumed more than same-sex vehicle controls the week of PNDs 52–58 ($p < 0.046$) and the 10.0 EE_2 males consumed less than same-sex vehicle controls the week of PNDs 45–51 ($p < 0.009$); however, there was nothing unusual about those 2 weeks. On any given week, females of the 5.0 EE_2 group consumed 3–14% more than same-sex vehicle controls and females of the 10.0 EE_2 group consumed −4 to 10% more than same-sex vehicle controls.

Analysis of water intake by females indicated a significant interaction of treatment with PND ($F(55, 696) = 2.74, p < 0.001$) as did the analysis of water intake by males ($F(55, 563) = 1.50, p < 0.015$) (data not shown). Pairwise comparisons indicated

TABLE 1
Sexual Dimorphisms in the Vehicle Control Group (Mean ± SE)

Assessment	Endpoint	Males	Females
Play behavior	Dorsal contact frequency*	63.2 ± 3.8	52.3 ± 2.5
	Pinning frequency	14.7 ± 2.6	10.9 ± 2.0
Residential running wheel activity (revolutions/12 h)	Light period activity*	216.7 ± 45.8	847.7 ± 107.2
	Dark period activity*	1368.5 ± 246.7	5447.0 ± 620.4
Flavored solution intake (ml consumed/kg body weight)	Saccharin solution*	0.1386 ± 0.0126	0.2426 ± 0.0219
	Sodium solution*	0.0587 ± 0.0062	0.0950 ± 0.0110
Flavored solution preference	Saccharin preference	88.01 ± 5.48	92.89 ± 2.55
	Sodium preference	63.52 ± 6.10	73.82 ± 4.56

*Indicates a significant difference between vehicle control males and vehicle control females.

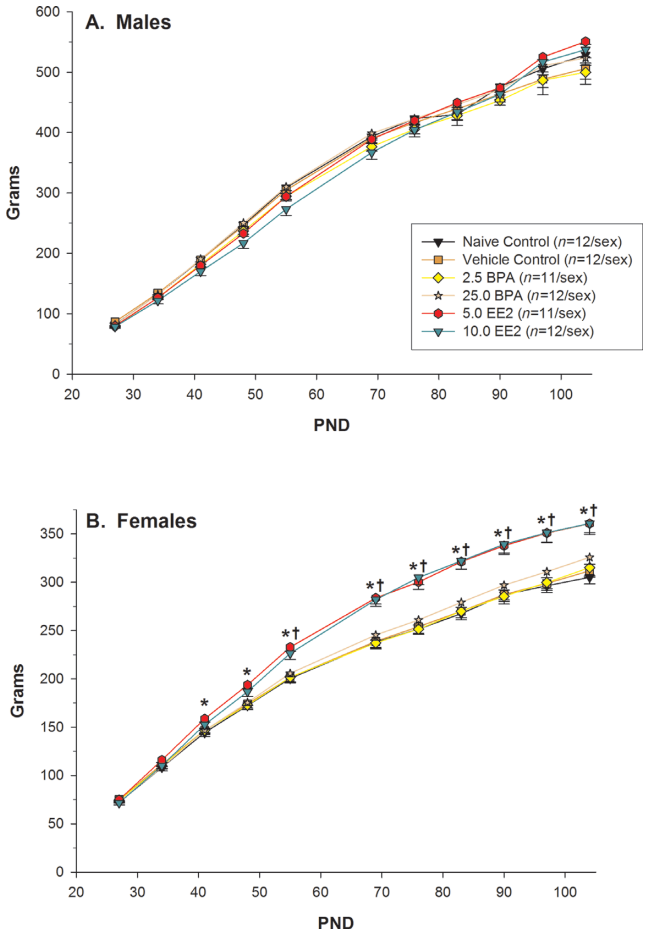

FIG. 1. Offspring body weight (mean ± SE). (A) Males: Body weights of males in the BPA or EE_2 groups did not differ significantly from same-sex vehicle controls at any age. (B) Females: (*) Body weights of 5.0 EE_2 females were significantly more than vehicle control females beginning at PND 41 and continuing throughout the study. (†) Body weights of 10.0 EE_2 females were significantly more than vehicle control females beginning at PND 55 and continuing throughout the study.

the 5.0 EE_2 females consumed more water than same-sex vehicle controls the week of PNDs 52–58 ($p < 0.009$) and the 10.0 EE_2 females consumed more water than same-sex vehicle controls the weeks of PNDs 66–72 and 73–79 ($p < 0.031$ for both). Pairwise comparisons of water intake by males indicated that the 5.0 EE_2 males consumed more water than same-sex vehicle controls the weeks of PNDs 66–72 and 108–110 (the last week included only 2 days) ($p < 0.046$ for both).

Pubertal Landmarks

Table 2 shows mean ± SE by treatment group and sex. Testes descent occurred in all male subjects. However, there was a main effect of treatment on PND of testes descent ($F(5, 64) = 41.16$, $p < 0.001$). Pairwise comparisons indicated that the 5.0 and 10.0 EE_2 groups experienced testes descent later than vehicle control males ($p < 0.001$ for both comparisons). There was a main effect of treatment on PND of preputial separation ($F(5, 63) = 34.03$, $p < 0.001$) (see Table 2). Pairwise comparisons indicated that the 5.0 and 10.0 EE_2 groups experienced preputial separation later than vehicle control males ($p < 0.020$ for both comparisons). Further, three males from two different 10.0 EE_2 litters never exhibited preputial separation. There was a main effect of treatment on PND of vaginal opening ($F(5, 56) = 4.88$, $p < 0.001$). Pairwise comparisons indicated that the 5.0 and 10.0 EE_2 groups experienced vaginal opening later than vehicle control females ($p < 0.023$ for both comparisons). Further, several females of 5.0 and 10.0 EE_2 litters never experienced vaginal opening (see Table 2 for details).

Figure 2 shows examples of normal genitalia in a vehicle control female (Fig. 2A) and the abnormal genitalia noted in some EE_2-treated females (Figs. 2B and 2C). Not all females were examined and the number affected in each treatment group is not known.

Serum Hormone Levels

Table 3 shows mean ± SE by treatment group and sex. There were significant main effects of sex in analyses of corticosterone ($F(1, 127) = 109.09$, $p < 0.001$) and T4 ($F(1, 127) = 45.68$, $p <$

TABLE 2
Pubertal Landmark Age (PND) (Mean ± SE)

Males	*n*	Testes descent	Preputial separation
Naïve control	12	22.79 ± 0.19	44.88 ± 0.80
Vehicle control	12	23.13 ± 0.21	45.21 ± 0.75
2.5 BPA	11	23.00 ± 0.32	44.45 ± 0.49
25.0 BPA	12	22.88 ± 0.22	44.25 ± 0.52
5.0 EE_2	11	27.27 ± 0.78*	52.50 ± 1.35*
10.0 EE_2	11–12[a]	32.88 ± 1.27*	70.45 ± 4.24*
Females	*n*	**Vaginal opening**	
Naïve control	12	35.96 ± 0.77	
Vehicle control	12	35.95 ± 0.88	
2.5 BPA	11	34.18 ± 0.66	
25.0 BPA	12	36.55 ± 0.69	
5.0 EE_2	7[b]	45.43 ± 5.98*	
10.0 EE_2	8[c]	42.94 ± 3.39*	

[a]Both male siblings from one 10.0 EE_2 dam never experienced preputial separation. One male from a separate 10.0 EE_2 dam also never experienced preputial separation, although its same-sex sibling did at PND 78. Thus, $n = 11$ for preputial separation.
[b]Both female siblings from four 5.0 EE_2 dams never experienced vaginal opening. One female each from three separate 5.0 EE_2 dams also never experienced vaginal opening, although their same-sex sibling did. Thus, $n = 7$ for this treatment group.
[c]Both female siblings from four 10.0 EE_2 dams never experienced vaginal opening. One female from a separate 10.0 EE_2 dam also never experienced vaginal opening, although its same-sex sibling did. Thus, $n = 8$ for this treatment group.
*Significantly later than same-sex vehicle controls.

0.001) levels. Corticosterone levels of females were higher than males (350.6 ± 12.3 and 196.3 ± 8.5 ng/ml, respectively) and T4 levels of males were higher than females (4.3 ± 0.1 and 3.2 ± 0.1 μg/dl, respectively). Analysis of testosterone levels indicated a significant interaction of treatment with sex ($F(5, 127) = 7.37$, $p < 0.001$). Pairwise comparisons did not indicate any significant differences in female groups; however, testosterone levels of males of both EE_2-treated groups were significantly lower than vehicle control males ($p < 0.001$ for both comparisons). There were no other significant main effects or interactions. Omitting data from the 15 females in which blood was obtained in the afternoon did not change the statistical results. Data from those subjects are included in Table 3.

Estrous Cyclicity

Table 4 shows proportion of days in each phase and percentage of extended estrus transitions by treatment group. Analysis of those proportions indicated a significant effect of treatment (Wilks' λ (15,141.2) = 2.533, $p < 0.003$), indicating that the distribution of phases differed between treatment groups. Contrasts indicated that the 10.0 EE_2 group experienced a significantly lower proportion of days in diestrus than the vehicle control group ($p < 0.002$).

Chi square analysis of all types of aberrant cycle transitions indicated significant differences between the vehicle control group and the two EE_2 groups (5.0 EE_2 group: $\chi^2 = 18.64$, df = 4, $p < 0.004$; 10.0 EE_2 group: $\chi^2 = 29.32$, df = 4, $p < 0.001$). There were no significant differences between the vehicle control group and any treated group in the analysis of extended diestrus transitions; however, both EE_2 groups had significantly more extended estrus transitions than the vehicle control group ($p < 0.005$ for both comparisons) (see Table 4).

Whole and Regional Brain Weights

Analyses of whole brain, frontal cortex, and hippocampal weights of males did not indicate any significant treatment effects, nor did analyses of ratios of those weights to body weight (data not shown).

Play Behavior

Analysis of frequency of pins/play behavior pair indicated a significant effect of treatment ($F(5, 127) = 6.59$, $p < 0.001$) (see Fig. 3A). Pairwise comparisons indicated that the 5.0 and 10.0 EE_2 groups engaged in more frequent pins than vehicle controls ($p < 0.012$ for both comparisons). Analysis of frequency of dorsal contacts/play behavior pair did not indicate any significant effects (see Fig. 3B).

Residential Running Wheel Activity

Although statistical analyses were conducted on averaged day or night activity, the online Supplementary data contains graphs of the activity by individual days and nights for each treatment group and sex to indicate the increasing activity with time. Analysis of average light period activity indicated a significant interaction of treatment with sex ($F(5, 128) = 7.83$, $p < 0.001$). There were no statistically significant comparisons between the vehicle control males and any same-sex BPA or EE_2 group (see Fig. 4A). However, pairwise comparisons indicated that average light period activity of females of the 5.0 and 10.0 EE_2 groups was less than that of vehicle control females ($p < 0.001$ for both comparisons) (see Fig. 4B).

TABLE 3
Serum Hormone Levels (Mean ± SE)*

	Estradiol (pg/ml)	Testosterone (ng/dl)	T3 (ng/dl)	T4 (μg/dl)[a]	Corticosterone (ng/ml)[a]	Ghrelin (ng/ml)	Leptin (ng/ml)
Males							
Naïve control ($n = 12$)	13.60 ± 2.67	359.020 ± 43.965	57.65 ± 3.72	4.198 ± 0.318	217.70 ± 17.00	1.01 ± 0.13	7.18 ± 1.50
Vehicle control ($n = 12$)	14.47 ± 3.15	339.730 ± 45.491	57.79 ± 4.22	4.180 ± 0.196	219.3 ± 19.0	0.99 ± 0.13	6.75 ± 1.32
2.5 BPA ($n = 11$)	15.21 ± 2.05	339.313 ± 63.267	47.81 ± 4.92	3.724 ± 0.311	193.1 ± 23.6	0.90 ± 0.11	5.62 ± 0.79
25.0 BPA ($n = 12$)	16.15 ± 2.50	442.343 ± 71.402	56.78 ± 4.43	4.578 ± 0.158	176.5 ± 22.4	1.13 ± 0.21	4.05 ± 0.57
5.0 EE_2 ($n = 11$)	15.23 ± 2.95	118.387 ± 26.074†	46.61 ± 5.02	4.277 ± 0.362	160.5 ± 19.5	1.11 ± 0.21	5.61 ± 0.84
10.0 EE_2 ($n = 12$)	16.29 ± 3.20	108.779 ± 19.916†	59.66 ± 4.67	4.764 ± 0.330	207.3 ± 18.5	0.99 ± 0.18	8.37 ± 1.75
Females							
Naïve control ($n = 12$)	15.88 ± 3.17	4.346 ± 0.29	55.37 ± 3.44	2.914 ± 0.191	385.9 ± 20.5	1.13 ± 0.24	4.09 ± 0.94
Vehicle control ($n = 12$)	11.96 ± 1.65	4.00 ± 0.00	49.27 ± 4.84	2.914 ± 0.170	334.7 ± 35.7	1.28 ± 0.30	4.20 ± 1.40
2.5 BPA ($n = 11$)	23.52 ± 4.57	4.97 ± 0.660	52.98 ± 5.45	3.029 ± 0.308	383.8 ± 19.7	1.22 ± 0.28	3.88 ± 0.83
25.0 BPA ($n = 12$)	15.34 ± 2.45	4.41 ± 0.34	63.32 ± 5.46	3.473 ± 0.231	358.2 ± 34.2	0.86 ± 0.17	6.43 ± 1.62
5.0 EE_2 ($n = 10$)	16.39 ± 2.43	4.35 ± 0.35	64.48 ± 8.45	3.2707 ± 0.2933	339.7 ± 26.6	1.02 ± 0.20	7.59 ± 1.92
10.0 EE_2 ($n = 12$)	18.96 ± 3.24	4.00 ± 0.00	58.24 ± 4.78	3.7478 ± 0.2486	313.7 ± 29.6	1.08 ± 0.25	5.75 ± 1.60

[a]Significant sex effects indicated higher T4 levels in males and higher corticosterone levels in females (neither interacted significantly with treatment).
*Data for LH are not shown as only one subject's level (a vehicle control female) was above the detection limit of 0.15 mIU/ml.
†Significantly lower than vehicle control males.

TABLE 4
Estrous Phase Proportions and Aberrant Transitions

Treatment	Proportion of days in each phase (mean ± SE)			% Extended estrus transitions			
	Diestrus	Proestrus	Estrus	EX to EX[b]	EX to N	N to EX	Total
Naïve control ($n = 12$)	0.57 ± 0.03	0.22 ± 0.02	0.21 ± 0.03	0.72%	1.44%	1.44%	3.60%
Vehicle control ($n = 12$)	0.59 ± 0.04	0.18 ± 0.02	0.24 ± 0.04	3.54%	0.39%	0.79%	4.72%
2.5 BPA ($n = 11$)	0.66 ± 0.04	0.16 ± 0.03	0.18 ± 0.03	0.00%	0.42%	0.42%	0.84%
25.0 BPA ($n = 12$)	0.67 ± 0.03	0.17 ± 0.01	0.16 ± 0.02	0.00%	0.00%	0.00%	0.00%
5.0 EE_2 ($n = 5$)[a]	0.57 ± 0.07	0.18 ± 0.03	0.25 ± 0.07	0.93%	3.74%	4.67%	9.34%†
10.0 EE_2 ($n = 7$)[a]	0.34 ± 0.06*	0.29 ± 0.10	0.37 ± 0.09	8.78%	6.76%	9.46%	25.00%†

[a]Not all females in the EE_2 groups experienced vaginal opening, thus, the number of subjects in these groups is less.
[b]EX to EX indicates a transition from extended estrus to extended estrus; N to EX indicates a transition from normal to extended estrus.
*Significantly less than vehicle control group.
†Significantly more than vehicle control group.

Analysis of average dark period activity indicated a significant interaction of treatment with sex ($F(5, 128) = 6.75$, $p < 0.001$). There were no significant comparisons among male treatment groups (see Fig. 4C). Pairwise comparisons indicated that females of the 5.0 and 10.0 EE_2 groups were less active than vehicle control females ($p < 0.012$ for both comparisons) (see Fig. 4D).

Table 5 shows the mean ± SE for the parameter estimates from the sigmoidally transformed cosine model of running wheel activity. Analyses of these parameters indicated that the amplitude of activity generally increased across days in all groups. The daily increase in amplitude was significantly higher for females than males ($F(1, 128) = 126.90$, $p < 0.001$), regardless of treatment. Additionally, t_{max} (the time each day at which maximum activity occurred) was significantly earlier for males than females ($F(1, 128) = 9.54$, $p < 0.002$) and $t_{1/2\ down}$ (the time at which activity began to slow down) was earlier for males than females ($F(1, 128) = 10.47$, $p < 0.001$). The daily increase in amplitude was significantly different among treatment groups ($F(5, 128) = 5.30$, $p < 0.001$), with a smaller increase in both EE_2 groups, particularly at the 10.0 dose, compared with the vehicle control group ($p < 0.050$). This effect did not interact with sex.

Intake of Flavored Solutions

Analyses were conducted on ml intake/g body weight and on preference endpoints. Table 6 shows saccharin and sodium preference by treatment group, sex and day. Analysis of regular water intake during the days that saccharin-flavored solution was available indicated a significant effect of day ($F(2, 256) = 4.72$, $p < 0.010$) (data not shown). Pairwise comparisons indicated that regular water intake on day 3 was higher than that on days 1 or 2 (both comparisons $p < 0.046$).

TABLE 5
Parameters Modeled from Residential Running Wheel Activity Dataset (Mean ± SE)

	Naïve control	Vehicle control	2.5 BPA	25.0 BPA	5.0 EE_2	10.0 EE_2
Males						
Initial amplitude	9.43 ± 2.57	2.57 ± 4.42	9.77 ± 4.49	4.21 ± 2.07	6.50 ± 4.10	18.87 ± 5.80
Daily increase in amplitude[*]	2.81 ± 0.46	4.99 ± 1.65	3.52 ± 1.18	4.31 ± 0.58	4.68 ± 1.79	1.96 ± 0.96
t_{max}[†a]	23.98 ± 0.32	23.33 ± 0.36	23.38 ± 0.49	24.22 ± 0.29	23.64 ± 0.27	24.52 ± 0.46
Beta	3.87 ± 1.98	4.87 ± 2.58	4.48 ± 1.36	2.09 ± 0.16	4.12 ± 1.64	3.35 ± 1.22
$t_{1/2\ up}$[b]	19.87 ± 0.30	19.24 ± 0.37	19.34 ± 0.46	20.11 ± 0.28	19.53 ± 0.27	20.44 ± 0.47
$t_{1/2\ down}$[#b]	4.10 ± 0.33	3.42 ± 0.35	3.43 ± 0.51	4.33 ± 0.29	3.75 ± 0.27	4.61 ± 0.46
Maximum activity	8.23 ± 0.04	8.18 ± 0.04	8.08 ± 0.10	8.22 ± 0.04	8.22 ± 0.06	8.17 ± 0.09
Minimum activity	15.77 ± 0.04	15.82 ± 0.04	15.92 ± 0.10	15.78 ± 0.04	15.78 ± 0.06	15.83 ± 0.09
Females						
Initial amplitude	10.99 ± 9.30	7.39 ± 10.22	15.21 ± 11.25	5.90 ± 6.32	6.38 ± 6.76	13.22 ± 5.34
Daily increase in amplitude[a]	20.57 ± 2.66	20.54 ± 3.19	21.75 ± 2.69	17.13 ± 1.92	13.18 ± 2.64	6.63 ± 1.94
t_{max}	24.69 ± 0.46	24.97 ± 0.53	24.65 ± 0.53	24.95 ± 0.45	23.76 ± 0.34	24.58 ± 0.47
Beta	2.90 ± 0.83	1.97 ± 0.14	1.94 ± 0.14	6.52 ± 4.82	6.99 ± 4.05	7.55 ± 3.71
$t_{1/2\ up}$	20.55 ± 0.49	18.87 ± 1.62	20.53 ± 0.53	20.83 ± 0.45	19.63 ± 0.34	20.46 ± 0.48
$t_{1/2\ down}$	4.84 ± 0.44	5.07 ± 0.51	4.76 ± 0.53	5.06 ± 0.45	3.90 ± 0.34	4.71 ± 0.46
Maximum activity	8.29 ± 0.16	8.20 ± 0.05	8.23 ± 0.02	8.23 ± 0.04	8.27 ± 0.05	8.25 ± 0.08
Minimum activity	15.71 ± 0.16	15.80 ± 0.05	15.77 ± 0.02	15.77 ± 0.04	15.73 ± 0.05	15.75 ± 0.08

[a] t_{max} was estimated as the time of day, on a 24-h clock, at which maximum activity occurred. The value, 24, was added to postmidnight t_{max} estimates to enable averaging with premidnight times. For example, a mean $t_{max} = 24.5$ is 30 min after midnight.
[b] $t_{1/2\ up}$ and $t_{1/2\ down}$ are estimated as the time of day, on a 24-h clock, at which half of the maximum activity was achieved, respectively, as activity increased or decreased.
[*] Significantly less for males relative to females ($p < 0.001$) and significantly less in the 5.0 and 10.0 EE2 groups ($p < 0.050$).
[†] Significantly earlier for males relative to females ($p < 0.002$).
[#] Significantly earlier for males relative to females ($p < 0.001$).

TABLE 6
Saccharin and Sodium Preference (Mean ± SE)

Males	Day 1	Day 2 Saccharin preference	Day 3	Day 1	Day 2 Sodium preference	Day 3
Males						
Naïve control	91.04 ± 2.45	81.48 ± 7.91	71.12 ± 9.60	68.47 ± 3.99	80.07 ± 3.27	59.16 ± 6.89
Vehicle control	93.05 ± 1.62	86.29 ± 7.58	84.69 ± 7.82	63.10 ± 7.61	71.05 ± 7.61	54.64 ± 6.36
2.5 BPA	87.92 ± 8.85	92.77 ± 3.86	82.37 ± 6.08	72.31 ± 8.60	73.81 ± 7.87	55.95 ± 11.63
25.0 BPA	86.56 ± 8.29	85.46 ± 8.40	80.86 ± 9.65	68.77 ± 6.10	75.80 ± 3.98	59.39 ± 6.17
5.0 EE_2	78.44 ± 10.91	68.69 ± 10.56	63.99 ± 11.06	72.10 ± 3.61	84.15 ± 1.85	67.95 ± 6.40
10.0 EE_2	86.80 ± 7.45	83.57 ± 7.27	76.76 ± 7.78	66.24 ± 3.47	78.15 ± 3.79	64.92 ± 3.34
Females						
Naïve control	91.79 ± 3.32	92.83 ± 2.40	83.17 ± 6.94	66.67 ± 7.05	80.73 ± 5.21	64.59 ± 5.96
Vehicle control	89.55 ± 7.12	96.39 ± 0.64	92.72 ± 1.31	76.74 ± 4.18	83.58 ± 3.89	61.15 ± 7.65
2.5 BPA	88.51 ± 8.00	90.35 ± 6.12	86.84 ± 8.02	75.93 ± 5.37	86.64 ± 3.02	69.91 ± 6.88
25.0 BPA	95.76 ± 0.89	95.27 ± 0.77	85.82 ± 6.82	84.20 ± 3.51	80.99 ± 3.99	77.69 ± 4.58
5.0 EE_2	79.85 ± 10.90	80.21 ± 10.45	82.41 ± 8.42	79.13 ± 3.29	82.78 ± 2.50	68.41 ± 3.93
10.0 EE_2	93.48 ± 3.02	92.52 ± 3.29	86.77 ± 6.49	62.71 ± 7.16	66.76 ± 6.85	53.26 ± 6.89

Analysis of saccharin-flavored intake indicated significant main effects of sex ($F(1, 128) = 53.54, p < 0.001$) and day ($F(2, 256) = 69.46, p < 0.001$) (see Fig. 5A). Females consumed significantly more than males on a ml/g basis. Intake on each of the 3 days differed from each other with the highest intake on the first day (all comparisons $p < 0.001$). Analysis of saccharin preference indicated a significant effect of day ($F(2, 251) = 7.82, p < 0.001$) and pairwise comparisons revealed that preference on days 1 and 2 was higher than on day 3 ($p < 0.001$).

Due to an inadvertent error, the sodium solution concentration for animals in replicate 1 was 3.0% and not 0.3% as intended. Therefore, data for regular water and sodium-flavored solution intake for those 14 subjects were deleted prior to analyses. Analysis of regular water intake during the time that sodium-flavored solution was available indicated significant effects of treatment with sex $F(5, 114) = 3.38, p < 0.008$) and day ($F(2, 226) = 16.73, p < 0.001$) (data not shown). Pairwise comparisons indicated that the 10.0 EE_2 females consumed more

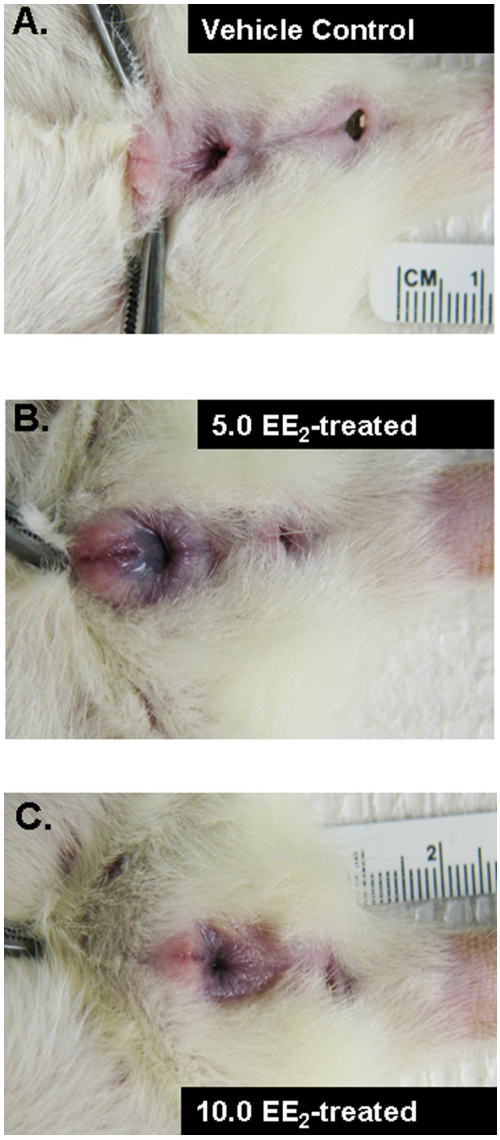

FIG. 2. External genitalia of three adult female rats. (A) Vehicle control female. (B) 5.0 EE_2-treated female. (C) 10.0 EE_2-treated female. Genitalia of the EE_2-treated females appear malformed and similar to what Sawaki *et al.* (see Figure 2 of Sawaki *et al.*, 2003b) described as "excessive cleavage of urethral slit and insufficient raphe formation".

regular water than did vehicle control females ($p < 0.001$). Intake of regular water during each of the three test days was significantly different with the highest intake on day 3 (all comparisons $p < 0.022$).

Analysis of sodium-flavored intake indicated significant effects of treatment with sex ($F(5, 114) = 2.96, p < 0.016$) and day ($F(2, 223) = 13.96, p < 0.001$) (see Fig. 5B). Pairwise comparisons indicated that the vehicle control females consumed more of the sodium-flavored solution than did vehicle control males ($p < 0.006$). Similarly, 25.0 BPA females consumed more of the sodium-flavored solution than 25.0 BPA males ($p < 0.001$); no other comparisons of this effect were significant. Intake on each of the 3 days differed as well (for all comparisons $p < 0.029$). Analysis of sodium preference indicated a significant interaction of treatment with sex ($F(5, 114) = 2.31, p < 0.049$); however, there were no significant pairwise comparisons to either the male or the female vehicle control groups. In addition, analysis of sodium preference indicated a significant effect of day ($F(2, 222) = 47.65, p < 0.001$) and pairwise comparisons revealed that preference on day 1 was less than day 2 ($p < 0.001$) but higher than day 3 ($p < 0.001$). Preference on day 2 was higher than on day 3 ($p < 0.001$).

Female Sex Behavior

There were no significant effects of treatment, test day, or the interaction in the analyses of lordosis postures, hops/darks, male aggression directed at female, female investigation of male anogenital area, or male investigation of female anogenital area. There was, however, a significant effect of treatment on the behavior of female aggression directed at male ($\chi^2 = 15.63$, 5 df, $p < 0.008$). Contrasts of this effect indicated that 10.0 EE_2 females exhibited more aggression directed at the male than did vehicle controls ($p < 0.007$). On average, 29% of the sessions with 10.0 EE_2 females contained at least one event of female aggression directed at the male whereas only 7% of the vehicle control female sessions contained a similar event. Each of the seven EE_2 females had at least one session in which there was female aggression directed at the male whereas only 4 of the 12 vehicle control female sessions exhibited a similar event.

Manual Lordosis

Analysis of lordosis responses did not indicate a significant treatment effect and excluding the EE_2 females without vaginal opening did not change the results (data not shown). When all females were included, the number (and percentage) of females in each group with a nonzero response on at least one of the three trials on each of the 5 days was: 9/12 (75.0%) for the naïve control group, 7/12 (58.3%) for the vehicle control group, 7/11 (63.6%) for the 2.5 BPA group, 8/12 (66.7%) for the 25.0 BPA group, 8/11 (72.7%) for the 5.0 EE_2 group, and 10/12 (83.3%) for the 10.0 EE_2 group. When the 5.0 and 10.0 EE_2 females that never experienced vaginal opening were excluded, the number (and percentage) of females in those two groups with a nonzero response on at least one of the three trials on each of the 5 days was: 3/5 (60.0%) for the 5.0 EE_2 group and 5/7 (71.4%) for the 10.0 EE_2 group.

DISCUSSION

Developmental BPA treatment at 2.5 or 25 μg/kg/day did not alter the physiological, pubertal, and sexually dimorphic behavioral endpoints measured here. However, those same endpoints were sensitive to developmental treatment with relatively high doses of the reference estrogen, ethinyl estradiol (EE_2).

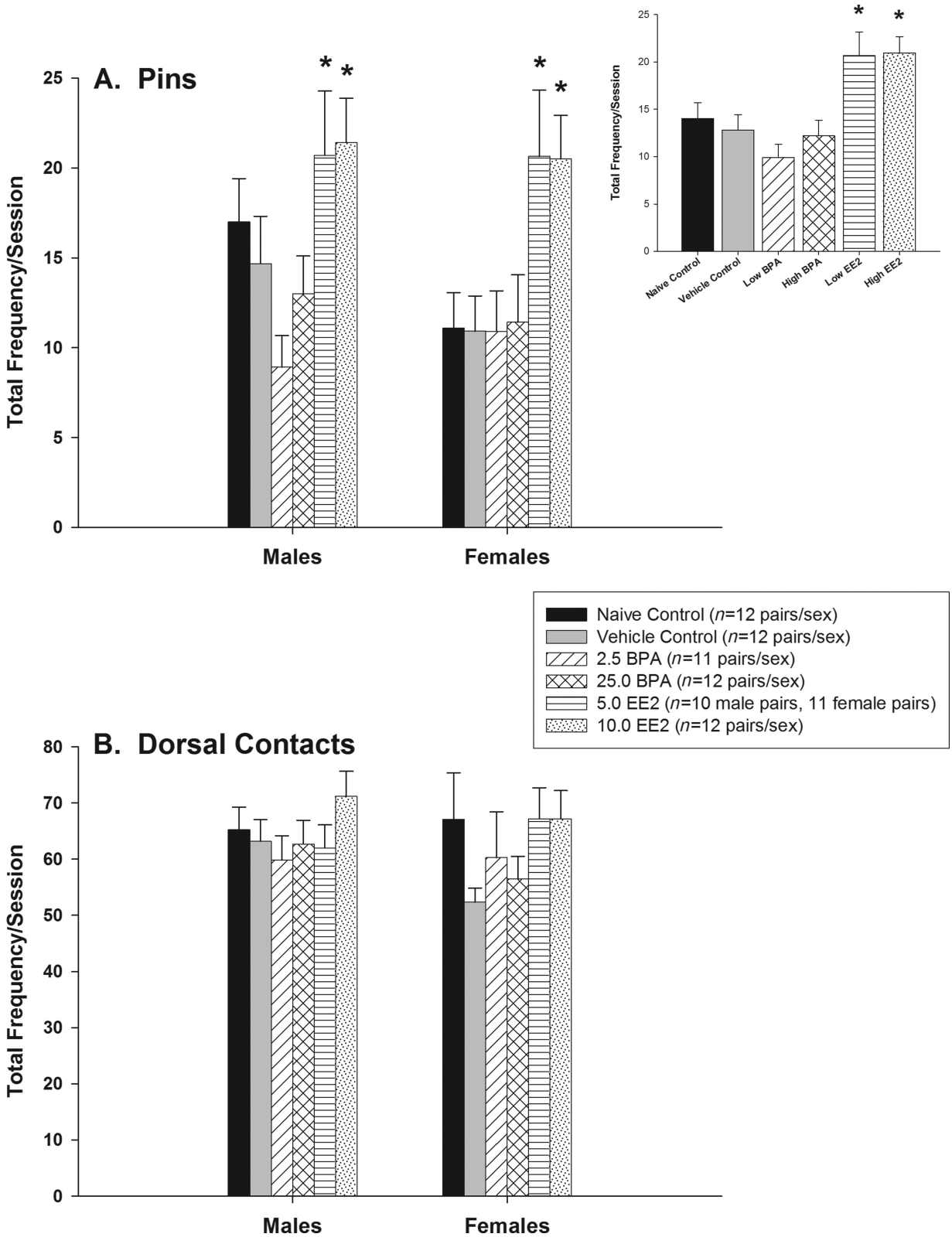

FIG. 3. Play behavior of male and female offspring (mean ± SE). (A) Frequency of pins by treatment group and sex. A significant effect of treatment indicated that the 5.0 and 10.0 EE_2 groups engaged in more pinning behavior than the vehicle control group. The treatment × sex interaction was not statistically significant ($p = 0.691$). Inset shows the effect by treatment group. (B) Frequency of dorsal contacts by treatment group and sex. There were no significant treatment group or sex differences on this play behavior endpoint.

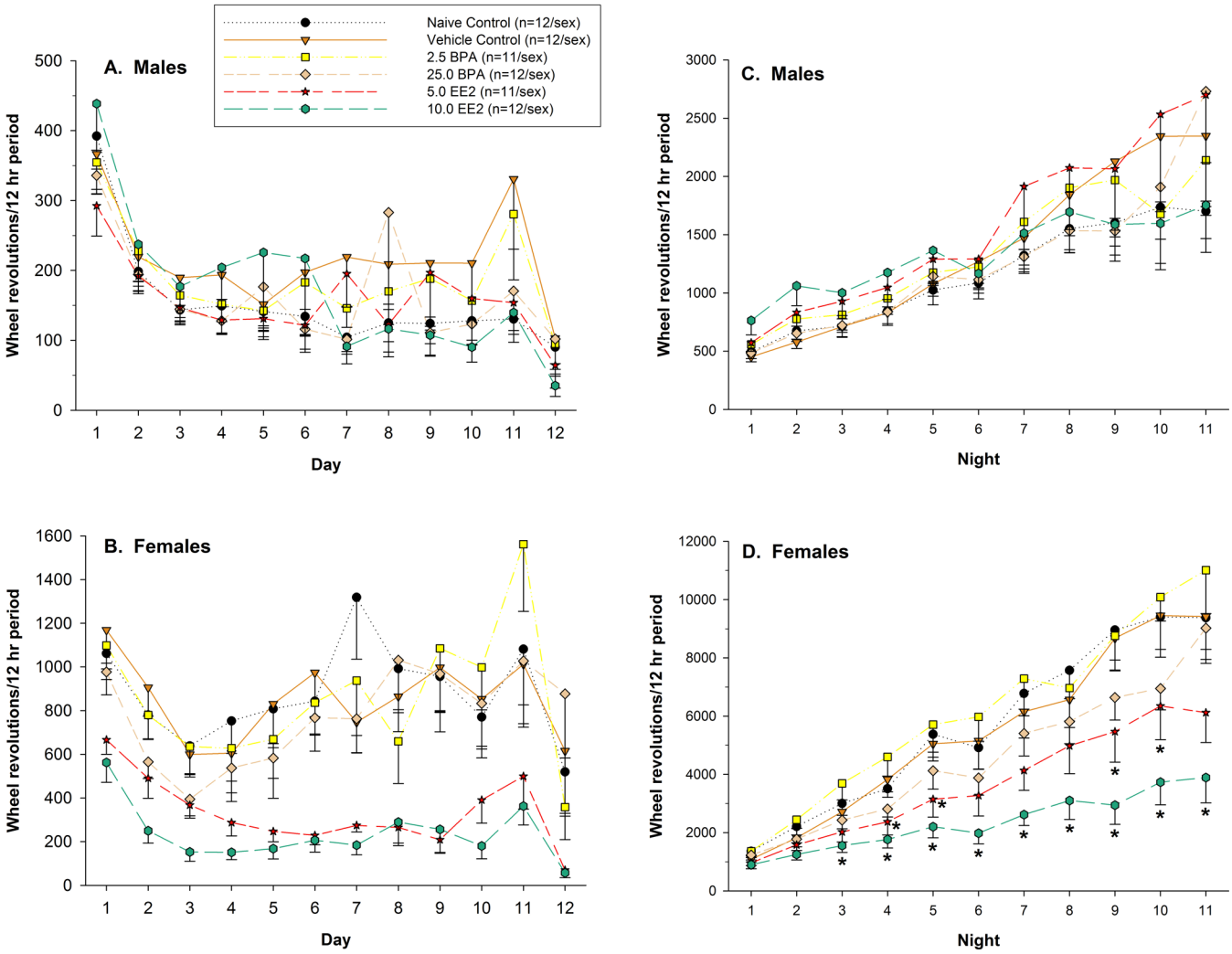

FIG. 4. Light and dark period residential running wheel activity (average number of wheel revolutions/12 h period) (mean ± SE). (A and B) Light period activity. *Pairwise comparisons of the significant interaction of treatment and sex indicated that females of the 5.0 and 10.0 EE_2 groups were significantly less active than vehicle control females. (C and D) Dark period activity. (*) Pairwise comparisons of the significant interaction of treatment and sex indicated that females of the 5.0 and 10.0 EE_2 groups were significantly less active than vehicle control females.

Multiple endpoints in both sexes were significantly affected by EE_2 treatment, although the number of affected endpoints and their severity were higher in females. Most of the alterations were consistent with previously reported effects of developmental estrogen treatment in laboratory rodents. Further, the EE_2-induced behavioral effects described here concur with our previously reported neuroanatomical alterations (i.e., EE_2-induced masculinization of females) collected from the same cohort as the current subjects (He *et al.*, 2012). Additionally, the lack of significance for BPA treatment here is consistent with our previous descriptions from the same cohort (Ferguson *et al.*, 2011, 2012).

The behavioral assessments in this study have been sensitive to sex differences in our laboratory (e.g., play behavior (Ferguson and Cada, 2004), light and dark period running wheel activity (Boctor and Ferguson, 2010; Ferguson *et al.*, 2003; Flynn *et al.*, 2000, 2001), saccharin solution intake (Ferguson and Boctor, 2010; Flynn *et al.*, 2000), and sodium solution intake (Ferguson *et al.*, 2003; Flynn *et al.*, 2000, 2005)). Similar sex differences in the expected directions were apparent in the vehicle control group of the current study. The historical literature from our laboratory and the effects in the vehicle control group here provide support for the treatment-related effects described here.

Although the EE_2 effects here replicate those previously described (discussed below), the lack of BPA effects at these doses is not entirely consistent with the literature. The methodology used here for assessing play behavior (i.e., a brief separation followed by reuniting a same-sex pair) is standard in the rodent literature, but others have used different methods for examin-

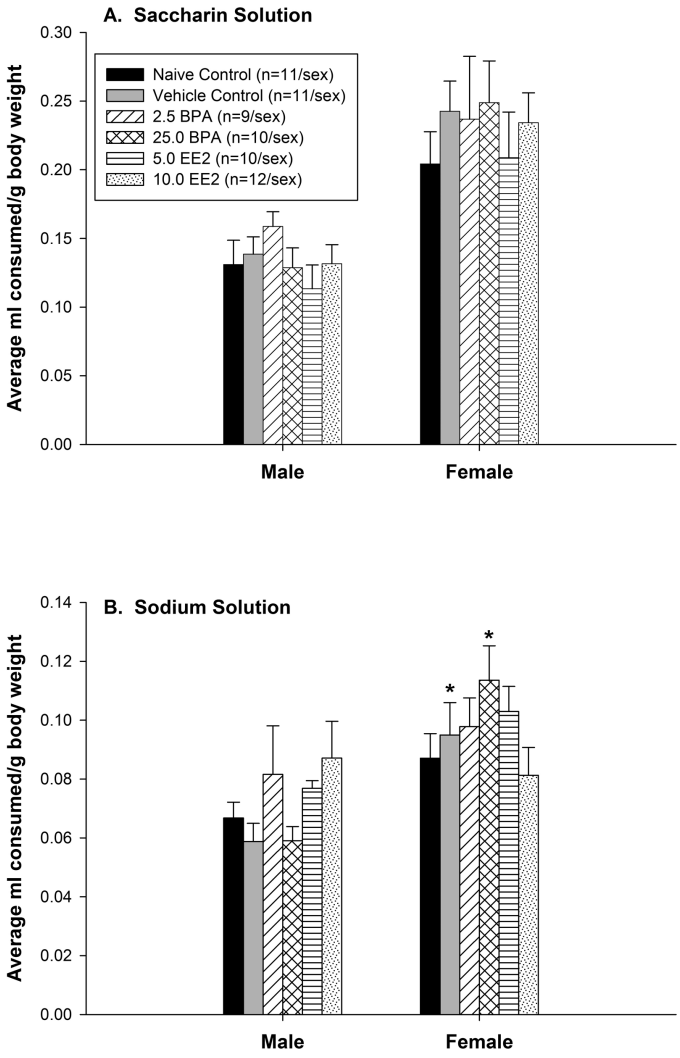

FIG. 5. (A) Saccharin-flavored solution intake, (B) sodium-flavored solution intake (mean ± SE). Data were first averaged over the 3 days of intake for each subject. There were no significant treatment-related effects in saccharin-flavored solution intake; however, females consumed significantly more than males. (*) For sodium-flavored solution intake, vehicle control females and 25.0 BPA females consumed more than same-treatment males.

ing BPA-induced alterations. Porrini *et al.* (2005) examined social behavior of female Sprague Dawley rats at PNDs 35, 45, and 55 after orally treating their dams from mating until pup weaning with 40 μg BPA/kg. The subjects were housed with males and females postweaning and prior to assessment, there was no social deprivation. BPA treatment caused increased exploration at PNDs 35 and 45 and decreased play with males (but not females) and social grooming at PND 45. The same lab had earlier reported BPA-induced masculinization of females when behavior was analyzed from minutes 2 and 3 only of a 7-min test session (Dessi-Fulgheri *et al.*, 2002). Dietary BPA exposure (≈5 μg BPA/day) during gestation and lactation appeared to increase the social behaviors of PND 21 female mice (Wolstenholme *et al.*, 2011). However, in none of those three studies was the litter statistically controlled. The multiple reports describing the severity of ignoring litter effects (e.g., Haseman *et al.*, 2001; Holson *et al.*, 2008) clearly indicate that statistical significance may be falsely inflated if the litter is not the unit of analysis.

There appear to be no reports of BPA effects on long-term activity assessments, such as residential running wheel activity. Ryan *et al.* (2010) described no alterations in the 10 h figure 8maze activity levels of adult male and female rats developmentally treated with BPA; however, the females in that study were ovariectomized at the time of assessment. Thus, the lack of BPA-induced long-term activity alterations reported here awaits replication.

Intake of saccharin or sodium flavored solutions was not altered at the BPA doses administered here. Preference for highly concentrated 0.25 and 0.50% saccharin or 10–15% sucrose solutions has been reported to be altered by developmental BPA treatment at doses of 0.1 and 1.0 mg/l of drinking water (Xu *et al.*, 2011) or subcutaneous injections of 40 μg BPA/kg during adolescence (Diaz Weinstein *et al.*, 2013). However, oral BPA doses of 2, 20, or 200 μg/kg from GD 7 through lactational day 18 to rats did not alter the preference for a 0.25% saccharin solution in their adult male and female offspring (Ryan *et al.*, 2010). There do not appear to be published descriptions of the effects of BPA treatment on intake or preference of sodium flavored solutions.

Developmental BPA treatment at these doses did not alter adult female sexual behavior. This is in contrast to the results of Naule *et al.* (2014) in which 0.050 (but not 5.0) mg/kg BPA administered orally to mice from GD 15 to lactational day 21 increased the lordosis quotient of their adult ovariectomized and hormonally primed female offspring on the first of two tests. However, similar to the results here, oral BPA doses of 20 or 200 μg/kg from GD 7 through lactational day 18 to rats did not alter the lordosis quotient of their adult ovariectomized and hormonally primed female offspring (Ryan *et al.*, 2010).

Although BPA treatment did not produce substantial alterations, prenatal EE_2 treatment followed by direct treatment from birth until weaning appeared to masculinize and/or defeminize females of the current study. These effects were expected given that estradiol, produced via aromatization of androgens, is essential for masculinization of the male rodent central nervous system (reviewed in Lenz and McCarthy, 2010). Here, the masculinization/defeminization of EE_2-treated females was apparent in a heavier body weight, delayed or complete lack of vaginal opening, aberrant estrous cyclicity, increased play behavior, decreased running wheel activity, and increased male-directed aggression. Further, most of those effects occurred in both EE_2 treatment groups (i.e., 5.0 and 10.0 μg/kg/day). With the possible exceptions of estrous cyclicity and dark period running wheel activity, there were no apparent indications of a dose-response as the effects seemed equally severe in both EE_2-treated groups.

Previous studies have described abnormal estrous cyclicity after developmental treatment with estrogens or estrogen-like compounds (e.g., diethylstilbestrol (DES)) (Berretti *et al.*, 2014; Delclos *et al.*, 2009; Kwon *et al.*, 2000; Levy *et al.*, 1995; Willoughby *et al.*, 2005). Here, EE_2-treated rats exhibited a significantly lower proportion of days in diestrus and a higher percentage of extended estrus transitions, similar to that reported for dietary EE_2 treatment in rats (Delclos *et al.*, 2009) and PNDs 15–18 DES treatment in mice (Nikaido *et al.*, 2005) as well as similar but chronic EE_2 treatment (Delclos *et al.*, 2014). Further, prenatal DES or neonatal estradiol benzoate treatment can produce increased body weight in female rats and mice (Donohoe and Stevens, 1983; Nikaido *et al.*, 2004).

The delayed vaginal opening described for the EE_2-treated females here was not unexpected as similar EE_2 (e.g., 0.5 or 5.0 μg/kg/day) or estradiol benzoate treatment also resulted in vaginal opening delays (Berretti *et al.*, 2014; Delclos *et al.*, 2012, 2014; Sawaki *et al.*, 2003a). Here, EE_2 treatment significantly delayed vaginal opening such that EE_2-treated rats experienced this landmark almost 7 days later than vehicle control females. Further, 4 of the 12 litters treated with 10.0 μg/kg/day EE_2 never experienced vaginal opening. Still, many studies report accelerated vaginal opening as a result of developmental estrogen or estrogen-like compound treatment (Ashby *et al.*, 1997; Nikaido *et al.*, 2004, 2005; Odum *et al.*, 2002; Rothschild *et al.*, 1988; Ryan *et al.*, 2010; Willoughby *et al.*, 2005). However, the compounds, doses, treatment ages/durations, and administration routes of those studies overlap with similar studies in which treatment did not alter age at vaginal opening (e.g., Kwon *et al.*, 2000; Shiorta *et al.*, 2012; Yamamoto *et al.*, 2003). As reviewed by Gorski (1986), even slight differences in the timing of exogenous estrogen treatment (e.g., PNDs 26–30 vs. PNDs 33–37) can affect vaginal opening.

The genital malformations in EE_2-treated females of the current study were comparable to those previously reported to be caused by similar EE_2 treatment in Long-Evans rats (see Figure 9 of Ryan *et al.*, 2010) and Sprague Dawley rats (see Figure 2 of Sawaki *et al.*, 2003b). Those abnormalities were described as cleft phallus with "excessive cleavage of urethral slit and insufficient raphe formation" (Sawaki *et al.*, 2003b). Such malformations in females can also result from a single injection of estradiol or DES on GD 19 (Henry *et al.*, 1984). Because not all females in the current study were carefully examined, it is not certain that similar abnormalities did not occur in BPA-treated females.

The behavioral masculinization of females by developmental EE_2 treatment was reflected in all behaviors assessed here, except flavored solution intake and manual elicitation of lordosis. For example, EE_2 treatment resulted in increased play behavior pinning frequencies by female rats similar to that caused by developmental androgen treatment (Meaney, 1989; Meaney and McEwen, 1986; Tonjes *et al.*, 1987). Here, the pinning frequency of EE_2-treated females was almost twice that of vehicle control females and nearly 40% more than that of vehicle control males, indicating pronounced masculinization. Masculinization of rodent play behavior had been thought to be due to direct effects on androgen receptors, rather than aromatization effects, as treatment of males with aromatase inhibitors or females with estradiol resulted in few play behavior alterations (Meaney and Stewart, 1981; Tonjes *et al.*, 1987). A more recent study, however, has demonstrated that neonatal estradiol treatment of females at doses which approximate male-typical levels can cause increased play behavior, although that increase was more related to wrestling/boxing and pouncing behaviors than to pinning behavior (Olesen *et al.*, 2005). A previous study of dietary EE_2 in our laboratory found no treatment effects on play behavior, despite the use of higher external doses than in the current study (Ferguson *et al.*, 2003). However, in that previous study, postnatal exposure to EE_2 occurred via lactation and it is likely that the offspring were exposed to a lower dose postnatally relative to the direct treatment in the current study. Given that the play behavior of rodents is organized during the early postnatal period (Beatty *et al.*, 1981), the direct EE_2 treatment used here is more likely to have had an effect than lactational transfer of EE_2.

Few studies have examined long-term activity levels, such as residential running wheel activity, after developmental estrogen treatment. In our previous study of dietary EE_2 treatment, dark period running wheel activity of female offspring was only mildly decreased in a dose-response manner by 1–200 ppb EE_2 in the diet (Ferguson *et al.*, 2003). Again, however, the postnatal EE_2 treatment occurred via lactational transfer in that study. Nevertheless, it is clear that loss of circulating estrogens (e.g., postovariectomy) drastically reduces running wheel activity in rats and mice, which can be restored by exogenous estradiol treatment (Gray *et al.*, 1989; Morgan and Pfaff, 2001, 2002; Rivera and Eckel, 2005; Ruiz de Elvira *et al.*, 1992). Adult ovariectomy, however, removes the potential activating effects of endogenous estrogens. In the current study, not only were all females gonadally intact, but serum estradiol levels were comparable in all treatment groups. Thus, the decreased running wheel activity levels may be due to an EE_2-induced alteration in hormonal organizational effects. The estrogen receptor alpha in the medial preoptic area seems crucially responsible for estrogen-induced increases in running wheel activity (Fahrbach *et al.*, 1985; Hertrampf *et al.*, 2008; Ogawa *et al.*, 2003; Spiteri *et al.*, 2012). However, estrogen receptor alpha expression in the medial preoptic area of PND 1 female siblings of these EE_2-treated subjects here was not significantly different from that of vehicle control females (Cao *et al.*, 2013). Thus, it may be that the continued direct EE_2 treatment on PNDs 1–21 contributed largely to the potential organizational alterations which then led to the decreased running wheel activity.

EE_2-treated females were more aggressive toward stimulus males during tests of sexual behavior. That aggression was typically expressed as biting and pouncing on top of the male. Those pounces were not misdirected mountings attempted by the female to the male. Similarly, adult female offspring of dams

treated with DES during gestation and via lactation performed more rejection behaviors, such as kicking or fighting, when tested with a sexually experienced male (Kubo *et al.*, 2003). Developmental EE_2 treatment also caused increased male-directed aggression during sexual behavior tests (Della Seta *et al.*, 2008). Increased agonistic behaviors during resident intruder tests were exhibited by adult female rats that had received an injection of estradiol benzoate at birth (Berretti *et al.*, 2014). Although lordosis postures were rare in our assessment paradigm, none of the EE_2-treated females exhibited a lordosis posture during any of the five test sessions. Likewise, EE_2 treatment (at doses >5.0 μg/kg/day) from GD 7 through lactational day 18 or an injection of estradiol benzoate at birth decreased the lordosis behavior of adult female offspring (Berretti *et al.*, 2014; Ryan *et al.*, 2010).

Developmental EE_2 treatment had few effects on male offspring. Males of both EE_2-treated groups experienced significant delays of 4–7 days in testes descent and 7–25 days in preputial separation. As adults, serum testosterone levels of males of both EE_2-treated groups were only 32–35% those of vehicle control males. Despite the magnitude of that decrease, there were few behavioral alterations. Pinning frequencies of EE_2-treated males during play behavior assessments were somewhat higher than those of vehicle control males, but saccharin- and sodium-flavored solution intake as well as running wheel activity were well within the range of vehicle control males. Because circulating androgen levels are critical for some of these behaviors, the apparent lack of treatment effects was somewhat surprising. For example, the necessity of normal circulating levels of testosterone or estradiol for sex-typical running wheel activity is clearly evident from the effects caused by adult or preweaning castration and/or exogenous replacement (Hagenauer *et al.*, 2011; Ibebunjo *et al.*, 2011; Jackson *et al.*, 2011; Rivera and Eckel, 2005). However, the minimum circulating levels necessary for normal male-like running wheel activity are not clear. Circulating levels that are 54%, but not 13%, of normal endogenous levels can maintain normal wheel running activity levels of male mice held under constant light or dark photoperiods (Butler *et al.*, 2012). Although low, perhaps the testosterone levels of the EE_2-treated males of the current study were sufficient to maintain normal wheel running activity. Further, there was no neuroanatomical evidence of demasculinization or feminization in PND 21 siblings of the male subjects assessed here. In contrast, males treated with 10 μg/kg/day EE_2 exhibited a significant increase in SDN-POA volume at PND 21 (He *et al.*, 2012). We previously reported that developmental treatment with 10–38 μg/kg/day of dietary EE_2 caused increased sodium-flavored solution intake at adulthood in males and females (Ferguson *et al.*, 2003), a finding not replicated here. However, in our previous study, that EE_2 treatment also decreased postweaning and adult body weights of both sexes, effects not seen here. Saccharin-flavored intake was not altered in either sex, a finding similar to that reported by McGivern and Henschel (1990) for adult male and female rats treated with estradiol benzoate on PNDs 2 and 3. The relatively few significant alterations in EE_2-treated males suggests that they may be less sensitive to developmental EE_2 treatment than females, a hypothesis that has been presented by Ryan *et al.* (Howdeshell *et al.*, 2008; Ryan *et al.*, 2010 and see Figure 11 in Ryan *et al.*, 2010).

To summarize, developmental EE_2 treatment affected physical, physiological, and behavioral endpoints. Although males were affected, females treated with EE_2 appeared more severely affected. Those treatment groups were specifically included to demonstrate sensitivity of this rat model to a reference estrogen; however, there is a need for such data by regulatory agencies (see evidence presented in Gray *et al.*, 2010). Further, although the EE_2 doses here were high enough to cause malformations, such doses are within the range of those that produce adverse effects in adult women (see argument presented in Gray *et al.*, 2010). Nevertheless, the main focus of these studies was to ascertain potential alterations caused by developmental BPA treatment. In the endpoints measured here, BPA treatment produced no significant effects, despite the significantly increased SDN-POA volume in PND 21 males of the 2.5 and 25.0 BPA groups (He *et al.*, 2012), siblings of the subjects described here. Our previous descriptions of significant EE_2, but few BPA, effects on pre- and postweaning behaviors (Ferguson *et al.*, 2011, 2012) add to the growing body of literature indicating lack of effect at these orally administered BPA doses.

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