Developmental Treatment with Bisphenol A or Ethinyl Estradiol Causes Few Alterations on Early Preweaning Measures

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Because bisphenol A (BPA) exposure is nearly ubiquitous, increased knowledge of its potential effects on development will enable better risk assessment and regulatory guidance. Here, Sprague-Dawley rats were reared in low exogenous estrogen environments. After breeding at adulthood, dams were gavaged on gestational days (GDs) 6–21 with vehicle (VEH), 2.5 or 25.0 μg/kg/day BPA, or 5.0 or 10.0 μg/kg/day ethinyl estradiol (EE2). Offspring were orally treated on postnatal days (PNDs) 1–21 with the same dose the dam received. Relative to the VEH group, dams of both EE2-treated groups weighed less throughout gestation and lactation. PND 1 absolute anogenital distance and anogenital index were unaltered by any treatment. Ages at fur development and eye and ear opening were unaffected by any treatment. Despite a significant treatment effect, no group was significantly different from VEH in PNDs 3–6 righting latencies; although males had shorter latencies and all latencies decreased with age. PNDs 8–11 slant board behavior was unaffected by any treatment; however, males had shorter turning latencies and latencies decreased with age. Preweaning body weights of BPA- and EE2-treated groups as well as naive controls were less than VEH. No treatment affected PND 21 whole or regional brain weights or levels of estradiol, testosterone, corticosterone, T3, T4, luteinizing hormone, ghrelin, or leptin. These results add to the literature indicating that developmental BPA treatment at these doses has no effects on gestational or lactational body weight, offspring anogenital distance, preweaning behaviors or hormone levels, and whole and regional brain weights measured at weaning.

Key Words: bisphenol A; developmental; rat; behavior; anogenital distance; righting reflex.

Human exposure to the plastic monomer bisphenol A (BPA) is nearly ubiquitous with more than 90% of the U.S. and Canadian populations exhibiting measurable urinary levels (Bushnik et al., 2010; Calafat et al., 2008). The primary route of BPA exposure appears to be oral from food contact materials (Kang et al., 2006; von Goetz et al., 2010), and daily intake for adults is estimated at 0.4–1.4 μg/kg/day (Food and Agriculture Organization of the United Nations, World Health Organization, 2010). Estimates of intake for infants are higher, however, ranging 0.01–2.4 μg/kg/day, with infants fed exclusively using polycarbonate baby bottles having higher levels (Food and Agriculture Organization of the United Nations, World Health Organization, 2010).

Although recent reviews and perspectives have noted varying levels of concern regarding the potential risks of BPA exposure (for a review of those risk assessments, see Beronius et al., 2010), most agree that there is at least some level of concern for neuro-behavioral effects which may result from developmental BPA exposure. In part, this concern is a consequence of the higher BPA exposure levels in infants and children. However, BPA is thought to act by perturbing endocrine systems (reviewed in Wolstenholme et al., 2011), and normal development of these systems is essential for appropriate neural and behavioral sexual differentiation and dimorphisms (for reviews, see Cooke et al., 1998; Morris et al., 2004). Thus, exposure to an endocrine disrupter during development could have severe and/or long-lasting effects.

There are many studies examining developmental BPA exposure in laboratory animals (for reviews of these, see Goodman et al., 2006; Palanza et al., 2008; Richter et al., 2007), but these have often generated inconsistent results. In response, explicit guidelines for future BPA research have been described. Those suggested standards include use of a sensitive positive control or reference estrogen (Hunt et al., 2009; Li et al., 2008) such as ethinyl estradiol (Richter et al., 2007), control of exogenous estrogens (Hunt et al., 2009), statistical control for litter effects (Goodman et al., 2006; Li et al., 2008; Richter et al., 2007), and use of oral administration for the most relevant extrapolation to humans (Li et al., 2008; Richter et al., 2007). That last recommendation could be troublesome for laboratory animal research. Orogastric gavage is the most typical form of oral administration for rodents but can be
stressful (Balcombe et al., 2004). Gestational stress can result in later alterations in offspring behavior (reviewed in Buynts and Mostofsky, 2009; Maccari and Morley-Fletcher, 2007; Weinstock, 2001); thus, control or evaluation of this potential confound would be beneficial.

The suggested guidances above were incorporated into the current study design in which pregnant Sprague-Dawley rats were housed in controlled environments, which contributed minimal levels of exogenous estrogen exposure. Dams were treated orally via gavage with vehicle, BPA (2.5 or 25.0 μg/kg/day), or EE2 (5.0 or 10.0 μg/kg/day). An additional control group was included (termed here, naive control) in which the animals were not gavaged to control for potential stress effects. Although lactational transfer of BPA has been demonstrated in Sprague-Dawley rats, the amount delivered to the offspring was reported as 300-fold lower than that administered to the dams (Doerge et al., 2010b). Thus, in order to more accurately control dose and better mimic bottle-fed infants who, relative to breast-fed infants, are estimated to have increased BPA intake (Lakind and Naiman, 2008; von Goetz et al., 2010), the offspring were directly orally treated beginning on postnatal day (PND) 1 until weaning at PND 21. Most lacking in the BPA literature are reports of potential BPA-induced alterations of early behaviors (but see Ema et al., 2001; Palanza et al., 2002; Stump et al., 2010 for lack of BPA effects on the righting reflex and cliff aversion). Here, we describe the effects of developmental BPA or EE2 treatment on dam and offspring body weight, dam food/water intake, offspring anogenital distance, offspring age at developmental landmark occurrence (i.e., ear and eye opening), offspring whole and regional brain weights and hormonal levels at weaning, and performance of two preweaning behaviors of offspring (righting reflex and slant board behavior). Those preweaning behaviors were previously shown to be sensitive to developmental EE2 treatment (Arabo et al., 2005). Internal measures of BPA are not described here; however, several studies have measured serum BPA levels after oral administration to adult and neonatal rats (Doerge et al., 2010a; Tominaga et al., 2006; Xiao et al., 2006).

**MATERIALS AND METHODS**

**Animals.** The National Center for Toxicological Research (NCTR) Breeding Colony supplied 364 female and 180 male Sprague-Dawley rats (derived from Charles River Crl: COBS CD (SD) BR Rat, Outbred) at weaning (PND 21). Upon reaching breeding age (see below), these rats became the dams and sires (i.e., F0 generation) of subsequent litters. Upon arrival to the vivarium at PND 21, each rat was tail tattooed for identification, weighed, and group-housed (three same-sex/cage). Ad lib food (see below for diet information) and water were provided at all times. Housing rooms were maintained on a 12/12-h light/dark cycle (6 a.m.–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 environments.** Upon arrival at PND 21, rats were housed in polysulfone cages with polysulfone microfilter tops, both of which were purchased new at the beginning of the study (Ancare, Bellmore, NY). Polysulfone caging was chosen as no detectable in vivo or in vitro estrogenic effects from BPA extracted from new polysulfone cages have been observed (Howdeshell et al., 2003). Analysis by high-performance liquid chromatography (HPLC) in the Division of Biochemical Toxicology/NCTR of new polysulfone cages filled with ammoniated water (pH 8.5, to duplicate urine) for 64 h indicated only a trace of BPA at a 1 ppb limit of quantitation (Siitonen, unpublished data). Further analysis by liquid chromatography tandem mass spectrometry indicated that new polysulfone cages leached 0.088 ppb BPA and, after three or nine washes, the leachate contained less than 0.030 ppb (Siitonen, unpublished data). Glass water bottles with food-grade silicone stoppers (Fisher Scientific, St Louis, MO) and metal sipper tubes were used to further reduce potential environmental estrogen exposure. Background BPA in the woodchip bedding material was determined in two different samples by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) utilizing d6-BPA and ranged 0.29–1.75 ppb (Siitonen, unpublished data). Metal mop buckets were used in the housing rooms, and all chow was stored in metal containers. This environment was maintained for the F0 generation beginning at PND 21 and throughout the remainder of the study.

**Diet.** Beginning upon arrival from the NCTR Breeding Colony at PND 21 and throughout the study, all animals were maintained on a low-phytoestrogen chow (TestDiet 5K06 [irradiated pellets]; Veriﬁed Casein Diet 10 IE; TestDiet, Richmond, IN). LC/MS analysis by the Division of Biochemical Toxicology indicated low levels of daidzein (< 0.34 ppm) and genistein (< 0.58 ppm) in three separate diet samples (Siitonen, unpublished data). Further, five different lots of diet were analyzed for BPA levels by LC-ESI-MS utilizing d6-BPA. These diets averaged 2.34 ± 0.43 (mean ± SE) ppb BPA (Siitonen, unpublished data).

**Breeding.** There were nine breeding rounds, each one consisting of a 24-h pairing of a male and female, with a 2-week interval between each round. Male and female breeders were those that were obtained at PND 21, housed in polysulfone cages with glass water bottles, and fed low-phytoestrogen chow as described above. Male/female pairings were random subject to the constraint that no grandparent could be shared within a pair (i.e., no pairings were done between siblings, first, or half cousins). Beginning on PND 87 or 90, each female was placed into a wire bottom cage with a PND 90 male that had been placed into the cage 72 h earlier for habituation. The pair was housed together for 24 h, after which the female and the cage bottom were visually examined for a sperm plug. If a sperm plug was detected, the female was removed, weighed, and housed individually, and this day was termed as gestational day (GD) 0. If no sperm plug was detected, the female was returned to her home cage and used in the next breeding round (2 weeks later). If no sperm plug was detected after two breeding rounds, the female was euthanized. Males were used for a maximum of three breeding rounds after which they were euthanized. Because all breeding occurred prior to any treatment, breeding success or failure was unrelated to treatment.

**Treatment assignment.** Females for which a sperm plug was detected were randomly assigned to treatment within their body weight stratum. The early strata were quartiles of all plug-positive dams. However, it became evident early in the study that probability of littering given sperm plug detection was related to body weight. Therefore, later strata were determined from the body weight quartiles of littering dams from the early breeding rounds. The six treatment groups were forced to be approximately balanced within each body weight stratum as the breeding rounds occurred.

**Drugs.** Bisphenol A (2,2’-Bis(4-hydroxyphenyl)propane, Product #B0494; TCI America) and EE2 (17β ethinyl estradiol, Product #E4876; Sigma, St Louis, MO) were each dissolved in a 0.3% (by weight) aqueous solution of carboxymethylcellulose sodium salt (CMC, high viscosity) (Product #C5013; Sigma). The purity of TCI America Lot #111909 BPA was determined to be > 99% by HPLC-PDA and proton NMR (Siitonen, unpublished data). Because of the lipophilic nature of BPA, oils (e.g., corn oil, tocopherol-stripped corn oil, arachis oil) are often used as vehicle. However, suggestions that such oils could possess
estrogenic activity (see Ashby and Lefèvre, 1997; Ryan, 2005) and their nutritive potential have discouraged their use (Ashby and Lefèvre, 1997). Thus, an aqueous vehicle was chosen for use here, which also offered the advantage of using automated dosing pumps (see below). Random BPA and EE2 gavage solutions were analyzed via HPLC-PDA and results ranged 105 ± 5% and 99 ± 8% (mean ± SD), respectively, of the intended targeted concentrations (Siitonen, unpublished data).

**BPA and EE2 treatment.** BPA dose selection was based on the estimated daily intake for adults and infants (see Introduction) as well as a “worst-case estimate” of intake of 11.0 µg BPA/kg/day for infants up to 6 months of age (Scientific Panel on Food Additives F, Processing Aids and Materials in Contact with Food, 2006). Here, 25.0 µg BPA/kg/day was selected as the higher dose and 2.5 µg BPA/kg/day as a lower dose.

Beginning on the morning of GD 6, dams were gavaged with 5.0 ml 0.3% CMC/kg/day (vehicle control group), 2.5 µg BPA/kg/day (low-BPA group), 25.0 µg BPA/kg/day (high-BPA group), 5.0 µg EE2/kg/day (low-EE2 group), or 10.0 µg EE2/kg/day (high-EE2 group) using a Hamilton Microlab 500 system (Hamilton Company, Reno, NV) interfaced with an animal weight scale and animal data collection software developed at the NCTR. An automated algorithm calculated the necessary volume (5 ml/kg) based on the daily body weight of each rat. Daily oral gavage of the dams continued through GD 21 (the day prior to parturition). Dams in the naive control group were removed from their home cage and restrained in the gavage position for the same duration as would be used for a gavage but were not gavaged. Thus, there were six treatment groups: (1) naive control, (2) vehicle control, (3) low-dose BPA, (4) high-dose BPA, (5) low-dose EE2, and (6) high-dose EE2.

Dams were not treated on the day of parturition (typically, GD 22) and were left undisturbed on that day. On the day after parturition (PND 1), the eight offspring in each litter (after culling, tattooing, and anogenital measures, see below) were orally treated with the same dose as their dam had received. Gavage volume remained the same (5 ml/kg). Daily oral treatment of offspring continued through PND 21. For offspring in the naive control group, each was removed from their home cage and restrained in the gavage position for the same duration as would be used for a gavage but were not gavaged. Offspring were directly treated, rather than continuing treatment of the dams to allow potential lactational transfer to the pups, because only small amounts of BPA are present in milk relative to concentrations in the serum of the dam (Doerge et al., 2010b). Furthermore, direct treatment of the offspring more closely mimics human BPA exposure through infant care bottles and infant formula cans as well as allowing increased dose accuracy.

**PND 1 litter treatment and measures.** Many of the assessments reported here require measurement over several consecutive days. For testing efficiency, any dam that littered early (before GD 22) or late (after GD 22) was not maintained beyond lactational day (LD) 1; however, litter data (e.g., total number of pups, sex ratio) were included for those subjects. Additionally, because treatment could affect gestation duration, those dams are included for that endpoint as well. On the day after parturition, all pups/litter were weighed, sexed, and each litter was randomly culled to eight pups (four-sex/litter where possible). Fostering was occasionally necessary and always done within treatment groups. Retained pups, including fostered pups, were paw tattooed on PND 1 for identification. Litter endpoints included gestation duration, total number of pups/litter, number of live pups/litter, live sex ratio (males/total live pups), and total sex ratio (including dead pups) (males/total pups). Birth weight (PND 1) analysis included all pups (pre-culling) for those litters on which parturition occurred on GD 22. Birth weights were first averaged for males/litter and females/litter prior to analysis.

Uteri of dams that did not litter by estimated GD 26 were examined at necropsy and numbers of grossly visible resorption sites (uterine scars) were counted. Subsequently, uteri were placed in 10% ammonium sulfide for at least 15 min, removed, rinsed, and placed on a light table for transillumination to determine any embryo implants that died before resorption sites were formed.

**Body weight and food and water intake.** Dams were weighed on GD 0 and daily beginning on GD 6 (before treatment) until pup weaning on LD 21. Food and water intake were measured daily beginning on GD 6 until LD 21. Body weight and food and water intake for those dams that did not litter or delivered early or late were deleted prior to statistical analyses. Offspring were weighed daily through PND 21.

**Anogenital distance and anogenital distance index.** Anogenital distance (AGD) (millimeters) of all retained offspring/litter was measured on PND 1 using a dissecting microscope by a trained technician blind to treatment group. Each subject was measured in triplicate, but the three readings were not done consecutively. Offspring in a litter were randomly selected for a first measurement until the litter was completed and then all offspring were remeasured again for the second reading and so on. Of 734 offspring measurements (or 2202 readings), 30% produced the same measure over the three readings. The maximum difference in any of the three individual subject readings was 0.8 mm, and this occurred in only two subjects, indicating very little variability in the three readings for each subject. The average of the three measures for each offspring was first calculated and then the average AGD for males/litter and females/litter was calculated. To normalize for potential differences in body weight, the AGD index (AGD distance in mm/cube root of body weight) (Christiansen et al., 2009) was calculated for each individual offspring using the average of the three AGD readings, followed by calculation of the average AGD index for males/litter and females/litter. Dividing by the cube root of body weight is a more appropriate normalization as it does not overcompensate for differences in body weight (Gallavan et al., 1999). Thus, each litter contributed two data points for absolute AGD (male and female) and two data points for AGD index (male and female).

**Developmental landmarks.** Beginning on PND 1, all offspring/litter were examined daily for bilateral ear canal opening, bilateral eye opening, and fur development. Age (PND) at reaching each of these milestones was recorded for each subject and then averaged for males/litter and females/litter such that each litter contributed two data points for each landmark. On PND 13, male pups were examined for nipple retention and recorded as either no retention or number of nipples retained.

**Righting reflex.** On PNDs 3–6, all offspring/litter were assessed for the righting reflex as previously described (Ferguson et al., 2010). Briefly, each pup was placed dorsal side down on a flat surface and the latency to right onto all four paws was recorded using a hand-held stopwatch (maximum latency 60 s). One trial/day was conducted by trained testers blind to treatment. Latency to right was averaged for males/litter and females/litter for each PND prior to analysis.

**Slant board behavior.** On PNDs 8–11, all offspring/litter were assessed for slant board behavior as previously described (Ferguson et al., 2010). Briefly, each pup was placed ventral side down on a sandpaper-covered board angled at 45° with the head pointed downward. Latency to turn 180° was measured using a hand-held stopwatch (maximum latency 60 s). Time of fall was recorded if the pup fell from the apparatus. One trial/day was conducted by trained testers blind to treatment.

**PND 21 serum hormone measures.** Beginning at 8:30 A.M. on PND 21 after the last oral treatment, one offspring/sex/litter was lightly anesthetized with CO2 after which blood was collected via cardiac puncture. The maximum duration between the first and last blood sample was 135 min (i.e., blood collection typically began at 8:30 A.M. and at its latest, ended at 10:45 A.M.). Euthanasia occurred with additional CO2. Samples were allowed to clot and then centrifuged. Serum was removed and stored at −80° C until analysis.

Many of the hormones measured here exhibit significant circadian rhythms (e.g., Kalra and Kalra, 1977; Mock et al., 1978) and some are quite low in young animals. For example, serum levels of luteinizing hormone (LH) are low in prepubertal male rats (Dohler and Wuttke, 1976). Still, given the previously described effects of developmental BPA on rodent serum LH levels (Akingbemi et al., 2004; Cardoso et al., 2010; Nakamura et al., 2010), it was important to obtain this measure at this age. Serum samples were obtained during the early portion of the light period during as short a duration as possible. Samples could not be obtained earlier on that day (i.e., PND 21) as the offspring must be orally gavaged prior to blood collection. The selection of a morning sample was done.
ANOVA) was conducted. Statistical significance was defined as distributed, a log transform or an ANOVA on ranks (i.e., Kruskal-Wallis weights adjusted for body weight (whole brain weight/body weight, frontal cortex brain, frontal cortex, and hippocampal weights were subjected to analysis as were correlation structure (Littell, 1996)). Appropriate comparisons to compare each treatment group with the vehicle control group for each day, and overall, were performed using Dunnett’s test. Developmental landmark data were analyzed for each sex separately using one-way ANOVAs with the factor of treatment. Pairwise comparisons to the vehicle control group were performed using Dunnett’s test. Nipple retention was not noted in any male offspring; thus, this endpoint was not analyzed.

Righting reflex data (i.e., latency to right) were not normally distributed; thus, a natural log transformation was applied to reduce skewness. A repeated measures ANOVA with factors of treatment, sex, PND, and all interactions was used to analyze the log latency time. Pairwise comparisons to the vehicle control group for each day, and overall, were performed using Dunnett’s test.

To analyze the latency to turn time on the slant board behavior test, a Cox proportional hazards model was performed with treatment, sex, PND, and all interactions as factors. This type of analysis adjusts for failure, here, defined as failure to turn or falling from the apparatus. Use of a Cox proportional hazards model is recommended for latency outcomes in behavioral endpoints as it appropriately handles failure and the underlying data distribution is not relevant (Jahn-Eimermacher et al., 2011). Pairwise comparisons to the vehicle control group for each day, and overall, were performed using Holm’s adjusted contrasts.

PND 21 serum hormone concentrations and whole and regional brain weights were analyzed via separate two-way ANOVAs with treatment and sex as factors. Some serum hormone concentrations were below the limit of detection. For analysis, these data were set to the detection limit.

RESULTS

Differences between the Vehicle Control and Naive Control Groups

Regardless of the overall significance of a main effect or interaction in the analysis, inspection of all pairwise comparisons indicated that there were only two statistically significant differences between the naive control and vehicle control groups. Preweaning body weight (i.e., PND 1–22) of offspring indicated a significant difference between the naive control and vehicle control groups (described below), and on GD 20, dams of the naive control group had a significantly higher water intake than the vehicle control group (described below). No other pairwise comparison yielded a significant difference between these two groups.

Pregnancy rates and early deaths. With the exception of one low-dose BPA-treated dam with three implantation sites, no sperm plug-positive female that did not litter exhibited any implantation or resorption sites. Thus, BPA and EE2 treatment, which began on GD 6, did not appear to affect pregnancy rates.

One high-dose BPA dam became moribund on GD 17. At necropsy, a pharynx perforation was present, likely due to a gavage accident. The uterus of this dam contained 13 fetuses and 2 implantation sites. Body weight and food and water
intake data from this dam were included in analyses through GD 17. One low-dose EE2 dam died during parturition. At necropsy, five full-term fetuses were present in the uterus, and the lumen of the stomach was dilated and contained gas and ingested placental fragments. The oral cavity was filled with a large placental fragment, strongly suggesting that the cause of death was choking. Data from this dam were included in gestational analyses only.

The litter of one low-dose EE2 dam contained only two pups (one per sex). The male pup was fostered after which the dam and female pup were euthanized. Two dams (one each from the naive control and high-dose BPA groups) killed all pups in their respective litters on PND 1 or 2 after culling and tattooing; these two dams were euthanized. Gestational and litter results data were retained in analyses from these three dams.

One dam in the low-dose BPA group with nine live offspring was euthanized, but gestational and litter results data were retained in analyses from these three dams.

Thirty-five offspring died or were missing (and presumed cannibalized) prior to PND 21: none in the naive control group, three males and five females in the vehicle control group, five males and eight females in the low-dose BPA group, four males and one female in the high-dose BPA group, one male in the low-dose EE2 group, and three males and five females in the high-dose EE2 group. Pairwise comparisons indicated that relative to the number of vehicle control female deaths, fewer naive control females died ($p < 0.001$) and fewer low EE2 females died ($p < 0.001$). Relative to the number of vehicle control male deaths, fewer naive control males died ($p < 0.001$). There were no other significant pairwise comparisons to the vehicle control group.

**Litter results.** Litter results are shown by treatment group in Table 1. Twenty-nine pups were fostered within treatment to maintain a 4/sex/litter composition. Litters that received fosters were three naive control, four vehicle control, four low-dose BPA, one high-dose BPA, four low-dose EE2, and two high-dose EE2. Duration of gestation was not normally distributed and an ANOVA performed on ranks indicated a significant effect of treatment ($H = 15.49, 5$ d.f., $p = 0.008$). However, Dunn’s method of multiple comparisons indicated that no treatment group differed significantly from the vehicle control group. Total pups/litter (alive and dead) and total live pups/litter were not normally distributed, and ANOVAs performed on ranks did not indicate a significant effect of treatment for either endpoint. Analysis of total sex ratio (including dead pups) indicated a significant treatment effect ($F(5, 117) = 2.43, p < 0.039$); however, no treated group was significantly different from the vehicle control group. Analysis of live sex ratio did not indicate a significant treatment effect.

Analysis of PND 1 offspring body weights (before culling) indicated significant effects of treatment ($F(5, 180) = 3.74, p < 0.003$) and sex ($F(1, 180) = 21.64, p < 0.001$). Pairwise comparisons indicated that pups of the high-dose EE2 group (10.0 μg/kg/day) weighed less than those of the vehicle control group ($p < 0.01$) and males weighed more than females (see Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Number of plug-positive dams</th>
<th>Number of pregnancies (including early and late litters)$^a$</th>
<th>Gestation duration (days)$^b$</th>
<th>Number of litters maintained until weaning</th>
<th>Total pups/litter</th>
<th>Live pups/litter</th>
<th>Total sex ratio (M/total live pups)$^c$</th>
<th>Live sex ratio (M/total pups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive control</td>
<td>24</td>
<td>22</td>
<td>21.7 ± 0.1</td>
<td>15$^d$</td>
<td>12.6 ± 0.5</td>
<td>12.0 ± 0.5</td>
<td>1.16 ± 0.14</td>
<td>0.50 ± 0.03</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>23</td>
<td>22</td>
<td>21.9 ± 0.1</td>
<td>19$^e$</td>
<td>12.5 ± 0.6</td>
<td>12.0 ± 0.5</td>
<td>1.10 ± 0.12</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>2.5 μg/kg BPA</td>
<td>25</td>
<td>19</td>
<td>22.0 ± 0.0</td>
<td>17$^f$</td>
<td>11.7 ± 0.9</td>
<td>11.1 ± 0.9</td>
<td>1.46 ± 0.22</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>25.0 μg/kg BPA</td>
<td>25</td>
<td>23</td>
<td>22.1 ± 0.1</td>
<td>14$^g$</td>
<td>12.8 ± 1.0</td>
<td>11.7 ± 0.9</td>
<td>0.96 ± 0.13</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>5.0 μg/kg EE2</td>
<td>25</td>
<td>21</td>
<td>21.9 ± 0.1</td>
<td>13$^h$</td>
<td>11.2 ± 1.0</td>
<td>10.7 ± 1.1</td>
<td>0.86 ± 0.10</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>10.0 μg/kg EE2</td>
<td>20</td>
<td>18</td>
<td>22.1 ± 0.1</td>
<td>15$^i$</td>
<td>13.1 ± 0.8</td>
<td>12.7 ± 0.8</td>
<td>1.33 ± 0.16</td>
<td>0.54 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$One 2.5 μg/kg BPA female that did not litter was found to have three implantation sites. All other females that did not litter showed no evidence of resorptions or implantations.

$^b$Analysis of total sex ratio indicated a significant treatment effect ($p < 0.039$); however, no group was significantly different from the vehicle control group.

$^c$Analysis of total sex ratio indicated a significant treatment effect ($p < 0.039$); however, no group was significantly different from the vehicle control group.

$^d$Six females littered 1 day early and were not maintained further; one female became moribund on LD 2.

$^e$Four females littered 1 day early and three females littered 1 day late and were not maintained further; one female became moribund on GD 12 (see text for details); one female cannibalized all pups on PND 1.

$^f$One 2.5 μg/kg BPA female that did not litter was found to have three implantation sites. All other females that did not litter showed no evidence of resorptions or implantations.

$^g$Analysis of total sex ratio indicated a significant treatment effect ($p < 0.039$); however, no group was significantly different from the vehicle control group.

$^h$Six females littered 1 day early and were not maintained further; one female killed all pups on PND 2.

$^i$Three females littered 1 day early and were not maintained further.

$^j$One female littered 1 day early and was not maintained further; one female delivered nine pups and became moribund on LD 1 (see text for details).

$^k$Five females littered 1 day early and one female littered 1 day late and were not maintained further; one female died during parturition (see text for details); one female delivered only two pups and was not maintained further.

$^l$Two females littered 1 day early and one female littered 1 day late and were not maintained further.
Dam body weight and food and water intake. The ANCOVA for gestational body weight indicated significant main effects of treatment ($F(5, 92) = 8.69, p < 0.001$) and GD ($F(14, 1297) = 206.64, p < 0.001$). Pairwise comparisons to the vehicle control group indicated that gestational body weights of the two EE$_2$ groups were significantly less than that of the vehicle control group ($p < 0.002$ for the 5.0 µg EE$_2$/kg/day and $p < 0.008$ for the 10.0 µg EE$_2$/kg/day) (see Fig. 1, top). The significant effect of GD indicated that all subjects gained weight with increasing gestation. Analysis of dam lactational body weight indicated significant main effects of treatment ($F(5, 86) = 5.39, p < 0.0003$) and day ($F(20, 1733) = 18.51, p < 0.0001$) (see Fig. 1, bottom). Pairwise comparisons to the vehicle control group indicated that the high-dose EE$_2$ group weighed less than the vehicle control group ($p < 0.002$). The significant effect of day indicated that all subjects gained weight during the first two lactational weeks.

Analysis of gestational food intake indicated a significant interaction of treatment × day ($F(70, 1291) = 1.33, p < 0.039$) (see Supplementary figure 1). Pairwise comparisons indicated that the two EE$_2$ groups consumed less food than the vehicle control group on GDs 8 and 10–13 ($p < 0.031$ for all comparisons). Similarly, analysis of lactational food intake indicated a significant interaction of treatment × day ($F(100, 1732) = 1.49, p < 0.002$) (see Supplementary figure 2). Pairwise comparisons indicated that the low-dose BPA group consumed less food than the vehicle control group on LDs 10, 15, 16, and 18 ($p < 0.027$ for all comparisons). On LDs 13 and 21, the high-dose EE$_2$ group consumed less food than the vehicle control group ($p < 0.046$ for both days).

Analysis of gestational water intake indicated a significant treatment × day interaction ($F(70, 1298) = 1.36, p < 0.029$) (data not shown). Pairwise comparisons indicated that the high-dose EE$_2$ group drank less water than the vehicle control group on GD 18 ($p < 0.025$). On GD 20, the naive control group drank less water than the vehicle control group ($p < 0.012$). Analysis of lactational water intake indicated a main effect of day ($F(20, 1738) = 113.42, p < 0.001$), indicating that water intake increased as the lactation period progressed.

PND 1–21 offspring body weight. Analysis of male offspring body weight indicated significant main effects of treatment ($F(5, 85) = 34.27, p < 0.001$) and PND ($F(20, 1700) = 2896.78, p < 0.001$) (Fig. 2, top). Similarly, analysis of female offspring body weight indicated significant main effects of treatment ($F(5, 84) = 10.90, p < 0.001$) and PND ($F(20, 1680) = 2286.41, p < 0.001$) (Fig. 2, bottom). Pairwise comparisons to the vehicle control male offspring group indicated that all other same-sex groups (naive control, low- and high-dose BPA, low- and high-dose EE$_2$) weighed significantly less ($p < 0.001$ for all). Pairwise comparisons to the vehicle control female offspring group indicated that the naive control, low- and high-dose BPA, and high EE$_2$ dose groups weighed significantly less ($p < 0.003$ for all) (see also Supplementary figure 3 for individual litter averages).

AGD and AGD index. There were no significant effects of treatment on absolute AGD of males or females. Analysis of AGD index (AGD/cube root of body weight) of males indicated a significant effect of treatment ($F(5, 89) = 2.43, p < 0.041$). However, pairwise comparisons indicated that no treatment group was significantly different from the vehicle control group. Analysis of AGD index in females did not indicate a significant treatment effect. Table 2 shows absolute AGD and AGD index by sex and treatment group.

**Developmental landmarks.** Age at bilateral eye opening, ear opening, and fur development were not significantly affected by treatment (see Supplementary table 1). No male offspring in any treatment group exhibited any retained nipples at PND 13.

**Righting reflex.** Analysis of latency to right indicated significant main effects of treatment ($F(5, 87) = 2.60, p < 0.031$), sex ($F(1, 87) = 7.93, p < 0.006$), and PND ($F(3, 261) = 109.57, p < 0.001$) (see Supplementary figure 4). Pairwise comparisons did not indicate that any treatment group was significantly different from the vehicle control group. Overall, however, males exhibited a significantly faster latency to right than females and latencies to right significantly decreased with age (i.e., increasing PND).

**Slant board behavior.** Analysis of latency to turn on the slant board indicated significant main effects of sex ($\chi^2(1) = 25.50, p < 0.001$) and PND ($\chi^2(3) = 254.05, p < 0.001$)

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Birth weight (g)$^b$</th>
<th>Absolute AGD (mm)$^c$</th>
<th>AGD index$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
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</tr>
<tr>
<td>Naive control</td>
<td>6.65 ± 0.13</td>
<td>4.29 ± 0.04</td>
<td>2.28 ± 0.02</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>7.00 ± 0.14</td>
<td>4.43 ± 0.07</td>
<td>2.32 ± 0.03</td>
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<tr>
<td>2.5 µg/kg BPA</td>
<td>6.67 ± 0.16</td>
<td>4.46 ± 0.06</td>
<td>2.37 ± 0.03</td>
</tr>
<tr>
<td>25.0 µg/kg BPA</td>
<td>6.97 ± 0.21</td>
<td>4.34 ± 0.07</td>
<td>2.28 ± 0.04</td>
</tr>
<tr>
<td>5.0 µg/kg EE$_2$</td>
<td>6.70 ± 0.20</td>
<td>4.36 ± 0.03</td>
<td>2.32 ± 0.03</td>
</tr>
<tr>
<td>10.0 µg/kg EE$_2$</td>
<td>6.28 ± 0.21$^d$</td>
<td>4.41 ± 0.05</td>
<td>2.39 ± 0.03</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Naive control</td>
<td>6.16 ± 0.10</td>
<td>2.69 ± 0.03</td>
<td>1.47 ± 0.01</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>6.49 ± 0.16</td>
<td>2.72 ± 0.03</td>
<td>1.46 ± 0.02</td>
</tr>
<tr>
<td>2.5 µg/kg BPA</td>
<td>6.22 ± 0.17</td>
<td>2.70 ± 0.03</td>
<td>1.47 ± 0.02</td>
</tr>
<tr>
<td>25.0 µg/kg BPA</td>
<td>6.35 ± 0.16</td>
<td>2.67 ± 0.04</td>
<td>1.44 ± 0.03</td>
</tr>
<tr>
<td>5.0 µg/kg EE$_2$</td>
<td>6.36 ± 0.18</td>
<td>2.62 ± 0.03</td>
<td>1.42 ± 0.02</td>
</tr>
<tr>
<td>10.0 µg/kg EE$_2$</td>
<td>5.92 ± 0.23$^d$</td>
<td>2.71 ± 0.02</td>
<td>1.50 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$Birth weights were determined pre-culling (all live pups/litter).

$^b$AGD and AGD index were measured on PND 1 after culling (only those pups maintained in each litter) ($n = 13–19$ litters/treatment group).

$^c$Analysis of AGD index of males indicated a significant effect of treatment ($p < 0.041$); however, no group was significantly different from the vehicle control group.

$^d$Pairwise comparisons of the main effect of treatment on birth weight indicated that offspring of the 10.0 µg/kg EE$_2$ group weighed significantly less than the vehicle group ($p < 0.01$).
Males turned significantly faster than females and latency to turn decreased with age (i.e., increasing PND). There were neither any effects of treatment nor any significant interactions with treatment.

PND 21 serum hormone measures. Mean ± SE of these data by treatment and sex are shown in Table 3. Analyses of serum concentrations of T4, E2, LH, ghrelin, and leptin did not indicate significant effects of treatment, sex, or a treatment × sex interaction. Analysis of T3 concentrations indicated a main effect of treatment \((F(5, 149) = 2.46, p < 0.035)\) but no significant effect of sex or a treatment × sex interaction; pairwise comparisons, however, indicated that no-treatment group was significantly different from the vehicle control group. Analysis of corticosterone levels indicated a marginal effect of treatment \((F(5, 149) = 2.25, p < 0.052)\) with somewhat higher levels in the high-dose EE2 group. A marginal effect of sex \((F(1, 149) = 3.74, p < 0.055)\) on testosterone levels indicated somewhat higher levels in males.

PND 21 whole and regional brain weights. Mean ± SE of these data by treatment and sex are shown in Supplementary table 2. Analysis of whole brain weight indicated a significant effect of sex \((F(1, 149) = 13.71, p < 0.001)\), indicating that brain weights of males were heavier than those of females. There was neither
a significant effect of treatment nor a significant treatment × sex interaction. Analysis of frontal cortex and hippocampal weights did not indicate significant effects of treatment, sex, or significant interactions of treatment × sex.

Analyzes of whole brain, frontal cortex, and hippocampal weights as ratios to body weight did not indicate any significant effects of treatment or sex or any significant interactions of treatment × sex.

**DISCUSSION**

Oral BPA treatment of pregnant Sprague-Dawley rats followed by direct oral treatment of their offspring had no effects on gestational or lactational body weight and only sporadic effects on food intake. Offspring AGD, AGD index, developmental landmarks, measures of serum hormones, and whole/regional brain weights were unaffected by BPA treatment. Two early behaviors (righting reflex and slant board behavior) were not altered by BPA treatment, although offspring preweaning body weight was significantly decreased by BPA treatment. In contrast, oral EE2 treatment significantly decreased gestational and lactational body weight, birth weights, and preweaning body weights. The general lack of BPA effects is consistent with results from previous studies of oral BPA treatment. However, several endpoints measured here have not been previously described. These results indicate that the preweaning measures assessed here appear resistant to interference by these oral BPA doses.

BPA treatment had no effect on gestational or lactational body weights. This finding is similar to those of others using oral BPA doses ranging 0.05–320,000 μg/kg/day to rats (Cagen *et al.*, 1999; Howdeshell *et al.*, 2008; Kim *et al.*, 2001; Kwon *et al.*, 2000; Somm *et al.*, 2009; Tyl *et al.*, 2002). Similarly, BPA treatment here had no effects on gestational food intake or gestational or lactational water intake. There were four LDs (10, 15–16, and 18) in which the low-BPA group consumed less food than the vehicle control group; however, this appeared to be neither a consistent effect nor dose related. This lack of BPA treatment effects on dam food and water intake is similar to those reports using BPA doses ranging 1.0–4022 μg/kg/day to rats (Cagen *et al.*, 1999; Kim *et al.*, 2001; Somm *et al.*, 2009).

Absolute AGD can be sensitive to prenatal treatment with endocrine disruptors (Ball *et al.*, 2010; Delclos *et al.*, 2009; Taxvig *et al.*, 2008); previous studies, however, have indicated no effects on absolute AGD of rats prenatally treated with 2–384,400 μg/kg/day BPA (Howdeshell *et al.*, 2008; Ryan *et al.*, 2010; Takagi *et al.*, 2004; Tinwell *et al.*, 2002). Furthermore, measures of AGD index, which normalizes AGD to weight, were not altered at birth by oral BPA treatment at doses ranging 0.2–200 μg/kg/day (Ema *et al.*, 2001). Thus, our finding that neither absolute AGD nor AGD index was sensitive to prenatal BPA treatment is confirmatory and not surprising.

Birth weights of offspring exposed in utero to BPA were not affected by treatment; however, preweaning body weights were decreased in both sexes relative to the vehicle control group (maximum 10% decrease in low-dose BPA PND 5 females). Because preweaning body weights of the ungavaged naive control group were decreased relative to vehicle controls, this

| TABLE 3 |
| PND 21 Serum Hormone Measures (mean ± SE) |

<table>
<thead>
<tr>
<th></th>
<th>T4, μg/dl</th>
<th>T3, ng/dl</th>
<th>Estradiol, pg/ml</th>
<th>Testosterone, ng/dl</th>
<th>Corticosterone, ng/ml</th>
<th>LH, mIU/ml</th>
<th>Leptin, ng/ml</th>
<th>Ghrelin, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
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<tr>
<td>Naive control</td>
<td>2.73 ± 0.26</td>
<td>87.447 ± 4.235</td>
<td>8.567 ± 0.379</td>
<td>4.513 ± 0.275</td>
<td>167.05 ± 33.21</td>
<td>0.1907 ± 0.0407</td>
<td>5.0 ± 1.0</td>
<td>1.913 ± 0.179</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>2.58 ± 0.19</td>
<td>96.394 ± 4.368</td>
<td>8.447 ± 0.323</td>
<td>4.894 ± 0.894</td>
<td>159.37 ± 27.85</td>
<td>0.1812 ± 0.0266</td>
<td>4.7 ± 0.6</td>
<td>1.688 ± 0.139</td>
</tr>
<tr>
<td>2.5 μg/kg BPA</td>
<td>2.59 ± 0.27</td>
<td>97.855 ± 5.814</td>
<td>8.227 ± 0.186</td>
<td>18.333 ± 11.488</td>
<td>162.44 ± 27.28</td>
<td>0.3520 ± 0.2020</td>
<td>4.2 ± 0.5</td>
<td>1.567 ± 0.227</td>
</tr>
<tr>
<td>25.0 μg/kg BPA</td>
<td>2.38 ± 0.31</td>
<td>79.630 ± 2.97</td>
<td>8.600 ± 0.600</td>
<td>5.040 ± 0.678</td>
<td>142.23 ± 20.62</td>
<td>0.2200 ± 0.0700</td>
<td>4.7 ± 1.7</td>
<td>1.760 ± 0.193</td>
</tr>
<tr>
<td>5.0 μg/kg EE2</td>
<td>2.90 ± 0.31</td>
<td>91.636 ± 5.065</td>
<td>8.191 ± 0.128</td>
<td>12.645 ± 4.037</td>
<td>168.05 ± 30.29</td>
<td>0.2191 ± 0.0643</td>
<td>3.8 ± 0.8</td>
<td>1.755 ± 0.210</td>
</tr>
<tr>
<td>10.0 μg/kg EE2</td>
<td>3.23 ± 0.53</td>
<td>98.658 ± 5.381</td>
<td>10.325 ± 1.937</td>
<td>5.433 ± 1.068</td>
<td>259.53 ± 39.11</td>
<td>0.2858 ± 0.0927</td>
<td>3.1 ± 0.4</td>
<td>1.667 ± 0.201</td>
</tr>
<tr>
<td><strong>Females</strong></td>
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<tr>
<td>Naive control</td>
<td>2.69 ± 0.19</td>
<td>91.400 ± 5.794</td>
<td>8.492 ± 0.476</td>
<td>4.046 ± 0.046</td>
<td>162.51 ± 34.53</td>
<td>0.1500 ± 0.0000</td>
<td>5.8 ± 1.1</td>
<td>2.085 ± 0.357</td>
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<tr>
<td>Vehicle control</td>
<td>2.69 ± 0.16</td>
<td>95.820 ± 3.933</td>
<td>8.000 ± 0.000</td>
<td>4.027 ± 0.027</td>
<td>151.85 ± 21.25</td>
<td>0.2313 ± 0.0581</td>
<td>5.5 ± 0.8</td>
<td>1.953 ± 0.250</td>
</tr>
<tr>
<td>2.5 μg/kg BPA</td>
<td>2.89 ± 0.36</td>
<td>97.179 ± 6.165</td>
<td>10.314 ± 1.441</td>
<td>4.000 ± 0.000</td>
<td>156.75 ± 26.10</td>
<td>0.1500 ± 0.0000</td>
<td>4.1 ± 0.7</td>
<td>1.693 ± 0.170</td>
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<tr>
<td>25.0 μg/kg BPA</td>
<td>2.64 ± 0.21</td>
<td>87.892 ± 4.171</td>
<td>8.346 ± 0.346</td>
<td>4.338 ± 0.266</td>
<td>182.56 ± 31.21</td>
<td>0.3715 ± 0.1500</td>
<td>3.3 ± 0.4</td>
<td>1.508 ± 0.140</td>
</tr>
<tr>
<td>5.0 μg/kg EE2</td>
<td>2.68 ± 0.20</td>
<td>99.908 ± 4.314</td>
<td>8.300 ± 0.300</td>
<td>4.269 ± 0.193</td>
<td>218.98 ± 33.52</td>
<td>0.1546 ± 0.0046</td>
<td>4.5 ± 1.2</td>
<td>1.823 ± 0.183</td>
</tr>
<tr>
<td>10.0 μg/kg EE2</td>
<td>2.58 ± 0.26</td>
<td>97.485 ± 4.286</td>
<td>0.400 ± 1.694</td>
<td>4.000 ± 0.000</td>
<td>223.46 ± 36.96</td>
<td>0.2946 ± 0.1285</td>
<td>3.2 ± 0.5</td>
<td>1.623 ± 0.184</td>
</tr>
</tbody>
</table>

*Note.* There was a significant effect of treatment on T3 concentrations (F(5, 149) = 2.46, p < 0.035); however, pairwise comparisons to the vehicle control group did not indicate that any treatment group was significantly different. A marginally significant treatment effect on corticosterone levels (F(5, 149) = 2.25, p < 0.052) indicated somewhat higher levels in the high-dose EE2 group. A marginal effect of sex (F(1, 149) = 3.74, p < 0.055) on testosterone levels indicated somewhat higher levels in males. Group sizes were 10–17 per group for males and 13–15 per group for females.

*Of the 15 low BPA male samples for testosterone, only 6 were above the detection limit (4.0 ng/dl) and of those 6, one sample was 176.1 and another was 37.8, thus resulting in the large SE. The sample that assayed at 37.8 had enough serum remaining to reassy several months later in duplicate and the levels were 37.8 and 36.6, indicating that the original high level was correct. The sample that originally assayed at 176.1 did not have enough remaining serum to reassay.*
requires a closer examination. Differences between offspring body weights of the naive control and vehicle control groups could be due to the carboxymethylcellulose vehicle. However, birth weights that included all pups/litter of the naive control and vehicle control groups were not significantly different. Still, the vehicle control group exhibited the highest birth weights of all groups, although these were only significantly different from the high-dose EE2 group. Thus, prenatal treatment with carboxymethylcellulose appeared to have no or only minor effects on birth weight. With direct oral treatment of the offspring on PNDs 1–21, the naive control group weighed significantly less than the vehicle control group.

An early study indicated that reproductive, developmental, and early behavioral outcomes (e.g., righting reflex) of rats were unaffected by oral treatment with a 2% aqueous CMC solution administered at 10 ml/kg/day (Fritz and Becker, 1981). In adult rats, dietary CMC resulted in few effects at doses of 1.36–1.57 g/kg/day (Bar et al., 1995). Here, the CMC dose was 0.3% CMC at 5 ml/kg/day or 0.015 g/kg/day. Thus, the unusual finding of higher preweaning body weights in the vehicle control group relative to the naive control group remains unexplained (maximum 8% decrease in PND 7 males). The most consistent effect associated with 1.36–1.57 g/kg/day of dietary CMC was increased water intake by adult males, but not females, throughout the 11-week exposure, likely related to the increased sodium of the CMC diets (Bar et al., 1995). Although it could be hypothesized that increased thirst in vehicle control offspring of the current study may have resulted in increased suckling and potentially increased body weight, four other treatment groups here also received the same daily dose of CMC (i.e., the BPA and EE2 groups). Similar to the naive control group, those groups also exhibited decreased preweaning body weights relative to vehicle controls. A preliminary examination of postweaning body weights of the subjects of this study indicates that there are few, if any, significant differences between the naive and vehicle control groups (unpublished data), which would indicate this effect is either restricted to the preweaning period or perhaps a spurious effect. A larger ongoing study using identical vehicle and naive control groups will determine the replicability of this effect (Delclos, in preparation).

Developmental landmarks (e.g., age at eye opening) were reported to be unaffected by oral BPA treatment at doses ranging 0.2–200 μg/kg/day to rats (Ema et al., 2001), and similar findings were noted here. Two additional landmarks measured here, age at bilateral ear canal opening and fur development for the Sprague-Dawley strain (e.g., Matta and Elberger, 2007; National Toxicology Program, 2010). Furthermore, no male offspring exhibited signs of nipple retention, replicating previous findings in rats using dietary BPA doses ranging 1–500,000 μg/kg/day (Tyl et al., 2002). Thus, the new findings here that BPA had no effects on ear canal opening or fur development provide additional support that oral BPA treatment does not affect these early landmarks.

Although performance of the righting reflex was previously investigated in BPA-treated rats (Ema et al., 2001; Stump et al., 2010), our laboratory uses a more detailed methodology. Specifically, rather than recording the behavior as a success or failure, the latency to complete the behavior over consecutive days was measured. This allows a more thorough detection of potential subtle alterations on specific days. Still BPA treatment had no significant effects on righting latencies. Thus, it would appear that this early behavior is resistant to oral BPA treatment in two rodent species at oral doses spanning 10–150,000 μg/kg/day (Ema et al., 2001; Stump et al., 2010). As expected and previously reported (Ferguson et al., 2003b, 2010), latencies here were shorter with increasing age and males were faster to right.

Similar to measurement of the righting reflex, latency to complete an 180° turn on the slant board over consecutive days was measured. However, BPA treatment did not alter the latency to turn, a finding similar to that of Ema et al. (2001) in which oral BPA doses of 0.2–200 μg/kg/day had no effect on age at achieving this behavior in rats. The finding here that oral BPA treatment did not alter slant board behavior is further strengthened by our previous reports indicating the sensitivity of this behavior to strain differences (Ferguson et al., 2003b), developmental synthetic glucocorticoid treatment (Ferguson et al., 2001), and developmental NMDA antagonist treatment (Boctor et al., 2008).

BPA treatment had no effects on hormonal measures or brain weights at weaning. Although measures of testosterone, estradiol, LH, T3, T4, and corticosterone are typical, levels of ghrelin and leptin were also measured here. Ghrelin and leptin are implicated in appetite and obesity (Dieguez et al., 2010; Hassouna et al., 2010; Myers et al., 2010; Suzuki et al., 2010), and BPA has been suggested to act as an obesogen (Grün, 2010; Newbold, 2010; Newbold et al., 2009). Furthermore, plasma leptin levels of mice were elevated during pregnancy after subcutaneous BPA treatment (100 μg/kg) (Alonso-Magdalena et al., 2010). Here, PND 21 serum levels of ghrelin and leptin in offspring were not altered by BPA treatment. Because hormone levels are age dependent, direct cross-study comparisons are difficult. However, similar to our findings, subcutaneous injections of 50 or 20,000 μg/kg/day BPA on PNDs 1–7 did not alter serum estradiol levels in female rats at weaning (Monje et al., 2007). In PND 30, male offspring of rats sc injected with 2 μg/kg/day BPA from GD 10 to LD 7, LH levels were higher and testosterone levels were lower (Bai et al., 2011). Similarly, PND 35 male offspring of rats that consumed approximately 2500 μg/kg/day BPA in drinking water throughout gestation and lactation had lower LH and testosterone levels (Cardoso et al., 2010). Here, there were no effects of BPA treatment on LH levels; however, differences in age and dose/route of BPA administration do not allow a straightforward comparison with the levels reported here.
EE₂ was selected as a reference estrogen for this study based on its previous use in studies of potential endocrine disrupters (Sahambi et al., 2010; Stoker et al., 2010). The majority of the statistically significant effects in this study were noted in one or both EE₂ groups, indicating sensitivity to treatment with this compound. Several reports, however, have criticized use of the Sprague-Dawley strain in studies of potential estrogenic substances (e.g., Richter et al., 2007), suggesting that this strain is less sensitive to estrogenic compounds. To the contrary, the current results imply this strain is quite sensitive to exogenously administered estrogens, and previous studies from the NCTR have indicated similar findings (Latendresse et al., 2009). Others have also discussed this issue and described the Sprague-Dawley strain as sensitive (Diel et al., 2004; Goodman et al., 2006).

The findings here for EE₂ were similar to those described by others using oral treatment. Specifically, oral doses of 1.5–50 μg/kg/day decreased gestational weight gain in rats (Howdeshell et al., 2008), and birth weight or PND 2 body weight of rats was decreased by oral doses of 10–50 μg/kg/day EE₂ (Ferguson et al., 2003a; Howdeshell et al., 2008; National Toxicology Program, 2010), although others have described a lack of treatment effects on female birth weights at doses of 0.05–15 μg/kg/day EE₂ (Ryan et al., 2010). Furthermore, oral treatment with 0.5–50 μg/kg/day EE₂ did not alter AGD in rats (Delclos et al., 2009; Howdeshell et al., 2008; Sawaki et al., 2003). Performance of the righting reflex or slant board turn behavior has not been described after developmental estrogen treatment; however, developmental treatment with the fungicide fenarimol that has estrogenic properties was described to alter the righting reflex in rats (de Castro et al., 2007), whereas exposure to different dietary levels of phytoestrogens had no effects on the same behavior in rats (Becker et al., 2005).

In summary, oral BPA treatment had no consistent or dose-related effects on various gestational or lactational measures. Furthermore, offspring physical and behavioral development seemed impervious to BPA-induced alterations, with perhaps the exception of preweaning body weight. Conversely, oral treatment with the reference estrogen EE₂ decreased gestational and lactational body weight and offspring birth weight but did not alter offspring preweaning behavior. These data provide additional evidence that relatively low oral doses of BPA are not associated with early alterations. A variety of behaviors were assessed in the offspring described here after weaning and through adulthood, including measures of spatial learning/ memory, play behavior, and activity. Those data will be reported separately. Such measures may prove to be more sensitive to BPA treatment.

SUPPLEMENTARY DATA

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

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FEW EFFECTS OF DEVELOPMENTAL BPA OR EE$_2$


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