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Research Article

Impact of Low-Dose Oral Exposure to Bisphenol A (BPA) on Juvenile and Adult Rat Exploratory and Anxiety Behavior: A CLARITY-BPA Consortium Study

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ABSTRACT

Bisphenol A (BPA) is a high volume production chemical and has been identified as an endocrine disruptor, prompting concern that developmental exposure could impact brain development and behavior. Rodent and human studies suggest that early life BPA exposure may result in an anxious, hyperactive phenotype but results are conflicting and data from studies using multiple doses below the no-observed-adverse-effect level are limited. To address this, the present studies were conducted as part of the CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) program. The impact of perinatal BPA exposure (2.5, 25, or 2500 μg/kg body weight (bw)/day) on behaviors related to anxiety and exploratory activity was assessed in juvenile (prepubertal) and adult NCTR Sprague-Dawley rats of both sexes. Ethinyl estradiol (0.5 μg/kg bw/day) was used as a reference estrogen. Exposure spanned gestation and lactation with dams gavaged from gestational day 6 until birth and then the offspring gavaged directly through weaning ($n = 12$/sex/group). Behavioral assessments included open field, elevated plus maze, and zero maze. Anticipated sex differences in behavior were statistically identified or suggested in most cases. No consistent effects of BPA were observed for any endpoint, in either sex, at either age compared to vehicle controls; however, significant differences between BPA-exposed and ethinyl estradiol-exposed groups were identified for some endpoints. Limitations of this study are discussed and include suboptimal statistical power and low concordance across behavioral tasks. These data do not indicate BPA-related effects on anxiety or exploratory activity in these developmentally exposed rats.

Key words: bisphenol A, CLARITY, behavior, anxiety, exploratory activity, endocrine disruption, EDC, sexually dimorphic, brain, BPA, plastic

Bisphenol A (BPA) is a high volume production industrial chemical now ubiquitous in the environment. It is the monomer for polycarbonate plastics and epoxy resins and incorporated in numerous products including food and beverage containers, food can linings, medical devices, and thermal paper. In developed countries, human exposure is nearly universal (Calafat *et al.*, 2008; Casas *et al.*, 2013) and occurs primarily via contaminated food and beverages (von Goetz *et al.*, 2010) but may also occur

from handling thermal receipts and occupational exposures (Ehrlich *et al.*, 2014; Li *et al.*, 2010). The EPA has set the reference dose for chronic oral BPA exposure at 50 μg/kg body weight (bw)/day (EPA, 1993). Average daily human BPA intake likely varies with lifestyle and age (exposure was reportedly as high as 1.6 μg/kg bw/day among infants fed with polycarbonate bottles containing BPA), but US intakes are estimated to be ~34 ng/kg bw/day (FAO/WHO, 2011; Lakind and Naiman, 2011). BPA has been extensively evaluated for potential adverse effects, but results have been mixed, and consensus on the potential health risks at human-relevant exposure levels has not been reached. Because BPA has been shown to interfere with endogenous hormone signaling and metabolism, the concern that exposure could impact the development of hormone-sensitive tissues, such as the brain, has garnered particular attention (Chapin *et al.*, 2008; FAO/WHO, 2011; NTP, 2008; Palanza *et al.*, 2008; Patisaul and Polston, 2008; Rosenfeld, 2012; vom Saal *et al.*, 2007; Wolstenholme *et al.*, 2011a). While numerous studies have evaluated the potential for BPA to impact the brain and behavior, data from studies specifically designed and conducted to be appropriate for human risk assessment are extremely limited. The present studies were conducted to fill that data gap.

Within the past decade, the National Toxicology Program (NTP), World Health Organization (WHO), and Food and Agricultural Organization (FAO) have expressed concern for effects on the brain and behavior (Beronius *et al.*, 2010; Chapin *et al.*, 2008; FAO/WHO, 2011; FDA, 2012; NTP, 2008). Notably, the WHO/FAO report highlighted concerns regarding the potential for BPA exposure to increase anxiety (FAO/WHO, 2011) and recommended further investigation. Comprehensive risk assessments and literature reviews have consistently identified limited replication across animal studies and insufficient epidemiological data as critical limitations (Beronius *et al.*, 2010; Rochester, 2013; Wolstenholme *et al.*, 2011a). Subsequent epidemiological and experimental animal studies have generated additional data suggesting a link between heightened anxiety and developmental BPA exposure (representative examples using rats include [Goncalves *et al.*, 2010; Jones *et al.*, 2010; Patisaul *et al.*, 2012]) but indicating anxiogenic and other behavioral effects vary across age and sex. For example, work by us and others in animal models has revealed that perinatal exposure to BPA alters sociosexual (Farabollini *et al.*, 2002; Wolstenholme *et al.*, 2013) and anxiety-related behaviors in rodents (including non-traditional animal models, such as the prairie vole) (Patisaul *et al.*, 2012; Sullivan *et al.*, 2014) and non-human primates (Nakagami *et al.*, 2009) in sex and age specific manners. Sex specific structural and molecular changes have been found within key brain regions critical for the etiology of these behaviors, including the hypothalamus (Adewale *et al.*, 2011; Patisaul *et al.*, 2006, 2007) and hippocampus (Leranth *et al.*, 2008a,b), further supporting the hypothesis that BPA can influence behavioral systems. Other studies, however, have not found evidence of anxiogenic effects (Viberg *et al.*, 2011; Wolstenholme *et al.*, 2011b). Differences in study design including dose, exposure window, species/strain, and diet likely contribute to literature inconsistencies. Altered exploratory activity has also emerged as a behavioral outcome of concern because epidemiological studies have associated BPA exposure with hyperactivity in children (Braun *et al.*, 2011b; Harley *et al.*, 2013; Perera *et al.*, 2012). Evidence for BPA-related hyperactivity in animal models is limited and mixed, but heightened activity is suggested in some publications (representative examples include [Anderson *et al.*, 2013; Kundakovic *et al.*, 2013]). This study investigated anxiety and activity-related behaviors in adolescent and adult rats of both sexes following developmental (gestation through lactation) exposure to low doses (2.5, 25, or 2500 μg/kg bw/day) of BPA.

The experiments herein were conducted as part of the CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) program (Birnbaum *et al.*, 2012; Schug *et al.*, 2013), a collaborative effort between academic and Federal Government scientists, coordinated by the NTP, the National Institute of Environmental Health Sciences (NIEHS), and the U.S. Food and Drug Administration (FDA) National Center for Toxicological Research (NCTR) to draw on the strengths of both guideline and academic studies to fill data gaps and incorporate research recommendations identified by the WHO and others (EFSA 2015; Beronius *et al.*, 2010; Chapin *et al.*, 2008; FAO/WHO, 2011; FDA, 2012; NTP, 2008). These recommendations include use of multiple, concordant assessments for anxiety and related behaviors in laboratory animals (the WHO report specifically suggested inclusion of the elevated plus and zero mazes [EPM and ZM]), testing and comparing effects in both sexes, testing at multiple ages, use of a low phytoestrogen diet, inclusion of a reference estrogen, and evaluation of multiple BPA doses, particularly doses at or below the no-observed-adverse-effect level (NOAEL) of 5 mg/kg bw/day. Thus, this program has the potential to identify novel endpoints and pathways sensitive to BPA exposure at doses above and below the NOAEL.

Through a similar collaborative effort, using a different set of animals, we previously showed that NCTR Sprague-Dawley (NCTR-SD) rats dosed with BPA, over the same developmental period as this study, exhibited altered estrogen receptor expression in hypothalamic structures fundamental for the manifestation of anxiety and related behaviors (Rebuli *et al.*, 2014). The brains for that study were collected from siblings of animals used in an NCTR 90-day subchronic study (a study which preceded the CLARITY-BPA studies), which assessed the effects of BPA on a wide range of non-neural endpoints including body and organ weights, puberty onset, histopathology, sperm parameters, gene expression, and internal dosimetry across life stages (Camacho *et al.*, 2015; Churchwell *et al.*, 2014; Delclos *et al.*, 2014). These results highlight the advantages of a multi-agency research approach and provide support for the hypothesis that developmental BPA exposure may result in anxiety and exploratory-related behavioral changes.

For the present studies, exposure began to the dam on gestational day 6 (GD 6) and, following parturition, direct dosing of pups proceeded through weaning (postnatal day 21 [PND21]) via orogastric gavage. Direct dosing of pups was employed to circumvent the possibility of poor lactational transfer of BPA (Doerge *et al.*, 2010). In addition to the vehicle control, exposure groups included 3 BPA doses (2.5, 25, 2,500 μg/kg bw/day) and ethinyl estradiol (EE) as a reference estrogen. Anxiety and activity levels were assessed in juveniles and adults using the EPM and the open field (OF). Adults were also tested using the ZM. Neural endpoints will ultimately be assessed and reported. Inclusion of a prepubertal cohort fills a critical gap in the existing literature regarding the impact of developmental BPA exposure because most published data were obtained from adult animals.

MATERIALS AND METHODS

This study is a component of the CLARITY-BPA program (Birnbaum *et al.*, 2012; Schug *et al.*, 2013). Comprehensive study design details are fully described in (Heindel *et al.*, 2015) so only applicable portions are summarized here. All procedures

involving animals were approved in advance by the NCTR Institutional Animal Care and Use Committee and conducted in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited facility.

Animal Husbandry

Throughout the study, animal rooms were maintained at 23 ± 3°C with a relative humidity of 50 ± 20% and a 12:12 h light/dark cycle. Food and water were available *ad libitum*. Dams and preweaned pups were housed with lights on at 6:00 AM. Animals were then moved at weaning to a different building with a shifted light cycle (off at 11:00, on at 23:00) to accommodate behavioral testing in the dark phase. The overall experimental design and timeline is depicted in Figure 1. Housing and diet were selected to minimize unintended exposure to BPA and other endocrine disruptors. The diet was soy- and alfalfa-free because these diets contain hormonally active compounds that may be a confounder and obfuscate behavioral sex differences and/or BPA-related effects (Patisaul *et al.*, 2005, 2012; Thigpen *et al.*, 2007, 2013) (5K96-verified casein diet 10 IF, round pellets, γ-irradiated [catalog no. 1810069], Test Diets, Purina Mills, Richmond, IN). Diet lots and other study materials were monitored for BPA by liquid chromatography/mass spectrometry as described previously (Delclos *et al.*, 2014; Rebuli *et al.*, 2014). As detailed in Heindel *et al.* (2015), no assayed lot of diet contained BPA above the protocol-specified limit of 5 ppb, no study materials, including cage leachates, drinking water, and bedding extracts, were found to have BPA detectable above the analytical method blank, and each lot of diet was further certified to contain less than 1 ppm genistein and daidzein, and less than 0.5 ppm zearalenone and coumestrol.

Dose Preparation and Administration

Five dose groups ($n = 12$ per sex per group) were generated for the studies reported herein vehicle, BPA 2.5, 25, and 2500 μg/kg bw/day and EE 0.5 μg/kg bw/day (note: the full CLARITY-BPA study has additional groups) (see Heindel *et al.*, 2015). Dosing began on GD 6 and terminated at weaning (PND 21).

Approximately 2 weeks prior to mating, female NCTR-SD breeders were randomized to exposure groups stratified by body weight to give approximately equivalent mean body weights in each group. No sibling or first cousin mating was permitted. Rats were mated in 5 loads (cohorts) spaced 4 weeks apart. Animals for this study came from loads 4 and 5 (Fig. 1). Mating was conducted as previously described (Delclos *et al.*, 2014), but solid-bottomed polysulfone caging with hardwood chip bedding was used in place of wire bottom cages. Daily gavage dosing for dams was done immediately after body weight collection (dose volume determined by that day's body weight) from GD 6 and continued until parturition began (neither dams nor pups were dosed on the day of birth [PND 0]). Direct gavage of the pups began on PND 1 after the litter was culled. For pups younger than PND 5, the gavage needle did not enter the esophagus. Pups were weighed and gavaged daily until PND 21 (weaning). This preweaning part of the study was good laboratory practices (GLP)-compliant.

BPA (CAS no. 80-05-7, TCI America, Portland, OR; catalog no. B0494, Lot no. 111909/AOHOK [air-milled], ≥99.9% purity) and EE (CAS no. 57-63-6, Sigma-Aldrich, St. Louis, MO; catalog no. E4876, Lot no. 071M1492V, ≥99.9% purity) were prepared in the vehicle, 0.3% aqueous carboxymethyl cellulose (Sigma-Aldrich, St. Louis, MO; catalog no. C5013, Lot no. 041M0105V) in water, and administered by gavage daily at a volume of 5 ml/kg bw using a modified Hamilton Microlab ML511C programmable 115 V pump (Hamilton Co., Reno, NV)(Lewis *et al.*, 2010).

Weaning and Transfer of Subjects

Offspring were weaned on PND 21, after their last daily gavage, and tattooed on the tail with a unique identification number. Animals used for this study were then transported to a different building for housing and behavioral testing (termed "behavioral building" in Fig. 1). The postweaning housing rooms were held under identical environmental conditions as the preweaning housing room described above, except for the light cycle (23:00–11:00), to accommodate testing in the dark phase. Only pups from litters with at least 9 live pups on PND 0 and a balanced sex ratio at birth (no litter had more than a 4 pup sex difference except for 2 litters in load 5, which had a 5 pup sex difference: 9 males and 4 females) were used in this study. Juvenile testing began on PND 25, allowing the animals from PND 21 to PND 25 to habituate to the new building. Juvenile and adult test subjects were siblings; that is, 1/sex/litter was assessed as juveniles and another 1/sex/litter was assessed in adulthood. At weaning, each subject was housed with 1 or 2 conspecifics (same-exposure group, same-sex, same-age, non-siblings). Where needed, treatment-naïve "companion" rats were used to provide cagemates for those study subjects that could not be housed with a conspecific (ie, those in which only 1 litter of that exposure group was born on that day). No data were collected from these "companion" rats.

Behavioral Testing

Rats were assessed either as juveniles on PND 25–27 (prepuberty) or at adulthood (Fig. 1) using a test battery selected because the tasks have high predictive validity for anxiety and generate corroborative results (Chadman *et al.*, 2009; Walf and Frye, 2007). Juveniles were assessed using the EPM and open field (OF). Adults were first assessed for 7 consecutive days using a Barnes Maze by another CLARITY-BPA consortium

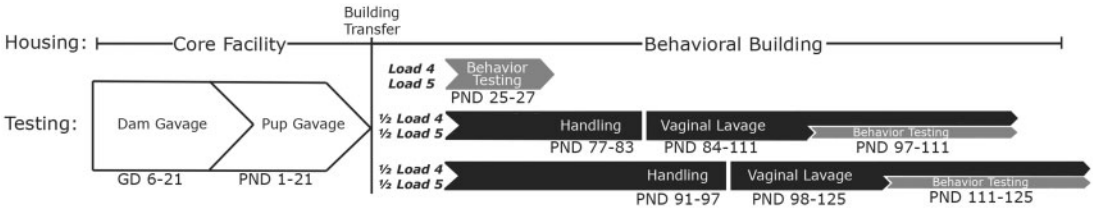

FIG. 1. Methods timeline. Visual depiction of the experimental methods timeline including dosing and housing. Dam and pup gavages occurred in the core animal facility (white arrows), and the experimental animals were transferred to a different animal facility on postnatal day (PND) 21 and acclimated to the new facility from PND 21 to 25 for subsequent testing. Juveniles (gray arrow) were tested prior to puberty. Adults were tested in 2 groups (black arrows) and the time of behavioral testing for each group is indicated.

team, then on the EPM, OF, and ZM. Testing procedures conformed to commonly used standards previously reported and used by us and others (Cao *et al.*, 2013; Ferguson and Boctor, 2010; Ferguson *et al.*, 2012; Hogg, 1996; Patisaul *et al.*, 2012; Pellow *et al.*, 1985; Shepherd *et al.*, 1994). Behavioral testing rooms (each containing only 1 type of maze) contained a white noise generator (producing ~66 dB; Marpac Dohm, Rocky Point, NC), and multiple apparatuses, half designated for males and half designated for females. All apparatuses were cleaned with 70% ethanol after each testing session. Subjects were preassigned to an apparatus such that approximately equal numbers of each exposure group were tested in each. When possible, cagemates were tested on the same day, but if not feasible (eg, when estrous cycle did not match testing protocol), cagemates were tested in sequential sessions (days between testing of cagemates ranged from 1 to 8). All assessments commenced after housing room lights were off (approximately 11:00) and were completed within 4 h. For testing, all subjects were transported to the nearby test room in their home cages on a rolling cart and remained on the cart until testing. The hallway between the housing room and test rooms was illuminated with red light. The OF was a beam break assay (PAS-Open Field, San Diego Instruments, San Diego, CA). All other tests were video recorded from overhead cameras under dim red lighting and analyzed from the video by TopScan software (Clever Sys Inc., Reston, VA) by the NCSU research team. For all tasks, the number of defecation events was counted because this behavior is sometimes interpreted to indicate anxiety, but these data were ultimately not used or analyzed because the majority of animals did not defecate during testing. Animals were weighed at the time of testing to look for signs of overt toxicity. As expected, a significant effect of sex on body weight was observed at both ages ($P \leq .001$ for both ages; data not shown) with juvenile and adult males weighing more than same-age females but exposure had no effect on body weight in either sex at either age.

Juvenile testing spanned PNDs 25–27 to minimize the likelihood that females would be tested after vaginal opening (pubertal onset). For adult behavioral testing, subjects from the 2 loads (4 and 5) were subdivided into testing intervals for logistical reasons. Both sexes (beginning at PND 77 for half of each load and PND 91 for the remainder) were handled daily to become habituated to human contact. Because behavior varies across the estrous cycle, monitoring and controlling for estrous cycle to the best degree possible is crucial for decreasing biological variability that could result from different estrous cycle phases at the time of the assessment and ensuring testing consistency (Anderson, 1940; Archer, 1975; Diaz-Veliz *et al.*, 1997; Frye *et al.*, 2000; Mora *et al.*, 1996; Patisaul *et al.*, 2005). Vaginal lavage began on PND 84 (for half of each load) or 98 (remaining animals) and continued daily until sacrifice. Estrous cycle stage was assessed each morning (between 7:30 and 8:00, or 3–3.5 h before testing) via vaginal lavage. Slides were categorized by 2 experienced testers blind to treatment. Rats were tested on the EPM and ZM on the day they were categorized to be in proestrus or any stage of estrus (early to late). OF testing was conducted the day after EPM testing, regardless of estrous stage. Thus, the testing sequence for females was (1) EPM during proestrus or estrus, (2) OF on the subsequent 2 days, and (3) ZM during proestrus or estrus. The testing sequence for males was 4 consecutive days (ie, EPM, 2 days of OF, and ZM). Adult testing spanned 11 days maximally from PND 97 (for half of the subjects) or PND 111 (for the remainder).

Elevated plus maze. Juveniles (PND 25) and adults were assessed for anxiety-like behavior during a 5-min test session using 1 of 4 EPMs, as previously described (Ferguson and Berry, 2010). Briefly, each apparatus consisted of 4 connected black Plexiglas arms, each 10 cm wide and 50 cm long, elevated 50 cm above the floor. Two arms were enclosed within 40 cm walls (closed arms) and 2 arms had a short (8 mm) ledge around the edge (open arms). Each subject was gently placed on the central area facing the closed arm closest to the room wall, and the home cage and rolling cart were moved outside the test room.

Open field (OF). Juveniles (PNDs 26–27) and adults were assessed for anxiety and locomotor activity during 2 30-min test sessions (over 2 consecutive days) using 1 of 8 OF apparatuses as previously described (Ferguson *et al.*, 2012). The clear Plexiglas arenas (each $40 \times 40 \times 40$ cm) had a 16×16 photo beam detector around the outside floor perimeter for detection of horizontal movements and an elevated photo beam detector to measure vertical activity. Opaque boards between adjacent apparatuses prevented visual contact. Each subject was introduced to the front left corner (same apparatus on both testing days).

For each of the 2 test days, activity was collected in 5-min intervals and summed over the entire 30-min testing session (total activity). Behaviors assessed were total distance traveled (cm), average speed (cm/s), resting time (total time with no activity for > 2 s), and time and entries into the center area (defined as the central 20×20 cm). An "entry" was defined as consecutive breaking of 2 beams. PAS-Reporter (San Diego Instruments, San Diego, CA) was used to convert the raw x,y beam break data into the distance, speed, resting, and zone data for statistical analyses.

Zero maze. Adults were assessed for anxiety-like behavior during a 5-min session using 1 of 2 ZM apparatuses, constructed to be consistent with those previously described (Cao *et al.*, 2013; Patisaul and Bateman, 2008; Shepherd *et al.*, 1994). Each maze consisted of 2 open arms (9.5 cm in width) and 2 closed arms (29.5 cm high walls), was 123 cm in diameter, and elevated 75.5 cm above the floor. Each of the 2 open arm areas had a 10 mm ledge around the edge (so as to be structurally similar to the open arms of the EPMs). The subject was gently placed onto an open arm facing a closed arm and left undisturbed for 5 min.

Summary of primary endpoints in the behavioral tasks. The strongest indices of anxiety in these tasks are open arm activity in the EPM and ZM (less = heightened anxiety) and center activity in the OF (less = heightened anxiety) (Bailey and Crawley, 2009; Gould *et al.*, 2010). The most robust indices of activity are closed arm exploration on the EPM and ZM and total distance traveled in the OF (over the full 30-min task). Habituation was assessed by comparing OF behavior across the 2 successive testing days (activity declines with experience) (Bailey and Crawley, 2009; Gould *et al.*, 2010). Results from all assessed endpoints are presented in the tables and the most commonly reported, salient endpoints for each testing apparatus depicted graphically.

Data decoding. All behavioral testing was completed and scored blind to exposure group. The blinded raw data were submitted to the NTP Chemical Effects in Biological Systems database. It was then independently verified to account for all expected data sets and data points, and "locked" such that data could not

be altered. The NCSU researchers were then provided with the exposure code for data analysis.

Statistical analysis. The statistical approach was developed to be consistent with prior work and using published guidelines for low-dose endocrine disrupting chemical studies with sample sizes in this range (Haseman *et al*., 2001). Main effects and their interactions were examined using ANOVA. A Fisher's protected least significant difference (LSD) was used as the *post hoc* test (when main effects or interactions were identified). While the Fisher's protected LSD does not provide the strong family-wise error control of alternative *post hoc* procedures, it was selected over a more conservative approach to minimize risk of Type II error (rejecting a meaningful effect). Because very few BPA effects (versus vehicle control) were identified, controlling for false positives was not considered of high concern, as doing so would not impact data interpretation. All statistical analyses were implemented in R (R Team, 2014) and adults and juveniles were analyzed separately. For all endpoints, significance was considered $P \leq .05$.

EPM data from 2 juvenile subjects (1 EE male and 1 BPA 2.5 female) and 1 adult vehicle control female were excluded from analyses because they fell from the apparatus. One adult EE male was excluded from the ZM analyses because it was an extreme outlier (greater than twice the number of open arm entries as the next highest data point for that sex and exposure group). This exclusion did not affect the statistical identification of any exposure effects. Four adult females (3 EE and 1 BPA 2.5) could not be included in the analysis for the second OF day, because the data collection software was not started. Because of the reduced sample size for the adult female EE group on the second OF test day, data from the second OF day were only used to assess the impact of test day on the outcomes. Data from the first OF day were analyzed in detail, graphed, and included in the figures and tables. For consistency, the juvenile OF data were approached the same way.

For EPM and ZM data sets, ANOVA models assessed effects of sex, exposure, and exposure by sex interactions. Significant main effects were followed up with a Fisher's protected LSD *post hoc* test. Because aspects of EPM and ZM behavior are sexually dimorphic, if a main effect of sex was found for any endpoint on that maze, all subsequent analyses for exposure-related effects on that maze were made within sex. Additionally, confirmation of known sex differences in the vehicle controls was considered to be an indication that the test was robust, powered sufficiently to detect a difference in the range of that effect size, and properly conducted. As commonly seen with a sample size of 12/sex/exposure group, achieving normality in all residual distributions within a given endpoint ANOVA model was rare. Because violations of this assumption tend to produce false positives and there were no consistent treatment-associated effects, we did not differentially perform non-parametric tests in cases where deviation from normality may have been present. Rather, we applied a consistent modeling approach to all endpoints across each maze type (Cohen *et al*., 2002).

OF data sets were analyzed in 2 ways: (1) for each endpoint the data were summed over the entire 30-min session and analyzed and (2) behavior was also assessed in 5-min intervals (ie, a separate ANOVA was conducted for each 5-min interval). Breaking the 30-min session down into 5-min intervals allows exploratory behavior to be assessed at different points across the session as behavior changes with experience (Bailey and Crawley, 2009; Goma and Tobena, 1978; Gould *et al*., 2010). The first 5 min of the test are thought to give the most informative general measures of anxiety (because novelty is highest). As the test progresses, activity declines as the animal becomes familiar with the arena; thus, differences in overall activity or center area behavior during the final intervals could be reflective of anxiety and/or exploratory behavior. Activity toward the end of the 30-min task is thought to reach a steady state so behavior in the final 5-min interval is considered to be the best indicator of general (not driven by novelty stress) locomotion (Gould *et al*., 2010). For both the 30 min and interval analyses, a 3-way ANOVA was conducted to test for main effects of sex, exposure, and test day (across the 2 days), and their interactions. Day 1 data were then further assessed using 2-way ANOVAs with sex and exposure as factors. Significant main effects and interactions were followed up with a Fisher's protected LSD *post hoc* test. All tables (including Supplementary Tables 1 and 2) report *P* values for the F-test associated with each endpoint across all factors tested.

Because effects were primarily negative, a *post hoc* power analysis for a range of treatment effect sizes was performed to evaluate possible risk of a Type II error (rejecting the null hypothesis when an effect is present). To parameterize these calculations, we used the experimental data (treatment group-wise means and variances) from the adult male EPM measure of time spent in the open arms. The power calculations were implemented using the G*Power software (Faul *et al*., 2009), then plotted using R.

Pairwise correlations between anxiety-related endpoints were conducted to assess data concordance across the OF, EPM, and ZM for the adult testing using methods similar to those described for characterizing intermaze relationships (Padilla *et al*., 2009).

RESULTS

Juveniles

In the EPM (Fig. 2), no significant main effect of exposure group was found for any endpoint. Main effects of sex were identified for 4 of 11 endpoints, females spent less time in the central area ($P \leq .015$), exhibited more stretch attend postures ($P \leq .001$), had a shorter latency to enter the open arms ($P \leq .034$), and traveled more distance in the closed arms ($P \leq .005$). No significant interaction of sex and exposure was found for any endpoint.

OF data were first analyzed by assessing total behavior over the entire 30-min session of the first testing day (Fig. 3). No significant main effects of exposure were found for any of the 4 endpoints. In contrast, significant main effects of sex were identified for all endpoints: females traveled less ($P \leq .006$), rested more ($P \leq .009$), made fewer center entries ($P \leq .014$), and spent less time in the center ($P \leq .011$). No significant interaction of sex and exposure was identified for any endpoint. Additionally, there was no significant effect of test day on any endpoint.

To obtain greater detail about possible impacts on behavior within the 30-min session, OF data from the first testing day were independently analyzed in 5-min intervals (Supplementary Table 1). Only the first day was analyzed to be consistent with the approach used for the adults (reported below) and because there were no significant effects of test day. Briefly, main effects of sex were found in 11 of 24 interval analyses, but no significant interaction of sex and exposure was identified. Main effects of exposure were identified in only 3 of 24 interval analyses (Supplementary Table 1). In the second 5-min interval of the first day, the 2.5 and 25 BPA rats spent more time resting than the vehicle controls ($P \leq .003$ and $P \leq .001$,

A **Juvenile Elevated Plus Maze ANOVA p-values**

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction
Time Spent - Closed Arms	0.509	0.381	F=M	0.362
Time Spent - Open Arms	0.282	0.728	F=M	0.713
Time Spent - Center	0.763	**0.015**	F<M	0.618
Stretch Attends	0.232	**0.001**	F>M	0.288
Latency to Enter Open Arms	0.777	**0.034**	F<M	0.485
Distance Traveled - Closed Arms	0.324	**0.005**	F>M	0.518
Distance Traveled - Open Arms	0.120	0.562	F=M	0.905
Distance Traveled - Center	0.552	0.175	F=M	0.922
Overall Distance Traveled	0.352	0.059	F=M	0.863
Open Arm Entries	0.336	0.507	F=M	0.820
Closed Arm Entries	0.948	0.127	F=M	0.507

F=M, no statistically significant sex difference

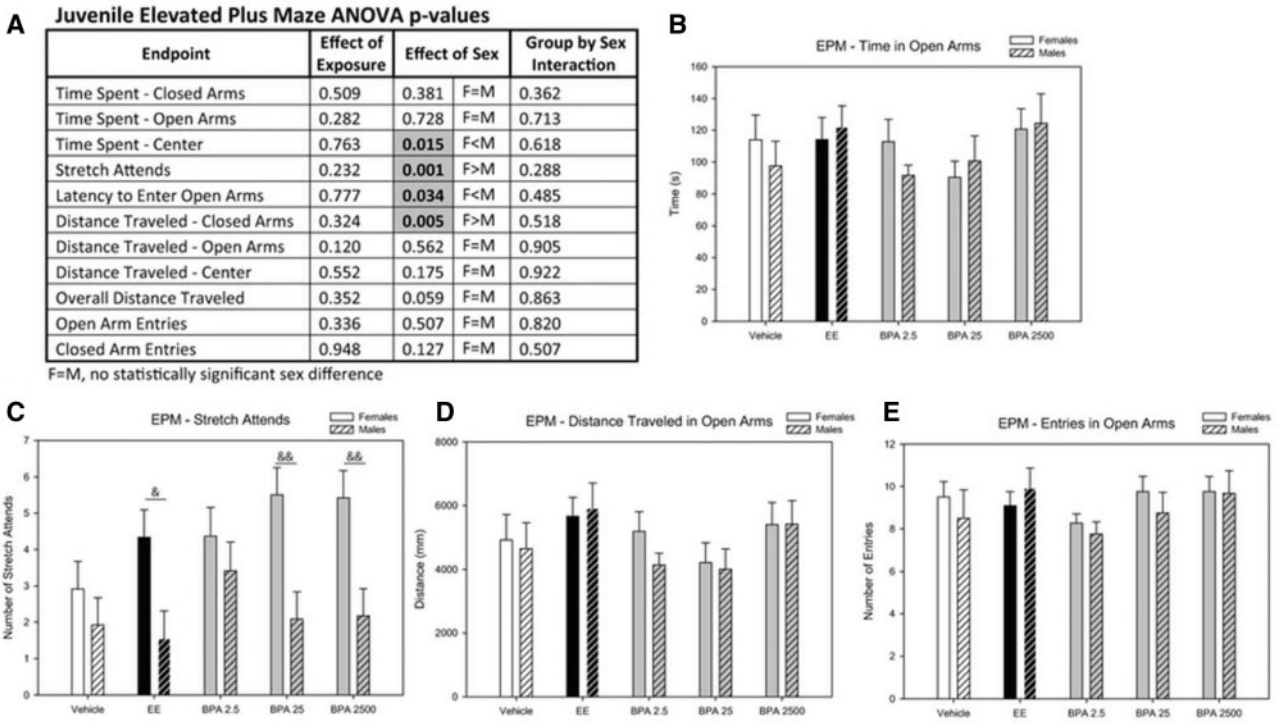

FIG. 2. Juvenile elevated plus maze (EPM). A, ANOVA P-values for main effects of exposure, sex, and their interaction for each endpoint. Significant effects are bolded and highlighted in gray and the direction of significant sex differences is indicated (M = male; F = female). B, Time in the open arms did not differ by sex or across exposure groups. C, Number of stretch attends was sexually dimorphic, with females performing more stretch attends than males. This sex difference was not statistically significant in the vehicle controls or the bisphenol A (BPA) 2.5 groups. No effects of ethinyl estradiol (EE) or BPA were observed versus vehicle control. Distance traveled on the open arms D, and number of open arm entries E, were not impacted by sex or exposure. Graphs depict mean ± SEM. For all graphs, females are depicted in open bars and males in striped bars. Sex differences within exposure group are indicated by &$P \leq .05$ and &&$P \leq .01$.

A

Juvenile Open Field ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction	Effect of Test Day	
Overall Distance Traveled	0.264	**0.006**	F<M	0.906	0.661	1=2
Overall Time Resting	0.071	**0.009**	F>M	0.980	0.516	1=2
Total Entries – Center	0.373	**0.014**	F<M	0.981	0.897	1=2
Overall Time – Center	0.373	**0.011**	F<M	0.990	0.401	1=2

1=2, no statistically significant difference

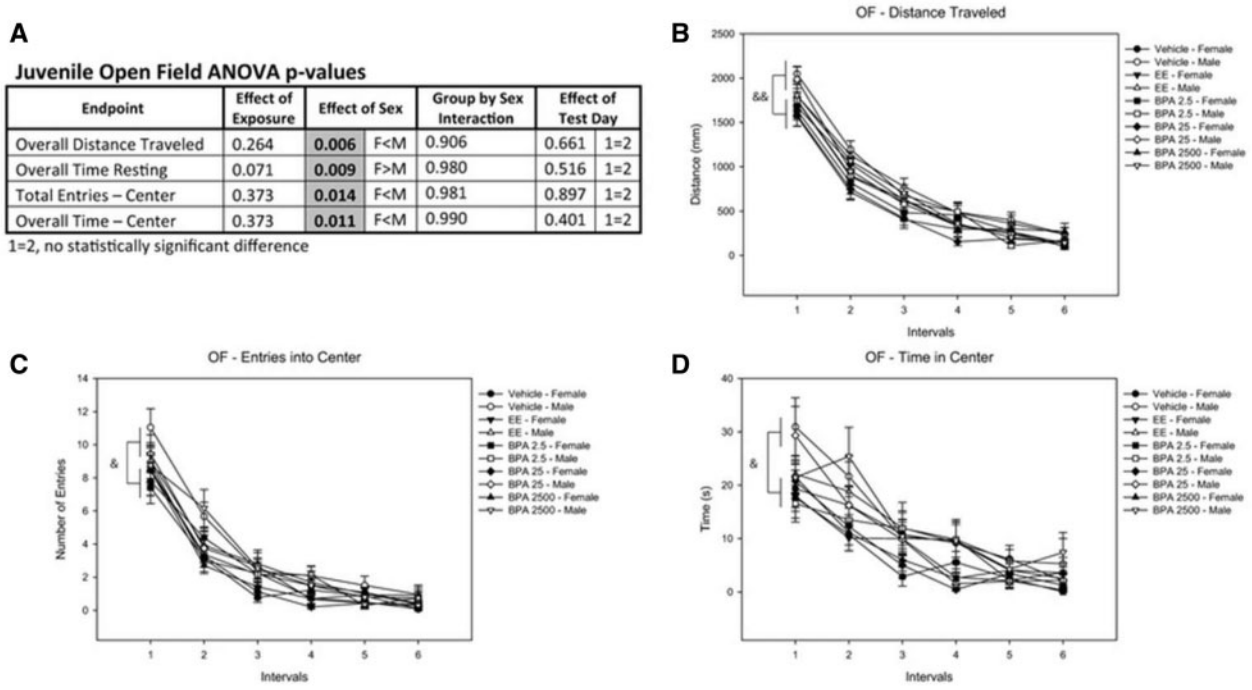

FIG. 3. Juvenile OF. A, ANOVA P-values for main effects of exposure, sex, their interaction, and test day for each endpoint. Significant effects are bolded and highlighted in gray. Sex and test day differences are indicated (M = male; F = female; 1 = first test day; 2 = second test day). Distance traveled B, entries into the center C, and time in the center D, differed by sex but did not differ across exposure groups. No effects of ethinyl estradiol (EE) or bisphenol A (BPA) were observed versus vehicle control. Graphs depict mean ± SEM. For all graphs, females are represented by open shapes and males by black, filled shapes. Each interval was 5 min; all graphs show results from the first day of testing (data from the second day are not shown). Main effect of sex denoted by &$P \leq .05$ and &&$P \leq .01$.

A

Adult Elevated Plus Maze ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction	Group Differences
Time Spent - Closed Arms	**0.005**	0.351	F=M	0.393	EE < BPA 2500*** BPA 25 < BPA 2500**
Time Spent - Open Arms	**0.025**	**0.001**	F>M	0.380	Vehicle < EE* BPA 2.5 < EE* BPA 2500 < EE**
Time Spent - Center	0.508	**0.001**	F<M	0.365	
Stretch Attends	0.302	0.267	F=M	0.908	
Latency to Enter Open Arms	0.544	0.061	F=M	0.406	
Distance Traveled - Closed Arms	**0.029**	**0.001**	F>M	0.583	Vehicle > EE ** BPA 2.5 > EE*
Distance Traveled - Open Arms	0.271	**0.001**	F>M	0.658	
Distance Traveled - Center	**0.011**	0.445	F=M	0.956	BPA 25 > EE** BPA 25 > BPA 2500**
Overall Distance Traveled	0.132	**0.001**	F>M	0.568	
Open Arm Entries	0.319	**0.007**	F>M	0.850	
Closed Arm Entries	**0.031**	0.184	F=M	0.954	BPA 25 > EE**

*=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$; F=M, no statistically significant sex difference

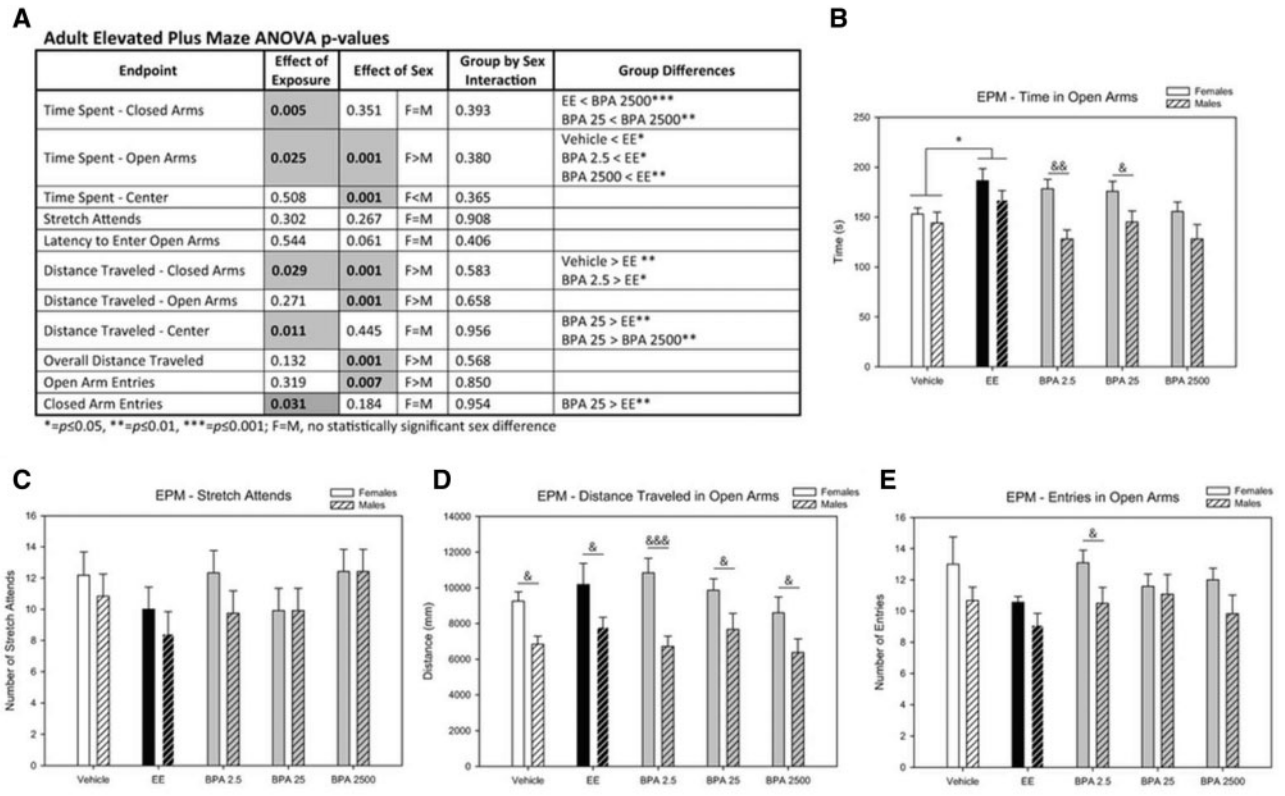

FIG. 4. Adult elevated plus maze (EPM). A, ANOVA P-values for main effects of exposure, sex, and their interaction for each endpoint. Significant effects are bolded and highlighted in gray. Sex and group differences are indicated (M = male; F = female). B, Time in the open arms differed by exposure and sex. The ethinyl estradiol (EE) group had a significantly longer time on the open arms than the vehicle group. Bisphenol A (BPA) 2.5 and 2500 groups differed significantly from the ethinyl estradiol (EE) group, but not the vehicle controls. Sex differences were identified in the BPA 2.5 and 25 groups, but not in the vehicle controls. C, Number of stretch attends was not impacted by sex or exposure. D, Distance traveled in the open arms was sexually dimorphic with females traveling farther. E, Open arm entries were not impacted by exposure but were sexually dimorphic. This sex difference was only statistically significant in the BPA 2.5 group. Graphs depict mean ± SEM. For all graphs, females are depicted in open bars and males in striped bars. Group differences compared to the vehicle control group are indicated with *$P \leq .05$. Sex differences within exposure group are indicated by &$P \leq .05$; &&$P \leq .01$; and &&&$P \leq .001$.

respectively). In the fourth interval, the BPA 25 group made fewer center entries ($P \leq .005$) and spent less time in the center ($P \leq .003$) than vehicle controls. Significant main effects of test day were detected in only 2 of 24 instances.

Adults

In the EPM, significant main effects of exposure group were found for 5 of 11 endpoints, but *post hoc* testing did not indicate that any BPA group was significantly different from the vehicle group. Instead, BPA and/or vehicle groups were significantly different from the EE group (Fig. 4). EE and BPA 25 rats spent less time on the closed arms than BPA 2500 rats ($P \leq .001$ and $P \leq .006$, respectively). EE animals spent significantly more time on the open arms than the vehicle control, BPA 2.5, and BPA 2500 groups ($P \leq .05$, $P \leq .05$, and $P \leq .01$, respectively). The vehicle control and BPA 2.5 groups traveled more distance in the closed arms than the EE group ($P \leq .006$ and $P \leq .037$, respectively). EE and BPA 2500 groups traveled less distance in the center than the BPA 25 group ($P \leq .004$ and $P \leq .008$, respectively). The EE group entered the closed arms fewer times than the BPA 25 group ($P \leq .008$). Main effects of sex were identified in 6 of 11 endpoints, females spent more time in the open arms ($P \leq .001$), less time in the center ($P \leq .001$), traveled more distance (closed arms ($P \leq .001$), open arms ($P \leq .001$), and overall ($P \leq .001$), and made more open arm entries ($P \leq .007$); effects consistent with known sex differences in rat EPM performance. No significant interactions of sex and exposure were identified for any endpoint.

No significant main effects of exposure were found for any OF endpoint when endpoints (day 1 only) were summed over the entire 30-min session (Fig. 5). Main effects of sex were found for all endpoints. Females traveled less distance overall ($P \leq .001$), spent more time resting ($P \leq .009$), made fewer center entries ($P \leq .003$), and spent less time in the center area ($P \leq .001$). No significant interaction of sex and exposure was found for any of those 4 day 1 endpoints. A significant main effect of test day was also identified for every overall endpoint; demonstrating that all groups habituated to the task. On the first test day, rats (regardless of sex or exposure group) traveled farther ($P \leq .001$), rested less ($P \leq .001$), made more center entries ($P \leq .001$), and spent more time in the center area ($P \leq .001$).

Data from day 1 were then analyzed using separate ANOVAs for each 5-min interval. No significant main effects of exposure were identified in any interval (Supplementary Table 2). Main effects of sex were found in 18 of 24 interval analyses (Supplementary Table 2), confirming the behavioral sex difference detected in the full session analysis. No significant interactions of sex and exposure were found. Comparing behavior in each interval across days 1 and 2, significant main effects of test day were found in 17 of 24 interval analyses (Supplementary Table 2) confirming across-session habituation regardless of sex or exposure.

A

Adult Open Field ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction	Effect of Test Day	
Overall Distance Traveled	0.227	**0.001**	F<M	0.793	**0.001**	1>2
Overall Time Resting	0.315	**0.009**	F>M	0.669	**0.001**	1<2
Total Entries – Center	0.300	**0.003**	F<M	0.813	**0.001**	1>2
Overall Time – Center	0.262	**0.001**	F<M	0.898	**0.001**	1>2

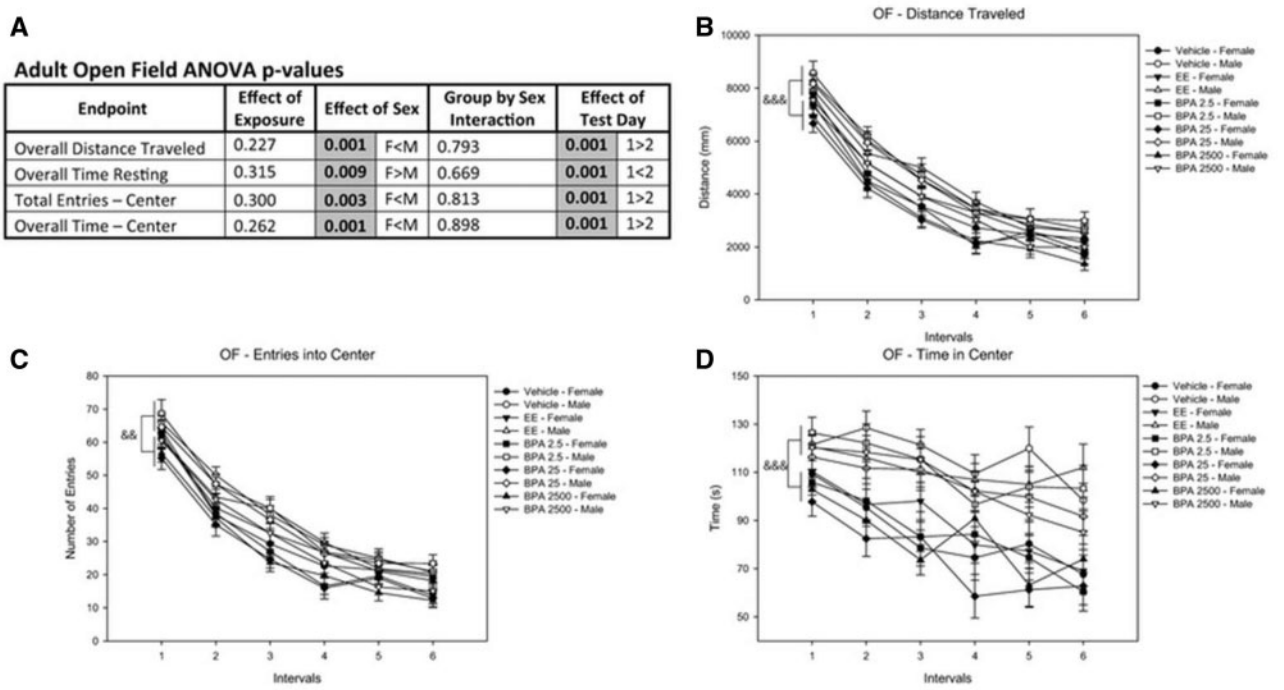

FIG. 5. Adult OF. A, ANOVA *P*-values for main effects of exposure, sex, their interaction, and test day for each endpoint. Significant effects are bolded and highlighted in gray. Sex and test day differences are indicated (M = male; F = female; 1 = first test day; 2 = second test day). Distance traveled B, entries into the center C, and time in the center D, differed by sex, but did not differ across exposure groups. No effects of ethinyl estradiol (EE) or bisphenol A (BPA) were observed. Graphs depict mean ± SEM. For all graphs, females are represented by open shapes and males by black, filled shapes. Each interval was 5 min; all graphs show results from the first day of testing (data from the second day are not shown). Main effect of sex denoted by && $P \leq .01$ and &&&$P \leq .001$.

In the ZM (Fig. 6), no significant main effect of exposure was found for any endpoint. A main effect of sex was identified for 1 of the 7 endpoints and indicated that females performed fewer stretch attends ($P \leq .041$). No significant interactions between exposure and sex were identified for any ZM endpoint.

Correlation Between Outcome Measures

Linear correlation patterns between anxiety endpoints were explored for the 3 adult testing arenas. The results are presented in Supplementary Figure 1. As expected, high empirical correlations (r) were found between related outcome measures within each apparatus (eg, measures of speed, time, and number of entries into a specific area or arm). This is reflected in the groupings of significant results for individual outcomes reported in all tables; however, lower than expected correlations were found between related measures across the different testing arenas (Supplementary Fig. 1), with the majority of across-assessment correlations $r < 0.15$. For example, concordance between time in the open ZM and EPM arms was a reasonable $r < 0.38$, but number of entries into the respective arms was poorly correlated at $r < 0.066$. Low concordance was observed in all exposure groups (depicted in Supplementary Fig. 1), so the overall effect was not impacted by exposure group.

Estimating Observed Effect Size of BPA and Detection Power

A subset of the experimental data was used to estimate the observed effect size of treatment and the associated detection power. The effect size, f, was defined as: $f = \sigma_m/\sigma$, where σ_m is the standard deviation of the group means and σ is the standard deviation within each group. Supplementary Figure 2 shows the estimated power (the probability of rejecting a null hypothesis given that it is truly false) for a range of effect sizes. The effect size, f, was solved for plotting as η^2, which is interpreted here as "proportion of variance explained by exposure group." Thus, the range of effect sizes plotted in Supplementary Figure 2 represents effect sizes, η^2, of 1% ($f = 0.1$) to 50% ($f = 1$). The data used to generate these curves were based upon observed data from the time in the open arms for adult males (vehicle and BPA 2500 groups) in the EPM. This data set was chosen for this analysis because main effects of exposure and sex were found for some of the endpoints on the EPM, including time in the open arms, and the variability was reasonably consistent across all exposure groups (regardless of sex). For this behavioral measure, our effect size was estimated as $f = 0.37$, which corresponds to an estimated power of 58% using 60 total animals (12 rats per each of 5 groups). Under this effect size (considered "moderate"), 95 total animals (19 rats per each of 5 groups) would be required to achieve 80% power. Note that these estimates do not account for any expected "ordering" of the treatment groups. If notions of non-monotonicity in complex behavioral responses were discarded, then alternative models might achieve slightly higher power estimates—given that other assumptions were held constant.

DISCUSSION

No systematic effects of BPA were observed on any endpoint in juveniles or adults. In juveniles, statistically significant effects of 2.5 and 25 μg/kg bw/day BPA were identified on a few endpoints in the interval OF analysis but, overall, evidence for BPA-related effects was minimal and inconsistent and thus not interpreted to be indicative of a biologically meaningful effect on either anxiety or activity. The reference estrogen, EE, also had no meaningful effects on behavior compared with

A

Adult Zero Maze ANOVA p-values

Endpoint	Effect of Exposure	Effect of Sex		Group by Sex Interaction
Time Spent - Closed Arms	0.629	0.074	F=M	0.101
Time Spent - Open Arms	0.636	0.077	F=M	0.098
Stretch Attends	0.451	**0.041**	F<M	0.077
Latency to Enter Open Arm	0.495	0.582	F=M	0.837
Distance Traveled - Closed Arm	0.650	0.630	F=M	0.708
Distance Traveled - Open Arms	0.965	0.673	F=M	0.839
Overall Distance Traveled	0.708	0.602	F=M	0.908

F=M, no statistically significant sex difference

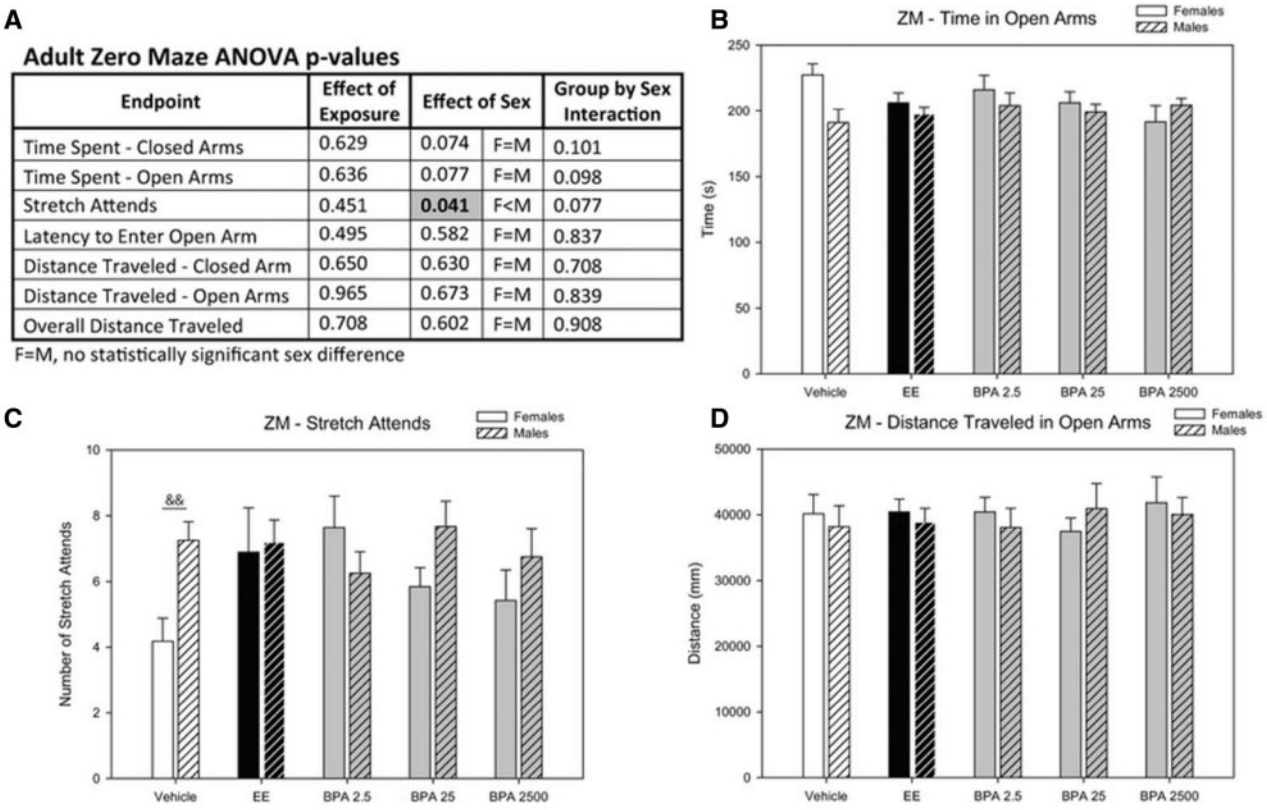

FIG. 6. Adult ZM. A, ANOVA *P*-values for main effects of exposure, sex, and their interaction for each endpoint. Significant effects are bolded and highlighted in gray. Sex and group differences are indicated (M = male; F = female). B, Time in the open arms was not impacted by sex or exposure group. C, Number of stretch attends was sexually dimorphic with females performing fewer stretch attends. No effects of ethinyl estradiol (EE) or bisphenol A (BPA) were identified. D, Distance traveled in the open arms was not impacted by sex or exposure group. Graphs depict mean ± SEM. For all graphs, females are depicted in open bars and males in striped bars. Sex differences within exposure group are indicated by &&$P \leq .01$.

the vehicle controls. Significant differences between BPA-exposed and EE-exposed groups were identified for several endpoints; an outcome suggesting BPA and EE may not have duplicative effects on behavioral tasks. Detection of expected sex differences was interpreted to signify that tasks were robust and sufficiently powered to identify these well-established differences. Expected sex differences in EPM performance (summarized in Simpson *et al.*, 2012) were statistically significant (main effect of sex in the ANOVA) or suggested for most endpoints, but not consistently observed within the vehicle (unexposed) control groups, and thus considered a potential limitation. Sex differences in the OF were consistent with what has previously been shown for the NCTR-SD strain (eg, Boctor and Ferguson, 2010; Ferguson and Berry, 2010; Ferguson *et al.*, 2012) but opposite of what has historically been reported across most rat strains (Frye *et al.*, 2000; Gould *et al.*, 2010; Padilla *et al.*, 2009; Valles, 1976). Sex differences on the ZM were not observed. Suboptimal statistical power, low concordance across behavioral tasks, and light cycle shift 4 days prior to juvenile testing were identified as study limitations. Subsequent studies using these animals will investigate the possibility that BPA exposure induced morphological, molecular, or epigenetic changes in rat brain regions fundamental to the coordination of these and other sexually dimorphic behaviors.

Published data regarding BPA-related effects on anxiety in juvenile rodents generally suggest anxiogenic activity, but available evidence is limited and sex-specific effects at this age are conflicting (Wolstenholme *et al.*, 2011a). For example, 2 mouse studies published since the WHO assessment reported increased anxiety-like behaviors in juvenile C57BL/6J males, but not in females (Cox *et al.*, 2010; Matsuda *et al.*, 2012), while another study reported the opposite in CD1 mice (Gioiosa *et al.*, 2013). Two studies reported no effects on anxiety in juvenile C57BL/6J mice (Wolstenholme *et al.*, 2011, 2013), while another observed decreased anxiety in both sexes of ICL mice (Nakamura *et al.*, 2012). Heightened anxiety was found in BPA-exposed juvenile Wistar rats (Patisaul *et al.*, 2012); an effect which was abrogated by soy diet, emphasizing that species, strain, and diet are all factors which likely contribute to outcome variability in the literature. Here, no effects of BPA were detected on any OF endpoint in the juvenile cohort when data from the 30-min sessions were assessed as a whole. In the interval analysis, however, some mid-trial effects were observed. Elevated overall time resting in the BPA 2.5 and 25 groups (Supplementary Table 1) was detected in the second interval (minutes 5–10 of the session). The BPA 25 group also displayed fewer center entries and less time in the center during the fourth interval. Although these results could potentially be interpreted as suggestive of heightened anxiety and reduced activity, mid-trial activity changes are more difficult to interpret than those at the start or end of the task, and these sporadic observations are likely due to chance and not indicative of a meaningful impact of BPA on anxiety or activity. Similarly no evidence of BPA-related effects was observed on EPM performance.

Shifting the animals to a new building and reversing the light cycle (to accommodate testing in the dark, when rats are naturally most active) 4 days prior to testing is a potential confound of the juvenile testing. Disruption of circadian rhythm is well recognized to alter behavioral patterns, including motor behaviors (Silver and Kriegsfeld, 2014; Yamazaki *et al.*, 2000), and sensitivity is age dependent (Albani *et al.*, 2015) and sexually dimorphic (Bailey and Silver, 2014). The testing window was selected to maximize recovery time from the shift but also complete assessment prior to pubertal onset.

There was no effect of test day on OF behavior in juveniles, indicating no across-session habituation. This observation is concordant with prior work in NCTR-SD rats (Ferguson *et al.*, 2012), but this age-dependent behavior typically emerges earlier in SD rats (Bronstein *et al.*, 1974; Laviola *et al.*, 1988). Only some sex differences were observed in the OF, but this finding is consistent with prior work showing that sex differences do not fully emerge until adulthood in rats (Masur *et al.*, 1980; Slob *et al.*, 1986; Valles, 1976).

In adults, numerous prior studies using a variety of exposure and testing paradigms have reported evidence of heightened anxiety following developmental exposure to BPA at dose levels approximate to the range used here (reviewed in Mileva *et al.*, 2014; Wolstenholme *et al.*, 2011a). These effects have been reported in both sexes across a variety of rodent species and strains, eliminating naturally occurring sex differences in some cases (examples from rats, mice, and prairie voles include [Kundakovic *et al.*, 2013; Matsuda *et al.*, 2012; Patisaul *et al.*, 2012; Sullivan *et al.*, 2014]). While the majority of available studies reveal anxiogenic effects, decreased anxiety has been observed in Long Evans rats (Jones and Watson, 2012), an effect the authors attributed to heightened general activity and demasculinization. Anxiolytic effects have also been reported in ICR mice exposed to 100 or 500 μg/kg bw/day from GD 7–PND 36 (Tian *et al.*, 2010). Adult behavioral assessments in this study revealed no BPA-related effects on the OF or ZM. On the EPM, main effects of exposure were identified for several endpoints (Fig. 4), but these effects were attributable to differences between the BPA and EE groups. We have previously reported differences between BPA and EE exposures (Cao *et al.*, 2013; Patisaul *et al.*, 2012; Rebuli *et al.*, 2014), and the present results reinforce our prior conclusion that BPA does not act strictly like an "estrogen" on the brain and behavior. Thus impacts on behavior may occur via alternative mechanisms (Kinch *et al.*, 2015; Wolstenholme *et al.*, 2011a; Yeo *et al.*, 2013) and ongoing studies assessing gene expression and methylation patterns in the brains of the animals used here should inform on these mechanisms.

Adult rat EPM behavior is typically sexually dimorphic, with females in behavioral estrus more active and exhibiting increased exploration of "high anxiety" areas (ie, open arms of the EPM and ZM) than females in other estrous phases or males (Anderson, 1940; Archer, 1975; Diaz-Veliz *et al.*, 1997; Frye *et al.*, 2000; Mora *et al.*, 1996; Patisaul *et al.*, 2005). Although main effects of sex were detected by ANOVA in the EPM (most importantly, for open arm entries and time on the open arms), with the exception of distance traveled in the open arms, those differences were not significant in the vehicle control groups leading to some concern about sensitivity and study power. Behavioral sex differences were also not detected in the ZM. In the OF, males were more active and more exploratory of regions considered high anxiety than females. This sex difference was robust but opposite of what is typically reported for rats, including SD rats (Frye *et al.*, 2000; Gould *et al.*, 2010; Padilla *et al.*, 2009; Valles, 1976). Prior OF work in this SD strain at NCTR has consistently found similar or no sex differences. (eg, Boctor and Ferguson, 2010; Ferguson and Berry, 2010; Ferguson *et al.*, 2012). Absent and opposite behavioral sex differences in the NCTR-SD rat population might possibly factor into why the results reported here differ from prior BPA studies in rats.

Because the data were primarily negative, *post hoc* power calculations were conducted to assess the potential for Type II error (false negatives). For this analysis, male performance on the EPM (vehicle and BPA 2500 groups) was used because this was an endpoint for which a main effect of sex and exposure were identified. A moderate power deficit, arising primarily from the high interindividual variability observed on these behavioral measures, was detected. Although the reliability of the experiments was high, in terms of measures within subjects and across test days, the possibility interindividual variability obscured an exposure-related signal cannot be entirely ruled out. A sufficiently powered study using these specific outcomes and exposure groups would minimally require a sample size of 19/sex/exposure group (7 additional subjects/sex/exposure group) to confidently rule out Type II error (Supplementary Fig. 2). This estimate is consistent with prior assertions that sample sizes of approximately 10–20 animals per sex per group are minimally needed to overcome interindividual behavioral variability in routine behavioral phenotyping (Chadman *et al.*, 2009). *Post hoc* power analyses have well-characterized limitations (Levine and Ensom, 2001; Wagenmakers *et al.*, 2014), and sample sizes for this study were within range of, or exceeded, historical norms for this laboratory and most others publishing on neurobehavioral effects of BPA. For example, EPA recommends a minimum of 10/sex/group for its guideline neurotoxicity studies and the Organization for Economic Cooperation and Development (OECD)'s guidance document recommends 10–20/sex/group. Nonetheless, insufficient power may at least partially account for why the present results contrast with prior work reporting evidence of heightened anxiety in developmentally BPA-exposed animals.

Use of a battery of corroborative behavioral tasks with high predictive and convergent validity (Chadman *et al.*, 2009; Cryan and Sweeney, 2011; Walf and Frye, 2007) was considered a strength of this design. Diagnostic behaviors, including open arm activity, are expected to be highly concordant across mazes and thus equivalently predictive of behavioral state ([Cryan and Sweeney, 2011] and as an example see Bell *et al.*, 2014). In this study, concordance between some EPM and ZM endpoints was markedly low, suggesting that performance on 1 task was neither predictive nor reflective of performance on the other. Percentage of time spent in the open arms (Supplementary Fig. 3) was consistently higher in the ZM than the EPM for both male and female adults in all exposure groups, an effect consistent with the idea that the ZM is a less aversive test than the EPM (Braun *et al.*, 2011a; Shepherd *et al.*, 1994). Sequential testing might have contributed to low concordance, as some studies indicate that prior exposure to a novel environment increases subsequent activity and open arm exploration, but we do not believe this to be the case, because this effect is not consistently observed (Pellow *et al.*, 1985; Walf and Frye, 2007; Weiss *et al.*, 1998), and test order was carefully considered and within historical norms for behavioral phenotyping (Chadman *et al.*, 2009). Numerous prior studies have reported BPA-related effects in rats (Patisaul and Bateman, 2008; Patisaul *et al.*, 2012) and other species in the EPM (Jasarevic *et al.*, 2011, 2013; Luo *et al.*, 2013; Wolstenholme *et al.*, 2011a) but, to our knowledge, the only prior study using ZM to investigate BPA-related outcomes exposed

the animals during adolescence (Luo *et al.*, 2013), so appropriately analogous historical data are unavailable for comparison.

The influence of gavage on behavioral performance is also a potential concern. It is well known that perinatal stress can remodel the stress axis; amplifying risk of abnormal stress-responsivity, including heightened anxiety, and depressive-like behavior in adulthood (Markham and Koenig, 2011; Russo *et al.*, 2012). In a related study, we showed that prenatal gavage alters estrogen receptor expression in neonatal brain regions fundamental to stress and fear-learning, anxiety, and activity (Cao *et al.*, 2013). Gavage effects eclipsed those of BPA in several instances, raising concerns that gavage itself may interact with BPA exposure to induce molecular, cellular, neural, and behavioral changes. Subsequent studies using their siblings, however, found no differences between gavaged and naïve controls (same strain, same housing facility) on preweaning behavior, OF activity, Barnes maze and water maze performance, novelty preference, motor coordination, adolescent play, running wheel activity, flavored solution intake, female sex behavior, manually elicited lordosis, or circulating corticosterone levels measured at weaning or adulthood (Ferguson *et al.*, 2011, 2012, 2014).

CONCLUSIONS

The present studies represent a portion of the data obtained under the CLARITY-BPA program (Birnbaum *et al.*, 2012; Schug *et al.*, 2013). No compelling evidence of BPA-related effects on anxiety or exploratory behavior was found in developmentally exposed adult NCTR-SD rats, and only limited, inconsistent evidence for heightened anxiety and activity was found in juveniles. Although it is perhaps parsimonious to conclude that perinatal exposure to BPA levels below the NOAEL has little to no impact on affective behaviors, limitations of this study include lower than required statistical power to confidently rule out Type II error, low maze concordance, incomplete statistical identification of all expected behavioral sex differences, light cycle reversal prior to juvenile testing, and potential stress-related effects of gavage (pre- and postnatal) (Cao *et al.*, 2013). Importantly, power calculations are specific to the experiments herein, and not intended nor anticipated to be indicative of probable power levels for other endpoints in the CLARITY-BPA program, particularly those for which effect size is greater, and interindividual variability is anticipated to be lower. Subsequent studies from other CLARITY-BPA projects will provide further resolution on the potential effects of BPA by providing data on other neural-related endpoints, both at the behavioral and molecular levels, and a wealth of other organ systems and outcomes.

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SUPPLEMENTARY DATA

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

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