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Effects of maternal or paternal bisphenol A exposure on offspring behavior

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ABSTRACT

Bisphenol A (BPA) is an endocrine disrupting chemical used in the production of polycarbonate plastics and resins. Exposure to BPA during gestation has been proposed as a risk factor for the development of neurobehavioral disorders, such as autism spectrum disorder. To address the behavioral impact of developmental exposure to BPA, we tested offspring of mice exposed to a daily low dose of BPA during pregnancy. We also asked if preconception exposure of the sire affected behaviors in offspring. Sires that consumed BPA for 50 days prior to mating weighed less than controls, but no effects on any reproductive measures were noted. Juvenile offspring exposed to BPA maternally, but not paternally, spent less time in the open arms of the elevated plus maze than controls, indicating increased anxiety-like behavior. However, neither parental exposure group differed significantly from controls in the social recognition task. We also assessed the behaviors of maternally exposed offspring in two novel tasks: ultrasonic vocalizations (USVs) in pups and operant reversal learning in adults. Maternal BPA exposure increased the duration and median frequency of USVs emitted by pups during maternal separation. In the reversal learning task, females responded more accurately and earned more rewards than males. Additionally, control females received more rewards than BPA females during the acquisition phase of the task. These are among the first studies conducted to ask if BPA exposure via the sire affects offspring behavior and the first study to report effects of gestational BPA exposure on pup USVs and adult operant responding.

1. Introduction

Endocrine disrupting chemicals (EDCs) interfere with the synthesis, secretion, transport, binding, and/or action of endogenous hormones (Gore et al., 2015). Bisphenol A (BPA) is an EDC commonly used in the production of polycarbonate plastics and epoxy resins (Michałowicz, 2014). Human exposure to BPA is widespread: the CDC reports detectable levels of BPA in over 93% of human urine samples (CDC, Centers for Disease Control and Prevention, 2009). BPA is also detected in serum, amniotic fluid, umbilical cord blood, and breast milk (Cao et al., 2015; Ikezuki et al., 2002). BPA is primarily considered a xenoestrogen, capable of binding to the receptors of endogenous estrogens (Kurosawa et al., 2002; Matthews et al., 2001). However, BPA can also bind to various other receptors and proteins to disrupt the functions of thyroid hormone (Chevrier et al., 2013; Moriyama et al., 2002), testosterone (Tanaka et al., 2006; Xu et al., 2005), and glucocorticoids (Poimenova et al., 2010). Hormones play an important role in shaping the developing brain, so it is crucial to understand how gestational exposure to EDCs, like BPA, can affect neurodevelopment and behavior in later life.

Studies in humans and animals have demonstrated associations between gestational exposure to BPA and adverse neurobehavioral outcomes (Mustieles et al., 2015; Palanza et al., 2016). Higher concentrations of BPA measured in urine during pregnancy have been correlated with sex-specific alterations in anxiety, aggression, hyperactivity, and externalizing behaviors in humans (Braun et al., 2011, 2009; Harley et al., 2013; Perera et al., 2012). Environmental factors, such as EDC exposure, have been implicated in the pathogenesis of neurodevelopmental disorders, such as autism spectrum disorder (ASD) (Schug et al., 2015). Two studies reported urinary concentrations of BPA metabolites in children with ASD are higher than in typically developing children (Kardas et al., 2015; Stein et al., 2015).

Similar outcomes have been reported in models of developmental BPA exposure in animals. Several groups have described differences in anxiety-like behavior (Chen et al., 2015; Gioiosa et al., 2013; Xu et al., 2015; Zhou et al., 2015), and motor activity (Anderson et al., 2013; Komada et al., 2014; Zhou et al., 2011), in response to developmental BPA exposure. Some studies report impairments in learning and memory (Kumar and Thakur, 2014; Tian et al., 2010; Xu et al., 2013), while others do not find any effect of BPA on spatial learning (Sadowski et al., 2014). Other types of learning, such as acquisition and reversal in an operant learning task have not been explored. Early-life exposure to

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BPA has also been shown to affect social behaviors in rats (Dessì-Fulgheri et al., 2002; Porrini et al., 2005), mice (Kundakovic et al., 2013; Wolstenholme et al., 2013, 2012, 2011a), prairie voles (Sullivan et al., 2014), and monkeys (Negishi et al., 2014). In our previous studies, we found significant effects of gestational exposure to BPA on social interactions, social preference, and social recognition behaviors in juvenile mice compared to controls (Wolstenholme et al., 2013, 2012, 2011b).

These studies focused on the effects of BPA during gestation or during early postnatal life. Growing evidence supports the notion that sires can transmit the effects of environmental exposures to their offspring, likely via sperm (Kundakovic and Champagne, 2015). For example, paternal exposure to chronic stress affects stress physiology and behavior in offspring (Rodgers et al., 2013), and these effects appear to be mediated by epigenetic mechanisms (Rodgers et al., 2015). Additionally, paternal exposure to BPA in zebrafish disrupts cardiac development in offspring (Lombó et al., 2015). While the negative reproductive consequences of adult exposure to BPA in males have been fairly well-studied (decreased spermatogenesis, lower sperm counts, and reduction in pregnancy rates) (Dobrzyńska et al., 2015; Jin et al., 2013; Liu et al., 2013; Qiu et al., 2013), it remains unclear how preconception exposure to BPA via the sire affects offspring behavior. To date, two studies have reported behavioral effects of preconception paternal BPA exposure (Luo et al., 2017; Fan et al., 2013).

In the current study, we expanded on previous studies by including a paternal exposure group. We administered BPA to the parents via daily treats, which avoids the stress related to oral gavage. Paternally exposed and maternally exposed offspring were tested for social recognition and anxiety-like behavior on the elevated plus maze as juveniles. In maternally exposed mice only, we examined two previously unreported behavioral endpoints in offspring: ultrasonic vocalizations (USVs) in pups and associative learning/perseverative behaviors in an operant reversal learning task (Heyser et al., 2000). The behavioral tests in this study were chosen to address the three core symptoms in ASD: communication difficulties, impaired social interactions, and perseverative behaviors (Crawley, 2007).

2. Methods

2.1. Animals

The mice were generated in our C57BL/6J breeding colony at the Biological Resources Facility at NC State University (Raleigh, NC, USA). The progenitor mice were purchased from Jackson Labs (Bar Harbor, ME). All animals were maintained on a 12:12 light/dark cycle (lights off at 1200) and provided food (soy-free Teklad 2020X; Madison, WI) and water ad libitum. The Institutional Animal Care and Use Committee at NC State University approved all procedures.

2.1.1. Exposure of breeders to BPA

Mice received BPA daily on a small chocolate-flavored treat that weighed approximately 0.5 g (Bio-serv chocolate treats F05472; Flemington, NJ). BPA (> 99% purity, Sigma-Aldrich; St. Louis, MO). BPA was initially dissolved in 100% ethanol, and diluted to a concentration of 2 mg/mL. Ten microlitres of this solution was pipetted onto the treat for a final dose of 20 μg BPA per day. For control treats, the ethanol solution contained no BPA. The ethanol was allowed to evaporate from the treats overnight. This dose was chosen based on the amount of BPA consumed per day using a custom diet with BPA incorporated into the chow (5 mg/kg diet BPA, Teklad 09386). Free BPA levels in the plasma of pregnant dams consuming the BPA diet averaged 3.9 ng/mL (Wolstenholme et al., 2012), which is within the range (0.3–4.0 ng/mL) reported in pregnant women (Schönfelder et al., 2002). Males and females were approximately 3 months of age when dosing began and had no prior breeding experience. For 5 days prior to dosing, all mice received a plain treat to acclimate them to the novel food. By the end of this period, all mice immediately approached and consumed the entire treat.

2.1.2. Maternal exposure

For maternal BPA exposure, singly housed females were randomly assigned to the control group (n = 15) or BPA group (n = 18). Dosing began one week prior to mating; treats were given 1 h after lights out each day. Each female was paired with a naïve male for six days. Males were briefly removed from the cage each day when the treat was presented to ensure the female consumed the entire treat (about 10 min). Daily dosing of the dam continued through gestation. Cages were checked for litters, and dosing ended on the day of birth.

2.1.3. Paternal exposure

To expose sires to BPA, males were randomly assigned to the control group (n = 8) or the BPA group (n = 12) and received a daily treat for 50 days. We chose this length of exposure in order to cover one cycle of spermatogenesis. Beginning on day 51, each male was paired with a naïve female (no exposure to treats). Females were checked daily for the presence of a mating plug. Males remained with females for up to seven days, or until a plug was observed, at which time the male was weighed, euthanized using CO_2, and the testes, seminal vesicles, and epididymis were weighed. The caudal epididymis was reserved for sperm collection (procedure below).

For both maternal and paternal exposure studies, all litters were culled to 6 pups with a balanced sex ratio on the day after birth (postnatal day 1, P1). Litters were weaned on P21 and housed in same-sex, same-treatment groups. No more than one mouse of each sex per litter was used in each behavior test.

2.1.4. F0 male sperm collection and counting

Sperm was collected from males used in the paternal exposure study, on the day the mating plug was detected. No mating plug was detected for one control male and two BPA males and their sperm counts were not included in the analysis. Briefly, the caudal epididymis was removed, placed in a petri dish containing 500 μL of warmed PBS and minced. The dish containing the minced sample was incubated at 37 °C for 15 min to allow sperm to swim out of the epididymis. After incubation, the remaining sperm was gently extruded from the caudal epididymis. The solution was aspirated from the dish with a 200 μL pipette tip and placed in a 1.5 mL centrifuge tube. The dish was rinsed with an additional 500 μL of warm PBS, which was then recovered and added to the same tube. Ten microliters of the sperm solution was loaded into a hemocytometer and counted. All cell counts were performed in triplicate and the average was used for statistics (Wang, 2003).

2.1.5. Social recognition

Each juvenile (postnatal day 28) was acclimated to a test cage (37 × 19 × 13 cm) for 20 min under red lights 1 h after lights off. An empty cylindrical metal holding cell (10 cm diameter × 14 cm tall) was placed in the test cage for the last 10 min of the acclimation period. The social recognition test consisted of two phases: habituation and dish-abituation, as previously described (Wolstenholme et al., 2013). During each one-minute trial, the time the juvenile spent investigating the stimulus mouse was measured. Investigation was defined as the nose of the test mouse within 1 cm of the head or body of the stimulus animal or directly touching the bars of the cylinder. Investigation was scored live during the test by an investigator blind to the treatment and sex of the mice. We tested 24 control mice (6 males and 6 females from the maternal exposure study and 7 males and 5 females from the paternal exposure study). We tested 12 maternally exposed BPA mice (6 males and 6 females) and 18 paternally exposed mice (9 males and 9 females).

2.1.6. Elevated plus maze

Juvenile (P28–32) mice were habituated to the dark testing room

for 1 h under red lights. Each mouse was gently placed in the center of the elevated plus maze (Columbus Instruments, Columbus, OH; wall height: 15.25 cm, arm length: 30 cm, arm width: 5 cm, height above ground: 32 cm) facing an open arm and recorded for 5 min. A trained observer, blind to treatment groups, watched the recordings and scored the time spent in each area of the maze (closed arms, open arms, center area) and the number of crosses between each area (defined as all four paws within the area) using Noldus Observer (Leesburg, VA). We tested 28 control mice (7 males and 7 females from maternal exposure study and 7 males and 7 females from paternal exposure study). We tested 18 maternally exposed BPA mice (7 males and 11 females) and 16 paternally exposed BPA mice (8 males and 8 females).

2.1.7. Ultrasonic vocalizations

Pup ultrasonic vocalizations were recorded on P8, the peak age for vocalization in C57BL/6 mouse pups (Shair, 2007; Young et al., 2010). Dams and their litters were moved to the testing room 30 min after lights-off and habituated for 1 h. After habituation, we tested two randomly selected pups (one of each sex) from each litter. Individual pups were placed in a small cup below an ultrasonic microphone (Avisoft-Bioacoustics CM16/CMPA; Glienicke, Germany) in a sound-attenuating chamber (Med Associates ENV-022S; Fairfax, VT). Ultrasonic vocalizations were recorded for 5 min. The tail of each pup was marked with a sharpie, to differentiate which pup had been recorded, and returned to the rest of the litter. Five minutes after the first recording, the pup was removed again recorded for an additional 5 min. This paradigm is referred to as maternal potentiation (Scattoni et al., 2009). After the second recording, the pup was anesthetized with isoflurane then euthanized by decapitation. We recorded vocalizations from 8 control pups (5 males and 3 females) and 10 maternally exposed pups (5 males and 5 females).

We recorded USVs up to 200 KHz and analyzed them using published methods (Young et al., 2010). The raw signal was cleaned by first filtering with a finite impulse response filter then performing spectral subtraction. In spectral subtraction, the average of the noise in each frequency band is subtracted from the sound (Liu et al., 2003). A sound envelope calculated using this "cleaned" sound was then passed through a thresholder to detect putative mouse calls. The sound files were thus segmented into two categories: putative calls and regions where no call was detected. A trained experimenter used a custom-made interface to ensure that all the putative calls were correctly identified and none were missed by the thresholding algorithm. We assessed the number of calls, call duration, and call median frequency. Call median frequency was calculated by examining the distribution of frequencies contained in a call. For each call, all frequencies in each time segment were counted in the frequency distribution if the power in that frequency and time bin exceeded 3 standard deviations above the mean noise level. The median of this distribution was defined as the median frequency of the call. A total of 6582 calls were analyzed. We also analyzed the number of bursts, which refers to a group of calls that are separated from another group of calls by an interval in time that is statistically longer the mean time between individual calls. Bursts were detected by defining a threshold on the inter-call intervals.

2.1.8. Operant reversal learning

At 15 weeks of age, one male and one female from each litter of maternally exposed offspring were weighed and paired with a same-sex, same-exposure partner from a different litter based on body weight. These matched pairs were housed together. To motivate responding in the operant task, animals were food-restricted to 85% of their initial body weight. Animals were weighed daily and each pair was given a measured amount of food to maintain the desired weights. To habituate to the reward pellets, mice were given pellets (Dustless precision pellets, 14 mg each [F05684]; Bioserv) in their cages for several days before the beginning of the training.

The 5-nose poke hole operant conditioning apparatus (Med Associates MED-NP5M-B1) was housed inside a ventilated, sound-attenuating chamber. Each session in the testing chamber lasted 15 min and took place during the dark. The testing schedule consisted of five consecutive days of training sessions followed by one day off.

During the habituation phase, none of the nose-poke holes were illuminated and no rewards could be earned. Habituation trials continued for three days, or until the mouse poked fewer than 10 times in any one hole, in order to ensure low baseline levels of nose poke activity. During the training phase, two of the five holes were illuminated. One illuminated hole was designated the "active" hole and the other illuminated hole was the "inactive" hole. Only a nose poke in the active hole triggered the release of a pellet into the hopper. The location of the active hole remained the same for each mouse across training sessions. A fixed-ratio schedule determined the number of active hole responses required for a reward pellet. For fixed-ratio 1 (FR1), one active hole response elicited one reward. For fixed-ratio 3 (FR3), three active hole responses were required for one reward, etc.

All animals were trained on an FR1 schedule for 7 days. FR3, FR5, and FR10 sessions each lasted for five days and FR15 was 10 days. Finally, during the reversal phase, the positions of the active and inactive holes for each mouse were reversed at an FR10 schedule for 8 sessions. During reversal, responding in the previously active hole resulted in no rewards, whereas 10 responses in the previously inactive hole was rewarded. Nose-pokes in the hopper and all holes (active, inactive, and unlit) were recorded during each session. The percent accuracy was calculated for each session: active responses/total (active responses + inactive responses) × 100% (Heyser et al., 2000). We tested 12 control mice (6 males and 6 females) and 12 maternal BPA mice (6 males and 6 females).

2.2. Statistics

All data were scored by observers "blind" to treatment conditions. To ensure unbiased scoring, the individual who scored the elevated plus maze videos was kept blind by using coded file names that did not indicate sex or treatment group. Social recognition tests were scored blind by experimenters using a numbered code for each juvenile. Statistics were analyzed using NCSS software. Pairwise interactions were evaluated by Bonferroni-corrected multiple comparisons tests. Partial eta squared values were reported as an estimate of effect size ($\eta_p^2 = SS_{Effect}/[SS_{Effect} + SS_{Error}]$). The control groups from the maternal and paternal exposure studies were combined for social recognition and elevated plus maze behavior, as there were no statistically significant differences between the two control groups. For social recognition, we analyzed the habituation (trials 1–8) and dishabituation (trials 8 and 9) phases separately using three-way repeated measures ANOVA. Elevated plus maze data were analyzed by two-way ANOVA with sex and BPA exposure as the two factors.

For ultrasonic vocalizations, data were collapsed across sexes and trials because we did not detect a statistically significant sex difference, nor maternal potentiation responses. The lack of maternal potentiation is not atypical in mouse studies (Branchi et al., 2004). Operant reversal learning data were analyzed by three-way repeated measures ANOVA and separated according to training schedule (FR1 versus reversal). Body weight was used as covariate in the analysis of reproductive organ weights in F0 males. Sperm counts and body weights were analyzed by pair tests.

3. Results

3.1. Social recognition

BPA exposure primarily affected investigation time during the habituation phase of the social recognition task. Investigation of the stimulus mouse decreased significantly across the habituation trials (1–8) ($F(7,432) = 56.8$, $p < 0.0001$, $\eta_p^2 = 0.54$; Fig. 1). Parent that received

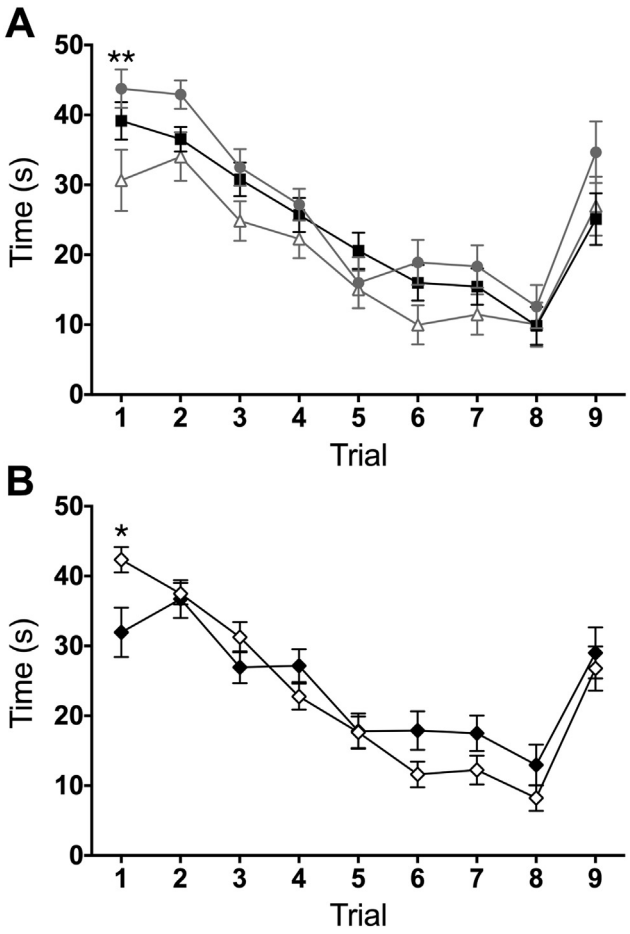

Fig. 1. Social recognition.
Mean ± SEM time (*sec*) spent investigating the stimulus mouse A) grouped by exposure. Black squares represent control males and females, black circles denote maternal BPA males and females, white triangles represent paternal BPA males and females. B) grouped by sex. Black diamonds denote females, white diamonds represent males.
**Maternal BPA group is significantly different from paternal BPA group on trial 1, $p < 0.05$.
*Significant sex difference on trial 1, $p < 0.05$.
Control Males n = 13, Control Females n = 11, Maternal BPA Males n = 6, Maternal BPA Females n = 6, Paternal BPA Males n = 9, Paternal BPA Females n = 9.

BPA exposure group affected the time offspring spent investigating the stimulus mouse ($F(7,432) = 3.3$, $p < 0.05$, $\eta_p^2 = 0.12$; Fig. 1A). Maternally exposed offspring spent more time investigating the stimulus mouse than paternally exposed offspring during the habituation phase. However, neither BPA exposure group was significantly different from the controls. An interaction between sex and trial ($F(7,432) = 4.1$, $p < 0.001$, $\eta_p^2 = 0.08$) revealed that females spent less time investigating a stranger than the males did on trial 1 ($p < 0.05$; Fig. 1B). A three-way interaction between exposure group, sex, and trial demonstrated that the difference between maternal and paternal BPA exposure was primarily caused by behavior of females, specifically on trial 1 ($F(14,432) = 1.81$, $p < 0.05$, $\eta_p^2 = 0.07$). In the dishabituation trials, social investigation increased in response to the novel stimulus female (trial 9 compared to trial 8) ($F(1,108) = 87.8$, $p < 0.0001$, $\eta_p^2 = 0.64$). However, we noted no other significant effects.

3.2. Elevated plus maze

Maternal, but not paternal BPA exposure, increased anxiety-like behavior on the elevated plus maze (EPM). We noted an overall effect of BPA exposure on time spent in the open arms of the EPM ($F(2,61) = 6.1$, $p < 0.01$, $\eta_p^2 = 0.18$; Fig. 2A). Juveniles from BPA-exposed dams spent less time in the open arms than control juveniles ($p < 0.05$). BPA exposure also affected time spent in the closed arms of the EPM ($F(2,61) = 4.1$, $p < 0.05$, $\eta_p^2 = 0.13$; Fig. 2B), and this effect was caused by the difference between maternal and paternal exposure. BPA exposure did not affect time spent in the center of the maze, nor the number of crosses between regions of the EPM ($F(2,61) = 3.0, 1.24$; $p > 0.05$; $\eta_p^2 = 0.10, 0.04$; Fig. 2C and D). No significant effects of sex were found for the time spent in any region (open, closed, or center) or the total number of crosses ($F(1,61) = 1.5, 2.7, 2.1, 0.8$; $p > 0.05$; $\eta_p^2 = 0.03, 0.05, 0.04, 0.01$).

3.3. Ultrasonic vocalizations

There were no significant differences in the number of calls between the first and second recordings, thus the two recordings for each pup were combined. Gestational BPA exposure significantly increased the median frequency and average duration of ultrasonic calls on postnatal day 8. The distribution of the median frequency (kHz) of calls emitted by BPA-exposed pups differed significantly from the median frequency distribution of control pups (interaction between frequency and exposure: $F(22,414) = 1.85$, $p < 0.01$, $\eta_p^2 = 0.10$; Fig. 3A). This was not due to an increase in overall number of calls, as BPA exposure alone did not significantly affect the total number of calls ($F(1,414) = 2.68$, $\eta_p^2 = 0.09$; Fig. 3B inset). BPA exposure also shifted the distribution of call durations towards longer durations ($F(10,198) = 2.17$, $p < 0.05$, $\eta_p^2 = 0.12$; Fig. 3B). The percentage of call durations at 0.01 s (a short call duration) was significantly higher for control pups as compared to BPA pups ($p < 0.05$). Pups exposed to BPA in utero also tended to have more call "bursts" than control pups (Control: 44.0 ± 11.1, n = 8; BPA: 87.2 ± 19.1, n = 10; $F(1,18) = 3.5$, $p = 0.08$, $\eta_p^2 = 0.17$).

3.4. Fixed ratio and reversal learning

There were two primary performance measures in the reversal learning task: accuracy, measured by the percent of correct responses, and number of rewards received. We noted substantial sex differences in the accuracy of responding (percent correct) and number of rewards delivered throughout the operant reversal learning task. Overall, females were significantly more accurate (Fig. 4A) and received more rewards (Fig. 4B) than males ($F(1,928) = 10.7, 31.8$; $p < 0.01$, 0.0001; $\eta_p^2 = 0.35, 0.59$ respectively). Restricting this analysis to the first twelve days of training (FR1 and FR3), correct responses and the number of rewards earned significantly increased over trials ($F(11,288) = 16.7, 34.0$; $p < 0.0001$, respectively; $\eta_p^2 = 0.46, 0.63$; Fig. 5A and B), particularly in females. An interaction between sex and trial was noted ($F(11,288) = 3.07$, $p < 0.001$, $\eta_p^2 = 0.13$; Fig. 5A). Females responded more accurately than males ($F(1,288) = 7.0$, $p < 0.05$, $\eta_p^2 = 0.26$).

Females also received more rewards than males in FR1 and FR3 ($F(1,288) = 68.5$, $p < 0.0001$, $\eta_p^2 = 0.77$) and again we found an interaction between sex and trial ($F(11,288) = 13.1$, $p < 0.0001$, $\eta_p^2 = 0.40$; Fig. 5B). Interestingly, the number of rewards per session was also affected by BPA exposure ($F(1,288) = 4.95$, $p < 0.05$, $\eta_p^2 = 0.20$) and we found an interaction between trial and exposure ($F(6,288) = 1.82$, $p = 0.052$, $\eta_p^2 = 0.08$). BPA-exposed mice earned fewer rewards than controls in FR1 and FR3, and post-tests revealed that this effect was limited to females ($p < 0.05$). Despite earning fewer rewards, there was no effect of BPA exposure on the accuracy of responses in FR1 and FR3 ($F(6,288) = 1.22$, $\eta_p^2 = 0.06$).

During the reversal phase, the accuracy of responding and number of rewards received increased over the 8 sessions ($F(7,192) = 174.4$, 66.81; $p < 0.0001$; $\eta_p^2 = 0.90, 0.77$ respectively). Females continued to be more accurate ($F(1,192) = 4.76$, $p < 0.05$, $\eta_p^2 = 0.19$; Fig. 5C) and receive more rewards than males during reversal ($F(1,192) = 11.29$, $p < 0.01$, $\eta_p^2 = 0.36$). An interaction between sex and trial

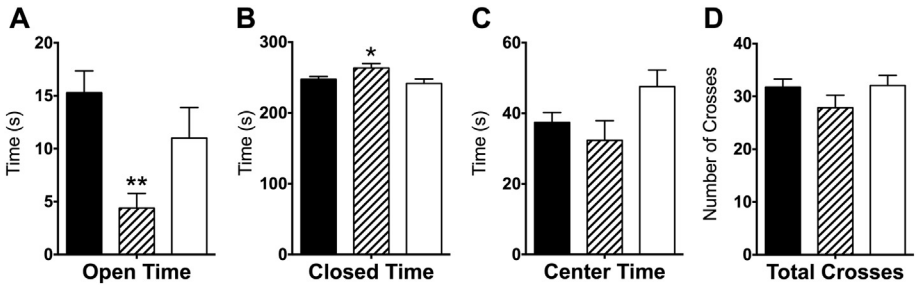

Fig. 2. Elevated plus maze.
Mean ± SEM of time (sec) spent in A) the open arms B) closed arms C) center portion of the maze and D) total number of crosses through the middle of the EPM.
Bars from left to right: Control (Black), Maternal BPA (Striped), Paternal BPA (White).
**Maternal BPA exposure group spend less time in the open arms than control mice, $p < 0.01$.
*Maternal BPA exposure group spend more time in closed arms than paternal BPA exposure group, $p < 0.05$.
Control Males n = 14, Control Female n = 14, Maternal BPA Male n = 7, Maternal BPA Females n = 11, Paternal BPA Males n = 8, Paternal BPA Females n = 8.

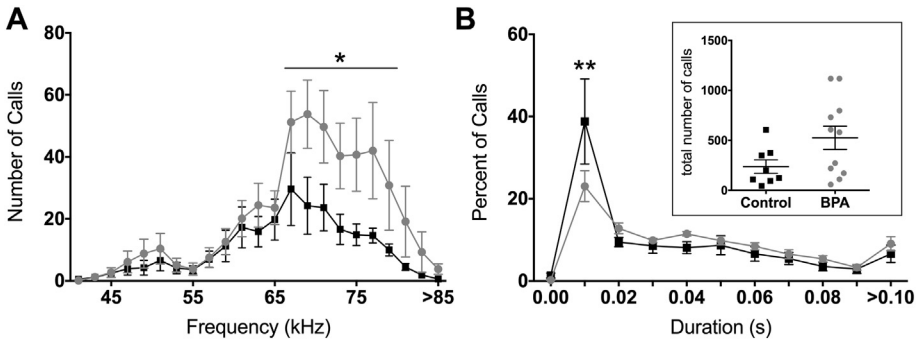

Fig. 3. Median frequency distribution and call durations of ultrasonic vocalizations.
A) Distribution of individual call median frequencies: Mean ± SEM number of calls per frequency bin (kHz) B) Relative distribution of call durations, Mean ± SEM percent of calls per duration bin; inserted graph in B: Mean ± SEM total number of calls, points represent individual pups.
Black squares represent control group, gray circles represent BPA group.
*Significant interaction between median frequency and BPA exposure, $p < 0.01$.
**Control pups display a significantly higher percentage of calls in the 0.01 duration bin than BPA-exposed pups, $p < 0.05$.

Control: n = 8 (5 males, 3 females), BPA: n = 10 (5 males, 5 females).

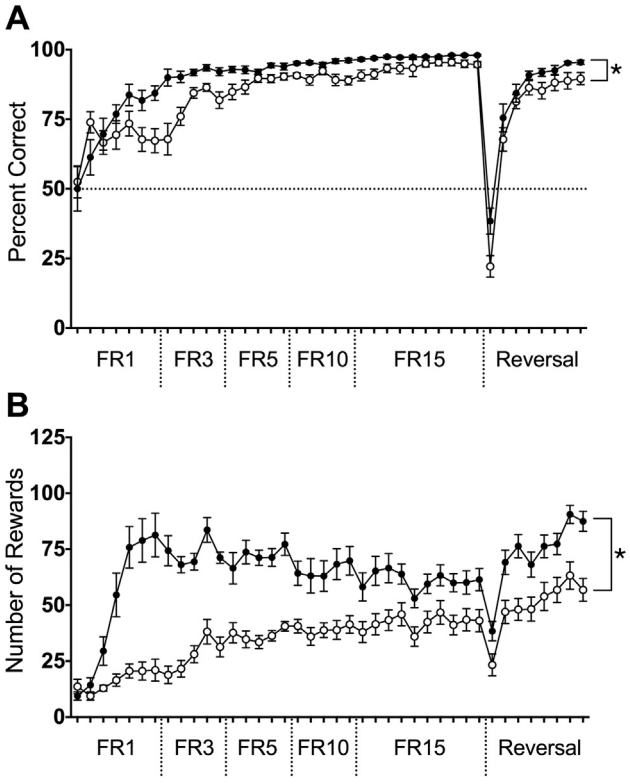

Fig. 4. Sex differences in reversal learning.
Mean ± SEM of A) Percent correct responses in the active hole B) Number of rewards earned per session. Filled circles represent the females and open circles represent the males. FR1 = fixed ratio 1, etc.
*Significant sex difference across all training sessions, $p < 0.001$.
Male n = 12 (6 control, 6 BPA); Female n = 12 (6 control, 6 BPA).

was present ($F(7,192) = 2.27$, $p < 0.05$, $\eta_p^2 = 0.10$; Fig. 5D). However, there were no effects of BPA on accuracy or rewards during reversal ($F(7,192) = 0.35, 0.97$; $\eta_p^2 = 0.02, 0.05$ respectively).

3.5. F0 males

BPA exposure for 50 days in adult males did not affect reproductive outcomes, but significantly impacted body weight. We detected mating plugs in 7 of 8 females paired with control males, but all 8 females paired with control males delivered litters. We found mating plugs in 10 of the 12 females paired with BPA-exposed males, and 11 females paired with BPA-exposed males delivered litters. Adult males consuming BPA for 50 days weighed significantly less than control males at the time of sacrifice ($F(1,20) = 14.9$, $p < 0.001$, $\eta_p^2 = 0.45$; Table 1). Sperm counts were not significantly affected by BPA exposure ($F(1,17) = 0.03$, $\eta_p^2 = 0.01$; Table 1). After adjusting for body weight as a covariate in the analysis, we found no effect of BPA exposure on the weights of seminal vesicles, testes, or epididymis ($F(1,20) = 0.68, 0.24, 0.23$; $\eta_p^2 = 0.04, 0.01, 0.01$ respectively; Table 1).

4. Discussion

Mouse pups exposed to BPA throughout gestation differed from controls in several behavioral measures examined here. However, preconception exposure of the sires to BPA did not change behavior of their offspring in social recognition or EPM. Comparing the effects of paternal and maternal exposure to BPA, we report that neither exposure had strong effects on juvenile social recognition. Juvenile mice maternally exposed to BPA spent less time in open arms of the EPM compared to controls, indicating increased anxiety-like behavior. Pups exposed to BPA in utero also emitted USVs with a higher median frequency distribution and longer duration than controls. We found a significant sex difference in an operant fixed ratio learning paradigm, and a decrease in the number of rewards earned per session by BPA females compared to control females during training.

4.1. Anxiety and social behavior

Juvenile mice exposed to BPA during gestation demonstrated increased anxiety-like behavior on the EPM. This finding has been noted in multiple studies that also report increased anxiety-like behaviors in adult offspring as a result of early life exposure to BPA at various doses. BPA exposure to the sire, however, did not significantly impact

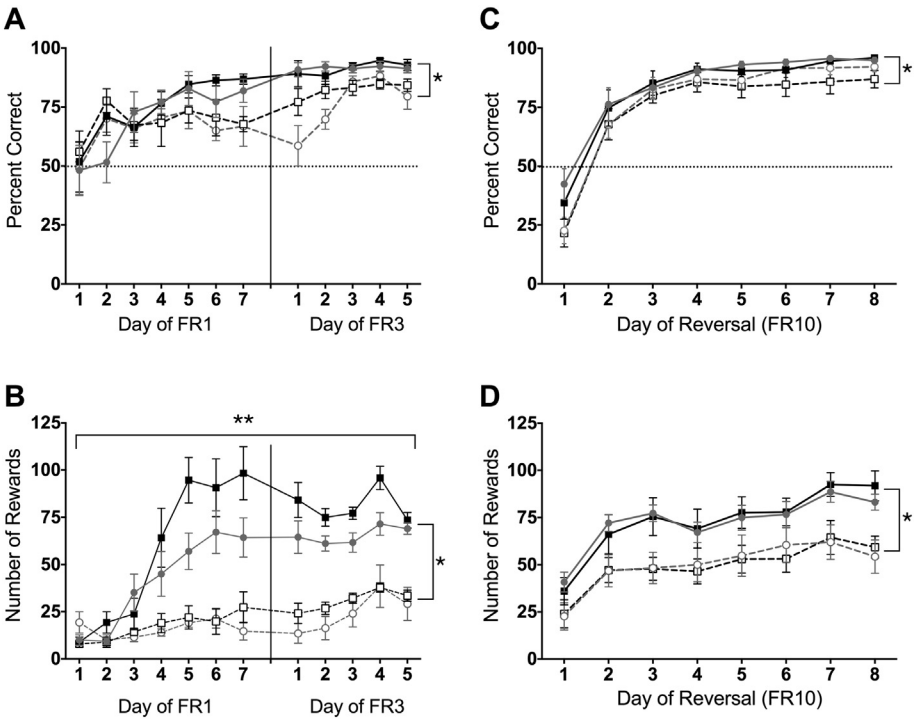

Fig. 5. Percent correct and rewards earned during Fixed Ratio (FR) 1 and 3 and reversal sessions.
Mean ± SEM of A) percent correct responses in active hole during FR1 and FR3 B) number of rewards earned per session in FR1 and FR3 C) percent correct responses in active hole during reversal D) number of rewards earned per session during reversal.
Black squares represent control groups, black circles represent BPA groups. Filled symbols with solid lines are females; unfilled symbols with dashed lines are males.
Dotted line at 50% in A and C represents chance responding.
*Significant sex difference, $p < 0.01$.
**Significant effect of BPA exposure on rewards earned during FR1 and FR3 training, $p < 0.05$.
Control Males n = 6, Control Females n = 6, Maternal BPA Males n = 6, Maternal BPA Females n = 6.

Table 1
F0 male body weights and reproductive organ weights.

	Control males			BPA males		
	Mean	± SEM	n	Mean	± SEM	n
Body weight (g)[a]	32.16	0.9	8	27.35	0.82	12
Sperm counts	2.98E + 06	1.84E + 05	7	2.94E + 06	1.31E + 05	10
Seminal vesicles (mg)	297.7	19.8	8	294.42	13.15	12
Testes (mg)	220.3	5.7	8	214.43	5.44	12
Epididymis (mg)	100.56	15.34	8	88.89	6.88	12

[a] Significant effect of BPA exposure on body weight at the time of sacrifice, $p < 0.001$.

behavior on the EPM of juvenile offspring. It is important to point out that the paternal and maternal BPA dosing paradigms in this study are not perfectly comparable. The paternal exposure was limited to preconception, and meant to affect about 1.3 sperm cycles. The maternal exposure period covered both a short pre- and longer post-conception interval. Furthermore, maternal exposure during gestation may also influence some aspect(s) of the postnatal experience for the pups (i.e. milk quality and maternal care). Perhaps it is not surprising that the behavioral outcomes of maternal and paternal exposures differed.

In our previous studies using gestational BPA exposure, females received a phytoestrogen free diet supplemented with 5 mg BPA per kg diet, this produces a daily dose of about 20 μg per day (approximately 500–800 μg/kg bodyweight per day, depending on the weight of the female). The mice in the current study received a daily treat with 20 μg of BPA. We chose this exposure method to more precisely control the timing and dose of BPA without causing stress to the animal, or having to food restrict mice during mating. Other researchers have used a similar daily oral dosing method and observed behavioral changes in offspring (Ogi et al., 2013; Palanza et al., 2002; Poimenova et al., 2010). Also, in contrast to our previous studies, pups remained with their biological dam instead of being fostered to a control dam at birth.

Exposure to BPA has been shown to affect maternal behavior in a dose-, timing-, and species/strain-dependent manner (Rosenfeld, 2015). However, results differ as to whether maternal behavior produces behavioral changes associated with BPA exposure (Kundakovic et al., 2013). We have previously shown that BPA exposure and cross-fostering interact to produce differing behavioral outcomes on the elevated plus maze (Cox et al., 2010). In that study, juvenile and adult offspring exposed in utero to a higher dose of BPA than we used here (50 mg BPA per kg diet) and raised by their biological dams spent less time in the open arms of the EPM, indicating increased anxiety-like behavior. However, when all mice were fostered to a control dam at birth, gestational BPA exposure did not affect anxiety-like behavior on the EPM in juveniles exposed to two lower BPA doses during gestation (5 mg and 1.25 mg BPA per kg diet) (Wolstenholme et al., 2012, 2011b). Similarly, no effect of BPA on anxiety-like behavior in the open field was found (Wolstenholme et al., 2013). In the current study, we reported a significant decrease in time spent in the open arms of the EPM in maternally, but not paternally, exposed juveniles in this study. This is in agreement with our previous work showing that pups exposed to BPA in utero and raised by their biological dams spend less time in the open arms of the EPM than controls. Likewise, studies that report increased anxiety-like behavior associated with early life BPA exposure (cited previously) did not foster pups to unexposed dams at birth.

In previous studies, we also examined the effect of BPA exposure on social behaviors. Juvenile offspring exposed during gestation to BPA showed significantly different behaviors from controls in a 30-minute social interaction task (Wolstenholme et al., 2012, 2011b). Notably, BPA-exposed mice spent less time engaged in side-by-side interactions and anogenital investigations of their test partner. We also found that gestational BPA exposure reversed sex differences in the social preference task. In the social recognition task used here, juvenile BPA-exposed offspring spent more time investigating a familiar stimulus female than controls during the habituation phase, but the dishabituation responses were unaffected (Wolstenholme et al., 2013). In the current study, neither maternal nor paternal exposure to BPA affected the habituation or dishabituation responses in social recognition. While maternal and paternal exposure groups were significantly different from each other, neither group differed from controls. However, regardless of exposure group, females spent less time than males investigating the stimulus mouse on the first trial. This could potentially indicate that the female juveniles were more reluctant to approach a novel mouse compared to males, but this hypothesis would need to be

tested further before drawing conclusions. We also acknowledge that the group sizes for BPA-exposed mice in this study are on the low end. We tested juveniles at an older age in this study because P21 juvenile control mice were less exploratory and did not exhibit the expected dishabituation response. Testing juveniles one week later may have had an impact on social investigation behavior in this test. However, it is more likely that fostering all pups to control dams at birth interacted with gestational BPA exposure to produce the previously reported differences in investigation during the habituation phase of social recognition (Wolstenholme et al., 2013).

4.2. Ultrasonic vocalizations

To our knowledge, this is the first report of in utero BPA exposure affecting USVs in pups. USVs in rodent pups have been proposed as sensitive behavioral measure in animal models of communication deficits in neurodevelopmental disorders (Scattoni et al., 2009). Mouse pups emit high frequency calls when isolated from the dam and nest as distress signals intended to elicit maternal approach and retrieval (Dirks et al., 2002). Multiple studies in genetic mouse models of neurodevelopmental disorders (*Fmr1*, *Mecp2*, *Foxp2*, etc.) show differences in the number, duration, and types of ultrasonic calls emitted during maternal separation (Lai et al., 2014; Scattoni et al., 2008; Williams et al., 1998, 1995; Young et al., 2010). Pup vocalizations can also be modified by prenatal manipulations like chronic stress, drug administration, and environmental contaminants (Dirks et al., 2002; Mychasiuk et al., 2011; Trezza et al., 2008; Venerosi et al., 2009).

In this study, the large degree of variability between pups in the total number of calls resulted in a non-significant difference between groups for that particular measure. However, separating the individual calls by median frequency revealed a specific increase in high-frequency calls emitted by BPA pups compared to control pups. In rat pups, the frequency distribution of calls emitted during isolation is indicative of the pup's affective state; stressful stimuli increased the number of higher frequency calls (Ise and Ohta, 2009). The rightward shift in median frequency distribution of BPA-exposed pups suggests a heightened sensitivity to stress. This hypothesis is supported by increased anxiety-like behavior in the EPM displayed by juveniles exposed maternally to BPA. In several studies, infant USVs during maternal separation are also correlated with anxiety/depressive-like behaviors in later life (Barua et al., 2014; Trezza et al., 2008; Veronesi et al., 2017). Rats selectively bred across many generations for high levels of USVs during maternal separation demonstrate significantly more anxiety and depressive-like behaviors in later life (Brunelli and Hofer, 2007; Dichter et al., 1996).

We also found that gestational BPA exposure tended to increase ultrasonic call bursts in pups during maternal isolation. Few studies have specifically quantified burst patterns of USVs in pups (Wiaderkiewicz et al., 2013; Young et al., 2010). In adults, bursts of USVs sometimes coincide with specific mating or social behaviors (Wang et al., 2008; White and Barfield, 1990), but the purpose of burst calling in infant rodents remains to be investigated. A recent study reported that prenatal exposure to polychlorinated biphenyls (PCBs) significantly affected USVs produced by rats during affiliative interactions in adolescence and sociosexual interactions in adulthood (Bell et al., 2016). Given the results of this study, future research should address whether gestational BPA exposure alters patterns of USVs emitted during juvenile and adult social interactions.

4.3. Reversal learning

Several studies have noted significant spatial learning and memory deficits in rodents developmentally exposed to BPA (Kumar and Thakur, 2014; Sadowski et al., 2014; Tian et al., 2010; Xu et al., 2013) as well as non-human primates (Elsworth et al., 2015). However, no studies have examined non-spatial, operant conditioning. In the current study, we examined how gestational BPA exposure impacted operant fixed ration leaning and reversal in both sexes in a non-spatial task. We hypothesized that BPA-exposed mice would be more perseverative in the reversal portion of the test than control mice: BPA mice would continue to poke in the previously active hole despite receiving no rewards. However, the main finding was a significant sex difference in the accuracy and number of rewards earned across all training and reversal sessions. Females were significantly more accurate and received more reward pellets per session than males, regardless of gestational BPA exposure. The significant difference between BPA and control females for rewards earned in FR1 did not coincide with decreased accuracy of responding, which likely indicates that BPA is affecting general activity rather than the acquisition of the task. However, we did not directly measure motor activity in adult offspring, so this result is difficult to interpret.

Studies in rats have shown that females outperform males in operant learning tasks unrelated to food such as shuttle box avoidance and passive avoidance (Dalla and Shors, 2009; Kokras and Dalla, 2014). Female mice also perform better than males in a more complex operant task, 5-choice serial reaction time (Groves and Burne, 2016). Appetitive operant learning is somewhat confounded by the necessity of food restriction in order to motivate responding. However, sexually dimorphic body weight and food consumption do not explain this behavioral sex difference. In a progressive ratio operant learning task, male and female rats do not differ in their motivation to respond for a food reward, even after food restriction (van Hest et al., 1988). The enhanced performance of females could be partially attributed to higher motor activity displayed by females as compared to males (van Haaren et al., 1990).

4.4. Paternal preconception exposure

This is one of the first reports to examine behavioral effect of preconception exposure to BPA. First, we established that BPA exposure in males at this dose did not affect sperm plugs, pregnancy rate, sperm counts, or reproductive organ weights. Effects of BPA on male reproductive outcomes vary based on timing and length of exposure, and strain of mouse or rat. We did not expect to find significant alterations in sperm quality or reproductive capacity at this dose, however, we were surprised to find that males exposed to BPA for 50 days weighed significantly less than controls. This is contrary to a recent study reporting an increase in body weight and fat mass of C57BL/6 male mice after 5 weeks of oral exposure to BPA at doses ranging from 5 to 5000 μg/kg body weight per day (Yang et al., 2016). However, other studies in C57BL/6 mice have found no differences in body weight after long-term exposure to BPA (Moon et al., 2015; Takao et al., 2003). Biomonitoring data from the National Health and Nutrition Examination Survey (NHANES) suggest that BPA exposure in adult humans is associated with metabolic syndromes (Teppala et al., 2012). Responses to EDCs like BPA are often non-monotonic, so varying doses of BPA may affect metabolic processes differently (Vandenberg et al., 2012).

We found no effect of preconception paternal BPA exposure on anxiety-like behavior or social recognition in juveniles. A recent study using CD1 sires fed a tenfold higher amount of BPA in diet than used here, reported behavioral effects in F1 offspring on three tasks: open field, elevated zero maze, and social preference (Luo et al., 2017). The results are exciting and support the hypothesis that paternal exposure to BPA can modify behaviors of offspring. The differences between our data are likely caused by differences in the tasks used, BPA dose, and/or mouse strains. It is possible that BPA exposure at this dose does not lead to molecular changes in sperm robust enough to be maintained throughout the extensive epigenetic reprogramming of germ cells that takes place post-fertilization. Therefore, the question of preconception parental exposure to BPA is still an important one that will require further study.

5. Conclusions

The results of this study add to the growing literature on BPA-induced increases in anxiety-like behavior in maternally exposed offspring and expand the sparse knowledge concerning behavioral effects of paternal BPA exposure. Most notably, ours is the first study to report effects of BPA exposure during gestation on ultrasonic vocalizations of pups and associative learning in adult offspring. This observation is confirmatory of the heightened anxiety we and others have noted in BPA-treated mice.

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