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Effects of developmental exposure to bisphenol A and ethinyl estradiol on spatial navigational learning and memory in painted turtles (*Chrysemys picta*)

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ABSTRACT

Developmental exposure of turtles and other reptiles to endocrine disrupting chemicals (EDCs), including bisphenol A (BPA) and ethinyl estradiol (EE2, estrogen present in birth control pills), can induce partial to full gonadal sex-reversal in males. No prior studies have considered whether *in ovo* exposure to EDCs disrupts normal brain sexual differentiation. Yet, rodent model studies indicate early exposure to these chemicals disturbs sexually selected behavioral traits, including spatial navigational learning and memory. Thus, we sought to determine whether developmental exposure of painted turtles (*Chrysemys picta*) to BPA and EE2 results in sex-dependent behavioral changes. At developmental stage 17, turtles incubated at 26°C (male-inducing temperature) were treated with 1) BPA High (100 μg /mL), 2) BPA Low (0.01 μg/mL), 3) EE2 (0.2 μg/mL), or 4) vehicle or no vehicle control groups. Five months after hatching, turtles were tested with a spatial navigational test that included four food containers, only one of which was baited with food. Each turtle was randomly assigned one container that did not change over the trial period. Each individual was tested for 14 consecutive days. Results show developmental exposure to BPA High and EE2 improved spatial navigational learning and memory, as evidenced by increased number of times spent in the correct target zone and greater likelihood of solving the maze compared to control turtles. This study is the first to show that in addition to overriding temperature sex determination (TSD) of the male gonad, these EDCs may induce sex-dependent behavioral changes in turtles.

1. Introduction

Endocrine disrupting chemicals (EDCs) are widely distributed throughout aquatic and terrestrial environments (Reviewed in (Bhandari et al., 2015)). Two abundant EDCs are bisphenol A (BPA) and ethinyl estradiol (EE2). As a plasticizer, BPA is present in a wide range of commonly used household items, including plastic storage containers and cardboard products. Current estimates indicate global production of BPA at 15 billion pounds annually (GrandViewResearch, 2014). Bisphenol A has been identified in almost all aquatic habitats tested to date (Reviewed in (Bhandari et al., 2015)), thus, causing concern for widespread and continued exposure of humans and wildlife. Ethinyl estradiol is the estrogen present in birth control pills. The unmetabolized form can be excreted in the urine, and thus, can also collect in aquatic ecosystems (Laurenson et al., 2014).

Many xenoestrogens can induce gonadal sex reversal in reptilian species that exhibit temperature sex determination (TSD) (Bergeron et al., 1999; Bull et al., 1988; Clairardin et al., 2013; Crews et al., 1991; Crews et al., 1995; Crews et al., 1996; Freedberg et al., 2006; Gutzke and Chymiy, 1988; Jandegian et al., 2015; Kohno et al., 2015; Sheehan et al., 1999; Willingham and Crews, 1999). Recently, we showed that *in ovo* exposure of eastern painted turtles (*Chrysemys picta*) to BPA and 17β-estradiol results in partial to full gonadal sex reversal in animals incubated at the male-inducing temperature, 26°C (Jandegian et al., 2015). In Olive Ridley sea turtles (*Lepidochelys olivacea*), estradiol

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treatment leads to hypoplastic ovaries but delayed remodeling of the medullary cords and suppression of testicular factor Sox9 (Diaz-Hernandez et al., 2015). While it is generally thought that *in ovo* exposure to exogenous estrogens fully replicate the effects of ovarian-promoting temperature incubation, this study showed that estradiol but not the female-permissive temperature causes an up-regulation of *FoxL2* prior to the expression of aromatase.

In mammalian species, brain sexual differentiation is driven by the organizational and activational effects of gonadal hormones (Arnold and Breedlove, 1985; Morris et al., 2004; Phoenix et al., 1959). Organizational and activational effects have also been reported in male leopard geckos (*Eublpharis macularius*) (Rhen and Crews, 2000). An initial prenatal surge of testosterone, which may be converted to estrogen in certain brain regions, guides the initial programming. Full elaboration of sex-dependent behaviors requires a later adult increase in testosterone (Arnold and Breedlove, 1985; Morris et al., 2004). As sexual differentiation of brain is guided by sex hormones, it is likely vulnerable to disruption by EDCs found in the environment.

Past rodent and other animal studies show that early exposure to BPA or EE2 can disrupt in a sex-dependent manner various behavioral domains, such as cognition, anxiety, and socio-sexual (Reviewed in (Rosenfeld and Trainor, 2014)). For instance, male deer mice (*Peromyscus maniculatus bairdii*) exposed to BPA or EE2, during the perinatal period through the maternal diet, demonstrate compromised spatial navigational learning and memory (Jasarevic et al., 2011); whereas, performance of exposed females was comparable to control females. In contrast, Sprague-Dawley female but not male rats developmentally exposed to BPA exhibited impairment in this behavior (Johnson et al., 2015). Exploratory behavior is reduced in BPA-exposed female California mice (*Peromyscus californicus*) (Williams et al., 2013), who rely on this behavior to locate food sources for their young.

To verify that endogenous or exogenous hormones alone can impact brain sexual differentiation, the behavioral effects of these chemicals should be tested in a species lacking sex chromosomes (e.g. painted turtles). While many turtles exhibit TSD instead of sex-chromosomal dependent gonadal sexual differentiation, there are similarities in the brain organization, such as the hippocampus, between the turtle/archosaur lineage and mammals (Striedter, 2015). During the temperature sensitive period (TSP) of embryogenesis, activity of brain aromatase, the enzyme that converts testosterone to estrogen, rises in potential female turtles relative to presumptive males and then declines prior to hatching (Willingham et al., 2000). This pattern of expression suggests that elevated brain estrogen concentrations during this critical window of time may orchestrate neurobehavioral programming.

The objective of the current study was to determine whether painted turtles incubated at the male-inducing temperature and exposed to BPA or EE2 would demonstrate changes in learning and memory compared to non-exposed, control males. To test for such effects, treated and control individuals were tested as juveniles with a spatial behavioral test designed for turtles (López et al., 2001). This semi-aquatic species has previously been shown to possess excellent spatial memory abilities (Bowne and White, 2004, Bowne, 2008; Krochmal et al., 2015; Petrillo et al., 1994; Roth and Krochmal, 2015, 2016).

2. Materials and Methods

1. Incubation of Turtle Eggs and Distribution of Treatments

Recently laid painted turtle eggs (203) were purchased from Louisiana Cypress Turtle Farms (Pierre Part, LA). The eggs were maintained and transported in clutches as laid. Beforehand, an import permit was obtained from the Missouri Department of Conservation (MDC), and in June 2014, eggs were transported by automobile from Louisiana to Columbia, Missouri. The experiments were performed in accordance with the NIH guidelines for the care and use of laboratory animals and under the approved MU ACUC animal protocol 8165.

Upon arrival at the Bond Life Sciences Center, University of Missouri, Columbia, MO, the eggs were candled to determine viability and estimate the developmental stage, as detailed in (Mahmoud et al., 1973). To avoid potential clutch bias effects, each egg was pre-assigned to one of the five treatments listed below. The egg was placed in moistened vermiculite (1:1 *v*/v autoclaved vermiculite:water) within a BPA-free incubation ("tackle") box made of polypropylene that allowed each egg to be incubated in individual compartments with each box holding ~20 eggs. While BPA has not been detected in polypropylene cages (Howdeshell et al., 2003), it remains to be determined whether this type of container releases BPS and BPF. Both of these BPA-replacement chemicals have come under recent scrutiny, especially in fish (Kinch et al., 2015; Tisler et al., 2016). Notwithstanding, polypropylene is to date considered one of the best types of housing material for various animal models used in endocrine disruptor studies. The boxes containing the eggs were then placed in an incubator at the male-producing temperature (26 ± 1°C). Two thermometers were used to ensure that the incubator maintained the correct temperature throughout the incubation period (June to late July/early August). A sign was placed on the outside of the incubator door to designate it as containing live turtle eggs.

The boxes were weighed on an Ohaus digital scale (Pleasant Prairie, WI) three times weekly and additional water was added to remoisten the vermiculite and maintain proper hydration of the eggs (Jandegian et al., 2015). The boxes were then rotated in the incubator to minimize any positional effects. Candling was used to monitor embryonic development and determine the developmental stage. When the turtles reached the TSP (~ Stage 17), the pre-assigned topical treatments were applied to the egg via a micropipette. The treatments included 1) BPA High (100 μg /mL), 2) BPA Low (0.01 μg/mL), 3) EE2 (0.2 μg/mL), or 4) vehicle control (Ethanol) or no treatment (both considered negative control groups) (Jandegian et al., 2015). The two doses of BPA and single dose of EE2 are reflective of that which may be encountered in the aquatic environment (Bhandari et al., 2015; Jandegian et al., 2015; Sheehan et al., 1999; Zhang et al., 2015). As discussed below, no differences were detected between the two control groups (ethanol and no treatment), and thus, these results were combined together.

Shortly after the turtles hatched (mid-July to early August 2014), they were weighed and given an identifying notch on the marginal scute of the carapace. Out of the original eggs, 112 turtles hatched (53% hatching success), and 108 survived throughout the experiment. Of these, there were 19 BPA High, 22 BPA Low, 23 EE2, 20 vehicle control, and 24 no treatment control. These hatching rates are less than our earlier study where we had 84% average hatching success (Jandegian et al., 2015). However, in the previous and current study, hatching success was not influenced by treatment. Both studies used eggs from the same commercial source and maintained the eggs under similar conditions. There could, however, be annual variability in hatching rates due to variation in rainfall and other environmental factors. It also has been shown that older painted turtle females produce eggs with reduced hatching success (Warner et al., 2016). Unfortunately, these data were recently published, and thus, we had not considered requesting the age of the breeder female for each clutch. In the future, such information will be requested.

Individuals were transported from the incubation boxes in the Bond Life Sciences Center to 100 gal BPA-free glass aquarium (Marineland, Blacksburg, VA) housed within the animal facility in Connway Hall on the MU campus. The turtles were at this facility for the entire duration of the study. On an every other day basis in the morning, the turtles were ad libitum fed Mazuri turtle pellets (Purina, Montgomery, MO). On the behavioral trial days, turtles were not fed until after the trials were completed.

For behavioral testing, 12 turtles from each of the five groups were randomly selected for a total of 60 turtles. This number of animals was

predicted to provide sufficient power based on our prior rodent and turtle studies (Jandegian et al., 2015; Jasarevic et al., 2011; Johnson et al., 2015; Williams et al., 2013). As we could not perform behavioral testing on all of the turtles at the same time, they were randomly divided into five testing loads each composed of 12 individuals, three exposed to ethanol (vehicle) or no vehicle treatments (Control group), three to BPA high, three to BPA low, and three to EE2. Therefore, each load had representative animals from all treatment groups. Each load was housed into individual 100 gal BPA-free glass aquarium from hatching to completion of trials.

2. Spatial Navigation Maze Design and Testing Room

The maze was a four-sided pool (1 m × 1 m × 20 cm) with curved corners composed of white BPA free polypropylene sheets purchased from US Plastic (Lima, OH). Care was taken to ensure that only BPA-free materials were used to construct the maze. Black Bull adhesive (DAO-P Inc., Bronx NY) with primer was used to adhere the polypropylene plates and metal fasteners. One pyramidal white polypropylene food container (3.25 in × 2.25 in at top, 5.5 in × 4 in at bottom × 5 in), with ¼ in. square aluminum metal mesh work on the sides extending from the top edge to 3.25 in down that allowed the turtles to grip onto the surface (Fig. 1), was located in the center of each side of the maze, approximately 2.25 in from the wall for a total of four food containers (Fig. 1). With this design, the food in the "baited" container is above the water level but stays within respective container. On the wall of the maze, above each polypropylene food container, removable shapes were placed such that each food container was delineated with its own intra-maze spatial cue. The shapes all painted in red included a star, square, circle, and triangle of similar sizes (approximately 2.5 in × 2.5 in placed 5.25 in from the bottom of the wall). We chose to use red shapes as extensive studies have shown that different turtle species, including painted turtles, can distinguish different colors with some being exquisitely sensitive to those in the red end of the spectrum (Arnold and Neumeyer, 1987; Graf, 1967; Twig and Perlman, 2004; Ventura et al., 1999; Zwick and Holst, 1976). The center of the maze was delineated by a 6 in × 6 in square, which is where the turtles were placed at the start of the maze. An 8 ft. tall metal stand held a Canon Vixia HF HD hand held camcorder (Canon, Melville, NY) and two 100-watt lights over the center of the maze. Lights were positioned to ensure no reflection was cast on the water to interfere with the video recording. The 10.5 ft. × 13.5 ft. room which held the maze had no markings on the walls and location of objects within the room was consistent for each trial day.

3. Habituation Period

Two wk. prior to behavioral testing, three food containers (similar to those used in the maze test) were refilled each day and added to the home aquaria to habituate the turtles to the food containers. Each trial day, the turtles within each load were brought into and habituated to the testing room 30 min prior to the start of the experiment. The two overhead lamps and video camera were turned on and the door was shut to reduce background noise. Electronics were silenced and a "Do Not Disturb" sign placed on the outside of the door. Two Nalgene BPA-free carboys filled with 20L of water, at room temperature (26 °C), were poured daily into the maze prior to the initiation of the trials. Water was previously tested by Dr. Christopher Kassotis in Dr. Susan Nagel's laboratory in accordance with their published methods (Kassotis et al., 2015), and no BPA was detected. The water level within the maze was brought to the rim of the food container to about 4.9 in in depth to keep the food from drifting off the container. After the maze was filled with water, frozen brine shrimp (Hikari, Hayward, CA) was rubbed 10 times on each food container rim to reduce any potential confounding olfactory cues, as was done previously (López et al., 2001). Mazuri® Aquatic Turtle Diet pellets and frozen brine shrimp (~three to four of each) were placed on top of the food container corresponding with the assigned hole for each of the turtles to be tested.

4. Maze Testing

The behavioral tests commenced each day at 09.00 h, and lasted for approximately 2 h to allow time to test all turtles within each load. The testing order for turtles within the load was randomized. The turtles were selected out of the tank and identifying notches were noted. Within each load, turtles were randomly assigned one of four removable intra-maze cues (i.e., circle, star, triangle, and square) for the entire duration of the testing period such that the assigned spatial cue remained the same for each turtle throughout the study period. The assigned intra-maze visual cue corresponded to the assigned container that was baited with food. The appropriate food container for the assigned turtle was then baited with the reward: Mazuri® Aquatic Turtle Diet pellets and frozen brine shrimp. Before each trial, the turtle was placed in a 4 in × 4 in × 5 in open-ended columnar holding container, in the center of the maze or "release zone" (Fig. 1A). Once the turtle was placed in this area, video recording was initiated and, the column was lifted to release the turtle (Fig. 1B, Supplemental Video S1), which designated the start time of the maze. The trial ended either after the turtle located the correct food container or the duration of 600 s elapsed without the turtle finding the correct food container. Latency (time to solve the maze) and trial number were recorded. If the turtle did not find the correct food container in the 600 s duration on the first day, the animal was gently guided to it to increase the likelihood of finding the correct food container on subsequent trial days. Between each trial, the water was stirred with a glass rod to dilute any potential olfactory cues in subsequent testing. After the trials for the day were completed, water within the maze was drained. Each turtle within a load was tested once daily for 14 consecutive days, and then the next load was tested. To test all five loads, maze testing was conducted from mid-January to early April 2015.

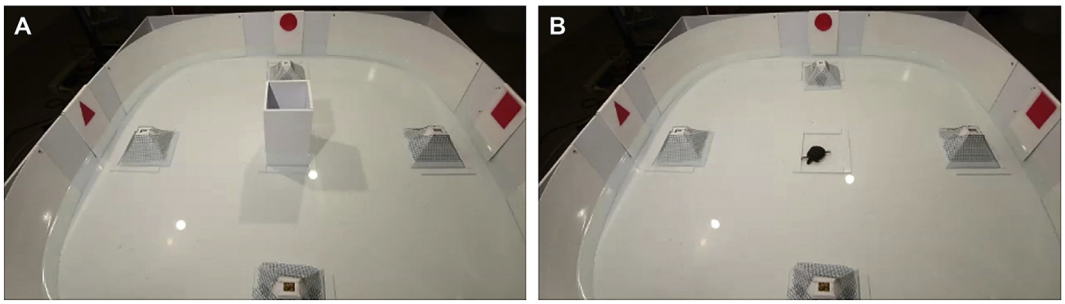

Fig. 1. Spatial navigation maze design for turtles. A) The picture shows the maze design with the four food containers and associated intra-maze visual cues. The turtle was placed in the center of the maze, and a holding container was placed over this region until the trial commenced. B) At the start of the trial, the container was lifted and the ability of the turtle to locate the correct food container within 600 s was assessed.

5. Video Trial Behavior Analysis

After testing was completed, the archived videos were imported into the ANY-maze (Stoetling Co. Wood Dale, IL) software analysis system for behavioral analyses. Indices that were measured included, total distance traveled from the initiation of the trial until the turtle located the correct food container, velocity during the trial period, latency to locate the food (as evidenced by the animal climbing up to the correct food container), and number of entries into the correct target zone or quadrant area, as determined by the ANY-maze program which divides the circular maze into four equal quadrants with only one being the correct one. The turtle only needs to enter this defined region for it to be considered a correct entry. The number of entries into the correct quadrant area is determined based on each trial day for a given turtle.

6. Statistics

Because each egg within a clutch was randomly assigned to one treatment group, the individual rather than the clutch was considered the statistical unit, i.e. the denominator of F. The trial days were grouped into early (1 to 6), mid (7 to 10), and late (11–13 or 14) testing stages. The rationale for this approach was that initial analyses with individual days suggested that behavioral performance for latency and incorrect *vs.* correct entries clustered into these three groupings. For load 1, the day 14 trials failed to be recorded; thus, the data for late trial days represent days 11–13. Because a stop-watch was used to record latency, these data include up through day 14. There were no statistical differences between the vehicle and no treatment control groups, and thus, these results were combined into one control group.

The total distance traveled, velocity, and number of entries into the correct target quadrant were analyzed with a split plot in time (a repeated measure ANOVA-based analysis), which included the effects of treatment and testing stages (early, mid, and late) and potential interactions of treatment and testing stages. Fisher's protected Least Significant Difference (LSD) was used to determine mean differences, and Fisher's posthoc analysis was used to follow up on significant interactions. SAS version 9.2 Software (SAS Institute, Cary, NC) was employed for these analyses.

Latency data were analyzed by using the PROC LIFETEST and Proportional Hazard Ratio (PROC PHREG) functions in the SAS version 9.2 software analyses, as detailed previously (Johnson et al., 2015). Within each testing stage, all groups were compared to each other to determine if one group had a greater likelihood of finding the correct container within the allotted time relative to all other groups tested. These analyses adjust for right-censoring (defined here as not locating the escape box within the allotted time) while still accommodating the study design of 600 s per trial. These methods had to be employed as not all turtles located the correct container within the allotted 600 s. It is not clear if animals were provided more time whether they would or would not locate it. Therefore, these individuals cannot be assigned the maximum time of 600 s and the data analyzed by conventional ANOVA methods. Data are reported as a hazard ratio that signifies the odds of a subject in a treatment group locating the correct food container compared to the other groups tested. A significant result indicates the odds are not 1:1. A result >1 indicates the test group finds the food container more quickly than all other groups tested. A result <1 indicates that the treatment group is less likely to locate the food container compared to the other study groups. In other words, an increased likelihood corresponds to shorter latency; whereas a decreased likelihood represents an increase in latency. As not all of the turtles located the correct food container within the allotted time, likelihood is the best descriptor. The individual, rather than the clutch, was again used as the denominator of *F* (within individual variability) to examine the effects of treatment and testing stages (early, mid, and late) and potential interactions between treatment and testing stages. Latency data are reported as the mean, 95% lower and upper confidence limit.

To determine if there were any differences at each test stage in actually locating the food container within the allotted time of 600 s, all of the groups were first analyzed together with Chi-Squared Analysis. To pinpoint differences between two groups, pairwise comparisons were then performed with a Continuity Adjusted Chi-Squared Analysis where a p value ≤0.05 was considered significant.

3. Results

1. Total Distance Traveled and Velocity

For distance traveled, there was a significant interaction between treatment and testing stages ($p = 0.009$). On the late trial days, BPA High animals traveled less distance than Control and EE2-exposed turtles ($p \leq 0.05$, Fig. 2A). For velocity, there were no main or interaction effects for treatment and testing stages (treatment*testing stages, $p = 0.7$, Fig. 2B).

2. Number of Entries into the Correct Target Zone and Latency

There was a significant two-way interaction (treatment*testing stages) for the number of times spent in the correct target zone ($p = 0.02$). On the early trial days, turtles in the BPA High group exhibited greater number of times spent in the correct target zone than Controls ($p = 0.01$, Fig. 3). On these same trial days, EE2-exposed animals showed greater number of times in the correct target zone than those in the Control and BPA Low groups (p value range = 0.0002 to 0.005). These same differences between EE2 compared to Controls and BPA Low animals were evident at the mid-trial days (p value range = 0.01 to 0.04). By the late trial days, no differences were evident between any of the groups for number of times spent in the correct target zone. However, for reasons that are not clear, individuals within the EE2 group show reduced entries into the correct target zone by the late stage relative to the early stage ($p = 0.009$).

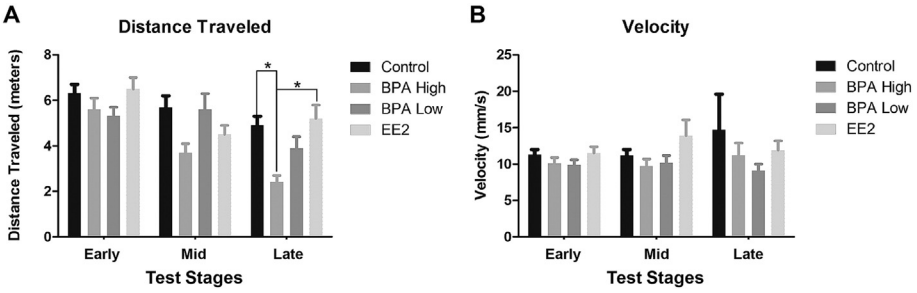

Fig. 2. Distance traveled and velocity within the maze. A) Distance traveled in the maze. No differences in distance traveled were observed at the early and mid-stages. However, individuals within the BPA High group traveled less distance than those in the control and EE2 at the late stage. B) Velocity. No differences in velocity were detected between any of the groups at any of the stages tested. *$p \leq 0.05$.

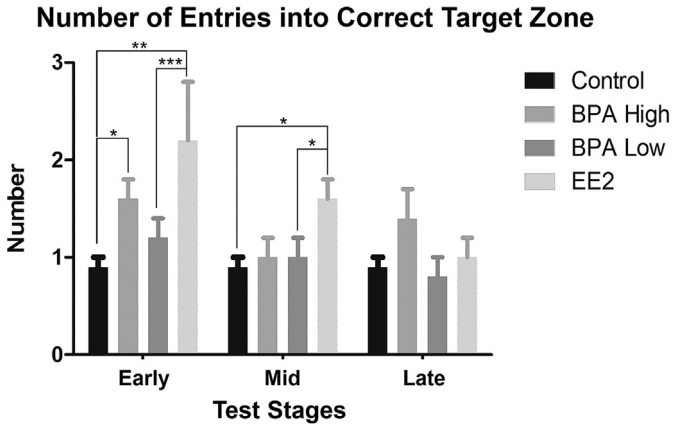

Fig. 3. Number of times spent in the correct target zone. At the early stage, BPA High individuals exhibited more correct entries than controls. At the early- and mid-stages, EE2 demonstrated greater correct entries than control and BPA Low groups. At the late stage, no differences were observed in correct entries between any of the groups. Individuals within the EE2 group show reduced entries by the late stage into the correct target zone relative to the early stage ($p = 0.009$). *p ≤ 0.05, **$p = 0.00002$, and ***$p = 0.005$.

Table 1
Percentage of each group that located the correct escape hole at each test stage

	Percentage (# that located the correct escape hole/total trials for each test stage)*		
	Early	Mid	Late
Control	6.94 (10/144)[a]	14.58% (14/96)[a]	15.63 (15/96)[a]
BPA High	18.06 (13/72)[b]	33.33 (16/48)[b]	47.92 (23/48)[b**]
BPA Low	8.33 (6/72)[a,b]	16.67 (8/48)[a,b]	22.92 (11/48)[a]
EE2	6.94 (5/72)[a,b]	33.33 (16/48)[b]	22.92 (11/48)[a]

* The total number includes all of days within each test stage. The total value for the control group is twice that of other groups as this group includes those individuals who received the ethanol vehicle control and no treatment.
[a,b] Values with different superscripts are significant at p ≤ 0.05, as determined by Continuity Adjusted Chi-Squared Analysis.
** For the late stage, the BPA High significantly differed from the Control group by $p < 0.0001$.

For latency, the PROC LIFETEST analysis showed a significant interaction between treatment and testing stages ($P < 0.0001$). The Proportional Hazard Ratio analysis was then used to determine which comparisons were significantly different. On the early trial days (1–6), the BPA High group demonstrated an increased likelihood or reduced latency of locating the correct food container relative to Controls ($p = 0.02$, Fig. 4). The differences between these two groups became even more significant in the mid (7–10) and late (11–14) trial days ($p = 0.009$ to <0.0001). Additionally, EE2-treated animals were more likely, decreased latency, to locate the correct food container at the mid-trial days than Controls ($p = 0.009$).

We also considered whether there were differences in number of individuals within each group that located the correct food container within the allotted time. BPA High individuals were more likely to locate the correct food container than controls at the early, mid, and late stages (Table 1). By the late stage, the differences between these two groups became quite pronounced (BPA High = 47.92% vs Control = 15.63%, $p < 0.0001$). At the late stage, the BPA High group was also more likely to locate the correct food container relative to the other two groups: BPA Low and EE2 ($p < 0.05$). EE2 individuals show improved responses relative to controls by the mid-stage ($p < 0.05$). However, by the late stage, there was no difference between these two groups with fewer individuals in the EE2 group locating the correct container in the allotted time. It is not clear why performance in this group seems to diminish at this stage.

4. Discussion

The primary goal of this study was to determine if in ovo exposure of painted turtles to BPA or EE2 would exhibit changes in spatial learning and memory relative to unexposed controls. We and others have previously shown that turtle eggs incubated at the male-inducing temperature that are developmentally exposed to EDCs are partially to fully sex-reversed to females (Bergeron et al., 1999; Bull et al., 1988; Clairardin et al., 2013; Crews et al., 1991; Crews et al., 1995; Crews et al., 1996; Freedberg et al., 2006; Gutzke and Chymiy, 1988; Jandegian et al., 2015; Kohno et al., 2015; Sheehan et al., 1999; Willingham and Crews, 1999), although developmental exposure to exogenous estrogens may not fully replicate all of the effects observed in eggs incubated at the female-inducing temperature (Diaz-Hernandez et al., 2015). Thus, we sought to determine whether these estrogenic chemicals could disrupt normal brain programming, which might be due to direct effect of these chemicals on the various brain regions and/or by inducing partial to full sex reversal to female gonads. If behavioral differences are observed, it may suggest that sexually dimorphic differences exist in spatial navigational learning and memory and the brain areas governing this behavior. However, there is a paucity of

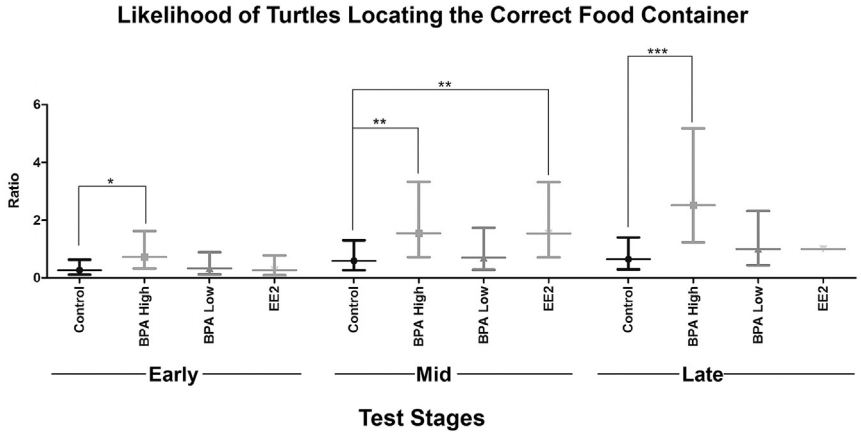

Fig. 4. Likelihood of turtles in each treatment group locating the correct food container in the allotted time. Note that increasing ratio corresponds to shorter latency. The upper, middle, and lower bars represent the ratio of locating the correct escape hole at the 95% upper confidence limit, mean, and 95% lower confidence limit, respectively. In the early stage (1–6), the BPA High group demonstrated an increased likelihood or reduced latency of locating the correct food container relative to controls. The differences between these two groups became even more significant in the mid (7–10) and late (11–14) stage. Additionally, EE2-treated animals were more likely (decreased latency) to locate the correct food container at the mid-trial stage than controls. *$p = 0.02$, **$p = 0.009$, ***$p < 0.0001$.

studies examining sex differences in navigational behavior in turtles and other reptilian species.

Species, such as painted turtles, that lack sex chromosomes are likely valuable models for testing predictions on whether sex steroid hormones alone are sufficient to stimulate sexual differentiation of the brain. A recent study with red-eared slider turtles (*Trachemys scripta*) who were subjected to one of three pre-hatching manipulations: estrogen sulfate or corticosterone exposure or thermal fluctuations revealed that none of these treatments affected hatchling behaviors, including "righting response" and exploration (Carter et al., 2016). However, early exposure to estrogenic chemicals may manifest later in life.

Painted turtles exhibit excellent spatial navigational abilities (Bowne and White, 2004; Bowne, 2008; Krochmal et al., 2015; Petrillo et al., 1994; Roth and Krochmal, 2015, 2016). They may have evolved this behavior such that they could rapidly navigate the terrain to avoid predation, hypo- or hyperthermia, and other stressors (Bowne, 2008; Roth and Krochmal, 2016). While this species may use multiple cues, vision, including UV vision, appears to help facilitate learning specific routes (Roth and Krochmal, 2015). There also appears to be a critical window of time during the juvenile period, i.e. prior to 4 years of age, when painted turtles must learn to navigate specific paths, otherwise they will never acquire this ability (Roth and Krochmal, 2015, 2016). However, most of the prior studies did not mention sex(es) of the turtles studied (Krochmal et al., 2015; Petrillo et al., 1994; Roth and Krochmal, 2015, 2016), and the two studies that examined both sexes did not comment on whether there were sex-dependent differences in this behavior (Bowne and White, 2004; Bowne, 2008). A meta-analysis of several studies of aquatic and semi-aquatic turtle species suggests sex and social structure do not influence home range size defined as the area traversed by an animal during normal activities (Slavenko et al., 2016). Instead, it is affected by diet (greatest in omnivores relative to herbivores and carnivores) and aquatic status (aquatic greater than semi-aquatic). Even though the home range size does not seemingly differ based on turtle sex, there still may be differences in spatial memory.

Notably, our data indicate that *in ovo* exposure of turtles to a high dose of BPA and to a lesser extent, EE2, improves spatial navigational learning and memory compared to controls and individuals exposed to a low dose of BPA, as evidenced by the greater number of entries into the correct target zone and increased likelihood of solving the maze in the latter groups. As shown in Table 1, BPA High individuals were more likely to locate the correct food container in the allotted 600 s than controls. By the late stage, the difference between these two groups becomes even more striking. Additionally, this is also accompanied by decreased distance traveled at the late stage for BPA high-exposed individuals. Performance by EE2 individuals seems to peak at the mid stage where they show greater number of entries into the correct target zone, are more likely to solve the maze, and more individuals in this group locate the correct food container within the test period. However, by the late stage, their performance declines. It is not clear why this is the case. Potential possibilities include that they were not motivated to locate the correct food container at the later trial days or they were distracted by other cues that were imperceptible to the other groups. To examine further how early exposure to EE2 affects spatial navigational learning and memory in turtles, eggs incubated at the female- inducing temperature and exposed to EE2 should be tested, as well as different doses for those incubated at both the male- and female-inducing temperatures.

The observed behavioral differences in the BPA and EE2 groups might be due to alterations in brain aromatase activity. Brain aromatase concentrations differ during the TSP in female compared to male red-eared slider turtles (*Trachemys scripta elegans*) (Willingham et al., 2000). Another study with Olive Ridley sea turtles (*Lepidochelys olivacea*) suggests that BPA exposure during the TSP only affects brain aromatase activity at the female, but not male, inducing temperature (Gomez-Picos et al., 2014).

In deer mice, early exposure of females to EE2 enhances spatial learning and memory or results in a masculinized response (Jasarevic et al., 2011). In contrast, male offspring exposed early on to EE2 show impaired spatial learning and memory abilities. In polygynous deer mice, spatial learning and memory is considered a sexually selected trait favoring males, who must locate potential female reproductive partners, who are widely dispersed throughout the environment (Galea et al., 1996; Galea et al., 1994). A similar masculinized response for spatial memory in radial arm and Barnes mazes has been reported in female mice developmentally exposed to EE2 (Ryan and Vandenbergh, 2006). In ovariectomized female rats, low levels of estradiol benzoate improve working memory performance in a radial arm maze but high levels of this chemical result in the opposite effect (Holmes et al., 2002). Therefore, levels of exposure to EE2 and BPA may be important determinants in the final behavioral outcome. Our prediction, based on the current findings, is that female painted turtles, produced by exposure to estrogenic chemicals or higher incubation temperature, will show improved spatial learning and memory relative to males, i.e. controls incubated at the male-inducing temperature.

Early exposure to BPA and EE2 might induce changes in the hippocampal region, which is a key brain region controlling spatial navigational learning and memory in mammalian taxa (Pyter et al., 2005; Pyter et al., 2006; Walton et al., 2011). The hippocampus of turtles shares similar features to that of mammals (Striedter, 2015). In rodents and non-human primate models, BPA exposure disturbs the hippocampal neural circuitry (Bowman et al., 2014; Elsworth et al., 2013; Hajszan and Leranth, 2010; Kim et al., 2011; Leranth et al., 2008a; Leranth et al., 2008b; Tiwari et al., 2014; Xu et al., 2013; Xu et al., 2014; Zhang et al., 2014). In turtles, early exposure to BPA or EE2 may reprogram the hippocampus and other brain regions (discussed below) such that they are feminized relative to control, non-exposed males. The estrogenic-improvements in spatial learning and memory might be attributed to sex-dependent epigenetic changes in the hippocampus and other brain regions (Jasarevic et al., 2012; Kundakovic et al., 2013; Yaoi et al., 2008).

In turtles, the dorsal cortex, which contains cholinergic cells projecting to the basal forebrain, might be even more important than the hippocampus in regulating various learned responses, including spatial navigation and pattern and spatial discrimination (Blau and Powers, 1989; Grisham and Powers, 1989, 1990; Petrillo et al., 1994). Lesions of the basal forebrain or dorsal cortex impair learning ability, including in spatial tests (Blau and Powers, 1989; Petrillo et al., 1994). Muscarinic acetylcholine receptors (mAChR) in these brain regions likely stimulate spatial memory in turtles (Petrillo et al., 1994; Powers et al., 2009). In support of this notion, pharmacological antagonism of mAChR with scopolamine but not methylscopalamine (which cannot cross the blood-brain barrier) disrupts spatial learning in adult painted turtles (Petrillo et al., 1994; Roth and Krochmal, 2016). To our knowledge, no prior studies have determined whether there are sexual dimorphic differences in the dorsal cortex and basal forebrain in any turtle species. Yet, estrogen receptors are located in the forebrain region of painted turtles (Mak et al., 1982). This is a critical area that merits further examination.

To determine if the behavioral differences are due to aromatase or other genes, such as *Esr*, *Esr2*, *Ar,* or mAchR genes (*Chrm2* and *Chrm4*), we are currently examining the neural expression pattern of these transcripts, along with performing global transcriptomic and DNA methylation studies, in those animals who underwent behavioral testing. Such studies are facilitated by the sequencing and annotation of the painted turtle genome (Badenhorst et al., 2015; Shaffer et al., 2013).

The handful of other studies examining for sex-dependent differences in cognitive and other behavioral responses in reptiles have yielded mixed results. Male eastern water skinks (*Eluamprus quoyii*) outperformed female skinks in a spatial learning task (Carazo et al., 2014). Conversely, two other studies suggest that incubation temperature rather than sex modulates cognitive and other behavioral changes (Amiel and Shine, 2012; Huang and Crews, 2012). Regardless of sex, three-lined skinks (*Bassiana duperreyi*) incubated at higher temperature

demonstrate greater learning ability compared to those incubated at a lower temperature (Amiel and Shine, 2012). Similarly, social association differences occur in male leopard geckos (*Eublepharis macularius*) incubated at different temperatures (Huang and Crews, 2012). Increased scent marking is observed in males treated with dihydrotestosterone, suggesting that exogenous hormones can underpin neurobehavioral changes. During the breeding season, captive female pink-eared turtles (*Emydura victoriae*) are reported to consume more food than males (Gaikhorst et al., 2011). The sex differences in feeding behavior likely reflect their divergent interests at this time with females under greater energy demands due to egg development and males spending a greater amount of time seeking out potential reproductive mates.

In summary, to our knowledge these are the first studies to show that *in ovo* exposure to BPA and EE2 can lead to later behavioral modifications in a reptilian species. Both xenoestrogens improved spatial navigational learning and memory in exposed painted turtles. These neurobehavioral improvements are likely due to BPA and EE2 inducing partial to full sex reversal to female gonads in turtles that would otherwise be male (Jandegian et al., 2015). The findings thus suggest that early exposure of painted turtles, and possibly other reptiles, to BPA and EE2 can potentially override TSD of the gonad and brain. The current turtle data also suggest that sex steroid hormones alone may guide sexual differentiation of the brain. Ongoing biomolecular studies with the same turtles that underwent behavioral testing will allow us to test predictions whether the enhanced cognitive abilities observed in the BPA- and EE2-exposed animals are attributed to epigenetic and gene expression changes, especially aromatase, in the hippocampus or other brain regions governing this trait. The effects of these chemicals on spatial learning and memory may also be due to sexually-dimorphic differences in social association, scent marking, and food consumption in turtles, which should be considered in future work. This study though is the first to demonstrate that developmental exposure to BPA and EE2 may induce sex-dependent changes in spatial learning and memory in a reptilian species.

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