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BISPHENOL A EXPOSURE DURING EARLY DEVELOPMENT INDUCES SEX-SPECIFIC CHANGES IN ADULT ZEBRAFISH SOCIAL INTERACTIONS

Daniel N. Weber^{1,*}, Raymond G. Hoffmann², Elizabeth S. Hoke³, and Robert L. Tanguay⁴

¹Children's Environmental Health Sciences Core Center, University of Wisconsin-Milwaukee

²Department of Pediatrics, Medical College of Wisconsin

³Union Grove High School, Union Grove, WI§

⁴College of Agricultural Sciences, Department of Environmental & Molecular Toxicology, Oregon State University

Abstract

Developmental bisphenol A (BPA) exposure is associated with adverse behavioral effects, although underlying modes of action remain unclear. Because BPA is a suspected xenoestrogen, the objective was to identify sex-based changes in adult zebrafish social behavior developmentally exposed to BPA (0.0, 0.1 or 1 μ M) or one of two control compounds (0.1 μ M 17 β -estradiol [E2], and 0.1 μ M GSK4716, a synthetic estrogen-related receptor γ ligand). A test chamber was divided lengthwise so each arena held one fish unable to detect the presence of the other fish. A mirror was inserted at one end of each arena; baseline activity levels were determined without mirror. Arenas were divided into 3, computer-generated zones to represent different distances from mirror image. Circadian rhythm patterns were evaluated at 1–3 (= AM) and 5–8 (= PM) hr postprandial. Adult zebrafish were placed into arenas and monitored by digital camera for 5 min. Total distance traveled, % time spent at mirror image, and number of attacks on mirror image were quantified. E2, GSK4716, and all BPA treatments dampened male activity and altered male circadian activity patterns; there was no marked effect on female activity. BPA induced non-monotonic effects (response curve changes direction within range of concentrations examined) on male % time at mirror only in AM. All treatments produced increased % time at the mirror during PM. Male attacks on the mirror were reduced by BPA exposure only during AM. There were sex-specific effects of developmental BPA on social interactions and time-of-day of observation affected results.

Keywords

agonistic behavior; bisphenol A; circadian rhythms; developmental exposure; sex-specific behavior; social behavior; zebrafish

*To Whom Correspondence Should Be Addressed: dweber@uwm.edu, 600 E. Greenfield Ave, Milwaukee, WI 53204, (414) 382-1726.

§Current Address: Institute on the Environment, University of Minnesota

INTRODUCTION

Bisphenol A (BPA) is produced primarily for the production of polycarbonate plastics used in food and drink packaging, compact discs, impact-resistant safety equipment, and medical devices, as well as epoxy resins that are used as lacquers to coat metal food cans, bottle tops, and water supply pipes. In addition, BPA is used to synthesize polyvinyl chloride (PVC), some dental sealants and composites, and thermal printing paper used for cash register receipts (Noonan et al. 2011; Biedermann et al. 2010; Welshons et al. 2006).

The primary source of human exposure to BPA is through food and beverage containers from which BPA has leached into the product. In addition, BPA was also detected in dairy products, fruits and vegetables, meat and fish, cereals, and drinking water, although it is not clear if these levels are hazardous to health (Liao and Kannan 2013; Cao et al. 2011; Wei et al. 2011; Thomson and Grounds 2005; Fent 2000; Theobald et al. 2000). The 2003–2004 National Health and Nutrition Examination Survey (NHANES III) conducted by the Center for Disease Control and Prevention found detectable levels of BPA in 93% of individuals 6 years and older (Calafat et al. 2008); no associations with health effects were discussed. However, social cognition, communication and awareness were poorer among children ages 7–9 prenatally exposed to levels of BPA similar to those found in the NHANES III study population (Miodovnik et al. 2011). Pre- and post-natal BPA levels were also reported in pregnant women. Such exposures were associated with altered birth weight and height (Lee et al. 2014), increased incidence of childhood asthma (Donohue et al. 2013), and sex-specific childhood behavioral problems (Perera et al. 2012; Braun et al. 2009). Other studies, however, did not find correlations between BPA body burdens and maternal or maternal-child health outcomes such as metabolic disorders, immunologic or neurologic disease, risk of carcinogenicity, or various measures of reproductive development (Robledo et al. 2013; Kasper-Sonnenberg 2012; Willhite et al. 2008). However, none of those studies investigated developmental BPA-induced changes in childhood neurodevelopment or adult behavior, the central focus of this study.

Because it is considered by many as a xenoestrogen, BPA has been associated with a range of molecular and physiological processes that may affect social behaviors, especially those linked to sex-specific activity patterns, e.g., alterations in gene expression (fish: Hafez et al. 2012; Ribeiro et al. 2012; rats: Li et al. 2014), organ development (general vertebrates: Gibert et al. 2011; Masuo and Ishido 2011; fish: Molina et al. 2013; Huang et al. 2012; Mihaich et al. 2012; Cao et al. 2010), and the hypothalamic-pituitary-gonadal axis activity (rats: Li et al. 2014). While it was shown to bind to a range of hormone receptors (human: Prasanth et al. 2010; fish: Jiao and Cheng 2010), there has been particular interest in BPA binding to estrogen receptors (ER), e.g., ER α , ER β , and estrogen-related receptor gamma, ERR γ (general vertebrate: Ben-Jonathan and Steinmetz 1998; human: Takayanagi et al. 2006; fish: Saili et al. 2012; mice: Kundakovic et al. 2013; rat: Cao et al. 2013;). To explain differences in behavioral responses to BPA exposure by males vs. females, Masuo and Ishido (2011) and Kubo et al. (2001) suggest BPA action in the locus ceruleus, especially in males. Estrogens are a class of hormones of which 17 β -estradiol (E2) is the most abundant and potent that are involved in brain neurodevelopment (McCarthy 2008). Disruptions in the normal binding activity of ER and ERR may be the basis for the observed alterations in the

sex-based social behavior of several vertebrate species, including human (general vertebrate: Galea and Barha 2011; human: Harley et al. 2013; Braun et al. 2011; fish: Saili et al. 2012; mice: Williams et al. 2013; Wolstenholme et al. 2011; monkeys: Nakagami et al. 2009). Therefore, in this study, the ER ligand E2, and a less commonly used synthetic ERR β / γ ligand, GSK4716 were used to explore the hypothesis that BPA-induced changes in social behavior are a result of xenoestrogenic activity. While there are few data correlating ERRs to social behavior, two lines of indirect evidence suggest possible effects. Exposures to BPA or GSK4716 during early development led to hypersensitivity in larval zebrafish to light-dark transitions followed by extended swim times (Saili et al. 2012). Since in the present study, distance traveled was one of the variables measured as a function of social interaction, changes in locomotor activity due to GSK4716 might be a possible result even for adults. In juvenile mice, maternal exposures to BPA induced a change in mRNA levels for epigenetic regulators of DNA methyltransferase 1 and 3 in the hypothalamus. These alterations paralleled changes in ERR γ (Kundakovic et al. 2013) that may be a basis for altered social behavior.

Even in non-social behaviors, e.g., learning, emotion and exploration, males and females displayed differential outcomes due to developmental BPA exposures (human: Perera et al. 2012; Braun et al. 2011, 2009; fish: Saili et al. 2012; mice: Jašarević et al. 2013; Kundakovic et al. 2013; Palanza et al. 2008 rats: Jones et al. 2011). These behavioral disruptions are strongly correlated with a range of molecular, physiological, and organ-level mechanisms involved in sex-dependent behaviors, e.g., brain ER gene expression (rat: Cao et al. 2013), fetal ovarian and gonadal development (fish: Chung et al. 2011; mice: Kundakovic et al. 2013; Tainaka et al. 2012; Xi et al. 2011a, 2011b; rat: Cao et al. 2013; sheep: Veifa-Lopez et al. 2013), pituitary and gonadotrophin development (mice: Brannick et al. 2012), brain and gonadal enzyme activity (rat: Nanjappa et al. 2012), altered hypothalamic-pituitary-gonadal (HPG) axis activity (mice: Xi et al. 2011a; rats: Ramos et al. 2003), and circulating testosterone levels (mice: Tanaka et al. 2006). In contrast, several studies were not able to identify correlations between BPA-induced mechanistic effects and behavioral disruptions (mice: Palanza et al. 2002; Cagan et al. 1999; rat: Kobayashi et al. 2012; Ryan et al. 2010) or differences between sexes in BPA-induced learning deficits (fish: Saili et al. 2012). Considerations, therefore, regarding differing experimental protocols, e.g., time of day of testing, age and length of exposure, specific behavioral outcome being examined, or genetic strain of test species, may be required to provide insights into mechanisms underlying behavioral toxicity. This study examined two of these possibilities, circadian variations in BPA-induced behavioral outcomes by including multiple times of day for testing and social interactions. Different outcomes and cause-and-effect interpretations may result if time-of-day of testing is not considered (Weber and Spieler 1994).

Mammals and fishes display parallel social behaviors that are governed by similar underlying mechanisms (Oliveira 2013). Zebrafish (*Danio rerio*) has been used extensively to elucidate basic mechanisms underlying behavioral toxicology (Bailey et al. 2013). In particular, zebrafish was employed as a model for identifying sex-specific effects on social interactions and their sensitivity to chemical exposures (Dalbohm et al. 2012) induced by developmental BPA exposure.

METHODS

Treatment of Glassware and Plasticware

All lab materials made of plastic were washed thoroughly in a 10% solution of a nontoxic, biodegradable detergent (Simple Green™; Sunshine Makers, Inc., Huntington Harbour, CA), rinsed repeatedly in ultrapure Milli-Q™ water (Millipore Corp., Medford, MA), and immersed in a 30mM Na₄EDTA (Fisher Scientific, Hanover Park, IL) solution overnight to remove all surface adsorbed metal ions; glassware was washed and rinsed similarly but immersed in a 10% HNO₃ (Fisher Scientific, Hanover Park, IL) solution overnight. Glass and plasticware were then rinsed in ultra-pure Milli-Q™ water.

Zebrafish rearing

Adult tropical 5D strain (wildtype) zebrafish were raised at the Sinnhuber Aquatic Research Laboratory (SARL) in the Aquatic Biomedical Models Facility Core of the Environmental Health Sciences Center at Oregon State University under standard conditions (28°C, 14 hr light/10 hr dark cycle) on a recirculating water system. Embryos obtained from group spawns were washed, screened for viability, and incubated in embryo medium (Westerfield, 2000) at 28°C. Larvae destined for adult behavior testing were exposed to 0 (0.1% DMSO only = control), 0.1, 1 µM BPA, 0.1 µM E2, or 0.1 µM GSK4716 from 8–120 h post fertilization (hpf), then removed from the exposure solution, thoroughly rinsed with water, and raised at the SARL under standard conditions until approximately 3 months of age, at which time they were shipped overnight to the Neurobehavioral Toxicology Facility, Children's Environmental Health Sciences Center, University of Wisconsin-Milwaukee, where they were raised under standard conditions prior to adult testing. Zebrafish husbandry and behavior testing was conducted in compliance with approved Oregon State University and University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee protocols.

Chemical preparation

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane; 99% purity, Tokyo Chemical Industry America (TCI), Portland, OR), GSK4716 (4-Hydroxy-2-[(1E)-[4-(1-methylethyl)phenyl]methylene]hydrazide; Tocris Bioscience, Ellisville, MO, USA), and 17β-estradiol (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). BPA stock concentration was confirmed by high-performance liquid chromatography (HPLC) analysis. Exposure solutions were prepared by diluting working stocks in buffered embryo medium at a final vehicle concentration of 0.1% DMSO.

Social Behavior Test

A. Testing Chamber—A white, plastic box (16 cm width × 25 cm length × 10 cm height) was constructed with interchangeable panels to allow for multiple testing designs. In the test described below a white plastic wall was inserted to split the chamber lengthwise so that each section of equal area would hold a single fish and each fish was unable to detect the presence of the other visually or by odor. The 10 cm height was necessary to prevent fish

from jumping out of the chamber. At one end of each section a mirror was inserted; the opposite end remained without a mirror. An individual male was placed in one section and a female in the other so that each sex was tested together within the same time frame. Each section was divided into three zones: 0–1 cm from the mirror (= mirror), 1–12.5 cm from the mirror (= transition), and 12.5–25 cm from the mirror (= opaque). These three zones represent different distances from the mirror image and provided, therefore, different intensity levels of social interaction.

Fish movements were recorded at a rate of 30 frames/sec with an infrared-sensitive digital camera (Ikegami Model ICD-49, Neuss, Germany) placed 45 cm above the bottom of the test chamber. Lighting was provided by a ring (diam. 10 cm) of 18 white light LED (EnvironmentalLights.com, San Diego, CA, USA) placed above the test chamber to provide even lighting intensity (200 lux) throughout the chamber to allow the fish to see the mirror image. Lighting for the camera was provided by an infrared lamp (IR—ROOM Ultra-Covert 940 nm Infrared Illuminator, Night Vision Experts.com, Buffalo, NY) placed 45 cm below the test chamber to prevent glare.

B. Testing Protocol—Experimental protocol follows that of Weber and Ghorai (2013). All tests used dechlorinated Lake Michigan tap water warmed to 28°C in an incubator. This represents the same water conditions used for maintaining the adult fish. The quality of City of Milwaukee water is rigorously and continuously tested and found to have undetectable levels of BPA, among many other chemical contaminants, that could potentially confound experimental variables (Milwaukee Water Works 2013). Water (1 L, 2.5 cm depth) was replaced after each trial to maintain equal temperatures between trials and remove any olfactory clues left by the previous fish. Before replacing with fresh water, chamber was rinsed three times with distilled water. To account for differential circadian social activity patterns entrained by feeding time, e.g., agonistic behavior (Weber and Spieler 1987) each fish was tested first within two hr of feeding time (0930 h) and again at 5 hr post feeding time (1400 h). Time required to test a 10-fish set was approximately 90 min.

Individual male and female adult zebrafish (12-months old; $n = 10$ of each sex) were placed into separate sections of the testing chamber and allowed to acclimate for 1 min before recording started. All fish were first tested in a chamber without a mirror to obtain baseline activity patterns; they were retested on a different day with the mirror inserted. Fish movements were monitored for 5 min. Using image analysis software (EthoVision v8.5, Noldus, Wageningen, The Netherlands), total distance traveled (cm), percent time spent in each zone (mirror, transition, and opaque), and number of attacks on the mirror image were recorded. Attacks were defined as movement into the zone where the mirror was located. While such movement may include both actual attacks on the mirror image it may also include general swimming motion into that zone. To minimize the second possibility, the zone was created to be no wider than $\frac{1}{2}$ the average snout-to-tail length of an adult zebrafish.

Statistical Analysis

Since none of the variables displayed a normal distribution, a generalized linear model, GLM, was used for each of the variables with a transform that was the most appropriate to model statistical comparisons, specifically for distance traveled a gamma distribution with a log transform, for time spent at the mirror a normal distribution with a log transform, and for attacks on the mirror a negative binomial (a Poisson allowing variability in the rate between subjects and assuming the same rate for each subject) distribution with a log transform was used. Multiple comparisons were made with the Duncan Empirical Bayes method (Dixon and Duncan 1975). Data were analyzed for AM and PM time points for each of the 3 variables. Statistical significance was set at $p < 0.05$.

RESULTS

Distance Travelled

AM Testing Time—If the mirror was absent (Figure 1), there was no significant difference in distance traveled by males between any treatment groups. However, control females traveled significantly more than control males (+871 cm). BPA-, E2 and GSK4716-exposed females traveled significantly less than control females: 0.1 μ M BPA (–496 cm), 1 μ M BPA (–476 cm), E2 (–1107 cm) and GSK4716 (–989). 0.1 μ M BPA differed markedly only from E2 and GSK4716.

After the mirror was inserted (Figure 1), distance traveled by BPA-, E2 and GSK4716-exposed males was significantly less than control. There was no significant difference between BPA-, E2 and GSK4716 treatments. While the distance traveled by control males was more than control females (+566 cm), it was less than females for exposures to 1 μ M BPA (–809 cm) and E2 (–655 cm). There were no significant differences between males and females for exposures to 0.1 μ M BPA or GSK4716. Distance traveled for females treated with 0.1 μ M BPA or GSK4716 was markedly less than control (–496 cm and –749 cm, respectively). Distance traveled by females treated with either 1 μ M BPA or E2 did not differ from control.

PM Testing Time—If the mirror was absent (Figure 1), females traveled a significantly greater distance vs. males (+211 cm) regardless of treatment. Neither BPA nor E2 produced significant changes in distance traveled vs. control for either females or males. GSK4716 induced a significant decrease in distance traveled (–301 cm).

After the mirror was inserted (Figure 1), control male activity was significantly higher than males treated with 1 μ M BPA (+434 cm), E2 (+546 cm) or GSK4716 (+592 cm); these 3 treatments did not significantly differ from each other. Travel distance for males treated with 0.1 μ M BPA was similar to control. For females, only GSK4716 treatment traveled significantly less than controls (–422 cm). The distance traveled for control males was significantly higher than females (+1421 vs. +1081 cm).

% Time Spent at Mirror

AM Testing Time—If the mirror was absent (Figure 2), there were no significant differences in % time spent at the mirror between controls vs. treatment or between sexes. After the mirror was inserted (Figure 2), there were significantly longer times spent at the mirror for control males and females. Significant differences in time spent at the mirror were observed for: 0.1 μ M BPA > control (+42%), E2 < control (−4%), and GSK4716 > control (+33%). While fish exposed to either 0.1 μ M BPA or GSK4716 spent equal amounts of time at the mirror, they were both significantly greater than for those individuals exposed to 1 μ M BPA or E2 (>35%).

PM Testing Time—When the mirror was absent (Figure 2), there was no marked treatment effect for males or females, i.e., no sex x treatment interaction. Females spent more time at the mirror than males (+7%). After the mirror was inserted (Figure 2), there were no significant gender effects. For this variable, however, both E2 (+13%) and GSK4716 (+27%) exerted an additive effect on time males spent at the mirror in addition to the effect of the mirror.

Attacks on Mirror

AM Testing Time—When the mirror was absent (Figure 3), there was no marked treatment effect on the number of attacks. There was, however, a significant gender effect (females > males). As there was no mirror, these “attacks” were more likely general swimming movements into the mirror zone. After the mirror was inserted (Figure 3), there was no gender effect but there was a treatment effect; among males each of the 4 treatments was significantly lower than control (−14.5 attacks, 0.1 μ M BPA < control; 1 μ M BPA < control, −10.5 attacks; E2 < control, −10.4 attacks; GSK4716 < control, −14.5 attacks), but none were significantly different from one another. Female zebrafish exposed to 0.1 μ M BPA or GSK4716 showed a significant decrease in attacks on the mirror image.

PM Testing Time—The dominant factor for changes in the number of attacks was the presence of the mirror (Figure 3). While there were no significant differences among treatments, or interactions of mirror x sex, the difference relative to control with the mirror was significantly greater than without the mirror (+13.8 attacks).

DISCUSSION

Zebrafish live in loose social groups (Spence et al. 2008) and, therefore, serve as a useful model to understand how xenoestrogenic compounds disrupt normal social interactions. In this study, mirrors were employed to elicit agonistic displays, which include ritualized defensive interactions as well as passive and active aggression (King 1973). These traits were measured among adult male and female fish that were developmentally exposed as embryos to varying concentrations of BPA. This exposure regimen was identical to the environmentally relevant levels used in a previous study (Saili et al. 2012) and compare favorably to the maternal delivery of BPA to a fetus (Zimmers et al. 2014; Aries 2013).

Male zebrafish not treated with BPA as embryos swam shorter total distances when no mirror was in the test chamber vs. when a mirror was present. The image of a same-sex and same-sized conspecific differs from those studies in which two live competitors are present in the same tank because body morphology affects mirror-elicited response outcomes (Holtby et al. 1993). Without a clear body morphology difference, the response of an adult zebrafish to its perceived equally sized competitor may be more intense and for a longer period of time. While there was decreased activity in the PM observation time, males still swam longer distances if a mirror was present. When the mirror was absent, male activity was not significantly different among treatments; when the mirror was present, distance traveled was significantly less among BPA-, estradiol-, or GSK4716-exposed individuals vs. controls. This was in contrast to several studies, including rat pups (Xu et al. 2007) and our previous investigations into the locomotor activity of zebrafish larvae (Saili et al. 2012), that indicated hyperactivity following BPA exposure. Larval activity is not sex-based, whereas social interactions with another adult of same sex and size potentially raise issues of territoriality, resource use, and reproductive status which have a strong sex-based linkage. Interestingly, prenatal BPA exposures increased depression in boys (Harley et al. 2013) and mice (Xu et al. 2012), a behavioral outcome that may parallel the changes observed in the depressed locomotor activity in male zebrafish.

Percent time spent in the mirror zone displayed a non-monotonic response (curve changes direction within range of concentrations examined), in this case the concentration-response curve was an inverted U-shape in which the intermediate exposure (0.1 μ M BPA) concentration produced a higher response than either 0 or 1 μ M BPA. Behavior within the mirror zone was most intense only when the mirror was inserted into the chamber and consisted of back-and-forth swimming in front of the mirror image with no apparent attack movements directed to the mirror image. This activity, therefore, was likely to be a combination of several agonistic behaviors including aggression and territorial displays, as documented by zebrafish in the wild (Spence et al. 2008). That developmental exposure to either 0.1 μ M BPA or 0.1 μ M GSK4716 resulted in equally intense activity in the mirror zone suggests that, as supported by our previous studies (Saili et al. 2012) BPA is acting as an ERR γ agonist, at least at the lower BPA concentration. Yet, developmental exposure to either 0.1 μ M E2 or 1 μ M BPA resulted in a decrease in the amount of time spent in the mirror zone suggesting differential, concentration-dependent sensitivity to multiple pathways during development. For example, Masuo and Ishido (2011) and Kubo et al. (2001) demonstrated an inverted U-shaped BPA effect on the locus ceruleus in response to stimuli. Performance was decreased at very low levels and high levels of LC tonic discharge due to drowsiness and being inattentiveness and at high. Performance on a task that required focused attention (possibly similar to that required for interacting with the mirror image) was highest at moderate LC tonic activity (Ashton-Jones et al. 2007). Whether this parallels the inverted U-shaped response curve for % time swimming in front of the mirror of the low vs. high BPA-exposed zebrafish in this study is unclear, although fishes do possess this structure, albeit much smaller (Ma 1994). However, estrogenic compounds, e.g., diethylstilbestrol, have been shown to alter locus ceruleus morphology (Kubo et al. 2001). The opposite effect of two compounds that interact with estrogen receptor sites as it relates to this one specific variable, % time spent at the mirror, suggests that ERs and ERRs may be

involved in regulating the direction of non-aggressive social behaviors and which receptor is being affected may be dependent on BPA exposure concentration.

Conversely, the number of attacks in the mirror zone showed a U-shaped concentration-dependent response for females, albeit statistically insignificant; a linear decrease in attacks with increasing exposure concentration was observed with males. Other non-monotonic responses due to BPA exposure were noted with learning tasks in male rats (Jones et al. 2011), metabolic function in mice (Angle et al. 2013), and protein regulation in a terrestrial isopod (Lemos et al. 2010). One functional basis for the non-monotonic effect is that at doses higher than those required for ER-mediated responses, BPA interacts with other hormone receptor sites. Alternatively, BPA may be interacting on neuroendocrine systems that impact physiological process underlying behavioral outcomes (Léon-Olea et al. 2014; Wayne and Trudeau 2011). These interactions also may affect neurodevelopment potentially confounding the ability to make predictions of neurobehavioral toxicity over a range of concentrations (vom Saal et al. 2007). This would imply that BPA is more than a xenoestrogen and that it interacts directly and/or indirectly with multiple classes of neural and endocrine receptors.

Another aspect of this study not generally included in other examinations of social interaction was the role of circadian rhythmicity in affecting outcomes and interpretations of behavioral toxicity. As demonstrated by each of the variables analyzed, the time-of-day when an experiment was conducted relative to a regularly occurring stimulus to which behavior was entrained (in this case feeding time) exerted a profound effect on behavioral outcomes. Examination of only the afternoon data would have forced the conclusion that BPA exerted little or no effect on social behavior. The morning data, however, indicated that BPA produced a substantial impact on zebrafish social behavior.

Circadian rhythms are important in regulating responses to the social stimulus used in this study, i.e., a social partner created by the mirror image. The presence of social partners might produce mutual behavioral synchronization in a wide range of species (Favreau et al. 2009). Such group enhancement of activity was observed in killifish (*Fundulus heteroclitus*) to be significantly higher than among solitary individuals (Kavaliers 1980). Social influences within a hierarchical relationship, i.e., relative dominance within the population, may induce mutual synchronization of activity rhythms. These, however, were not accounted for in this study and may explain the high variability in the behavioral outcomes. Circadian rhythms direct locomotor activity patterns in fish (Weber and Spieler 1994), which, in turn, alter the intensity of social behavior and sensitivity to mutual synchronization (Pankseep et al. 2008). It was observed that the afternoon swimming distance was less intense than the morning activity level and this, in turn, may play an important role in the unequal number of attacks during each observation time. Studies cited earlier that support the null hypothesis of BPA not inducing changes in specific behaviors and their underlying mechanisms (mice: Palanza et al. 2002; Cagan et al. 1999; rat: Kobayashi et al. 2012; Ryan et al. 2010) may be due to recording observations at times-of-day in which activity levels were less intense. While the light-dark cycle entrains pineal 5-HT rhythms (Ceinos et al. 2005), feeding time entrains both locomotor activity (Weber and Spieler 1987) and circulating 5-HT levels (Ho et al. 1985). Without knowledge as to when the tests were

conducted relative to these variables, it becomes difficult to compare other studies to our data. Complicating the picture of locomotor activity patterns even further, some studies fed their test animals ad libitum rather than at a single time of day, thus altering the times and intensity of peak activity.

Social behaviors are dependent upon neural and endocrine signaling as influenced by external cues. Disruptions in normal patterns of these behaviors may result from changes induced by environmental stressors on the complex interplay between neural and endocrine systems. The mirror-elicited behavior of the adult zebrafish involved two aspects of agonistic display, ritualized territorial displays and direct attacks. By measuring total distance traveled, total % time spent at the mirror (or the zone in which the mirror would be placed), and number of attacks on the mirror (or the zone in which the mirror would be placed), specific aspects of social interaction with a conspecific of the same sex and morphology (general activity level, degree of interaction or association, and aggressive behavior, respectively), insights into other mechanisms that were possibly affected by developmental BPA exposure are suggested.

Ovarian steroids, which are affected by BPA exposure (fish: Hatef et al. 2012; mice: Xi et al. 2011; rats: Li et al. 2014), regulate the serotonin system, especially in regions critical to controlling social behaviors, e.g., raphe nuclei and hypothalamus. In rodent brains, treatment with estrogens increased levels of dorsal raphe 5HT_{2A} mRNA (McEwen 2002) and increased the density of 5-HT_{2A} binding sites in other brain regions associated with emotion and behavior (Fink et al. 1996). Since agonistic displays may be markedly affected by this interaction, xenoestrogenic activity of BPA as it relates to ER and ER γ agonism (general: Zuercher et al. 2005; Ben-Jonathan and Steinmetz 1998; human: Okada et al. 2008; rat: Washington et al. 2001), and the consequent effects on the serotonergic system during development may be an example of BPA-induced neuroendocrine disruption. Because serotonin is important during brain development and in controlling levels of aggression, and BPA enhances 5-HT activity, BPA-induced changes in embryonic 5-HT levels may be responsible for altered brain development (fish: Elipot et al. 2013; Dahlbom et al. 2012; Clotfelter et al. 2007; mice: Rood and Beck 2013; rat: Cao et al. 2013; Donner and Handa 2011; Matsuda et al. 2010; González et al. 2008; Honma et al. 2006; Orozco-Suárez 2003; Persico et al. 2000; Yan et al. 1997).

It is still an open question whether, in fact, altered embryonic 5-HT dynamics explain the changes in adult behavior observed in this study. Other investigations, however, using fish exposed to fluoxetine (FLX), a selective serotonin re-uptake inhibitor that is found as a contaminant in some aquatic systems suggest this may be a useful avenue of research. Behavioral disruptions occurred in fathead minnows (*Pimephales promelas*) of the 5-HT system after short-term, adult exposures to FLX (Weinberger and Klaper 2014). While at FLX concentrations higher than used in this study male aggression toward females increased, fish exposed to concentrations similar to this study caused a concentration-dependent decrease in total swimming distance. Other studies using fish models involve short-term larval FLX exposure followed by larval locomotor behavioral observations (Airhart et al. 2007) or 4-wk adult exposure followed by analyses of reproductive physiology and behavior (Foran et al. 2004) also demonstrated behavioral effects

comparable to this study. Full comparisons, however, are limited due to different exposure regimens between studies. The embryonic exposures used in this study suggest that adult behavioral effects may be the result of permanent neurological alterations caused by early (first 24 hpf) developmental exposures to low-level, environmentally-relevant concentrations of BPA. The underlying mechanisms of this behavior remain to be elucidated.

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Figure 1.

Effect of exposures to bisphenol A (BPA) during early development on activity level of adult zebrafish (12 months). Distance traveled by male and female zebrafish within test chamber over a 5 min period after a 1 min acclimation period was compared with and without the presence of a mirror. Changes in circadian rhythm patterns were evaluated by testing each fish at two times of day: morning immediately after feeding time at 0900 hr and again at 1400 hr. Developmental exposure regimen (2–48 hours post fertilization) consisted of: ■ = 0.0 μ M BPA; ■ = 0.1 μ M BPA; ■ = 1.0 μ M BPA; □ = 0.1 μ M estradiol; and □ = 0.1 μ M GSK4716.

* = significantly different from control ($p < 0.05$) of same sex.

Figure 2.

Effect of exposures to bisphenol A (BPA) during early development on % time spent interacting with the mirror image by adult zebrafish (12 months). Percent time spent by male and female zebrafish in the test chamber mirror zone (2 cm away from wall where mirror was placed) over a 5 min period after a 1 min acclimation period was compared with and without the presence of a mirror. Changes in circadian rhythm patterns were evaluated by testing each fish at two times of day: morning immediately after feeding time at 0900 hr and again at 1400 hr. Developmental exposure regimen (2–48 hours post fertilization) consisted of: ■ = 0.0 μ M BPA; ■ = 0.1 μ M BPA; □ = 1.0 μ M BPA; ■ = 0.1 μ M estradiol; and □ = 0.1 μ M GSK4716.

* = significantly different from control ($p < 0.05$) of same sex.

Figure 3.

Effect of exposures to bisphenol A (BPA) during early development on the number of attacks on the mirror image by adult zebrafish (12 months). The number of attacks on the wall where the mirror was placed by male and female zebrafish in the test chamber over a 5 min period after a 1 min acclimation period was compared with and without the presence of a mirror. Changes in circadian rhythm patterns were evaluated by testing each fish at two times of day: morning immediately after feeding time at 0900 hr and again at 1400 hr.

Developmental exposure regimen (2–48 hours post fertilization) consisted of:

■ = 0.0 μ M BPA; □ = 0.1 μ M BPA; ▒ = 1.0 μ M BPA; □ = 0.1 μ M estradiol; and □ = 0.1 μ M GSK4716.

* = significantly different from control ($p < 0.05$) of same sex.