Bisphenol A Exposure During Early Development Induces Sex-Specific Changes in Adult Zebrafish Social Interactions

1 BISPHENOL A EXPOSURE DURING EARLY DEVELOPMENT

2 INDUCES SEX-SPECIFIC CHANGES

3 IN ADULT ZEBRAFISH SOCIAL INTERACTIONS

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25   ABSTRACT

26   Developmental bisphenol A (BPA) exposure is associated with adverse behavioral

27   effects, although underlying modes of action remain unclear. Because BPA is a

28   suspected xenoestrogen, the objective was to identify sex-based changes in adult

29   zebrafish social behavior developmentally exposed to BPA (0.0, 0.1 or 1 µM) or one of

30   two control compounds (0.1µM 17β-estradiol [E2], and 0.1 µM GSK4716, a synthetic

31   estrogen-related receptor γ ligand). A test chamber was divided lengthwise so each

32   arena held one fish unable to detect the presence of the other fish. A mirror was

33   inserted at one end of each arena; baseline activity levels were determined without

34   mirror. Arenas were divided into 3, computer-generated zones to represent different

35   distances from mirror image. Circadian rhythm patterns were evaluated at 1-3 (= AM)

36   and 5-8 (= PM) hr postprandial. Adult zebrafish were placed into arenas and monitored

37   by digital camera for 5 min. Total distance traveled, % time spent at mirror image, and

38   number of attacks on mirror image were quantified. E2, GSK4716, and all BPA

39   treatments dampened male activity and altered male circadian activity patterns; there

40   was no marked effect on female activity. BPA induced non-monotonic effects (response

41   curve changes direction within range of concentrations examined) on male % time at

42   mirror only in AM. All treatments produced increased % time at the mirror during PM.

43   Male attacks on the mirror were reduced by BPA exposure only during AM. There were

44   sex-specific effects of developmental BPA on social interactions and time-of-day of

45   observation affected results.

46

47   Key words: agonistic behavior, bisphenol A, circadian rhythms, developmental

48   exposure, sex-specific behavior, social behavior, zebrafish

49

50   Running Head: BPA AFFECTS SOCIAL BEHAVIOR

51

52   INTRODUCTION

53   Bisphenol A (BPA) is produced primarily for the production of polycarbonate

54   plastics used in food and drink packaging, compact discs, impact-resistant safety

55   equipment, and medical devices, as well as epoxy resins that are used as lacquers to

56   coat metal food cans, bottle tops, and water supply pipes. In addition, BPA is used to

57   synthesize polyvinyl chloride (PVC), some dental sealants and composites, and thermal

58   printing paper used for cash register receipts (Noonan et al. 2011; Biedermann et al.

59   2010; Welshons et al. 2006).

60   The primary source of human exposure to BPA is through food and beverage

61   containers from which BPA has leached into the product. In addition, BPA was also

62   detected in dairy products, fruits and vegetables, meat and fish, cereals, and drinking

63   water, although it is not clear if these levels are hazardous to health (Liao and Kannan

64   2013; Cao et al. 2011; Wei et al. 2011; Thomson and Grounds 2005; Fent 2000;

65   Theobald et al. 2000). The 2003-2004 National Health and Nutrition Examination

66   Survey (NHANES III) conducted by the Center for Disease Control and Prevention

67   found detectable levels of BPA in 93% of individuals 6 years and older (Calafat et al.

68   2008); no associations with health effects were discussed. However, social cognition,

69   communication and awareness were poorer among children ages 7-9 prenatally

70   exposed to levels of BPA similar to those found in the NHANES III study population

71   (Miodovnik et al. 2011). Pre- and post-natal BPA levels were also reported in pregnant

72   women. Such exposures were associated with altered birth weight and height (Lee et al.

73   2014), increased incidence of childhood asthma (Donohue et al. 2013), and sex-specific

74   childhood behavioral problems (Perera et al. 2012; Braun et al. 2009). Other studies,

75 however, did not find correlations between BPA body burdens and maternal or

76 maternal-child health outcomes such as metabolic disorders, immunologic or neurologic

77 disease, risk of carcinogenicity, or various measures of reproductive development

78 (Robledo et al. 2013; Kasper-Sonnenberg 2012; Willhite et al. 2008). However, none of

79 those studies investigated developmental BPA-induced changes in childhood

80 neurodevelopment or adult behavior, the central focus of this study.

81   Because it is considered by many as a xenoestrogen, BPA has been associated

82 with a range of molecular and physiological processes that may affect social behaviors,

83 especially those linked to sex-specific activity patterns, e.g., alterations in gene

84 expression (fish: Hatef et al. 2012; Ribeiro et al. 2012; rats: Li et al 2014), organ

85 development (general vertebrates: Gibert et al. 2011; Masuo and Ishido 2011; fish:

86 Molina et al. 2013; Huang et al 2012; Mihaich et al. 2012; Cao et al. 2010), and the

87 hypothalamic-pituitary-gonadal axis activity (rats: Li et al. 2014). While it was shown to

88 bind to a range of hormone receptors (human: Prasanth et al. 2010; fish: Jiao and

89 Cheng 2010), there has been particular interest in BPA binding to estrogen receptors

90 (ER), e.g., ERα, ERβ, and estrogen-related receptor gamma, ERRγ (general vertebrate:

91 Ben-Jonathan and Steinmetz 1998; human: Takayanagi et al. 2006; fish: Saili et al.

92 2012; mice: Kundakovic et al. 2013; rat: Cao et al. 2013;). To explain differences in

93 behavioral responses to BPA exposure by males vs. females, Masuo and Ishido (2011)

94 and Kubo et al. (2001) suggest BPA action in the locus ceruleus, especially in males.

95 Estrogens are a class of hormones of which 17β-estradiol (E2) is the most abundant

96 and potent that are involved in brain neurodevelopment (McCarthy 2008). Disruptions in

97 the normal binding activity of ER and ERR may be the basis for the observed alterations

98   in the sex-based social behavior of several vertebrate species, including human

99   (general vertebrate: Galea and Barha 2011; human: Harley et al. 2013; Braun et al.

100   2011; fish: Saili et al. 2012; mice: Williams et al 2013; Wolstenholme et al. 2011;

101   monkeys: Nakagami et al. 2009). Therefore, in this study, the ER ligand E2, and a less

102   commonly used synthetic ERRβ/γ ligand, GSK4716 were used to explore the

103   hypothesis that BPA-induced changes in social behavior are a result of xenoestrogenic

104   activity. While there are few data correlating ERRs to social behavior, two lines of

105   indirect evidence suggest possible effects. Exposures to BPA or GSK4716 during early

106   development led to hypersensitivity in larval zebrafish to light-dark transitions followed

107   by extended swim times (Saili et al. 2012). Since in the present study, distance traveled

108   was one of the variables measured as a function of social interaction, changes in

109   locomotor activity due to GSK4716 might be a possible result even for adults. In juvenile

110   mice, maternal exposures to BPA induced a change in mRNA levels for epigenetic

111   regulators of DNA methyltransferase 1 and 3 in the hypothalamus. These alterations

112   paralleled changes in ERRγ (Kundakovic et al. 2013) that may be a basis for altered

113   social behavior.

114   Even in non-social behaviors, e.g., learning, emotion and exploration, males and

115   females displayed differential outcomes due to developmental BPA exposures (human:

116   Perera et al. 2012; Braun et al. 2011, 2009; fish: Saili et al. 2012; mice: Jašareviɠ et al.

117   2013; Kundakovic et al. 2013; Palanza et al. 2008 rats: Jones et al. 2011).   These

118   behavioral disruptions are strongly correlated with a range of molecular, physiological,

119   and organ-level mechanisms involved in sex-dependent behaviors, e.g., brain ER gene

120   expression (rat: Cao et al. 2013), fetal ovarian and gonadal development (fish: Chung et

121 al. 2011; mice: Kundakovic et al. 2013; Tainaka et al. 2012; Xi et al. 2011a, 2011b; rat:

122 Cao et al. 2013; sheep: Veifa-Lopez et al. 2013), pituitary and gonadotrophin

123 development (mice: Brannick et al. 2012), brain and gonadal enzyme activity (rat:

124 Nanjappa et al. 2012), altered hypothalamic-pituitary-gonadal (HPG) axis activity (mice:

125 Xi et al. 2011a; rats: Ramos et al. 2003), and circulating testosterone levels (mice:

126 Tanaka et al. 2006). In contrast, several studies were not able to identify correlations

127 between BPA-induced mechanistic effects and behavioral disruptions (mice: Palanza et

128 al. 2002; Cagan et al. 1999; rat: Kobayashi et al. 2012; Ryan et al. 2010) or differences

129 between sexes in BPA-induced learning deficits (fish: Saili et al. 2012). Considerations,

130 therefore, regarding differing experimental protocols, e.g., time of day of testing, age

131 and length of exposure, specific behavioral outcome being examined, or genetic strain

132 of test species, may be required to provide insights into mechanisms underlying

133 behavioral toxicity. This study examined two of these possibilities, circadian variations in

134 BPA-induced behavioral outcomes by including multiple times of day for testing and

135 social interactions. Different outcomes and cause-and-effect interpretations may result if

136 time-of-day of testing is not considered (Weber and Spieler 1994).

137 Mammals and fishes display parallel social behaviors that are governed by

138 similar underlying mechanisms (Oliveira 2013). Zebrafish (*Danio rerio*) has been used

139 extensively to elucidate basic mechanisms underlying behavioral toxicology (Bailey et

140 al. 2013). In particular, zebrafish was employed as a model for identifying sex-specific

141 effects on social interactions and their sensitivity to chemical exposures (Dalbohm et al.

142 2012) induced by developmental BPA exposure.

143

144

145   METHODS

146

147   **Treatment of Glassware and Plasticware:**   All lab materials made of plastic

148   were washed thoroughly in a 10% solution of a nontoxic, biodegradable detergent

149   (Simple Green[TM]; Sunshine Makers, Inc., Huntington Harbour, CA), rinsed repeatedly in

150   ultrapure Milli-Q[TM] water (Millipore Corp., Medford, MA), and immersed in a 30mM

151   Na_4EDTA (Fisher Scientific, Hanover Park, IL) solution overnight to remove all surface

152   adsorbed metal ions; glassware was washed and rinsed similarly but immersed in a

153   10% HNO_3 (Fisher Scientific, Hanover Park, IL) solution overnight.   Glass and

154   plasticware were then rinsed in ultra-pure Milli-Q[TM] water.

155   **Zebrafish rearing:** Adult tropical 5D strain (wildtype) zebrafish were raised at the

156   Sinnhuber Aquatic Research Laboratory (SARL) in the Aquatic Biomedical Models

157   Facility Core of the Environmental Health Sciences Center at Oregon State University

158   under standard conditions (28°C, 14 hr light/10 hr dark cycle) on a recirculating water

159   system. Embryos obtained from group spawns were washed, screened for viability, and

160   incubated in embryo medium (Westerfield, 2000) at 28°C. Larvae destined for adult

161   behavior testing were exposed to 0 (0.1% DMSO only = control), 0.1, 1 µM BPA, 0.1 µM

162   E2, or 0.1 µM GSK4716 from 8-120 h post fertilization (hpf), then removed from the

163   exposure solution, thoroughly rinsed with water, and raised at the SARL under standard

164   conditions until approximately 3 months of age, at which time they were shipped

165   overnight to the Neurobehavioral Toxicology Facility, Children's Environmental Health

166   Sciences Center, University of Wisconsin-Milwaukee, where they were raised under

167 standard conditions prior to adult testing. Zebrafish husbandry and behavior testing was

168 conducted in compliance with approved Oregon State University and University of

169 Wisconsin-Milwaukee Institutional Animal Care and Use Committee protocols.

170 **Chemical preparation:** Bisphenol A (2,2-bis(4-hydroxyphenyl)propane; 99%

171 purity, Tokyo Chemical Industry America (TCI), Portland, OR), GSK4716 (4-Hydroxy-2-

172 [(1E)-[4-(1-methylethyl)phenyl]methylene]hydrazide; Tocris Bioscience, Ellisville, MO,

173 USA), and 17β-estradiol (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl

174 sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). BPA stock concentration was

175 confirmed by high-performance liquid chromatography (HPLC) analysis. Exposure

176 solutions were prepared by diluting working stocks in buffered embryo medium at a final

177 vehicle concentration of 0.1% DMSO.

178 **Social Behavior Test:**

179 *A. Testing Chamber:* A white, plastic box (16 cm width x 25 cm length x 10 cm

180 height) was constructed with interchangeable panels to allow for multiple testing

181 designs. In the test described below a white plastic wall was inserted to split the

182 chamber lengthwise so that each section of equal area would hold a single fish and

183 each fish was unable to detect the presence of the other visually or by odor. The 10 cm

184 height was necessary to prevent fish from jumping out of the chamber. At one end of

185 each section a mirror was inserted; the opposite end remained without a mirror. An

186 individual male was placed in one section and a female in the other so that each sex

187 was tested together within the same time frame. Each section was divided into three

188 zones: 0-1 cm from the mirror (= mirror), 1-12.5 cm from the mirror (= transition), and

189 12.5-25 cm from the mirror (= opaque). These three zones represent different distances

190 from the mirror image and provided, therefore, different intensity levels of social

191 interaction.

192   Fish movements were recorded at a rate of 30 frames/sec with an infrared-

193 sensitive digital camera (Ikegami Model ICD-49, Neuss, Germany) placed 45 cm above

194 the bottom of the test chamber. Lighting was provided by a ring (diam. 10 cm) of 18

195 white light LED (EnvironmentalLights.com, San Diego, CA, USA) placed above the test

196 chamber to provide even lighting intensity (200 lux) throughout the chamber to allow the

197 fish to see the mirror image. Lighting for the camera was provided by an infrared lamp

198 (IR—ROOM Ultra-Covert 940 nm Infrared Illuminator, Night Vision Experts.com,

199 Buffalo, NY) placed 45 cm below the test chamber to prevent glare.

200   *B. Testing Protocol:* Experimental protocol follows that of Weber and Ghorai

201 (2013). All tests used dechlorinated Lake Michigan tap water warmed to 28°C in an

202 incubator. This represents the same water conditions used for maintaining the adult fish.

203 The quality of City of Milwaukee water is rigorously and continuously tested and found

204 to have undetectable levels of BPA, among many other chemical contaminants, that

205 could potentially confound experimental variables (Milwaukee Water Works 2013).

206 Water (1 L, 2.5 cm depth) was replaced after each trial to maintain equal temperatures

207 between trials and remove any olfactory clues left by the previous fish. Before replacing

208 with fresh water, chamber was rinsed three times with distilled water. To account for

209 differential circadian social activity patterns entrained by feeding time, e.g., agonistic

210 behavior (Weber and Spieler 1987) each fish was tested first within two hr of feeding

211 time (0930 h) and again at 5 hr post feeding time (1400 h). Time required to test a 10-

212 fish set was approximately 90 min.

213   Individual male and female adult zebrafish (12-months old; n = 10 of each sex)

214   were placed into separate sections of the testing chamber and allowed to acclimate for

215   1 min before recording started. All fish were first tested in a chamber without a mirror to

216   obtain baseline activity patterns; they were retested on a different day with the mirror

217   inserted. Fish movements were monitored for 5 min. Using image analysis software

218   (EthoVision v8.5, Noldus, Wageningin, The Netherlands), total distance traveled (cm),

219   percent time spent in each zone (mirror, transition, and opaque), and number of attacks

220   on the mirror image were recorded. Attacks were defined as movement into the zone

221   where the mirror was located. While such movement may include both actual attacks on

222   the mirror image it may also include general swimming motion into that zone. To

223   minimize the second possibility, the zone was created to be no wider than ½ the

224   average snout-to-tail length of an adult zebrafish.

225   <u>Statistical Analysis:</u> Since none of the variables displayed a normal distribution, a

226   generalized linear model, GLM, was used for each of the variables with a transform that

227   was the most appropriate to model statistical comparisons, specifically for distance

228   traveled a gamma distribution with a log transform, for time spent at the mirror a normal

229   distribution with a log transform, and for attacks on the mirror a negative binomial (a

230   Poisson allowing variability in the rate between subjects and assuming the same rate for

231   each subject) distribution with a log transform was used. Multiple comparisons were

232   made with the Duncan Empirical Bayes method (Dixon and Duncan1975). Data were

233   analyzed for AM and PM time points for each of the 3 variables. Statistical significance

234   was set at $p < 0.05$.

235

236   RESULTS

237

238   **Distance Travelled:** *AM Testing Time:* If the mirror was absent (Figure 1), there

239   was no significant difference in distance traveled by males between any treatment

240   groups. However, control females traveled significantly more than control males (+871

241   cm,). BPA-, E2 and GSK4716-exposed females traveled significantly less than control

242   females: 0.1 µM BPA (-496 cm), 1 µM BPA (-476 cm), E2 (-1107 cm) and GSK4716 (-

243   989). 0.1 µM BPA differed markedly only from E2 and GSK4716.

244   After the mirror was inserted (Figure 1), distance traveled by BPA-, E2 and

245   GSK4716-exposed males was significantly less than control. There was no significant

246   difference between BPA-, E2 and GSK4716 treatments. While the distance traveled by

247   control males was more than control females (+566 cm), it was less than females for

248   exposures to 1 µM BPA (-809 cm) and E2 (-655 cm). There were no significant

249   differences between males and females for exposures to 0.1 µM BPA or GSK4716.

250   Distance traveled for females treated with 0.1 µM BPA or GSK4716 was markedly less

251   than control (-496 cm and -749 cm, respectively). Distance traveled by females treated

252   with either 1 µM BPA or E2 did not differ from control.

253   *PM Testing Time:* If the mirror was absent (Figure 1), females traveled a

254   significantly greater distance vs. males (+211 cm) regardless of treatment. Neither BPA

255   nor E2 produced significant changes in distance traveled vs. control for either females

256   or males. GSK4716 induced a significant decrease in distance traveled (-301 cm).

257   After the mirror was inserted (Figure 1), control male activity was significantly

258   higher than males treated with 1 µM BPA (+434 cm), E2 (+546 cm) or GSK4716 (+592

259 cm); these 3 treatments did not significantly differ from each other. Travel distance for

260 males treated with 0.1 µM BPA was similar to control. For females, only GSK4716

261 treatment traveled significantly less than controls (-422 cm). The distance traveled for

262 control males was significantly higher than females (+1421 vs. +1081 cm).

263 **% Time Spent at Mirror:** *AM Testing Time:* If the mirror was absent (Figure 2),

264 there were no significant differences in % time spent at the mirror between controls vs.

265 treatment or between sexes. After the mirror was inserted (Figure 2), there were

266 significantly longer times spent at the mirror for control males and females. Significant

267 differences in time spent at the mirror were observed for: 0.1 µM BPA > control (+42%),

268 E2 < control (-4%), and GSK4716 > control (+33%). While fish exposed to either 0.1 µM

269 BPA or GSK4716 spent equal amounts of time at the mirror, they were both significantly

270 greater than for those individuals exposed to 1 µM BPA or E2 (>35%).

271 *PM Testing Time:* When the mirror was absent (Figure 2), there was no marked

272 treatment effect for males or females, i.e., no sex x treatment interaction. Females spent

273 more time at the mirror than males (+7%). After the mirror was inserted (Figure 2), there

274 were no significant gender effects. For this variable, however, both E2 (+13%) and

275 GSK4716 (+27%) exerted an additive effect on time males spent at the mirror time in

276 addition to the effect of the mirror.

277 **Attacks on Mirror:** *AM Testing Time:* When the mirror was absent (Figure 3),

278 there was no marked treatment effect on the number of attacks. There was, however, a

279 significant gender effect (females > males). As there was no mirror, these "attacks" were

280 more likely general swimming movements into the mirror zone. After the mirror was

281 inserted (Figure 3), there was no gender effect but there was a treatment effect; among

282   males each of the 4 treatments was significantly lower than control (-14.5 attacks, 0.1

283   μM BPA < control; 1 μM BPA < control, -10.5 attacks; E2 < control, -10.4 attacks;

284   GSK4716 < control, -14.5 attacks), but none were significantly different from one

285   another. Female zebrafish exposed to 0.1 μM BPA or GSK4716 showed a significant

286   decrease in attacks on the mirror image.

287   *PM Testing Time:* The dominant factor for changes in the number of attacks was

288   the presence of the mirror (Figure 3). While there were no significant differences among

289   treatments, or interactions of mirror x sex, the difference relative to control with the

290   mirror was significantly greater than without the mirror (+13.8 attacks).

291

292   DISCUSSION

293

294   Zebrafish live in loose social groups (Spence et al. 2008) and, therefore, serve as

295   a useful model to understand how xenoestrogenic compounds disrupt normal social

296   interactions. In this study, mirrors were employed to elicit agonistic displays, which

297   include ritualized defensive interactions as well as passive and active aggression (King

298   1973). These traits were measured among adult male and female fish that were

299   developmentally exposed as embryos to varying concentrations of BPA. This exposure

300   regimen was identical to the environmentally relevant levels used in a previous study

301   (Saili et al. 2012) and compare favorably to the maternal delivery of BPA to a fetus

302   (Zimmers et al. 2014; Aries 2013).

303   Male zebrafish not treated with BPA as embryos swam shorter total distances

304   when no mirror was in the test chamber vs. when a mirror was present. The image of a

305   same-sex and same-sized conspecific differs from those studies in which two live

306   competitors are present in the same tank because body morphology affects mirror-

307   elicited response outcomes (Holtby et al. 1993). Without a clear body morphology

308   difference, the response of an adult zebrafish to its perceived equally sized competitor

309   may be more intense and for a longer period of time. While there was decreased activity

310   in the PM observation time, males still swam longer distances if a mirror was present.

311   When the mirror was absent, male activity was not significantly different among

312   treatments; when the mirror was present, distance traveled was significantly less among

313   BPA-, estradiol-, or GSK4716-exposed individuals vs. controls. This was in contrast to

314   several studies, including rat pups (Xu et al. 2007) and our previous investigations into

315   the locomotor activity of zebrafish larvae (Saili et al. 2012), that indicated hyperactivity

316   following BPA exposure. Larval activity is not sex-based, whereas social interactions

317   with another adult of same sex and size potentially raise issues of territoriality, resource

318   use, and reproductive status which have a strong sex-based linkage. Interestingly,

319   prenatal BPA exposures increased depression in boys (Harley et al. 2013) and mice (Xu

320   et al. 2012), a behavioral outcome that may parallel the changes observed in the

321   depressed locomotor activity in male zebrafish.

322   Percent time spent in the mirror zone displayed a non-monotonic response

323   (curve changes direction within range of concentrations examined), in this case the

324   concentration-response curve was an inverted U-shape in which the intermediate

325   exposure (0.1 µM BPA) concentration produced a higher response than either 0 or 1 µM

326   BPA. Behavior within the mirror zone was most intense only when the mirror was

327   inserted into the chamber and consisted of back-and-forth swimming in front of the

328 mirror image with no apparent attack movements directed to the mirror image. This

329 activity, therefore, was likely to be a combination of several agonistic behaviors

330 including aggression and territorial displays, as documented by zebrafish in the wild

331 (Spence et al. 2008). That developmental exposure to either 0.1 μM BPA or 0.1 μM

332 GSK4716 resulted in equally intense activity in the mirror zone suggests that, as

333 supported by our previous studies (Saili et al. 2012) BPA is acting as an ERRγ agonist,

334 at least at the lower BPA concentration. Yet, developmental exposure to either 0.1 μM

335 E2 or 1 μM BPA resulted in a decrease in the amount of time spent in the mirror zone

336 suggesting differential, concentration-dependent sensitivity to multiple pathways during

337 development. For example, Masuo and Ishido (2011) and Kubo et al. (2001)

338 demonstrated an inverted U-shaped BPA effect on the locus ceruleus in response to

339 stimuli. Performance was decreased at very low levels and high levels of LC tonic

340 discharge due to drowsiness and being inattentiveness and at high. Performance on a

341 task that required focused attention (possibly similar to that required for interacting with

342 the mirror image) was highest at moderate LC tonic activity (Ashton-Jones et al. 2007).

343 Whether this parallels the inverted U-shaped response curve for % time swimming in

344 front of the mirror of the low vs. high BPA-exposed zebrafish in this study is unclear,

345 although fishes do possess this structure, albeit much smaller (Ma 1994). However,

346 estrogenic compounds, e.g., diethylstilbestrol, have been shown to alter locus ceruleus

347 morphology (Kubo et al. 2001). The opposite effect of two compounds that interact with

348 estrogen receptor sites as it relates to this one specific variable, % time spent at the

349 mirror, suggests that ERs and ERRs may be involved in regulating the direction of non-

350 aggressive social behaviors and which receptor is being affected may be dependent on

351 BPA exposure concentration.

352   Conversely, the number of attacks in the mirror zone showed a U-shaped

353 concentration-dependent response for females, albeit statistically insignificant; a linear

354 decrease in attacks with increasing exposure concentration was observed with males.

355 Other non-monotonic responses due to BPA exposure were noted with learning tasks in

356 male rats (Jones et al. 2011), metabolic function in mice (Angle et al. 2013), and protein

357 regulation in a terrestrial isopod (Lemos et al. 2010). One functional basis for the non-

358 monotonic effect is that at doses higher than those required for ER-mediated

359 responses, BPA interacts with other hormone receptor sites. Alternatively, BPA may be

360 interacting on neuroendocrine systems that impact physiological process underlying

361 behavioral outcomes (Léon-Olea et al. 2014; Wayne and Trudeau 2011). These

362 interactions also may affect neurodevelopment potentially confounding the ability to

363 make predictions of neurobehavioral toxicity over a range of concentrations (vom Saal

364 et al. 2007). This would imply that BPA is more than a xenoestrogen and that it interacts

365 directly and/or indirectly with multiple classes of neural and endocrine receptors.

366   Another aspect of this study not generally included in other examinations of

367 social interaction was the role of circadian rhythmicity in affecting outcomes and

368 interpretations of behavioral toxicity. As demonstrated by each of the variables

369 analyzed, the time-of-day when an experiment was conducted relative to a regularly

370 occurring stimulus to which behavior was entrained (in this case feeding time) exerted a

371 profound effect on behavioral outcomes. Examination of only the afternoon data would

372 have forced the conclusion that BPA exerted little or no effect on social behavior. The

373 morning data, however, indicated that BPA produced a substantial impact on zebrafish

374 social behavior.

375   Circadian rhythms are important in regulating responses to the social stimulus

376 used in this study, i.e., a social partner created by the mirror image. The presence of

377 social partners might produce mutual behavioral synchronization in a wide range of

378 species (Favreau et al. 2009). Such group enhancement of activity was observed in

379 killifish (Fundulus heteroclitus) to be significantly higher than among solitary individuals

380 (Kavaliers 1980). Social influences within a hierarchical relationship, i.e., relative

381 dominance within the population, may induce mutual synchronization of activity

382 rhythms. These, however, were not accounted for in this study and may explain the high

383 variability in the behavioral outcomes. Circadian rhythms direct locomotor activity

384 patterns in fish (Weber and Spieler 1994), which, in turn, alter the intensity of social

385 behavior and sensitivity to mutual synchronization (Pankseep et al. 2008). It was

386 observed that the afternoon swimming distance was less intense than the morning

387 activity level and this, in turn, may play an important role in the unequal number of

388 attacks during each observation time. Studies cited earlier that support the null

389 hypothesis of BPA not inducing changes in specific behaviors and their underlying

390 mechanisms (mice: Palanza et al. 2002; Cagan et al. 1999; rat: Kobayashi et al. 2012;

391 Ryan et al. 2010) may be due to recording observations at times-of-day in which activity

392 levels were less intense. While the light-dark cycle entrains pineal 5-HT rhythms

393 (Ceinos et al. 2005), feeding time entrains both locomotor activity (Weber and Spieler

394 1987) and circulating 5-HT levels (Ho et al. 1985). Without knowledge as to when the

395 tests were conducted relative to these variables, it becomes difficult to compare other

396   studies to our data. Complicating the picture of locomotor activity patterns even further,

397   some studies fed their test animals ad libitum rather than at a single time of day, thus

398   altering the times and intensity of peak activity.

399   Social behaviors are dependent upon neural and endocrine signaling as

400   influenced by external cues. Disruptions in normal patterns of these behaviors may

401   result from changes induced by environmental stressors on the complex interplay

402   between neural and endocrine systems. The mirror-elicited behavior of the adult

403   zebrafish involved two aspects of agonistic display, ritualized territorial displays and

404   direct attacks. By measuring total distance traveled, total % time spent at the mirror (or

405   the zone in which the mirror would be placed), and number of attacks on the mirror (or

406   the zone in which the mirror would be placed), specific aspects of social interaction with

407   a conspecific of the same sex and morphology (general activity level, degree of

408   interaction or association, and aggressive behavior, respectively), insights into other

409   mechanisms that were possibly affected by developmental BPA exposure are

410   suggested.

411   Ovarian steroids, which are affected by BPA exposure (fish: Hatef et al. 2012;

412   mice: Xi et al. 2011; rats: Li et al. 2014), regulate the serotonin system, especially in

413   regions critical to controlling social behaviors, e.g., raphe nuclei and hypothalamus. In

414   rodent brains, treatment with estrogens increased levels of dorsal raphe 5HT2A mRNA

415   (McEwen 2002) and increased the density of 5-HT2A binding sites in other brain

416   regions associated with emotion and behavior (Fink et al. 1996). Since agonistic

417   displays may be markedly affected by this interaction, xenoestrogenic activity of BPA as

418   it relates to ER and ERRγ agonism (general: Zuercher et al. 2005; Ben-Jonathan and

419 Steinmetz 1998; human: Okada et al. 2008; rat: Washington et al. 2001), and the

420 consequent effects on the serotonergic system during development may be an example

421 of BPA-induced neuroendocrine disruption. Because serotonin is important during brain

422 development and in controlling levels of aggression, and BPA enhances 5-HT activity,

423 BPA-induced changes in embryonic 5-HT levels may be responsible for altered brain

424 development (fish: Elipot et al. 2013; Dahlbom et al 2012; Clotfelter et al. 2007; mice:

425 Rood and Beck 2013; rat: Cao et al. 2013; Donner and Handa 2011; Matsuda et al.

426 2010; González et al. 2008; Honma et al. 2006; Orozco-Suárez 2003; Persico et al.

427 2000; Yan et al. 1997).

428   It is still an open question whether, in fact, altered embryonic 5-HT dynamics

429 explain the changes in adult behavior observed in this study. Other investigations,

430 however, using fish exposed to fluoxetine (FLX), a selective serotonin re-uptake

431 inhibitor that is found as a contaminant in some aquatic systems suggest this may be a

432 useful avenue of research. Behavioral disruptions occurred in fathead minnows

433 (*Pimephales promelas*) of the 5-HT system after short-term, adult exposures to FLX

434 (Weinberger and Klaper 2014). While at FLX concentrations higher than used in this

435 study male aggression toward females increased, fish exposed to concentrations similar

436 to this study caused a concentration-dependent decrease in total swimming distance.

437 Other studies using fish models involve short-term larval FLX exposure followed by

438 larval locomotor behavioral observations (Airhart et al. 2007) or 4-wk adult exposure

439 followed by analyses of reproductive physiology and behavior (Foran et al. 2004) also

440 demonstrated behavioral effects comparable to this study. Full comparisons, however,

441 are limited due to different exposure regimens between studies. The embryonic

442 exposures used in this study suggest that adult behavioral effects may be the result of

443 permanent neurological alterations caused by early (first 24 hpf) developmental

444 exposures to low-level, environmentally-relevant concentrations of BPA. The underlying

445 mechanisms of this behavior remain to be elucidated.

446

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448

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873   Figure Legends:

874   Figure 1: Effect of exposures to bisphenol A (BPA) during early development on activity

875   level of adult zebrafish (12 months). Distance traveled by male and female zebrafish

876   within test chamber over a 5 min period after a 1 min acclimation period was compared

877   with and without the presence of a mirror. Changes in circadian rhythm patterns were

878   evaluated by testing each fish at two times of day: morning immediately after feeding

879   time at 0900 hr and again at 1400 hr. Developmental exposure regimen (2-48 hours

880   post fertilization) consisted of: ■ = 0.0 µM BPA;   ▨ = 0.1 µM BPA;   □ = 1.0 µM

881   BPA;   □ = 0.1 µM estradiol; and   ▨ = 0.1 µM GSK4716.

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

883

884   Figure 2: Effect of exposures to bisphenol A (BPA) during early development on % time

885   spent interacting with the mirror image by adult zebrafish (12 months). Percent time

886   spent by male and female zebrafish in the test chamber mirror zone (≤2 cm away from

887   wall where mirror was placed) over a 5 min period after a 1 min acclimation period was

888   compared with and without the presence of a mirror. Changes in circadian rhythm

889   patterns were evaluated by testing each fish at two times of day: morning immediately

890   after feeding time at 0900 hr and again at 1400 hr. Developmental exposure regimen (2-

891   48 hours post fertilization) consisted of: ■ = 0.0 µM BPA; ▨ = 0.1 µM BPA;

892   ▨ = 1.0 µM BPA; □ = 0.1 µM estradiol; and ▨ = 0.1 µM GSK4716.

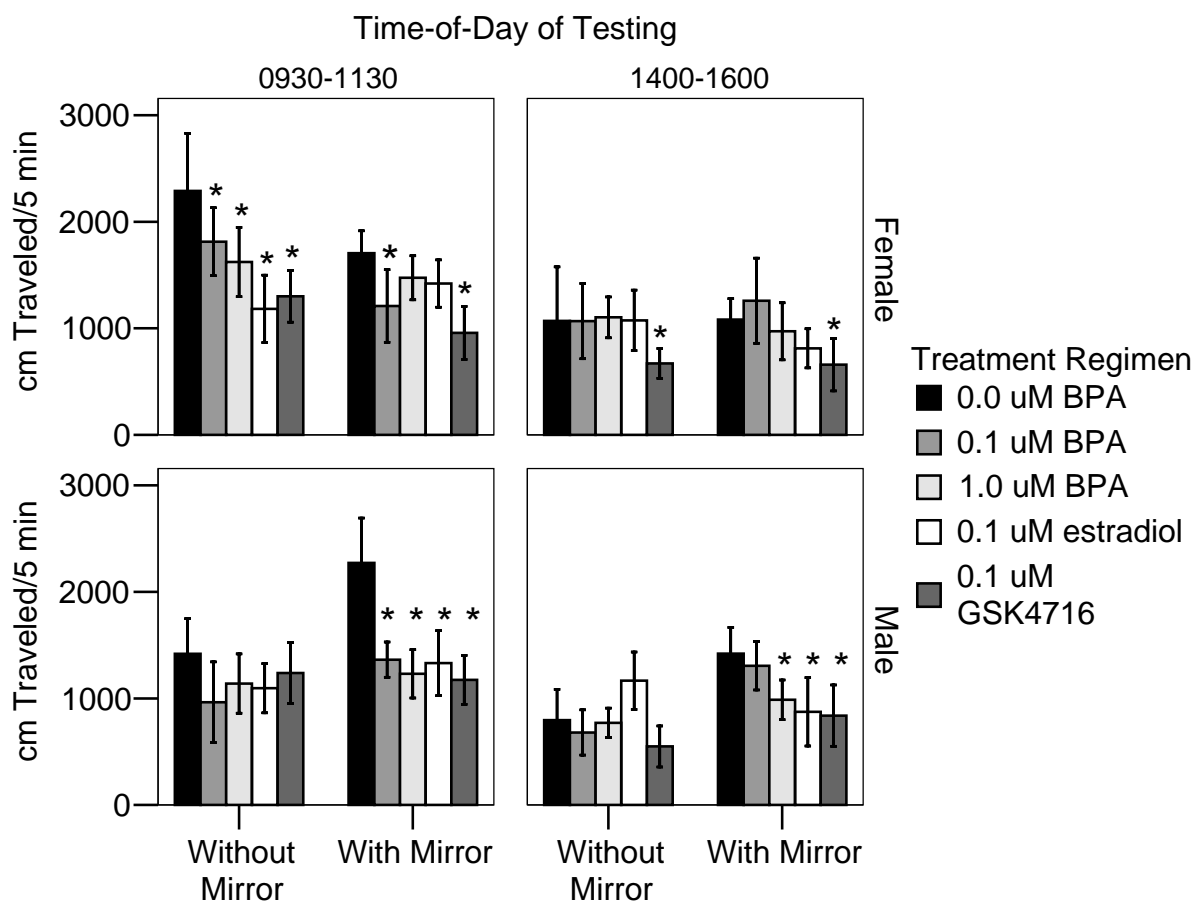893   * = significantly different from control (p < 0.05) of same sex.

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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 µM BPA; ▨ = 0.1 µM BPA; ☐ = 1.0 µM BPA; ☐ = 0.1 µM estradiol; and ▦ = 0.1 µM GSK4716.

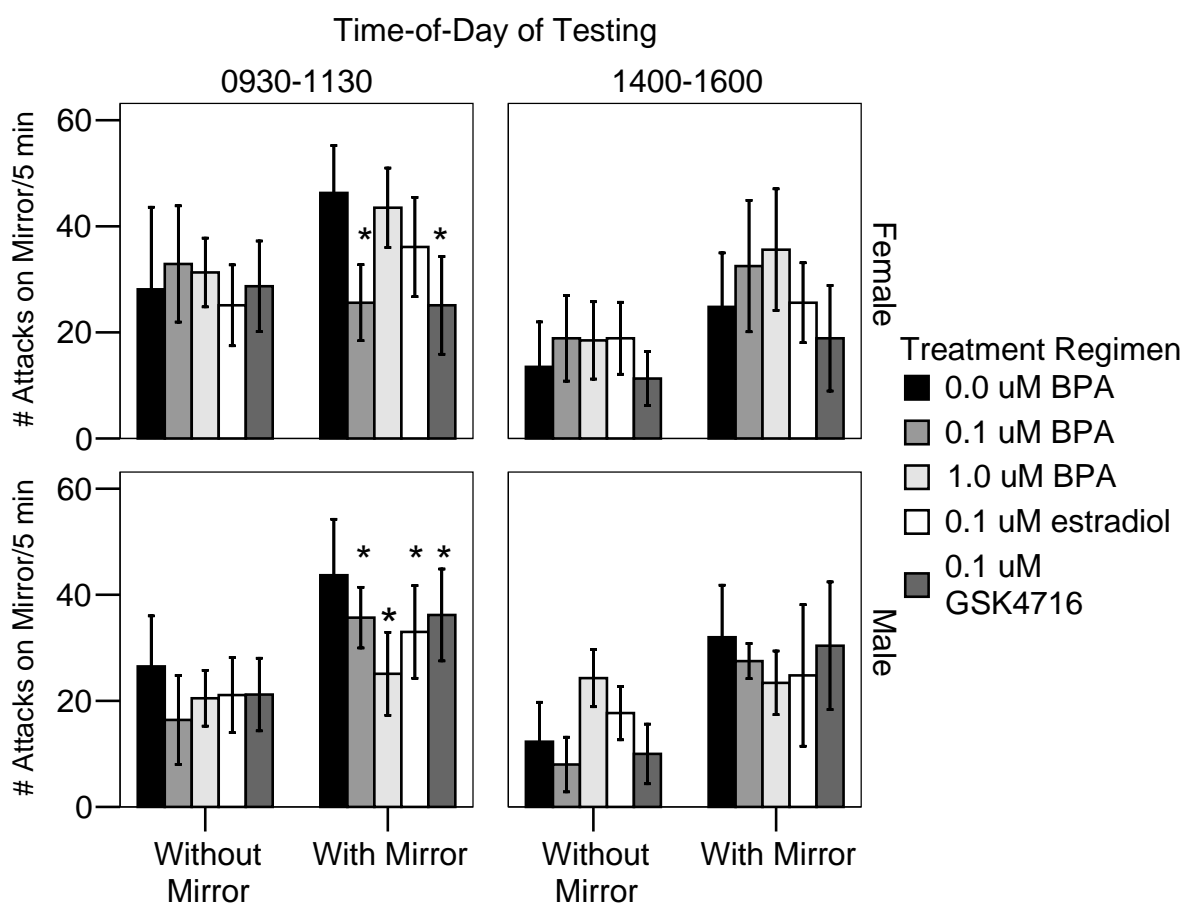* = significantly different from control ($p < 0.05$) of same sex.

906   Figure 1:

Time-of-Day of Testing

908   Figure 2:

910   Figure 3:

Time-of-Day of Testing

911