Hypothalamic-pituitary-ovarian axis perturbation in the basis of bisphenol A (BPA) reproductive toxicity in female zebrafish (*Danio rerio*)

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Abstract

Thousands of safety-related studies have been published on bisphenol A (BPA), a ubiquitous environmental pollutant with estrogenic activity and many other potential biological effects. In recent years, BPA exposure has been shown to cause anovulation and infertility through irreversible alteration of the hypothalamic-pituitary-gonadal axis. Recently, the European Chemical Agency classified BPA as a "substance of very high concern" because of its endocrine-disrupting properties, which have serious effects on human health. Given the risk of exposure to BPA as a pollutant in the environment, food, and water, the objective of our study was to assess the effects of that compound on the pituitary gland by means of a histopathological and morphometric study of the gonadotroph cells. In addition, using quantitative real-time PCR (qRT-PCR) assays, we analyzed the changes in the expression of Cyp19b (an aromatase gene). Absolute expression of Cyp19b was found to be diminished in BPA-exposed fish, indicating downregulation of gonadotropic hormones, in agreement with the observed degeneration of gonadotropic cells. This is the first study in which the gonadotroph cells have been evaluated using histomorphological endpoints after BPA exposure in zebrafish.

Keywords: Bisphenol A; zebrafish; pituitary gland; Cyp19b; gonadotroph cell

1. Introduction

There is a growing body of evidence that environmental exposure to endocrine-disrupting chemicals (EDCs) is ubiquitous and exerts potentially adverse health effects on the human organism. Consequently, there is an increasing need for consistent screening assays that specifically detect and characterize EDCs present in the environment (Sun et al., 2010; Flint et al., 2012). Bisphenol A (BPA), considered an EDC, is one of the most abundantly produced chemicals worldwide and a usual component of plastics and food containers. Over 3.5 million tons of BPA are produced each year, and more than 100 tons are released into the atmosphere. Changes in pH, mechanical abrasions, and heat accelerate hydrolysis of the ester bond linking BPA molecules in polycarbonate plastic, and repeated washing of polycarbonate products causes BPA to easily leach out and spread through the environment, even at moderate temperatures (Hoekstra and Simoneau 2013; Huo et al., 2015; Inagaki et al., 2016). In the past few years, interest has increased because of its ubiquitous presence and thus the increasing exposure of humans and other organisms from the environment and food, which has become a public health problem (Shi et al., 2015); aquatic wildlife, in particular, appears to be at an increased risk, but humans and all animals may suffer adverse health effects from current BPA levels (Chapin et al., 2008; Sun et al., 2010; Rochester et al., 2013; Teeguarden et al., 2013; Canesi and Fabbri, 2015). In June 2017, the European Chemical Agency (ECHA), supporting the decision made by France, classified BPA as a “substance of very high concern” because of its endocrine-disrupting properties, which have serious effects on human health. Because of its ability to mimic the body's own hormones and bind estrogen receptors, BPA interferes with the function of the endocrine system and alters overall female reproductive capacity by

91 affecting the morphology and function of the oviduct, uterus, ovaries, and
92 hypothalamus-pituitary-ovarian axis. Normal folliculogenesis depends upon intra-
93 ovarian androgens for the synthesis of estradiol, regulated by the neuroendocrine
94 hypothalamic-pituitary-ovarian (HPO) axis driven by the gonadotropin-releasing
95 hormone (GnRH) neuronal network. The coordinated action of two gonadotropins,
96 namely, luteinizing hormone (LH, GTHII in fish) and follicle-stimulating hormone
97 (FSH, GTHI in fish), controls the levels of androgen and their conversion to estradiol.
98 LH acts on the theca cells, inducing secretion of androstenedione. FSH acts on
99 granulosa cells, promoting the conversion of androstenedione to estradiol by the action
100 of aromatase. Estradiol levels must increase and predominate over androgen
101 concentrations in order for follicles to develop. A fine balance exists, and excess
102 production of androgens as a consequence of disordered folliculogenesis results in poor
103 follicle maturation and increased follicular atresia (reviewed in Baskind and Balen
104 2016). The phenolic structure of BPA allows this compound to interact with estrogen
105 receptors and estrogen signaling pathways, and it has been reported that BPA may be as
106 effective as estradiol in triggering some receptor responses; it may also act as an
107 androgen receptor antagonist (reviewed in Flint et al., 2012). Rodent and fish studies
108 indicate that BPA exposure also affects FSH and LH levels, although the results are
109 limited and inconclusive (Ziv-Gal and Flaws, 2016). The objective of the present study
110 was to investigate the toxic effects of a wide range of BPA dosages, including a 'safe'
111 dose of 1 μg/L, at the pituitary level, which could render female *Danio rerio* unable to
112 reach normal reproductive capacity. We assessed the histopathological consequences of
113 BPA exposure for gonadotropic cells and analyzed the gene expression of brain
114 aromatase (Cyp19b) at the transcript level, using both parameters as biomarkers of BPA
115 exposure.
116

117 **2. Material and methods**

118 **2.1. Fish exposure and sampling protocol**

119 Sixteen-week-old female zebrafish *(Danio rerio)* (n=105) were used. The experimental
120 procedure was conducted at the Experimental Animal Service of the University of
121 Córdoba, in concordance with the European Regulations for the Protection of
122 Experimental Animals (Directive 2010/63/EU). The treated groups were exposed for 14
123 days (Organization for Economic Co-operation and Development -OECD- Guideline
124 No. 204) to graded concentrations (1, 10, 100 and 1000 µg/L) of BPA (Sigma-
125 Aldrich®). A control group (kept in unchlorinated tap water) completed the exposure
126 design. After 2 weeks of exposure, the zebrafish were sacrificed by an overdose of an
127 anesthetic solution of tricaine methanesulfonate (MS-222® 500 mg/L; Sigma-Aldrich)
128 buffered with sodium bicarbonate (300 mg/L; Sigma-Aldrich). The heads of 30 animals
129 (n=6 per group) were dissected and fixed for histological analysis for the qualitative and
130 quantitative evaluations. The heads of 45 additional zebrafish (n=9 per group) were
131 removed, immediately immersed in liquid nitrogen, and stored at -80°C for qRT-PCR.
132 The fish (n=6 per group) used for the toxicological analysis (analytical BPA
133 determinations) were dried with sterile gauze and then frozen and stored at -80°C.

134 **2.2. Analysis of BPA content**

135 Whole-body homogenates were obtained for BPA quantification. They were
136 homogenized with a ribolyser to 10.000 G in 1 ml tubes at a 1:2 ratio of tissue wet
137 weight to buffer (50 mM Tris-HCl pH 7.4).

138 Samples were processed for extraction and purification and finally transferred into vials.
139 A volume of 20 µl was injected into a LC-MS/MS system for BPA quantification using
140 the technique employed by Molina et al. (2013).

141 **2.3. Histological evaluation**

142 *Qualitative study:* For the structural evaluation, samples were routinely processed for
143 paraffin sections by fixation in 10% buffered-formalin solution, dehydration in a graded
144 ethanol series, immersion in xylol and embedding in paraffin wax. Sections (4 µm
145 thick) of each block were stained with hematoxylin and eosin and used for the
146 morphological evaluation.

147 For the ultrastructural study, small, randomly selected samples were first fixed in a 2%
148 glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4, 4ºC overnight) and then
149 refixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 30 min. After
150 the samples were dehydrated in a graded ethanol series and embedded in Araldite,
151 semithin and ultrathin sections were cut on an LKB ultramicrotome at the Central
152 Microscopy Research Facilities, University of Córdoba, Spain. Ultrathin sections were
153 double stained with uranyl acetate and lead citrate. For the scanning study, the sample
154 was critical point dried and then coated in colloidal gold. Ultrathin sections were
155 viewed and photographed with a JEM 1400 transmission electron microscope.

156 *Quantitative study:* The morphometric study of the gonadotroph cells was conducted
157 directly on images taken of the cephalic lobe of the pituitary gland under the electron
158 microscope, quantifying both the number and the size of the cells. Twenty small squares
159 per animal were quantified for a total of 100 squares per group, each grid square being
160 100 µm per side, the total surface area analyzed per group being 100x100=10000 μm^2.
161 We based our counts on cell identification by the pleomorphism of granules, measuring
162 the nucleus surface so that we obtained the number of cells observed on that surface.

163 **2.4. Isolation of RNA and synthesis of cDNA**

164 Total RNA was isolated using Isol-RNA Lysis Reagent (5PRIME) and the Total RNA
165 Cleanup/DNase Digestion kit to remove genomic DNA contamination; the success of

166 the isolation and purification was verified by PCR amplification of the RNA samples
167 without previous reverse transcription, using specific intraexonic primers designed for
168 GAPDH (F: 5´-CCAGTACGACTCCACCCATGGAAA-3´; R: 5´-CGCTATAGACTG
169 TGATTGCATGACCA-3´). PCR was performed using the following program: 95°C for
170 2 min; 40 cycles of denaturing at 94°C for 15 s, annealing at 65°C for 30 s, and
171 extension at 72°C for 30 s; and a final extension step at 72°C for 10 min.
172 Quantification of RNA was conducted by measuring optical density at 260 nm. An
173 absorbance ratio of 2:1 at 260 nm and 280 nm was considered indicative of protein-free
174 samples. The integrity of the RNA samples was determined with an Agilent 2100
175 Bioanalyzer (Agilent Technologies) and only samples with RIN values >8.5 were used.
176 cDNA was generated from 1 µg total RNA per reaction with the QuantiTect Reverse
177 Transcription Kit (Qiagen), according to the manufacturer's protocol.

178 **2.5. Primer design and amplification efficiency**

179 Primers for the genes ActB, B2m, Hprt1, GusB, Nono and Rpl13 have been previously
180 described (own data). To design specific primers to quantify the mRNA transcripts of
181 *Cyp19b*, we obtained the sequences from the GenBank database
182 (http://www.ncbi.nlm.nih.gov/gene) and used the software Oligo 7 software (Molecular
183 Biology Insights, Inc.). The *Cyp19b* primers (F: 5´-ACGTTACTGCTGCTG
184 ACGGGAACCTTAATG-3´; R: 5´-TCATTGTAGTAGTTGCACGCCGTGCCGATG-
185 3´) had high Tm (≥80°C) and a 3′-ΔG ≥−5 kcal/mol and were free of hairpin and duplex
186 structures. Their amplification efficiency was 100%, and they generated one specific
187 PCR product, as confirmed by PCR product sequencing.

188 **2.6. qRT-PCR analysis**

189 Real-time RT-PCR was performed in quadruplicate with Platinum Taq DNA
190 polymerase (0.75 units/reaction; Thermo-Fisher) and 1:100,000 SYBR Green I dye

191 (Roche) in a Cycler Q Real-Time PCR System (Bio-Rad). The 25 µL reaction volume
192 also contained 50 ng of cDNA template, 0.3 µM of each primer, 3 mM $MgCl_2$ and 250
193 µM of each dNTP. The cycling conditions were set as follows: 95°C for 2 min; 40 two-
194 step cycles of denaturing at 95°C for 15 s and annealing/extension at 70°C for 30 s; and
195 a final melting curve (60°C to 95°C) analysis to verify the specificity of the amplicons.
196 The linear regression of a calibration curve (Jurado et al., 2003; Prieto-Alamo et al.,
197 2003) was used to calculate the absolute number of transcript molecules.
198

199 **2.7. Statistical analysis**

200 Data were analyzed using the statistical program Statgraphics Centurion XVI®
201 (Statgraphic. com) to determine the effects of BPA on each exposed group. ANOVA (F-
202 test) was used to demonstrate whether there were any significant differences between
203 the averages. Fisher's LSD post hoc test was performed for multiple comparisons
204 between groups. The results are expressed as the mean values ± standard deviation
205 (SD), and $P<0.05$ was considered significant. In the transcriptional analysis, the
206 statistically significant differences between the control and experimental samples were
207 determined by using a Student´s *t*-test and were expressed as * ($P< 0.05$) and *** ($P<$
208 0.001). The statistics program SigmaStat 5.1 (GraphPad Prism) was used throughout the
209 study.

210 **3. Results**

211 **3.1. Analysis of BPA content**

212 Table 1 shows the BPA concentrations in the zebrafish at 14 days after BPA exposure. A
213 statistically significant increase in BPA concentration was observed in the zebrafish
214 exposed to increasing concentrations of BPA, while the BPA levels in the control group
215 were not detectable. There was also a statistically significant difference between the

216 low-dose groups (treated with 1 and 10 µg/L BPA) and the high-dose groups (100 and
217 1000 µg/L) at the $P< 0.05$ level.

218 **3.2. Histological evaluation**

219 In the control group, all the adenohypophyseal cells appeared to be normal (Figure 1 A-
220 D). However, in the 1 µg/L group, the gonadotroph cells were activated, showing an
221 abundant Golgi complex, a dilated rough endoplasmic reticulum and secretory granules.
222 The reticulum was distributed all over the cytoplasm and had protein content inside it;
223 the Golgi complex had copious condensation granules that ultimately formed secretory
224 granules (Figure 1 E-H). In the 10 µg/L group, two types of gonadotroph cells found at
225 different phases stood out. The first type had apparently normal morphology, but other
226 gonadotroph cells, designated Gn1, showed some features that indicated a clear loss of
227 activity. In the Gn1 cells there was an increase in the number of rough endoplasmic
228 reticulum cisternae, which showed dilated lumens indicative of gonadotroph cells
229 inactivation (Figure 2 A-D).

230 In the 100 µg/L group, the Gn1 cells were prominent because of their increased size and
231 hypertrophied appearing, albeit they retained a scant functionality. Some of these cells
232 degraded their secretory granules, showing irregular morphology with dense concentric
233 lines indicating autophagy (Figure 2 E-H).

234 In the 1000 µg/L group, abundant large gonadotroph cells were noted, this cellular
235 hypertrophy being due to an increase in the size of the rough endoplasmic reticulum
236 because of the dilatation of its sacculations. In specific cases, the dilated cisternae
237 formed large vacuoles that ended up occupying the whole cytoplasm, and the dilated
238 sacculations frequently joined together and ultimately produced a large sac that covered
239 practically the whole of the cytoplasm, turning the cells into so-called maximally
240 degenerated gonadotroph cells (Figure 3). This vacuole ended up displacing the nucleus

241 towards the membrane, and the little remaining cytoplasm was left surrounding the
242 nucleus. This cell phase is considered a third stage of the inactive, degenerated
243 gonadotroph cell.

244 In the morphometric evaluation, the percentages of cells (gonadotroph, degenerated
245 gonadotroph, maximally degenerated gonadotroph and the rest of the pituitary cells) in
246 each study group (Table 2) were determined. Significant differences, $P<0.05$, were
247 obtained in all of them with respect to the control. Similarly, there were significant
248 differences, $P<0.05$, between all the exposure groups except in the case of degenerated
249 gonadotroph cells, which did not exhibit significant differences between the two groups
250 with the highest exposure concentrations.

251 **3.3. Real-time qRT-PCR quantification of *Cyp19b* transcripts in the heads of**
252 **female zebrafish**

253 ***3.3.1. Identification of Valid Reference Genes for the Normalization of***
254 ***the qRT-PCR Expression Studies.***

255 The expression level stability of six candidate reference genes (ActB, B2m, Hprt1,
256 GusB, Nono and Rpl13) was evaluated by determining their Ct (threshold cycle) values
257 in all the samples (Figure 4). The software RefFinder (Xie et al. 2012) ranked the six
258 genes by the geometric mean of their weights and identified GusB as the most stable
259 reference gene. We included this gene in our absolute quantification experiments to
260 evaluate the quality of the qRT-PCR determinations.

261

262 ***3.3.2. Quantification of Cyp19b transcripts in the heads of BPA-treated***
263 ***zebrafish.***

264 We analyzed the effects of a wide range (0, 1, 10, 100 or 1000 µg/L in the water) of
265 BPA doses on the transcript levels of aromatase (Cyp19b) in the brains of female
266 zebrafish by using real-time qRT-PCR. We pooled equal amounts of RNA from 10

heads in each experimental group into three mini-pools to diminish cost and time while preserving the ability to analyze interindividual variation. Preliminary analysis clearly indicates that the results obtained from the mixed samples of multiple individuals are not particularly prone to misinterpretations due to the interindividual differences (data not shown). Figure 5 shows the absolute numbers obtained for the transcripts of the Cyp19b gene in the zebrafish head and the changes caused in these basal counts by BPA exposure.

As described for Cyp19a mRNA in ovaries (Molina et al., manuscript under revision), the increasing doses of BPA significantly altered the number of Cyp19b mRNA molecules following a non-monotonic curve that peaked at 10 µg/L. The lowest BPA dose (1 µg/L) caused a >2-fold decrease in the amount of Cyp19b transcript. In contrast, the levels of this transcript increased significantly (≥1.5-fold increase compared with the control) in zebrafish treated with 10-100 µg/L and decreased again with the 1000 µg/L doses of BPA.

4. Discussion

EDCs are natural or synthetic compounds that alter endocrine functions by mimicking or blocking endogenous hormones. These compounds affect the expression of an estrogen-related target gene, aromatase *CYP19b*, in the brain (Chung et al., 2011).

BPA is a well-known EDC and is one of the most prolifically produced chemicals worldwide. It enters the aquatic environment through landfill leachate, sewage treatment effluent, or natural degradation of polycarbonate plastics (Meesters and Schroder, 2002; Wintgens et al., 2003; Gatidou et al., 2007) and gains access to the food chain, constituting a serious health hazard. Among its multiple harmful effects, it has been shown that BPA induces strong brain-specific overexpression of aromatase (Chung et al., 2011).

292 Due to its importance as an environmental and fundamentally aquatic pollutant, the
293 action of BPA as a neuroendocrine disruptor has been widely studied (Molina et al.,
294 2013; Huo et al., 2015; Inagaki et al., 2016; Jedeon et al., 2016; Rhaman et al., 2016). In
295 the present study, we proposed to evaluate its pituitary effects, for which purpose we
296 exposed the animals in a standardized manner to concentrations of 1, 10, 100, and 1000
297 µg/L. The concentrations selected for this study are found naturally in the environment
298 or are detected in areas close to places where the compound is produced (Belfroid et al.,
299 2002; Viganò et al., 2006; Crain et al., 2007; Huang et al., 2012); these BPA
300 concentrations were chosen for previous studies for the same reason (Mandich et al.,
301 2007; Villanueve et al., 2012; Molina et al., 2013). On analyzing the results, we
302 observed that no deaths occurred during the study in any of the groups our data
303 coincided with previous studies that used similar experimental conditions (Mandich et
304 al., 2007; Hatef et al., 2012; Molina et al., 2013). BPA does not affect this parameter
305 even at the highest concentration (1000 µg/L), the level that might be found in industrial
306 areas where this compound is manufactured.

307 Upon analyzing the BPA content in fish, we observed that there was an increase in its
308 concentration as the exposure concentration was augmented from one study group to the
309 next. All the groups treated displayed significant differences ($P<0.05$) with respect to
310 the control group, and no BPA concentration could be detected in the latter. Similarly,
311 there were significant differences ($P<0.05$) between all of the exposed groups. These
312 data coincide with what is expected from a dose-dependent increase in the BPA
313 concentration in whole fish. It is appropriate to use the whole body to detect BPA levels
314 instead of using just one tissue, which might only give us a partial view of its
315 concentration.

316 In the evaluation of the effects of endocrine disruptors, and specifically of BPA, it was
317 observed that alterations are generated at both the gonadal and pituitary levels, but in
318 analyzing the doses to which the animals were exposed, it was verified that the
319 histological modifications appearing in the gonads occurred at higher concentrations
320 than in the case of the pituitary gland (starting from 100 μg/L). Similarly, in previous
321 studies, it has been found that aromatases are more sensitive than histological signs as
322 biomarkers of exposure. All the above leads us to think that BPA would directly affect
323 the pituitary gland, especially the gonadotroph cells, and that, as a result, this action
324 would generate alterations that could turn out to be very severe in the gonads both of
325 males and females (Brannick et al., 2012; Molina et al., 2013; Zhou et al., 2016).

326 All types of apparently normal cells were perceived in the control group. However, in
327 the exposure groups, although all the expected cells were identified, some alterations
328 could be observed that were intensified as the exposure dose was increased. At the
329 lowest exposure dose (1 μg/L), it was observed that, as a consequence of BPA, the
330 gonadotroph cells were activated, probably in response to endocrine alterations. To
331 counteract the latter, the gonadotroph cells increased the sizes of the organelles involved
332 in hormonal synthesis (hypertrophy), and there was even a rise in the number of those
333 cells, giving rise to hyperplasia. The action of BPA on gonadotroph cells at the
334 histological level was so intense that, at the lowest exposure dose, significant
335 differences appeared ($P<0.05$) in all the cell types studied in all the groups evaluated.
336 This differs from what was observed at a gonadal level, in which, at the lowest dose, no
337 apparent histological modifications were produced (Mandich et al., 2007; Molina et al.,
338 2013). These results reinforced our theory that BPA acts primarily at a central level,
339 triggering the rest of its effects secondarily.

340 It was observed that, after exposure to the 10 µg/L concentration of BPA, the
341 gonadotroph cells were exhausted; they ended up losing their functionality, and a large
342 number of these cells degenerated, producing a second type of cell. The latter showed
343 significant differences, $P<0.05$, with respect to the percentage of all the cells evaluated
344 in the study groups.

345 In the study groups exposed to higher concentrations of BPA (100 and 1000 µg/L), the
346 gonadotroph cells appeared extremely altered and they were considered as maximally
347 degenerated cells (Gn-DgM), characterized by profoundly modified structures. They
348 lost all their pituitary hormone synthesis activity, and this gonadotroph cell
349 degeneration was irreversible. Both groups showed significant differences, $P<0.05$, in
350 the cell percentages studied with respect to all the exposure and control groups except
351 for the degenerated cells, in which there were no significant differences between the 100
352 and 1000 µg/L groups. This was probably because they degenerated so much that the
353 process did not evolve further. The appearance of maximally degenerated (Gn-DgM)
354 cells at a percentage of 14.3% in the group with the highest exposure concentration
355 signified the appearance of these nonfunctional cells. Thus, the HPO axis was blocked,
356 which led to severe ovarian alterations, as various studies have demonstrated (Mandich
357 et al., 2007; Molina et al., 2013; Zhou et al., 2016). Few studies have examined the
358 gonadotroph cells after BPA exposure, although our work partly coincides with that of
359 Brannick et al. (2012), who, after exposing female rats to BPA at 0.5 and 50 µg/kg/day
360 during gestation, observed a significant increase in the number of cells both compared
361 with the control and between the study groups. In our case, the number of degenerated
362 (Gn-Dg) and Gn-DgM cells increased as the exposure concentration did. By contrast,
363 the number of gonadotroph (Gn) cells diminished as the BPA concentration increased.
364 This coincided with the work of Singh et al. (2008), who, after exposing fish to another

365 EDC (cypermethrin) for 45 days, reported a decrease in the size of Gn cells, along with
366 very little granulation and cytoplasmic vacuolization.

367 Aromatase, a member of the cytochrome P450 superfamily encoded by *Cyp19*,
368 catalyzes the synthesis of the steroid hormone 17β-estradiol (E2). Aromatase is
369 expressed not only in the gonads of humans, rodents and fish but also in their brains,
370 where it converts circulating androgens to active estrogenic metabolites in specific
371 neural target tissues and supplies estrogen directly to specific regions of the brain,
372 acting as a regulator of brain differentiation, neuroendocrine function, and sexual
373 behavior, and protects the brain against a variety of neurological and neurodegenerative
374 disorders (Roselli 2007; Saldanha et al., 2009). Because androgens are substrates of
375 brain aromatase, this enzyme has androgen-signaling functions that are important for
376 reproduction: hypothalamic aromatase is important for negative feedback regulation of
377 LH secretion by testosterone (Roselli, 2007). In contrast to non-teleost vertebrates, in
378 which a multiple-promoter region regulates tissue-specific expression of the single gene
379 coding for aromatase, zebrafish, similar to other teleosts, have two paralogues: *Cyp19a*,
380 which is mostly expressed in the gonads, and *Cyp19b*, which is predominantly
381 expressed in the brain (Chiang et al., 2001). The presence of functional estrogen
382 response elements in the *Cyp19b* proximal promoter region allows strong transcriptional
383 upregulation by estrogens and xenoestrogens, including BPA, in zebrafish embryos
384 (Chung et al., 2011), and it has been proposed that changes in the expression of this
385 gene can be used as biomarker for xenoestrogen exposure (rev. in Cano-Nicolau et al.,
386 2016).

387 In our work, zebrafish were exposed to a wide range of doses, including one (1 μg/L)
388 considered a 'low' dose, others (10 μg/L and 100 μg/L) that are environmentally
389 relevant and one (1000 μg/L) that is high but not lethal for fish (Canesi and Fabbri

390 2015). The effects of the different doses followed a non-monotonic curve (Figure 5),
391 with the 1 µg/L dose causing a dramatic decrease in the number of *Cyp19b* transcripts
392 while the higher doses (10, 100 and 1000 µg/L) caused increases of more than 50% in
393 the level of *Cyp19b* mRNA, peaking at 10 µg/L. The inclusion of GusB as a reference
394 allowed us to assure that this non-monotonic response could not be attributed to
395 inaccuracy in the quantification. Our group and others have described a nonlinear
396 relationship between EDC dose and various reproductive parameters, including ovarian
397 *Cyp19a* transcript variation response (Molina et al., 2017), and non-monotonic
398 responses to EDCs (effects at low doses that are not predicted by effects at higher
399 doses) have been well documented (Vandenberg et al., 2007). Nevertheless, there is a
400 lack of mechanistic understanding of non-monotonic responses that sometimes weakens
401 the reliability of the results. We have found similar responses by the two zebrafish
402 *Cyp19* genes to BPA exposure in the ovary (*Cyp19a*) (Molina et al., 2017) and in the
403 brain (*Cyp19b*). In this study, the lower BPA dose reduced the number of aromatase
404 transcripts by 50%, which probably resulted in increased levels of testosterone, while all
405 the other doses increased the *Cyp19b* transcript counts, favoring the conversion of
406 testosterone to estradiol. It has been reported that adult female mice treated with low
407 doses of BPA show an increased level of *Kiss1* mRNA. This gene codes for the
408 hormone kisspeptin, which stimulates the secretion of gonadotropin-releasing hormone
409 (GnRH). GnRH stimulates the anterior pituitary to secrete the gonadotrophic hormones
410 LH/GTHII and FSH/GTHI, which act on the ovary to support folliculogenesis. In
411 contrast, other reports demonstrated that exposure to a medium-high dose of BPA
412 causes alteration in hypothalamic-pituitary-gonadal axis (Fernandez et al., 2010). In this
413 case, the proposed underlying mechanism involved diminished levels of *Kiss1* mRNA
414 expression in the hypothalamus, with reduced secretion of LH/GTHII and FSH/GTHI

415 (Knez, 2013), which would result in diminished levels of *Kiss1* mRNA. These data
416 would be in agreement with the degenerative changes in gonadotropic cells observed in
417 this work.
418
419 In conclusion, our data show that exposure to low doses of BPA for 14 days affects the
420 adult neuroendocrine system, with modifications in the pituitary gland being observed.
421 BPA had an estrogenic effect at the lowest concentration (1 μg/L), and the expression of
422 *CYP19b* was significantly reduced ($P<0.001$) with respect to the control group. The
423 gonadotroph cells were activated and began to display signs of hypertrophy and
424 hyperplasia, yielding significant differences between all the study groups. Our results
425 reported irreversible alterations at higher doses (100 and 1000 μg/L), with the
426 histological images showing severe damage to gonadotroph cells. Such modification
427 could generate changes in the functionality of the gonadal neuroendocrine axis since the
428 effects observed in the pituitary gland indicated that it had lost its gonadotrophic
429 pituitary hormone-synthesizing activity. This would lead to direct repercussions on
430 female fertility and reproduction by interfering with correct ovarian functionality, with
431 the follicular atresia rate significantly increasing as the exposure dose increased. To our
432 knowledge, this is the first study in which gonadotroph cells have been evaluated using
433 histomorphological endpoints after BPA exposure in zebrafish.
434
435
436 **Acknowledgments**
437 The authors wish to thank the Spanish Junta de Andalucía (P09-AGR-514) for
438 financially supporting this study.

439 The authors wish to thank the Institud für Pathologie Stiftung Tierázliche Hochschule
440 Hannover (Germany), especially Dr. Wolfgang Baumgärtner and the electron
441 microscopy technician for their assistance during the ultrastructural image evaluation.
442
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594 **Figure legends:**
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596 **Figure 1. Control group: A-D. 1 µg/L group: E-H. Bars for H&E images=20 µm.**
597 **Bars for TEM images=10 µm.** **(A)** Image of the pituitary with a mosaic of abundant
598 gonadotroph cells; H&E. **(B)** Control group. Apparently normal gonadotroph cell with a
599 large quantity of organelles, mainly reticulum (R); TEM. **(C)** Control group. Image of
600 gonadotroph cell with an abundant reticulum (R); TEM. **(D)** Control group. Image of
601 gonadotroph cell with Golgi complex and abundant reticulum (R); TEM. **(E)** 1 µg/L
602 group. Image of pituitary gland with abundant gonadotroph cells in a mosaic formation
603 (GC); H&E. **(F)** 1 µg/L group. Very active gonadotroph cells (GC) with abundant
604 organelles, among which the reticulum stands out (R); TEM. **(G)** 1 µg/L group.
605 Gonadotroph cell (GC) with scant activity, few organelles and a greatly dilated
606 reticulum (DR); TEM. **(H)** 1 µg/L group. Image of gonadotroph cell (GC) with a greatly
607 dilated reticulum (DR) standing out; TEM.

608

609 **Figure 2. 10 µg/L group: A-D. 100 µg/L group: E-H Bars for H&E images=20 µm.**
610 **Bars for TEM images=10 µm.** **(A)** 10 µg/L group. Image of pituitary gland with its
611 gonadotroph cells arranged in extensive cumuli; H&E. **(B)** 10 µg/L group. Image of
612 active gonadotroph cell (GC) with abundant cytoplasmic organelles; TEM. **(C)** 10 µg/L
613 group. Gonadotroph cell (GC) with loss of activity and with only its greatly dilated
614 reticulum (DR) prominent; TEM. **(D)** 10 µg/L group. Image of gonadotroph cell (GC)
615 with loss of activity and with greatly dilated reticulum cisternae (R) apparent; TEM. **(E)**
616 100 µg/L group. Image of pituitary in which there are gonadotroph cells (GC) whose
617 cytoplasm is occupied by large vacuoles (V); H&E. **(F)** 100 µg/L group. Gonadotroph
618 cell (GC) with loss of activity, showing a somewhat dilated reticulum (DR); TEM. **(G)**

619 100 µg/L group. Gonadotroph cell (GC), showing great dilation and degeneration of
620 structures such as the reticulum (RD); TEM. **(H)** 100 µg/L group. Image of a
621 gonadotroph cell converted into a maximally degenerated cell (Gn-DgM) with a dilated
622 reticulum (DR) and a large cistern producing a vacuole (V); TEM.

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624 **Figure 3. 1000 µg/L group: A-D. Bars for H&E images=20 µm. Bars for TEM**
625 **images=10 µm. (A)** 1000 µg/L group. Gonadotroph cells including some maximally
626 degenerated ones (Gn-DgM), with large vacuoles occupying the cytoplasm (V); H&E.
627 **(B)** 1000 µg/L group. Image of gonadotroph cell (GC) with abundant reticulum
628 cisternae, TEM. **(C)** 1000 µg/L group. Maximally degenerated cell (Gn-DgM) with all
629 its cisternae dilated into a vacuole (V); TEM. **(D)** 1000 µg/L group. Maximally
630 degenerated cell (Gn-DgM) with large vacuoles (V); TEM.

631

632 **Figure 4. Stability ranking of six candidate reference genes across all zebrafish**
633 **samples as analyzed by the RefFinder algorithm.** This algorithm integrates the
634 comparative ΔCt, BestKeeper, Normfinder and geNorm methods to produce a
635 comprehensive ranking of the studied genes.

636

637 **Figure 5**. **Changes in the abundance of Cyp19b mRNA molecules in the brains of**
638 **female *D. rerio* exposed to different doses of BPA.** Transcript molecules were
639 quantified by absolute real-time qRT-PCR with three biological replicates (3-4
640 individuals in each). Each measurement was performed in three technical replicates in
641 two different experiments. Bars represent the average ± SEM. The absolute
642 quantification of *GusB* transcripts, used as a reference for normalization, has been

643 included for comparison. The statistically significant differences between the control

644 and experimental samples were determined using Student's *t*-test and were expressed as

645 follows: **, $P < 0.01$; ***, $P < 0.001$.