Evaluation of toxicological endpoints in female zebrafish after bisphenol A exposure

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25 **ABSTRACT**

26 Given the importance of bisphenol A (BPA) as a xenoestrogen and its potential effects
27 on human and animal health, we evaluated BPA exposure's short-term effects on
28 follicular development, yolk protein vitellogenin (VTG) production and aromatase
29 expression in female zebrafish. Histological modifications were observed along with
30 increased presence of atretic follicles. Whole-body VTG concentration increased with the
31 dose of BPA exposure. In contrast, expression of *Cyp19a* mRNA in the ovaries of BPA-
32 exposed fish exhibited an apparent non-monotonic response curve, marked by
33 downregulation at 1 μg/L BPA, upregulation at 10 μg/L BPA, and a return to
34 downregulation at 100 μg/L BPA and higher doses. Ovaries only exhibited significant
35 increases in follicular atresia and VTG concentration after exposure to 100 μg/L BPA and
36 higher doses. Ovarian histopathology, aromatase *Cyp19a* transcript levels and whole-
37 body VTG protein abundance may be good biomarkers for early detection of
38 environmental BPA exposure.

39

40 **Keywords:** bisphenol A; zebrafish; aromatase; vitellogenin; atretic follicle

41

42 # 1. Introduction

43 Endocrine-disrupting chemicals (EDCs) have attracted considerable worldwide scientific
44 and public attention due to their potential adverse effects on exposed organisms.
45 Xenoestrogenic endocrine-disrupting compounds are found in the environment as a result
46 of industrial and manufacturing activities (Cheng et al., 2011). Among these compounds,
47 bisphenol A (BPA) is one of the most highly produced chemicals worldwide. BPA is
48 ubiquitously present as an environmental contaminant in rivers and drinking water, most
49 likely due to the migration of plastic containers from industrial waste sites. In river water
50 BPA levels were reported of 0.01-21 μg/L while higher concentrations were also detected
51 near wastewater treatment plants or landfills (Crain et al., 2007; Kang et al., 2007; Naderi
52 et al., 2014). BPA is widely used in the manufacturing of various polycarbonate plastics
53 used in lacquer coatings of food cans and food and beverage containers. The widespread
54 distribution and environmental persistence of xenoestrogen BPA indicates a strong
55 potential for human and animal exposure. Because humans are exposed to this compound
56 daily, the effects of BPA are relevant to public health (Graselli et al., 2010). This exposure
57 primarily occurs via the hydrolysis of polycarbonate plastics and epoxy resins and results
58 in low concentrations of free BPA in food and liquids. The presence of endocrine-
59 disrupting chemicals in the environment reportedly disturbs the normal endogenous
60 hormone pathway and interrupts reproductive development in wildlife species. These
61 compounds can affect the reproductive regulation of the neuroendocrine system, often by
62 mimicking or blocking endogenous hormones (Qin et al., 2013; Naderi et al., 2014).

63 BPA acts as an EDC by causing adverse biochemical and physiological changes that alter
64 the histological structure of cells and modify the function of tissues and organs,
65 interfering with reproductive efficiency. Within the reproductive system, ovarian
66 granulosa cells have been documented as a target of BPA action (Graselli et al., 2010). It

67 is well-known that granulosa cells play a crucial role in ovarian physiology through the
68 production of estrogens, which depends on androgen production in theca cells in addition
69 to other factors that interact with the oocyte during its development. Therefore, the
70 disruption of their functional activities by BPA could have a significant impact on fertility
71 (Graselli et al., 2010). Therefore, BPA's effects in ovaries could be evaluated by
72 histology. However, molecular responses usually occur earlier than histological
73 perturbations, which are considered to be higher-level biological responses. The yolk
74 protein vitellogenin (VTG) has been widely used as an endpoint for many exposure
75 studies on the estrogenic effect of BPA in fish (Holbech et al., 2006). Changes in VTG
76 levels in females have been suggested as a complementary biomarker of potential
77 reproductive disruption, as well as modifications in the sex hormone balance (Mandich
78 et al., 2007).

79 Conversely, BPA, similar to some other EDCs, could also act as anti-androgens by
80 binding to the androgen receptor and producing specific alterations in gene expression
81 (López-Casas et al., 2012). The cytochrome P_{450} CYP19 (aromatase) is involved in the
82 generation of estradiol from testosterone. In this way, CYP19 is considered a potential
83 EDC target because the modulation of its expression and function can dramatically alter
84 the rate of estrogen production (Cheshenko et al., 2008). Two different *Cyp19* genes,
85 *Cyp19a* and *Cyp19b*, are expressed in many teleost fish, preferentially in the ovary and
86 the brain, respectively. Recent *in vitro* studies showed that BPA downregulates *Cyp19a*
87 mRNA and protein expression in rats (Lee et al., 2013), which is relevant to the evaluation
88 of the expression of the aromatase genes in ovaries as a biomarker of BPA exposure.

89 The purpose of our study was to evaluate the estrogenic activity of BPA as a ubiquitous
90 environmental contaminant through the assessment of different biomarkers in the

91 zebrafish ovary. The toxicological endpoints were selected at different levels of biological
92 organization and included histological modifications in ovaries affecting the number of
93 atretic follicles, VTG measurements, and determinations of the aromatase *Cyp19a* mRNA
94 levels in zebrafish exposed to BPA (1, 10, 100 and 1000 μg/L) for 14 days.
95

96 # 2. Materials and Methods

97 ## 2.1. Fish exposure and sampling protocol

98 Sixteen-week-old female zebrafish *(Danio rerio)* (n=150; standard length: 4.17 ± 0.24
99 cm; 0.57±0.14 g wet weight) were used. The treated groups were exposed (OECD
100 Guideline No. 204) to graded concentrations (1, 10, 100 and 1000 µg/L) of BPA (Sigma-
101 Aldrich®) for 14 days. A control group exposed to only unchlorinated tap water completed
102 the exposure design. The research procedure was conducted in the Experimental Animal
103 Service of the University of Córdoba (Spain) after approval by the animal care committee
104 of the University of Córdoba (Spain) and in accordance with the European Regulations
105 for the Protection of Experimental Animals (Directive 2010/63/EU).

106 After 2 weeks of exposure, the zebrafish were sacrificed by an overdose of an anesthetic
107 solution of tricaine methanesulfonate (MS-222® 500 mg/L; Sigma-Aldrich) buffered with
108 sodium bicarbonate (300 mg/L; Sigma-Aldrich). Immediately afterward, the standard
109 length (SL) and body weight (BW) of each animal were measured. The gonads from 30
110 animals (n=6 per group) were dissected and fixed for histological analysis for qualitative
111 and quantitative evaluations. To complete these experiments, each fish was necropsied
112 by placing it in right lateral recumbency on the stage of a dissecting microscope. The
113 ovaries of 45 additional zebrafish (n=9 per group) were removed, immediately immersed
114 in liquid nitrogen, and stored at -80°C for qRT-PCR. For the vitellogenin analysis, another
115 45 fish were frozen and stored at -80°C until further analysis. The final 30 fish (n=6 per
116 group) were dried with sterile gauze, frozen and stored at -80°C until use in the
117 toxicological analysis (analytical BPA determinations).

118 **2.2. Light and electron microscopy**

119 For light microscopy, the fixed ovaries of 6 animals from each experimental group were
120 routinely processed for paraffin sectioning by fixation in 10% buffered formalin solution,
121 dehydration in a graded series of ethanol, immersion in xylol and embedding in paraffin
122 wax by routine techniques. Tissue sections of 4 μm were mounted. After
123 deparaffinization, the sections were rehydrated, stained with hematoxylin and eosin, and
124 mounted on microscope slides with Cristal/Mount (Paraplast, Oxford Labware, St. Louis,
125 MO).

126 For electron microscopy, randomly selected gonad samples were first fixed in a 2%
127 glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4, 4°C, overnight) and later fixed
128 in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 30 min. After dehydration
129 in a graded ethanol series and embedding in Araldite, semi-thin and ultra-thin sections
130 were cut on an LKB ultramicrotome (LKB). The semi-thin sections were stained with
131 toluidine blue, whereas the ultra-thin sections were double-stained with uranyl acetate
132 and lead citrate. Tissue sections were examined in a JEM 1400 transmission electron
133 microscope (TEM; JEOL, Ltd.).

134 **2.3. Morphometric study**

135 For the structural quantifications, the fixed gonads were cut into three sections. Each
136 portion was later processed and embedded in paraffin, as for routine histology. The first
137 section (4 μm thick) of each block was stained with hematoxylin and eosin and used for
138 the stereological study.

139 The quantitative study was performed according to Molina et al. (2013). Each
140 microscopic image was processed using the Visilog 5® software (Noesis). Quantification
141 was performed in a blind manner by an observer experienced in the use of the analysis

142 system (J.G.M.). The system was initially and regularly calibrated using a millimeter
143 slide.

144 Atretic follicle identification was based on the determination of non-physiological
145 alterations in four follicular components: oocyte, follicular cells, and pellucid zone. The
146 numerical density (Q_A) of the atretic follicles in the plane was estimated using a test
147 system consisting of sixteen rectangular counting frames superimposed onto each
148 microscopic image. Thus, the number of profiles per area Q_A *(nucl/tis)* was estimated
149 using the following equation:

150 $$estQ_A(nucl/tis) = \sum Q(nucl) / (\sum P(tis) \bullet a/p),$$

151 where Q_A *(nucl/tis)* is the numerical density of follicular nuclei per ovary, *∑Q(nucl)* is the
152 total number of nuclear profiles counted within the counting frames of the area obtained
153 from *∑P(tis)* (i.e., the total number of points in the tissue), and *a/p* is the area associated
154 with one point in the test system (in our study, $a/p = 125$ µm^2).

155 **2.4. Whole-body vitellogenin measurements**

156 The VTG levels in whole-body homogenates (n=9 per experimental group) were
157 determined using a commercial enzyme-linked immunosorbent assay developed for
158 *Danio rerio* (ELISA, Biosense Laboratories, Bergen, Norway) following the
159 manufacturer's indications. A multiple range test was used to make multiple comparisons
160 between groups.

161 **2.5. *Cyp19a* transcript quantification by real-time qRT-PCR**

162 ***2.5.1. RNA isolation***

163 Nine zebrafish from each experimental condition were selected for this experiment. The
164 total RNA from the ovaries of each animal was isolated using the Isol-RNA Lysis Reagent
165 (5PRIME). Genomic DNA was removed by the Total RNA Cleanup with DNase

166 Digestion kit using the Qiagen RNeasy Protocol (Qiagen). The absence of any remaining
167 gDNA contamination was confirmed by PCR amplification of the RNA samples without
168 previous retrotranscription. An Agilent 2100 Bioanalyzer (Agilent Technologies) was
169 used to determine the RNA integrity number (RIN) (Schroeder et al., 2006). The RNA
170 purity and concentrations were determined by spectrophotometry. Only high-quality
171 RNAs with RIN values > 8.5 and A260:A280 ratios close to 2.0 were used for the
172 subsequent experiments (Fleige and Pfaffl 2006; Taylor and Mrkusich 2014).

173 cDNAs was generated from total RNA (1 µg/rx) by using the QuantiTect Reverse
174 Transcription Kit (Qiagen), according to the manufacturer's protocol.

175 ***2.5.2. Primer design and amplification efficiency***

176 The *Danio rerio* sequences for *Cyp19a* and six candidate reference genes (*ActB*, *B2m*,
177 *Hprt1*, *GusB*, *Nono* and *Rpl13*) were obtained from GenBank
178 (http://www.ncbi.nlm.nih.gov/gene) to design the primers pairs. The primers (Supp. Inf.
179 Table 1) were designed with Oligo 7 software (Molecular Biology Insights, Inc.), as
180 previously described (Pueyo et al., 2002). To obtain high specificity and performance, the
181 primers were required to have high Tm (≥80°C), optimal 3′-ΔG (≥−5 kcal/mol) values
182 and no hairpin or duplex structures.

183 The amplification efficiency curves were obtained by preparing ten-fold serial dilutions
184 from 2 x 10^5 to 0.02 pg of total RNA input, which were subsequently reverse transcribed
185 and amplified by real-time PCR. The log of the RNA input versus the Ct value was
186 plotted, and the efficiency value was estimated from the slope of the efficiency curve. All
187 primer pairs amplified the selected targets with optimal (≈100%) PCR efficiencies (Supp.
188 Inf. Table 1 and Supp. Inf. Fig. 1) and produced specific PCR products, as confirmed by
189 PCR product sequencing.

190 ***2.5.3. qRT-PCR***

191 Real-time PCR reactions were performed in quadruplicate using 50 ng of cDNA template,
192 0.3 µM of each primer, 3 mM $MgCl_2$, 250 µM of each dNTP, 0.75 units of Platinum Taq
193 DNA polymerase, and 1:100,000 SYBR Green I dye (Roche) in a volume of 25 µl. The
194 reactions were analyzed on a Cycler Q Real-Time PCR System (Bio-Rad). The cycling
195 conditions were as follows: 2 min at 95°C for Platinum Taq activation and 40 cycles for
196 the melting (15 s, 95°C) and annealing/extension (30 s, 70°C) steps. Next, a melting curve
197 analysis was performed (60°C to 95°C) to verify the specificity of the amplicons.
198 For absolute quantification, the number of transcript molecules was calculated from the
199 linear regression of a calibration curve (Jurado et al., 2003; Prieto-Alamo et al., 2003).
200

201 **2.6. Statistics**

202 The data were analyzed using the statistical program Statgraphic (Centurion XVI®) to
203 determine the effects of BPA in each of the treated groups. ANOVA (F-test) was used to
204 determine significant differences between the mean values of each group. Fisher's LSD
205 post hoc test was used to perform multiple comparisons between groups. The results are
206 expressed as the mean ± standard deviation (SD), and $P < 0.05$ was considered to be
207 significant. In the transcriptional analysis, the statistically significant differences between
208 the control and experimental samples were determined using Student´s *t*-test and were
209 expressed by * to denote $P < 0.05$; and ***, $P < 0.001$. The statistics program SigmaStat
210 5.1 (GraphPad Prism) was used throughout the study.

211 # 3. Results

212 None of the fish died during the study. There were no significant differences in the mean
213 BW and SL between the control and BPA-exposed groups.

214 ## 3.1. Histopathology

215 Follicular atresia (Fig. 1) was variable, depending on the type of affected follicle and the
216 study group. In the control group, all follicles showed normal appearance under both the
217 light and electron microscopes (Fig. 1 cA, cB). In the exposed groups, different levels of
218 atresia were observed in the various follicular populations. Primordial follicles (Fovg)
219 showed modifications, with atretic degeneration being visible at the intersection between
220 follicular cells and oocytes. Moreover, both the optical and electronic microscope
221 preparations showed total or partial vacuolization that may even be irregular (Fig. 1A, B).

222 The alveolar cortical follicles (Fc) exhibited a poor formation of the pellucid zone, leading
223 to egg–follicular cell separation due to vacuolization (Fig. 1C, D). In these follicles, a
224 high amount of modifications was observed, especially under the light microscope, which
225 triggered follicular atresia with a total disintegration of the follicular components and
226 hyalinization in the oocyte cytoplasm. The vitellogenic follicles (Fv) exhibited full
227 vacuolization due to modifications affecting the oocyte, the pellucid zone and the
228 follicular cells. The poor formation of the pellucid zone caused the complete separation
229 of the oocyte from the follicle cells (Fig. 1E, F), similar to that observed in Fc. Finally,
230 mature follicles (Fm) showed a complete degeneration of all components (Fig. 1G, H),
231 yielding a breakdown of all organelles, primarily in the oocyte, as the cellular debris
232 remained within the follicle until it further disintegrated. As the BPA concentration
233 increased, we observed a concomitant increase in the proportion of follicular atresia
234 affecting the different types of follicles. This trend of increasing follicular atresia was

235 observed in the Fc, the Fv and the Fm. Considering the total amount of follicular atresia
236 in each group, significant differences (P < 0.05) between the control group and other
237 study groups were observed. Similarly, significant differences (P < 0.05) were observed
238 between the groups treated with 1, 10, 100 and 1000 µg/L BPA (Fig. 2).

239

240 **3.2. Whole-body VTG levels**

241

242 The whole-body VTG concentration in the control group was 21.13 ± 2.16 ng/g. These
243 levels significantly increased (P < 0.05) in the exposed animals, even at the lowest BPA
244 dose assayed (1 µg/L), in a dose-dependent manner (Fig. 3), reaching a 12-fold increase
245 at the highest assayed dose.

246

247

248 **3.3. Real-time PCR**

249 ***3.3.1. Identification of valid reference genes for the normalization of the qRT-PCR***
250 ***expression studies.***

251 The expression levels of six candidate reference genes (*ActB*, *B2m*, *Hprt1*, *GusB*, *Nono*
252 and *Rpl13)* were evaluated using the threshold cycle (Ct) values from nine biological and
253 three technical replicates from the five different experimental conditions. The box plot of
254 the Ct values obtained is shown in Suppl. Inf. Fig. 2.

255 To identify the most stable reference genes, we used the RefFinder software
256 (http://fulxie.0fees.us), which integrates the currently available major computational
257 programs—geNorm (Vandesompele et al, 2002), Normfinder (Andersen et al., 2004),
258 BestKeeper (Pfaffl et al., 2004), and the comparative ΔCt method (Silver et al, 2006)—
259 to compare, rank and assign an appropriate weight to an individual gene and calculate the

260 geometric mean of their weights for overall final ranking. The recommended
261 comprehensive rankings are shown in Suppl. Inf. Table 2. *Nono*, *Hprt1* and *GusB*,
262 identified as the most stably expressed genes, were included in qRT-PCR experiments.

263 ***3.3.2. Quantification of Cyp19a transcripts in the ovaries of BPA-treated zebrafish.***

264 We analyzed the changes in the levels of transcripts for the aromatase *Cyp19a* gene in the
265 ovaries of BPA-treated zebrafish by real-time qRT-PCR. The relative quantification of
266 *Cyp19a* mRNA is shown in Fig. 4 using *GusB*, *Nono* or *Hprt1* as internal reference genes.
267 The lowest variations were obtained when the aromatase Ct values were normalized
268 against *GusB*, although no significant differences were observed among any of the three
269 reference genes. The results confirm that *Cyp19a* is a detectable but non-abundant
270 transcript in the *D. rerio* ovary.

271 The expression of *Cyp19a* mRNA in the ovaries of BPA-exposed fish was significantly
272 altered, following a possible non-monotonic curve, where the slope sign changed from
273 negative to positive and back to negative as the dose increased (Fig. 4). The lowest BPA
274 dose (1 μg/L) caused a 3.2-fold decrease in *Cyp19a* transcript levels. In contrast, the
275 levels of this transcript increased significantly (a 1.6-fold increase compared to the
276 control) in zebrafish treated with 10 μg/L but later decrease again with higher doses of
277 BPA in a dose-dependent manner (1.5- and 5-fold in the animals treated with the 100 and
278 1000 μg/L doses, respectively).

279 **4. Discussion**

280 BPA is one of the highest volume chemicals produced worldwide. As with many other
281 endocrine-disrupting chemicals (EDCs), BPA interferes with the body's endocrine
282 system by binding to α- and β-estrogen receptors (ERs) (Kuiper et al., 1998; Metcalfe et
283 al., 2001; Qin et al., 2013) and may produce adverse developmental, reproductive,

284 neurological, and immune effects in both humans and wildlife. Given its prevalence in
285 the environment, identification of BPA exposure biomarkers is urgent for predicting its
286 detrimental effects. The toxicological endpoints of this work in zebrafish exposed to BPA
287 were selected at different levels of biological organization and included histological
288 evaluation of the ovaries, determination of whole-body VTG concentration and analysis
289 of the ovarian aromatase *Cyp19a* mRNA abundance. We chose these parameters for the
290 following reasons: (*i*) environmental chemical exposure may result in changes in the
291 histological structure of cells, and consequently, histopathological analysis is considered
292 an important biomarker in evaluating the toxicological pathology of different compounds
293 in fish; (*ii)* VTG has been used as a biomarker of exposure to (anti)estrogenic compounds
294 in a number of *in vivo* and *in vitro* studies with fish (i.e., Rankouhi, 2002; Navas, 2006;
295 Sun, 2010); and (*iii*) it has been reported that exposure to low doses of BPA causes a
296 decrease in 17β-estradiol (E2) serum concentration linked to ovarian aromatase
297 downregulation (Lee et al, 2013).

298 The primary route of BPA contamination is through ingestion. However, the transdermal
299 route could also contribute to BPA exposure when direct contact with BPA occurs. Hence,
300 this work exposed female zebrafish to different concentrations of BPA (1, 10, 100 and
301 1000 µg/L) introduced into the water of the aquariums by using a continuous flow system.
302 Doses were chosen after reviewing the literature, as they have been reported by previous
303 studies in diverse fish species (Ishibashi et al., 2005; Mandich et al., 2007; Villeneuve et
304 al., 2012; Molina et al., 2013). Doses under 200 ng/mL (< 1 uM) are considered low-dose
305 for aquatic organisms (vom Saal, 2006) but still have important physiological effects
306 (Inagaki, 2016). Because a non-monotonic dose-response curve has been previously
307 described for BPA exposure, we decided to evaluate the effect of a 5-fold higher dose
308 (1000 µg/L) over the toxicological endpoints addressed in this work. No differences in

309 mortality, body weight or size were observed in the animals used in this study, in keeping
310 with previously reported data (Mandich et al., 2007; Hatef et al., 2012; Villaneuve et al.,
311 2012; Molina et al., 2013).

312 Histopathological analysis of BPA-exposed zebrafish ovaries revealed an increase in the
313 number of atretic follicles as BPA concentration rose (Fig. 1). Previtellogenic oocyte
314 atresia has reportedly affected approximately 10% of female carps exposed to
315 environmental concentrations (1 and 10 μg/L) of BPA (Mandich et al., 2007). These data
316 were partially consistent with those presented here, as they indicated that BPA exposure
317 did not equally affect all types of the follicle. The proportion of atretic *Fovg* (primordial)
318 follicles was less than 10% at lower concentrations (1 and 10 μg/L) but increased to levels
319 over 10% at higher concentrations (100 and 1000 μg/L) (Fig. 2). In fact, more than 10%
320 of the *Fovg*, *Fc* (alveolar cortical), *Fv* (vitellogenic) and *Fm* (mature) follicles were atretic
321 in the study groups exposed to higher concentrations of BPA (100 and 1000 μg/L),
322 showing a dose-dependent increase in the number of atretic follicles (Fig. 2). The fact that
323 the *Fc* showed significant differences ($P < 0.05$) with respect to the *Fovg* in all study
324 groups could indicate that BPA disrupted follicular development at the primary stages.
325 Currently, there is no study that has evaluated this hypothesis. Moustafa et al. (2016)
326 reported follicles degeneration, congestion blood vessels, and hemorrhage in ovaries,
327 after rats exposure to BPA higher doses in a two generational study. The onset of
328 follicular atresia in response to exposure to different concentrations of BPA has been
329 studied in depth (Weber et al., 2003; Wolf et al., 2004; Mandich et al., 2007; Molina et
330 al., 2013; Moustafa et al., 2016), but none of these studies addressed which follicular
331 population was the most affected in response to BPA exposure dose. The proportion of
332 atretic follicles increased in the groups that were exposed to higher BPA concentrations

333 (100 and 1000 μg/L) compared to that in the control group ($P < 0.05$), which suggests that
334 BPA affects reproductive function at these doses.

335 Another biomarker that was evaluated during this study was VTG, which has been widely
336 used as an endpoint for estrogen exposure in fish (Orn et al., 2003; Spano et al., 2004;
337 Mandich et al., 2007). VTG is secreted by hepatocytes in the liver. In mature females,
338 VTG is generally synthesized in response to endogenous estrogens, such as 17β-estradiol
339 (E2), which are released into the bloodstream and transported to the ovary, where they
340 are stored in the developing oocytes (Van der Belt et al., 2003; Matozzo et al., 2008). The
341 animals from the control group exhibited whole-body VTG levels of ≈ 20 μg/g (Fig. 2).
342 After two weeks of exposure, VTG concentrations in all study groups increased ($P < 0.05$)
343 in a dose-dependent manner, reaching values 500-fold higher at the highest BPA dose
344 tested. This finding could confirm the estrogenic potential of BPA, as previous reports
345 have shown a dose-dependent increase in VTG production (Mandich et al., 2007; Naderi
346 et al., 2014). Even the group exposed to the lowest concentration of BPA (1 μg/L) showed
347 a significant ($P < 0.05$) 2-fold increase in VTG concentration after 14 days of exposure.
348 This result indicates that zebrafish respond to low BPA concentrations by synthesizing
349 VTG (Mandich et al., 2007; Naderi et al., 2014) and suggests that VTG is a very sensitive
350 biomarker of BPA exposure, even at environmentally relevant BPA concentrations.

351 The observed increase in follicular atresia and in VTG synthesis depending on BPA
352 concentration, suggests that estrogenic action of BPA is mediated by an abnormal
353 synthesis of estrogen and VTG by the follicles in a dose-dependent manner. The increase
354 in VTG concentration in BPA-exposed zebrafish ovaries might be related to the
355 significant increment of Fv with respect to the control group that we reported in a previous
356 work (Molina et al., 2013).

357 The most accepted hypothesis states that EDCs interfere with steroid hormone action by
358 disrupting steroid biosynthesis with consequences for downstream signaling pathways
359 (López-Casas et al., 2012). In the ovary, androgens synthesized in theca cells are
360 converted into estrogens by ovarian aromatase *CYP19a* in the local granulosa cells
361 (Cheshenko et al., 2008). Endocrine disruptors have challenged the toxicological
362 assumptions related to dose-response relationships. Non-linear relationships between the
363 dose and the response have been observed for certain EDCs. Using a quantitative RT-
364 PCR assay, the kinetics of *Cyp19a* transcript abundance variation was investigated in
365 zebrafish ovaries after BPA exposure. Data in Fig. 4 show that *Cyp19a* transcript
366 abundance was significantly diminished ($P < 0.001$) in response to exposure at the lowest
367 BPA concentration (1 μg/L), a dose that produced no important histological changes or
368 VTG increases. The peak abundance of *Cyp19a* mRNA was detected at the dose of 1
369 μg/L BPA and decreased afterward. This type of non-monotonic dose-response curve has
370 been reported for several endocrine-disrupting compounds, including BPA and many
371 other hormonally active compounds, and has been attributed to multiple (yet
372 undetermined) mechanisms stimulated when the assimilated dose is low but able to inhibit
373 toxic effects of ulterior higher doses of the compound (see Myers et al., 2009; Vandenberg
374 et al., 2009, for reviews; Renieri et al., 2017). However, it must be taken in consideration
375 that the apparent non-monotonic dose response is based on a single low dose, and testing
376 more low doses is needed to confirm this trend. Data presented in this study corroborated
377 that measurement of transcriptional profiles variations are very suitable early exposure
378 biomarkers, as transcription is affected prior to protein synthesis. These results are in
379 agreement with previous reports indicating changes in *Cyp19a* expression by even very
380 low concentrations of EDCs (Flint et al., 2012). However, in our experimental model, the
381 decrease in *Cyp19a* transcripts at the 1 μg /L BPA dose had no effect on histological

382 organization of the ovary (Fig. 1). Only when exposed to 100 μg/L BPA or higher doses
383 did the ovaries show significant histological changes with significant increases in the
384 follicular atresia (over 10% compared to the control group, Molina et al., 2013). This
385 result coincides with the inhibition of *Cyp19a* expression, which was accentuated in
386 response to exposure to higher BPA concentrations (100 and 1000 μg/L).

387 Although the precise cellular and biochemical mechanism(s) underlying these changes in
388 *Cyp19a* transcript levels after BPA exposure are currently unknown, low BPA doses
389 presumably exert a direct negative action on the transcriptional regulation of *Cyp19a*
390 expression in granulosa cells. This effect would lead to a reduced number of *Cyp9a*
391 mRNA molecules but also to the onset of a stress response that stimulates *Cyp19a*
392 transcript synthesis. In accord with Lee et al. (2013), the results presented in this study
393 suggest that the low-dose BPA exposure in zebrafish females initially reduces E2
394 synthesis and thus disrupts ovarian steroidogenesis by inhibiting *Cyp19a*. BPA has been
395 reported to regulate the expression of the Aryl hydrocarbon receptor (AhR) mRNA
396 (Kharrazian, 2014), a key transcription factor (TF) in the xenobiotic response that
397 activates the transcription of a battery of genes, including *Cyp19a* (Baba et al., 2005).
398 Similar to aromatase, VTG synthesis is induced by estrogen-dependent stimulation of
399 gene expression via AhR cross-talk. However, the estrogenic potency of BPA is
400 significantly increased by bioactivation, depending on the microsomal and cytosolic
401 constituents. It is likely, in contrast with the direct BPA inhibition of *Cyp19a* expression,
402 that BPA doses over 1 μg/L are required to induce the production of biotransformation
403 enzymes and significantly increase the VTG levels in the zebrafish ovary.

404

405 **5. Conclusions**

406 We observed a good correlation between BPA exposure and the possibility of
407 reproductive dysfunction. The results of this work indicate that aromatase *Cyp19a* mRNA
408 quantifications, whole-body VTG measurements and morphological endpoints are
409 sufficiently sensitive biomarkers to identify the precocious effects of environmental
410 concentrations of BPA on the ovaries after two weeks of BPA exposure. In accord with
411 previous studies demonstrating the utility of the aromatase as a biomarker in rats, we
412 show the relevance of determining the changes in transcriptional profiles of the *Cyp19*a
413 gene in the zebrafish ovary as an early biomarker of BPA exposure. Additionally, we
414 observed that variations in the VTG protein are also indicative of endocrine alterations.
415 Both *Cyp19a* and VTG are more sensitive biomarkers than histological evaluations, as
416 their abundance changes at lower doses than those required to produce morphological
417 alterations. Data suggest that low BPA doses exert a direct negative action on the *Cyp19a*
418 expression, but higher doses are needed to observe BPA acting as a positive inductor of
419 VTG synthesis.

420

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588 **Figure captions**

589 **Figure 1: BPA-exposure-induced histological changes in zebrafish ovaries.** Images
590 obtained under light microscope (left) are marked as cA (control), A, C, E, G (groups
591 treated with increasing doses of BPA); for these images, bars represent 50 μm.
592 Ultrastructural observations (right) are marked as cB (control), B, D, F, H (exposure
593 groups); in these images, bars represent 10 μm. *Control group:* Sections shown all types
594 of ovarian follicles (FO) (cA) interstitial tissue and ovg follicles (Fovg) (cB) with their
595 normal appearance. *Group 1 μg/L*: A) Vacuolizations (V) are visible between the oocyte
596 and the follicular cells (Fovg). B) Detachment of an ovg follicle (Fovg) from the follicular
597 cells of the oocyte. *Group 10 μg/L*: C) Vacuolizations (V) of the pellucid zone (ZP) can
598 be seen in the Fc, particularly follicular atresia (FA), with the disintegration of all its
599 components. D) Vacuolizations (V) were observed in the pellucid zone (ZP) of the Fc.
600 *Group 100 μg/L*: E) Strong vacuolizations (V) and separations of the pellucid zone (ZP)
601 were observed in the Fv, with evident follicular atresia (FA). F) Disorganization and
602 vacuolization (circle) of the dense and light pellucid zone (ZP) of the Fv. *Group 1000*
603 *μg/L*: G) Follicular atresia (FA) was observed in the Fm, along with the production of
604 cellular debris (RC). H) Follicular atresia of Fm, with the disintegration of cellular
605 components and production of cellular debris (RC).

606 **Figure 2. Follicular atresia caused by BPA exposure.** The percentage (numbers inside
607 the bars) of atretic follicles in the different follicular populations in each study group is
608 represented. Fm: mature follicles; Fv: vitellogenic follicles; Fc: cortical alveolar follicles;
609 Fovg: primordial follicles.

610 **Figure 3. VTG levels in the whole-body extracts of zebrafish exposed to increasing**
611 **concentrations of BPA.** The vitellogenin (VTG) concentrations (μg/g) were determined

612 in whole-body extracts of zebrafish (n = 9 per experimental group) exposed to increasing
613 amounts of BPA using a specific commercial enzyme-linked immunosorbent assay. The
614 data are presented as the means ± SD of 5 independent determinations. All treatment
615 groups were significantly different from the control at the $P < 0.001$ level; significant
616 differences at the $P \leq 0.05$ level were also observed for the comparisons between each
617 two consecutive treatment doses.

618 **Figure 4. Changes in the number of *Cyp19a* transcripts in the zebrafish ovary after**
619 **different concentrations BPA exposure.** The relative quantification of the levels of the
620 *Cyp19a* transcripts was calculated by using the $2^{-\Delta\Delta Ct}$ method and the *GusB*, *Nono* or
621 *Hprt1* transcripts as internal references. Numbers over the bars indicate the fold change
622 in relation to the control counts. The significant differences between the control and
623 experimental samples were determined using Student´s *t*-test and were expressed by **
624 to denote $P < 0.01$; and ***, $P < 0.001$. The statistics program SigmaStat 5.1 (GraphPad
625 Prism) was used throughout the study.

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627