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1 **Differential activity of BPA, BPAF and BPC on zebrafish estrogen receptors *in vitro* and *in***
2 ***vivo***

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23

24 **Abstract**

25 The high volume production compound bisphenol A (BPA) is of environmental concern largely
26 because of its estrogenic activity. Consequently, BPA analogues have been synthesized to be
27 considered as replacement molecules for BPA. These analogues need to be thoroughly evaluated
28 for their estrogenic activity. Here, we combined mechanism zebrafish-based assays to examine
29 estrogenic and anti-estrogenic activities of BPA and two of its analogues, bisphenol AF (BPAF)
30 and bisphenol C (BPC) *in vitro* and *in vivo*. *In vitro* reporter cell lines were used to investigate
31 agonistic and antagonistic effects of the three bisphenols on the three zebrafish estrogen
32 receptors. The transgenic *Tg(5×ERE:GFP)* and *Cyp19a1b-GFP* zebrafish lines were then used
33 to analyze estrogenic and anti-estrogenic responses of the three bisphenols *in vivo*. BPA, BPAF
34 and BPC were agonists with different potencies for the three zebrafish estrogen receptors *in*
35 *vitro*. The potent *zfERα*-mediated activity of BPA and BPAF *in vitro* resulted *in vivo* by
36 activation of GFP expression in zebrafish larvae in the heart (*zfERα*-dependent) at lower
37 concentrations, and in the liver (*zfERβ*-dependent) at higher concentrations. BPC induced
38 *zfERβ*-mediated luciferase expression *in vitro*, and the *zfERβ* agonism led to activation of GFP
39 expression in the liver and the brain *in vivo*. In addition, BPC acted as a full antagonist on
40 *zfERα*, and completely inhibited estrogen-induced GFP expression in the heart of the zebrafish
41 larvae. To summarize, applying a combination of zebrafish-based *in vitro* and *in vivo* methods to
42 evaluate bisphenol analogues for estrogenic activity will facilitate the prioritization of these
43 chemicals for further analysis in higher vertebrates as well as the risk assessment in humans.

44

45 **Keywords:** zebrafish, bisphenol, estrogen receptor, transgenic fish, reporter cell lines

46

47 **Introduction**

48 The environmental pollutant bisphenol A (BPA) has been widely studied during the past decade.
49 BPA has since the 1950s been used to make epoxy resin and polycarbonate plastics, and it is one
50 of the highest volume chemicals produced worldwide. BPA is found in products such as reusable
51 food and beverage containers, lining of cans, dental materials, computers, and thermal paper.
52 Through its estrogenic activity, BPA has been shown to cause deleterious effects in laboratory
53 animals, such as abnormal fetal development (Palanza *et al.*, 2008), alterations in sex
54 differentiation (Rubin *et al.*, 2006), reproduction (Honma *et al.*, 2002), brain function and mood
55 (Leranth *et al.*, 2008; Elsworth *et al.*, 2013) as well as promotion of certain types of cancers
56 (Markey *et al.*, 2001; Munoz-de-Toro *et al.*, 2005; Ho *et al.*, 2006). In humans, epidemiological
57 studies have shown that high BPA levels are linked to heart disease, diabetes and liver toxicity
58 ((Lang *et al.*, 2008) and reviewed in (Vandenberg *et al.*, 2012)). Food, indoor air, indoor dust,
59 wastewater and dermal absorption are all important sources of human exposure to BPA
60 (Vandenberg *et al.*, 2007; Zalko *et al.*, 2011). In addition, BPA is commonly found as a
61 contaminant in wastewater, and may as such have deleterious effects on wildlife (Santos *et al.*,
62 2016; Xu *et al.*, 2016).

63 Concurrent with the increasing awareness of potential health problems linked to BPA, a search
64 for safer alternatives has begun. Two of these BPA alternatives, bisphenol AF (BPAF) and
65 bisphenol C (BPC), are the focus of this study. BPAF is a fluorinated derivative of BPA, and it is
66 used both as a crosslinking agent and for polymer synthesis in plastics industry. It has an annual
67 estimated production of 10,000–500,000 pounds in the United States (NTP-NIEHS, 2008).
68 BPAF has been detected in wastewater from water treatment plants and was also shown to be

69 resistant to biodegradation (Sun *et al.*, 2017). BPC is used in the preparation of flame-resistant
70 polycarbonates, and it has also been detected in wastewater (Cesen *et al.*, 2018).

71 In humans, BPA interferes with estrogen signaling mediated by the two estrogen receptors, ER α
72 and ER β . The canonical estrogen signaling pathway involves estrogen binding to the ligand
73 binding domain (LBD) of the ERs, translocation of the ER-ligand complexes into the nucleus,
74 followed by recruitment of co-regulator complexes and docking to the estrogen response element
75 (ERE) of target gene promoters (reviewed in (Nilsson *et al.*, 2001)). Similarly to BPA, *in vitro*
76 studies show that several bisphenol analogues are able to bind and activate the human ERs
77 [(Delfosse *et al.*, 2012; Delfosse *et al.*, 2014; Grimaldi *et al.*, 2019) and reviewed in (Chen *et al.*,
78 2016)]. BPAF has been repeatedly reported to activate ER-dependent transcription at lower
79 concentrations than BPA and other bisphenol derivatives *in vitro* ((Delfosse *et al.*, 2012) and
80 reviewed in (Chen *et al.*, 2016)). While fewer studies have investigated BPC, this chlorinated
81 analogue has been described to have stronger affinity for the ERs than BPA, acting as a full
82 agonist on MCF7 proliferation, and as both an agonist and antagonist in HELN (HeLa ER
83 negative cells stably transfected by the ERE-Luc plasmid) cells stably-expressing the human
84 ERs (HELN hERs cells) (Delfosse *et al.*, 2012).

85 Zebrafish (*Danio rerio*) is an *in vivo* model to study environmental pollutants. There are three
86 ERs (zfER α , zfER β 2 and zfER β 1) in zebrafish, with an approximate 50% amino-acid sequence
87 identity to the human ER orthologues (Menuet *et al.*, 2002). We have previously generated cell
88 lines stably-transfected with the zfER subtypes to study the potential estrogenic activity of
89 environmental pollutants in zebrafish (Pinto *et al.*, 2014). We demonstrated with these cell
90 models that mammalian estrogenic ligands also modulate the activity of the zfERs, although the
91 selectivity and potency of the ligand often varied for the human and zebrafish orthologues. In

92 zebrafish, the three ERs are all expressed from embryonic stages to adulthood, but with different
93 temporal and organ distributions (Bardet *et al.*, 2002; Chandrasekar *et al.*, 2010; Mouriec *et al.*,
94 2009). zfER α is detected in the heart valves of the developing zebrafish larvae, while the zfER β 1
95 subtype is robustly expressed in the liver (Gorelick *et al.*, 2014). In the developing brain,
96 zebrafish embryos express detectable levels of zfER β 2 and zfER β 1, but not zfER α , in different
97 brain regions. The expression of *cyp19a1b* gene was temporally correlated with that of zfER β 2
98 and zfER β 1 (Mouriec *et al.*, 2009) suggesting that these receptors drive the *cyp19a1b* expression.

99 During the past few years, several transgenic zebrafish models have been developed to
100 investigate the cell- and/or tissue-specific effects of chemicals on estrogen signaling pathways in
101 developing fish. For instance, in transgenic zebrafish model containing five EREs
102 (*Tg(5 \times ERE:GFP)*) (Gorelick and Halpern, 2011), the GFP expression is clearly detected in
103 tissues expressing the estrogen receptors. Thus, compounds with *in vitro* selectivity for zfER α
104 and zfER β s induce expression of the fluorescent protein in the heart and liver, respectively
105 (Gorelick *et al.*, 2016). For example, PPT specifically activates the zfER β s receptors *in vitro* and
106 induces GFP expression only in the liver of zebrafish larvae, whereas the pan-agonist 17 α -
107 ethinylestradiol induces GFP in both heart and liver (Gorelick and Halpern, 2011; Pinto *et al.*,
108 2014; Gorelick *et al.*, 2016). Another reporter fish model, the transgenic *cyp19a1b-GFP*
109 zebrafish, expresses GFP under the control of the ER-regulated *cyp19a1b* (brain aromatase) gene
110 in radial glial cells in the brain (Tong *et al.*, 2009). This fish model has been thoroughly
111 evaluated for assessing the estrogenic activity of a panel of environmental contaminants -
112 including natural and synthetic hormones, pharmaceuticals, pesticides, industrial chemicals- in
113 embryos and allowed to demonstrate that numerous contaminants were able to disrupt the brain-

114 specific expression of the *cyp19a1b* gene in an ER-dependent manner (Brion *et al.*, 2012; Cano-
115 Nicolau, et al., 2016; Le Fol et al., 2017).

116 In this article, we combined *in vitro* and *in vivo* zebrafish mechanism-based reporter gene assays
117 as screening tools to assess the estrogenic properties of BPA, BPAF and BPC. HELN cells
118 expressing the zfERs (HELN zfERs cells) were first used to evaluate the capacity of the selected
119 bisphenols to bind to the three zfERs subtypes and to transactivate zfERs to induce luciferase
120 activity. To further evaluate their estrogenic properties at the organism level, (*Tg(5×ERE:GFP)*)
121 and *tg(cyp19a1b-GFP)* zebrafish embryos were exposed to BPA, BPAF and BPC and the tissue-
122 specific estrogenic responses in the brain, heart and liver of zebrafish embryos were observed
123 and/or quantified and interpreted in light of their *in vitro* estrogenic activities towards the zfER
124 subtypes.

125

126 **Materials and Methods**

127 *Materials*

128 Tissue culture plates were obtained from Greiner Bio-one (Monroe, NC, USA), and media was
129 purchased from Invitrogen (Grand Island, NY, USA). Luciferin (sodium salt) was purchased
130 from Promega (Charbonnières, France). The chemicals 17 β -estradiol (E2), 17 α -ethinylestradiol
131 (EE2), bisphenol A (CAS no. 80-05-7), bisphenol AF (BPAF; CAS no. 1478-61-1), and
132 bisphenol C (BPC; CAS no. 14868-03-2) were purchased from Sigma-Aldrich (Saint-Quentin
133 Fallavier, France). ICI 182,780 (7 α ,17 β -[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-
134 1,3,5(10)-triene-3,17-diol, fulvestrant) was obtained from Zeneca (Macclesfield, UK), and 3,17-
135 Dihydroxy-19-nor-17 α -pregna-1,3,5(10)-triene-21,16 α -lactone (16 α -LE2) was a kind gift of

136 Peter Muhn from Research Laboratories of Schering AG (Berlin, Germany). Compounds were
137 dissolved in dimethyl sulfoxide (DMSO) as 10^{-2} M stock solutions.

138

139 *Cell lines*

140 The establishment and culturing of the HELN zfER α (Esr1), zfER β 1 (Esr2b) and zfER β 2 (Esr2a)
141 reporter cell lines have been previously described (Pinto et al. 2014). Briefly, the HELN-zfER α ,
142 β 1 and β 2 cell lines were obtained by transfection of HELN cells (HeLa cells stably transfected
143 with the ERE- β Globin-Luc-SVNeo plasmid) (Delfosse *et al.*, 2012) by the corresponding pSG5-
144 puro plasmids (pSG5-zfER α -puro, pSG5-zfER β 1-puro and pSG5-zfER β 2-puro, respectively).

145 The HELN zfER cells were maintained in phenol red-free Dulbecco's Modified Eagle's
146 Medium/F12 (DMEM/F12) and supplemented with 5% dextran-coated charcoal-treated fetal
147 bovine serum (FBS-DCC), 1% antibiotic (penicillin/streptomycin), geneticin (1 mg/mL) and
148 puromycin (0.25 μ g/mL) in a 5% CO₂ humidified atmosphere at 37°C.

149

150 *zfERs binding assays*

151 The assay for measuring zfER binding affinity has been previously described (Pinto *et al.*, 2014).
152 Briefly, the HELN-zfERs cell lines were treated with 3 nM [³H]-E2 in the absence or in the
153 presence of chemicals for 4h at 28°C. At the end of the incubation period, unbound material was
154 aspirated, cells washed with PBS, and [³H]-bound radioactivity counted. Protein concentrations
155 were used to normalize bound radioactivity values expressed in desintegrations per minute
156 (dpm). Experiments were performed in quadruplicate in at least two independent experiments.

157

158 *Transactivation assays*

159 Chemicals successive dilutions were performed in cell culture medium, using the same protocol
160 for treatments as described previously (Pinto *et al.*, 2014). The compounds were added 8 h after
161 seeding cells into 96-well plates, and cells were incubated with compounds for 16h at 28°C.
162 Luciferase assays were performed as previously published (Pinto *et al.*, 2014), and experiments
163 were performed in quadruplicates in three independent experiments. Data were expressed as
164 percentage of the maximal activity induced by E2 10 nM. EC50 values were measured using
165 GraphPad Prism (GraphPad Software Inc). Datas were analyzed for significant differences using
166 one-way ANOVA followed by Dunnet's post-comparison test (vs.control) (***p<0.001,
167 **p<0.01, *p<0.05). Differences were considered statistically significant at P <0.05.

168

169 *Zebrafish maintenance and transgenic zebrafish Tg(5xERE:GFP) treatment*

170 Transgenic fish *Tg(5xERE:GFP)* (Gorelick and Halpern, 2011) were used according to the
171 maintenance and experimental protocols approved by the Institutional Animal Care and Use
172 Committee at the University of Houston. Adult zebrafish were maintained and fed as described
173 previously (Hao *et al.*, 2013).

174 Embryos from several pairs of adult fish were collected after spawning and cultured in a Petri
175 dish in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) at
176 28.5°C with 14:10 LD. For fluorescent imaging experiments, *Tg(5xERE:GFP)* zebrafish
177 embryos were incubated in E3 medium with 0.003% 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich,

178 St. Louis, MO) starting at 24h post fertilization (hpf) to inhibit pigment formation. Samples of 10
179 zebrafish larvae at 3 days post fertilization (dpf) were treated for 24 h with EE2, 16 α -LE2 and
180 bisphenols in 24-well plate, and transferred to 96-well plates at a density of one larva per well for
181 imaging. EE2, 16 α -LE2, ICI 182,780 and bisphenols in 100% DMSO were diluted in embryo
182 media, and vehicle concentration did not exceed 0.2% DMSO in treatments. Vehicle only was
183 used as a negative control. The larvae were imaged under 10X magnification lenses using a
184 fluorescent microscope (Olympus IX51) equipped with an Olympus XM10 camera (Olympus,
185 Center Valley, PA).

186

187 *Treatment of cyp19a1b-GFP transgenic zebrafish embryos*

188 Newly fertilized transgenic *cyp19a1b-GFP* zebrafish eggs were collected and treated as
189 previously described (Brion *et al.*, 2012). Briefly, 20 embryos (< 4 h post-fertilization) were
190 exposed from 0 to 4 dpf to DMSO (0.01% v:v), 0.05 nM EE2 (used as positive control) or a
191 range of concentrations of BPA, BPAF or BPC. The exposure medium was renewed totally
192 every 24 h. At the end of the exposure period, the zebrafish embryos were collected for *in vivo*
193 fluorescence imaging of the brain. Each 4 dpf-old zebrafish was photographed in dorsal view
194 under a fluorescence microscope connected to an AxioCam Mrm camera (Zeiss GmbH,
195 Gottingen, Germany). All photographs were taken with the same parameters. The fluorescence
196 of the regions of interest was then quantified by image analysis using an ImageJ macro as
197 previously described (Brion *et al.*, 2012).

198

199 *RNA extraction and quantitative real-time PCR*

200 Zebrafish larvae were treated with bisphenols and controls from 4 dpf to 6 dpf. Treatments were
201 repeated once at 5 dpf. Total RNA from 40 pooled embryos were collected at 6 dpf using Trizol
202 (Invitrogen Corporation, Carlsbad, CA) and RNeasy spin columns (Qiagen, Chatsworth, CA)
203 according to manufacturer's protocols. DNase I (Qiagen, Chatsworth, CA) was used to remove
204 any DNA in the column. RNA concentration was measured using NanoDrop 1000
205 spectrophotometer (Agilent Technologies, Palo Alto, CA) and 100 ng of total RNA was used for
206 cDNA synthesis using Superscript III reverse transcriptase (Invitrogen Corporation, Carlsbad,
207 CA) with random hexamer primers.

208 Expression of Cyp19a1b and Vtg1 were measured by qPCR using an ABI 7500 Fast Real-Time
209 PCR system (Applied Biosystems, Foster City, CA) with iTaq Universal SYBR Green Supermix
210 (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Primers were synthesized by
211 Integrated DNA Technologies, Inc (Coralville, IA). Primer sequences were for vtg1 Forward (5'-
212 3') ACTACCAACTGGCTGCTTAC and Reverse (5'-3') ACCATCGGCACAGATCTTC; and
213 for cyp19a1b Forward (5'-3') AAAGAGTTACTAATAAAGATCCACCGGTAT and Reverse
214 (5'-3') TCCACAAGCTTTCCCATTTCA (Hao *et al.*, 2013). Expression levels were normalized
215 to zebrafish β -actin using the $\Delta\Delta$ Ct method. Student's t-test was performed for statistical
216 analysis using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA).

217

218 **Results**

219 **Transcriptional activity of bisphenols mediated by zfERs *in vitro***

220 The transcriptional activity of BPA, BPAF and BPC have been reported for human estrogen
221 receptors in HELN cells (Delfosse et al, 2012). To investigate the activity of BPA and its

222 analogues on zebrafish ERs, we treated the HELN cell lines expressing the zfER α , zfER β 1 and
223 zfER β 2 receptors and an ERE-driven luciferase reporter with increasing concentrations of the
224 bisphenols. BPA and BPAF activated the three zfER reporter cells, reaching approximately 53-
225 89% of E2-induced luciferase expression (Figure 1A, B). These bisphenols displayed higher
226 selectivity for zfER α , than for the zfER β subtypes. BPAF was more potent than BPA in
227 inducing luciferase expression, and displayed approximately 3.1-5.6-fold greater potency on the
228 three zfER cells than BPA, with EC50s of 84, 758 and 897 nM for zfER α , zfER β 1 and zfER β 2,
229 respectively (Table 1). BPC activated both zfER α and zfER β 1 at low concentrations, but the
230 luciferase activity reached only 31 and 36% of the maximal luciferase activity, respectively. BPC
231 induced transcription to 59% of maximal activation on zfER β 2 (Figure 1C). The measured
232 EC50s and maximal activation for the BPA, BPAF, BPC and controls are shown in Table 1.

233 BPA, BPAF and BPC were identified as partial agonists in HELN hERs cells (Delfosse *et al.*,
234 2012). The partial bisphenols induced-luciferase activity in the human ER reporter cells and the
235 marginal effect of BPC on zfER α and zfER β 1 (figure 1C) suggest that BPC can act as an
236 antagonist for these zfERs. We therefore evaluated the ability of BPC to antagonize the activity
237 of E2. We found that BPC clearly reduced E2-induced transactivation in the zfER α and zfER β
238 cell lines with IC50s of 46, 160 and 136 nM on zfER α , zfER β 1 and zfER β 2, respectively
239 (Figure 1D; Table 1) thereby revealing a stronger antagonistic activity of BPC towards zfER α
240 and zfER β 1.

241 The selectivity of BPA, BPAF and BPC for zfER α was confirmed in competitive binding assays
242 with radiolabeled E2 (Table 2). As previously demonstrated for the selective zfER α compound
243 16 α -LE2 (Pinto *et al.*, 2014), bisphenols displayed stronger binding affinity for zfER α

244 compared to the zfER β subtypes. Additionally, BPAF and BPC displaced radiolabeled E2 at
245 lower concentrations than BPA, confirming the stronger affinity of BPAF and BPC for the
246 zebrafish ERs relative to BPA (Table 2).

247

248 **Activation of estrogen signaling by bisphenols in *tg ERE-GFP* embryos**

249 To assess the estrogenic effects of BPA, BPAF and BPC *in vivo* in zebrafish embryos, the
250 transgenic fish line *Tg(5xERE:GFP)* containing 5xERE upstream of a *c-fos* promoter and a GFP
251 reporter was used (Gorelick and Halpern, 2011). In order to qualitatively investigate the tissue-
252 specific activity of the bisphenols, we treated 3 dpf zebrafish larvae for 24 h with BPA, BPAF
253 and BPC (Figure 2). The ligand 16 α -LE2, whose zfER α selectivity *in vitro* is associated with *in*
254 *vivo* GFP expression exclusively in the heart valves at lower doses (10-100 nM) (Figure 2C-D),
255 was used to compare the tissue-specific effects resulting from treatment with the bisphenols. The
256 zfER α -selective agonist activity of BPA and BPAF was confirmed in the zebrafish embryos. At
257 lower concentrations, BPA and BPAF only activated GFP expression in the heart valves, while
258 higher doses of the ligands were required for induction of liver expression (Figure 2G-J and 2K-
259 N). Treatment with BPC induced GFP expression in the liver at 1 μ M, and no heart GFP
260 expression was observed, likely due to its only marginal activity on zfER α (Figure 2O-R).

261 The stronger potency of BPAF and BPC on the zfERs compared to BPA observed *in our*
262 *experiments vitro* was confirmed *in vivo*. Treatment with 100 nM BPAF induced GFP expression
263 in the heart, whereas a dose of 1 μ M BPA was required to achieve a similar effect in the
264 zebrafish embryos. At the hepatic level, GFP expression was induced after exposure to 1 μ M
265 BPAF or 1 μ M BPC, while BPA was only able to activate hepatic GFP expression at 10 μ M

266 (Figure 2G-R). Therefore, the responses observed *in vivo* seems to reflect both the estrogenic
267 activity reported *in vitro* as well as the selectivity of the compounds towards the zfERs. Thus, the
268 stronger potencies of BPAF and BPC compared to BPA seen *in vitro* was also observed at the *in*
269 *vivo* level.

270 To investigate the antagonist activity of BPC *in vivo*, we co-treated zebrafish embryos with the
271 estrogenic ligand EE2, which is more stable than E2 *in vivo*, or 16 α -LE2 in combination with
272 BPC (Figure 3 and Figure 4). Treatment with 100 pM EE2 induced GFP expression both in the
273 heart and liver of the embryos, in accordance with its pan-agonist ER activity (Figure 3C) (Pinto
274 *et al.*, 2014; Gorelick *et al.*, 2016). The pure antagonist ICI 182,780 completely blocked both
275 heart and liver GFP expression (Figure 3E). Treatment with 1 μ M BPC induced GFP expression
276 in the liver of the embryos in accordance with its zfER β 2 activity. Co-treatment of EE2 with 1
277 μ M BPC completely blocked EE2-induced GFP heart expression whereas it only slightly
278 reduced EE2-induced GFP liver expression (Figure 3G). Likewise, BPC was capable of
279 completely inhibiting the GFP expression induced by 10 nM 16 α -LE2 in the heart (Figure 4).
280 These results are in accordance with the *in vitro* antagonist/agonist activity of BPC, indicating
281 that BPC is an antagonist on zfER α and partial agonist on zfER β s *in vivo*.

282

283 **Activation of the brain aromatase promoter in the transgenic cyp19a1b-** 284 **GFP zebrafish embryos by the three bisphenols**

285 To assess the effect of bisphenols on the expression of the estrogen-sensitive brain aromatase
286 gene, the fluorescence signal in the developing brain of transgenic cyp19a1b-GFP zebrafish
287 embryos was quantified after a 4-day treatment with BPA, BPAF and BPC. All bisphenols

288 induced GFP expression in the brain of the zebrafish embryos (Figure 5A-C). BPA at 10 μ M
289 promoted a 28-fold induction in the GFP signal, whereas no or very weak activity was observed
290 at lower concentrations. Exposure to BPAF or BPC at 1 μ M was sufficient to promote a 18-fold
291 induction in the GFP expression, approximately, and a 2.9-fold and 5.5-fold GFP activation was
292 observed at 0.1 μ M for BPAF and BPC, respectively (Figure 5A).

293 To assess the potential for the bisphenols to inhibit estrogen-mediated activation of the brain
294 aromatase promoter, the *cyp19a1b*-GFP zebrafish embryos were treated with 0.05 nM EE2 in the
295 presence of the bisphenols. Under these experimental conditions, BPA, BPAF and BPC were
296 unable to inhibit the EE2-mediated induction of GFP in the zebrafish embryos. These results
297 indicate that BPA, BPAF and BPC act as agonists to induce brain aromatase expression within
298 the radial glial cell context but that the bisphenols lack of ER antagonist activity on the brain
299 aromatase of zebrafish embryos (Figure 5B-C).

300

301 **Endogenous estrogen target gene activation by bisphenols *in vivo***

302 To analyze the effect of BPA, BPAF and BPC exposure on the expression of endogenous
303 estrogen target genes, we performed quantitative PCR. We investigated the expression of the
304 estrogen-sensitive markers *vtg1* gene, highly expressed in the liver, and the brain *cyp19a1b*. In
305 concordance with the *in vivo* GFP expression data, BPAF and BPC activated *vtg1* and *cyp19a1b*
306 at lower concentrations than BPA (clearly detected at 1 μ M vs. 10 μ M, respectively) (Figures 6A
307 and 6B). We conclude that the *in vivo* endogenous activation of estrogenic target genes by BPA
308 analogs is in accordance with both the brain and liver *in vivo* GFP activation.

309

310 **Discussion**

311 Evidence in the literature showing that exposure to BPA causes harmful health effects in humans
312 and wildlife has prompted a search for safer bisphenol alternatives. We assessed the effects of
313 BPA, BPAF and BPC using zfER reporter cell lines, and found that BPAF and BPC in
314 comparison to BPA have a stronger binding affinity for the zebrafish ERs, and a lower EC50 to
315 activate them. *In vitro*, BPAF and BPA activated luciferase expression mediated by the three
316 zebrafish estrogen receptors, with stronger affinity for zfER α . This zfER α selectivity was
317 confirmed by the preferential activation of the GFP reporter in the heart valves *in vivo* in
318 zebrafish larvae. BPC only minimally stimulated the activity of zfER α *in vitro*, and partially
319 activated zfER β -mediated transcription to levels ranging from 40-60% of the activity of E2. In
320 accordance with its agonist profile *in vitro*, exposure to BPC induced GFP expression in the
321 brain and in the liver of the zebrafish larvae. Additionally, the stronger affinity of BPAF and
322 BPC for the zebrafish receptors compared to BPA *in vitro* was confirmed *in vivo*, as lower
323 concentrations of these bisphenol analogues were necessary to activate liver and brain GFP
324 expression, as well as promote the induction of vtg1 and cyp19a1b gene expression in zebrafish
325 embryos.

326 The estrogenic activities of bisphenols have previously been analyzed for human ERs in reporter
327 cell lines (Kitamura *et al.*, 2005; Matsushima *et al.*, 2010; Delfosse *et al.*, 2012; Chen *et al.*,
328 2016; Grimaldi *et al.*, 2019). The pronounced agonist activity of BPAF has been previously
329 reported for human ERs, with several studies demonstrating that BPAF is more estrogen-active
330 than BPA. In HELN cells expressing the human ERs, both BPAF and BPC are more potent
331 ligands than BPA (Delfosse *et al.*, 2012). Bisphenols show a slightly better affinity for human
332 ER β . This opposite ER selectivity profile between human and zebrafish ERs also observed for

333 several other xenoestrogens, could be due to differences in the constitution and interaction with
334 amino acids in the LBD of human and zebrafish receptors (Pinto *et al.*, 2014).

335 The *in vivo* estrogenic activity of bisphenols have been studied in fish and rodents. In adult male
336 medaka, BPAF and BPC are more potent than BPA in inducing hepatic upregulation of the
337 expression of vtg and other estrogenic biomarkers (Yamaguchi *et al.*, 2015). Moreover, the
338 bisphenols induce uterine weight gain in ovariectomized rats, and the uterotrophic effect is more
339 pronounced for BPC and BPAF compared to BPA (Conley *et al.*, 2016). These *in vivo* results are
340 in accordance with our results from *in vitro* and transgenic zebrafish models, and provide
341 substantial data to support that BPC and BPAF have a stronger ability than BPA to disrupt
342 estrogen signaling across different vertebrate species.

343 The present study is, to our knowledge, the first to demonstrate that the chlorinated bisphenol
344 analogue, BPC, has pronounced antagonistic zfER α activity in zebrafish both *in vitro* and *in*
345 *vivo*. Exposure to BPC resulted in clear inhibition of the EE2- and 16 α -LE2-induced GFP
346 expression in the heart of the zebrafish larvae, in accordance with its zfER α -mediated antagonist
347 activity in the HELN reporter cells. The outcomes of activation or inactivation of estrogen
348 signaling by bisphenols on the development and physiology of the zebrafish heart are unknown.
349 However, it has been reported that estrogenic ligands can alter the basal heart rate in zebrafish
350 embryos. Exposure to estradiol and ICI 182,780 resulted in increased heart rate in zebrafish, an
351 effect that was attributed to modulation of the G protein-coupled estrogen receptor (GPER)
352 pathway (Romano *et al.*, 2016), while treatment with the anti-estrogen tamoxifen caused a
353 decrease in the heart rate of zebrafish embryos (Xia *et al.*, 2016). BPA treatment has been shown
354 to impair the cardiac contractile performance of rodent hearts, an effect associated with altered
355 intracellular calcium handling (Posnack *et al.*, 2015).

356 Although ER antagonist activity was observed for BPC in the ERE-driven activation of
357 fluorescence in the zebrafish heart, BPC, as well as BPA and BPAF, were unable to inhibit the
358 EE2-induced brain aromatase promoter. zfER β 2 has been reported to be the main ER subtype
359 detected in the brain during zebrafish early developmental stages, while zfER α and zfER β 1
360 mRNA are in contrast much lower (Mouriec *et al.*, 2009). Studies have also shown that
361 morpholino knockdown of zfER β 2, but not zfER α or zfER β 1, completely blocks estrogen-
362 mediated upregulation of the aromatase mRNA (Griffin *et al.*, 2013). This data suggests that
363 zfER β 2 is the main subtype controlling the activation of brain aromatase during zebrafish early
364 development. The results with BPC support this hypothesis, as BPC would be expected to inhibit
365 the estrogen-mediated activation of aromatase in the cyp19a1b-GFP zebrafish embryos if
366 aromatase expression was indeed controlled by zfER α . Inferring from this data, BPC will act as
367 both an agonist and antagonist *in vivo* in a tissue-specific manner.

368 In addition to perturbing the ER pathway, BPAF and BPC have been reported to interact with
369 other endocrine targets (Delfosse *et al.*, 2014; Grimaldi *et al.*, 2019; Lei *et al.*, 2017; Wang *et al.*,
370 2014). The increasing and widespread use of these bisphenol analogues and their ability to
371 interact with endocrine molecular targets at lower concentrations than BPA pose a serious
372 concern associated with potential health risks for humans and wildlife. Implementation of green
373 chemistry practices for identification of safer alternatives will improve characterization of
374 chemical hazard and should be useful to guide manufacturers on more sustainable bisphenol
375 alternatives.

376 In conclusion, we show that a combined strategy using cell-based assays and transgenic zebrafish
377 models can be used to thoroughly assess estrogenic properties of bisphenol derivatives. This

378 approach allows for the quick screening of both estrogenic and anti-estrogenic activities of
379 environmental pollutants, and are sensitive tools to inform dose-response relationships and
380 tissue-specific effects. Characterization of species differences in response to estrogenic
381 compounds can be addressed in the HELN cells expressing human or zebrafish ERs. The
382 transgenic zebrafish models could also be used to address the influence of metabolism and
383 pharmacokinetic parameters on chemical activity. Moreover, these tools are amenable to high-
384 throughput screening, and can be incorporated into screening platforms currently applied to
385 inform perturbations in ER signaling, such as the ones of the U.S. Environmental Protection
386 Agency (EPA)'s ToxCast/Tox21 program.

387

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395

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539

540

541 **Figure legends**

542 Fig. 1. Agonistic and antagonistic activity of bisphenols in HELN zfER reporter cell lines. (A-C)
543 Dose-response curves for BPA, BPAF, and BPC in HELN zfER reporter cell lines. Cells were
544 incubated for 16h with various concentrations of BPA, BPAF, and BPC. (D) Dose responsive
545 curves of BPC in HELN zfER reporter cell lines. Cells were incubated for 16h with various
546 concentrations of BPC in presence of in presence of 1 nM E2. Data were expressed as percentage
547 of the maximal activity induced by E2 10 nM. EC50 values were measured using GraphPad
548 Prism (GraphPad Software Inc). Datas were analyzed for significant differences using one-way
549 ANOVA followed by Dunnet's post-comparison test (vs.control) (***p<0.001, **p<0.01,
550 *p<0.05).

551

552 Fig. 2. *In vivo* estrogenic activities of bisphenols on ERE reporter fish. *Tg (ERE:GFP)* larvae
553 were treated with 16 α -LE2 or bisphenols at 3 dpf for 24 h. Images were taken at 4 dpf. Lateral
554 view showing GFP expression in the heart valves (red arrows) and liver (white arrow). A.
555 Bright-field image; B. GFP fluorescence image of vehicle-treated zebrafish larvae; C-F. GFP
556 images of zebrafish larvae exposed to increasing concentrations of 16 α -LE2; G-J. GFP images of

557 zebrafish treated with BPA; K-N. Images of zebrafish exposed to BPAF; O-R. GFP images of
558 zebrafish exposed to BPC. *Scale bars*, 200 μm .

559

560 Fig. 3. Anti-estrogenic effects of BPC on ERE reporter fish co-treated with EE2. A. Bright-field
561 image; B. GFP fluorescence image of vehicle-treated zebrafish larvae; C. GFP images of
562 zebrafish larvae exposed to 100 pm EE2; D. Zebrafish exposed to 1 μM ICI 182,780; E. GFP
563 images of zebrafish co-treated with EE2 and ICI; F. Images of zebrafish exposed to 1 μM BPC;
564 G. GFP images of zebrafish co-treated with EE2 and BPC. *Scale bars*, 100 μm .

565

566 Fig. 4. Anti-estrogenic effects of BPC on ERE reporter fish co-treated with 16 α -LE2. A. Bright-
567 field image; B. GFP fluorescence image of vehicle-treated zebrafish larvae; C. GFP images of
568 zebrafish larvae exposed to 10 nM 16 α -LE2; D. GFP images of zebrafish exposed to 3 μM BPC;
569 E. GFP images of zebrafish larvae co-treated with 16 α -LE2 and BPC. *Scale bars*, 200 μm .

570

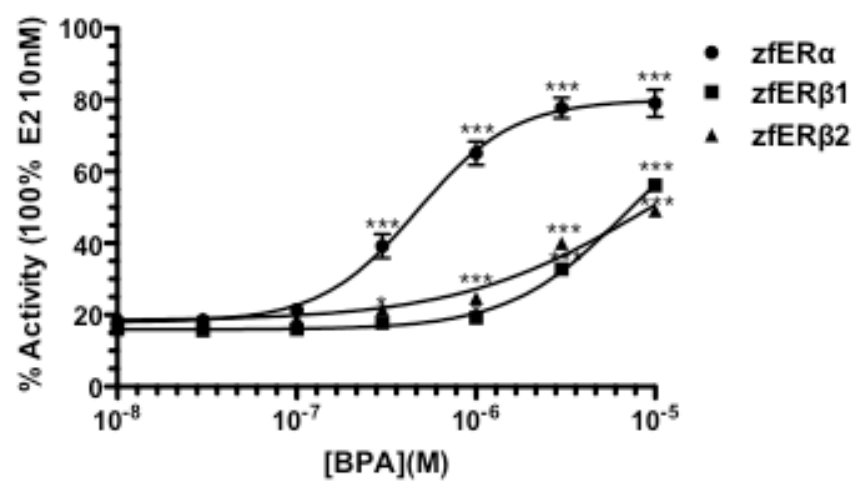
571 Fig. 5. *In vivo* estrogenic activity of bisphenols in the *cyp19a1b-GFP* zebrafish. A.
572 Concentration-dependent induction of GFP by BPA, BPAF and BPC in *cyp19a1b-GFP*
573 transgenic larvae after exposure to the bisphenols from 0 to 4 dpf. Values are expressed as mean
574 +/- SEM (n = 4-13). B. Effect of BPA, BPAF and BPC on EE2-mediated GFP induction in
575 *cyp19a1b-GFP* transgenic larva after exposure from 0 to 4 dpf. Values are expressed as mean +/-
576 SEM (n = 8-14). C. Fluorescence images of *cyp19a1b-GFP* transgenic larvae treated with 0.05

577 nM EE2, 10 μ M BPA, 1 μ M BPAF and 1 μ M BPC alone and co-treatments of EE2 with the
578 bisphenols. Images were taken in the dorsal view, anterior side to the top.

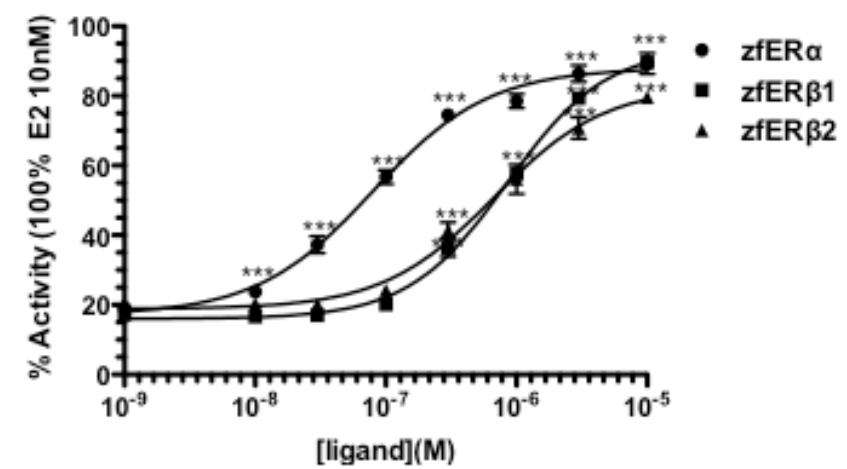
579

580 Fig. 6. Endogenous estrogen target gene activation by bisphenols *in vivo*. Concentration-
581 dependent induction of *vtg1* (A) and *cyp19a1b* (B) mRNA levels after treatment of zebrafish
582 embryos with BPA, BPAF and BPC from 4 to 6 dpf. Transcript levels were normalized to
583 zebrafish β -actin using the $\Delta\Delta$ Ct method.

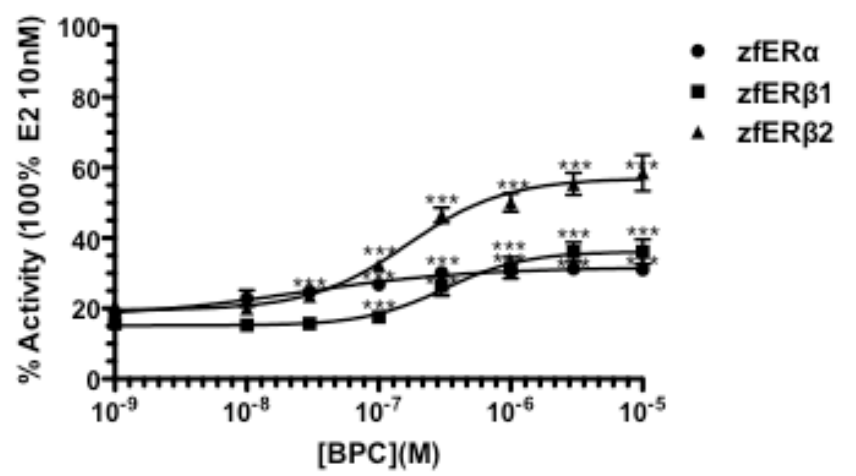
1A



1B



1C



1D

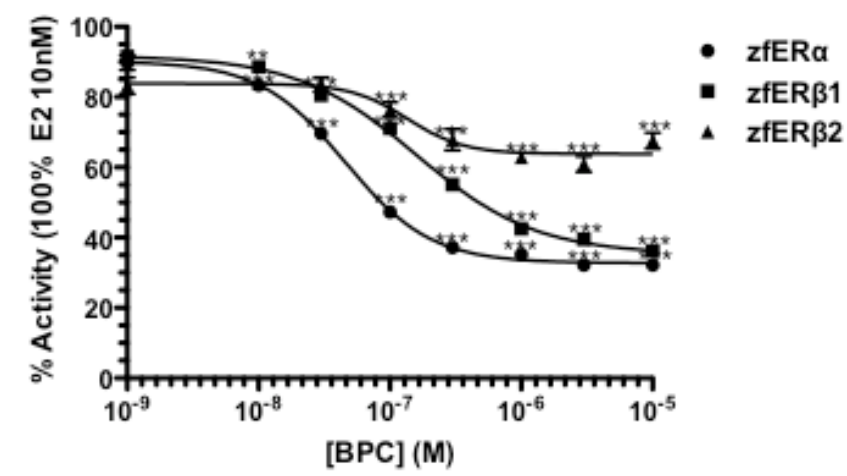


Figure 1

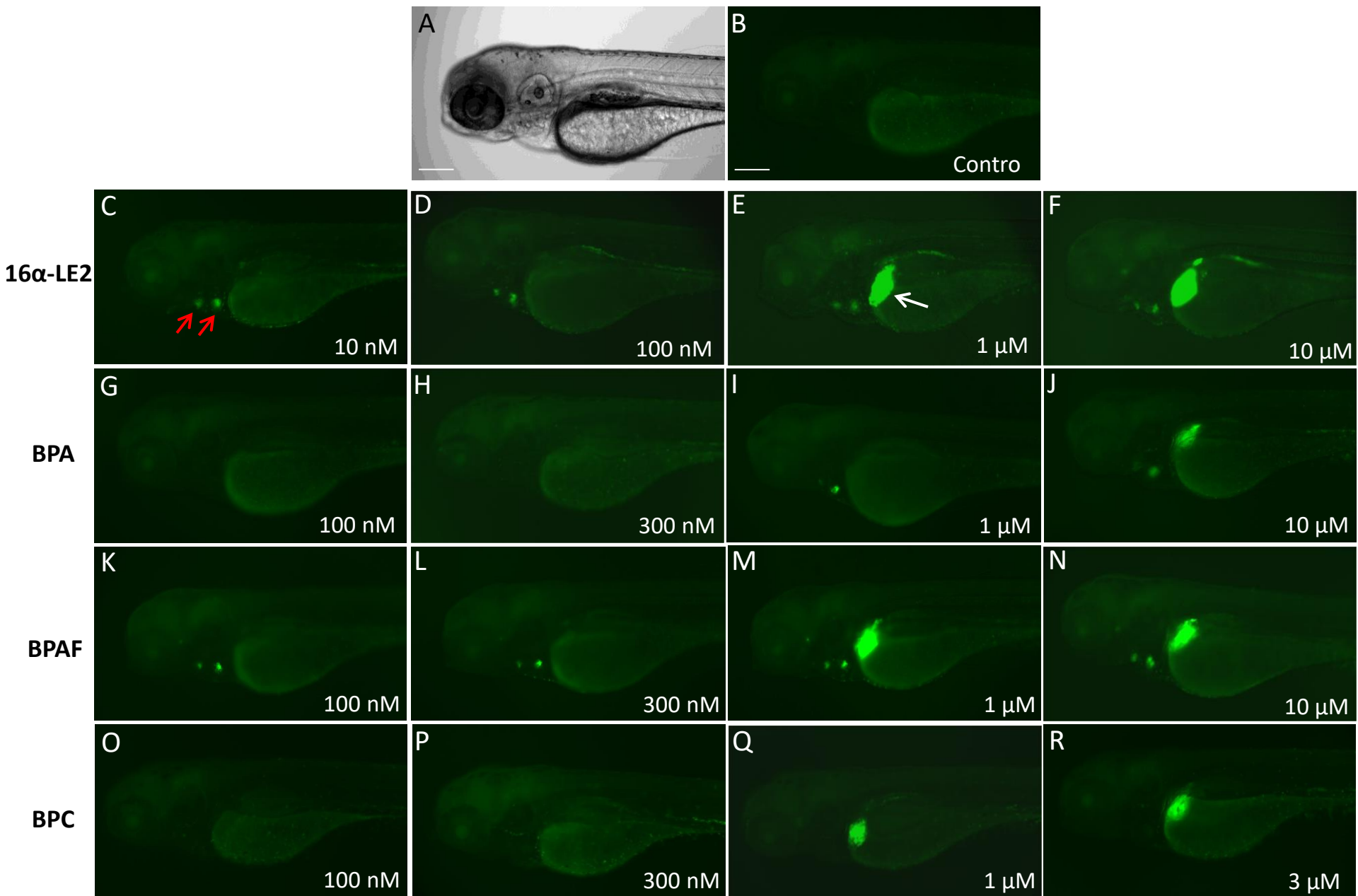


Figure 2

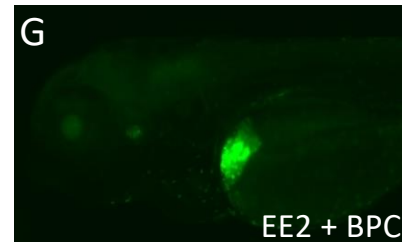
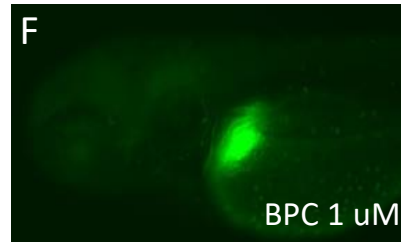
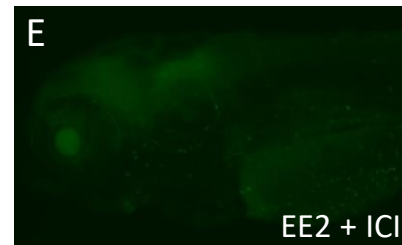
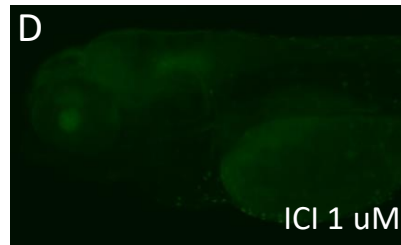
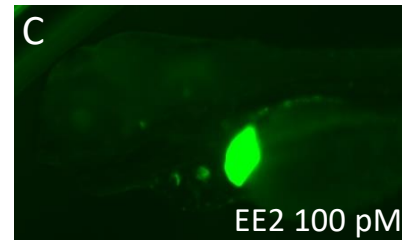
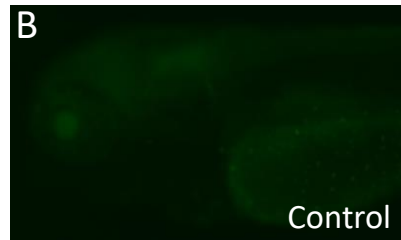
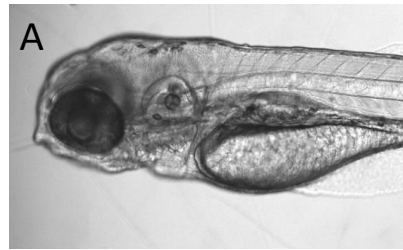


Figure 3

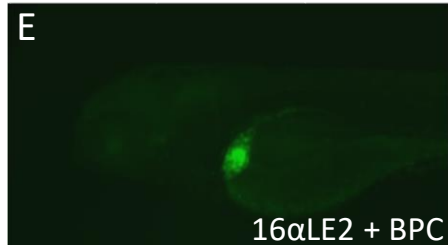
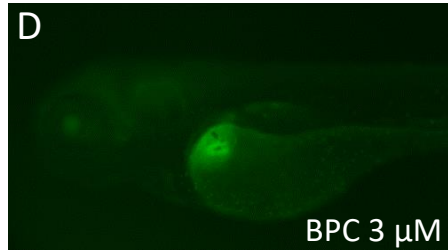
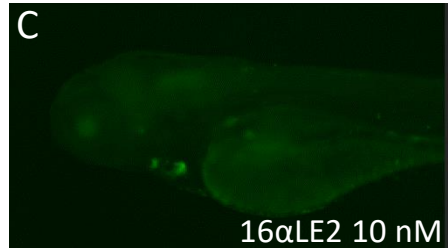
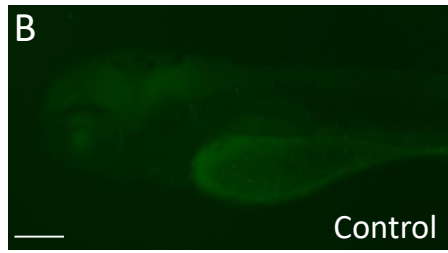
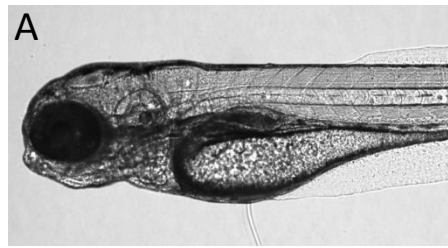
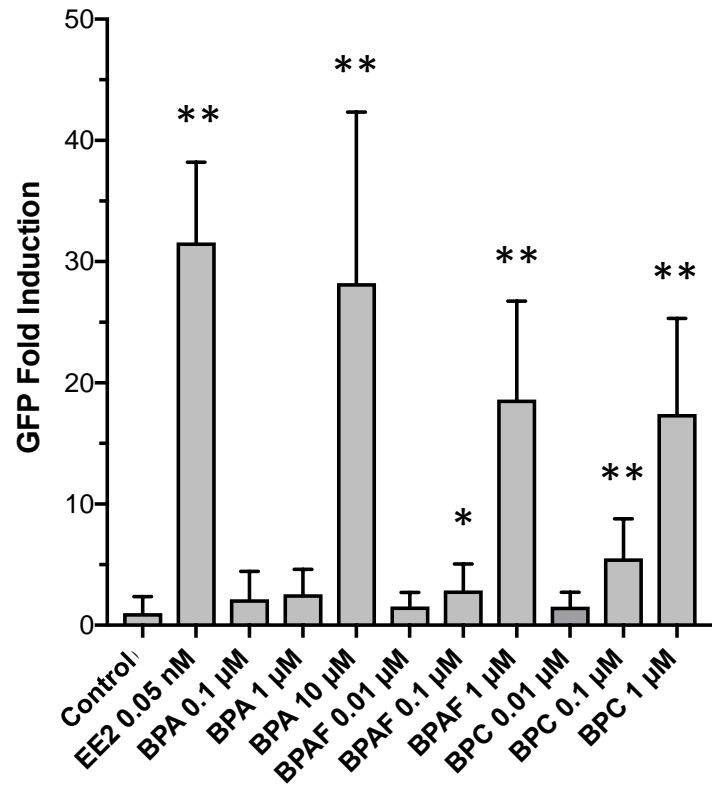
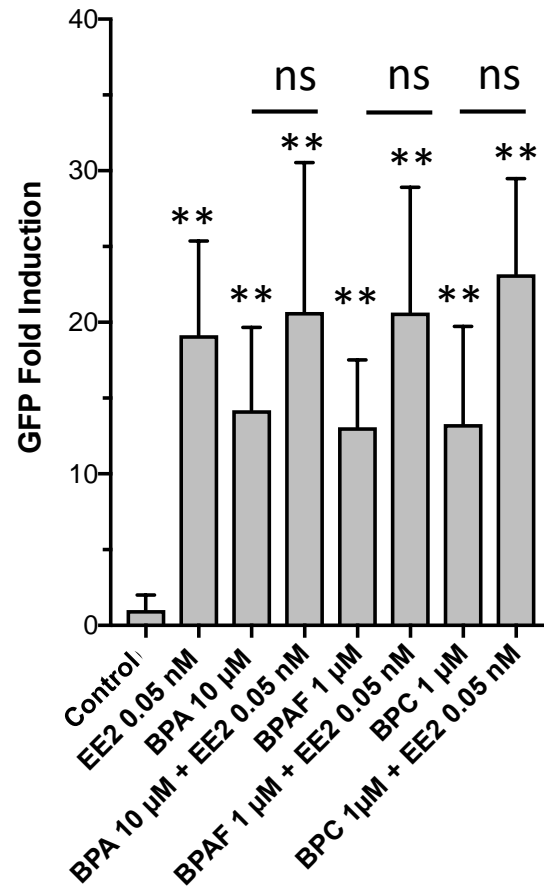


Figure 4

A



B



C

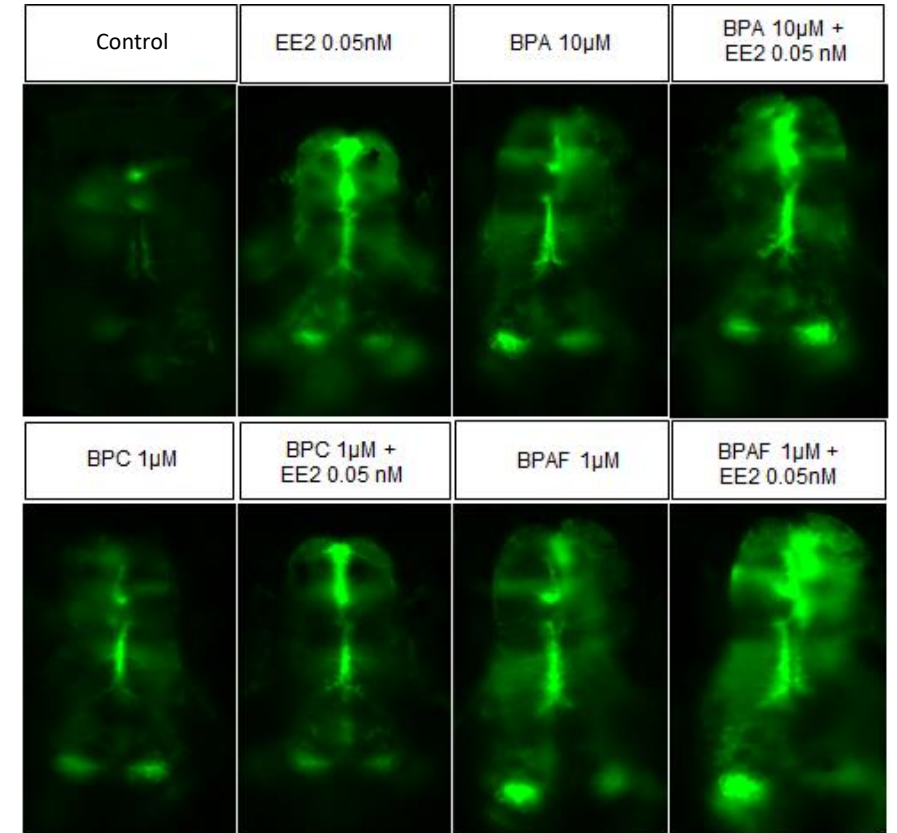


Figure 5

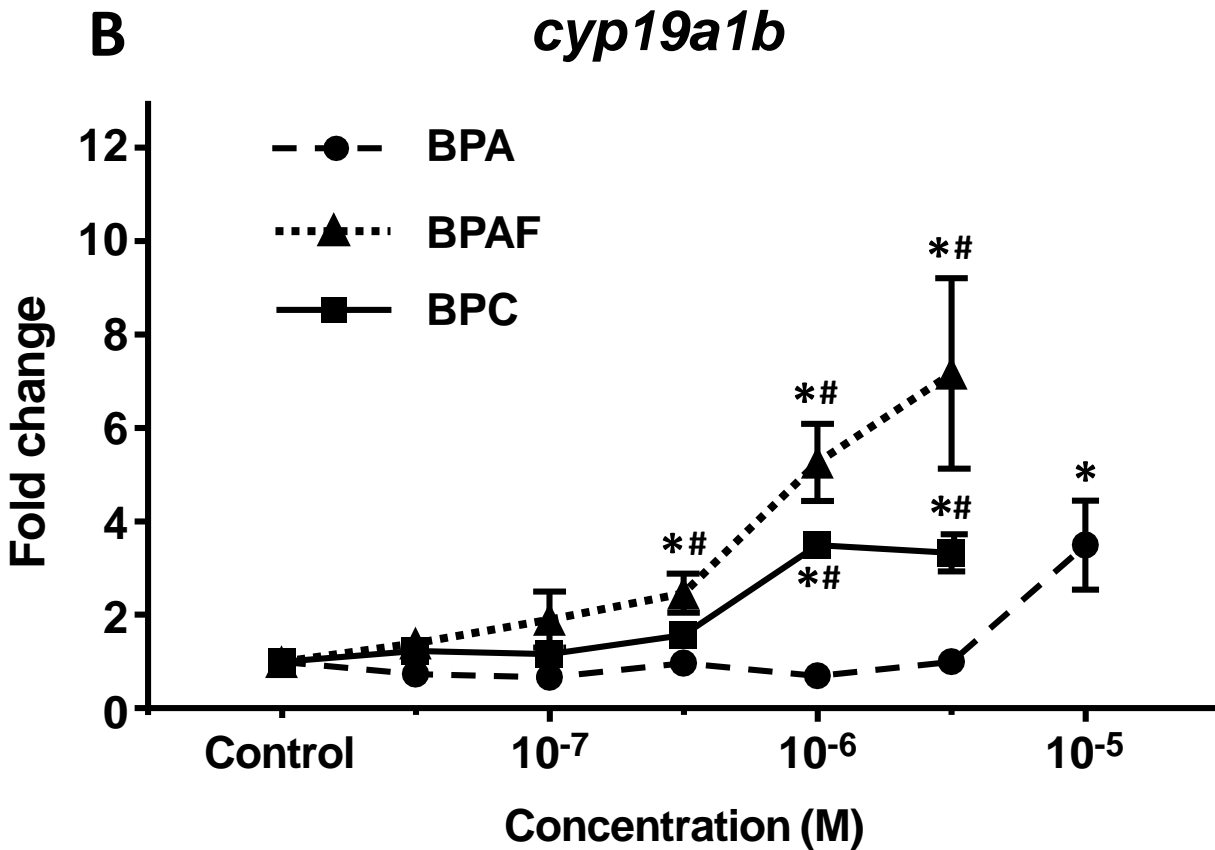
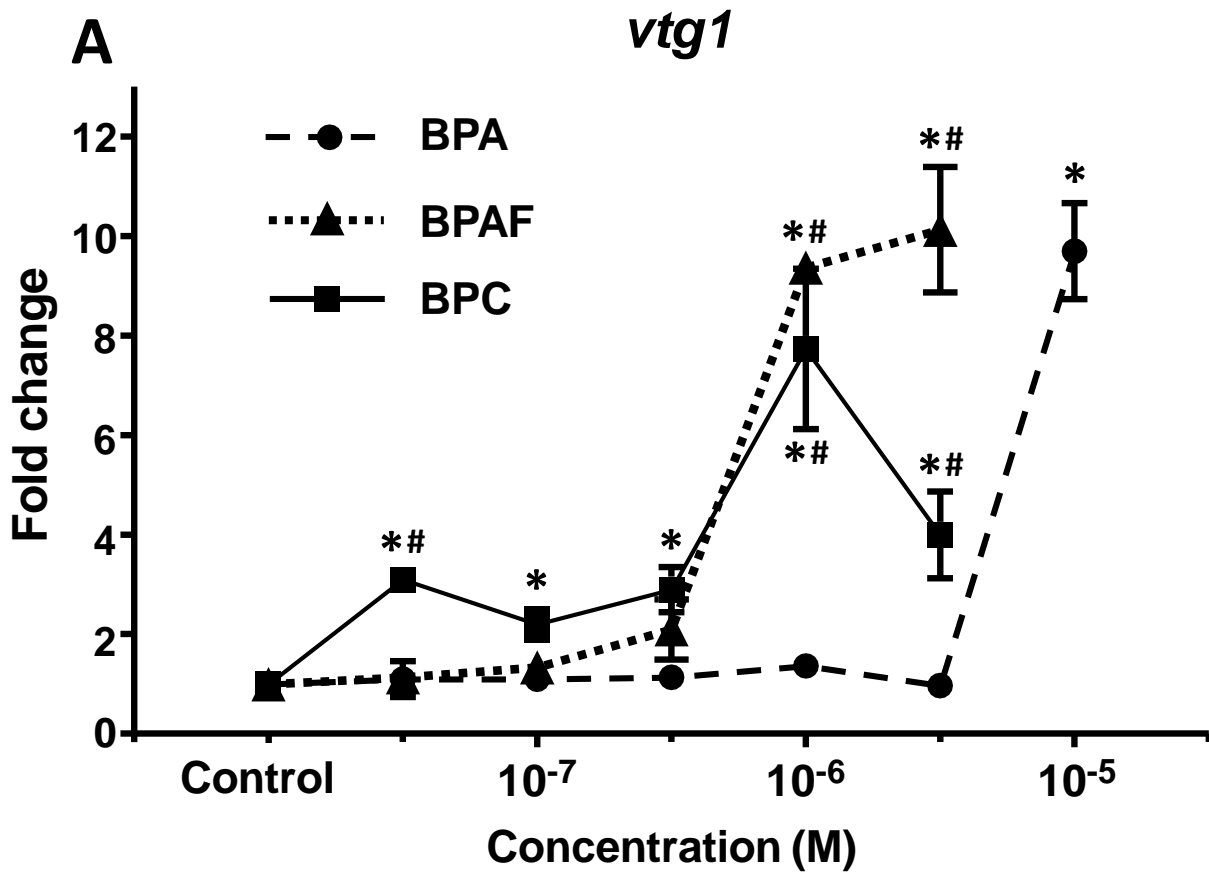


Figure 6

Table 1. Effective concentration (EC50 in nM), IC50 in nM), relative estrogenic potency (REP) and maximal activity (%) of 17 β -E2, 16 α -LE2, BPA and the bisphenol analogues on the zfERs.

	zfER α		zfER β 1		zfER β 2	
Basal activity	18.8 \pm 0.3		16.5 \pm 0.7		20.2 \pm 0.6	
Agonism						
Ligand	EC50 (REP)	Maximal activity	EC50	Maximal activity	EC50	Maximal activity
E2	0.08 \pm 0.03 (1)	100	0.04 \pm 0.01 (1)	100	0.12 \pm 0.03 (1)	100
16 α -LE2	0.65 \pm 0.1 (1.2 $\cdot 10^{-1}$)	100	271 \pm 45 (1.5 $\cdot 10^{-4}$)	100	69.5 \pm 17.5 (1.7 $\cdot 10^{-3}$)	100
BPA	469 \pm 42 (1.7 $\cdot 10^{-4}$)	79	3326 \pm 381 (1.2 $\cdot 10^{-5}$)	53.1	2800 \pm 562 (4.3 $\cdot 10^{-5}$)	56.1
BPAF	84 \pm 27 (9.5 $\cdot 10^{-4}$)	89.3	758 \pm 131 (5.3 $\cdot 10^{-5}$)	89.6	897 \pm 268 (1.3 $\cdot 10^{-4}$)	79.6
BPC	25 \pm 7.5 (3.2 $\cdot 10^{-3}$)	31	316 \pm 110 (1.3 $\cdot 10^{-4}$)	36.1	167 \pm 72 (7.2 $\cdot 10^{-4}$)	58.5
Antagonism						
	IC50		IC50		IC50	
BPC	46 \pm 6.4		160 \pm 42		136 \pm 75	

Values are the mean \pm standard deviations from three separate experiments. Maximal activity is expressed as percentage of luciferase activity induced by 10 nM E2. REPs were determined as the ratio of E2 EC50 to that of bisphenol.

Table 2. Dissociation constant (Kd) values for E2, 16 α -LE2, BPA, BPAF and BPC on the zfERs. Values are the mean \pm standard deviations from three separate experiments.

Ligands	zfER α Kd (nM)	zfER β 1 Kd (nM)	zfER β 2 Kd (nM)
E2	2.6 \pm 0.6	1.1 \pm 0.2	2.6 \pm 0.7
16 α -LE2	9.4 \pm 0.9	1109 \pm 126	351 \pm 44
BPA	1245 \pm 265	10040 \pm 2919	6884 \pm 1270
BPAF	428 \pm 85	1023 \pm 184	1993 \pm 628
BPC	78 \pm 16	678 \pm 124	402 \pm 89