

1 Estrogenic mechanisms and cardiac responses
2 following early life exposure to Bisphenol A
3 (BPA) and its metabolite 4-methyl-2,4-bis(*p*-
4 hydroxyphenyl)pent-1-ene (MBP) in zebrafish.

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14 **ABSTRACT** Environmental exposure to Bisphenol A (BPA) has been associated with a
15 range of adverse health effects, including on the cardiovascular system in humans. Lack
16 of agreement on its mechanism(s) of action likely stem from comparisons between *in vivo*
17 and *in vitro* test systems and potential multiple effects pathways. In rodents, *in vivo*,
18 metabolic activation of BPA produces 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene
19 (MBP), which is reported to be up to 1000 times more potent as an estrogen than BPA.
20 We investigated the estrogenic effects and estrogen receptor signaling pathway(s) of BPA
21 and MBP following early life exposure using a transgenic, estrogen responsive (ERE-
22 TG) zebrafish and a targeted morpholino approach to knockdown the three fish estrogen
23 receptor (ER) subtypes. The functional consequences of BPA exposure on the
24 cardiovascular system of zebrafish larvae were also examined. The heart atrioventricular
25 valves and the *bulbus arteriosus* were primary target tissues for both BPA and MBP in
26 the ERE-TG zebrafish, and MBP was approximately 1000-fold more potent than BPA as
27 an estrogen in these tissues. Estrogen receptor knockdown with morpholinos indicated
28 that the estrogenic responses in the heart for both BPA and MBP were mediated via an
29 estrogen receptor 1 (*esr1*) dependent pathway. At the highest BPA concentration tested
30 (2500 µg/L), alterations in the atrial:ventricular beat ratio indicated a functional impact
31 on the heart of 5 days post fertilization (dpf) larvae, and there was also a significantly
32 reduced heart rate in these larvae at 14 dpf. Our findings indicate that some of the reported
33 adverse effects on heart function associated with BPA exposure (in mammals) may act
34 through an estrogenic mechanism, but that fish are unlikely to be susceptible to adverse
35 effects on heart development for environmentally relevant exposures.

36

37 INTRODUCTION

38 Bisphenol A (BPA) was originally developed as a synthetic estrogen¹ but has
39 subsequently been used as a monomer for the production of plastics and resins. These
40 materials are used in a wide range of consumer products, including plastic drink bottles,
41 food and beverage can linings and thermal paper². BPA can leach from these materials
42 and is taken up into the human body either via dermal contact or ingestion via the gut.
43 BPA enters aquatic environments predominantly through effluent discharge from
44 wastewater treatment plants but also directly from manufacturing plants, landfill leachate,
45 and degradation of plastic litter³. Concentrations of BPA in the aquatic environment are
46 generally below 1 µg/L, but have been reported up to 21 µg/L in river water and as high
47 as 17 200 µg/L in landfill leachate⁴.

48 BPA binds to and activates estrogen receptors (ERs), mimicking the actions of the
49 endogenous estrogen 17β-estradiol. Adverse health effects attributed to BPA exposure in
50 mammals include decreases in sperm production and fertility⁵, polycystic ovarian
51 syndrome⁶, obesity and diabetes⁷ and cancer⁸. Epidemiological data indicate positive
52 correlations between BPA exposure in human populations and various risk factors
53 pertaining to cardiovascular disease⁹⁻¹² although BPA has not been proven to be the
54 causative factor. Human health concerns relating to BPA exposure have led to its ban
55 from infant feeding bottles in Europe and America^{13, 14} and its listing as a Substance of
56 Very High Concern (SVHC) under REACH in 2017¹⁵. In 2020, BPA will also be banned
57 in thermal paper in Europe¹⁶.

58 Several experimental studies have reported effects of BPA on the cardiovascular system
59 in rodents, *in vitro* cell lines and *ex vivo* hearts. Lifelong exposure to BPA (0.5 – 5
60 mg/kg/day) in mice was shown to modify cardiac structure and function with sex specific
61 effects¹⁷. In males there was concentric re-modelling, whereas in females there were

62 increases in systolic and diastolic blood pressure. Further female specific effects reported
63 include arrhythmia which was exacerbated in the presence of 17 β -estradiol^{18, 19}. This
64 arrhythmic effect was abolished when animals were treated with an ER antagonist,
65 suggesting mediation via ER signaling¹⁸. BPA has also been shown to decrease atrial
66 contraction rate and force in *ex vivo* rat hearts at exposure concentrations of 0.23 and 23
67 mg/L²⁰.

68 Studies in mammals have also shown that BPA can affect cardiac electrophysiology.
69 Acute exposure to BPA was shown to decrease ventricular conduction velocity and
70 increase action potential duration in female hearts²¹, however, the underlying mechanism
71 for these effects was not explored.

72 The molecular mechanisms for BPA effects in the heart in general are not well
73 established. BPA has been demonstrated to act through a variety of different mechanisms
74 including via the nuclear estrogen receptors ER α and ER β (three isoforms exist in fish
75 *esr1* (ER α), *esr2a* (ER β 2) and *esr2b* (ER β 1))^{22, 23} and the orphan receptor Estrogen
76 Related Receptor γ (ERR γ). BPA activates the ERR γ receptor more effectively than 17 β -
77 estradiol²⁴.

78 Estrogen receptors occur in a wide range of body tissues but the abundance of different
79 isoforms varies, which can affect the cell sensitivity to a particular ligand²⁵. In
80 mammalian models, moderate to high-level expression of ER α occurs in the uterus, testis,
81 pituitary, ovary, kidney and epididymis, while high expression for ER β occurs in prostate,
82 ovary, lung, bladder, brain, uterus and testis²⁶. Both ER subtypes occur in the rodent heart
83 and in human cardiomyocytes^{25, 27}. In fish ER subtype localisation in body tissues also
84 varies. In the fathead minnow (*Pimephales promelas*) every ER isoform is expressed in
85 the brain, pituitary, liver, gonad, intestine, and gill. In the liver *esr1* and *esr2b* are
86 predominantly expressed, while *esr2a* is expressed principally in the intestine²⁸. Zebrafish

87 embryos at 5 days post-fertilisation (dpf) have been shown to express *esr1* transcripts in
88 the heart while *esr2a* (reported as *esr2b*) transcripts appear to be expressed in the liver²⁹.
89 To add to this complexity, expression of the ER subtypes can also vary during ontogeny
90 in fish³⁰.

91 Findings for responses to BPA *in vivo* are not always supported by *in vitro* studies. A
92 possible explanation for this in mammals is that metabolic activation of BPA *in vivo*
93 produces 4-methyl-2,4-bis(*p*-hydroxyphenyl)pent-1-ene (MBP) which is a more potent
94 estrogen than BPA³¹. Metabolic activation may also help to explain non-monotonic
95 responses that are sometimes observed following BPA exposure³². Incubation of BPA
96 with S9 liver fractions from mouse, monkey or humans all produce metabolites more
97 potent as estrogens than the parent BPA compound which are also thought to be MBP³¹.
98 In rats and medaka (*Oryzias latipes*) estrogenic effects of MBP *in vivo* are between 400 -
99 1000 times greater than for BPA³³⁻³⁶. Three-dimensional modelling of human ERs
100 together with binding strength assessments for BPA, MBP and 17 β -estradiol suggest that
101 structural differences account for the increased effectiveness of MBP over BPA³⁷. Despite
102 these findings MBP has not yet been measured as a natural *in vivo* metabolite in any
103 environmental samples. There is little information on the metabolism of BPA to MBP or
104 otherwise *in vivo* in fish, but one report indicates an inability to produce the conjugated
105 metabolite bisphenol A glucuronic acid (BPA-GA) in juvenile trout³⁸.

106 In this study we investigated the estrogenic responses and estrogen receptor signaling
107 mechanisms by which BPA and its metabolite in mammals MBP act in an estrogenic
108 responsive transgenic zebrafish. Estrogen Response Element (ERE)-TG zebrafish were
109 exposed to estrogenic chemicals in combination with injected morpholino antisense
110 oligomers i specific to three individual ER subtypes to knockdown function. Responses
111 to the potent steroidal estrogen 17 α -ethinylestradiol (EE2) in the ERE-TG zebrafish were

112 used as a positive control for estrogenic activation. We further assessed whether effects
113 on heart function in juvenile fish may result from early life developmental exposure to
114 BPA.
115

116 **MATERIALS AND METHODS**

117 **Fish source, culture and husbandry.** All zebrafish embryos used in this study were
118 obtained from Tg(ERE:Gal4ff)(UAS:GFP) transgenic zebrafish adults³⁹. Full details of
119 fish husbandry can be found in the Supporting Information.

120 **Chemical preparations.** Bisphenol A (purity >99%) and 17 α -Ethinylestradiol (\geq 98%)
121 (EE2) were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK). 4-methyl-
122 2,4-bis(4-hydroxyphenol)pent-1-ene (MBP) was synthesized at the University of Exeter
123 as described by Cipelli *et al*⁴⁰. Stock chemicals were prepared as described previously by
124 Moreman *et al*⁴¹.

125
126 **Tissue responses to chemical treatments.** All exposures were conducted in a
127 temperature controlled laboratory held at 28 ± 1 °C, in glass vessels with daily renewal
128 of the media. To determine tissue differences in response to BPA, MBP and EE2, embryos
129 were exposed to these chemicals at a series of different concentrations or to solvent
130 control. BPA and MBP have similar molecular weights (228.3 g and 268.4 g respectively)
131 and effects comparisons are made on a w/v basis: 100 μ g/L = approx. 0.4 μ M for both
132 materials). Concentration ranges based on preliminary investigations for EE2 were 2.0
133 and 20 ng/L, for BPA 10, 100 and 1000 μ g/L, and for MBP 0.025, 0.25 and 2.5 μ g/L.
134 Each experimental group consisted of 20 embryos exposed in 100 mL of ISO water and
135 each treatment group was run in triplicate. The exposures were conducted from 0 hpf to
136 120 hpf. At the end of the exposure period, 120 hpf old larvae were processed for
137 fluorescent imaging analysis.

138 To investigate responses to BPA and MBP specifically in the heart of developing
139 zebrafish a similar exposure regime as described above was used but across a narrower
140 chemical exposure range. To better visualise the location of GFP expression in the heart,

141 larvae were stained with MF-20 monoclonal antibody, which stains cardiac myosin, and
142 hearts were then excised from stained larvae for imaging. Full details of the
143 immunostaining technique can be found in the Supporting Information.

144 **Morpholino analysis and injection.** Morpholino (MO) design is described in the
145 Supporting Information. Using a microinjector (INTRACEL, PICOSPRITZER®III) 2nL
146 of 0.25 mM MO solution was injected into the yolk proximal to the blastomeres of the
147 embryo at the one- to four-cell stage, control embryos were injected with 2 nL of solution
148 containing no MO. Fluorescence imaging of MO injected and chemically exposed larvae
149 was conducted at 72 hpf as pilot studies demonstrated that for a combination of all three
150 esr morpholinos the maximum inhibitory effect occurred up to 72 hpf.

151 **Image analysis of Tg(ERE:Gal4ff)(UAS:GFP) zebrafish.** All larval images were
152 obtained using a Zeiss Axio Observer.Z1 equipped with an AxioCam Mrm camera (Zeiss,
153 Cambridge, UK) as described in the Supporting Information.

154 Confocal microscopy was used on stained dissected hearts. The hearts were mounted
155 in 0.7 % agarose (low melting point). Hearts were photographed under X20 magnification
156 on a confocal-laser scanning microscope (ZEISS LSM510). Images were reconstituted
157 using LSM510 Meta.

158 **Cardiovascular functional analysis.** To assess for effects on heart function and blood
159 flow, non-invasive video analysis of the heart and dorsal aorta was used to measure
160 multiple cardiovascular endpoints; including beat rate, atrial:ventricular beat ratio, blood
161 flow/velocity, stroke volume and blood vessel diameter⁴². Studies were conducted on fish
162 at 5 dpf and 14 dpf following exposures to both 1000 µg/L BPA, and 2500 µg/L BPA.

163 Following chemical exposure, two cameras captured separate videos of larvae to
164 measure the cardiovascular endpoints. One camera (Grasshopper® GRAS-50S5C-C,
165 Point Grey, Richmond, Canada) was positioned to capture the whole heart at 30 frames

166 per second (fps) and the second (Grasshopper® GRAS-03K2M-C, Point Grey,
167 Richmond, Canada) to capture the dorsal aorta, caudal to the swim bladder, at 120 fps.
168 Videos were analysed using MicroZebraLab™ (v3.5, ViewPoint, Lyon, France) or
169 ZebraBlood™ (v1.3.2, ViewPoint, Lyon, France) software. Full details of cardiovascular
170 functional analysis are provided in the Supporting Information.

171 **Measured chemical exposure concentrations.** For water samples measured
172 concentrations were determined for 1000 µg/L BPA and 0.25, 2.5 and 25 µg/L MBP using
173 LC-MS/MS analysis. . For uptake analysis embryo-larvae were exposed to 1000 µg/L
174 BPA and 0.25, 2.5 and 25 µg/L MBP. See Supporting Information for full details on
175 chemical analysis and larval preparation.

176 **Data analysis.** Fluorescence data are expressed as mean fold induction above the
177 solvent control, ± standard error of the mean (SEM). Data quoted in the text, or shown
178 graphically, for cardiac function parameters are presented as the mean ± SEM over the 5
179 minute observation period. Data for A:V beat ratio at 5 dpf were separated into 0.5 minute
180 time segments to clearly visualise observed fluctuations in A:V ratio in exposure groups.
181 Significant differences between groups were analysed using a one-way ANOVA, and a
182 Tukey posthoc test was carried out where significant differences were detected ($p < 0.05$).
183 A Kolmogorov-Smirnov test was used to compare the distribution curves between
184 treatments for A:V beat rate data and differences were reported as significant at $p < 0.05$.
185 Statistical analyses of data were performed in IBM SPSS Statistics 22.

186

187 **RESULTS**

188 **Uptake and metabolism of BPA in exposed embryos.** Accurate dosing of test
189 chemicals was confirmed at the start of the exposures (Supporting Information Table S2).
190 At a concentration of 0.25 µg/L, MBP was below the limit of quantification (LOQ) of the
191 analytical method (0.4 µg/L). Larvae exposed to BPA at a concentration of 1000 µg/L
192 resulted in an internal BPA load of 2.3 ± 0.2 ng/larvae (Table S2), equating to an
193 estimated bioconcentration factor (BCF) of 2.3 (assuming a larval volume of 1 µL). MBP
194 was not detected in 5 dpf zebrafish embryos exposed to BPA. Similarly, MBP was not
195 detected in larvae exposed to 0.25 µg/L MBP and 2.5 µg/L MBP with LC-MS analysis.
196 MBP was, however, detected in larvae exposed to the highest MBP concentration (25
197 µg/L) with an internal MBP concentration of 0.66 ± 0.16 ng/ larvae equating to a BCF of
198 26.4.

199 **Tissue expression of GFP in response to BPA, MBP and EE2 in ERE-TG zebrafish**
200 **larvae.** Some ERE-TG zebrafish had detectable GFP expression in the otic vesicle in the
201 absence of chemical treatment (Figure 1A). This fluorescence was not inducible by the
202 chemicals tested and had a similar level of occurrence across all treatments. No GFP
203 fluorescence was detected in other tissues of control larvae.

204 For exposure to EE2 the most responsive tissue was the liver; an exposure concentration
205 of 2 ng/L resulting in an 8.6 ± 2.1 - fold increase in GFP fluorescence above controls. At
206 20 ng µg/L EE2, increased fluorescence was detected in the heart (9.6 ± 0.9 -fold
207 increase), liver (128 ± 16 -fold increase) and muscle somites in tail region (13.2 ± 0.6 -fold
208 increase). Responses to EE2 were much more pronounced in liver and muscle somites
209 compared with in the heart.

210 Responses to the phenolic compounds differed compared with that for EE2. No
211 induction of GFP was detected in ERE-TG zebrafish exposed to 10 µg/L BPA (Figure 1),

212 but there was a significant increase in GFP fluorescence in the heart at 100 $\mu\text{g/L}$ BPA
213 (3.2 ± 0.7 -fold above control) (Figure 1B), but not in other tissues (Figure 1C and D). In
214 larvae exposed to 1000 $\mu\text{g/L}$ BPA a GFP fluorescence signal was detected in the heart,
215 liver and muscles somites in the tail region (Figure 1B - D), with fold increases above
216 control of 25.7 ± 2.0 , 55.6 ± 5.5 and 10.4 ± 1.8 , respectively. Fluorescence in the tail
217 region was associated with individual muscle myotubes, expressed in a mosaic pattern,
218 and in the corpuscles of Stannius (Figure 1A).

219 Exposure to MBP resulted in a very similar pattern of fluorescence expression to that
220 for BPA, but MBP produced a stronger response. A response in the heart was detected at
221 an MBP exposure concentration of 0.25 $\mu\text{g/L}$ (3.5 ± 0.6 -fold increase above control)
222 increasing to 32.6 ± 1.6 -fold above controls at 2.5 $\mu\text{g/L}$. At 2.5 $\mu\text{g/L}$ MBP fluorescence
223 was also detected in the liver and tail regions at 78.4 ± 13.1 and 12.1 ± 0.9 -fold above the
224 controls, respectively.

225 **Heart valves responses to BPA and MBP.** Examples of hearts from larval zebrafish
226 exposed to BPA and MBP and stained with MF-20 (to identify myocardium tissue) for
227 microscopy analysis are shown in Figure 2. The main GFP expression domains within the
228 heart were the atrioventricular valve and the *bulbus arteriosus*, structural features that are
229 essential for regulating blood flow in fish. At the higher exposure concentrations used
230 (1000 $\mu\text{g/L}$ BPA and 2.5 $\mu\text{g/L}$ MBP) GFP fluorescence was seen to extend from the
231 atrioventricular valve throughout the ventricular tissue. There was no evidence that the
232 overall morphology of valves or *bulbus arteriosus* were affected by the chemical
233 treatments.

234 Concentration dependent responses for GFP fluorescence induction in the heart were
235 observed for both BPA and MBP. Equivalent fold-increases in fluorescence above control
236 for BPA and MBP occurred at 1000 $\mu\text{g/L}$ (23.6 ± 2.6 fold) and 1 $\mu\text{g/L}$ (23.4 ± 3.5 fold)

237 respectively, indicating MBP (based on GFP fluorescence intensity) had a relative
238 potency 1000 times greater than BPA for responses in the heart valves.

239 **Estrogen Receptor signaling pathways for BPA and MBP in ERE-TG zebrafish.**

240 To determine which ERs were responsible for the observed tissue specific responses,
241 ERE-TG zebrafish larvae were injected with antisense morpholinos for the different ER
242 subtypes and subsequently treated with BPA, MBP or EE2.

243 Negative-morpholino control fish exposed to BPA (1000 µg/L), MBP (2.5 µg/L) and
244 EE2 (20 ng/L) showed significant increases in GFP fluorescence intensity in the heart,
245 liver, anterior trunk and tail regions (Figure 3). Injection with the morpholino for *esr1*,
246 resulted in a suppression of GFP signal in the heart, liver and somite muscles in the trunk
247 region of the body (Figure 3B-D). GFP fluorescence intensity in *esr1*-MO injected fish
248 was reduced for all chemical exposures in the heart and liver to levels that did not differ
249 from controls (i.e. complete suppression of the GFP) (Figure 3B, 3C). GFP fluorescence
250 intensity in *esr1*-MO injected embryo was reduced in the trunk region in fish exposed to
251 BPA. Although reduced compared to the negative morpholino control, the *esr1* morphant
252 fish exposed to MBP and EE2 still demonstrated significantly higher fluorescence than
253 unexposed fish (Figure 3D).

254 In contrast, zebrafish injected with morpholinos for *esr2a* and *esr2b* continued to exhibit
255 increased GFP fluorescence in the heart when exposed to the estrogenic chemicals. There
256 was no significant increase observed in GFP fluorescence for BPA, MBP and EE2 *esr2a*
257 & *esr2b* exposed morphants in the liver, trunk, and tail regions when compared to the
258 controls (Figure 3 C-E). This indicates that a combined knockdown of *esr2a* and *esr2b*
259 may prevent estrogen signaling in these tissues.

260 **Effects of BPA on heart function in 5 and 14 dpf zebrafish.** For 5 dpf fish GFP
261 fluorescence intensity in the heart was 8.9 ± 1.2 -fold above controls for 1000 µg/L BPA,

262 and 10.8 ± 1.4 -fold above controls for 2500 $\mu\text{g/L}$ BPA; and for the 14 dpf fish, $50.7 \pm$
263 11.5 -fold above controls for 1000 $\mu\text{g/L}$ BPA and 44.7 ± 7.7 -fold above controls for 2500
264 $\mu\text{g/L}$ BPA (Figure 4).

265 At 5 dpf, there were no significant effects for either of the two BPA exposure
266 concentrations on heart beat rate, dorsal aorta diameter, the speed or volume of blood
267 flow or the surrogate stroke volume. No significant differences were found between the
268 means of the atrium to ventricle (A:V) beat ratio values (data not shown). Comparing the
269 distribution pattern around the means (using a Kolmogorov-Smirnov analysis) showed
270 that in the 2500 $\mu\text{g/L}$ BPA treatment group there was a significantly different pattern in
271 the frequency of the heart beat from the control and 1000 $\mu\text{g/L}$ BPA treatment group.
272 When the A:V beat ratio measurements were divided into 30 second time bins
273 representing means \pm SEMs (Figure 4E) for those time intervals, the variation in beat
274 ratio was be greater for the higher BPA treatment compared with controls, ($p < 0.05$,
275 Kolmogorov-Smirnov test), varying more across the 30 second time intervals indicating
276 a more erratic A:V beat ratio for those fish.

277 Pigment formation at 14 days precluded accurate detection of atrial and ventricular beat
278 rates due to an inability to clearly visualise the heart. Heart beat rate, however, was
279 estimated from the pulse rate of the dorsal aorta. There was no effect of BPA exposure
280 on volume or speed of blood flow, dorsal aorta diameter, or surrogate stroke volume.
281 However, there was a significant reduction in the arterial beat rate of fish exposed to 2500
282 $\mu\text{g/L}$ BPA (184 ± 3.5 bpm compared with 201 ± 4.6 bpm in controls, $p < 0.05$, Figure
283 4D).

284

285 **DISCUSSION**

286 Estrogens have widespread effects including in the heart. Estrogen effects on
287 circulatory function, mediated through genomic and non-genomic mechanisms, are well
288 documented⁴³, leading to suggestions that BPA and other xenoestrogens may exert
289 adverse effects on the cardiovascular system. Several epidemiological studies have
290 reported that BPA exposure in humans is associated with an increased occurrence of
291 cardiovascular disease⁹⁻¹², however, there is little direct experimental evidence to support
292 this, and the mechanisms by which this may occur are not known. The zebrafish heart
293 differs structurally in some aspects from the mammalian heart but the molecular
294 mechanisms underlying cardiac development and function are highly conserved between
295 mammals and fish, and with phenotypic similarities between cardiac mutants in fish and
296 human diseases also, the zebrafish model has become widely used in cardiac research⁴⁴.
297 Here we used an ERE-TG zebrafish, in combination with morpholino knockdown of ER
298 subtypes, to identify potential signaling mechanisms for BPA and its metabolite MBP in
299 the heart and across other body tissues, and assessed for effects of early life exposure to
300 BPA on subsequent heart function.

301 Estrogenic responses to BPA occurred in the heart, liver, muscle somites and corpuscles
302 of Stannius, as identified previously in different lines of estrogen responsive transgenic
303 zebrafish^{29, 39}. In the heart, the atrioventricular valves and *bulbus arteriosus* were most
304 responsive to BPA exposure (Figure 2). MBP showed similar spatial patterns of ER
305 activation, as indicated by increased GFP-fluorescence in the ERE-TG zebrafish,
306 including for the heart valves and *bulbus arteriosus*, but with apparent higher potency.
307 Across the different body tissues, MBP was between 400 - 1000 times more potent than
308 BPA, which is consistent with findings reported for relative estrogenic potencies in
309 mammals *in vivo*³³⁻³⁶. For the heart, the threshold concentration for induction of

310 measurable GFP fluorescence was 100 $\mu\text{g/L}$ for BPA, compared with 0.25 $\mu\text{g/L}$ for MBP.
311 It is possible that some effects for BPA in the ERE-TG zebrafish derived from its *in vivo*
312 metabolism to MBP, however we did not detect MBP in 5 dpf zebrafish exposed to BPA,
313 even at 1000 $\mu\text{g/L}$. This may indicate that zebrafish larvae do not possess the effective
314 liver function to metabolise BPA to MBP, or that the level of MBP in these larvae was
315 below the detection limit for our analysis method (0.4 $\mu\text{g/L}$). MBP was detectable in
316 larvae on exposure to the highest concentration of MBP tested (25 $\mu\text{g/L}$).

317 Induction of GFP by both BPA and MBP was inhibited in the hearts of ERE-TG larvae
318 injected with morpholinos against *esr1* (Figure 3). In liver, morpholinos against *esr1* and
319 against *esr2a* and *esr2b* together inhibited GFP expression, whereas in the somite
320 myotubes (most notably in the tail region) the combined *esr2a* and *esr2b* morpholinos
321 were effective, but the *esr1* morpholino was not. These data indicate that different ER
322 response pathways occur for the bisphenols within different body tissues. Studies on a
323 zebrafish liver cell line, transfected with different ERs found that BPA most strongly
324 activated ER α (*esr1*), with some activation of ER β 2 (*esr2a*) and no detectable effect on
325 ER β 1 (*esr2b*)⁴⁵. It is possible that the preferences indicated for different ERs could lead
326 to different chemicals targeting specific tissues, resulting in the differing response
327 patterns observed. Studies *in vitro* in mammalian-based systems including human ER α
328 and rat ER β protein synthesized in reticulocyte lysates⁴¹ and rat liver S9 liver fractions,
329 indicate that BPA preferentially binds to and activates ER β but this has not been
330 confirmed *in vivo*.

331 Whole mount *in situ* hybridisation conducted in 5 dpf zebrafish has shown the presence
332 of *esr1* transcripts in the atrioventricular valves and *bulbus arteriosus* and *esr2a* (reported
333 as *esr2b*) transcripts in the liver²⁹. In that study, *esr2b* (reported as *esr2a*) transcripts were
334 not detected²⁹. This suggests that *esr1* plays an important functional role in transducing

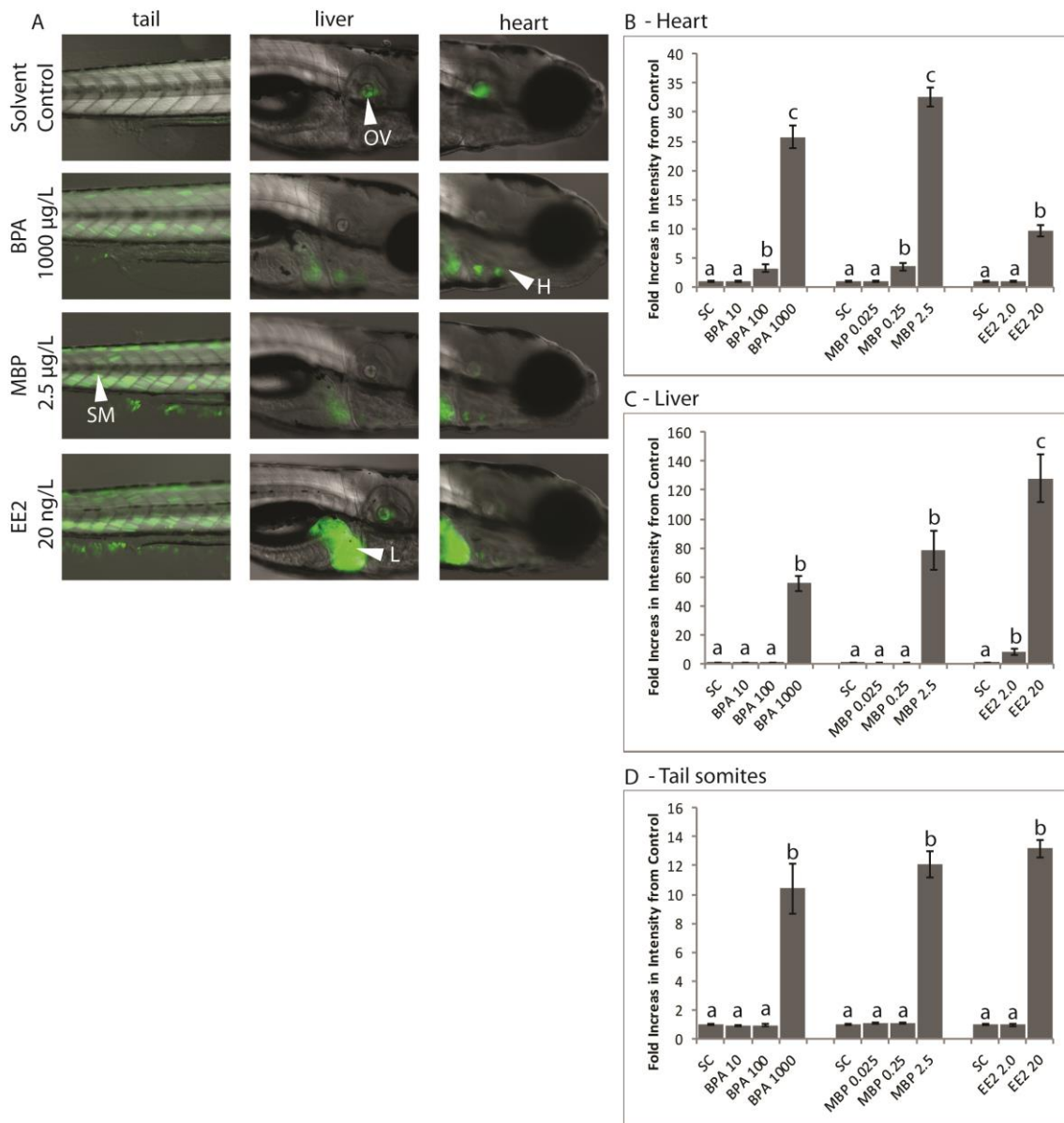
335 the estrogenic signal in the cardiac tissue during early life. This may explain why
336 estrogenic chemicals, such as BPA, with a higher affinity for *esr1*, show activation
337 responses in the heart at concentrations lower than those seen in other tissues. In our study
338 the GFP signal in the liver was knocked down using the *esr1* morpholino (as well as the
339 morpholinos for *esr2a* and *esr2b*) indicating hepatic expression of *esr1* transcripts, which
340 contrasts with studies reported for another transgenic zebrafish line for the same life
341 stage²⁹. It is well established, however, that *esr1* is expressed in the liver in many fish
342 species (including the zebrafish) at later life stages, where it plays a role in regulating the
343 expression of estrogen responsive genes, including signaling pathways for induction of
344 vitellogenin⁴⁶. Cross-talk between the *esr* sub-types is likely to occur in the liver (and
345 other body tissues) and there may be involvement of other factors in estrogen signaling,
346 that lead to a reduced GFP signal when either receptor subtype is knocked down/out.

347 Both BPA and MBP targeted the atrioventricular valves and *bulbus arteriosus*, tissues
348 responsible for regulating blood flow from early life. The valves of the atrioventricular
349 canal are crucial for maintaining unidirectional blood flow in the heart, completely
350 blocking retrograde blood flow by 72 hpf⁴⁷ with the valve leaflets developed by 102 hpf⁴⁸.
351 The *bulbus arteriosus* functions as a type of capacitor to maintain a continuous blood
352 flow. The first five days of life are crucial for correct valve development in zebrafish with
353 the valves forming from migrating endocardial cells at this time⁴⁹. In humans, it has been
354 estimated that 5% of children may carry some form of congenital valve deformation, and
355 10 - 20% of all congenital heart disease is caused by defects in the atrioventricular canal⁵⁰,
356 ⁵¹. This emphasises the need to establish whether chemicals, such as BPA, known to act
357 on these tissues during development, effect valve function contributing to congenital heart
358 disease, and to establish the mechanisms for any such effects.

359 Non-invasive video analysis has previously been used in the assessment of integrated
360 cardiovascular function following drug treatment in zebrafish⁴². Endocardial cushion
361 (precursors to functioning valves) development is thought to be complete around 5 dpf
362 while valve elongation is thought to occur from around 6 dpf, completing at around 14
363 dpf⁴⁸. If BPA significantly impacts heart valve function in zebrafish larvae, effects should
364 therefore be detectable at this time. At 5 dpf the only significant effect measured was the
365 difference in A:V beat ratio. This ratio was relatively conserved at 1:1 in control larvae
366 with little variation observed throughout the 5 minute observation window. In fish
367 exposed to 2500 µg/L BPA, A:V beat ratio was more variable over the assessment period
368 (Figure 4E), indicating that although there was no uniform decoupling of atrial and
369 ventricular beat rates, BPA exposure at high concentrations can cause erratic beat in
370 either, or both, heart chambers. It is unlikely that A:V beat ratio would be linked to a
371 disruption of the heart valves, however in dissected hearts fluorescent cells extending into
372 the ventricle were observed (Figure 2A), possibly indicating BPA action, but more
373 detailed and higher resolution microscope analyses would be required to develop a better
374 understanding on this possibility. Previous studies have indicated that BPA may promote
375 arrhythmia in mammalian hearts by alteration of myocyte Ca²⁺ handling, mediated by
376 ERβ-signaling^{18, 19, 52}. Estrogen-responsive fluorescent signaling in our ERE-TG fish
377 appears to be mediated by an *esr1*-dependent pathway but at the higher concentration of
378 2500 µg/L BPA further pathways may also be induced. The only significant effect
379 observed at 14 dpf was a reduction in heart rate (as indicated using arterial pulse data) in
380 zebrafish exposed to 2500 µg/L BPA although the mechanism for this was not
381 determined. BPA, however, has been shown to affect thyroid signaling⁵³ and reduced
382 circulating thyroid hormones can lead to a reduction in heart rate in humans⁵⁴.

383 In conclusion, we demonstrate that the estrogenic BPA metabolite MBP targets the
384 same tissues as its parent compound, BPA, and distinct from that of the pharmaceutical
385 steroidal estrogen EE2. In common with data from mammals, the potency of MBP in the
386 zebrafish is several orders of magnitude higher than for BPA. We demonstrate that
387 developing atrioventricular valves and *bulbus arteriosus* are key targets in the heart for
388 both BPA and MBP and the estrogenic signal transduction for both bisphenols are
389 mediated via an *esr1* dependent pathway. Although we show the potential for ER-
390 mediated mechanisms to account for the reported cardiovascular effects in humans for
391 these bisphenols, for fish functional effect concentrations for early life stage exposure far
392 exceed those for most environments by several orders of magnitude. From these data it
393 seems unlikely that chronic exposure to BPA and MBP in natural environment will have
394 functional consequences for the heart in fish.

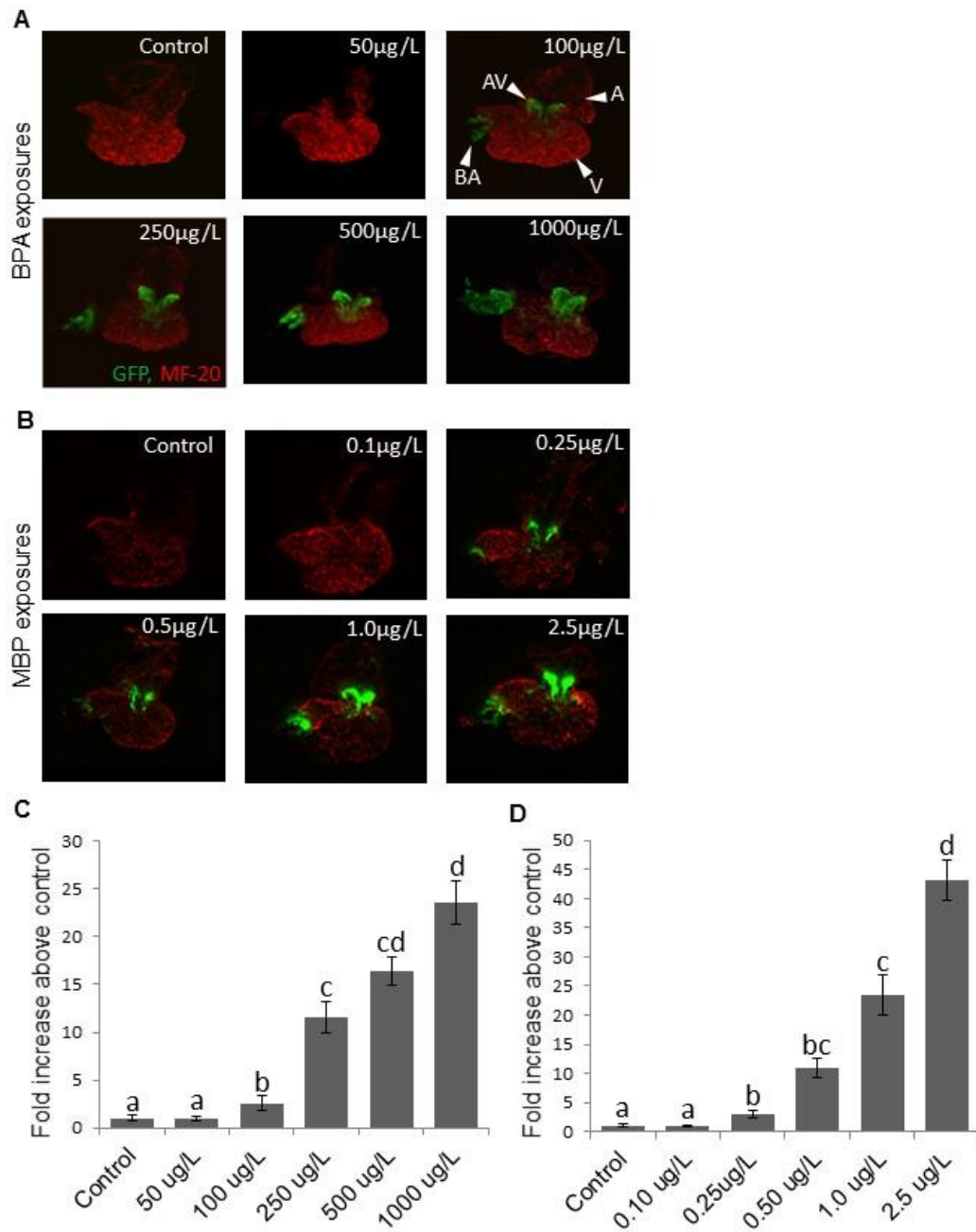
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397 **Figure 1.** Tissue responses in ERE-TG zebrafish larvae exposed to BPA and MBP. (A)
 398 Representative images of 120 hpf ERE-TG zebrafish larvae for controls and exposures to
 399 1000 µg/L BPA, 2.5 µg/L MBP and 20 ng µg/L EE2 (images for lower exposure
 400 concentrations are not shown; OV=otic vesicle, H=heart, SM=somite muscle, L=liver).
 401 (B-D) GFP induction in 120 hpf ERE-TG zebrafish larvae exposed to BPA and MBP
 402 (concentrations indicated in µg/L) and EE2 (concentrations in ng/L). Data presented as
 403 fold-increase above controls for the (B) heart, (C) liver, (D) tail somites. Letters represent
 404 significant differences ($p < 0.05$) between the chemical treatment and control groups.

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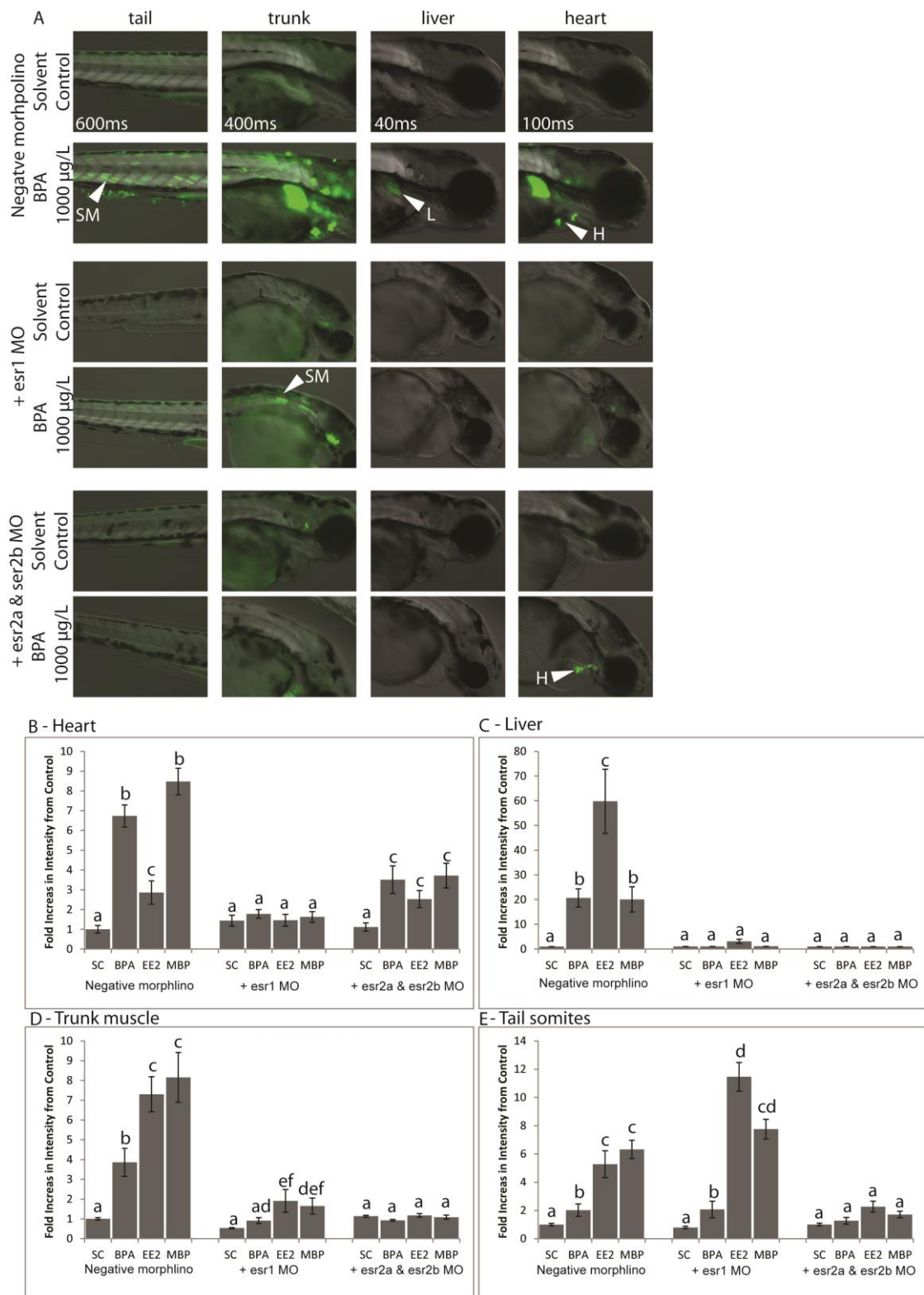


406

407 **Figure 2.** Composite z stacks of hearts dissected from 120 hfp ERE-TG zebrafish larvae
 408 exposed to a gradient of concentrations of (A) BPA and (B) MBP (A=atrium,

409 V=ventricle, AV=atrioventricular valve, BA=*bulbus arteriosus*. Hearts were
410 immunostained with MF-20 to identify cardiac tissue. GFP induction (fluorescence
411 intensity) was measured and quantified using fluorescence microscopy and image
412 analysis, (C) BPA and (D) MBP exposures Letters represent significant differences ($p <$
413 0.05) between the chemically dosed and control groups.

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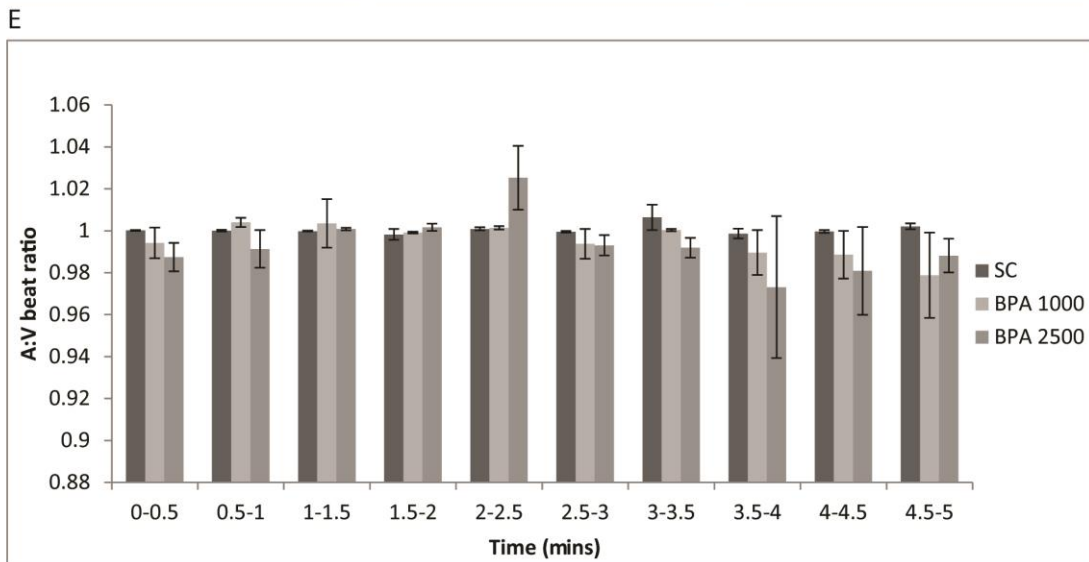
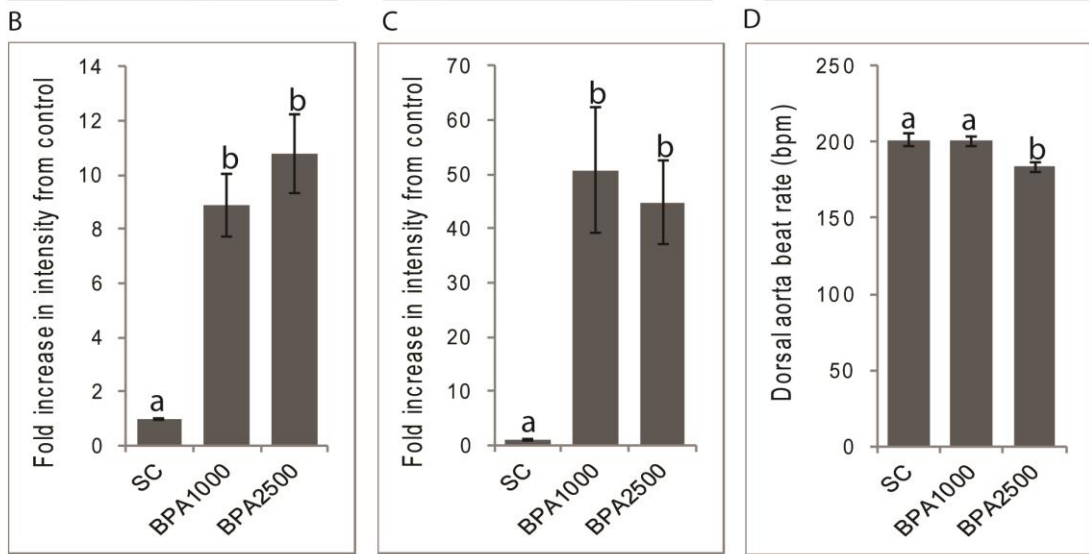
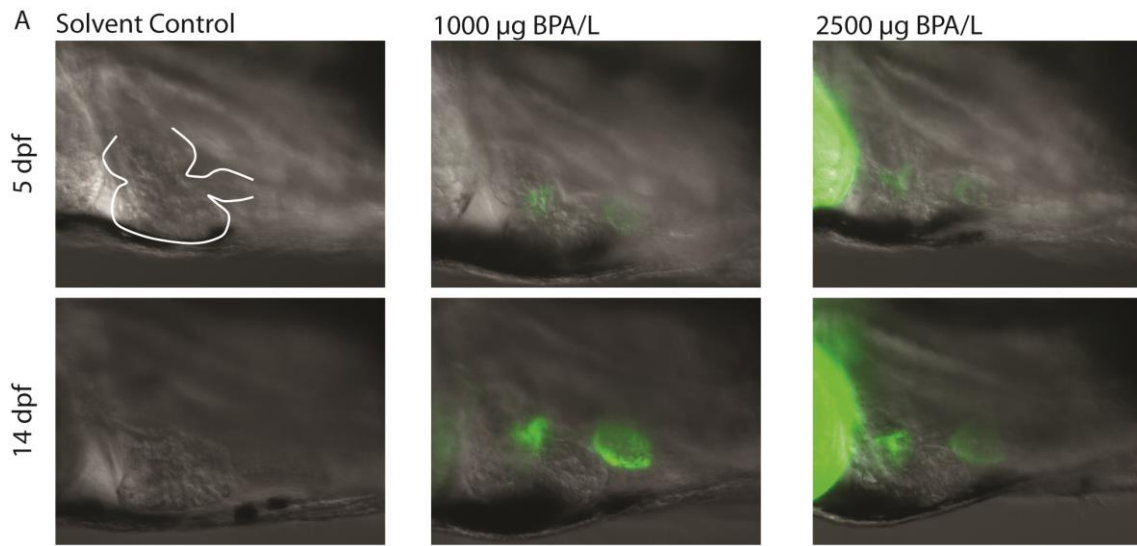
417 **Figure 3.** Effects of ER morpholino knockdowns on ERE-TG zebrafish larvae exposed

418 to BPA and MBP. (A) Images of ERE-TG zebrafish larvae at 72 hpf for controls and

419 larvae exposed to 1000 µg/L BPA, 2.5 µg/L MBP and 20 ng µg/L EE2 injected with
420 morpholinos against *esr1*, or *esr2a* & *esr2b* at 1-4 cell stage to knockdown the specific
421 ERs (H=heart, L=liver, SM=somite muscle; numerical values represent differing
422 exposure times across different regions due to present levels of fluorescence). (B-E)
423 Fluorescence response in 72 hpf ERE-TG zebrafish larvae exposed to BPA, MBP and
424 EE2 in combination with the injected morpholinos against *esr1* or *esr2a* & *esr2b*
425 expressed as fold increase above controls for target tissues; (B) heart, (C) liver, (D)
426 muscle somites in the trunk region, and (E) muscle somites in the tail region. Letters
427 represent significant differences ($p < 0.05$) between the chemically dosed and control
428 groups.

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433 **Figure 4.** Images of hearts in ERE-TG zebrafish exposed to 1000 and 2500 $\mu\text{g/L}$ BPA at
434 5 dpf and 14 dpf. (A) Heart outline shown on 5 dpf control. GFP induction in hearts of
435 ERE-TG zebrafish used for analysis of cardiac function at (B) 5 dpf and (C) 14 dpf
436 measured as fold- increase above controls. (D) Estimated heart rate based on arterial
437 blood flow at 14 dpf. Letters represent significant differences ($p < 0.05$) between the
438 chemically dosed and control groups. (E) Atrial to ventricular beat ratio in 5dpf zebrafish
439 data split in 30s time segments and shown as means \pm SEMs.

440

441 **ASSOCIATED CONTENT**

442 **Supporting Information.**

443 Protocols for fish culture and husbandry; LC-MS method used for chemical detection of
444 bisphenolic chemicals in exposure medium and fraction taken up in 120 hour post-
445 fertilisation (hpf) zebrafish larvae; morpholino (MO) design; image analysis of
446 Tg(ERE:Gal4ff)(UAS:GFP) zebrafish and immunostaining techniques for heart
447 visualization and methods for cardiovascular function capture and analysis. Table S1
448 describes transitions used to detect chemicals in LC-MS methods, Table S2 includes the
449 measurements of these chemicals in exposure medium and larvae.

450

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454

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