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

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

INTRODUCTION

Bisphenol A (BPA) was originally developed as a synthetic estrogen[1] but has subsequently been used as a monomer for the production of plastics and resins. These materials are used in a wide range of consumer products, including plastic drink bottles, food and beverage can linings and thermal paper[2]. BPA can leach from these materials and is taken up into the human body either via dermal contact or ingestion via the gut. BPA enters aquatic environments predominantly through effluent discharge from wastewater treatment plants but also directly from manufacturing plants, landfill leachate, and degradation of plastic litter[3]. Concentrations of BPA in the aquatic environment are generally below 1 µg/L, but have been reported up to 21 µg/L in river water and as high as 17 200 µg/L in landfill leachate[4].

BPA binds to and activates estrogen receptors (ERs), mimicking the actions of the endogenous estrogen 17β-estradiol. Adverse health effects attributed to BPA exposure in mammals include decreases in sperm production and fertility[5], polycystic ovarian syndrome[6], obesity and diabetes[7] and cancer[8]. Epidemiological data indicate positive correlations between BPA exposure in human populations and various risk factors pertaining to cardiovascular disease[9-12] although BPA has not been proven to be the causative factor. Human health concerns relating to BPA exposure have led to its ban from infant feeding bottles in Europe and America[13, 14] and its listing as a Substance of Very High Concern (SVHC) under REACH in 2017[15]. In 2020, BPA will also be banned in thermal paper in Europe[16].

Several experimental studies have reported effects of BPA on the cardiovascular system in rodents, *in vitro* cell lines and *ex vivo* hearts. Lifelong exposure to BPA (0.5 – 5 mg/kg/day) in mice was shown to modify cardiac structure and function with sex specific effects[17]. In males there was concentric re-modelling, whereas in females there were

increases in systolic and diastolic blood pressure. Further female specific effects reported include arrhythmia which was exacerbated in the presence of 17β-estradiol[18, 19]. This arrhythmic effect was abolished when animals were treated with an ER antagonist, suggesting mediation via ER signaling[18]. BPA has also been shown to decrease atrial contraction rate and force in *ex vivo* rat hearts at exposure concentrations of 0.23 and 23 mg/L[20].

Studies in mammals have also shown that BPA can affect cardiac electrophysiology. Acute exposure to BPA was shown to decrease ventricular conduction velocity and increase action potential duration in female hearts[21], however, the underlying mechanism for these effects was not explored.

The molecular mechanisms for BPA effects in the heart in general are not well established. BPA has been demonstrated to act through a variety of different mechanisms including via the nuclear estrogen receptors ERα and ERβ (three isoforms exist in fish esr1 (ERα), esr2a (ERβ2) and esr2b (ERβ1))[22, 23] and the orphan receptor Estrogen Related Receptor γ (ERRγ). BPA activates the ERRγ receptor more effectively than 17β-estradiol[24].

Estrogen receptors occur in a wide range of body tissues but the abundance of different isoforms varies, which can affect the cell sensitivity to a particular ligand[25]. In mammalian models, moderate to high-level expression of ERα occurs in the uterus, testis, pituitary, ovary, kidney and epididymis, while high expression for ERβ occurs in prostate, ovary, lung, bladder, brain, uterus and testis[26]. Both ER subtypes occur in the rodent heart and in human cardiomyocytes[25, 27]. In fish ER subtype localisation in body tissues also varies. In the fathead minnow (*Pimephales promelas*) every ER isoform is expressed in the brain, pituitary, liver, gonad, intestine, and gill. In the liver esr1 and esr2b are predominantly expressed, while esr2a is expressed principally in the intestine[28]. 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 liver[29].
89 To add to this complexity, expression of the ER subtypes can also vary during ontogeny
90 in fish[30].

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 BPA[31]. Metabolic activation may also help to explain non-monotonic
95 responses that are sometimes observed following BPA exposure[32]. 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 MBP[31].
98 In rats and medaka (*Oryzias latipes*) estrogenic effects of MBP *in vivo* are between 400 -
99 1000 times greater than for BPA[33-36]. Three-dimensional modelling of human ERs
100 together with binding strength assessments for BPA, MBP and 17β-estradiol suggest that
101 structural differences account for the increased effectiveness of MBP over BPA[37]. 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 trout[38].

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 17α-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

MATERIALS AND METHODS

Fish source, culture and husbandry. All zebrafish embryos used in this study were obtained from Tg(ERE:Gal4ff)(UAS:GFP) transgenic zebrafish adults[39]. Full details of fish husbandry can be found in the Supporting Information.

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

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

To investigate responses to BPA and MBP specifically in the heart of developing zebrafish a similar exposure regime as described above was used but across a narrower 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 diameter[42]. 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

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

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

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

RESULTS

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

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

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

Responses to the phenolic compounds differed compared with that for EE2. No 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 µg/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 µg/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 µg/L (3.5 ± 0.6 -fold increase above control)
222 increasing to 32.6 ± 1.6-fold above controls at 2.5 µg/L. At 2.5 µg/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 µg/L BPA and 2.5 µg/L MBP) GFP fluorescence was seen to extend from the
231 atrioventricular value 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 µg/L (23.6 ± 2.6 fold) and 1 µg/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 esr 1 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 µg/L BPA; and for the 14 dpf fish, 50.7 ±
263 11.5-fold above controls for 1000 µg/L BPA and 44.7 ± 7.7-fold above controls for 2500
264 µg/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 µg/L BPA treatment group there was a significantly different pattern in
271 the frequency of the heart beat from the control and 1000 µg/L BPA treatment group.
272 When the A:V beat ratio measurements were divided into 30 second time bins
273 representing means +/-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 µg/L BPA (184 ± 3.5 bpm compared with 201 ± 4.6 bpm in controls, $p < 0.05$, Figure
283 4D).

284

DISCUSSION

Estrogens have widespread effects including in the heart. Estrogen effects on circulatory function, mediated through genomic and non-genomic mechanisms, are well documented[43], leading to suggestions that BPA and other xenoestrogens may exert adverse effects on the cardiovascular system. Several epidemiological studies have reported that BPA exposure in humans is associated with an increased occurrence of cardiovascular disease[9-12], however, there is little direct experimental evidence to support this, and the mechanisms by which this may occur are not known. The zebrafish heart differs structurally in some aspects from the mammalian heart but the molecular mechanisms underlying cardiac development and function are highly conserved between mammals and fish, and with phenotypic similarities between cardiac mutants in fish and human diseases also, the zebrafish model has become widely used in cardiac research[44]. Here we used an ERE-TG zebrafish, in combination with morpholino knockdown of ER subtypes, to identify potential signaling mechanisms for BPA and its metabolite MBP in the heart and across other body tissues, and assessed for effects of early life exposure to BPA on subsequent heart function.

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

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

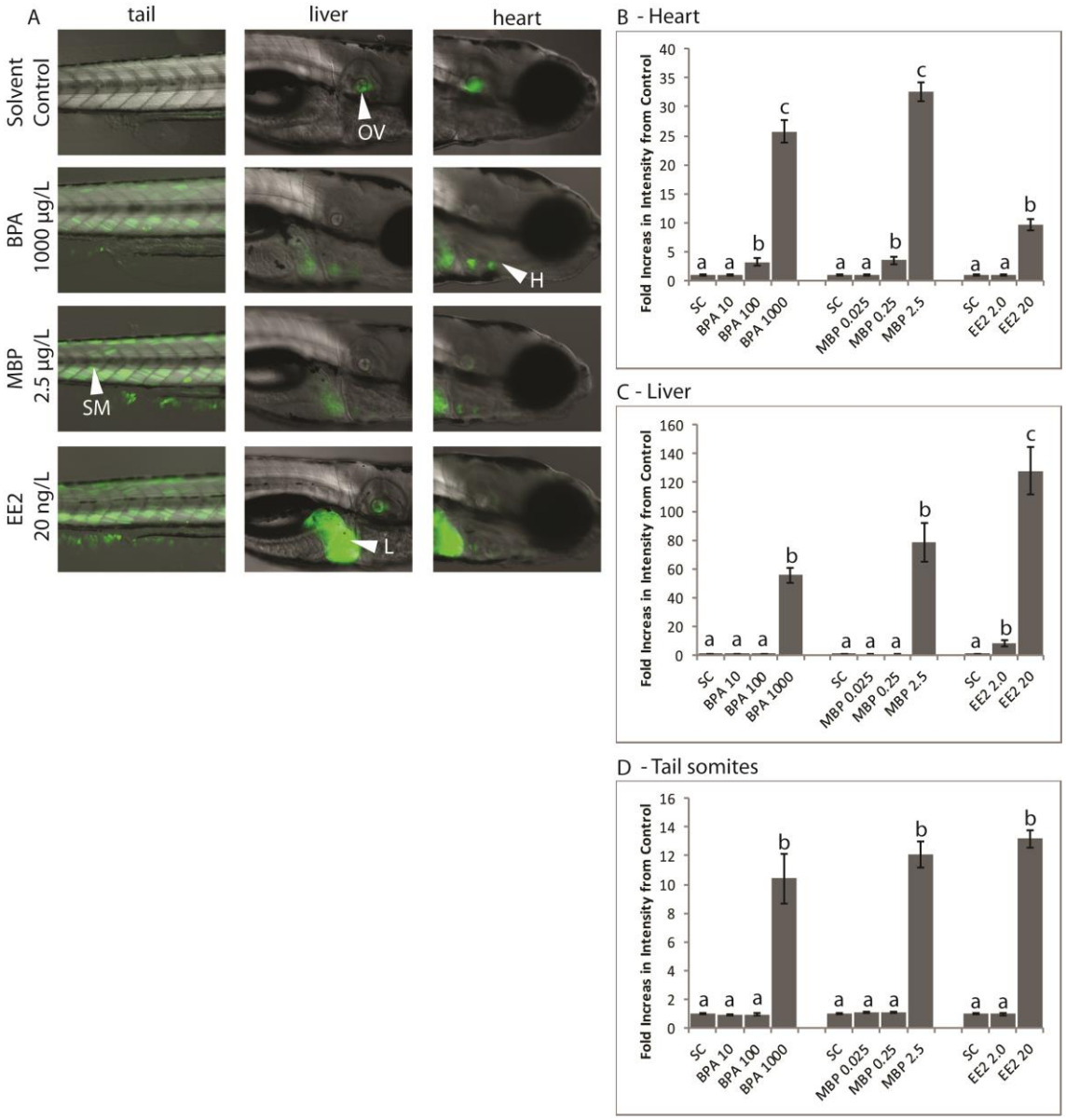
Induction of GFP by both BPA and MBP was inhibited in the hearts of ERE-TG larvae injected with morpholinos against esr1 (Figure 3). In liver, morpholinos against esr1 and against esr2a and esr2b together inhibited GFP expression, whereas in the somite myotubes (most notably in the tail region) the combined esr2a and esr2b morpholinos were effective, but the esr1 morpholino was not. These data indicate that different ER response pathways occur for the bisphenols within different body tissues. Studies on a zebrafish liver cell line, transfected with different ERs found that BPA most strongly activated ERα (esr1), with some activation of ERβ2 (esr2a) and no detectable effect on ERβ1 (esr2b)[45]. It is possible that the preferences indicated for different ERs could lead to different chemicals targeting specific tissues, resulting in the differing response patterns observed. Studies *in vitro* in mammalian-based systems including human ERα and rat ERβ protein synthesized in reticulocyte lysates[41] and rat liver S9 liver fractions, indicate that BPA preferentially binds to and activates ERβ but this has not been confirmed *in vivo*.

Whole mount *in situ* hybridisation conducted in 5 dpf zebrafish has shown the presence of esr1 transcripts in the atrioventricular valves and *bulbus arteriosus* and esr2a (reported as esr2b) transcripts in the liver[29]. In that study, esr2b (reported as esr2a) transcripts were not detected[29]. 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 stage[29]. It is well estabslished, 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 vitellogenin[46]. 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 hpf[47] with the valve leaflets developed by 102 hpf[48].
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 time[49]. 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 canal[50,
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.

Non-invasive video analysis has previously been used in the assessment of integrated cardiovascular function following drug treatment in zebrafish[42]. Endocardial cushion (precursors to functioning valves) development is thought to be complete around 5 dpf while valve elongation is thought to occur from around 6 dfp, completing at around 14 dpf[48]. If BPA significantly impacts heart valve function in zebrafish larvae, effects should therefore be detectable at this time. At 5 dpf the only significant effect measured was the difference in A:V beat ratio. This ratio was relatively conserved at 1:1 in control larvae with little variation observed throughout the 5 minute observation window. In fish exposed to 2500 µg/L BPA, A:V beat ratio was more variable over the assessment period (Figure 4E), indicating that although there was no uniform decoupling of atrial and ventricular beat rates, BPA exposure at high concentrations can cause erratic beat in either, or both, heart chambers. It is unlikely that A:V beat ratio would be linked to a disruption of the heart valves, however in dissected hearts fluorescent cells extending into the ventricle were observed (Figure 2A), possibly indicating BPA action, but more detailed and higher resolution microscope analyses would be required to develop a better understanding on this possibility. Previous studies have indicated that BPA may promote arrhythmia in mammalian hearts by alteration of myocyte Ca^{2+} handling, mediated by ERβ-signaling[18, 19, 52]. Estrogen-responsive fluorescent signaling in our ERE-TG fish appears to be mediated by an esr1-dependent pathway but at the higher concentration of 2500 µg/L BPA further pathways may also be induced. The only significant effect observed at 14 dpf was a reduction in heart rate (as indicated using arteria; pulse data) in zebrafish exposed to 2500 µg/L BPA although the mechanism for this was not determined. BPA, however, has been shown to affect thyroid signaling[53] and reduced circulating thyroid hormones can lead to a reduction in heart rate in humans[54].

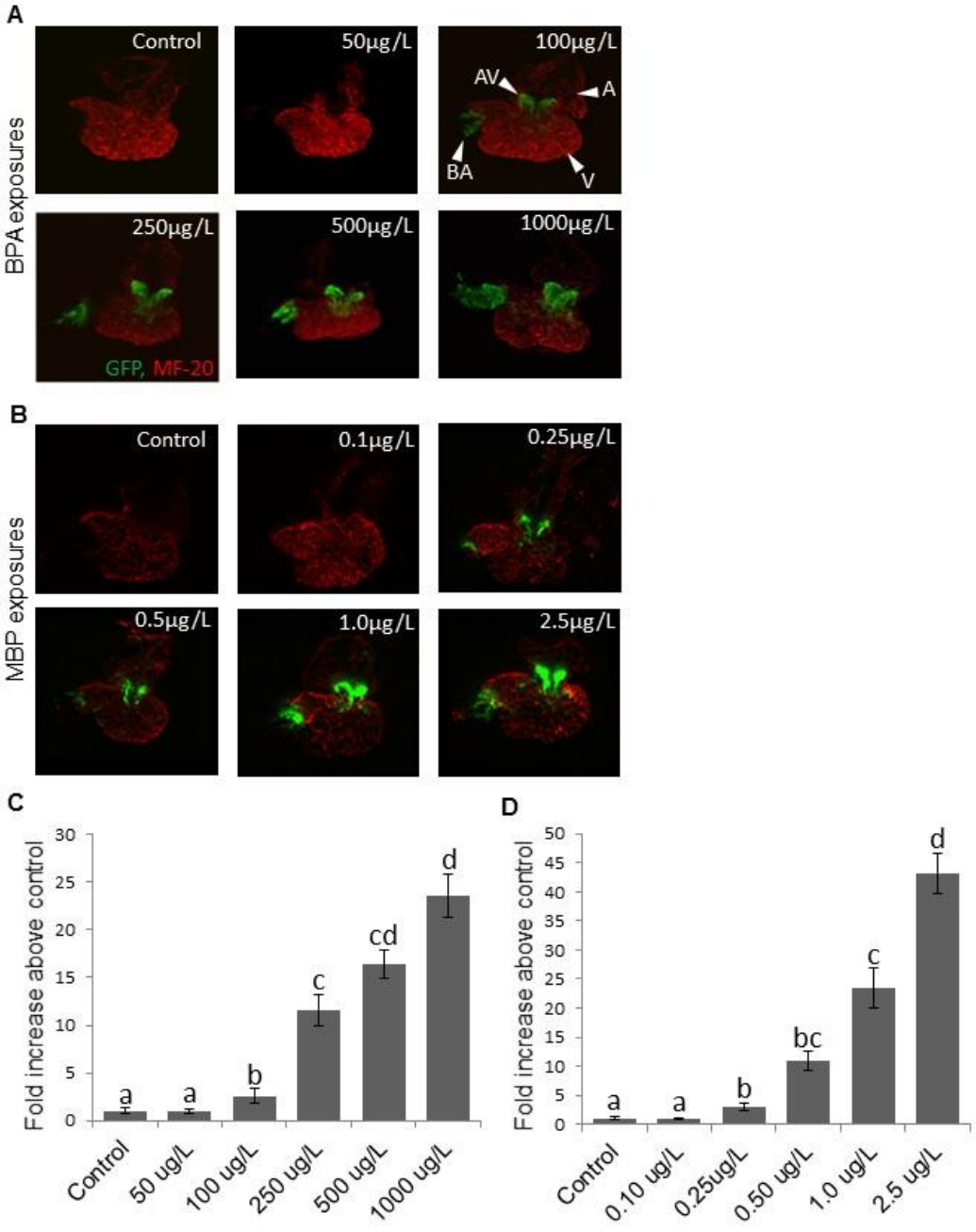
In conclusion, we demonstrate that the estrogenic BPA metabolite MBP targets the same tissues as its parent compound, BPA, and distinct from that of the pharmaceutical steroidal estrogen EE2. In common with data from mammals, the potency of MBP in the zebrafish is several orders of magnitude higher than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



396

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.

405

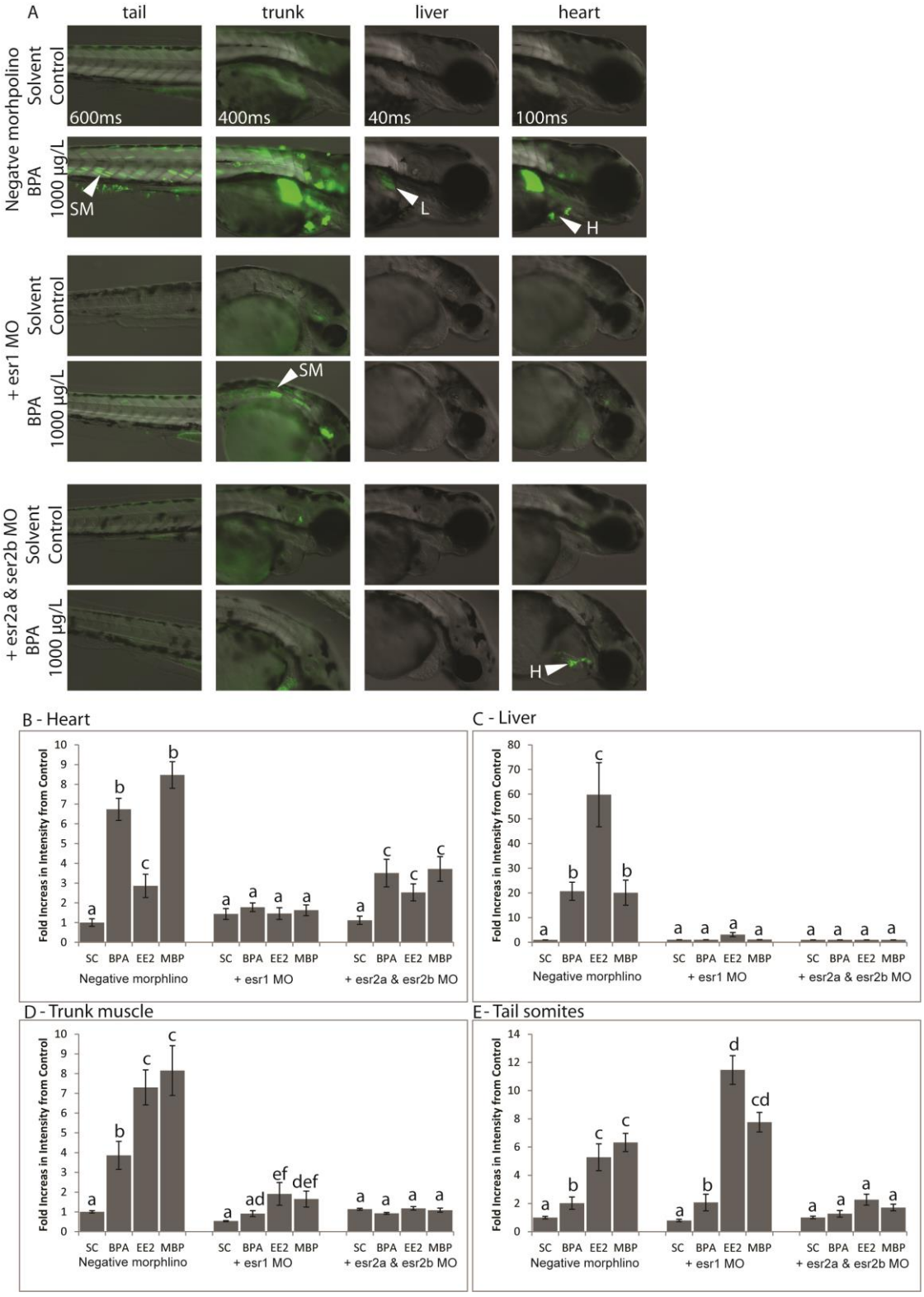


406

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.

414



415

416

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.

429

430

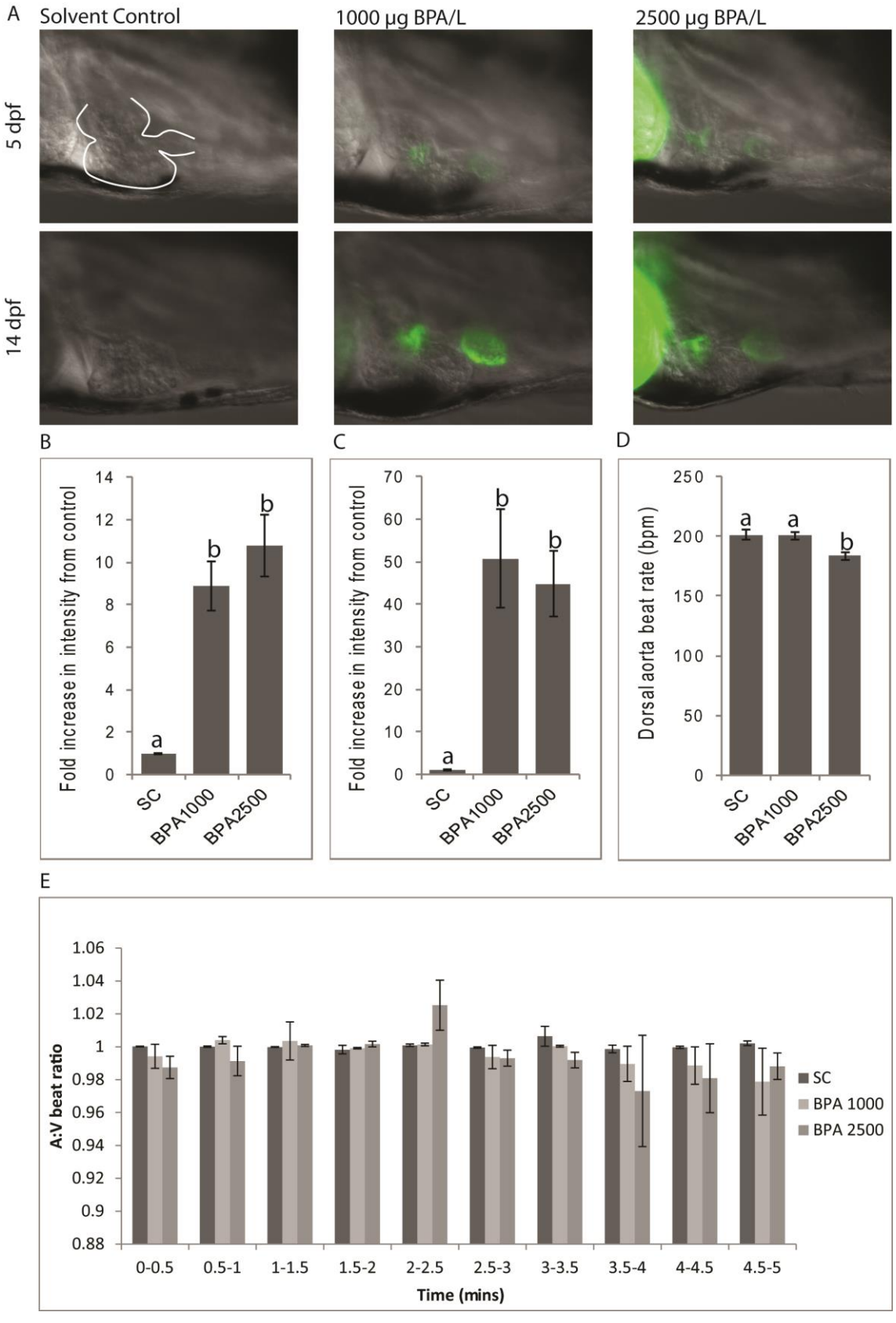

A
Solvent Control
1000 µg BPA/L
2500 µg BPA/L
5 dpf
14 dpf
B
Fold increase in intensity from control
SC
BPA1000
BPA2500
C
Fold increase in intensity from control
SC
BPA1000
BPA2500
D
Dorsal aorta beat rate (bpm)
SC
BPA1000
BPA2500
E
A:V beat ratio
Time (mins)
0-0.5
0.5-1
1-1.5
1.5-2
2-2.5
2.5-3
3-3.5
3.5-4
4-4.5
4.5-5
SC
BPA 1000
BPA 2500

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

ASSOCIATED CONTENT

Supporting Information.

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

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