1 # Cardiovascular effects and molecular mechanisms of

2 # bisphenol A and its metabolite MBP in zebrafish

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10 **KEYWORDS**

11 BPA, metabolite, MBP, endocrine, effects, estrogenic, heart valves, transgenic, zebrafish

12 **ABSTRACT**

13 The plastic monomer bisphenol A (BPA) is one of the highest production volume chemicals in the

14 world and is frequently detected in wildlife and humans, particularly children. BPA has been

15 associated with numerous adverse health outcomes relating to its estrogenic and other hormonal

16 properties, but direct causal links are unclear in humans and animal models. Here we simulated

17 measured (1×) and predicted worst-case (10×) maximum foetal exposures for BPA, or equivalent

1

18   concentrations of its metabolite MBP, using fluorescent reporter embryo-larval zebrafish, capable

19   of quantifying Estrogen Response Element (ERE) activation throughout the body. Heart valves

20   were primary sites for ERE activation by BPA and MBP, and transcriptomic analysis of micro-

21   dissected heart tissues showed that both chemicals targeted several molecular pathways

22   constituting biomarkers for calcific aortic valve disease (CAVD), including extra-cellular matrix

23   (ECM) alteration. ECM collagen deficiency and impact on heart valve structural integrity were

24   confirmed by histopathology for high-level MBP exposure, and structural defects (abnormal

25   curvature) of the atrio-ventricular valves corresponded with impaired cardiovascular function

26   (reduced ventricular beat rate and blood flow). Our results are the first to demonstrate plausible

27   mechanistic links between ERE activation in the heart valves by BPA's reactive metabolite MBP

28   and the development of valvular-cardiovascular disease states.

29   **INTRODUCTION**

30   Over 1400 chemicals have been identified as potential endocrine disrupting chemicals (EDCs) (1)

31   with potential to "alter function(s) of the endocrine system and consequently cause adverse health

32   effects in an intact organism, or its progeny, or (sub)populations" (2-5). Over 100 of these

33   chemicals are regarded internationally as priority EDCs and almost half (45%) are estrogenic i.e.

34   estrogen receptor (ER) and/or estrogen-related receptor (ERR) agonists (6-8). Estrogens play a

35   fundamental role in the formation and function of numerous organs and systems (9) and

36   imbalances are known to increase risks of cancers and disorders of reproductive, nervous,

37   metabolic, immune and cardiovascular systems in various animal models and humans (10-14).

38   However, linking cause and effect remains a major challenge in chemical risk/safety assessment.

39   Bisphenol A (BPA) is associated with the above disorders and is one of the world's highest

40 production volume chemicals (15), to which humans are continually exposed via plastic and other

41 products (16-23). Reported BPA-effect mechanisms include agonism of nuclear ERs (24), ERRs

42 (25,26), membrane ERs (27,28), epigenetic modulation of estrogen response elements (EREs)

43 (29,30) and weak agonism of androgen and thyroid (T3) receptors (31,32). Over 2700 peer-

44 reviewed papers have been published on the endocrine effects of BPA (Scopus search, September

45 2018) illustrating the level of scientific interest in BPA. Despite this high number of studies, 10%

46 of which are *in vivo* studies, regulatory authorities have concluded that there is insufficient

47 evidence to establish causal links between BPA and adverse effects on human health (21,33,34).

48 Nevertheless, public pressure has prompted the removal of BPA from baby products in Canada,

49 Europe and North America (35,36), due to higher exposures and lower competence for

50 metabolising BPA in infants (17,37). Some of the replacement products for BPA are also

51 estrogenic in mammals (38) and fish (39). Furthermore, the reactive BPA metabolite 4-methyl-

52 2,4-bis(p-hydroxyphenyl)pent-1-ene (MBP) has been shown to be far more potent than the parent

53 BPA in terms of: estrogen receptor binding and activation *in vitro* ($\times$10-1000) (40); stimulation of

54 uterine growth in rats ($\times$500) (41); elevated estrogen receptor (*esr1*) and vitellogenin (*vtg1, vtg2*)

55 gene and protein expression in medaka (*Oryzias latipes*) ($\times$250-400) (42,43); ERE activation in

56 zebrafish (*Danio rerio*) ($\times$1000) via *esr1* (44). These findings indicate an urgent need for more

57 integrative test systems capable of evaluating multiple effect levels, linking key molecular events

58 and adverse outcomes for chemicals like BPA and its analogues and metabolites.

59 Transgenic (TG) zebrafish models offer suitable integrative test systems, whereby key molecular

60 events (e.g. (ant)agonism of hormone receptors, or hormone metabolism) can be identified and

61 quantified by fluorescent protein reporters linked to specific enzymes, receptors or response

62 elements (45). Spatial and temporal resolution of key molecular events in TG zebrafish can

63 facilitate the detection of chemical effects throughout the body *in vivo*, in real time (45-49) and

64 can help establish causal links with subsequent adverse effects on biological development and/or

65 function (39,44). Here we exploit TG(ERE:GFP)Casper zebrafish to study the effects of BPA and

66 its highly estrogenic metabolite MBP on cardiovascular (CV) development and function, building

67 on previous work using this model, which highlighted the heart, and heart valves in particular, as

68 being key targets for these compounds (39,44,47,48).

69

70 **MATERIALS AND METHODS**

71 **Test substances**

72 Bisphenol A or BPA: 2,2-bis(4-hydroxyphenyl)propane (99% pure, CAS No. 80-05-7) was

73 obtained from Sigma-Aldrich Company Ltd., Dorset, UK.

74 The BPA derivative MBP: 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (99% pure, CAS No.

75 13464-24-9) was synthesised at the University of Exeter (SI (Figure S1).

76

77 **Test organisms**

78 Test organisms were 3rd generation homozygous TG(ERE:GFP)Casper zebrafish (*Danio rerio*)

79 (48), combining a green fluorescent protein (GFP) reporter system for estrogen response element

80 (ERE) activation (45) in a translucent Casper phenotype (50). This translucent model extends the

81 use of fluorescent reporters to life-stages >5 dpf, which would otherwise gain skin pigmentation

82 that interferes with GFP detection (48). This is an important feature of the model, as EDC effects

83 may vary both within a tissue and between body tissues at different life-stages (4,51,52). The life-

84 stages selected for this study represent two key landmarks in CV development: 5 days post

85 fertilisation (dpf) marking formation of the endocardial rings (precursors to the heart valve

86 leaflets); 15 dpf marking elongation of the valve leaflets (53-55). The latter life-stage also

87 corresponds with the depletion of the egg yolk and peaks in metabolism (oxygen consumption)

88 and heart beat rate (56).

89 The zebrafish is an established model for biomedical research on CV development, function and

90 disease (53,55,57), including heart valve (mal)formation (58-61). Despite some basic anatomical

91 differences from the human heart (58,62), the cellular and molecular mechanisms of heart

92 development are highly conserved between zebrafish and humans (55,63) and zebrafish have

93 several major advantages over other vertebrate models. Heart formation (including valvulogenesis

94 and remodelling, outlined in detail in Supporting Information SI Table S1) is completed by 35 dpf

95 (62,63), but a heartbeat is detectable as early as 1 dpf, with blood circulation beginning soon

96 thereafter (64). Standard (resting) heart beat rate in 5-15 dpf embryo-larval zebrafish (160–260

97 beats per minute; bpm) is much closer to resting human foetal heart rate (130–170 bpm) than in

98 rodents (300–600 bpm) (56,65-67). Furthermore, respiration in embryo-larval stages relies mainly

99 on cutaneous diffusion of O_2 and CO_2, rather than transport by convective blood circulation (68),

100 enabling the *in vivo* study of late phenotypes of congenital CV malformations, which would be

101 lethal in mammals (57).

102

103 **Ethical statement**

104 All experimental procedures with zebrafish were conducted in accordance with UK Home Office

105 regulations for the use of animals in scientific procedures and followed local ethical review

106 guidelines and approval processes. Water quality was assessed daily (SI Table S2).

107

108 **Chemical exposure**

109   TG zebrafish embryos/larvae were exposed in the laboratory from 6 hours post fertilisation (hpf)

110   to 5 days post fertilisation (dpf) or from 6 hpf to 15 dpf, to aqueous concentrations of BPA (solvent

111   (0.5% DMSO) control 0; low 100; high 1000 µg/L) or its metabolite MBP (solvent control 0; low

112   2.5; high 25 µg/L) in glass 1L aquaria (n=6 aquaria per exposure treatment), positioned in random

113   order and each containing ~120 randomly assigned embryos. The lower BPA concentration was

114   expected to represent maximum measured human maternal-foetal-placental unit concentrations of

115   up to 105 ng/g (69,70), based on bioconcentration factors ranging from 0.25 to 5.7 in larval and

116   adult zebrafish (39,71,72). Both lower and 10× higher (worst case) BPA concentrations were

117   substantially below maximum tolerable concentrations (48). MBP exposure concentrations were

118   based on a relative potency of 250× compared to BPA, measured *in vivo* in juvenile medaka (43).

119   Stock solutions were prepared by dissolving pure test chemicals in analytical grade dimethyl

120   sulfoxide (DMSO) and then diluting (200×) in 400 mL of embryo culture water (73) to give the

121   desired nominal exposure concentrations in 0.5% DMSO. The pH of stock solutions was checked

122   and adjusted to 7.5, as necessary. For the longer-term (0-15 dpf) exposure, 90% water changes

123   were undertaken every 2 days, after commencing feeding twice a day at 6 dpf with excess <100

124   µm particulate fish food (ZM000) and with *Artemia salinus* nauplii from 10 dpf. Exposure

125   solutions were maintained at 28°C, under a 16h:8h light : dark photoperiod cycle with a 15 min

126   dawn/dusk transition.

127   Concentrations of exposure solutions were measured in three replicate aquaria per exposure

128   treatment at the start and end of each exposure period. Chemical body burden was also measured

129   in whole zebrafish embryo-larvae at 5 dpf, and in composite samples of ×30 hearts extracted from

130   5 dpf embryos (n=3 composite samples per treatment). Heart extraction was performed *en masse*:

131   ×50 larvae per aquarium were disrupted in ice-cold Leibovitz's L-15 Medium (Invitrogen, UK)

132   containing 10% foetal bovine serum, using a 6 ml syringe and 19 gauge needle (74), and ×30 hearts

133   were isolated using a 30 µm mesh sieve followed by manual sorting in ice-cold Leibovitz's L-15

134   Medium under a 5× objective on an Olympus SZX16 microscope (Olympus, UK). Fish/hearts

135   were placed in embryo culture water with a terminal dose of anaesthetic of 2 mg/mL tricaine

136   methanesulfonate (MS222) at pH 7.5, then dried under vacuum, macerated and extracted in a

137   solution of 80:20 water:acetonitrile containing an internal standard. Details of chromatographic

138   separation and mass spectrometry analysis of BPA and MBP in water and fish tissues are provided

139   in the SI (Table S3). The limit of quantitation (LOQ) was 0.05 µg BPA/L and 0.05 µg MBP/L for

140   water, and 0.5 ng BPA/g and 0.5 ng MBP/g for fish tissue. Bioconcentration factors ($BCF_{whole\ body}$

141   and BCF_{heart}) were calculated based on a mean whole body wet weight of 1200 µg for 5 dpf

142   zebrafish larvae and a ventricle weight of 10% of the whole body weight at 5 dpf (75).

143   Following chemical exposure, TG zebrafish larvae were selected randomly from each aquarium

144   and were subject to the following effects analyses, which were conducted at 28°C.

145

146   **Quantifying ERE activation (estrogenicity)**

147   ERE activation was quantified in the atrio-ventricular (AV) and ventricular-bulbus (VB) valves as

148   follows. At 5 and 15 dpf ×6 larvae per replicate aquaria (n=6) were washed and then anaesthetised

149   in 0.1 mg/mL MS222 at pH 7.5 and mounted *in vivo* in 1% low melting agarose in embryo culture

150   water with MS222, and then placed into a glass bottom 35 mm dish (MatTek, Ashland, MA, USA).

151   Larvae were orientated right side down and images were obtained using an inverted compound

152   microscope (Zeiss Axio Observer, Cambridge, UK) with a 10× objective, under consistent GFP

153   excitation for a scanning time of 180 ms, using filter set 38 HE: Excitation BP 470/40 nm, Beam

154   splitter FT 495 nm, Emission BP 525/50 nm. GFP expression was quantified as the relative mean

155   pixel intensity of green fluorescence (relative to the solvent control) in a defined region of interest

156   (ROI) encompassing the heart, using ImageJ software (76).

157

158   **Histopathology of the heart and heart valve leaflets**

159   Histopathology of the heart and heart valve leaflets was conducted on ×6 whole zebrafish larvae

160   per replicate aquaria (n=6) at 5 and 15 dpf, following terminal anaesthesia in 2 mg/mL MS222 at

161   pH 7.5, and destruction of the brain. For visible light microscopy, zebrafish were fixed in Bouin's

162   solution (Sigma Aldrich, Dorset, UK), progressively dehydrated in 70-100% industrial methylated

163   spirits and embedded in paraffin wax. For transmission electron microscopy (TEM), zebrafish

164   were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1M PIPES buffer (pH 7.2) for 24

165   h, washed with buffer (3×5 min) and post-fixed for 1 h in 1% osmium tetroxide (reduced with

166   1.5% w/v potassium ferrocyanide) in 0.1M sodium cacodylate buffer (pH 7.2). After a series of

167   washes in deionised water (3×5 min) the larvae were subject to in-block staining with 1% uranyl

168   acetate for 30 mins, then 3×5 min washes with deionised water and then the larvae were

169   dehydrated through an ethanol gradient and embedded in Spurr resin (TAAB Laboratories,

170   Aldermaston, UK). Sagittal sections were obtained through the midline of the atrium and ventricle

171   (to examine the AV valves). For visible light microscopy, serial sections (5 μm) were obtained

172   and placed on glass slides, stained using Masson's trichrome and examined using a Leitz Diaplan

173   light microscope [×(10-100) magnification] to assess for structural pathologies (focusing on

174   valvular cells and interstitial extracellular matrix). For TEM, 70 nm ultrathin sections were

175   collected on pioloform-coated EM copper slot grids (Agar Scientific, Stansted, UK) and were

176   analysed using a JEOL JEM 1400 operated at 120kV, and images taken [×(3000-20000)

177   magnification] with a digital camera (ES 100W CCD, Gatan, Abingdon, UK) to assess for any

178   ultra-structural effects on the heart valves.

179

180   **Quantifying CV function**

181   Non-invasive video analysis of the heart and dorsal aorta was used to measure multiple

182   cardiovascular (CV) endpoints simultaneously (67). At 15 dpf ×6 larvae per replicate aquaria

183   (n=6), were anaesthetized in 0.1 mg/mL MS222 and embedded right side down in 1% agarose in

184   a single well of a press-to-seal silicon isolator (Sigma-Aldrich, Poole, UK) on a clear microscope

185   slide. The slide was then viewed using an inverted light microscope (Leica DM IRB, Leica

186   Microsystems UK Ltd., Milton Keynes, UK, 5× objective) fitted with two high speed video

187   cameras. The first camera (Grasshopper® GRAS-50S5C-C, Richmond, Canada) recorded

188   ventricular heart beat at 30 frames per second (the atrium was obscured at 15 dpf). The second

189   camera recorded blood flow in the dorsal aorta, caudal to the swim bladder, at 120 frames per

190   second (Grasshopper® GRAS-03K2M-C, Richmond, Canada). Both cameras were focused

191   independently on their respective regions of interest, to ensure optimal image quality, and set to

192   record simultaneously for 5 min; recordings from the last 3 mins were subject to image analysis

193   as follows. Resting (standard) ventricular beat rate (bpm - beats per minute) was measured using

194   MicroZebraLab™ image analysis software (v3.5, ViewPoint, Lyon, France). Resting (standard)

195   aortic blood flow rate (nL/s) was measured using ZebraBlood™ (v1.3.2, ViewPoint, Lyon,

196   France).

197

198   **Quantifying metabolic scope**

199   Metabolic scope, combining scope for growth and scope for movement, were measured in terms

200   of specific growth rate (SGR) (77) and critical swimming speed (U_{crit}) (78), respectively. SGR

201   was calculated by measuring individual standard body length [±0.01 mm from snout to caudal

202   peduncle] of TG zebrafish larvae (×10 per aquarium) under anaesthetic (0.1 mg/mL MS222) at 5

203   and 15 dpf, using an Olympus SZX16 microscope and cellSens^{TM} image analysis software. Since

204   individual fish were not tagged, mean specific growth rate (mean SGR as % standard body length

205   per day) was calculated for each aquarium (Equation 1). Critical swimming speed (U_{critb} as

206   standard body lengths/sec) was then assessed at 15 dpf, by placing ×5 larvae at a time in a

207   cylindrical swim flume of length 25 cm, internal diameter 2 cm and volume 78.55 mL, and

208   increasing laminar water flow incrementally by 1.33 cm/sec every 300 secs (5 mins) until the time

209   to exhaustion for all individual larvae. U_{critb} was calculated based on aquarium-mean standard

210   body length at 15 dpf (Equation 2).

211   **Equations**

212   1) Mean SGR = ((ln SL @15 dpf) – ln SL @ 5 dpf) / T)*100

213   Where SL is mean standard length per aquarium (mm); T is time interval (days)

214

215   2) $U_{critb} = U + (t / t_i * U_i)$

216   Where U is penultimate swimming speed (mean standard body lengths/sec); U_i is velocity

217   increment (1.33 cm/sec); t is time swum in final velocity increment (secs); t_i is time interval for

218   each increment (300 secs).

219

220   **Transcriptomic profiling of heart tissues**

221   Hearts were removed from TG zebrafish larvae following terminal anaesthesia in 2 mg/mL MS222

222   at pH 7.5, followed by destruction of the brain. At 5 dpf ×50 larvae per replicate aquaria (n=4)

223   were disrupted in ice-cold Leibovitz's L-15 Medium (Invitrogen, UK) containing 10% foetal

224   bovine serum, and hearts were extracted *en masse*, as described above. Hearts from larvae of 15

225   dpf were micro-dissected individually using fine tip forceps (Dumont Inox #5SF) and an Olympus

226   SZX16 microscope. Hearts for the 15 dpf larvae were pooled (×30 per aquarium) and then snap

227   frozen in liquid nitrogen. Total mRNA was extracted from each pool of 30 hearts using RNeasy

228   micro-kits with on-column DNase treatment (Qiagen, UK) and RNA integrity (RIN) scores were

229   confirmed to be in the range 7.4-8.7 using an Agilent 2200 TapeStation (Agilent Technologies

230   Ltd. Berkshire, UK).   cDNA libraries were prepared with polyA isolation using Illumina

231   TruSeqTM 2 Stranded mRNA Library Preparation kits and subsequent cluster generation was

232   conducted using TruSeqTM Paired-End Cluster Generation kits (Illumina, San Diego CA, USA).

233   Up to 24 cDNA libraries were prepared for sequencing for each test substance (BPA and MBP):

234   nominally n=4 replicate pooled samples from each of 3 treatment groups (solvent control, low,

235   high exposure), for two time points (5 dpf and 15 dpf). Libraries were sequenced with 12 per lane

236   using an Illumina HiSeq 2500 in standard mode, generating 100 base pair reads (paired-end).

237

238   **Statistical analysis**

239   Prior to statistical analysis, phenotypic data were tested for normality (Anderson–Darling test) and

240   homogeneity of variance (Bartlett's or Levene's tests) using Minitab 16 (Minitab, Coventry, UK).

241   Statistical analysis of phenotypic/functional endpoints was performed using linear mixed effects

242   (lme) models (R-statistics version 3.3.2, R Foundation for Statistical Computing) and all lme

243   models included aquarium as a random effect. A multi-variate MANOVA was used to assess the

244   fixed effect of exposure treatment on cardiovascular function, combining both ventricular heart

245   beat rate and blood flow (using the function 'cbind'). Statistical significance ($p<0.05$) of treatment

246   effects on these and other individual phenotypic endpoints was also determined by 'one-way'

247   comparisons of untransformed heart beat or blood flow data, and lme model fit was measured

248   using the Akaike Information Criterion (AIC). Phenotypic/functional effects data are presented in

249   the text or shown graphically as the mean ± 95% confidence interval.

250   Transcriptomic data were quality-trimmed and filtered (to remove sequencing adapters) and then

251   processed using the TopHat/ Cufflinks pipeline (79), followed by differential gene expression

252   analysis comparing chemical exposure treatments with control treatments using DESeq2 v3.6 and

253   an adjusted p-value of <0.05 set as the false discovery rate (80).

254   Gene Set Enrichment Analysis (GSEA) using DAVID v6.8 (81), Enrichr (82) and Reactome v66

255   (83) was conducted on sets of differentially expressed genes (for each chemical exposure

256   treatment) to identify over-represented Gene Ontology (GO) terms for biological processes and

257   functional pathways, including KEGG v88 (84) and Reactome v66 pathways (83) referenced to

258   the zebrafish (GRCz10) and the human genome (GRCh38.p12).

259   Transcription Factor Binding Site (TFBS) motif enrichment analysis was conducted on 5 and 50

260   kilobase (kB) long DNA flanking sequences both up and downstream of the differentially

261   expressed genes to quantify the potential for estrogen receptor interactions; flanking sequences

262   were retrieved using BioMart from Ensembl v94 (85). Over-represented motifs in each gene set

263   were identified using AME (86) in MEME Suite v5.0.2 (http://meme.nbcr.net). Each gene set was

264   'shuffled' to generate a control gene set (with matched GC content), and enriched TFBS motifs

265   were subsequently identified using the JASPAR 2018 (CORE:vertebrate) database (87).

266   Gene groups were considered to be enriched when enrichment scores (EASE scores) were >1.3,

267   and when *p*-values adjusted for multiple-testing (Benjamini-Hochberg) were < 0.1.

268

269   **RESULTS AND DISCUSSION**

270

271   **Chemical exposure**

272   Mean measured concentrations of aqueous exposure solutions were 104-128% of nominal values

273   for 100 and 1000 µg/L BPA (117 ± 4, 1028 ± 23 µg/L) and 82-113% of nominals for 2.5 and 25

274   µg/L MBP (2.1 ± 0.1, 28.2 ± 0.35), and both sets of controls contained no measurable test chemical

275   (SI Table S4). Mean measured bioconcentration factors for BPA were: 5 day $BCF_{whole\ body}$ = 2.5

276   and 3.8 for 100 and 1000 µg/L BPA exposures, respectively, corresponding with whole body

277   concentrations of ~250 and ~3700 ng/g, which are equivalent to ×2.5 and ×37 maximum human

278   maternal-foetal-placental unit concentrations of 105 ng/g (69,70). Mean measured 5 day BCF_{heart}

279   = 0.09 for the 1000 µg/L BPA exposure was substantially lower than the corresponding BCF_{whole}

280     $_{body}$. The mean measured bioconcentration factor for MBP was: 5 day $BCF_{whole\ body}$ = 27 for the 25

281   µg/L MBP exposure, corresponding with a whole body concentration of 675 ng/g. MBP was not

282   detectable above the LOQ (0.5 ng/g) in heart tissue, so a 5 day BCF_{heart} could not be determined.

283

284   **ERE activation**

285   Exposure to BPA or MBP induced fluorescence from the ERE:GFP reporter in the liver and in the

286   region of interest (ROI) encompassing the heart, specifically in the atrio-ventricular (AV) and the

287   ventricular-bulbus (VB) valves (Figure 1), which is consistent with previous studies (44,48).

288   Higher fluorescence intensity in the heart/valves was not due to chemical partitioning, since heart

289   tissue concentrations for BPA were more than an order of magnitude lower than in whole body

290   tissue (and the water concentration). Instead, greater ERE activation and fluorescence in the heart

291   valves may be due to tissue-specific expression of different ERs and receptor sub-types. Relative

292   mean fluorescence intensity in the ROI (relative to the solvent control) increased with BPA

293   exposure concentration (100 to 1000 µg/L) from 2.2 ± 0.3 to 31 ± 2 at 5 dpf, and showed a similar

294   concentration-related response, increasing from 15.3 ± 1.1 to 26.9 ± 0.2, at 15 dpf. There was a

295   similar response pattern in relative mean fluorescence intensity in the ROI for MBP, increasing

296   with MBP exposure (2.5 to 25 µg/L) from 39 ± 3 to 54 ± 3 at 5 dpf, and from 3.0 ± 0.4 to $14.6 \pm$

297   1.5 at 15 dpf (SI Figure S2). Comparing relative mean fluorescence of MBP versus BPA for the

298   lower-level exposure treatments, which can be assumed to lie on the linear sections of the dose

299   response curves (44,48), we calculated the relative potency of MBP compared to BPA to be 710×

300   at 5 dpf and 23× at 15 dpf (SI Table S5), which is consistent with results from previous *in vivo*

301   studies on zebrafish (39,44), medaka (42,43) and rats (41).

302   Greater potency of MBP compared with BPA may be explained, at least in part, by the

303   bioconcentration of MBP from water (5 day $BCF_{\text{whole body}} = 27$), which was one order of magnitude

304   greater than for BPA. Chemical exposure level- and time- related changes in ERE/GFP expression

305   in zebrafish heart valves are likely to be influenced by range of interacting factors. The lower

306   relative potency of MBP in larvae at 15 dpf, compared with that in 5dpf larvae, followed a net

307   reduction in ERE:GFP fluorescence intensity, possibly corresponding (to some extent) with tissue

308   thickening in older fish. Furthermore, the reduction in fluorescence intensity over time was greater

309   in MBP compared to BPA treatments. These results are consistent with metabolic activation of

310   BPA in fish (42,43,88) and greater metabolic activity in 15 dpf compared to 5 dpf zebrafish larvae

311   (56). The observed variation in levels of ERE activation at these different life stages may also be

312   due in part to age-related variation in the expression of different ERs and receptor sub-types (52).

313

314   **Effects on the structure of the heart valve leaflets**

315   There was qualitative evidence of ultra-structural changes in the AV valve leaflets in both BPA

316   and MBP high-level exposure treatments at 15 dpf. TEM images ($\times$3000-20000 magnification)

317   showed the dislocation of valvular cells and the loss of collagen filaments from the interstitial

318   extra-cellular spaces (Figure 2). In the high-level MBP exposure there were also gross-structural

319   changes in the AV valve leaflets. Light microscopy images ($\times$100 magnification), taken following

320   Masson's trichromatic staining, showed that valve leaflets were misshapen (bent) and that the

321   extra-cellular matrix between the bilayer of valvular cells was narrower and lacked collagen

322   (indicated by the lack of blue stain) compared to solvent controls (Figure 2). Although these ultra-

323   and gross- structural effects were clearly evident, they could not be quantified morphometrically,

324   due to the limited number (n=2 or 3 per treatment) of sagittal sections in which the AV valve

325   leaflets were discernible. No other heart valve pathologies were detected in any other treatment

326   or time point, and there was no evidence of edema to indicate general cardiotoxicity. Histological

327   assessment of ventricular-bulbus (VB) valves was not possible from the sagittal sections taken to

328   assess the AV valves, due to their alternative orientation/alignment.

329

330   **Effects on CV function**

331   There were no discernible effects of BPA exposure on CV function in agreement with a previous

332   study (44), but high-level MBP exposure resulted in a statistically significant reduction (versus

333   solvent controls) in ventricular heart beat rate (218 $\pm$ 4 versus 249 $\pm$ 7 bpm; $p = 0.023$) and aortic

334   blood flow rate (1.55 ± 0.07 versus 1.95 ± 0.06 nL/s; $p = 0.03$) in 15 dpf zebrafish, according to

335   the linear mixed effect model lme(Rate ~ Treatment, random=~1|Tank1) (Figure 3; SI Table S6).

336   These results fall within ranges reported at 28°C for zebrafish aged 3.5 to 21 dpf for resting heart

337   beat rate (160 to 260 bpm) (56,65-67) and resting blood flow rate (500 to 1860 μm/s; equivalent

338   to 0.25 to 2.1 nL/s) (67,89,90). Ontogeny of embryo-larval development in zebrafish is such that

339   heart beat and blood flow rate vary substantially with development time and peak at 10–20 dpf, a

340   period which corresponds with a peak in aerobic metabolism (56). Therefore CV performance is

341   likely to be most critical for our selected life-stage (15 dpf) and reduced CV performance (20%

342   reduction in blood flow rate) could be biologically significant. The proportional reduction in blood

343   flow ($20 \pm 3.6\%$) was greater than for heart beat rate ($12 \pm 2.3\%$), potentially indicating valve

344   prolapse (although note the variation in these CV parameters). We were unable to observe valve

345   function directly because of tissue thickening, nor were we able to demonstrate AV decoupling as

346   being symptomatic of valve prolapse (91), since organ growth prevented imaging of both atrial

347   and ventricular beating at 15 dpf. Nevertheless BPA exposures up to 2500 μg/L have been shown

348   to induce erratic AV beat ratios in 5 dpf zebrafish (44).

349

350   **Effects on metabolic scope**

351   No effects of BPA or MBP were seen on metabolic scope (i.e. SGR and U_{critb}) (SI Figure S3 and

352   Table S7). These energetic measures provide a general indication of individual metabolic scope,

353   i.e. energetic reserves beyond basal metabolism (92). U_{critb} integrates anaerobic as well as aerobic

354   scope for movement (93) and also cardio-respiratory performance (65). The lack of significant

355   effects (despite downward trends for MBP) may have been due to substantial inter-individual

356   variation across the exposure treatments. Inter-individual differences in locomotory performance

357 traits have been observed in larval zebrafish between 3 to 21 dpf, and shown to be due to heritable

358 genetic factors not necessarily related to body size (65). Our test organisms were 3rd generation

359 transgenic zebrafish maintained in up to six spawning groups with 30 fish in each group, therefore

360 genetic variation may have been a confounding factor, but this was not quantified. High inter-

361 individual variation may also be related to larval swimming behaviour, which is characterized by

362 intermittent bursts of swimming (94), such that prolonged swim challenge tests >10 mins may not

363 reliably indicate swimming stamina (65). Our swim challenge tests typically ran for 5-10 mins

364 and mean U_{critb} ranged from 10.7 to 12.6 body lengths/sec, which is comparable to values reported

365 elsewhere, ranging from 13 to 18 body lengths/sec in juvenile and adult zebrafish (95,96).

366

367 **Effects on the heart transcriptome**

368 BPA (100, 1000 µg/L) and MBP (2.5, 25 µg/L) exposures at 5 and 15 dpf resulted in significant

369 (adjusted p-value < 0.1) differential expression (predominantly down-regulated expression,

370 compared to solvent controls) in a range of genes governing heart valve development and function

371 (SI Figures S4-S5; SI Tables S8-S10). The down-regulation of a range of genes by ER signaling

372 (via *esr1, esr2* and heterodimers of these) has been demonstrated elsewhere in humans and

373 mammalian models (97,98). The number of genes affected and the level of effect were greatest at

374 5 dpf for high-level BPA exposure (371 genes: 329 down-regulated by log_2 fold changes of -0.5

375 to -9), followed by low-level BPA exposure (131 genes: 115 down-regulated by log_2 fold changes

376 of -0.9 to -9) and high-level MBP exposure (127 genes: 101 down-regulated by log_2 fold changes

377 of -2.5 to -9). There was some overlap between high and low-level BPA exposures at 5 dpf, with

378 62 genes being common to both treatments, whereas only one gene (elastin microfibril interfacer

379 3 - *emilin3*) was common to both MBP treatments at 5 dpf, due in part to the low number of genes

17

380   (8 genes) that were differentially expressed in the low-level MBP exposure (SI Figure S6 - S7).

381   Collectively however, the genes affected by both BPA and MBP at 5 dpf shared significant

382   enrichment for molecular pathways concerning i) nuclear receptor and calcium signalling

383   (estrogen and (para)thyroid), ii) lipid metabolism and iii) extra-cellular matrix (ECM) interactions

384   (99) (Figure 4; SI Figure S8; SI Tables S8 – S10). ECM-related pathways comprising collagen and

385   cartilage formation and organization were particularly prominent for the high-level MBP exposure,

386   and can be related directly to phenotypic effects on the heart valves observed therein. ECM

387   interactions were found to involve Notch signalling (dre04330) and calcium ion binding

388   (GO:0005509) (SI Table S9) which, along with the afore mentioned pathways (i-iii), have

389   previously been linked to the progression of calcific aortic valve disease (99,100) (CAVD -

390   fibrosis, and subsequent calcification and thickening of the aortic valves), which affects up to 13%

391   of human populations in the western world (99) (see later discussion). At 15 dpf fewer genes were

392   differentially expressed compared to 5 dpf. Although only 5 genes overlapped between time points

393   for the high-level BPA exposure treatment (SI Figure S7), there was gene set enrichment for

394   thyroid hormone synthesis (also seen at 5 dpf for MBP) and for estrogen signalling for the 15 dpf

395   high-level BPA exposure (Figure 4). Both hormone signaling pathways have been highlighted in

396   mammalian studies on BPA (31,101). Overall, gene sets from 15 dpf represented pathways

397   associated with heart function (more so than development), particularly cardiac muscle contraction

398   (SI Tables S8 – S9). Four of the 32 genes down-regulated by the high-level BPA exposure: actinin

399   alpha 3b (*actn3b*), myosin light chain, phosphorylatable, fast skeletal muscle a (*mylpfa*), myosin,

400   heavy polypeptide 2, fast muscle specific (*myhz2*) and troponin I type 2a (skeletal, fast), tandem

401   duplicate 4 (*tnni2a.4*) have previously been identified as potential biomarkers for cardiac disease

402   in animal models, including zebrafish (102). Low level BPA exposure at 15 dpf led to the

403   differential expression of only gene: apolipoprotein Da, duplicate 2 (*apoda.2* transport protein).

404   The transcriptomic effects of high-level MBP exposure at 15 dpf could not be established due to

405   problems encountered in sample processing (PCR amplification during library preparation), while

406   low-level MBP exposure at 15 dpf led to the differential expression of only one (unannotated) gene

407   (*si:dkey-7c18.24*) (SI Figure S7, SI Table S8).

408

409   Analysis of TFBS motif enrichment showed that differentially expressed genes in both BPA and

410   MBP exposures were similarly enriched and were flanked (within 5 kB) by binding sites for

411   transcription factors associated with estrogen receptor signaling, including: estrogen receptor 2

412   (*esr2*); specificity proteins constituting ERE tethering factors (*sp1, sp3, sp4*); pioneer factors

413   facilitating ERE binding (*foxa1, nfkb2, pbx1, runx1*); CAMP responsive element binding proteins

414   (*creb1, creb5*) (SI Figure S9, SI Tables S11 – S13). There was also enrichment for estrogen

415   receptors (*esr1, esr2*) beyond proximal promoter regions, up to 50 kB from differentially expressed

416   genes (SI Table S13). Examination of these findings versus the established roles of estrogen in

417   normal (and abnormal) heart valve development indicate plausible links between substantial ERE

418   activation by BPA and MBP and the observed transcriptomic and phenotypic effects on the heart

419   valves in our zebrafish. Endogenous estrogen (17β-estradiol) is known to be protective of the CV

420   system in later life, which is one of the reasons for hormone replacement therapy in post-

421   menopausal women. The results of studies on the pathogenesis of CAVD share some similarities

422   with the findings in our study (in terms of effect pathways) and also show that major risk factors

423   include polymorphisms of the estrogen receptor (103). Inactivation of estrogen receptors is

424   generally associated with increased calcification of the valve leaflets in diseased patients

425   (104,105). Empirical studies in mammalian models have shown that estrogen inhibits collagen

426 synthesis in rat cardiac fibroblasts (106) and, more specifically, estrogen decreases collagen I and

427 III gene expression in fibroblasts from female rats (107). BPA has also been shown to cause sex-

428 specific alterations in gene expression profiles, changes in the composition of the cardiac collagen

429 extracellular matrix and altered CV function in CD-1 mice (101). Furthermore, disruption of

430 collagen in the extracellular matrix has been shown to directly promote valve leaflet calcification

431 *in vitro* (108). Based on these studies it is entirely plausible that the estrogenic action of high-

432 level MBP exposure led to collagen deficiency and valve weakening in our larval zebrafish. This

433 preliminary (pre-calcification) condition resembles 'myxomatous degeneration' characterized by

434 collagen degradation and elastic fibre fragmentation, resulting in a "floppy" valve that is prone to

435 prolapse and regurgitation (109). As with CAVD, it may be speculated that more progressive

436 effects of MBP (and BPA) on heart valve development and function may occur in the longer- term

437 in zebrafish, but this remains to be proven.

438

439 **Linking cause and effect**

440 Although estrogen is known to be cardio-protective in later life, inappropriate estrogenic

441 exposures, including from estrogenic chemicals, in early life can lead to cardiac malformation

442 (110). We demonstrate that aqueous exposure to the weak estrogen BPA at $1\times$ and $10\times$ maximum

443 human maternal-foetal-placental unit concentrations, or its more potent metabolite MBP, at

444 equivalent potency concentrations, activate estrogen responsive elements (EREs via estrogen

445 receptor signalling) in the heart valves and alter the expression of a range of genes, including

446 several governing CV development and function in embryo-larval zebrafish. BPA and MBP

447 perturbed similar downstream effect pathways, but only the high-level MBP exposure resulted in

448 gross phenotypic effects including, malformation of the AV valves, and reduced heart beat and

449   blood flow, at 15 dpf. Our findings provide a substantial chain of evidence, but further work is

450   required to fully define adverse outcome pathways for the effects of BPA and its metabolites,

451   including MBP, on heart development and function. Longer-term studies, including lower level

452   chemical exposures, are needed, since heart valve formation and remodelling (e.g. AV valve

453   transitioning from two to four leaflets) is not complete in zebrafish until 35 dpf (62,63) and effects

454   may not become functionally manifest until in later life. Vertebrate models with short life spans,

455   such as zebrafish are highly amenable for this work.

456

FIGURES

458

459
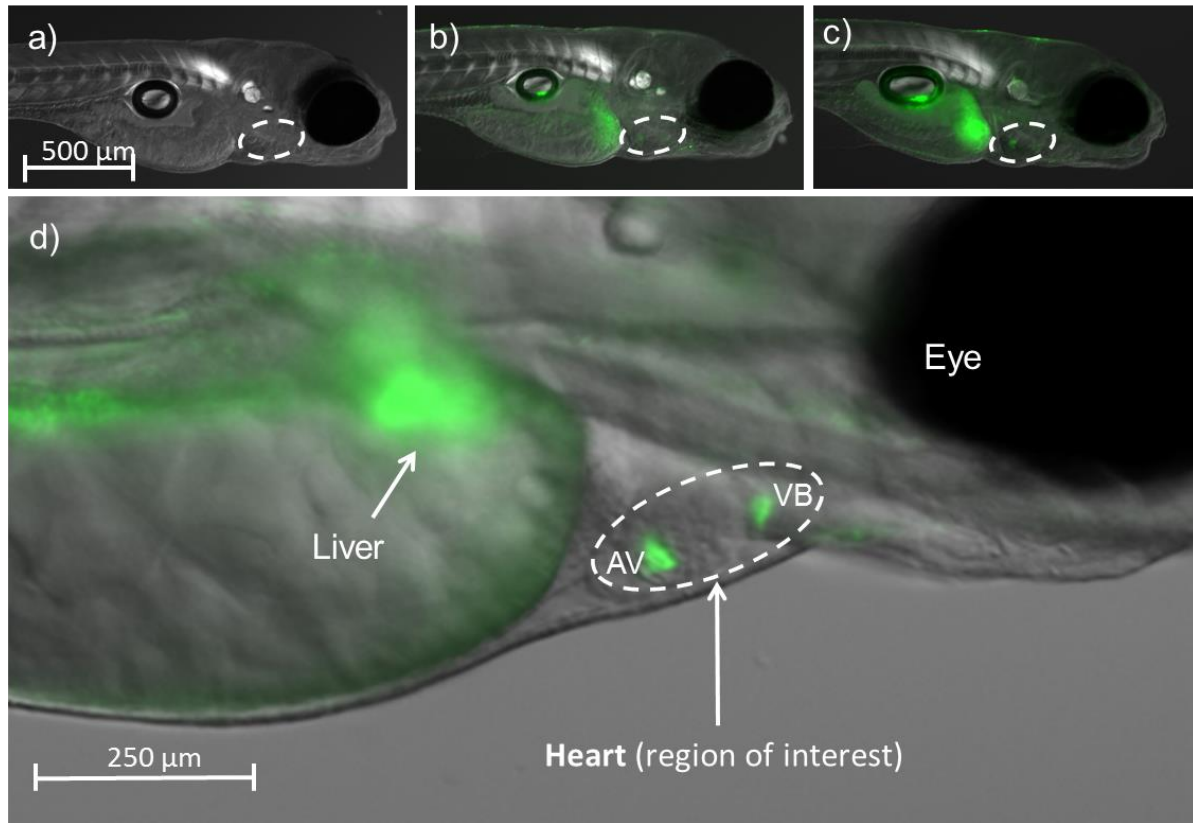
Figure 1. GFP fluorescence locating and quantifying ERE activation by BPA and MBP in the

heart in embryo-larval zebrafish

Embryo-larval zebrafish at 5 dpf: a) Solvent control; b) 1000 µg/L BPA; c) 25 µg/L MBP;
d) Close up of the region of interest showing the heart (encircled).

Fluorescence indicating Estrogen Response Element (ERE) activation was concentrated in the
Atrio-Ventricular (AV) and Ventricular-Bulbous (VB) valves.

466
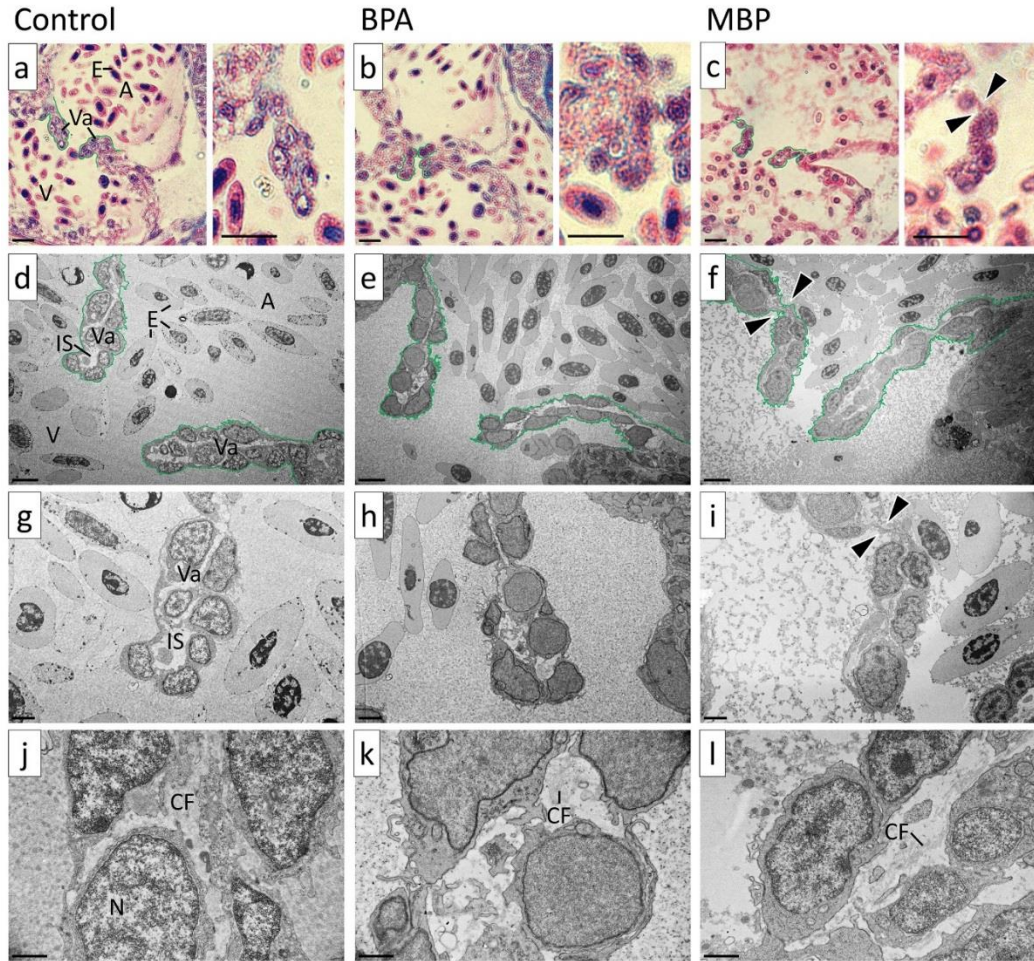
Figure 2. Images of atrio-ventricular (AV) valve leaflets from high-level BPA and MBP exposure treatments versus solvent controls at 15 days post fertilisation (dpf)

Bright field images a-c are shown at ×100 magnification: AV valve leaflets were bent in the high-level MBP exposure. TEM images d-l are shown at ×3000, ×6000 and 20000 magnification: The extra-cellular matrix between the bilayer of valvular cells was narrower and lacked collagen in the high-level BPA and MBP exposure treatments compared to solvent controls (qualitative assessment). Annotations: A = Atrium, V = Ventricle, E = Erythrocytes, Va = Valve leaflet, IS = Interstitial Space, CF = Collagen Fibres, N = Nucleus, Arrow heads indicate bent AV valve leaflet. Scale bar (bottom left in each image): a-c = 10 μm; d-f = 5 μm; g-i = 2 μm; j-l = 1 μm.
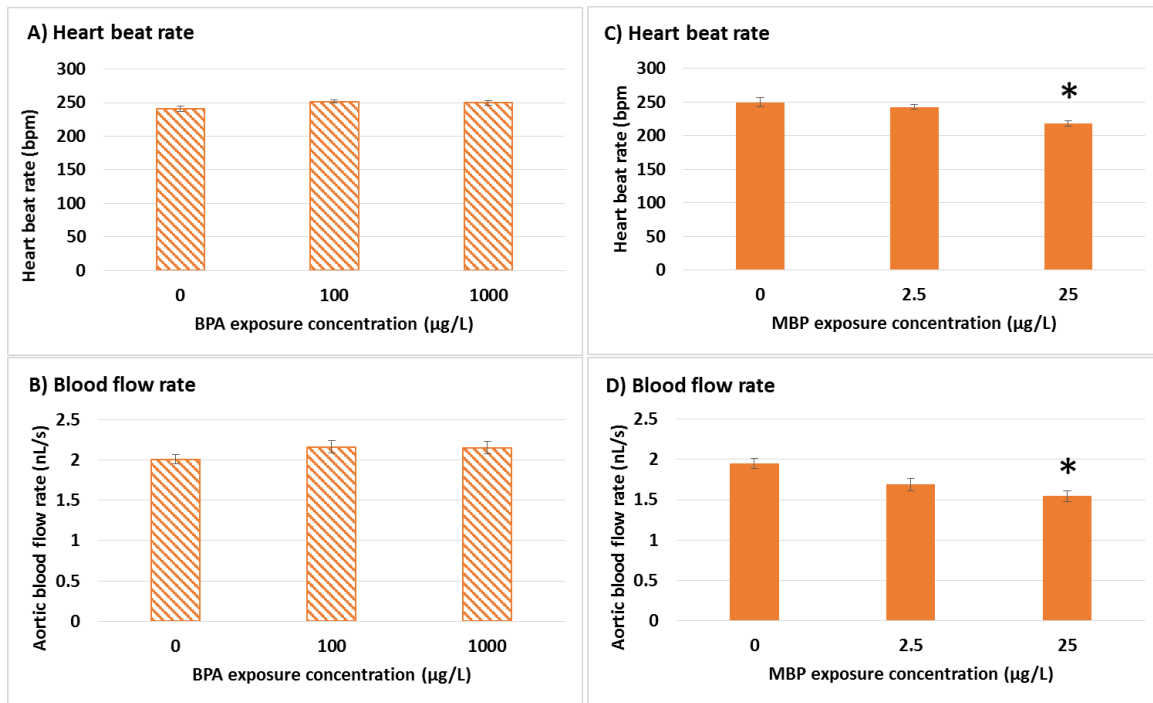
Figure 3. Effects of BPA and MBP exposure on cardiovascular function in zebrafish larvae at 15 days post fertilisation (dpf)

Hatched bar charts (A-B) represent BPA, solid bar charts (C-D) represent MBP. Data represent 6 individual fish taken randomly from each of 6 separate aquaria (n=6 experimental replicates) per exposure treatment. Bar heights represent means, error bars represent 95% confidence intervals. Significant differences from 0 µg/L solvent control ($p>0.05$) are highlighted with an asterisk.
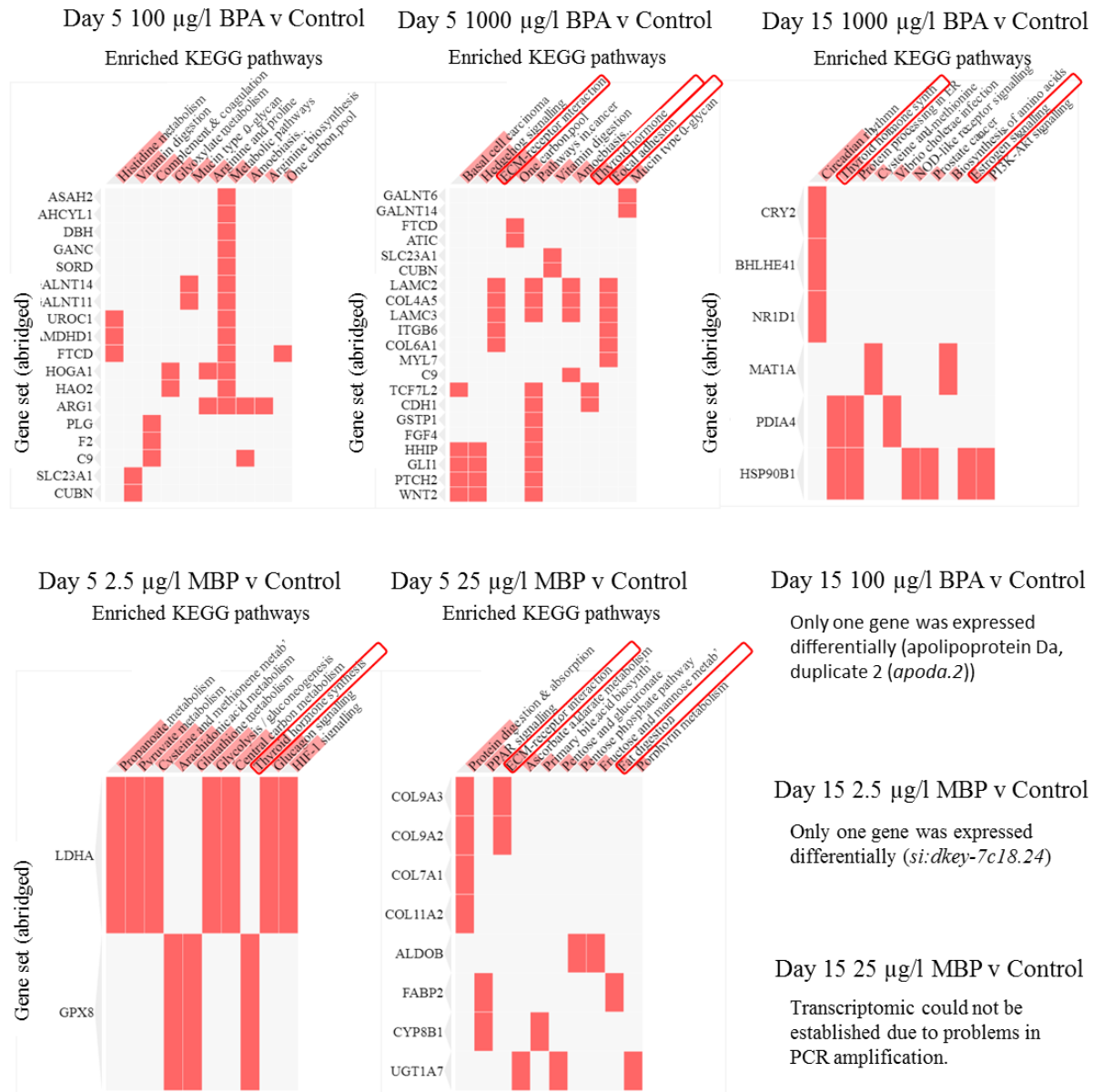
Figure 4: Gene set enrichment for KEGG pathways in heart tissues from 5 and 15 day old larval zebrafish in BPA and MBP exposure treatments (versus solvent controls).

Sequence data were generated from hearts pooled from ~30 individuals from each of 4 separate aquaria (nominally n=4 experimental replicates) per exposure treatment. Enriched pathways were identified using Enrichr and referenced to the KEGG database (2016). Pathways highlighted in red boxes are calcific aortic valve disease (CAVD) biomarkers. (Also see enriched Reactome pathways (in SI Figure S10).

500
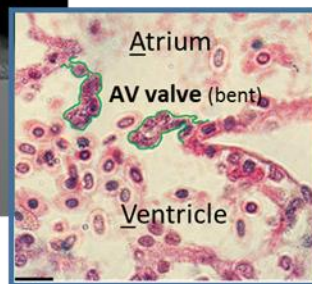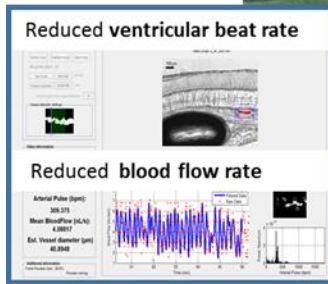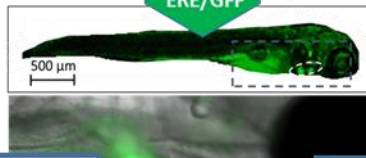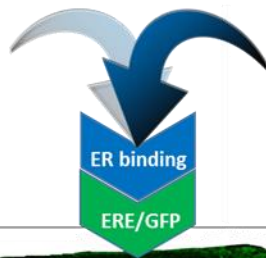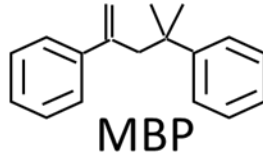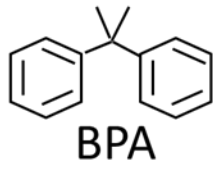
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532

533   **SUPPORTING INFORMATION**

534   Tables describing: zebrafish heart and heart valve morphogenesis (valvulogenesis); water quality;

535   test substance analysis; ERE response - relative mean fluorescence intensity induced by BPA and

536   MBP in the heart valves; effects on CV function; specific growth rate (SGR) and critical swimming

537   speed (U_{critb}); differentially expressed genes and enrichment for GO terms, KEGG and Reactome

538   pathways and TFBS motifs in BPA and MBP exposure treatments.

539   Figures showing: Nuclear Magnetic Resonance Spectrum for MBP; relative fluorescence in ERE-

540   GFP transgenic zebrafish larvae following exposure to BPA and MBP; effects of BPA and MBP

541   exposure on specific growth rate (SGR) and critical swimming speed (U_{critb}); heat maps showing

542   differentially expressed genes; Venn diagrams showing overlap in differentially expressed genes

543 for BPA and for MBP exposure treatments and for time points 5 and 15 dpf; cluster plots showing

544 enrichment of Reactome pathways and TFBS motifs in BPA and MBP exposure treatments.

545

546 **ABBREVIATIONS**

547 AIC - Akaike information criterion

548 AV - atrio-ventricular (valves)

549 BCF - bio-concentration factor

550 BPA - bisphenol A

551 CAVD - calcific aortic valve disease

552 CV - cardiovascular

553 DMSO - dimethyl sulfoxide

554 ECM - extra-cellular matrix

555 EDC - endocrine disrupting chemical

556 ER - estrogen receptor

557 ERE - estrogen response element

558 GFP - green fluorescent protein

559 LOQ - limit of quantitation

560 MBP - 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene

561 PCR - polymerase chain reaction

562 ROI - region of interest

563 SGR - specific growth rate

564 TEM - transmission electron microscopy

565 TFBS - transcription factor binding site

566   TG - transgenic

567   VB - ventricular-bulbus (valves)

568

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