# Cardiovascular effects and molecular mechanisms of bisphenol A and its metabolite MBP in zebrafish

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**KEYWORDS**

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**ABSTRACT**

The plastic monomer bisphenol A (BPA) is one of the highest production volume chemicals in the world and is frequently detected in wildlife and humans, particularly children. BPA has been associated with numerous adverse health outcomes relating to its estrogenic and other hormonal properties, but direct causal links are unclear in humans and animal models. Here we simulated measured (1×) and predicted worst-case (10×) maximum foetal exposures for BPA, or equivalent

concentrations of its metabolite MBP, using fluorescent reporter embryo-larval zebrafish, capable of quantifying Estrogen Response Element (ERE) activation throughout the body. Heart valves were primary sites for ERE activation by BPA and MBP, and transcriptomic analysis of micro-dissected heart tissues showed that both chemicals targeted several molecular pathways constituting biomarkers for calcific aortic valve disease (CAVD), including extra-cellular matrix (ECM) alteration. ECM collagen deficiency and impact on heart valve structural integrity were confirmed by histopathology for high-level MBP exposure, and structural defects (abnormal curvature) of the atrio-ventricular valves corresponded with impaired cardiovascular function (reduced ventricular beat rate and blood flow). Our results are the first to demonstrate plausible mechanistic links between ERE activation in the heart valves by BPA's reactive metabolite MBP and the development of valvular-cardiovascular disease states.

## INTRODUCTION

Over 1400 chemicals have been identified as potential endocrine disrupting chemicals (EDCs) (1) with potential to "alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations" (2-5). Over 100 of these chemicals are regarded internationally as priority EDCs and almost half (45%) are estrogenic i.e. estrogen receptor (ER) and/or estrogen-related receptor (ERR) agonists (6-8). Estrogens play a fundamental role in the formation and function of numerous organs and systems (9) and imbalances are known to increase risks of cancers and disorders of reproductive, nervous, metabolic, immune and cardiovascular systems in various animal models and humans (10-14). However, linking cause and effect remains a major challenge in chemical risk/safety assessment. 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* (×10-1000) (40); stimulation of
54 uterine growth in rats (×500) (41); elevated estrogen receptor (*esr1*) and vitellogenin (*vtg1, vtg2*)
55 gene and protein expression in medaka (*Oryzias latipes*) (×250-400) (42,43); ERE activation in
56 zebrafish (*Danio rerio*) (×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

facilitate the detection of chemical effects throughout the body *in vivo*, in real time (45-49) and can help establish causal links with subsequent adverse effects on biological development and/or function (39,44). Here we exploit TG(ERE:GFP)Casper zebrafish to study the effects of BPA and its highly estrogenic metabolite MBP on cardiovascular (CV) development and function, building on previous work using this model, which highlighted the heart, and heart valves in particular, as being key targets for these compounds (39,44,47,48).

## MATERIALS AND METHODS

### Test substances

Bisphenol A or BPA: 2,2-bis(4-hydroxyphenyl)propane (99% pure, CAS No. 80-05-7) was obtained from Sigma-Aldrich Company Ltd., Dorset, UK.

The BPA derivative MBP: 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (99% pure, CAS No. 13464-24-9) was synthesised at the University of Exeter (SI (Figure S1).

### Test organisms

Test organisms were 3$^{rd}$ generation homozygous TG(ERE:GFP)Casper zebrafish (*Danio rerio*) (48), combining a green fluorescent protein (GFP) reporter system for estrogen response element (ERE) activation (45) in a translucent Casper phenotype (50). This translucent model extends the use of fluorescent reporters to life-stages >5 dpf, which would otherwise gain skin pigmentation that interferes with GFP detection (48). This is an important feature of the model, as EDC effects may vary both within a tissue and between body tissues at different life-stages (4,51,52). The life-stages selected for this study represent two key landmarks in CV development: 5 days post fertilisation (dpf) marking formation of the endocardial rings (precursors to the heart valve

leaflets); 15 dpf marking elongation of the valve leaflets (53-55). The latter life-stage also corresponds with the depletion of the egg yolk and peaks in metabolism (oxygen consumption) and heart beat rate (56).

The zebrafish is an established model for biomedical research on CV development, function and disease (53,55,57), including heart valve (mal)formation (58-61). Despite some basic anatomical differences from the human heart (58,62), the cellular and molecular mechanisms of heart development are highly conserved between zebrafish and humans (55,63) and zebrafish have several major advantages over other vertebrate models. Heart formation (including valvulogenesis and remodelling, outlined in detail in Supporting Information SI Table S1) is completed by 35 dpf (62,63), but a heartbeat is detectable as early as 1 dpf, with blood circulation beginning soon thereafter (64). Standard (resting) heart beat rate in 5-15 dpf embryo-larval zebrafish (160–260 beats per minute; bpm) is much closer to resting human foetal heart rate (130–170 bpm) than in rodents (300–600 bpm) (56,65-67). Furthermore, respiration in embryo-larval stages relies mainly on cutaneous diffusion of $O_2$ and $CO_2$, rather than transport by convective blood circulation (68), enabling the *in vivo* study of late phenotypes of congenital CV malformations, which would be lethal in mammals (57).

**Ethical statement**

All experimental procedures with zebrafish were conducted in accordance with UK Home Office regulations for the use of animals in scientific procedures and followed local ethical review guidelines and approval processes. Water quality was assessed daily (SI Table S2).

**Chemical exposure**

TG zebrafish embryos/larvae were exposed in the laboratory from 6 hours post fertilisation (hpf) to 5 days post fertilisation (dpf) or from 6 hpf to 15 dpf, to aqueous concentrations of BPA (solvent (0.5% DMSO) control 0; low 100; high 1000 µg/L) or its metabolite MBP (solvent control 0; low 2.5; high 25 µg/L) in glass 1L aquaria (n=6 aquaria per exposure treatment), positioned in random order and each containing ~120 randomly assigned embryos. The lower BPA concentration was expected to represent maximum measured human maternal-foetal-placental unit concentrations of up to 105 ng/g (69,70), based on bioconcentration factors ranging from 0.25 to 5.7 in larval and adult zebrafish (39,71,72). Both lower and 10× higher (worst case) BPA concentrations were substantially below maximum tolerable concentrations (48). MBP exposure concentrations were based on a relative potency of 250× compared to BPA, measured *in vivo* in juvenile medaka (43). Stock solutions were prepared by dissolving pure test chemicals in analytical grade dimethyl sulfoxide (DMSO) and then diluting (200×) in 400 mL of embryo culture water (73) to give the desired nominal exposure concentrations in 0.5% DMSO. The pH of stock solutions was checked and adjusted to 7.5, as necessary. For the longer-term (0-15 dpf) exposure, 90% water changes were undertaken every 2 days, after commencing feeding twice a day at 6 dpf with excess <100 µm particulate fish food (ZM000) and with *Artemia salinus* nauplii from 10 dpf. Exposure solutions were maintained at 28°C, under a 16h:8h light : dark photoperiod cycle with a 15 min dawn/dusk transition.

Concentrations of exposure solutions were measured in three replicate aquaria per exposure treatment at the start and end of each exposure period. Chemical body burden was also measured in whole zebrafish embryo-larvae at 5 dpf, and in composite samples of ×30 hearts extracted from 5 dpf embryos (n=3 composite samples per treatment). Heart extraction was performed *en masse*: ×50 larvae per aquarium were disrupted in ice-cold Leibovitz's L-15 Medium (Invitrogen, UK)

containing 10% foetal bovine serum, using a 6 ml syringe and 19 gauge needle (74), and ×30 hearts were isolated using a 30 µm mesh sieve followed by manual sorting in ice-cold Leibovitz's L-15 Medium under a 5× objective on an Olympus SZX16 microscope (Olympus, UK). Fish/hearts were placed in embryo culture water with a terminal dose of anaesthetic of 2 mg/mL tricaine methanesulfonate (MS222) at pH 7.5, then dried under vacuum, macerated and extracted in a solution of 80:20 water:acetonitrile containing an internal standard. Details of chromatographic separation and mass spectrometry analysis of BPA and MBP in water and fish tissues are provided in the SI (Table S3). The limit of quantitation (LOQ) was 0.05 µg BPA/L and 0.05 µg MBP/L for water, and 0.5 ng BPA/g and 0.5 ng MBP/g for fish tissue. Bioconcentration factors ($BCF_{whole\ body}$ and $BCF_{heart}$) were calculated based on a mean whole body wet weight of 1200 µg for 5 dpf zebrafish larvae and a ventricle weight of 10% of the whole body weight at 5 dpf (75).

Following chemical exposure, TG zebrafish larvae were selected randomly from each aquarium and were subject to the following effects analyses, which were conducted at 28°C.

**Quantifying ERE activation (estrogenicity)**

ERE activation was quantified in the atrio-ventricular (AV) and ventricular-bulbus (VB) valves as follows. At 5 and 15 dpf ×6 larvae per replicate aquaria (n=6) were washed and then anaesthetised in 0.1 mg/mL MS222 at pH 7.5 and mounted *in vivo* in 1% low melting agarose in embryo culture water with MS222, and then placed into a glass bottom 35 mm dish (MatTek, Ashland, MA, USA). Larvae were orientated right side down and images were obtained using an inverted compound microscope (Zeiss Axio Observer, Cambridge, UK) with a 10× objective, under consistent GFP excitation for a scanning time of 180 ms, using filter set 38 HE: Excitation BP 470/40 nm, Beam splitter FT 495 nm, Emission BP 525/50 nm. GFP expression was quantified as the relative mean

pixel intensity of green fluorescence (relative to the solvent control) in a defined region of interest (ROI) encompassing the heart, using ImageJ software (76).

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

Histopathology of the heart and heart valve leaflets was conducted on ×6 whole zebrafish larvae per replicate aquaria (n=6) at 5 and 15 dpf, following terminal anaesthesia in 2 mg/mL MS222 at pH 7.5, and destruction of the brain. For visible light microscopy, zebrafish were fixed in Bouin's solution (Sigma Aldrich, Dorset, UK), progressively dehydrated in 70-100% industrial methylated spirits and embedded in paraffin wax. For transmission electron microscopy (TEM), zebrafish were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1M PIPES buffer (pH 7.2) for 24 h, washed with buffer (3 × 5 min) and post-fixed for 1 h in 1% osmium tetroxide (reduced with 1.5% w/v potassium ferrocyanide) in 0.1M sodium cacodylate buffer (pH 7.2). After a series of washes in deionised water (3 × 5 min) the larvae were subject to in-block staining with 1% uranyl acetate for 30 mins, then 3 × 5 min washes with deionised water and then the larvae were dehydrated through an ethanol gradient and embedded in Spurr resin (TAAB Laboratories, Aldermaston, UK). Sagittal sections were obtained through the midline of the atrium and ventricle (to examine the AV valves). For visible light microscopy, serial sections (5 μm) were obtained and placed on glass slides, stained using Masson's trichrome and examined using a Leitz Diaplan light microscope [×(10-100) magnification] to assess for structural pathologies (focusing on valvular cells and interstitial extracellular matrix). For TEM, 70 nm ultrathin sections were collected on pioloform-coated EM copper slot grids (Agar Scientific, Stansted, UK) and were analysed using a JEOL JEM 1400 operated at 120kV, and images taken [×(3000-20000)

magnification] with a digital camera (ES 100W CCD, Gatan, Abingdon, UK) to assess for any ultra-structural effects on the heart valves.

**Quantifying CV function**

Non-invasive video analysis of the heart and dorsal aorta was used to measure multiple cardiovascular (CV) endpoints simultaneously (67). At 15 dpf ×6 larvae per replicate aquaria (n=6), were anaesthetized in 0.1 mg/mL MS222 and embedded right side down in 1% agarose in a single well of a press-to-seal silicon isolator (Sigma-Aldrich, Poole, UK) on a clear microscope slide. The slide was then viewed using an inverted light microscope (Leica DM IRB, Leica Microsystems UK Ltd., Milton Keynes, UK, 5× objective) fitted with two high speed video cameras. The first camera (Grasshopper® GRAS-50S5C-C, Richmond, Canada) recorded ventricular heart beat at 30 frames per second (the atrium was obscured at 15 dpf). The second camera recorded blood flow in the dorsal aorta, caudal to the swim bladder, at 120 frames per second (Grasshopper® GRAS-03K2M-C, Richmond, Canada). Both cameras were focused independently on their respective regions of interest, to ensure optimal image quality, and set to record simultaneously for 5 min; recordings from the last 3 mins were subject to image analysis as follows. Resting (standard) ventricular beat rate (bpm - beats per minute) was measured using MicroZebraLab™ image analysis software (v3.5, ViewPoint, Lyon, France). Resting (standard) aortic blood flow rate (nL/s) was measured using ZebraBlood™ (v1.3.2, ViewPoint, Lyon, France).

**Quantifying metabolic scope**

Metabolic scope, combining scope for growth and scope for movement, were measured in terms of specific growth rate (SGR) (77) and critical swimming speed ($U_{crit}$) (78), respectively. SGR was calculated by measuring individual standard body length [±0.01 mm from snout to caudal peduncle] of TG zebrafish larvae (×10 per aquarium) under anaesthetic (0.1 mg/mL MS222) at 5 and 15 dpf, using an Olympus SZX16 microscope and cellSens$^{TM}$ image analysis software. Since individual fish were not tagged, mean specific growth rate (mean SGR as % standard body length per day) was calculated for each aquarium (Equation 1). Critical swimming speed ($U_{critb}$ as standard body lengths/sec) was then assessed at 15 dpf, by placing ×5 larvae at a time in a cylindrical swim flume of length 25 cm, internal diameter 2 cm and volume 78.55 mL, and increasing laminar water flow incrementally by 1.33 cm/sec every 300 secs (5 mins) until the time to exhaustion for all individual larvae. $U_{critb}$ was calculated based on aquarium-mean standard body length at 15 dpf (Equation 2).

**Equations**

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

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

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

Where U is penultimate swimming speed (mean standard body lengths/sec); $U_i$ is velocity increment (1.33 cm/sec); t is time swum in final velocity increment (secs); $t_i$ is time interval for each increment (300 secs).

**Transcriptomic profiling of heart tissues**

Hearts were removed from TG zebrafish larvae following terminal anaesthesia in 2 mg/mL MS222 at pH 7.5, followed by destruction of the brain. At 5 dpf ×50 larvae per replicate aquaria (n=4) were disrupted in ice-cold Leibovitz's L-15 Medium (Invitrogen, UK) containing 10% foetal bovine serum, and hearts were extracted *en masse*, as described above. Hearts from larvae of 15 dpf were micro-dissected individually using fine tip forceps (Dumont Inox #5SF) and an Olympus SZX16 microscope. Hearts for the 15 dpf larvae were pooled (×30 per aquarium) and then snap frozen in liquid nitrogen. Total mRNA was extracted from each pool of 30 hearts using RNeasy micro-kits with on-column DNase treatment (Qiagen, UK) and RNA integrity (RIN) scores were confirmed to be in the range 7.4-8.7 using an Agilent 2200 TapeStation (Agilent Technologies Ltd. Berkshire, UK). cDNA libraries were prepared with polyA isolation using Illumina TruSeqTM 2 Stranded mRNA Library Preparation kits and subsequent cluster generation was conducted using TruSeqTM Paired-End Cluster Generation kits (Illumina, San Diego CA, USA). Up to 24 cDNA libraries were prepared for sequencing for each test substance (BPA and MBP): nominally n=4 replicate pooled samples from each of 3 treatment groups (solvent control, low, high exposure), for two time points (5 dpf and 15 dpf). Libraries were sequenced with 12 per lane using an Illumina HiSeq 2500 in standard mode, generating 100 base pair reads (paired-end).

**Statistical analysis**

Prior to statistical analysis, phenotypic data were tested for normality (Anderson–Darling test) and homogeneity of variance (Bartlett's or Levene's tests) using Minitab 16 (Minitab, Coventry, UK). Statistical analysis of phenotypic/functional endpoints was performed using linear mixed effects (lme) models (R-statistics version 3.3.2, R Foundation for Statistical Computing) and all lme models included aquarium as a random effect. A multi-variate MANOVA was used to assess the

fixed effect of exposure treatment on cardiovascular function, combining both ventricular heart beat rate and blood flow (using the function 'cbind'). Statistical significance ($p<0.05$) of treatment effects on these and other individual phenotypic endpoints was also determined by 'one-way' comparisons of untransformed heart beat or blood flow data, and lme model fit was measured using the Akaike Information Criterion (AIC). Phenotypic/functional effects data are presented in the text or shown graphically as the mean ± 95% confidence interval.

Transcriptomic data were quality-trimmed and filtered (to remove sequencing adapters) and then processed using the TopHat/ Cufflinks pipeline (79), followed by differential gene expression analysis comparing chemical exposure treatments with control treatments using DESeq2 v3.6 and an adjusted *p*-value of <0.05 set as the false discovery rate (80).

Gene Set Enrichment Analysis (GSEA) using DAVID v6.8 (81), Enrichr (82) and Reactome v66 (83) was conducted on sets of differentially expressed genes (for each chemical exposure treatment) to identify over-represented Gene Ontology (GO) terms for biological processes and functional pathways, including KEGG v88 (84) and Reactome v66 pathways (83) referenced to the zebrafish (GRCz10) and the human genome (GRCh38.p12).

Transcription Factor Binding Site (TFBS) motif enrichment analysis was conducted on 5 and 50 kilobase (kB) long DNA flanking sequences both up and downstream of the differentially expressed genes to quantify the potential for estrogen receptor interactions; flanking sequences were retrieved using BioMart from Ensembl v94 (85). Over-represented motifs in each gene set were identified using AME (86) in MEME Suite v5.0.2 (http://meme.nbcr.net). Each gene set was 'shuffled' to generate a control gene set (with matched GC content), and enriched TFBS motifs were subsequently identified using the JASPAR 2018 (CORE:vertebrate) database (87).

Gene groups were considered to be enriched when enrichment scores (EASE scores) were >1.3, and when *p*-values adjusted for multiple-testing (Benjamini-Hochberg) were < 0.1.

## RESULTS AND DISCUSSION

### Chemical exposure

Mean measured concentrations of aqueous exposure solutions were 104-128% of nominal values for 100 and 1000 µg/L BPA (117 ± 4, 1028 ± 23 µg/L) and 82-113% of nominals for 2.5 and 25 µg/L MBP (2.1 ± 0.1, 28.2 ± 0.35), and both sets of controls contained no measurable test chemical (SI Table S4). Mean measured bioconcentration factors for BPA were: 5 day $BCF_{whole\ body}$ = 2.5 and 3.8 for 100 and 1000 µg/L BPA exposures, respectively, corresponding with whole body concentrations of ~250 and ~3700 ng/g, which are equivalent to ×2.5 and ×37 maximum human maternal-foetal-placental unit concentrations of 105 ng/g (69,70). Mean measured 5 day $BCF_{heart}$ = 0.09 for the 1000 µg/L BPA exposure was substantially lower than the corresponding $BCF_{whole\ body}$. The mean measured bioconcentration factor for MBP was: 5 day $BCF_{whole\ body}$ = 27 for the 25 µg/L MBP exposure, corresponding with a whole body concentration of 675 ng/g. MBP was not detectable above the LOQ (0.5 ng/g) in heart tissue, so a 5 day $BCF_{heart}$ could not be determined.

### ERE activation

Exposure to BPA or MBP induced fluorescence from the ERE:GFP reporter in the liver and in the region of interest (ROI) encompassing the heart, specifically in the atrio-ventricular (AV) and the ventricular-bulbus (VB) valves (Figure 1), which is consistent with previous studies (44,48). 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 ±
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 $BCF_{whole\ body} = 27$), 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 (×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 (×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 ± 4 versus 249 ± 7 bpm; $p = 0.023$) and aortic

blood flow rate (1.55 ± 0.07 versus 1.95 ± 0.06 nL/s; $p = 0.03$) in 15 dpf zebrafish, according to the linear mixed effect model lme(Rate ~ Treatment, random=~1|Tank1) (Figure 3; SI Table S6). These results fall within ranges reported at 28°C for zebrafish aged 3.5 to 21 dpf for resting heart beat rate (160 to 260 bpm) (56,65-67) and resting blood flow rate (500 to 1860 µm/s; equivalent to 0.25 to 2.1 nL/s) (67,89,90). Ontogeny of embryo-larval development in zebrafish is such that heart beat and blood flow rate vary substantially with development time and peak at 10–20 dpf, a period which corresponds with a peak in aerobic metabolism (56). Therefore CV performance is likely to be most critical for our selected life-stage (15 dpf) and reduced CV performance (20% reduction in blood flow rate) could be biologically significant. The proportional reduction in blood flow (20 ± 3.6%) was greater than for heart beat rate (12 ± 2.3%), potentially indicating valve prolapse (although note the variation in these CV parameters). We were unable to observe valve function directly because of tissue thickening, nor were we able to demonstrate AV decoupling as being symptomatic of valve prolapse (91), since organ growth prevented imaging of both atrial and ventricular beating at 15 dpf. Nevertheless BPA exposures up to 2500 µg/L have been shown to induce erratic AV beat ratios in 5 dpf zebrafish (44).

**Effects on metabolic scope**

No effects of BPA or MBP were seen on metabolic scope (i.e. SGR and $U_{critb}$) (SI Figure S3 and Table S7). These energetic measures provide a general indication of individual metabolic scope, i.e. energetic reserves beyond basal metabolism (92). $U_{critb}$ integrates anaerobic as well as aerobic scope for movement (93) and also cardio-respiratory performance (65). The lack of significant effects (despite downward trends for MBP) may have been due to substantial inter-individual 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

(8 genes) that were differentially expressed in the low-level MBP exposure (SI Figure S6 - S7). Collectively however, the genes affected by both BPA and MBP at 5 dpf shared significant enrichment for molecular pathways concerning i) nuclear receptor and calcium signalling (estrogen and (para)thyroid), ii) lipid metabolism and iii) extra-cellular matrix (ECM) interactions (99) (Figure 4; SI Figure S8; SI Tables S8 – S10). ECM-related pathways comprising collagen and cartilage formation and organization were particularly prominent for the high-level MBP exposure, and can be related directly to phenotypic effects on the heart valves observed therein. ECM interactions were found to involve Notch signalling (dre04330) and calcium ion binding (GO:0005509) (SI Table S9) which, along with the afore mentioned pathways (i-iii), have previously been linked to the progression of calcific aortic valve disease (99,100) (CAVD - fibrosis, and subsequent calcification and thickening of the aortic valves), which affects up to 13% of human populations in the western world (99) (see later discussion). At 15 dpf fewer genes were differentially expressed compared to 5 dpf. Although only 5 genes overlapped between time points for the high-level BPA exposure treatment (SI Figure S7), there was gene set enrichment for thyroid hormone synthesis (also seen at 5 dpf for MBP) and for estrogen signalling for the 15 dpf high-level BPA exposure (Figure 4). Both hormone signaling pathways have been highlighted in mammalian studies on BPA (31,101). Overall, gene sets from 15 dpf represented pathways associated with heart function (more so than development), particularly cardiac muscle contraction (SI Tables S8 – S9). Four of the 32 genes down-regulated by the high-level BPA exposure: actinin alpha 3b (*actn3b*), myosin light chain, phosphorylatable, fast skeletal muscle a (*mylpfa*), myosin, heavy polypeptide 2, fast muscle specific (*myhz2*) and troponin I type 2a (skeletal, fast), tandem duplicate 4 (*tnni2a.4*) have previously been identified as potential biomarkers for cardiac disease 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

synthesis in rat cardiac fibroblasts (106) and, more specifically, estrogen decreases collagen I and III gene expression in fibroblasts from female rats (107). BPA has also been shown to cause sex-specific alterations in gene expression profiles, changes in the composition of the cardiac collagen extracellular matrix and altered CV function in CD-1 mice (101). Furthermore, disruption of collagen in the extracellular matrix has been shown to directly promote valve leaflet calcification *in vitro* (108). Based on these studies it is entirely plausible that the estrogenic action of high-level MBP exposure led to collagen deficiency and valve weakening in our larval zebrafish. This preliminary (pre-calcification) condition resembles 'myxomatous degeneration' characterized by collagen degradation and elastic fibre fragmentation, resulting in a "floppy" valve that is prone to prolapse and regurgitation (109). As with CAVD, it may be speculated that more progressive effects of MBP (and BPA) on heart valve development and function may occur in the longer- term in zebrafish, but this remains to be proven.

**Linking cause and effect**

Although estrogen is known to be cardio-protective in later life, inappropriate estrogenic exposures, including from estrogenic chemicals, in early life can lead to cardiac malformation (110). We demonstrate that aqueous exposure to the weak estrogen BPA at 1× and 10× maximum human maternal-foetal-placental unit concentrations, or its more potent metabolite MBP, at equivalent potency concentrations, activate estrogen responsive elements (EREs via estrogen receptor signalling) in the heart valves and alter the expression of a range of genes, including several governing CV development and function in embryo-larval zebrafish. BPA and MBP perturbed similar downstream effect pathways, but only the high-level MBP exposure resulted in 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

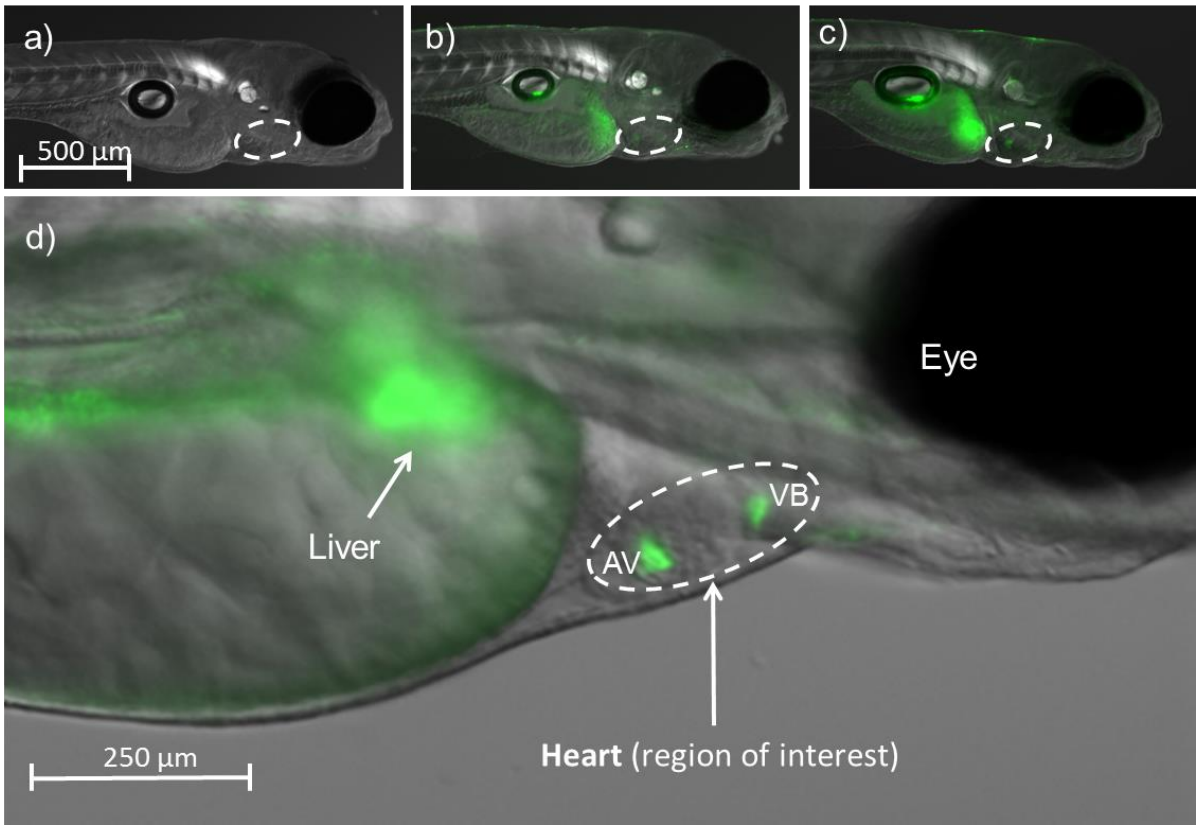



**Figure 1.** GFP fluorescence locating and quantifying ERE activation by BPA and MBP in the heart in embryo-larval zebrafish

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.

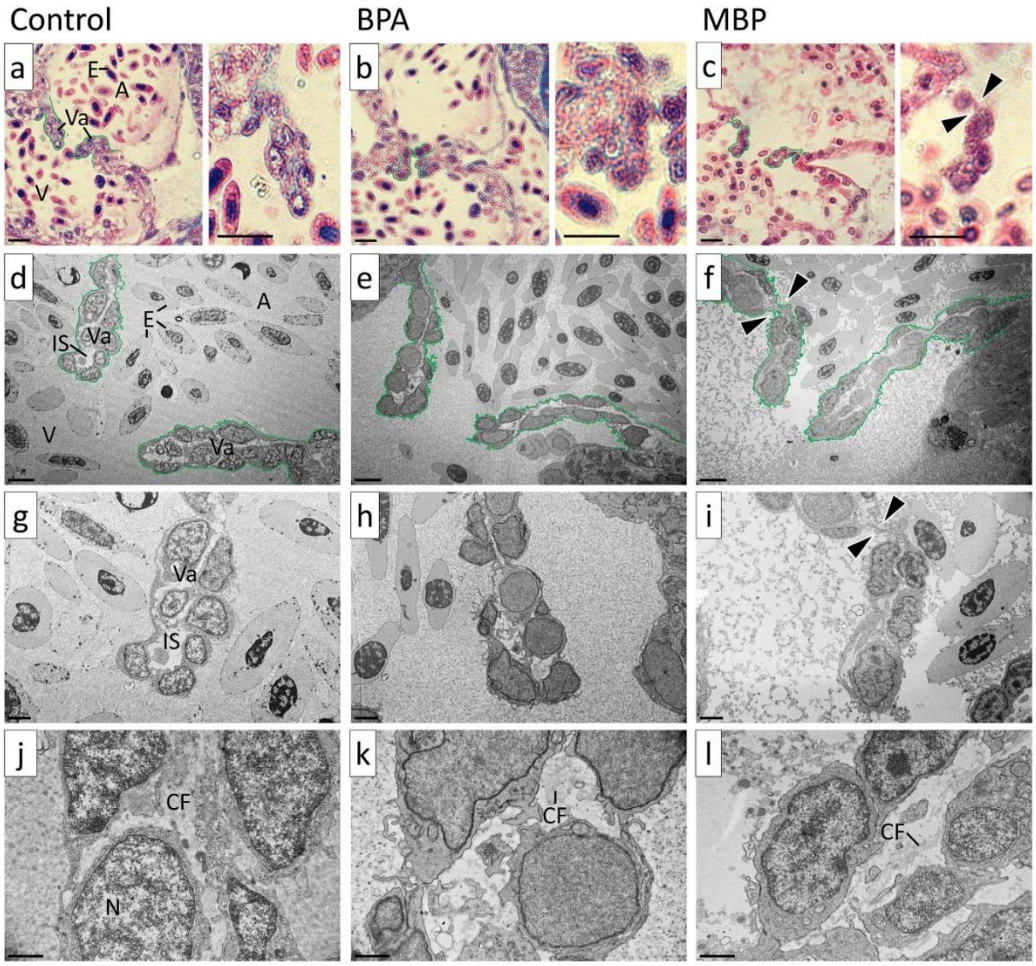



**Figure 2.** Images of atrio-ventricular (AV) valve leaflets from high-level BPA and MBP exposure treatments versus solvent controls at 15 days post fertilisation (dpf)

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.

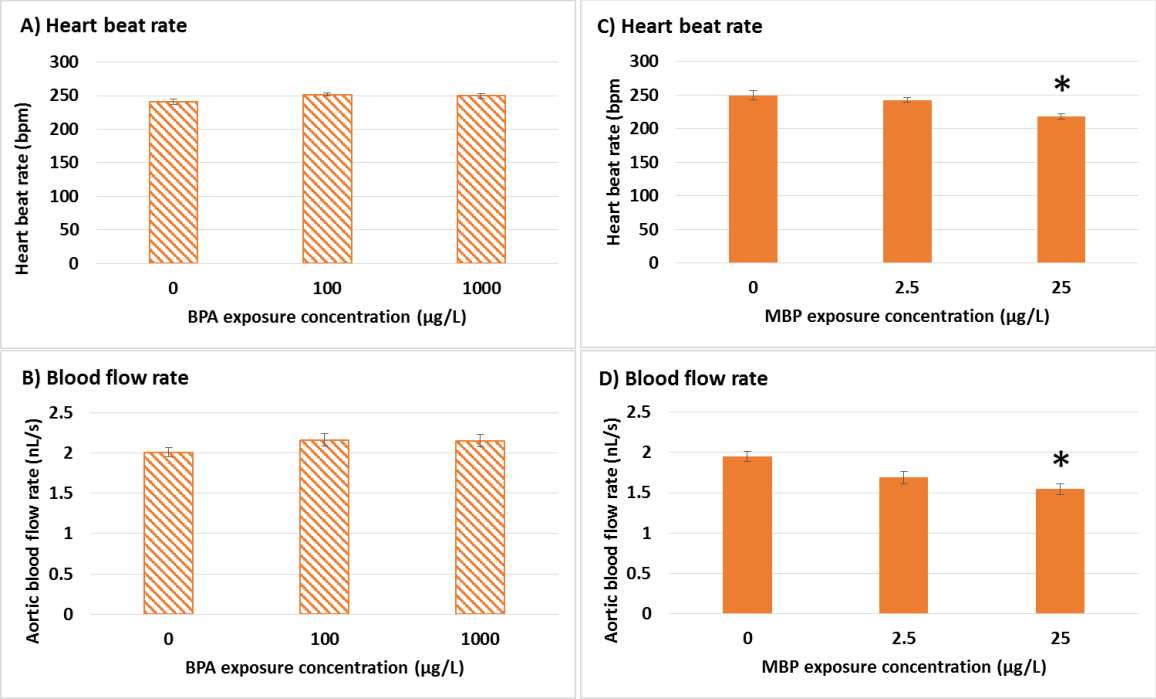



**Figure 3.** Effects of BPA and MBP exposure on cardiovascular function in zebrafish larvae at 15 days post fertilisation (dpf)

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.

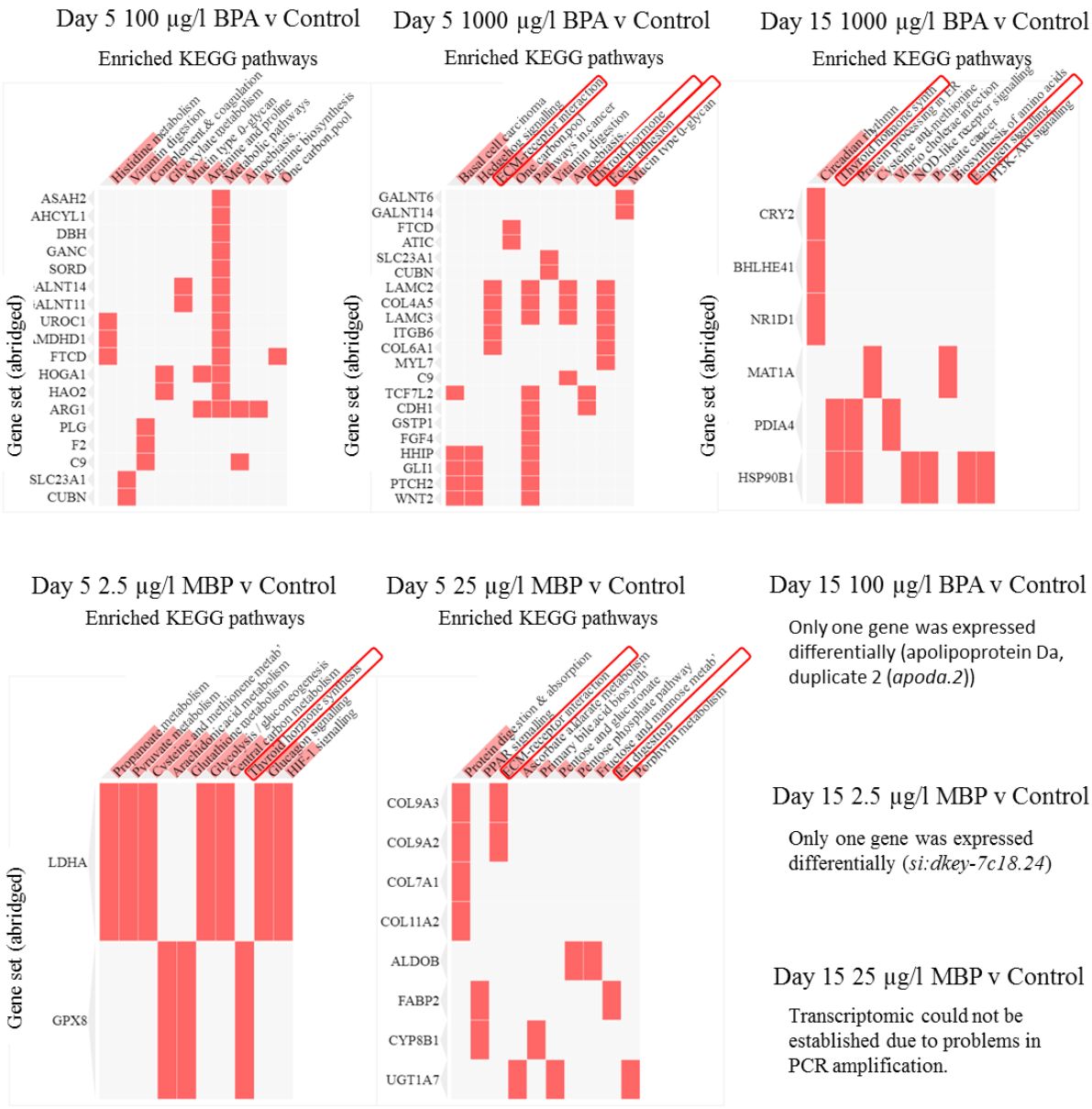
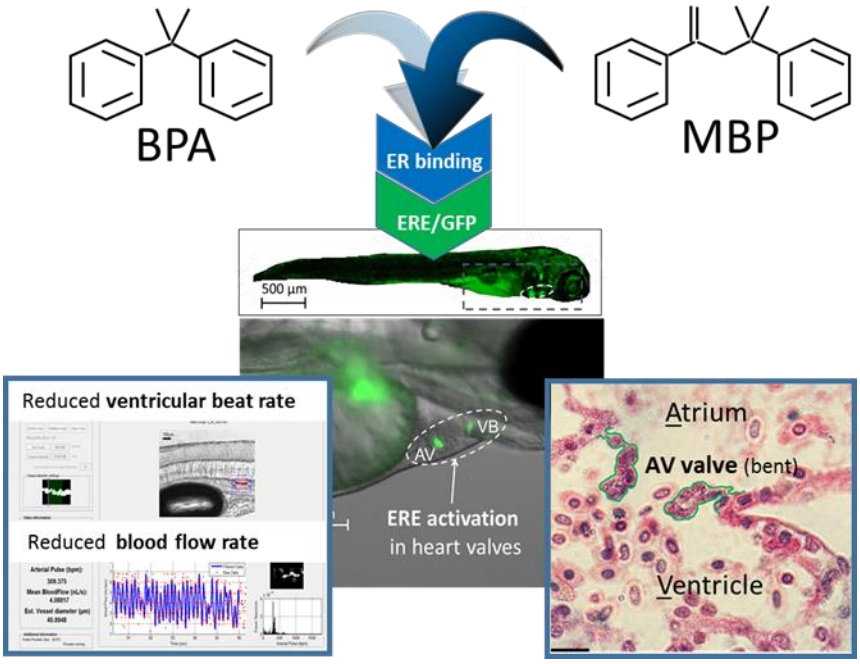



**Figure 4: Gene set enrichment for KEGG pathways in heart tissues from 5 and 15 day old larval zebrafish in BPA and MBP exposure treatments (versus solvent controls).**

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).


BPA
MBP
ER binding
ERE/GFP
500 µm
Reduced ventricular beat rate
Reduced blood flow rate
VB
AV
ERE activation
in heart valves
Atrium
AV valve (bent)
Ventricle

500

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### Author Contributions

The overall study was designed and implemented by ARB, RC, MJH and CRT. ARB ran the study, and analysed all the data presented in the manuscript; JMG helped to evaluate ERE activation via image analysis of GFP reporters and to measure chemical effects on specific growth rate and critical swimming speed; JM, LG and SM helped undertake microdissection of zebrafish hearts, DNA extraction and library preparation for sequencing; JB and MJW helped to design and undertake assays to evaluate CV function; MT undertook chemical analysis of water and zebrafish embryo-larvae; AC and CH undertook microscope and TEM work to assess for pathologies of the heart valves, AP and MEW synthesised the MBP used in the study; MJH, RAC and CRT helped to manage the overall study. The manuscript was written through the contributions of each author, all of whom have given approval to the final version of the manuscript.

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## SUPPORTING INFORMATION

Tables describing: zebrafish heart and heart valve morphogenesis (valvulogenesis); water quality; test substance analysis; ERE response - relative mean fluorescence intensity induced by BPA and MBP in the heart valves; effects on CV function; specific growth rate (SGR) and critical swimming speed ($U_{critb}$); differentially expressed genes and enrichment for GO terms, KEGG and Reactome pathways and TFBS motifs in BPA and MBP exposure treatments.

Figures showing: Nuclear Magnetic Resonance Spectrum for MBP; relative fluorescence in ERE-GFP transgenic zebrafish larvae following exposure to BPA and MBP; effects of BPA and MBP exposure on specific growth rate (SGR) and critical swimming speed ($U_{critb}$); heat maps showing differentially expressed genes; Venn diagrams showing overlap in differentially expressed genes

for BPA and for MBP exposure treatments and for time points 5 and 15 dpf; cluster plots showing enrichment of Reactome pathways and TFBS motifs in BPA and MBP exposure treatments.

## ABBREVIATIONS

AIC - Akaike information criterion

AV - atrio-ventricular (valves)

BCF - bio-concentration factor

BPA - bisphenol A

CAVD - calcific aortic valve disease

CV - cardiovascular

DMSO - dimethyl sulfoxide

ECM - extra-cellular matrix

EDC - endocrine disrupting chemical

ER - estrogen receptor

ERE - estrogen response element

GFP - green fluorescent protein

LOQ - limit of quantitation

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

PCR - polymerase chain reaction

ROI - region of interest

SGR - specific growth rate

TEM - transmission electron microscopy

TFBS - transcription factor binding site

TG - transgenic

VB - ventricular-bulbus (valves)

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