Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription and reduces global DNA methylation in breeding zebrafish (*Danio rerio*).

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Abbreviations: 17β-estradiol, E2; 5-formylcytosine, 5fC; 5-hydroxymethylcytosine, 5hmC; androgen receptor, *ar*; anti-Müllerian hormone, *amh*; aromatase, *cyp19a1a;* bisphenol A, BPA; DNA (cytosine-5)-methyltransferase 3, *dnmt3*; DNA methyltransferase 1, *dnmt1*; estrogen receptor 1, *esr1*; estrogen receptor 2a, *esr2a*; estrogen receptor 2b, *esr2b*; estrogen receptor, ER; gonadosomatic index, GSI; hepatosomatic index, HSI; histone deacetylase 1, *hdac1*; histone deacetylase 3, *hdac3*; methyl CpG binding protein 2, *mecp2*; methyl-CpG-binding domain protein 2, *mbd2*; methyl-CpG-binding domain protein 3a, *mbd3a*; methylcytosine, 5mC; principal component analysis, PCA; ribosomal protein L8, *rpl8*; vitellogenin 1, *vtg1*;

Abstract:

Bisphenol A (BPA) is a commercially important high production chemical widely used in epoxy resins and polycarbonate plastics, and is ubiquitous in the environment. Previous studies demonstrated that BPA activates estrogenic signalling pathways associated with adverse effects on reproduction in vertebrates and that exposure can induce epigenetic changes. We aimed to investigate the reproductive effects of BPA in a fish model and to document its mechanisms of toxicity. We exposed breeding groups of zebrafish (*Danio rerio*) to 0.01, 0.1 and 1mg/L BPA for 15 days, and observed a significant increase in egg production together with a reduced rate of fertilization in fish exposed to 1mg/L BPA was associated with significant alterations in the transcription of genes involved in reproductive function and epigenetic processes, in both liver and gonad tissue at concentrations representing hotspots of environmental contamination (0.1mg/L) and above. Of note, we observed reduced expression of DNA methyltransferase 1 (*dnmt1*) at environmentally-relevant concentrations of BPA, along with a significant reduction in global DNA methylation in testes and ovaries following exposure to 1mg/L BPA. Our findings demonstrate that BPA disrupts reproductive processes in zebrafish, likely via estrogenic mechanisms, and that environmentally-relevant concentrations of BPA are associated with altered transcription of key enzymes involved in DNA methylation maintenance. These findings provide evidence of the mechanisms of action of BPA in a model vertebrate and advocate for its reduction in the environment.

Introduction:

Bisphenol A (BPA) is a commercially important high production chemical widely used in the production of epoxy resins, utilized in food and beverage packaging, dental sealants and as a monomer component of polycarbonate plastics [1,2]. With over three million tons produced globally per annum, environmental exposure is common [3], and in the USA BPA was measurable in 75% of food products tested [4]. Human exposure occurs predominantly via ingestion of contaminated food, caused by leaching of BPA from linings of canned goods and polycarbonate packaging. BPA has also been detected in drinking water at concentrations up to 15 ng/L [5]. In addition, inhalation is thought to be a plausible secondary route of exposure [3], with BPA found to be present in 86% of domestic dust samples at concentrations ranging from 0.2 to 17.6 μg/g [6]. BPA has been detected in the urine of ~95% of adults in the USA and Asia [7,8]. It has also been measured in the serum of adult men and women [9] and in breast milk, fetal plasma and placental tissue, raising concerns about human exposures during critical periods of development [1,10].

BPA is moderately water soluble, entering the environment via direct discharge from BPA production and processing industries, wastewater treatment plants and leachate from landfill sites [11]. Its presence is ubiquitous in the aquatic environment and surface water concentrations have been detected up to the low μg/L range, with peak concentrations reaching up to 21 μg/L [12]. Concentrations in landfill leachate have been reported to reach up to 17,200μg/L [1]. Due to its ubiquitous nature, the potential for environmental exposure in wildlife populations, including fish, is very significant. Levels of BPA reported in fish vary, and 1-11ng BPA/g dry weight in the muscle and 2-75ng BPA/g dry weight in the liver have been reported [13].

BPA has been shown to act as an estrogen receptor (ER) agonist [14,15], able to bind to ERs, resulting in feminizing effects [16,17]. A study using the human cell line HepG2, found that BPA strongly activated estrogen receptor 1 (ESR1; previously known as ERα) mediated responses, but did not activate ESR2 (previously known as ERβ), while in the cell line HeLa, BPA was found to activate both ESR1 and ESR2

[14]. In fish, BPA induced *esr1* expression in the livers of male fathead minnows *(Pimephales promelas)* exposed for 4 days to 10µg BPA/L, consistent with an estrogenic mode-of-action [18]. BPA has also been shown to alter the transcriptional profile of steroidogenic enzyme genes in a time-dependent manner, including aromatase (*cyp19a1a*), which is responsible for the irreversible conversion of androgens into estrogens and is a key regulator of estrogen synthesis in the gonads. This enzyme was significantly upregulated in both the ovary and testis of *Gobiocypris rarus* exposed to 15 µg/L BPA for 7 days, followed by suppression after 35 days exposure [19].

Adverse impacts on reproduction have been observed in several fish models. A multi-generational study in fathead minnow showed that BPA reduced gonadal growth in males and females, reduced hatching in F1 offspring of fish exposed to 640 µg/L and induced the estrogen regulated egg yolk protein, vitellogenin, a well established biomarker of xenoestrogen exposure, in the liver of male fish exposed to 640 and 1280 µg/L BPA [20]. Further multigenerational studies have demonstrated the potential adverse effects associated with exposure to BPA [21,22]. Exposure to BPA in guppies has been associated with reduced sperm quality [23] and the presence of necrotic cells in the seminiferous tubules of *Xiphophorus helleri* was also reported [24]. Together, these studies demonstrate the potential reproductive consequences following exposure to relatively high concentrations of BPA in fish.

Evidence also exists supporting the involvement of BPA in the etiology of a range of human disease phenotypes including cardiovascular disease [25], altered behaviour in children [26], prostate cancer [27], and recurrent miscarriage [28]. In addition to the well-established estrogenic mode-of-action, additional mechanisms have been proposed, including potential anti-androgenic activity [29]. Low dose effects and non-monotonic dose response curves have been reported[30,31]. More recently, increasing evidence suggests that BPA may alter the epigenetic regulation of gene expression; for example, altered DNA methylation patterns have been observed both globally (i.e. changes to the total genomic content of DNA methylation) and at the regulatory regions of specific genes (i.e. locus-

specific) in mammals [32–36]. In humans, exposure to BPA in the workplace has been associated with hypomethylation of LINE-1 in spermatozoa, a marker of global DNA methylation levels in the genome [37]. Understanding the effects of BPA exposure on epigenetic processes, and how these alterations perturb expression of genes that are related to development and reproduction, are important to the evaluation of adverse effects associated with BPA exposure, both in humans and wildlife, particularly for exposures at environmentally-relevant concentrations.

To date, few studies have investigated the potential for BPA to induce epigenetic and transcriptional changes in fish. A study in *Gobiocypris rarus* found BPA exposure to be associated with altered DNA methylation in the 5' flanking region of *cyp19a1a* (aromatase), and the effects to be time-dependent [19]. In addition, a significant decrease in the expression of DNA methyltransferase 1 (*dnmt1*) in ovarian tissue has been reported, with a significant decrease in global DNA methylation [19].

Given the extensive use and ubiquity of BPA, it is important to understand the mechanisms mediating its toxic effects and the impacts these can have on both wild populations and human health. The present study aims to investigate the effects of BPA on reproduction in the zebrafish model and identify epigenetic and transcriptional changes associated with BPA exposure. We exposed breeding groups of zebrafish to BPA for 15 days to determine if reproduction was affected by the exposure. The concentrations tested included environmentally-relevant concentrations found world-wide (0.01mg/L) and at point sources (0.1mg/L) [12,38]. The highest concentration tested (1mg/L) has only been reported in landfill leachate and is unlikely to occur in surface waters, but it was included to enable a mechanistic analysis of BPA toxicity. We quantified the transcription of genes involved in epigenetic signalling and reproductive function, together with global and locus-specific DNA methylation in exposed fish.

Results:

Water Chemistry

The mean measured concentrations of BPA in the tank water were between 100 and 139% of the nominal concentrations for all treatments, and are presented in Supporting Information Table S1.

Effects of BPA on Morphometric Parameters

The mean mass and length of male and female fish were 460.0 ± 0.008 mg and 36.5 ± 0.02 mm, and 480.6 ± 0.01 mg and 35.7 ± 0.03 mm, respectively. There were no significant differences in size or condition factor (mean 0.95 and 1.05 for males and females respectively) between treatment groups.

No alterations in general feeding and swimming behaviour were observed in any spawning group, with the exception of the mortality of one female in the 0.1mg/L BPA treatment. The egg output calculations for that group were adjusted accordingly. Hepatosomatic index (HSI; the ratio of liver weight to body weight) in males was significantly increased in fish exposed to 1mg/L BPA, but no effects of BPA were observed in females (Supporting Information Figure S1). There were no significant differences in the gonadosomatic index (GSI; the ratio of gonad weight to body weight) of males or females as a result of the BPA exposure.

Effects of Bisphenol A on Reproduction

During the 10 day pre-exposure period there were no differences in cumulative egg production between treatment groups ($P = 0.098$). During the exposure, groups treated with 1mg/L BPA spawned a significantly greater number of eggs per female when compared to all other treatment groups ($P \leq 0.01$), and this increased egg production intensified throughout the exposure period

147 (Figure 1A). During the pre-exposure, fertilization success remained consistently high with no
148 significant differences between groups and an overall mean fertilization rate of 85.6%. During the 15
149 day exposure, fertilization success in colonies exposed to 1mg/L BPA significantly declined (P =
150 0.001; Figure 1B). Additionally, for this treatment group, there was a significant negative correlation
151 between the length of the exposure (number of days) and the average percentage of fertilization (R^2
152 = 0.80; P = < 0.001), indicating that the effects of BPA on fertilization became progressively more
153 pronounced over the exposure period.

154

155 *Effects of Bisphenol A on Gene Transcription*

156 Analysis of genes involved in reproductive processes in the liver revealed that *vtg1* and *esr2b* were
157 significantly up-regulated in males following exposure to 1mg/L BPA when compared to the solvent
158 control group (fold-change = 172.90, P =0.009 and fold-change = 5.40, P = 0.014, respectively). In
159 females, *esr2b* was significantly upregulated following exposure to 0.01mg/L BPA (P = 0.044). For
160 genes involved in epigenetic regulation, the most pronounced changes observed were for *dnmt1*
161 which was significantly down-regulated in the livers of females exposed to 0.01mg/L BPA (P = 0.040)
162 and in both males and females exposed to 0.1 (males: P = 0.020; females: P = 0.005) and 1mg/L BPA
163 (males: P = 0.020; females: P = 0.005). In addition, changes were also observed for histone
164 deacetylase 3 (*hdac3*), methyl-CpG-binding domain protein 2 (*mbd2*) and methyl CpG binding
165 protein 2 (*mecp2*) in males, and for *mbd2* in females (Figure 2A and B; Supporting Information
166 Figures S2 and 3).

167 In the gonads, BPA exposure was also associated with significant changes in transcription for genes
168 involved in reproductive function and on epigenetic pathways (Figure 2, 3). Principal component
169 analysis (PCA) for the testis indicated clear separations between the transcription profiles of fish
170 exposed to the solvent control and fish exposed 1mg/L BPA, based on the data for all genes

quantified (Figure 3). For ovaries, changes were more pronounced and PCA revealed a separation between fish exposed to 0.1 and 1mg/L BPA and the solvent control (Figure 3).

In the testis, the transcript encoding *esr2a* and *cyp19a1a* were significantly down-regulated in response to 1mg/L BPA (P = 0.002 and 0.018 respectively; Figure 2; Supporting Information Figure S4). There was also a significant association between the concentration of BPA and the level of transcription for *cyp19a1a* (P= 0.025; Supporting Information Table S4), which decreased with increasing concentrations of BPA. In addition, for *amh* (anti-Müllerian hormone) BPA affected gene transcription (P = <0.05) and a decreasing trend across all concentrations was observed, but this was not statistically significant (P = 0.094; Supporting Information Figure S4). Similarly to the testis, in the ovaries of exposed females, the transcript encoding *esr2a* was significantly down-regulated following exposure to 1mg/L BPA (P = < 0.001). In addition, there were similar (but non-significant) trends for other genes involved in reproductive function including *esr1* and *ar,* which appeared to decrease with increasing exposure concentrations (Figure 2; Supporting Information Figure S5).

As in the liver, *dnmt1* was significantly down-regulated in ovaries following exposure to all three BPA concentrations tested (P = 0.032, 0.032, 0.032). Although no significant group-wise changes in *dnmt1* transcription were observed in the testis (Figure 2; Supporting Information Figure S4), the expression of *dnmt1* in the testis was associated with BPA exposure concentration (R^2= 0.110; P= 0.046; Supporting Information Table S4). In addition, changes in *mbd2* transcription were observed in the testis, with a significant increase in transcription measured in males exposed to 0.01mg/L BPA (P= 0.020), but reduced expression in males exposed to 1mg/L BPA (P= 0.030; Figure 2; Supporting Information Figure S4).

Effects of Bisphenol A on Global DNA Methylation

Analysis of global DNA methylation in the gonads revealed significant decreases in the proportion of global methylation following exposure to 1mg/L BPA in both males (by 3.2%; P =0.029; Figure 4A) and females (by 4.92%; P =0.041, Figure 4B).

Effects of Bisphenol A on gene-specific DNA Methylation

Targeted DNA methylation profiling in the promoter region of *amh* revealed that exposure to 1mg/L BPA caused a small but significant increase in methylation compared to the solvent control for the first of the three CpG sites assessed in the testes (P =0.032, Figure 5, see Supporting Information Figure S6 for the position of this CpG site), with DNA methylation at this site being significantly correlated with BPA exposure concentration (R^2=0.1625; P=0.013). No differences in DNA methylation were seen for this region in ovaries from exposed female fish (Figure 5). BPA was also not associated with altered DNA methylation at two CpG sites in the 5' flanking region of the *esr1* gene in either the liver or gonads (Supporting Information Figure S7). The analysis of 11 CpG sites across the promoter of *dnmt1* identified significant increases in DNA methylation for a number of sites in the liver (in both males and females) and the testes (males). Although group-wise comparisons of this region revealed no significant differences in the female ovaries (Figure 6 and 7), *dnmt1* promoter methylation was significantly correlated with BPA exposure at various sites (positions 4, 5, 6 and 8; Supporting Information Table S4).

Discussion:

Exposure to BPA resulted in a consistent down-regulation of *dnmt1* transcription in the ovary and in the liver of both males and females following exposure to BPA, including at environmentally-relevant concentrations in females. In association with this, we found a reduction in global DNA methylation, probably due to the decrease in *dnmt1* expression. At the highest concentration tested, BPA caused reduced fertilization, potentially via estrogenic mechanisms. Together, our data provide evidence of the molecular mechanisms of action of BPA and the potential for it to cause adverse health impacts in vertebrates.

Reproductive Effects of BPA on Adult Zebrafish

We provide evidence that BPA exposure results in an impairment of reproductive function in breeding zebrafish. These effects included an increase in the number of eggs spawned and a decrease in fertilization success in groups exposed to 1mg/L BPA. A number of mechanisms may contribute to the observed effect of BPA on reproduction, including stimulation of estrogen responsive processes via the interaction of BPA or its metabolites with estrogen signalling pathways, as previously reported for a range of organisms [39–41]. We have investigated the effects of BPA on the expression of transcripts involved in reproductive function and known to be directly or indirectly regulated by estrogens.

We found no evidence for significant alterations in the transcription of *esr1* or DNA methylation across the *esr1* promoter in the gonads and livers of both sexes, but a significant association between BPA concentration and decreased transcription was found for the livers of females, and a trend for reduced expression was also observed in the ovaries and testis, similar to that described previously [31]. Disruption of ESR1 has been associated with alterations of spermatogenesis and subsequently infertility in mice [42,43], therefore suggesting that the apparent decrease in *esr1*

transcript in the testis may contribute towards the observed decline in fertilization success at this concentration.

BPA was found to down-regulate *esr2a* in both ovaries and testes, but not in the liver. Similarly, a decrease in *esr2a* transcription was reported in ovaries of *Gobiocypris rarus* exposed to 0.05mg/L BPA for 35 days, and was associated with disruption of oogenesis and the occurrence of atretic follicles [31]. These findings concur with previous studies reporting that *esr2a* is more sensitive compared to *esr1,* to the natural estrogen, 17β-estradiol (E2) [41]. In contrast, BPA caused increased transcription of *esr2b* in the livers of males and females but not in the gonads, and, importantly, for females this effect was observed at the environmentally-relevant concentration of 0.01mg/L BPA. In parallel, BPA induced a significant increase in the transcription of the egg yolk protein, *vtg1,* and an increase in HSI in males, likely as a result of increased vitellogenin production in hepatocytes, indicating an association between the induction of *esr2b* in males and the induction of *vtg1*, as previously reported for fathead minnows [44]. Together, these findings suggest that the effects of BPA on reproduction involve disruption of estrogen receptor signalling principally via *esr1* and *esr2b* in the liver, and *esr2a* in the gonads.

In addition to the disruption in estrogen receptor signalling, changes in sex steroid biosynthesis may have contributed to the observed disruption of reproduction in colonies exposed to 1mg/L BPA. We found a significant decrease in *cyp19a1a* transcript in the testis of males exposed to 1mg/L BPA, and a significant association between transcription and BPA exposure concentration. In ovaries, a decreasing trend was also observed. These findings suggest potential feedback mechanisms were activated to counteract the estrogen/androgen ratio imbalance caused by BPA, through reducing the irreversible conversion of testosterone into estrogens. Similar findings have recently been reported for *Gobiocypris rarus* following a long term exposure to BPA [19], and studies using the aromatase knockout (ArKO) mouse found ArKO males to have reduced fertility [45], demonstrating the critical role of aromatase in gametogenesis in males.

In the testis, a decrease in *amh* transcription was associated with increased BPA exposure concentrations. Similarly, in mammals down-regulation of AMH has been reported following exposure to BPA [46 47]. Exposure to 1mg/L BPA also caused significant DNA hypermethylation in the *amh* promoter in the testis (CpG 1), demonstrating that exposure to BPA caused epigenetic alterations at this specific gene locus. There was also a significant correlation between the level of methylation in CpG 1 and *amh* transcription, and with BPA exposure concentration. This suggests that epigenetic mechanisms may be playing a role in the observed decline in *amh* transcript in testis tissue, which in turn could have consequences for the functioning of the testis, resulting in de-masculinization.

Fertilization success decreased over time with the mean fertilization rate dropping from 89% on day 1 to 69% by day 15. These findings are consistent with those of Haubruge et al., who reported declines in sperm count of 40-75% in guppies exposed to 0.274 or 0.549mg/L BPA [23]. BPA exposure has been linked to male sexual dysfunction in humans, and urinary concentrations of BPA have been associated with declines in sperm concentration, motility, and morphology in men [48]. The mechanism by which disruption of normal spermatogenesis takes place is hypothesized to be via disruption of the Sertoli cells, which are directly sensitive to xenobiotic chemicals, and whose functions are essential during spermatogenesis [23]. Our data are in agreement with these findings and further document the importance of Sertoli cells as targets for BPA toxicity, by demonstrating its effects on *amh* and *cyp19a1a*, both expressed in these cells in the testis.

Changes in fertilization success may have occurred not only due to effects of BPA on spermatogenesis but also due to BPA-induced alterations in egg quality. Females exposed to 1mg/L BPA produced an increased number of eggs, but these eggs may have lacked the quality required for fertilization success and embryo survival. Many factors contribute to egg quality, of which the hormonal environment during oogenesis is a critical one [49]. The observed changes in the expression of estrogen receptors and the trends observed for *cyp19a1a* in females indicate a disruption of the

285 estrogen/androgen balance within ovaries and consequent alterations in sex steroid signalling
286 pathways, putatively leading to alterations in oogenesis and oocyte quality. This hypothesis is
287 supported by previous studies in which BPA was shown to affect oogenesis [50]. In addition, a study in
288 pregnant mice exposed to BPA found gross abnormalities in the meiotic prophase of oogenesis,
289 including synaptic defects, which were suggested to occur via Esr2 (ERβ) signalling [51]. Interestingly, in
290 the present study, changes were also observed in the expression of an ERβ subtype (*esr2a*) in the
291 gonads of both sexes, suggesting similar mechanisms could be occurring.

292

293 *Effects of BPA on Epigenetic Regulation*

294 There is now strong evidence demonstrating that BPA has the potential to induce changes in DNA
295 methylation at both gene-specific and genome-wide levels in exposed organisms [32,33], however this
296 has rarely been studied in fish.

297 In our study, we found a significant decrease in the expression of the DNA methylation maintenance
298 enzyme, *dnmt1,* for all three BPA concentrations tested in ovaries of females, including at
299 environmentally-relevant concentrations, and the DNA methylation pattern in the promoter region
300 of the *dnmt1* gene was found to be significantly associated with BPA exposure concentrations for
301 four CpG sites. The expression of *dnmt1* is known to be associated with changes in global DNA
302 methylation, and inactivation of *dnmt1* has been shown to cause global demethylation of the
303 genome. [52]. In this regard, it was interesting that global DNA methylation levels were significantly
304 decreased in ovarian tissue of fish exposed to 1mg/L BPA, potentially as a consequence of the
305 suppression in *dnmt1* transcription. In contrast, previous studies in *Gobiocypris rarus*, have reported
306 global DNA hypermethylation in ovaries exposed to 0.015mg/L BPA for 35 days [19], suggesting these
307 epigenetic effects may be concentration and time dependant, and potentially vary across vertebrate
308 species. Importantly, *dnmt1* is reported to be an important maternal transcript involved in the

309 regulation of DNA methylation during the first stages of embryo development, particularly prior to
310 the zygote genome activation [53,54]. Therefore, the significant decrease in the expression of *dnmt1*
311 observed in ovaries of females exposed to all three concentrations of BPA could have potential
312 consequences for the appropriate development of offspring, in addition to influencing the level of
313 DNA methylation in the ovary of exposed females

314 For males, *dnmt1* transcription was also negatively associated with BPA exposure concentrations
315 and a significant hypermethylation of two CpG sites in the promoter region of the *dnmt1* gene in fish
316 exposed to 0.1mg/L BPA was observed. In addition, we measured a significant decrease in global
317 DNA methylation in the testis of fish exposed to 1mg/L BPA, suggesting that the BPA-induced
318 reduction in global methylation is likely to be functionally linked to the decrease in *dnmt1*
319 transcription. These data align with the reported hypomethylation of sperm associated with the
320 presence of BPA in urine, in a study of male factory workers in China [37]. There is evidence to suggest
321 that DNA demethylation and methylation establishment events during early development are guided
322 by the paternal DNA methylation program instructed by the sperm chromosomes [55,56]. Therefore, it
323 is plausible that changes to the global DNA methylation pattern in testes such as those reported for
324 fish exposed to 1mg/L BPA may have the potential to impact on the epigenetic reprogramming of
325 embryos, with potential consequences for their subsequent development.

326 In the liver, we observed a significant decrease in *dnmt1* transcription in males and females,
327 including at environmentally-relevant concentrations, demonstrating the very significant impact of
328 BPA on the expression of this key DNA methylation maintenance enzyme. In addition, we report
329 significant hypermethylation of the promoter region of the *dnmt1* gene in both male and female
330 livers. Based on the positive association between the expression of this gene and global DNA
331 methylation, it is plausible that the suppression of *dnmt1* may impact on global methylation as seen
332 in other tissues. However this could not be measured in the liver due to technical limitations related
333 to the amount of DNA obtained from this tissue. The fact that changes in the transcript and

methylation profile for *dnmt1* occur at environmentally-relevant concentrations highlights the potential for BPA to cause epigenetic effects in exposed organisms within current exposure scenarios.

It is important to note that global DNA methylation in this study, measured using the LUMA assay, provides only an estimate of the total DNA methylation across all areas of the genome and all cell types in a given tissue. Decreased *dnmt1* transcription may be causing demethylation of specific areas of the genome or within specific cell types, but this may not be detectable by a global measurement of DNA methylation including all cell types simultaneously. This may explain why *dnmt1* transcription appears to be more sensitive to BPA exposure compared to global methylation measurements.

The transcript profile for *mbd2* was significantly altered following exposure to BPA in both the testis and the livers of females. *mbd2* belongs to a family of nuclear proteins capable of binding specifically to methylated DNA, and may also function to repress transcription from methylated gene promoters [57]. We found also a significant decrease in *mecp2* transcription in male livers, a gene involved in transcriptional repression by associating with methylated CpG dinucleotides where it silences transcription by recruiting histone deacetylases, resulting in chromatin remodelling [58]. In addition, in male livers a significant decrease in *hdac3* transcription was also observed. These findings suggest BPA is not only interacting with the processes linked to DNA methylation, but also has the potential to disrupt processes linked to chromatin structure and potentially impact on gene function via these mechanisms.

Despite the advances in our understanding of the epigenetic and transcriptional consequences of BPA in a model vertebrate, there are some limitations to the methodologies used: the locus-specific methylation measurements conducted were based on the sodium bisulphite treatment of genomic DNA, and therefore cannot distinguish between DNA modifications such as 5-hydroxymethylcytosine (5hmC) and 5-formylcytosine (5fC) and methylcytosine (5mC), which have unknown functional

359 significance [59]. In addition, we explored the methylation status of specific CpG positions, within the
360 regulatory regions of select target genes, hypothesized to be targets of BPA toxicity. This hypothesis-
361 driven approach was successful in identifying some important mechanisms of BPA toxicity but may
362 have missed other interesting effects outside these targeted regions, as suggested by the effects of
363 BPA on global methylation levels. In addition, the global and locus-specific methylation
364 measurements reported in this study are single measurements of DNA methylation across multiple
365 cellular populations and cell types within each tissue. Both the gonad and liver are comprised of a
366 mixture of cell types, whose genomic methylation and transcriptional activity is unique to the
367 function of each cell type. In the testis for example, a large percentage of the cellular composition is
368 made up of sperm cells containing very little cytoplasm and limited transcriptional activity, and the
369 genomic DNA of sperm cells is also known to be hypermethylated. In contrast, the ovary contains
370 oocytes characterized by very large cytoplasm where transcripts are stored to support the initial
371 stages of embryogenesis before embryonic genome activation. Therefore, the datasets collected for
372 these tissues are strongly dependent on the cellular composition of the tissue. In future studies, a
373 genome-wide approach to measure methylation and also histone modifications, as well as analysis
374 of single cells or pure populations of cells, may help to further characterize the effects of BPA on
375 epigenetic signalling pathways.

Conclusions

Overall, we have found evidence that BPA caused significant disruption to reproduction in breeding zebrafish exposed to 1mg/L BPA, likely via estrogenic mechanisms. The potential for BPA to cause disruption of reproduction shown here raises concerns for its toxicity when organisms are exposed to BPA in environments affected by other stressors including other environmental endocrine disruptors with similar mechanistic pathways that may act additively to cause reproductive disruption. Importantly, BPA also caused significant alterations in the transcription of a number of genes involved in epigenetic regulation in both liver and gonad tissue, most notably on *dnmt1*, which occurred in conjunction with decreases in global DNA methylation. Of note, some changes were observed after exposure to environmentally-relevant concentrations of BPA (0.01mg/L), corresponding to current exposure scenarios for both humans and for wildlife. These findings provide evidence of the adverse effects of BPA in a model vertebrate, and advocate for its replacement within consumer products and its reduction in the environment.

Materials and Methods:

Chemicals

All chemicals were obtained from Sigma-Aldrich, UK, unless stated otherwise.

Fish husbandry

Wild-type WIK strain adult zebrafish (originating from a stock population at the University of Exeter) were maintained according to the conditions reported in Paull et al. [60]. Prior to the start of the experiment, fish were randomly allocated into 18 breeding groups of 4 males and 4 females, kept in individual 15L flow-through tanks and were allowed to breed naturally during an acclimation period of 7 days. After this period, colonies that failed to spawn consistently were removed prior to the start of the experiment. Mains tap water was filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts to standardized synthetic freshwater (final concentrations to give a conductivity of 300mS: 122mg/L $CaCl_2 2H_2O$, 9.4mg/L $NaHCO_3$, 50mg/L $MgSO_4 7H_2O$, 2.5mg/L KCl, 50mg/L Tropic Marin Sea Salt), aerated, and heated to 28°C in a reservoir, before it was supplied to each aquarium using a flow-through system. Tanks were aerated and supplied with a flow rate of 48 L/day of water [60]. Tank water was maintained at 28 ± 0.5 °C and pH 7-7.5 and fish were maintained under a 12h light:dark cycle, including dawn and dusk transition periods of 30 minutes. Fish were fed live *Artemia nauplii* once daily (ZM Premium Grade Artemia; ZM Ltd.) and TetraMin tropical flake food (Tetra; Melle, Germany) twice daily, to satiation.

Exposures of breeding zebrafish to Bisphenol A

The selected 15 groups that showed consistent breeding and behavioural patterns during the initial

acclimation period were subjected to a 10 day pre-exposure period, followed by a 15 day exposure period. Reproductive data for the 10 day pre-exposure period were collected to ensure that all breeding groups were reproducing consistently and there were no differences between reproductive measurements for any of the breeding groups prior to the chemical exposure period. Three independent replicate breeding groups were assigned at random to each treatment. A flow-through system was used to dose the tanks for 15 days with three concentrations of BPA; 0.01, 0.1 and 1mg/L, using ethanol (0.0005%) as a solvent. An absolute control receiving water alone and a solvent control receiving the same concentration of ethanol as the chemical exposures were also included.

On day one of the exposure period, tanks were spiked with the appropriate amount of BPA to achieve the required exposure concentrations. Flow rates were monitored daily to ensure the chemical concentrations remained consistent and dosing stocks were replaced every day. Water samples from each tank were collected on days 5, 10 and 15 of the exposure, and were stored at -20 °C until chemical analysis.

The effects of BPA on reproduction were determined by measuring the egg production and fertilization success of individual groups. Eggs were collected each morning approximately one hour post-fertilization (hpf), washed and transferred to petri dishes for analysis. The numbers of fertilized and unfertilized eggs were determined by visual inspection for each treatment using a dissection microscope (Motic DM143, Hong Kong).

On day 15 of the exposure period, all fish were sacrificed humanely using a lethal dose of benzocaine followed by destruction of the brain, in accordance with UK Home Office regulations. The wet weight and fork length were recorded, and the condition factor for each fish was calculated (k= (weight (g) x 100)/ (fork length (cm))^3). The gonads and livers were dissected and weighed, and the gonadosomatic index (GSI = (gonad weight (mg)/ (total weight (mg)- gonad weight (mg)) x 100)) and hepatosomatic index (HSI = (liver weight (mg)/ (total weight (mg)- liver weight (mg)) x 100)) were

calculated. Gonads and livers were collected, snap frozen in liquid nitrogen and stored at -80°C until analysis for transcript profiling and DNA methylation.

Transcript profiling

Transcript profiling of genes encoding epigenetic regulatory proteins and genes involved in reproductive function was conducted using real-time quantitative PCR (RT-QPCR) as previously described [61]. Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) was used for designing primers for each target gene using zebrafish NCBI RefSeq sequences, and primers were purchased from MWG-Biotech (Ebersburg, Germany). Assays for each transcript were optimized and standard curves were generated as previously described [61]. Primer specificity was confirmed by observation of a single amplification product of the expected melting temperature throughout the range of detection. The linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was > 0.99 in each case, and efficiencies were between 1.86-2.24. The primer sequences, annealing temperatures, PCR product sizes and PCR efficiencies for each primer pair are shown in Supporting Information Table S2.

RNA and DNA were extracted together from the livers and gonads of 8 male and 8 female fish from each treatment group using the AllPrep DNA/RNA Micro Qiagen Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, which allows for extraction of both RNA and DNA from the same tissue sample. NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) was used to assess RNA and DNA purity and concentration. RNA was treated with DNase I (Qiagen) to remove any potential DNA contamination. cDNA was synthesized from 2 µg of total RNA using random hexamers (MWG-Biotech, Ebersberg, Germany) and M-MLV reverse transcriptase (Promega, Madison, USA), according to manufacturer's instructions. cDNA was then diluted 1:2 and RT-QPCR was performed in duplicate using an iCycler iQ Real-time Detection System

(Bio-Rad Laboratories, Hercules, CA) and SYBR Green chemistry as previously described [61]. On each plate, a template-minus negative control was run in duplicate to verify the absence of cDNA contamination. Efficiency-corrected relative expression levels were determined after normalization to a control gene, ribosomal protein l8 (*rpl8*), which has been shown to have stable expression in the livers and gonads following exposures to estrogens in another cyprinid fish species [44,62].

Bisulfite-Pyrosequencing

DNA sequence data for the promoter regions of *esr1*, *amh* and *dnmt1* were obtained from Ensembl (release 83; Cunningham et al 2015) [63] using the Biomart portal [64]. Zebrafish *esr1* (ENSDARG00000004111) has 3 known transcripts (*esr1*-001 (3449 bp), *esr1*-201 (3502 bp) and *esr1*-202 (212 bp)) and 2 transcription start sites (TSSs). The *dnmt1* gene (ENSDARG00000030756) also has 2 TSSs and 3 transcripts (*dnmt1*-001 (4896 bp), *dnmt1*-201 (4893 bp) and *dnmt1*-202 (5031 bp)). *amh* (ENSDARG00000014357) has one transcript (*amh*-001, 3243 bp) and one TSS (Supporting Information Figure S6). Target sites within the promoter sequences were chosen based on their proximity to the TSSs and estrogen-responsive elements (EREs), identified using JASPAR [65], and the matrix models ESR1 (MA0112) and ESR2 (MA0258). PCR and bisulfite-Pyrosequencing assays were designed using the PyroMark Assay design software (Qiagen, Hilden, Germany). Pyrosequencing primers and their corresponding target sequences are shown in Supporting Information Table S3.

Template preparation and pyrosequencing was carried out as described by Tost and Gut (2007) [66] on bisulfite-treated DNA from 8 individual fish (gonads and livers) per treatment group. Briefly, genomic DNA (500ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, CA, USA) according to the manufacturers' standard protocol. Water negative controls were run in duplicate to verify the absence of DNA contamination. Bisulfite-PCR amplification was performed in duplicate using the primers and assay conditions provided in Supporting Information

485 Table S3. Unmodified DNA samples were included during primer optimization to confirm primer
486 specificity for bisulfite-modified DNA.

487

488 *Luminometric-Based Assay (LUMA) for Global DNA Methylation*

489 The LUMA assay was performed as described by Karimi *et al.* (2006) using DNA extracted from gonad
490 samples from 8 individual fish per treatment [67]. Sufficient quantities of DNA were not available to
491 perform the LUMA assay in liver samples, therefore analysis of global DNA methylation were
492 conducted only for gonad samples. 250ng of each DNA sample was digested in duplicate with HpaII
493 and MspI, and data were normalized to the EcoRI peak to account for any technical differences
494 between samples [68]. Global DNA methylation values were calculated according to the formula
495 (HpaII(G)/EcoRI(T))/(MspI(G)/EcoRI(T)), where G and T refer to the peak heights for HpaII or MspI
496 (methylation) and EcoRI (input DNA), respectively.

497

498 *Water chemistry*

499 For analysis of the concentrations of BPA in the exposure water, methanol, acetonitrile and water,
500 both HPLC and LC-MS grade, HiPerSolv CHROMANORM®, were purchased from VWR Int. One mL of
501 each water sample was added to a glass vial and mixed with 1 mL of HPLC-grade acetonitrile. Before
502 LC-MS/MS analysis, aliquots were vortexed and diluted in a mixture of acetonitrile and water (1:3
503 v/v). Analyses were performed using a Surveyor MS Pump Plus HPLC pump with an HTC PAL
504 autosampler coupled to a TSQ Vantage triple quadrupole mass spectrometer equipped with heated
505 electrospray (HESI II) source (ThermoFisher Scientific, Hemel Hempstead, UK). Chromatographic
506 separation was achieved using a reversed-phase, 3 µm particle size, C18 Hypersil GOLD column 50
507 mm × 2.1 mm i.d. (Thermo Scientific, San Jose CA, USA). Analytes were separated using a linear
508 gradient of water and methanol. The initial conditions for the gradient consisted of 10% methanol,

509 which was increased to 100% in 4.5 minutes and maintained for 1 minute before returning to the
510 initial 10% methanol. The flow rate was 500µL/minute. The temperature of the autosampler was set
511 at 8˚C, and the column was kept at a room temperature. The HESI probe was operating in the
512 negative mode and an ion-spray voltage of -4.0 kV was applied. The heated capillary temperature
513 was set at 275 °C and the vaporizer temperature was 60 °C. Nitrogen was employed as sheath and
514 auxiliary gas at a pressure of 30 and 5 arbitrary units, respectively. The argon CID gas was used at a
515 pressure of 1.5 mTorr and the optimum collision energy (CE) for each transition was selected.
516 Quantification of BPA was performed using two characteristic multiple reaction monitoring (MRM)
517 transitions of precursor ion 227.1→ 212.1 (CE: 20 V) and 227.1 → 133.1 (CE: 28 V).

518

519 *Statistical analysis*

520 Statistical analyses were carried out using R (version 3.0.2) [69]. Prior to analysis, data were tested for
521 equal variance and for normality using the Shapiro–Wilk test. Proportional data and variables with
522 non-Gaussian distributions or non-homogeneous variances were subjected to variance-stabilizing
523 arcsine transformations or log transformations. Non-parametric statistics were used when
524 transformations did not result in distributions meeting the assumptions for parametric tests. All
525 graphs were plotted using untransformed data for ease of interpretation. For the mean fertilization
526 rates, comparisons between treatments were performed using Kruskal-Wallis tests followed by the
527 Wilcoxon signed rank test. The Regression coefficient (R^2) was calculated using linear modelling for
528 fertilization rates. Linear mixed effects models were generated using the lme4 package [70] in order to
529 explore the effect of BPA concentration and length of exposure on egg numbers. Non-significant
530 terms were removed from models; models were compared based on likelihood ratio testing to give
531 the appropriate minimum adequate model. Model results were inspected to ensure residuals were
532 normally distributed.

In order to determine the effects of BPA on the reproductive and molecular endpoints measured, statistical comparisons were performed between the solvent control and the groups exposed to BPA, and comparisons between the water control and the solvent control were also conducted to confirm that no significant differences occurred as a result of the presence of the solvent. Comparisons between treatments were performed using one-way analysis of variance (ANOVA) and Kruskal-Wallis tests. Where ANOVA analysis found a $P \leq 0.05$, post-hoc testing was carried out using the pairwise multiple comparisons of means method with false discovery rate P value adjustment. Where the Kruskal-Wallis test was used, post-hoc testing was carried out using the Wilcoxon signed rank test accounting for repeated measures within the datasets. P values of ≤0.05 were considered to be significant. All data are presented as mean ± SEM.

For transcript profiles, data points classified as outliers (using Chauvenet's criterion) and data points for which the expression was below the assay detection limit were excluded from analysis. Where amplification was detected in more than 70% of individuals, data were represented as fold-change relative to the expression in the water control group and groups were then compared using one-way ANOVA and Kruskal-Wallis tests with post-hoc tests as described previously. Where amplification was detected in less than 70% of individuals, data were represented as the proportion of individuals for which the target genes were detected, and analysis was conducted using a binomial Generalised Linear Model. In the gonadal data sets, PCA was also performed using the prcomp function to identify the main trends in gene expression.

In order to determine if there were associations between the methylation levels for specific loci in the promoter regions of genes of interest and their transcription, correlation analysis was conducted. Where data was normally distributed Pearson correlation was used, and where data did not meet the assumptions of parametric testing, Spearman correlation analysis was performed. Correlation analyses were also conducted to determine the relationship between global methylation and *dnmt1* transcription, as above. The relationship between BPA concentration and transcript

expression or methylation was also determined using regression analysis, calculated using linear modelling.

All graphs were plotted using untransformed data for ease of interpretation, and were created using the R packages ggplot2 [71], gplots [72], beeswarm [73] and ggbiplot [74]

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FIGURE LEGENDS

Figure 1. A) Cumulative number of eggs per female per day in breeding groups exposed to 0.01, 0.1 and 1 mg/L BPA. Data is presented for a 10 day pre-exposure followed by a 15 day chemical exposure periods (n=3 replicate groups per treatment). Statistical comparisons were conducted in R (version 3.0.2), and the lme4 package was used to fit mixed effects linear models, followed by repeated measures ANOVA and Chi-squared Wald test to determine the effects of the exposure to BPA compared to the solvent control.
B) Mean fertilization success (%) during the 15 day chemical exposure period (n= 3 replicate groups per treatment). Statistical analyses were conducted using R (version 3.0.2); the Regression coefficient (R^2) was calculated using linear modelling. Asterisks indicate significant differences between treatment groups (**p<0.01; ***p<0.001).

Figure 2. Transcript profiles for target genes in the livers of females **(A)** and males **(B)**, and in the ovary **(C)** and testis **(D)** following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data were collected for 6-8 fish per treatment, and data points classified as outliers (using the Chauvenet's criterion) and for which the expression was below the detection limit of the assay were excluded from analysis. Where amplification was detected in more than 70% of individuals, data are represented as fold-change relative to the expression in the solvent control group. Where amplification was detected in less than 70% of individuals, data are presented as the proportion of individuals for which the target genes were amplified. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05, **P<0.01, ***P<0.001).

Figure 3. Principal components (PC) score plots showing the relative similarity of gonadal transcription profiles for zebrafish exposed to solvent, 0.01, 0.1 & 1mg/L BPA for 15 days. **A)** Ovary

B) Testis. Points represent PC scores for individual fish along PCs 1 and 2. Circles represent a general characterization of the PC space occupied by each treatment group and were calculated using the prcomp package in R (version 3.0.2).

Figure 4. Global DNA methylation profiles in the gonads of adult zebrafish following exposure to 0.01, 0.1 and 1 mg/L BPA. Graphs present the percentage of global DNA methylation in ovaries **(A)** and testis **(B)**. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control (*P<0.05, **P<0.01, ***P<0.001).

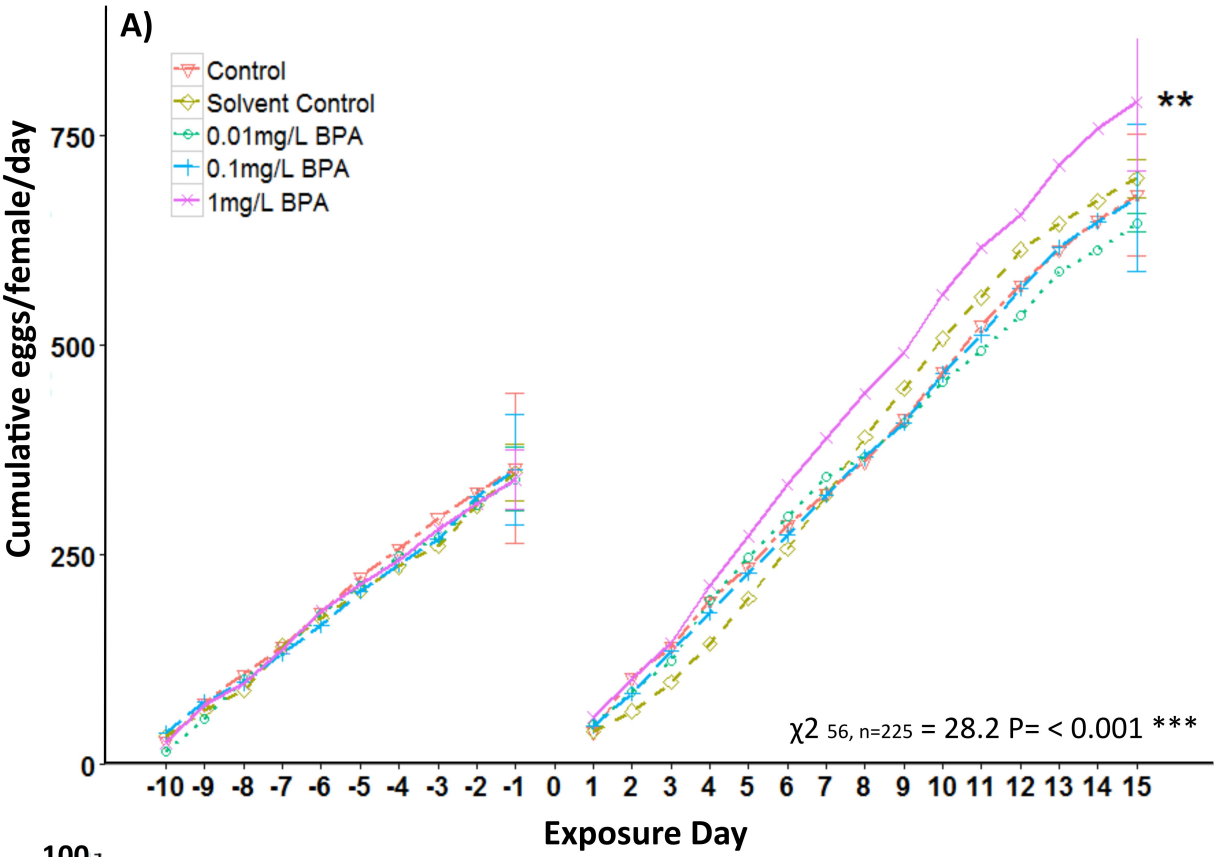
Figure 5. Gene specific DNA methylation profiles for three CpG sites in the promoter region of anti-Müllerian hormone (*amh*) in the ovaries **(A)** and testes **(B)** of adult zebrafish following exposure to 0.01, 0.1 and 1 mg/L BPA. **C)** Example pyrogram of three CpG sites in the 5' flanking regions of the *amh* gene. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control (*P<0.05 **P<0.01 ***P<0.001).

Figure 6. Gene-specific DNA methylation profiles for 11 CpG sites in the promoter region of DNA (cytosine-5)-methyltransferase 1 (*dnmt1*) in the ovaries **(A)** and testis **(B)** of adult zebrafish following exposure to 0.01, 0.1 and 1 mg/L BPA. **C)** Example pyrogram of 11 CpG sites in the 5' flanking regions of the *dnmt1* gene. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control (*P<0.05, **P<0.01, ***P<0.001).

Figure 7. Gene specific DNA methylation profiles for 11 CpG sites in the promoter region of DNA (cytosine-5)-methyltransferase 1 (*dnmt1*) in the livers of female **(A)** and male **(B)** adult zebrafish

following exposure to 0.01, 0.1 and 1 mg/L BPA. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control (*P<0.05 **P<0.01 ***P<0.001).

Fig. 1


A)
Control
Solvent Control
0.01mg/L BPA
0.1mg/L BPA
1mg/L BPA
**
Cumulative eggs/female/day
0
250
500
750
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Exposure Day
χ2 56, n=225 = 28.2 P= < 0.001 ***

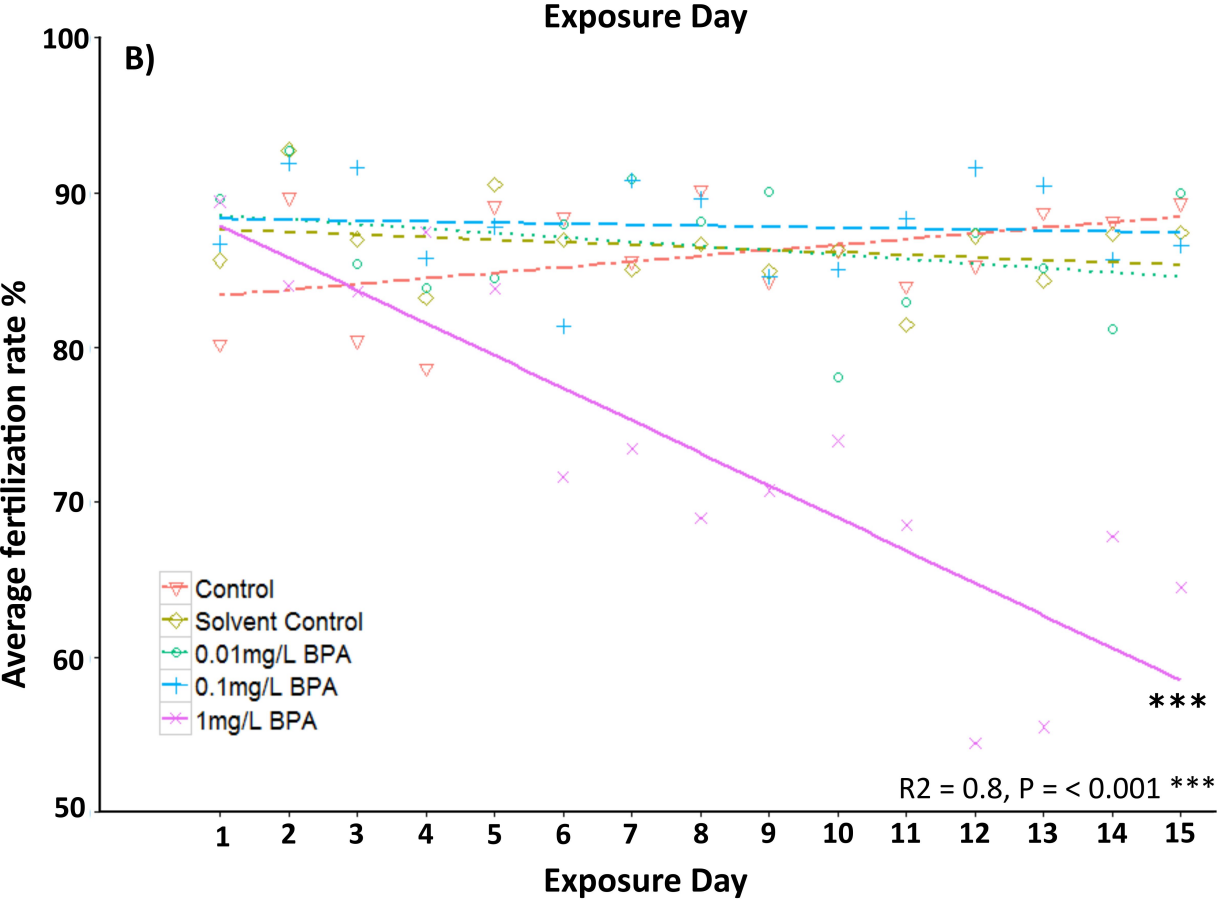

B)
Average fertilization rate %
50
60
70
80
90
100
Control
Solvent Control
0.01mg/L BPA
0.1mg/L BPA
1mg/L BPA
***
R2 = 0.8, P = < 0.001 ***
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Exposure Day

Fig. 2

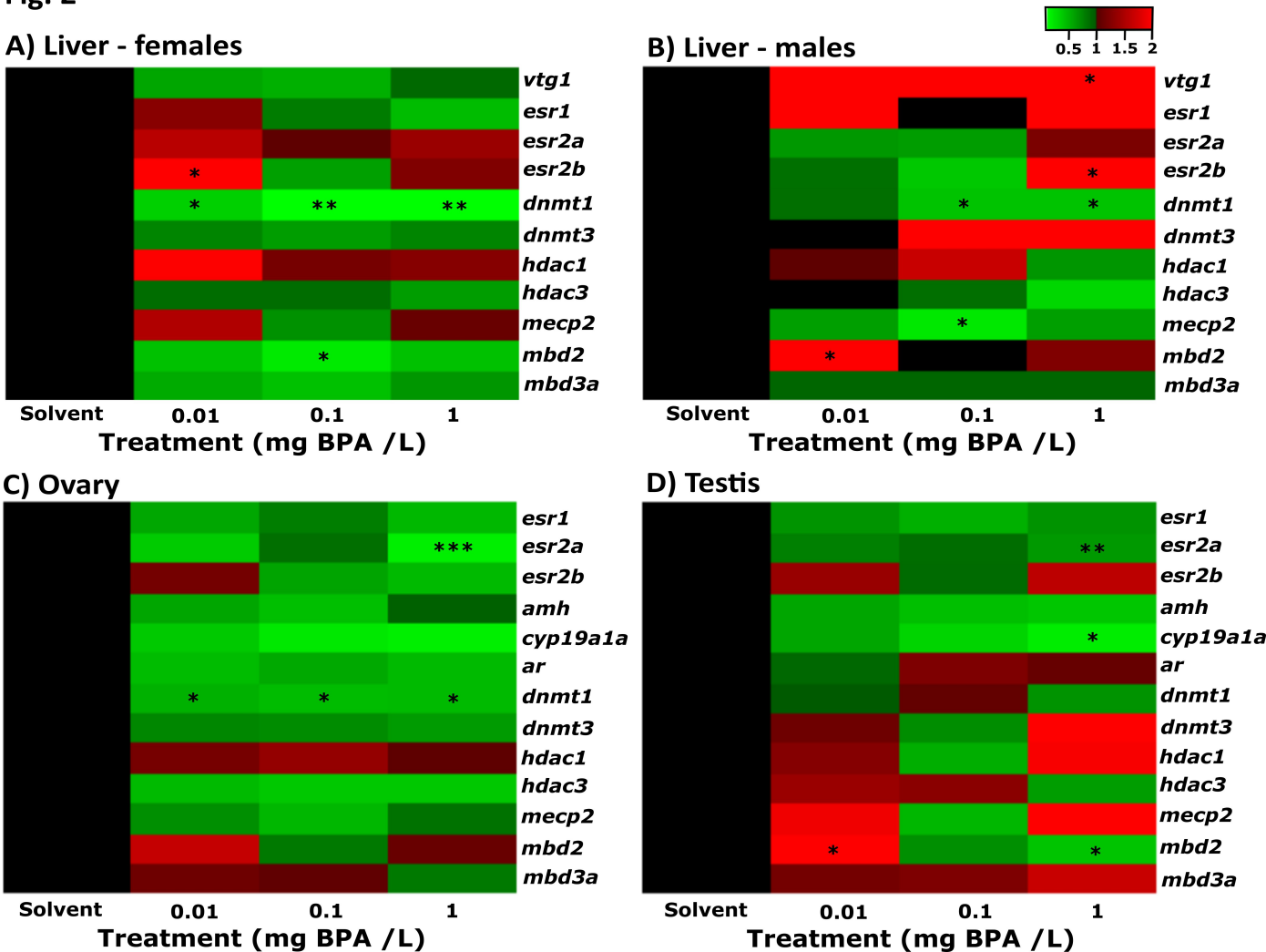

A) Liver - females
B) Liver - males
0.5 1 1.5 2
vtg1
esr1
esr2a
esr2b
dnmt1
dnmt3
hdac1
hdac3
mecp2
mbd2
mbd3a
Solvent
0.01
0.1
1
Treatment (mg BPA /L)
C) Ovary
D) Testis
esr1
esr2a
esr2b
amh
cyp19a1a
ar
dnmt1
dnmt3
hdac1
hdac3
mecp2
mbd2
mbd3a
Solvent
0.01
0.1
1
Treatment (mg BPA /L)

Fig. 3

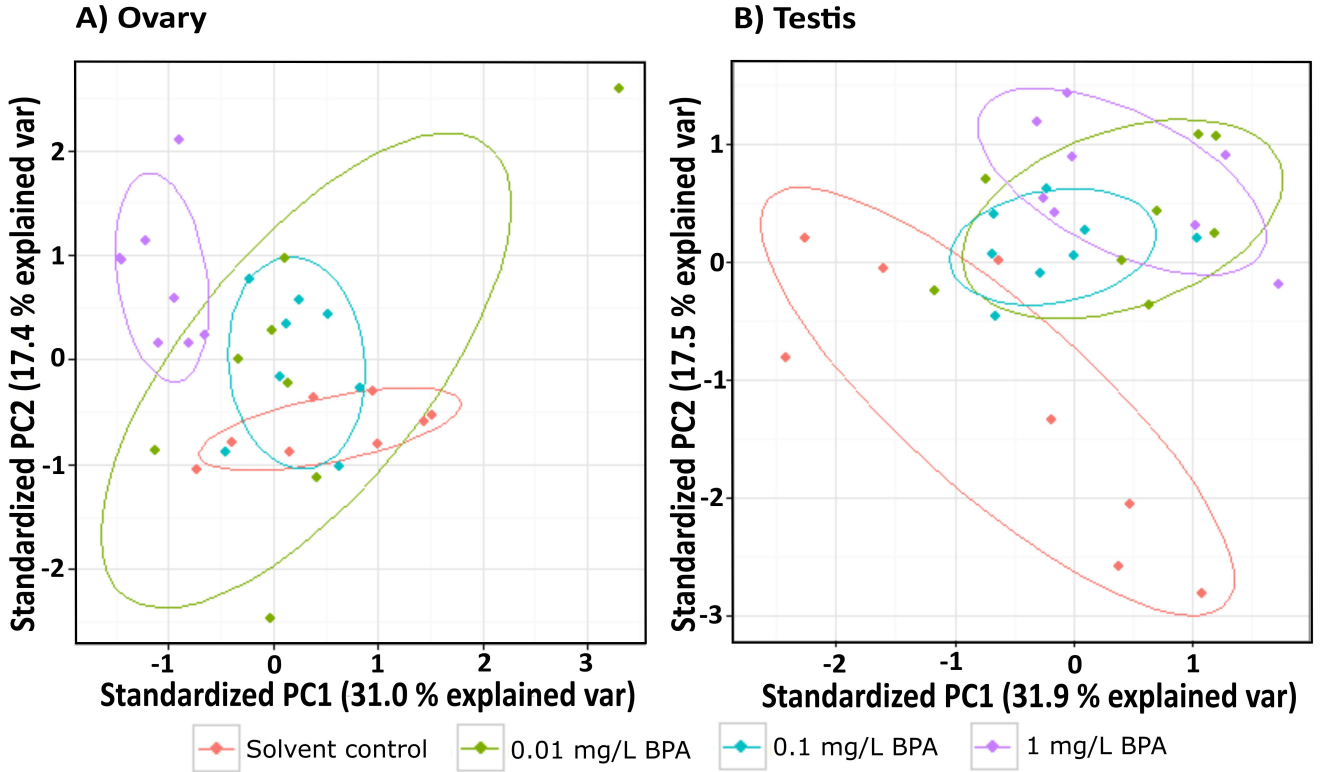

A) Ovary
B) Testis
Standardized PC2 (17.4 % explained var)
Standardized PC1 (31.0 % explained var)
Standardized PC2 (17.5 % explained var)
Standardized PC1 (31.9 % explained var)
Solvent control
0.01 mg/L BPA
0.1 mg/L BPA
1 mg/L BPA

Fig. 4

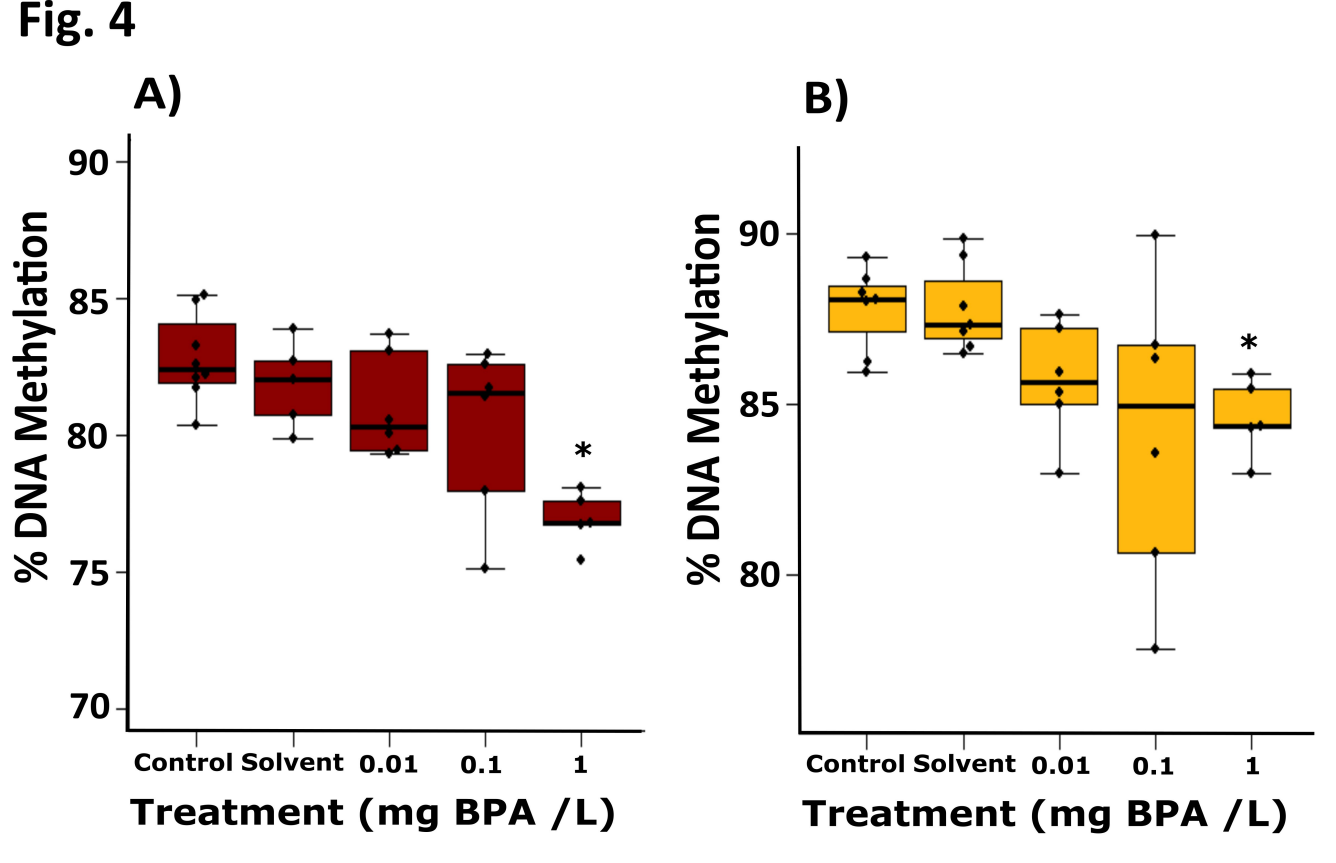

A)
% DNA Methylation
90
85
80
75
70
*
Control
Solvent
0.01
0.1
1
Treatment (mg BPA /L)
B)
% DNA Methylation
90
85
80
*
Control
Solvent
0.01
0.1
1
Treatment (mg BPA /L)

Fig. 5

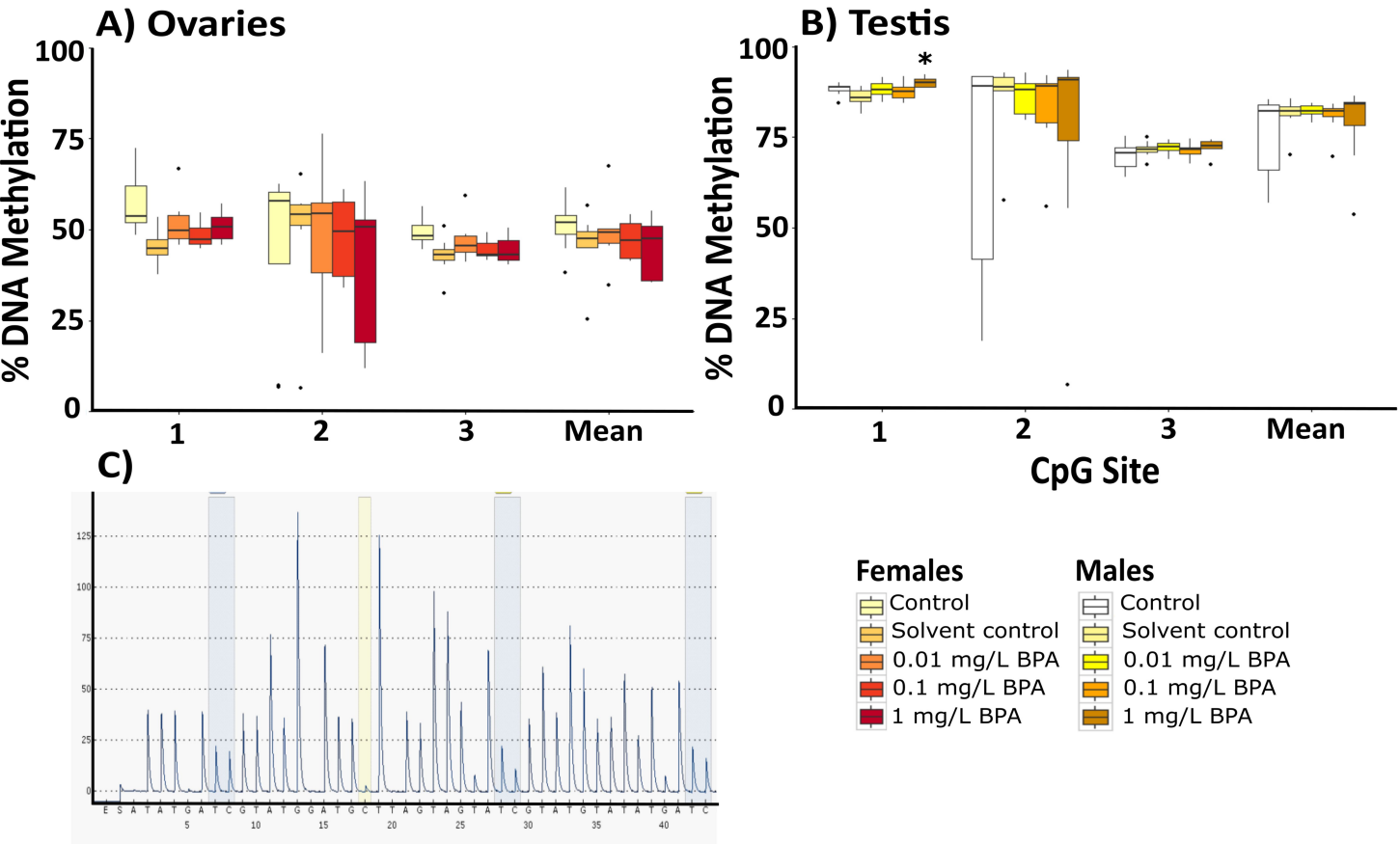

A) Ovaries
B) Testis
% DNA Methylation
100
75
50
25
0
1
2
3
Mean
*
CpG Site
C)
Females
Control
Solvent control
0.01 mg/L BPA
0.1 mg/L BPA
1 mg/L BPA
Males
Control
Solvent control
0.01 mg/L BPA
0.1 mg/L BPA
1 mg/L BPA

Fig. 6

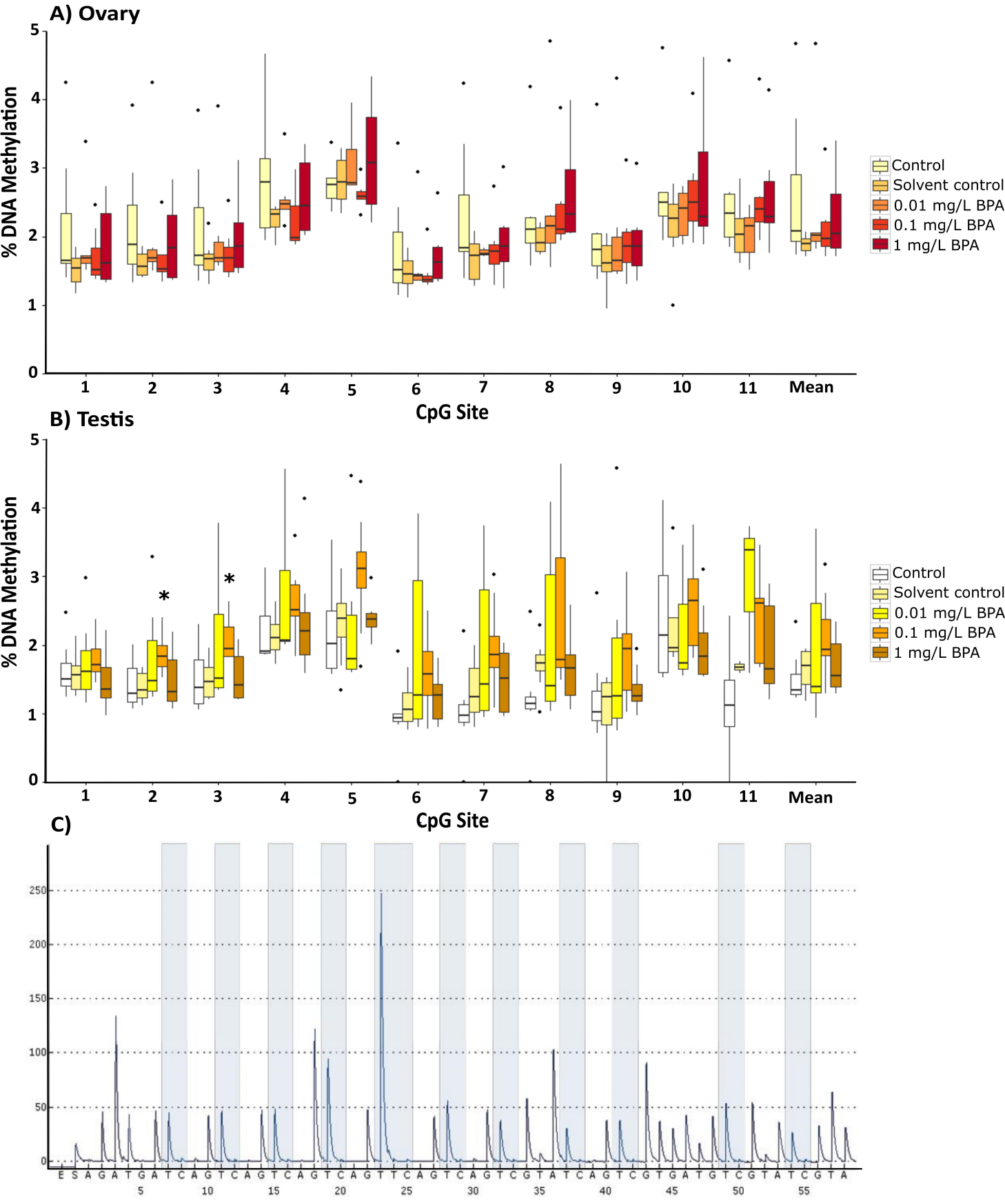
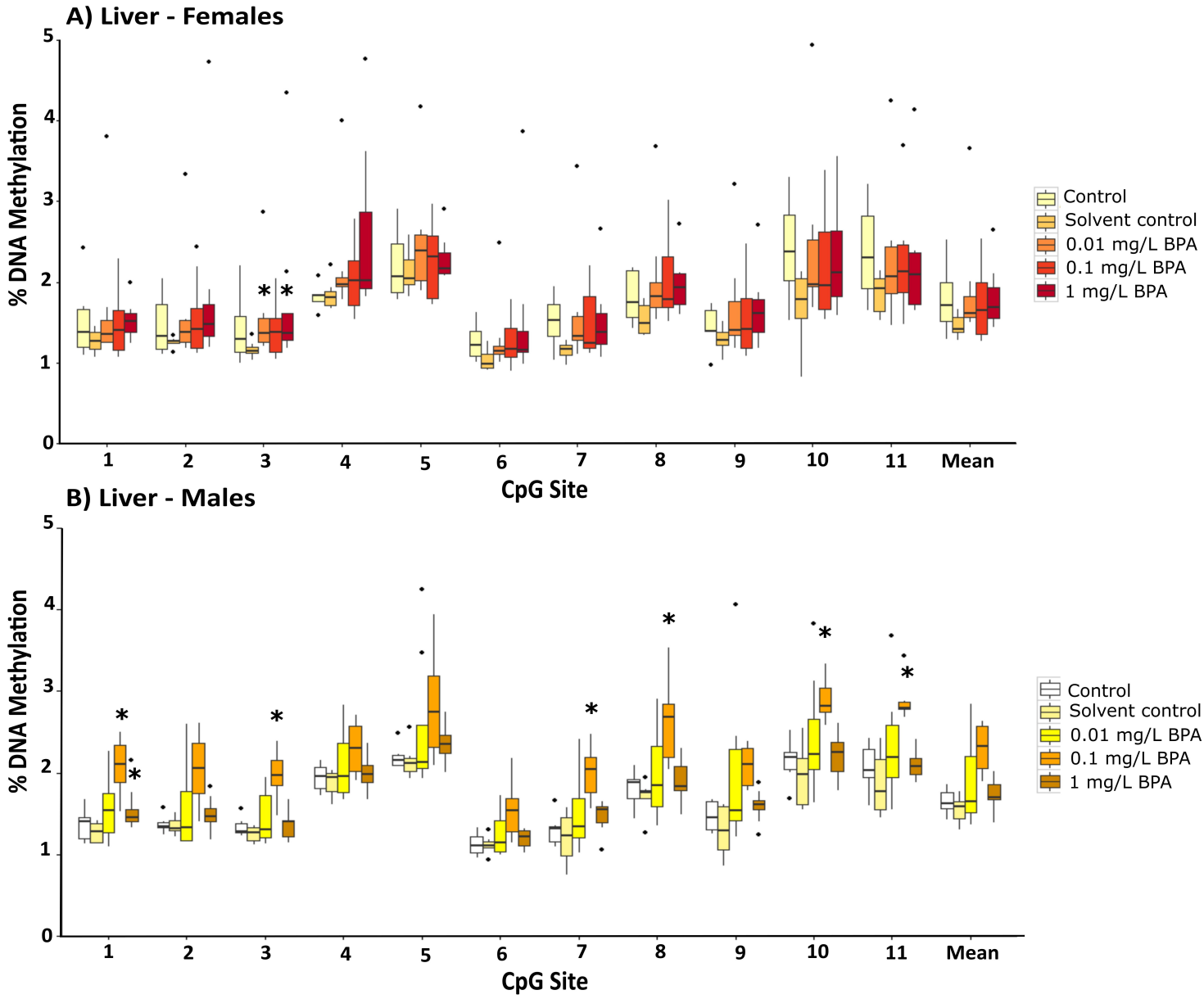

A) Ovary
% DNA Methylation
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
Mean
CpG Site
Control
Solvent control
0.01 mg/L BPA
0.1 mg/L BPA
1 mg/L BPA
B) Testis
% DNA Methylation
0
1
2
3
4
5
*
*
1
2
3
4
5
6
7
8
9
10
11
Mean
CpG Site
Control
Solvent control
0.01 mg/L BPA
0.1 mg/L BPA
1 mg/L BPA
C)
0
50
100
150
200
250
E S A G A T G A T C A G T C A G T C A G T C A G T T C A G T C A G T C G T A T C A G T C G T G A T G T C G T A T C G T A
5
10
15
20
25
30
35
40
45
50
55

Fig. 7


A) Liver - Females
% DNA Methylation
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
Mean
CpG Site
Control
Solvent control
0.01 mg/L BPA
0.1 mg/L BPA
1 mg/L BPA
B) Liver - Males
% DNA Methylation
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
Mean
CpG Site
Control
Solvent control
0.01 mg/L BPA
0.1 mg/L BPA
1 mg/L BPA

SUPPORTING INFORMATION

Bisphenol A causes reproductive toxicity, decreases *dnmt1* transcription and reduces global DNA methylation in breeding zebrafish (*Danio rerio*).

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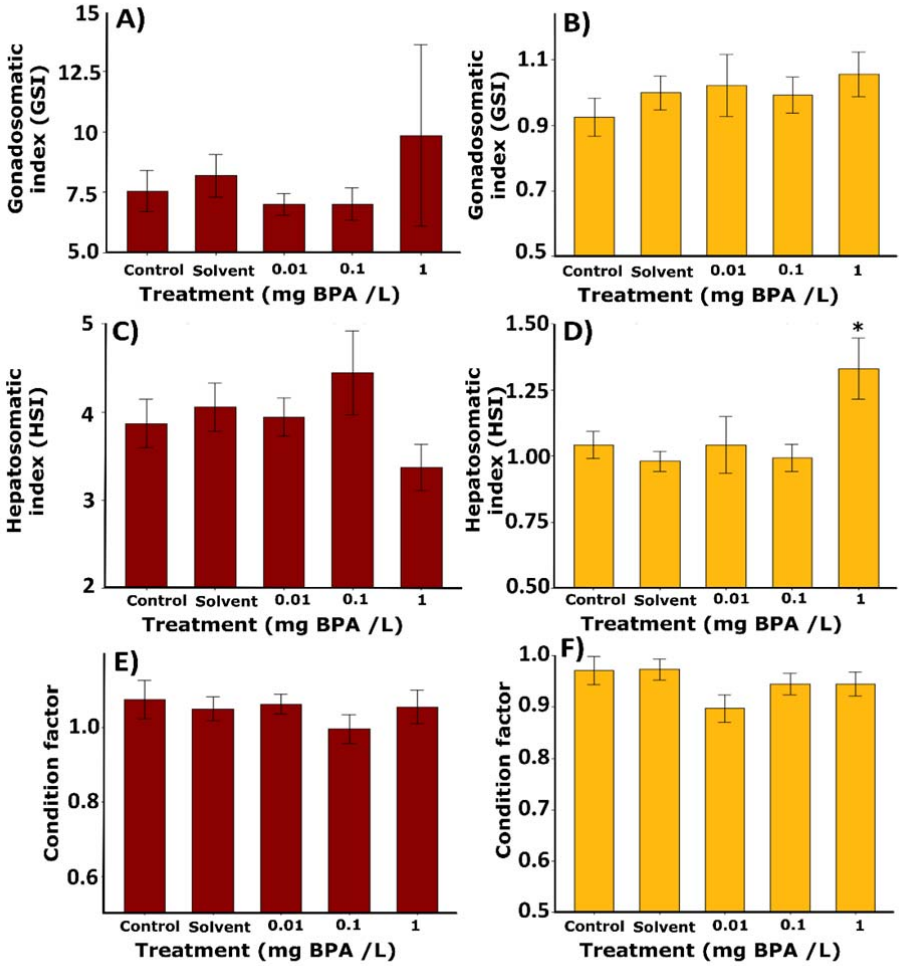
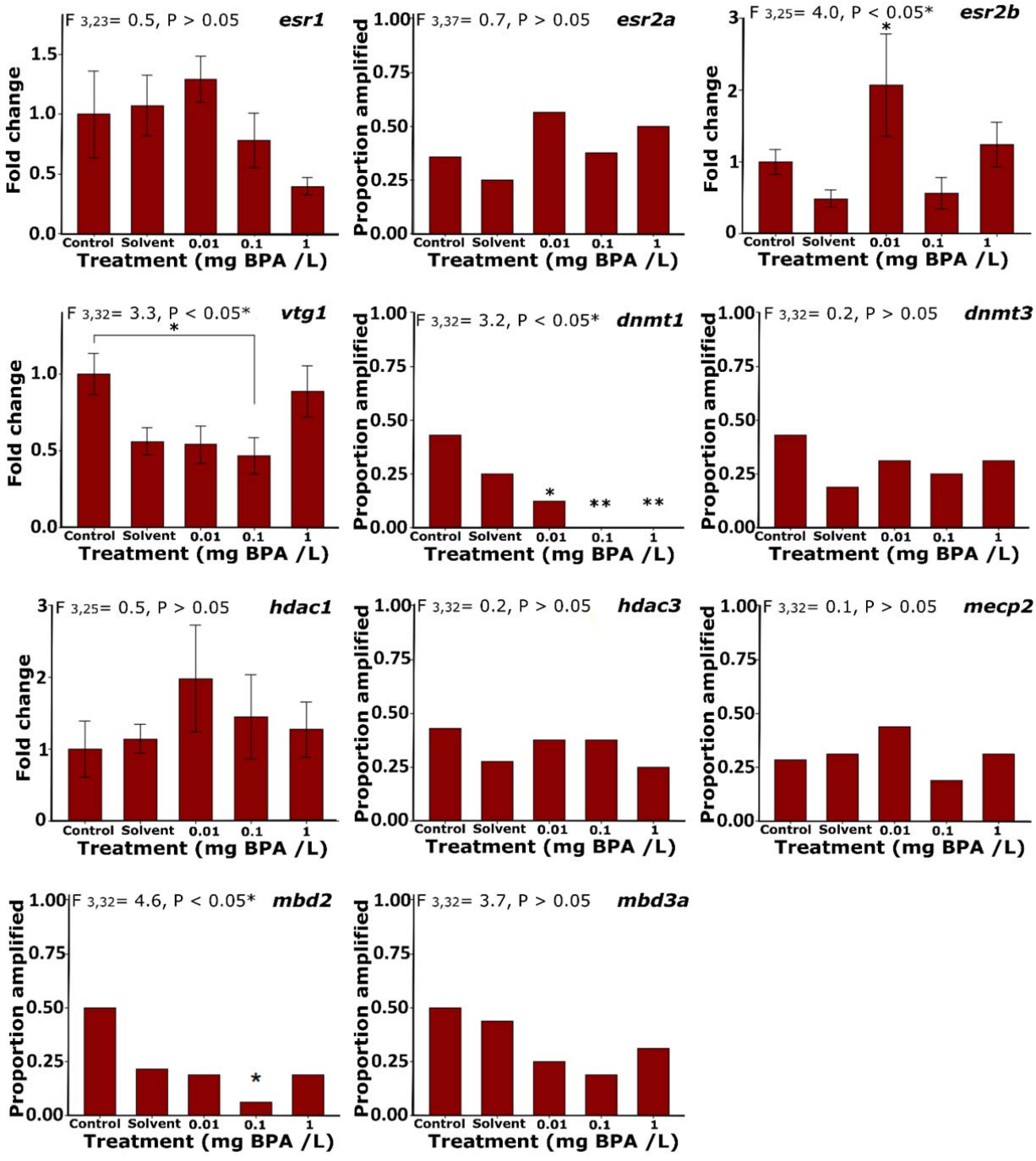
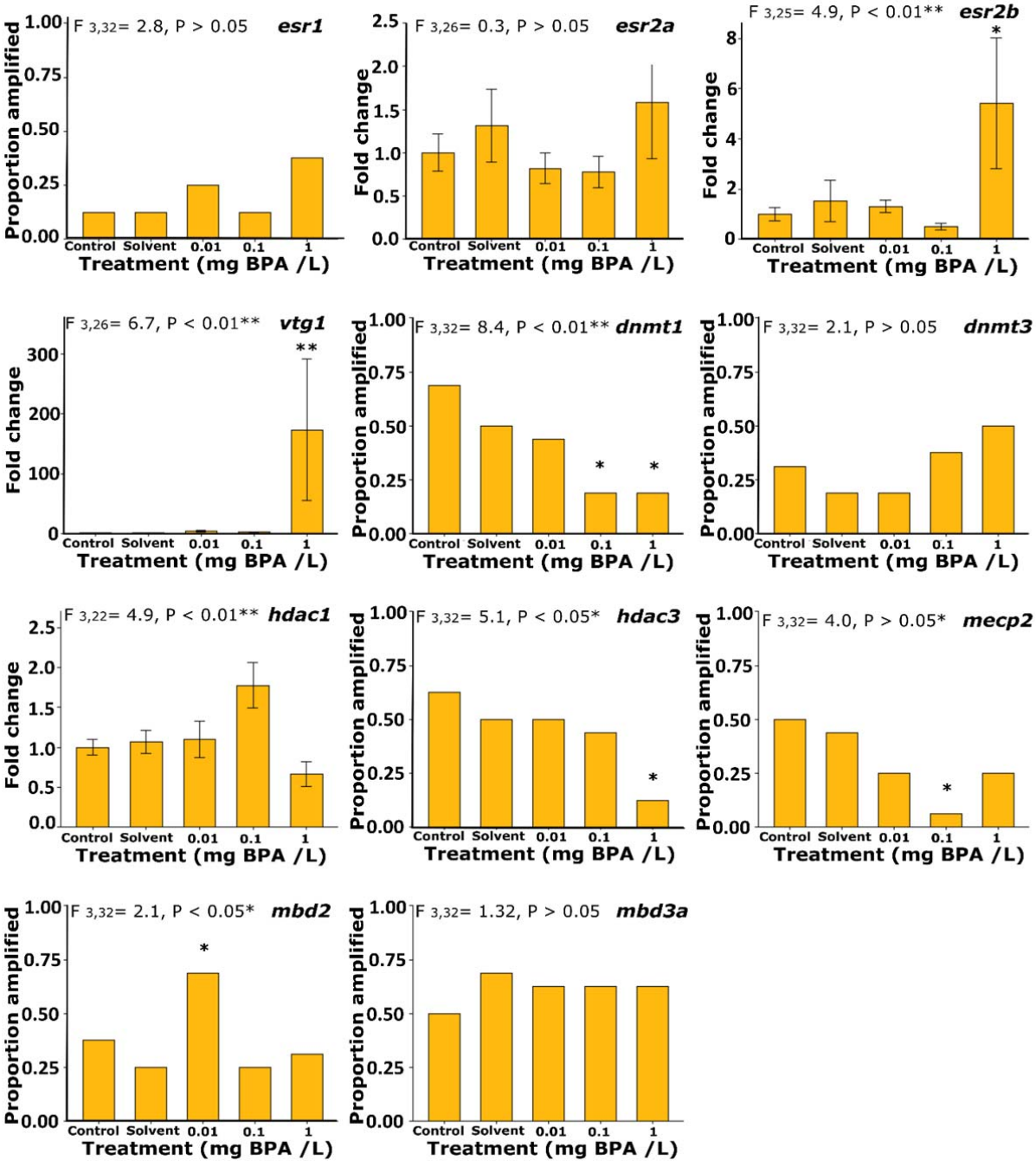


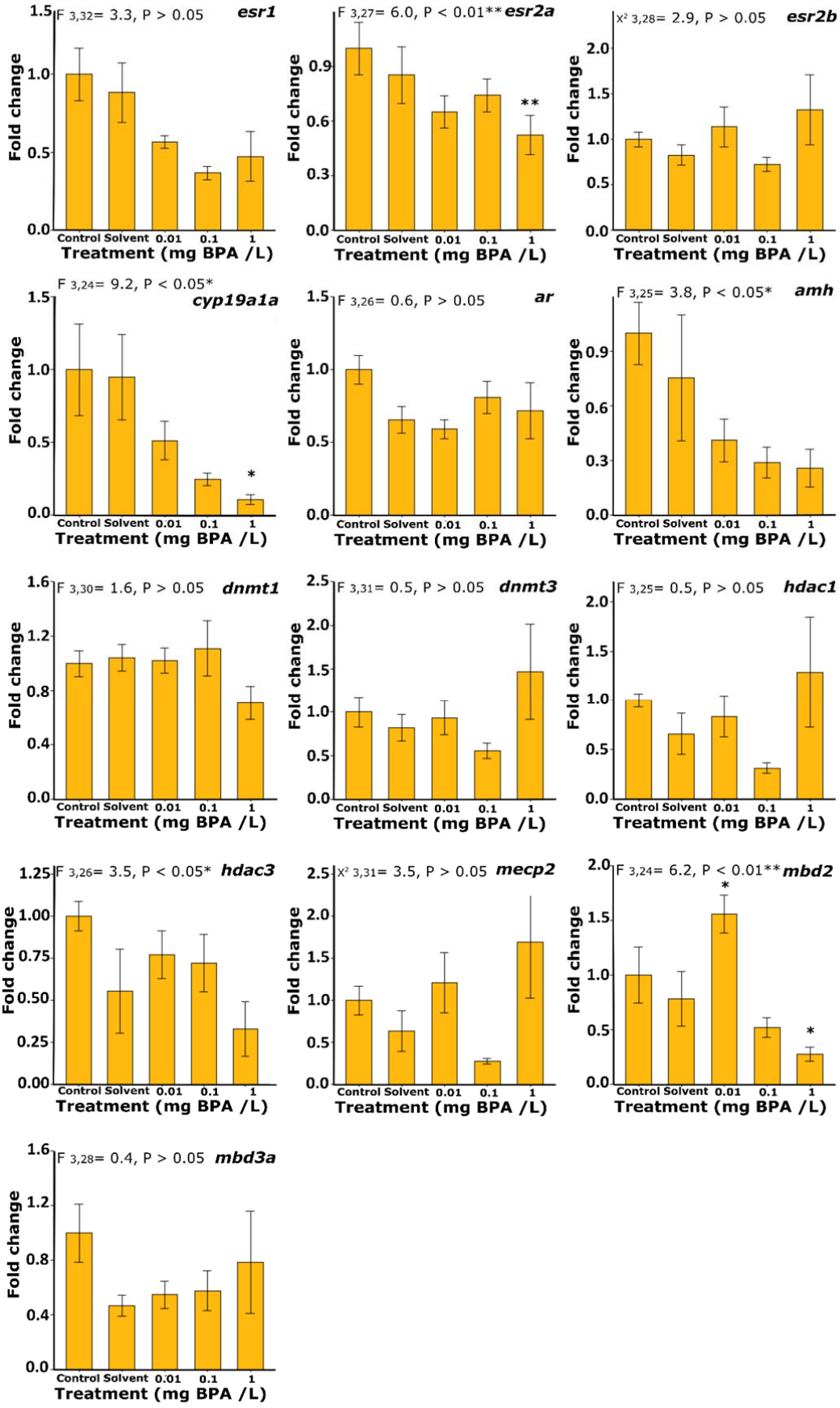
Supporting Information Figure S1. Morphometric parameters for males and females exposed to 0.01, 0.1 and 1 mg/L BPA (n=12 individuals per treatment). Individual plots represent the gonadosomatic index for females **(A)** and males **(B),** hepatosomatic index for females **(C)** and males **(D),** and the mean condition factor for females **(D)** and males **(E).** Statistical comparisons were conducted using Kruskal-Wallis one-way ANOVA on ranks followed by the pairwise Wilcox test, in R (version 3.0.2). All data are presented as mean ±SEM. Asterisks indicate significant differences compared to the solvent treatment (*p<0.05).


F 3,23= 0.5, P > 0.05
esr1
Fold change
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)
F 3,37= 0.7, P > 0.05
esr2a
Proportion amplified
F 3,25= 4.0, P < 0.05*
esr2b
F 3,32= 3.3, P < 0.05*
vtg1
F 3,32= 3.2, P < 0.05*
dnmt1
F 3,32= 0.2, P > 0.05
dnmt3
F 3,25= 0.5, P > 0.05
hdac1
F 3,32= 0.2, P > 0.05
hdac3
F 3,32= 0.1, P > 0.05
mecp2
F 3,32= 4.6, P < 0.05*
mbd2
F 3,32= 3.7, P > 0.05
mbd3a

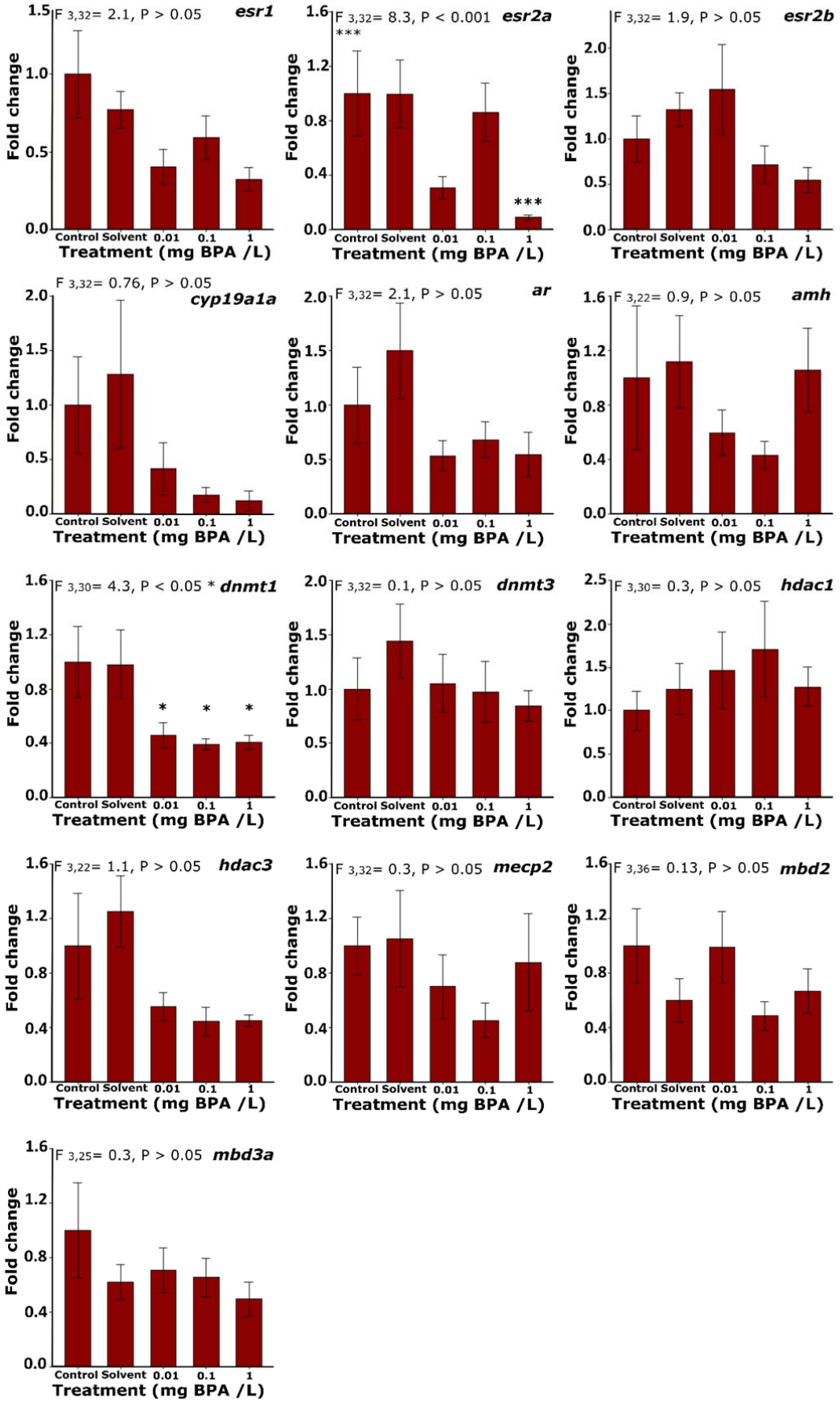
Supporting Information Figure S2. Transcript profiling of target genes in female livers following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data were collected for 6-8 fish per treatment, and data points classified as outliers (using Chauvenet's criterion) and for which the expression was below the detection limit of the assay were excluded from analysis. Where amplification was detected in more than 70% individuals, data are represented as fold- change relative to the expression in the control group. Where amplification was detected in less than 70% individuals data are presented as the proportion of individuals for which the target genes were detected. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05 **P<0.01 ***P<0.001).


F 3,32= 2.8, P > 0.05 esr1
Proportion amplified
F 3,26= 0.3, P > 0.05 esr2a
Fold change
F 3,25= 4.9, P < 0.01** esr2b
F 3,26= 6.7, P < 0.01** vtg1
F 3,32= 8.4, P < 0.01** dnmt1
F 3,32= 2.1, P > 0.05 dnmt3
F 3,22= 4.9, P < 0.01** hdac1
F 3,32= 5.1, P < 0.05* hdac3
F 3,32= 4.0, P > 0.05* mecp2
F 3,32= 2.1, P < 0.05* mbd2
F 3,32= 1.32, P > 0.05 mbd3a
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)

Supporting Information Figure S3. Transcript profiling of target genes in male livers following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data were collected for 6-8 fish per treatment, and data points classified as outliers (using Chauvenet's criterion) and for which the expression was below the detection limit of the assay were excluded from analysis. Where amplification was detected in more than 70% individuals, data are represented as fold-change relative to the expression in the control group. Where amplification was detected in less than 70% individuals data are presented as the proportion of individuals for which the target genes were detected. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05 **P<0.01 ***P<0.001).

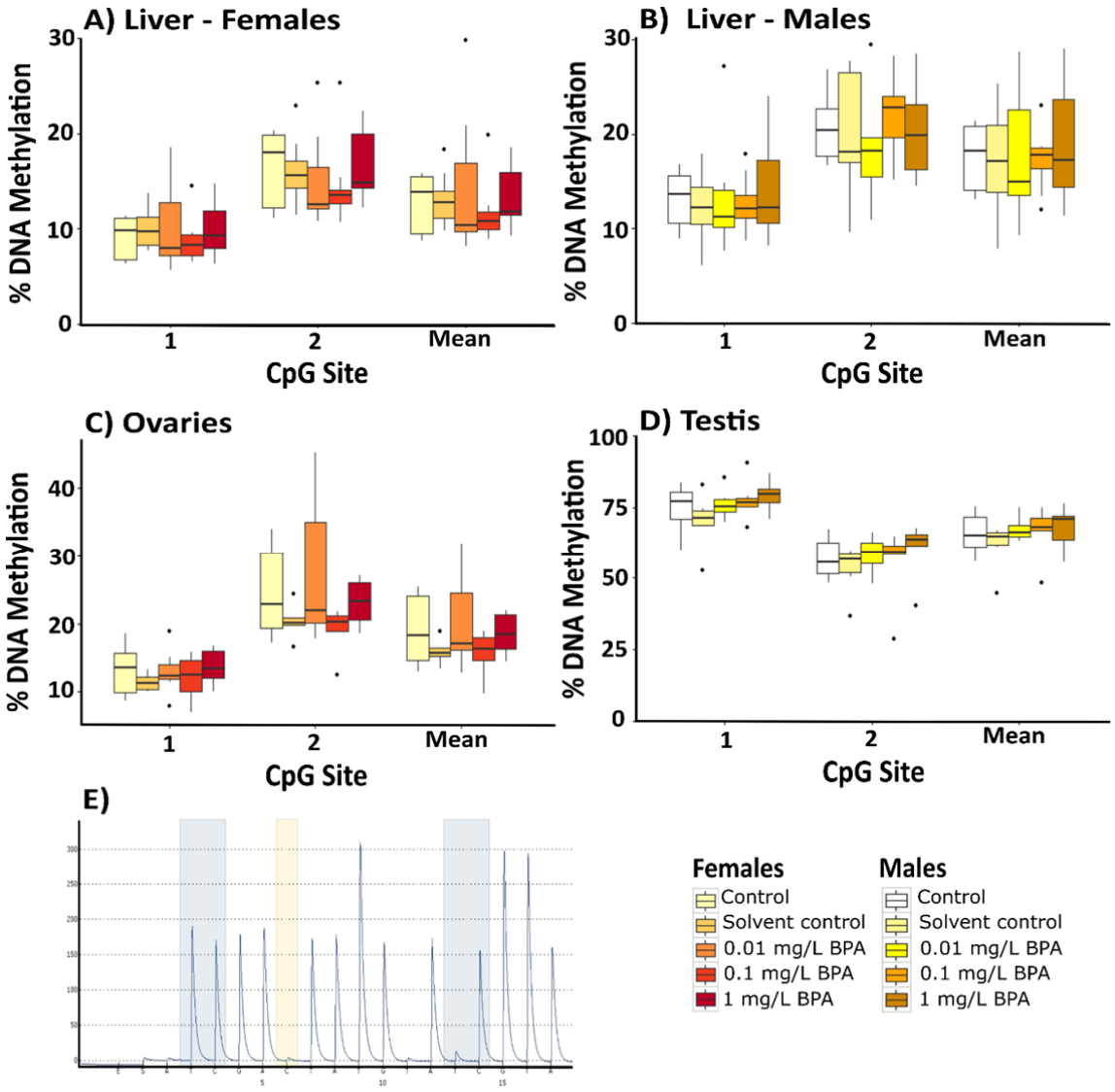

F 3,32= 3.3, P > 0.05
esr1
F 3,27= 6.0, P < 0.01**
esr2a
X² 3,28= 2.9, P > 0.05
esr2b
F 3,24= 9.2, P < 0.05*
cyp19a1a
F 3,26= 0.6, P > 0.05
ar
F 3,25= 3.8, P < 0.05*
amh
F 3,30= 1.6, P > 0.05
dnmt1
F 3,31= 0.5, P > 0.05
dnmt3
F 3,25= 0.5, P > 0.05
hdac1
F 3,26= 3.5, P < 0.05*
hdac3
X² 3,31= 3.5, P > 0.05
mecp2
F 3,24= 6.2, P < 0.01**
mbd2
F 3,28= 0.4, P > 0.05
mbd3a
Fold change
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)

Supporting Information Figure S4. Transcript profiling of target genes in the testis following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data are presented as fold-change relative to the control group. Relative expression was calculated as a ratio of the efficiency corrected expression data for the target gene / efficiency corrected expression data for *rpl8*. For each treatment, data were obtained for 6–8 individual fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis using the Chauvenet's criteria. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05 **P<0.01 ***P<0.001).


F 3,32= 2.1, P > 0.05 esr1
F 3,32= 8.3, P < 0.001 esr2a
***
***
F 3,32= 1.9, P > 0.05 esr2b
F 3,32= 0.76, P > 0.05 cyp19a1a
F 3,32= 2.1, P > 0.05 ar
F 3,22= 0.9, P > 0.05 amh
F 3,30= 4.3, P < 0.05 * dnmt1
* * *
F 3,32= 0.1, P > 0.05 dnmt3
F 3,30= 0.3, P > 0.05 hdac1
F 3,22= 1.1, P > 0.05 hdac3
F 3,32= 0.3, P > 0.05 mecp2
F 3,36= 0.13, P > 0.05 mbd2
F 3,25= 0.3, P > 0.05 mbd3a
Fold change
Control Solvent 0.01 0.1 1
Treatment (mg BPA /L)

Supporting Information Figure S5. Transcript profiles of target genes in ovaries following exposure to 0.01, 0.1 and 1mg/L BPA for 15 days. Data are presented as fold-change relative to the control group. Relative expression was calculated as a ratio of the efficiency corrected expression data for the target gene / efficiency corrected expression data for *rpl8*. For each treatment, data were obtained for 6–8 individual fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis using the Chauvenet's criteria. Asterisks represent significant differences between treatment groups compared to the solvent control group (*P<0.05 **P<0.01 ***P<0.001).

Supporting Information Figure S6. Promoter regions of *amh*, *dnmt1* and *esr1*, showing the location of the CpG sites (indicated in bold), the target sequences used for pyrosequencing (underlined) and putative EREs (highlighted in blue) in relation to the transcription start sites (TSSs; highlighted in red). The sequences shown were derived from Ensembl Zv9 (release 83; assembly GRCz10) and correspond to the following genomic positions: chr22:20,736,779-20,737,279 (*amh*), chr3:54,352,519-54,352,819 (*dnmt1*) and chr20:26,483,369-26,484,513 (*esr1*).



Supporting Information Figure S7. Gene specific DNA methylation profiles for a series of two CpG sites in the promoter region of estrogen receptor 1 (*esr1*) in the liver of female **(A)** and male **(B)** adult zebrafish, and in the ovaries **(C)** and testes **(D)** of adult zebrafish following exposure to 0.01, 0.1 and 1 mg/L BPA. **E)** Example pyrogram of two CpG sites in the 5' flanking regions of the *esr1* gene. Data are presented as boxplots (n = 6-8 for each group). Asterisks indicate significant differences compared to the solvent control (*P<0.05 **P<0.01 ***P<0.001).

Supporting Information Table 1. Measured concentrations of BPA in the exposure water, using HPLC-MS. Concentrations were measured for the three replicate treatment tanks on days 5, 10 and 15 of the exposure and are presented as mean values ± SEM.

Nominal concentration	**Control**	**Solvent control**	**0.01 mg/L BPA**	**0.1 mg/L BPA**	**1 mg/L BPA**
Day 5	< 0.001	< 0.001	0.02 ± 0.00	0.14 ± 0.01	1.28 ± 0.05
Day 10	< 0.001	< 0.001	0.01 ± 0.00	0.14 ± 0.01	1.20 ± 0.14
Day 15	< 0.001	< 0.001	0.01 ± 0.00	0.09 ± 0.03	1.43 ± 0.06
Mean	**< 0.001**	**< 0.001**	**0.01**	**0.12**	**1.30**

Supporting Information Table 2. Target genes, primer sequences and assay details for the RT-QPCR analysis.

Name	Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta (°C)	PCR efficiency
Ribosomal protein L8	*rpl8*	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	1.95
Aromatase	*cyp19a1a*	AGCCGTCCAGCCTCAG	ATCCAAAAGCAGAAGCAGTAG	101	61.5	2.06
Estrogen receptor 1	*esr1*	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	2.14
Estrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	1.86
Estrogen receptor 2b	*esr2b*	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	2.18
Androgen receptor	*ar*	ACGAGGGTGTTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	1.97
Anti-Mullerian hormone	*amh*	TGTCTCAACCATCGTCTTCAG	CAGTCAATCCATCCATCCAAAC	124	61.0	2.24
Vitellogenin	*vtg1*	AGCAGCAGCAGTCGTAAC	CAATGATGGTGGCAGTCTTAG	148	57.5	1.84
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	CGCTGTCGTGTTGAGTATGC	TCCCTTGCCCTTTCCTTTCC	180	58.5	2.06
DNA (cytosine-5)-methyltransferase 3	*dnmt3*	TGATGCCGTGAAAGTGAGTC	TTGCCGTGTAGTGATAGTGC	172	58.5	2.19
Histone deacetylase 1	*hdac1*	TGACAAACGCATCTCCATTCG	CTCTTCTCCATCCTTCTCTTCTTC	157	58.0	2.04
Histone deacetylase 3	*hdac3*	GAATGTGTGGAGTTTGTGAAGG	CTGGATGAAGTGTGAAGTCTGG	190	57.0	1.98
Methyl CpG binding protein 2	*mecp2*	GAGGCAGAAACAGGACAG	TGGTGGTGATGATGATGG	176	58.0	2.13
Methyl-CpG-binding domain protein 2	*mbd2*	AACAGCCTCCATCTTCAAG	CGTCCTCAGCACTTCTTC	166	59.0	2.19
Methyl-CpG-binding domain protein 3a	*mbd3a*	ACTCTTCTTTCGGCTCTG	TCTTCCTGCTTCCTGATG	164	57.0	1.99

Supporting Information Table 3. Bisulfite-pyrosequencing primers and assay details for the gene promoters analyzed.

Name	Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Sequence Primer (5'-3')	Sequence analysed (5'-3')	Ta (°C)
Estrogen receptor 1	*esr1*	AGAGGAGGTAAATAAATTAAAGATAGTTAG	Biotin-TACTCCTTTAACATATAATTTCCCATAACA	GGTAAATAAATTAAAGATAGTTAGG	TYGATATTGAYGGTTATTTTTTAGAGTAGGTTATGGTAATTAG	58.0
Anti-Mullerian hormone	*amh*	GTTTTTTATTTTTATGGGATGGTAGTTAGG	Biotin-AAACACAACTTAAAAACTTCCACTTATAT	TTGTTTTGAAGTATATTTGGAT	TATAYGTAATGGGGAATGTTTTAGTTTAAGGAAYGGTATTTGGTATTATTAAYGGGTTATTTATAAAATAATGTTTTTA	58.0
DNA (cytosine-5)-methyltransferase 1	*dnmt1*	GGGTATTAATATGTGATAGTGTTAATTGTAG	Biotin - TAAACCCAAATACACTCACAACAC	TTATGAATTGTAGTTAGTAGTTGA	GAAATAYGYGYGGGTYGTTTTTTYGYGYGGAAAYGYGGGTGAGTYGGAYGTTATT	58.0

Supporting Information Table 4. Statistical associations between **a)** BPA concentration and transcription; **b)** BPA concentration and global methylation; **c)** *dnmt1* transcription and global methylation; **d)** BPA concentration and specific CpG loci methylation; **e)** transcript expression and specific CpG loci methylation.

Table 4a. Regression analysis between BPA concentration and transcription .

Tissue	Gene	-	Adjusted R2	P value
Liver Female	*vtg1*	-	0.155	**0.018**
	esr1	-	0.142	**0.049**
	esr2b	-	-0.036	0.896
	hdac1	-	-0.045	0.718
Liver Male	*vtg1*	-	0.181	**0.012**
	esr2a	-	0.021	0.246
	esr2b	-	0.117	0.057
	hdac1	-	0.141	**0.033**
Ovary	*esr1*	-	0.046	0.161

	esr2a	-	0.238	**0.017**
	esr2b	-	0.081	0.086
	amh	-	0.021	0.248
	cyp19a1a	-	0.031	0.220
	ar	-	0.020	0.245
	dnmt1	-	-0.021	0.449
	dnmt3	-	0.036	0.186
	hdac1	-	-0.035	0.674
	hdac3	-	0.048	0.166
	mecp2	-	-0.043	0.751
	mbd2	-	-0.039	0.722
	mbd3a	-	0.005	0.303
Testis	*esr1*	-	-0.031	0.619
	esr2a	-	0.053	0.121
	esr2b	-	0.049	0.148
	amh	-	0.075	0.094
	cyp19a1a	-	0.189	**0.025**
	ar	-	-0.032	0.754
	dnmt1	-	0.111	**0.046**
	dnmt3	-	0.132	**0.033**
	hdac1	-	0.059	0.117
	hdac3	-	0.080	0.092
	mecp2	-	0.083	0.072
	mbd2	-	0.135	**0.048**
	mbd3a	-	0.022	0.226

Table 4b. Regression analysis between BPA concentration and global methylation.

Tissue	Gene	-	Adjusted R2	P value
Testis	-	-	0.033	0.949
Ovary	-	-	0.051	0.121

Table 4c. Correlation analysis between *dnmt1* trancript expression and global methylation.

Tissue	Gene	-	Correlation coefficient	P value
Testis	*dnmt1*	-	0.110	0.576
Ovary	*dnmt1*	-	0.293	0.198

Table 4d. Regression analysis between BPA concentration and specific CpG loci methylation.

Tissue	Gene	CpG Position	Adjusted R2	P value
Liver Female	*esr1*	1	-0.031	0.075
		2	-0.033	0.834

	dnmt1	1	0.054	0.109
		2	0.029	0.179
		3	0.046	0.128
		4	0.076	0.073
		5	0.024	0.197
		6	0.040	0.144
		7	0.065	0.089
		8	0.085	0.069
		9	0.079	0.069
		10	0.070	0.081
		11	0.051	0.133
		Mean	0.063	0.093
Liver Male	*esr1*	1	-0.025	0.649
		2	-0.030	0.779
	dnmt1	1	-0.036	0.905
		2	-0.026	0.597
		3	-0.003	0.348
		4	-0.030	0.681
		5	-0.031	0.700
		6	-0.024	0.565
		7	-0.035	0.820
		8	-0.025	0.581
		9	-0.233	0.552
		10	-0.023	0.551
		11	-0.017	0.465
		Mean	-0.023	0.541
Ovary	*esr1*	1	0.052	0.115
		2	-0.024	0.583
	amh	1	0.005	0.295
		2	0.144	0.246
		3	-0.034	0.836
	dnmt1	1	0.082	0.068
		2	0.087	0.063
		3	0.092	0.057
		4	0.105	**0.045**
		5	0.114	**0.038**
		6	0.100	**0.049**
		7	0.044	0.137
		8	0.115	**0.038**
		9	0.091	0.058
		10	0.098	0.051
		11	0.061	0.100
		Mean	0.094	0.055
Testis	*esr1*	1	-0.016	0.465
		2	-0.009	0.397

	amh	1	0.163	**0.013**
		2	0.036	0.152
		3	-0.017	0.497
	dnmt1	1	-0.380	**0.047**
		2	0.003	0.304
		3	0.011	0.255
		4	-0.016	0.480
		5	0.000	0.318
		6	0.000	0.325
		7	-0.016	0.471
		8	-0.001	0.334
		9	0.006	0.290
		10	0.038	0.182
		11	0.005	0.313
		Mean	-0.001	0.335

Table 4e. Correlation analysis between trancript expression and specific CpG loci methylation.

Tissue	Gene	CpG Position	Correlation coefficient	P value
Ovary	*esr1*	1	-0.229	0.281
		2	-0.225	0.289
	amh	1	-0.323	0.164
		2	-0.286	0.235
		3	-0.286	0.221
	dnmt1	1	-0.050	0.830
		2	-0.026	0.912
		3	-0.003	0.991
		4	-0.142	0.540
		5	0.097	0.674
		6	0.082	0.724
		7	0.192	0.404
		8	0.065	0.780
		9	-0.033	0.887
		10	-0.055	0.814
		11	-0.068	0.771
		Mean	0.023	0.921
Testis	*esr1*	1	0.095	0.653
		2	0.386	**0.035**
	amh	1	-0.452	**0.014**
		2	-0.047	0.815
		3	-0.214	0.285
	dnmt1	1	-0.024	0.903
		2	-0.180	0.359
		3	-0.204	0.306
		4	-0.157	0.425

		5	-0.523	**0.004**
		6	-0.514	**0.006**
		7	-0.475	**0.014**
		8	-0.435	**0.023**
		9	-0.382	0.066
		10	-0.035	0.886
		11	-0.039	0.889
		Mean	-0.380	**0.047**