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Reproductive toxicity of low level bisphenol A exposures in a two-generation zebrafish assay: Evidence of male-specific effects

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ABSTRACT

Bisphenol A (BPA), a high-volume chemical used to make polycarbonate plastic and epoxy resins, is a ubiquitous contaminant in environment and human body. To investigate the reproductive effects of long-term exposure to low concentrations of BPA, a two-generation study was conducted using the aquatic model species of zebrafish. Our findings revealed that exposure to 1 nM (0.228 μg/L) BPA for continuous two generations resulted in female-biased sex ratio in both F1 and F2 adult population, decreased sperm density, and decreased sperm quality as measured by motility, velocity, ATP content and lipid peroxidation in F1 and F2 males. Females were less sensitive to BPA exposures than males as no adverse effects were found in female gonads or gametes. Delayed hatching at 48 hpf and increased malformation and mortality were found in the offspring from BPA exposed F2, but not F1 parents. Most importantly, the adverse effect on larval development and survival from BPA exposed F2 parents was paternal-specific, resulting mainly from BPA exposed males. Subsequent transcription analysis of F2 male gonads revealed dysregulated mitochondrial biogenesis and significant activation of non-canonical Wnt/planar cell polarity and Wnt/Calcium signaling pathways. Gene expression analysis of larvae from BPA exposed F2 parents showed significant reduced expression of DNA methyltransferases such as *dnmt1*, *dnmt3*, and *dnmt5*. In conclusion, low level BPA exposures for continuous two generations not only affects sex ratio and sperm quantity/quality in F1 and F2 adults, reproductive success in offspring from F2 parents, but also perturbs various molecular pathways potentially contributing to these BPA induced male-specific reproductive defects.

1. Introduction

Bisphenol A (BPA) is widely used as an intermediate to manufacture polyvinylchloride (PVC) film, polycarbonate plastic, and epoxy resin-lined food containers (Biedermann et al., 2010; Welshons et al., 2006). Incomplete polymerization during large-scale production or gradual breakdown of numerous BPA-containing products has resulted in BPA released to various environmental media. Aquatic environments are the ultimate reservoirs for many anthropogenic chemicals including BPA (Bhandari et al., 2015b). The median and maximum reported BPA concentrations in 85 stream sites in the United States are 0.14 and 12 μg/L (0.6 and 52.6 nM), respectively (Kolpin et al., 2004). A review article published in 2007 examining BPA level in river water samples collected from the United States, China, Germany, Japan, Spain, and the Netherlands showed a concentration range of 0.02–8 μg/L (0.1–35 nM) for the maximum values reported in all those studies (Kang et al., 2007). Recently, Bhandari et al. reviewed existing literature on BPA level in surface water across multiple countries including the United States, China, Japan, Korea, Indian, and 23 EU countries and reported a median concentration range of 3–30 ng/L (0.01–0.1 nM) across all these different studies, and a concentration range of 1–28 μg/L (4.4–122.8 nM) for contaminated sites (Bhandari et al., 2015a).

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BPA is well known as an endocrine disrupting chemical (EDC) and its adverse effect on reproduction has been well documented. Perinatal exposure to environmentally relevant levels of BPA decreased fertility and fecundity in CD-1 mice (Cabaton et al., 2011), induced female offspring earlier vaginal opening and exhibited advanced puberty (Durando et al., 2007), decreased the anogenital distance of male offspring (Miao et al., 2011), and decreased embryo implantation rate (Berger and Foster, 2010). In the terrestrial isopod *Porcellio*, BPA exposure elicited reproductive toxicity by decreasing female reproductive allocation and increased abortions rate (Lemos et al., 2010). In fish, vitellogenin induction in males is one of important biomarkers associated with high level BPA exposures and has been reported in zebrafish (1000 μg/L or 4.4 μM for 3 weeks), swordtail *Xiphophorus helleri* (2000 μg/L or 8.8 μM for 3 days), fathead minnow *Pimephales promelas* (640 and 1280 μg/L or 2.8 and 5.6 μM for 43 days), medaka *Oryzias latipes* (1000 μg/L or 4.4 μM for 5 weeks), and rainbow trout *Oncorhynchus mykiss* (70–500 μg/L or 0.3–2.2 μM for 6 and 12 days) [see (Kang et al., 2007) for review]. Disruption of gametogenesis is another adverse reproductive outcome associated with BPA exposure in fish. In fathead minnow, changes in spermatogenesis (increases in spermatogonia and decreases in spermatocyte or spermatozoa) were observed at 16 μg/L (70 nM) BPA in one study (Sohoni et al., 2001a) and at 160 μg/L (700 nM) in another study (Mihaich et al., 2012) with same exposure duration of 164 days. In brown trout (*Salmotrutta f. fario*), a low level BPA exposure of 2.4 μg/L (10.5 nM) delayed the spermiation and decreased sperm density, motility and velocity (Lahnsteiner et al., 2005). In goldfish (*Carassius auratus* L.), BPA exposure at 0.6–11 μg/L (2.6–48.2 nM) adversely affected sperm motility and velocity (Hatef et al., 2012a). *In vitro*, Eurasian perch (*Perca fluviatilis* L.) sperm exposed to high concentrations of BPA (1 mM) affected sperm morphology, motility and velocity (Hatef et al., 2010). In addition, BPA exposure also led to formation of testis-ova, decreased androgens, induction of apoptosis in testis, and changes in sex ratios (Crane et al., 2007; Kang et al., 2007).

Clearly, BPA exposure at a wide range of concentrations affected various stages of reproduction development including sex determination and differentiation, gonad maturation, gametogenesis, and gamete quality. However, many of these adverse effects have concentration thresholds that are well above expected environmental concentrations likely to be encountered by aquatic organisms (Bhandari et al., 2015a; Kang et al., 2007). Moreover, for those studies that did evaluate BPA toxicity at concentrations that are closer to environmentally relevant levels (e.g., 1–10 μg/L), few of them look into the underlying molecular mechanism (Hatef et al., 2012b). Characterization of BPA-induced reproductive toxicity at environmentally relevant concentrations and understanding the mechanism basis for its associated adverse outcomes would not only allow better ecological risk assessment, but may also help delineate BPA's adverse effect on human health. In the present study we examined whether low level BPA (0.228 μg/L or 1 nM) exposures for continuous two-generations would exhibit similar reproductive toxicity as those observed at higher concentrations using zebrafish as a model species. Specifically, we evaluated the effect of BPA exposure on sex ratio, gonad histology, gamete quantity and quality, and reproductive success as measured by fertilization, hatching, and larval survival in F1 and F2 generations. Our findings revealed altered sex ratio, decreased sperm counts and quality, and male-specific reproductive failure in F2 offspring. We further examined the transcriptional changes of F2 male gonads using RNA-Seq and gene expression changes of larvae from F2 parents using qRT-PCR to explore the underlying mechanisms responsible for low concentration BPA exposure-induced reproductive toxicity.

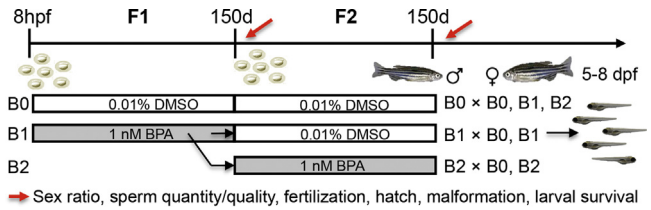

Fig. 1. A schematic diagram showing zebrafish embryos at 8 h post-fertilization (hpf) continuously exposed to 1 nM (0.228 μg/L) BPA for two generations (F1 and F2). Embryos exposed to 0.01% DMSO served as no BPA exposure control (B0). F2 embryos at 8 hpf from F1 BPA exposed group were either exposed to 0.01% DMSO, serving as one generation BPA exposure with 150 d recovery period (B1) or continuously exposed to 1 nM BPA for a total of two generations without any recovery period (B2). Various reproductive endpoints were assessed for F1 and F2 adults at 150 days post fertilization (dpf) and for offspring from F1 and F2 adults at 0–8 dpf.

2. Materials and methods

2.1. Fish husbandry and embryo collection

Zebrafish care and use were conducted according to established guidelines approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University. Adult zebrafish (*Danio rerio*) of the wildtype (AB strain) were raised and kept at standard laboratory conditions of 28 °C on a 14:10 dark/light photoperiod in a recirculation system according to standard zebrafish breeding protocols (Westerfield, 1995). Water supplied to the system was filtered by reverse osmosis (pH 7.0–7.5), and Instant Ocean® salt was added to the water to raise the conductivity to 450–1000 μS/cm (system water). The adult fish were fed twice daily with zebrafish diet (Zeigler, Aquatic Habitats, Apopka Florida) and a live artemia (Jiahong Feed Co., Tianjin, China).

Zebrafish embryos were obtained from spawning adults in tanks overnight with a sex ratio of 1:1. Embryos were collected within 0.5 h of spawning and rinsed in an embryo medium (EM: 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$) (Westerfield, 1995). Fertilized embryos with normal morphology were staged under a dissecting microscope SMZ1500 (Nikon, Japan) according to the standard methods (Kimmel et al., 1995).

2.2. BPA stock solutions and exposure protocols

BPA [2,2-bis (4-hydroxyphenyl) propane, CAS# 80-05-7, purity > 99%, Sigma] stock solution (10 μM BPA) were prepared by dissolving BPA in 100% dimethyl sulfoxide (DMSO) and stored at −20 °C. Working solution was prepared by dilution of stock solution immediately prior to experimental use. Embryos harvested from same batch of parental stocks were used to start the first generation (F1): 0.01% DMSO solvent control vs. 1 nM (0.228 μg/L) BPA in 0.01% DMSO exposures. Adult F1 fish (150 d) within the same treatment group were mated (4 females × 4 males/replicate × 3 replicates) to produce F2 embryos. The mating was conducted in clean water without BPA and embryos were subject to continuous treatment at 8 h post fertilization (hpf). In detail, the F2 embryos at 8 hpf from F1 DMSO control group were continuously exposed to 0.01% DMSO for 150 days, serving as no BPA exposure control (B0). The F2 embryos at 8 hpf from F1 BPA exposed group were either exposed to 0.01% DMSO, serving as one generation BPA exposure with 150 d recovery period (B1) or continuously exposed to 1 nM BPA for a total of two generations without any recovery period (B2) (Fig. 1). In total, our exposure regimens consist of two treatment groups (control vs. BPA) exposed for one generation, and three treatment groups (B0, B1, and B2) exposed for two generations (Fig. 1). The whole experiment was repeated three times each starting with a new batch of embryos. Reproductive endpoints such as sex ratio, sperm quan-

tity/quality, egg production, fertilization, hatch, malformation and larval survival were assessed at the end of each exposure generation (Fig. 1). BPA concentration in exposed water was measured based on our previous method (Lin et al., 2013). The measured BPA concentration in DMSO control fish tanks was 0.032 μg/L, and in tanks dosed with 1 nM BPA was 0.372 μg/L.

2.3. Sex ratio, sperm quantity and quality

At the end of each exposure generation (~150 d), all fish were visually checked for sex by an observer blind to the treatment to calculate sex ratio. A randomly selected subsample of 10 male and female fish from each replicate was used for measurement of standard body length (from snout to the fork point of caudal fin, cm) and wet weight (g). Condition factor (K = weight × 100/length^3) was also tabulated to determine their overall fitness. To evaluate sperm quality in male fish after chronic BPA exposure, sperm were collected by surgical removal of testis and prepared in Hanks' balanced salt solution [see (Jing et al., 2009) for details]. Sperm numbers were obtained from the average of duplicate counts using a hemocytometer. Sperm motility was determined by a computer assisted sperm analysis system (CASA, IVOS version 12.0, Hamilton Thorne Bioscience) following our previous published method (Wang et al., 2010). The total motility (TM) indicates the percentage of all motile sperm, and the progressive motility (PM) indicates the percentage of motile sperm that expressed vigorous swimming. The sperm swimming velocity analysis includes velocity average path (VAP), velocity straight line (VSL) and velocity curvilinear (VCL).

The measurement of adenosine triphosphate (ATP) in sperm was performed in a luminometer (Berthold, Wildbad, Germany), using an assay kit based on the luciferin–luciferase reaction, following the manufacturer's recommendations. A 6-point standard curve (from 0.5 to 50 nM) was included in each assay.

Lipid peroxidation was detected as malondialdehyde (MDA) reacting with thiobarbituric acid to form a colored complex by spectrofluorometric analysis (M5, ThermoFisher), as described in (Gong et al., 2012). The level of MDA was presented as nmol mg^{-1} protein.

2.4. Egg production, fertilization, hatch, larval malformation and survival

At the end of each exposure duration (~150 d), breeding trials (4 females × 4 males/replicate × 3 replicates) within the same treatment groups were carried out to produce offspring for assessment of egg production, fertilization, hatch, and larval malformation and survival, which are ultimate measurements of reproductive success. Embryos were collected continuously for 3 d, and a subsample of 270 embryos per spawning were assessed for these endpoints. The hatch rate was assessed at 48 and 72 h post fertilization, malformation and mortality were assessed at 6, 7, 8 dpf. Because we observed increased malformation and mortality in offspring from F2 parents, we also paired F2 females from B1 and B2 with males from B0, and females from B0 with males from B1 and B2 to differentiate maternal or paternal derived adverse effects.

2.5. RNA-Seq of F2 adult male gonads

Total RNA was extracted from 150 dpf F2 male gonads (5 gonads pooled as one replicate × 3 replicates) from B0 and B2 groups by Trizol reagent (Invitrogen). The RNA quality was checked by Bioanalyzer 2200 (Aligent) and kept at −80 °C. The RNA with RIN > 8.0 were used for cDNA library construction. The cDNA libraries for single-end sequencing were prepared using Ion Total RNA-Seq Kit v2.0 (Life Technologies) according to the manufacturer's instructions. The cDNA libraries were then processed for the Proton sequencing process according to the commercially available protocols. Samples were diluted and mixed, the mixture was processed on a OneTouch 2 instrument (Life Technologies) and enriched on a OneTouch 2 ES station (Life Technologies) for preparing the template-positive Ion PI™ Ion Sphere™ Particles (Life Technologies) according to Ion PI™ Template OT2 200 Kit v2.0 (Life Technologies). After enrichment, the mixed template-positive Ion PI™ Ion Sphere™ Particles of samples was loaded on to 1 P1v2 Proton Chip (Life Technologies) and sequenced on Proton Sequencers according to Ion PI Sequencing 200 Kit v2.0 (Life Technologies).

Before mapping, clean reads were obtained from the raw reads by removing the adaptor sequences, reads with >5% ambiguous bases (noted as N) and low-quality reads containing more than 20 percent of bases with qualities of <13. The clean reads were then aligned to zebrafish genome (version: Zv9_year_2013) using the MapSplice program (v2.1.6). In alignment, preliminary experiments were performed to optimize the alignment parameters (-s 22 -p 15 –ins 6 –del 6 –non-canonical) to provide the largest information on the AS events (Alonso-Magdalena et al., 2006). Deseq was used to call the differentially expressed genes between control and BPA treated samples. In addition, we used a multiple test correction to reduce the number of false positives. Specifically, FDR correction with a p-value of <0.5 was performed. Genes with fold change >2 or <0.5 and FDR adjusted p-value <0.05 were exported as differentially expressed genes (Anders and Huber, 2010). For zebrafish mRNAs without gene annotation, we searched their human orthologs using blast at the NCBI website. Then all genes with annotation were loaded into IPA analysis to do network, pathway and GO anlaysis (IPA, Ingenuity® Systems, http://www.ingenuity.com).

2.6. qRT-PCR validation

Quantitative real time PCR (qRT-PCR) was used as an alternative method to validate gene expression changes of the Wnt signaling pathway found from the RNA-Seq analysis. A subset of RNAs from the same samples used for the RNA-Seqanalysis was used for qRT-PCR evaluation. cDNA was prepared from 5 μg of total RNA per group using a Prime Script® RT reagent Kit (Takara, Japan). qRT-PCR using gene-specific primers (Table 1, Sunny Biotechnology) was conducted on an EppendorfMastercycler® Realplex2 and the detail protocol was stated in our previous paper (Chen et al., 2014).

2.7. Expression of genes associated with DNA methylation in larvae derived from F2 parents

To evaluate why parental BPA exposure for two generations affects larval survive, we further examined transcriptional changes of genes associated with DNA methylation such as DNA methyltransferase 1 (*dnmt1*), *dnmt3*, *dnmt3b*, *dnmt5*, and transcription factor sp3 (*sp3*) in 120 hpf larvae derived from F2 parents between B0 and B2 groups. Forty larvae from each treatment group were collected for each replicate and the experiments were replicated three times. The primers are listed in Table 1 and the qRT-PCR protocol is detailed above.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to compare the differences among treatment groups. For gene expression test, an unpaired t-test with 5% FDR was performed. All data are reported as means ± standard error unless otherwise stated and $P < 0.05$ was set as the significance level.

Table 1
Primers used for qRT-PCR expression.

Target	Forward sequence	Reverse sequence
actin	AAGCAGGAGTACGATGAGTC	TGGAGTCCTCAGATGCATTG
wnt10b	GTTAACCCAGCTCCAGCTTC	CAGCATTGTGACCTCATCCG
wnt5b	GCGCATTAACCGACGTGGA	TACCCTCCGAGGTCTTGTTG
wnt9a	GGATGGACACCTTCTCCTGG	CACACAGCTTGTAGTGCGAT
wnt4a	ACGGAGTCAGTCCAGAAGGTTTCC	ACTCCGTTCCCTGATGTCCACG
wnt8a	GCAAGCATCAGTTCGCATGG	TCCGATCTTGGAGTCGTCAC
fzd2	AGCATAGTTTGCCGGACTCA	CGAAACAGCACGGCAGTAAC
fzd3b	GGCATCCCGTCAGTCTTCTG	GCGAGACTTGCGGACGATG
fzd6	TGTGATGCACACCTTCGACA	CTTTAGCTGGAGCGGACACC
fzd7a	CGTTTGCCTAGCATGATCGC	CGGTCCGAATGAAACGAAGC
fzd7b	ACCATCGTCATTGCGTGTTAC	AGAATCCTGATGTTATGCCTACGA
fzd8b	TCCGGTGAATACAGGCTATGC	TGTGTAGGCGAGTCCATCCT
fzd9	GCACACAGCAGCTACTTCCA	CGTCCCGACGATGAGATAGC
fzd10	GGCCAGGTAACAGCCAAGAA	CCCTGGCTCCAGTACACATC
lgr6	GTTGATGCCCGGACATTTGG	CGGCCAAAGTCAGGAAGAGTA
damm1b	GATCACCTACCGTCTTCGGG	AAGGCAAACATGGCCTCTCT
pfn1	GGAGTCCATTTGGGCGTCAG	CCATCGTGAATTGACCGTCC
rhoaa	GTAAGAAGCTTGTAATCGTTGGAGATG	CCACCTCAATATCAGCGACATA
rac1a	TGGTATCCGGAGGTCAGACA	AAGGCAGAGCACTCCAGGTA
rac2	CTACACTACCAATGCGTTCCC	ATGCTGGGCTCACCAAAGAG
junb	CGTCATCACATCACCCACGC	CGCCACCGAACAACTCGAC
plcd3a	GGTGGAAGATCATGACCACAA	CCGTACCACCTTGACATGGA
lrp5	TCACCGCATGGATGGACACATCA	ACGGGCCGGATCAAACTGGT
sfrp5	TGTTGGCAACTGTATCTGCAT	TGATTGAAAGGGTCGTCAATGT
plc12	CGGGAATACGCCAGTATGAGG	GGGACAGAGCCAGAATACACT
nfatc1	TGTGAAAGCACTAGCGGGAG	TGGTAGAATGCGTGAGGTCG
dnmt1	AAGACCTCATTGTCTCTGCCA	GTCCTGAGTATCAGCGAGCA
dnmt3	TGGCAGATGTAAAGATCGGAGA	ACTGACAAAAAGCAGCACCTG
dnmt3b	TACTCAAGTGGAGCAATGTCG	GCTGCCTGAAGAAGACCGTA
dnmt5	GTTATCTCTGAAGTGCCAATGTAA	GCAACGACAGCAAAAATAGAGA
sp3	GCGACCGCTGATATGTTTGT	GCTCCCTGACTGAGGTATCC

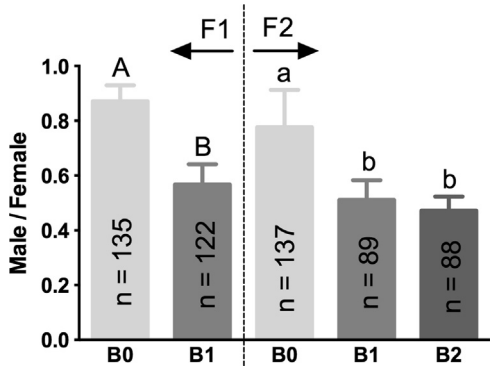

Fig. 2. Sex ratio in adult F1 population for control (B0; $n = 135$ from 4 replicates) and BPA exposed group (B1; $n = 122$ from 4 replicates), and adult F2 population for control (B0; $n = 137$ from 4 replicates) and BPA exposure for one generation (B1; $n = 89$ from 3 replicates) and two generations (B2; $n = 88$ from 3 replicates). Values plotted are mean ± SE and bars sharing the same letter indicate no significant difference.

3. Results

3.1. BPA exposure leads to female-biased sex ratio in F1 and F2 population

Chronic exposure to 1 nM BPA for only F1 generation or both F1 and F2 generations had no effect on adult fish survival and overall fitness measured by condition factor (data not shown). We also did not observe any obvious malformations in parental F1 and F2 fish during the whole exposure period. However, chronic BPA exposure significantly altered sex ratio (male/female) of the F1 and F2 population. In F1 adults, the sex ratio in control B0 group (0.87 ± 0.06) was higher than those in B1 (0.57 ± 0.07), and in F2 adults, the sex ratio in control B0 group (0.78 ± 0.14) was higher than those in B1 (0.51 ± 0.07) and B2 (0.47 ± 0.05) (Fig. 2).

3.2. BPA exposure reduces sperm counts and quality of F1 and F2 males

Chronic BPA exposure reduced sperm counts and quality for both F1 and F2 generations. Sperm density of F1 males from B1 group ($2.25 \pm 0.21 \times 10^9$/g) was significantly lower than that from B0 controls ($2.80 \pm 0.32 \times 10^9$/g) (Fig. 3). Similarly, sperm densities of F2 males from B1 ($2.34 \pm 0.0.44 \times 10^9$/g) and B2 ($1.94 \pm 0.32 \times 10^9$/g) groups were significantly lower than that from B0 controls ($3.07 \pm 0.52 \times 10^9$/g) (Fig. 3). Continuous BPA exposure for one or two generations resulted in significant reductions in sperm motility (%TM and %PM), sperm ATP production, and significant increase in sperm lipid peroxidation (MDA), however, sperm swimming velocity (VCL) was significantly reduced only in the two generation exposed B2 group (Fig. 3). Except for the reduction in ATP production, when exposures were restricted to only one generation with a subsequent recovery period of 150 days, there was no resulting adverse effect on sperm motility, velocity, and lipid peroxidation (Fig. 3). Sperm swimming velocity measured as VAP or VSL, however, was not different among sperm samples collected from F1 adult males in B0 and B1, F2 adult males in B0, B1 and B2 (data not shown).

3.3. BPA exposure affects larval malformation and survival in F2 offspring

Spawning trials showed no significant difference in egg production and fertilization of F1 and F2 females among all groups (data not shown). We also did not observe any adverse effect of embryo hatching or subsequent larval development and survival in offspring from F1 parents (Fig. 4). For offspring from F2 parents, parental BPA exposure delayed hatching rate at 48 hpf in embryos derived from intragroup mating parents within B1 ($20 \pm 2\%$) and B2 ($18 \pm 13\%$) groups when compared with B0 control ($49 \pm 6\%$). However, hatching rate was similar across all mating schemes at

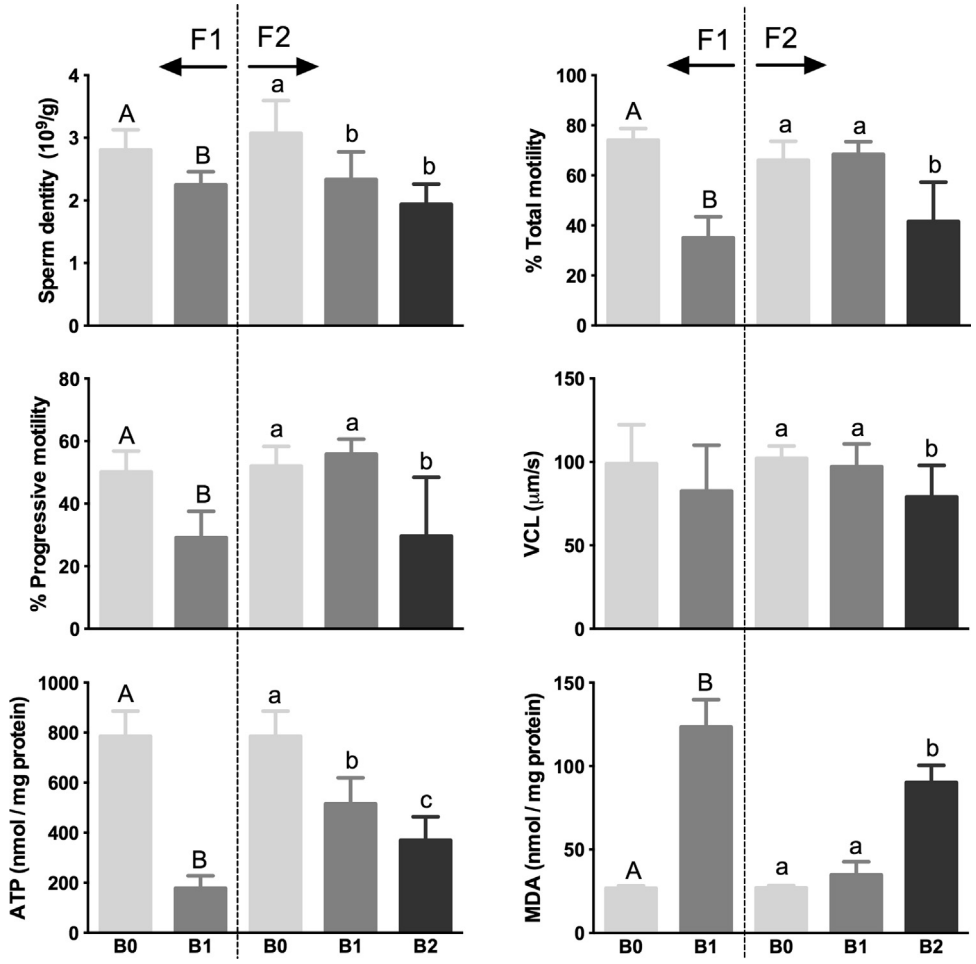

Fig. 3. Sperm parameters of F1 adult males between control (B0) and BPA exposed group (B1), and F2 adult males among control (B0), BPA exposure for one generation (B1) and two generations (B2). Assessed sperm parameters include sperm density ($n = 6$), total motility ($n = 6$), progressive motility ($n = 6$), velocity curvilinear (VCL; $n = 6$), sperm adenosine triphosphate content (ATP; $n = 9$), and lipid peroxidation expressed as malondialdehyde (MDA; $n = 3$). Values plotted are mean ± SE and bars sharing the same letter indicate no significant difference.

72 hpf (Fig. S1). In general, lower hatch rates were found in embryos derived from maternal BPA exposed groups (Fig. 4). In contrast, paternal BPA exposure had a significant adverse effect on malformation (e.g., uninflated swim bladder, pericardial edema and bent body) and mortality at 8 dpf. For example, % malformation in larvae derived from females paired with males from B0 was in a range of 7 ~ 17%, which was increased to ~30% in those paired with males from B1 and ~46% in those paired with males from B2 (Fig. 4). Similarly, % mortality increased from 2 to 18% in larvae derived from females paired with males from B0 to ~24% and ~44% in those paired with males from B1 and B2, respectively (Fig. 4).

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2015.10.020.

3.4. BPA exposure leads to mitochondrial dysfunction in F2 male gonads

To identify gene expression changes in male gonads following BPA continuous exposure for two generations, RNA-Seq analysis was conducted using RNAs isolated from 5-month-old F2 adult testis from B0 and B2 groups. Mapping of single-end reads indicated that the mapping rate of the six samples is high ranging from 0.828 to 0.881. The principal component analysis (PCA) of all genes on the RNA-Seq shows separation of samples from B0 and B2 groups into distinct clusters (data not shown). DEseq algorithm was used to filter the differentially expressed genes, and the level of expression of 1320 genes were significantly changed at least 2 fold by BPA as compared to the control group ($P < 0.05$ and 5% FDR) (Fig. 5A).

The top two pathways that perturbed by BPA exposure are the mitochondrial biogenesis and oxidative phosphorylation (Fig. S2). Genes that involved in these two pathways are overlap substantially and mainly related to enzymatic activities such as ATP synthase (*atp5c1*, *atp5o*), cytochorome c oxidase (*cox11*, *cox17*, *cox5aa*, *cox7a2*), glutathione peroxidase (*gpx4a*, *gpx4b*, *gpx7*), NADH dehydrogenase (*ndufa10*, *ndufab1*, *ndufaf1*, *ndufaf2*, *ndufb5*, *ndufb8*, *ndufs3*, *ndufs4*, *ndufs5*, *ndufs6*, *ndufs8a*), superoxide dismutase (*sox2*), and ubiquinol-cytochrome c reductase (*uqcrc1*, *uqcrc2b*, *uqcrfs1*, *uqcrh*). Except for a few genes, the expression of the majority of these genes was elevated by BPA exposure (Table 2), indicating activated mitochondrial biogenesis. Other significantly altered pathways include remodeling of epithelial adherens junctions, signaling by Rho family GTPases, EIF2 signaling, RhoGDI signaling, sertoli cell-sertoli cell junction signaling, integrin signaling, regulation of actin-based motility by Rho, germ cell-sertoli cell junction signaling, PPARa/RXRa activation, cyclins and cell cycle regulation, TGF-β signaling, protein ubiquitination pathway, estrogen-mediated S-phase entry, NRF2-mediated oxidative stress response, RhoA signaling, CXCR4 signaling, wnt/β-catenin signaling, and role of NFAT in regulation of the immune response (Fig. S2).

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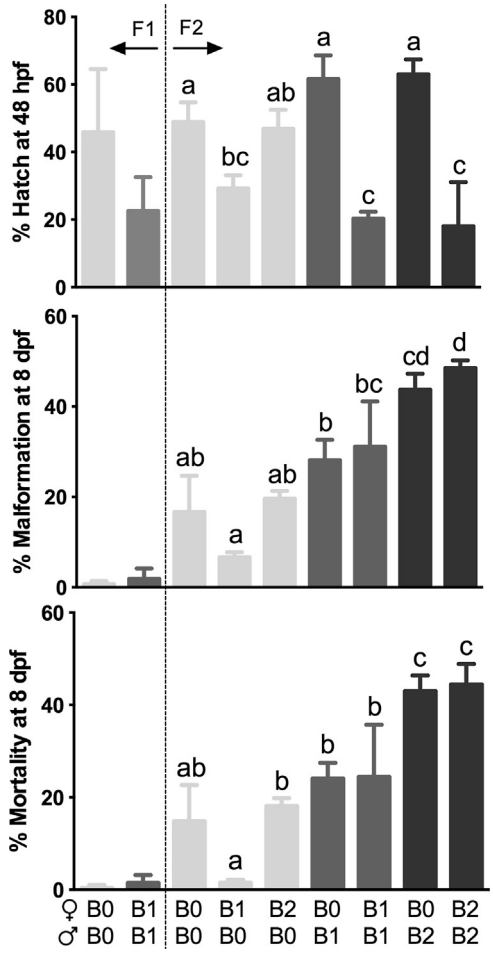

Fig. 4. Hatch (at 48 hpf), malformation and mortality (at 8 dpf) of offspring derived from control and BPA exposed F1 (B0 vs. B1) and F2 (B0, B1, B2) parents. All experiments were repeated three times with a minimum of 90 embryos per replicate. Values plotted are mean ± SE and bars sharing the same letter indicate no significant difference.

3.5. BPA exposure leads to dysregulated Wnt signaling in F2 male gonads

Further examination of specific transcripts related to reproductive development revealed significant perturbation of Wnt signaling pathway such as wingless-type MMTV integration site family member 5b (*wnt5b*), frizzled class receptor 2 (*fzd2*), *fzd8b*, *fzd9*, and *fzd10* (Fig. 5B). Pathway analysis revealed reduced expression of canonical Wnt/β-catenin pathway (Fig. S3) and an elevation in the expression of non-canonical Wnt/planar cell polarity (PCR, Fig. S4) and Wnt/Calcium pathways (Fig. S5). For example, besides *wnt5b* and various frizzled class receptors mentioned above, disheveled-associated activator of morphogenesis 1 (*daam-1*), profilin (*pfn*), Ras homolog gene family member A (*rhoa*), Ras homolog gene family, member A (*rac1*), *jun*, phospholipase C (*plc*), *axin*, nuclear factor of activated T-cells (*nfat*) were all elevated upon BPA exposure. We further validated differentially expressed genes constituting of Wnt ligands, receptors and downstream factors with qRT-PCR. Except for *wnt4a*, *wnt8a*, *wnt10b* and *irp5*, all other genes showed consistent expression between RNA-Seq and qRT-PCR (Fig. 6). These findings confirmed a general upregulation of non-canonical Wnt signaling pathway in testis upon two generation of BPA exposure (Fig. 6).

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3.6. BPA affects gene expression in F2 larvae

Further examination of genes associated with DNA methylation in larvae derived from F2 parents between B0 and B2 showed significant reduced expression of DNA methyltransferases *dnmt1*, *dnmt3*, *dnmt5* and transcription factor *sp3* in larvae from two generation continuous BPA exposed parents (Fig. 7).

4. Discussion

In the present study, a low level BPA exposure of 1 nM for two generations resulted in female-biased sex ratio, decreased sperm quantity, decreased sperm quality as measured by motility, velocity, ATP content and lipid peroxidation, delayed hatching at 48 hpf, and ultimately increased malformation and mortality in offspring from F2 parents. In particular, the adverse effect on larval development and survival from BPA exposed F2 parents was paternal-specific, resulting mainly from BPA exposed males. Subsequent transcription analysis of F2 male gonads revealed dysregulated mitochondrial biogenesis and significant activation of non-canonical wnt signaling pathways. Gene expression analysis of F2 larvae showed significant reduced expression of DNA methyltransferases. Alternation of these molecular pathways and gene expression may play a significant role in BPA induced reproductive toxicity in zebrafish.

It is known that low dose BPA exposure seldom caused any adverse effect on survival or overall body conditions. For example, dietary BPA supplement of less than 5 mg/kg/day had no impact on body weight or organ weights of Sprague-Dawley rats (Tyl et al., 2002). Waterborne BPA exposure of concentrations lower than 640 μg/L (2.8 μM) had no effect on somatic growth of fathead minnows (Sohoni et al., 2001a). In zebrafish, exposure to 10 μg/L (44 nM) BPA for 30–180 days had no adverse effect on body weight and body length in the F1 generation, however, this exposure paradigm resulted in a significant decrease of somatic growth in adult F2 fish sampled at 30 and 90 dpf, but surprisingly not those sampled at 180 dpf (Keiter et al., 2012). In the present study, although we observed a slight decrease of body length and weight in BPA exposed 5-month old F1 adult fish, the decrease was not statistically significant. We did not observe any adverse effect of BPA exposure on adult fish survival.

The effect of long-term BPA exposure on sex ratio has been reported in other fish species. In medaka, continuous BPA exposure for 60 d led to female-biased sex differentiation at the exposure concentration of 355 μg/L (1.6 μM) and essentially all females at the concentration of 1820 μg/L (8 μM) (Yokota et al., 2000). Shorter exposure time of 3 weeks at a similar exposure concentration range (837, 1720, 3120 μg/L or 3.7, 7.5, 13.7 μM) also induced testis-ova formation in 13–80% males, indicating significant effect on sexual development (Kang et al., 2002). However, alteration of sex ratio has not been reported in long-term BPA exposure at exposure concentrations as low as in our study (Bhandari et al., 2015a; Kang et al., 2007). The observation that 1 nM (0.228 μg/L) BPA exposure for one or two generations resulted significant female-biased sex ratio change indicated that BPA, at current environmental levels, could have an effect on fish sex differentiation (Fig. 2). Regulation of sex related genes such as male dominant anti-Mullerian hormone (*amh*), double sex and mab-3 related gene (*dmrt*), Sry-related HMG box-9 (*sox9a*), androgen receptor (*ar*), and female-dominant aromatase (*cyp19a1a*) and factor in the germline alpha (*figa*) have been proposed to play an important role in zebrafish sex differentiation (Jorgensen et al., 2008; von Hofsten and Olsson, 2005). In rat testicular leydig cells, BPA exposure increased aromatase gene expression and its enzyme and promoter activity, and reduced testosterone synthesis (Kim et al., 2010). In fathead minnows

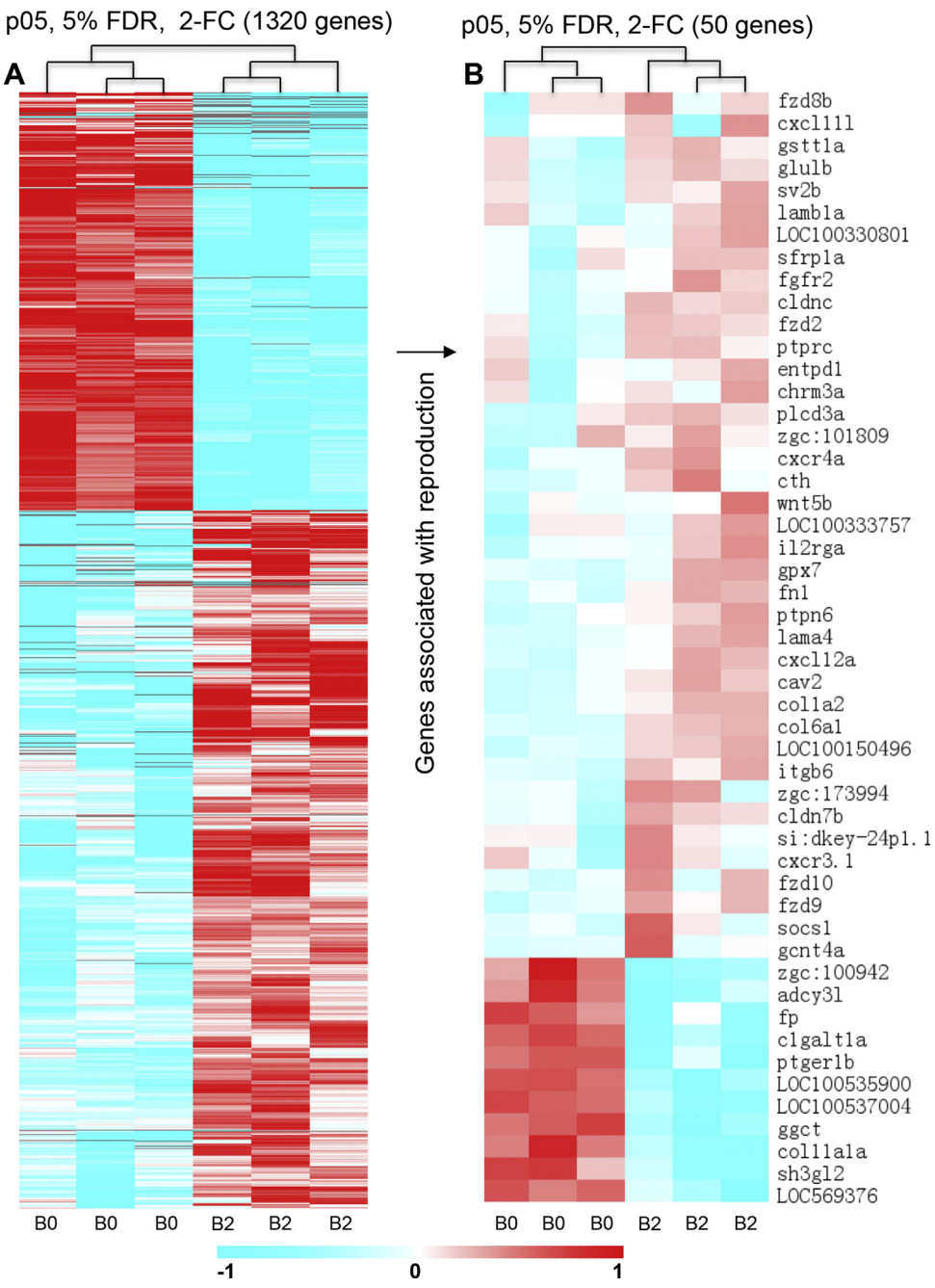

Fig. 5. Hierarchical clustering of differentially expressed genes between control (B0) and BPA exposed (B2) F2 male gonads ($n = 3$ with 5 gonads pooled as one replicate). (A) Total changed transcripts and (B) those involved in reproductive pathway ($P < 0.05$, FDR < 0.05 and >2 fold). Heatmap was generated with Log2-transformed ratio. Color is represented by *z*-score across samples, where red represents relative over-expression and cyan for under-expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(*Pimephales promelas*), 17α-ethinylestradiol, a synthetic estrogen, increased the expression of *cyp19a* and suppressed the expression of *amh* and *dmrt* (Filby et al., 2007). Our RNA-Seq data revealed significant reduced expression of *amh* and elevated expression of *figa*, which could contribute to female-biased sex differentiation. However, zebrafish sex determination is a complex process that involves the primordial germ cell initiation and migration in early development, and subsequent female germ cell growth, juvenile oocyte differentiation, and somatic differentiation at 3 weeks post fertilization (Tong et al., 2010). Since our study involves life-long BPA exposure, the altered sex ratio could have resulted from the effect of BPA exposure on a particular sensitive window or cumulative effect from life-long BPA exposure. Future studies are necessary to further clarify this to fully unravel the underlying mechanism for BPA induced sex ratio change in zebrafish.

Declines of sperm density and quality are the main adverse reproductive effects observed in males exposed to low levels of BPA in aquatic organisms (Crane et al., 2007). For example, sperm counts in male guppies (*Poecilia reticulata*) are decreased by 40–75% after exposure to 1.2 and 2.4 μM (274 and 574 μg/L) BPA for 21 days (Haubruge et al., 2000). Brown trout (*Salmotrutta f. fario*) exposed to environment relative concentrations of BPA (1.75–5 μg/L or 8–22 nM) had reduced sperm density, motility and swimming velocity (Lahnsteiner et al., 2005). In humans, increased urinary BPA concentration was found to be associated with declined semen quality (concentration, motility and morphology) and increased

Table 2
Genes associated with mitochondrial biogenesis.

Symbol	Gene annotation	Log ratio	*P*-value
atp5c1	ATP synthase, mitochondrial F1 complex, gamma polypeptide 1	0.256	5.19E-07
atp5o	ATP synthase, mitochondrial F1 complex, O subunit	0.505	1.60E-06
cox11	COX11 cytochrome c oxidase copper chaperone	0.381	2.65E-03
cox17	COX17 cytochrome c oxidase copper chaperone	1.118	1.81E-04
cox5a	Cytochrome c oxidase subunit Va	0.596	3.47E-03
cox7a2	Cytochrome c oxidase subunit VIIa polypeptide 2 (liver)	0.776	8.37E-09
cyc1	Cytochrome c-1	0.204	3.28E-04
furin	Furin (paired basic amino acid cleaving enzyme)	-0.335	4.58E-03
gpx4	Glutathione peroxidase 4	0.440	2.22E-03
gpx7	Glutathione peroxidase 7	2.240	1.22E-02
map2k4	Mitogen-activated protein kinase kinase 4	-0.936	1.54E-05
mt-co2	Cytochrome c oxidase subunit II	1.448	2.03E-02
mt-co3	Cytochrome c oxidase III	1.229	6.78E-03
mt-cyb	Cytochrome b	1.584	8.84E-03
mt-nd1	NADH dehydrogenase, subunit 1 (complex I)	1.447	4.86E-02
ndufa10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	0.404	5.49E-16
ndufab1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex 1	0.877	4.13E-03
ndufaf1	NADH dehydrogenase (ubiquinone) complex I, assembly factor 1	0.337	1.74E-02
ndufaf2	NADH dehydrogenase (ubiquinone) complex I, assembly factor 2	0.754	1.34E-04
ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 5	0.825	2.83E-03
ndufb8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8	0.433	4.48E-02
ndufs3	NADH dehydrogenase (ubiquinone) Fe–S protein 3	0.466	9.23E-06
ndufs4	NADH dehydrogenase (ubiquinone) Fe–S protein 4	0.727	9.38E-05
ndufs5	NADH dehydrogenase (ubiquinone) Fe–S protein 5	0.426	3.11E-03
ndufs6	NADH dehydrogenase (ubiquinone) Fe–S protein 6	1.066	2.33E-03
ndufs8	NADH dehydrogenase (ubiquinone) Fe–S protein 8	0.452	6.51E-06
park7	Parkinson protein 7	0.624	2.58E-12
prdx3	Peroxiredoxin 3	0.702	2.03E-31
prdx5	Peroxiredoxin 5	1.045	1.40E-06
sdhc	Succinate dehydrogenase complex, subunit C	0.635	9.82E-08
sod2	Superoxide dismutase 2, mitochondrial	0.778	5.71E-05
surf1	Surfeit 1	0.621	4.51E-04
uqcrc1	Ubiquinol-cytochrome c reductase core protein I	0.451	4.15E-24
uqcrc2	Ubiquinol-cytochrome c reductase core protein II	0.235	3.52E-02
uqcrfs1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	0.563	9.33E-04
uqcrh	Ubiquinol-cytochrome c reductase hinge protein	0.409	7.68E-08

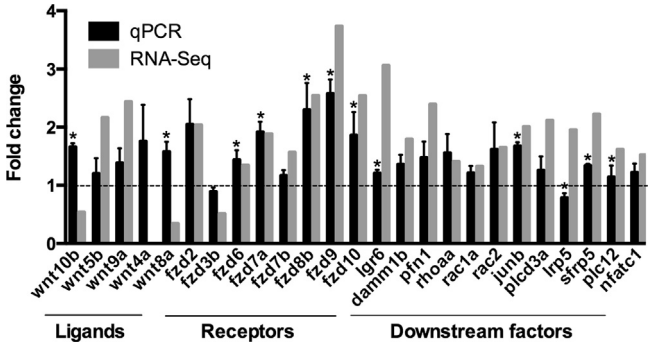

Fig. 6. Expression levels of genes associated with Wnt signaling pathway quantified by qRT-PCR in comparison with values obtained by RNA-Seq. The same mRNA samples collected from F2 male gonads used for RNA-Seq were used for qRT-PCR analysis. The mean fold change in BPA exposed group (B2) relative to the controls (B0) for the RNA-Seq and qRT-PCR are graphed for comparison. Data are representative of 3 biological replicates with 5 testis pooled as one replicate. Values plotted are mean ± SE and bars with asterisk indicate significant difference between control and BPA treated samples.

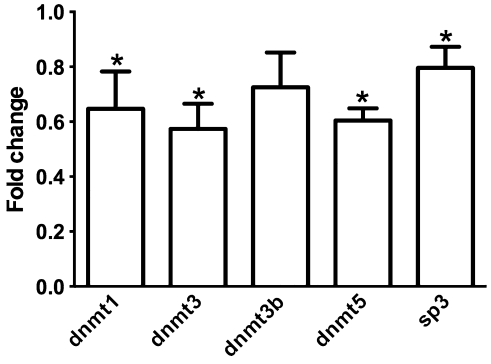

Fig. 7. Expression levels of genes associated with DNA methyltransferase and its transcriptional factor in larvae (120 hpf) collected from BPA exposed F2 parents in comparison with control F2 parents ($n = 3$ with 40 larvae per replicate). Values plotted are mean ± SE and bars with asterisk indicate significant difference between control and BPA treated samples.

sperm DNA damage (Meeker et al., 2010). In the present study, 1 nM BPA exposure reduced sperm density, motility, velocity and ATP production and increased lipid peroxidation in both F1 and F2 adult males (Fig. 3). Discontinuous of BPA exposure after first generation (B1 group) recovered sperm motility, velocity, and lipid peroxidation in F2 males (Fig. 3). The significant reduction of sperm motility along with increased lipid peroxidation upon BPA exposure suggested possible effect from oxidative stress, which was evidenced by increased mitochondrial biogenesis, oxidative phosphorylation as well as NRF2-mediated oxidative stress response in our RNA-Seq data (Fig. S2). Increased mitochondrial biogenesis can lead to excessive production of reactive oxygen species (ROS), which is known to be detrimental to sperm quality (Bansal and Bilaspuri, 2011). Recently, in vitro BPA exposure (0.5–10 μg/L or 2–44 nM) of sterlet (*Acipenser ruthenus*) spermatozoa for 2 h induced oxidative stress, leading to impaired sperm quality, DNA fragmentation and intracellular ATP content (Hulak et al., 2013). Given the important role of mitochondria in sperm quality and the overwhelming representation of mitochondrial signature genes in transcriptional changes associated with BPA exposed testicular tissue, we hypothesize that continuous low level BPA exposure induced sperm density and quality decline is mediated through ROS produced by dysfunc-

tional mitochondria. Future studies are necessary to validate this hypothesis.

In addition to mitochondrial biogenesis, another important signaling pathway perturbed by BPA exposure in adult male testicular tissue in the present study is the Wnt pathway. A recent review indicates that Wnt signaling functions in every aspect of testis development (Dong et al., 2015). It is commonly perceived that the canonical Wnt/β-catenin pathway play a negative role in testis determination, but there are evidence also suggests the opposite (Dong et al., 2015). In the present study, we found reduced expression of canonical Wnt signaling in BPA exposed male gonads, which contradicts to the suppressive role of canonical Wnt/β-catenin on spermatogenesis since lower sperm counts and quality were observed in BPA exposed group. Rather, we found increased expression of non-canonical Wnt/PCP and Wnt/Ca signaling in BPA exposed male gonads (Figs. S3–S5). In the Wnt/PCP pathway, binding of Wnt to Fz receptors recruits Dvl, which then activates the small GTPases Rho and Rac. These GTPases then activate downstream factors to regulate the cytoskeletal rearrangement and cell polarity (Veeman et al., 2003). Our pathway analysis also indicates significant activation of signaling by Rho family GTPase and suppression of its negative regulator RhoGDI signaling, confirming activation of the PCP pathway (Fig. S4). In the non-canonical Wnt/Ca pathway, we observed significant elevation of *plc* and *nfat* (Fig. S5). Activated PLC can lead to calcium release, which in turn activates calcineurin and induces activation of the transcription factor NFAT (Komiya and Habas, 2008). This pathway helps to regulate calcium release from the endoplasmic reticulum and thus control intracellular calcium levels, which are known to play an important role in sperm motility (Parodi, 2014). However, the role of non-canonical Wnt signaling in testis development is less well understood, and our findings suggest possible regulative functions of these non-canonical Wnt signaling in spermatogenesis.

Early studies using the C2C12 mouse muscle cell line and primary mouse embryonic fibroblasts (MEFs) revealed a direct modulation of the Wnt signaling pathway on mitochondrial biogenesis and oxidative phosphorylation (Yoon et al., 2010). In their study, addition of recombinant Wnt3A protein to the C2C12 or MEF cell culture media activated mitochondrial biogenesis and induced the expression of key mitochondrial oxidative phosphorylation genes such as ATP synthase gsubunit (ATP5g1), cytochrome c (Cyc1), and mitochondrially encoded cytochrome oxidase subunit 2 (Cox2). Although the canonical Wnt/β-catenin was verified to be the signaling pathway responsible for this Wnt modulated mitochondrial activation, it is possible that non-canonical Wnt signaling may play a similar role in modulating mitochondrial biogenesis in the present study as both signals were activated upon BPA exposure (Table 2 and Fig. 6). Future studies examining the connection between non-canonical Wnt signaling and mitochondrial dysfunction will help elucidate the underlying causes for BPA induced sperm quality deterioration in males.

One of our most striking results was that continuous BPA exposure for two generations resulted in significant high malformation and mortality in the following generation, and this effect was primarily due to BPA-exposed males (Fig. 4). Although it is well known that males are usually far more sensitive to BPA than females (Labadie and Budzinski, 2006; Sohoni et al., 2001b), our study for the first time discovered that sperm from BPA exposed males was the major contributor of malformation and mortality in offspring. This finding is similar to an early study with dioxin where zebrafish embryos exposure to 50 pg/ml 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at 4 hpf for 1 h tended to produce abnormalities in the following generation (Baker et al., 2013). In particular, the effect on males was greater as TCDD-exposed males fertilizing only 60% of the eggs released by control females when compared with 86% fertilization rate in control males crossed with TCDD-exposed females and 93% fertilization rate in control pairs (Baker et al., 2013). In addition, this TCDD study showed a transgenerational effect associated with developmental exposure of endocrine disrupting chemicals. Recently, medaka exposed to either 100 μg/L (0.44 μM) BPA or 0.05 μg/L 17α-ethinylestradiol during embryonic development at F0 (first 7 days) also demonstrated the transgenerational effects of reduced fertilization success and embryo survival in offspring two or three generations later (F2 and F3) (Bhandari et al., 2015b). Although our study employed a scenario of two-generation life time exposure, this exposure period spanned the critical window of germ cell development, thus our findings also support the paradigm of developmental origins of adult or offspring disease and health (Newbold, 2004).

Despite the observation that BPA exposure reduced sperm density and quality, we found no evidence of histological lesions in testes of exposed fish (data not shown). This effect was similar to earlier studies with BPA exposure at 10 μg/L (44 nM) or TCDD exposure where low dose EDCs had negligible effect on testis histology (Baker et al., 2013; Keiter et al., 2012). We also checked gene expression changes in steroidogenic pathway, in particular those involved in androgen production such as steroidogenic acute regulatory protein (*star*), cholesterol side chain cleavage cytochrome P450 (*cyp11a1*), 3β-hydroxysteroid deshydrogenase (*hsd3b*), steroid 11-beta-hydroxylase (*cyp11b1*), 5α reductase (*srd5a*), 11β-hydroxysteroid deshydrogenase (*hsd11b*) as well as androgen receptor (*ar*). However, we did not find any significant alteration of these genes between control and BPA exposed male gonads.

It is worthy noting that fertilization and hatching at 72 hpf in B1 and B2 were comparable to B0 controls (Fig. S1), suggesting that sperm from BPA exposed group were capable of successful fertilization. A practical observation in fact is that the sperm cells that successfully fertilized eggs are more likely to be normal in motility because only one sperm is required to fertilize one egg. If we assume a typical spawn contains 7.9×10^5 of motile sperm (Jing et al., 2009) and BPA exposure led to 50% reduction of motile sperm, we will still have approximately 4×10^5 sperm with high motility, which is more than sufficient to fertilize a typical spawn of eggs (100–200 eggs per female) from the females. In a natural spawning setting (females paired with males in a spawning tank), those sperm have vigorous motility are more likely to fertilize the eggs, thus we deduced that sperm that can successfully fertilize eggs must have normal morphology and retain good motility. This also explains why we obtained equal fertilization success of embryos derived from control and BPA exposed pairs. Therefore, sperm from BPA exposed males exerted their toxic effect on post fertilization processes. Coincidently, Nerin et al. recently found that pig semen stored in multilayer plastic bags containing bisphenol A diglycidylether (BADGE, a cyclic lactone) showed no alterations in any of the routine quality tests (motility, viability, hypoosmotic swelling test, and osmotic resistance test), in vitro fecundation test, and endocrine profiler panel analysis, yet caused significant reduction in fertilization and live born when assessed with the in vivo fecundation test (Nerin et al., 2014). Their findings together with ours revealed that reproductive failures could occur even when routine quality assays were not able to detect any defects of sperm quality.

Since we obtained equal fertilization success between control and BPA exposed pairs, we deduced that the subsequent increase of malformation and mortality in F2 offspring from BPA exposed males are most likely due to the effect of BPA on sperm DNA. However, we did not observe DNA damage in sperm samples collected from F1 and F2 adult males using the comet assay (data not shown). It is known that DNA methylation plays a critical role in embryonic development, and DNA methyltransferases (Dnmts)

are the key players in regulating DNA methylation marks (Singal and Ginder, 1999). An early study revealed that neonatal exposure of male rats to BPA down regulates the gene expression of *Dnmt1*, *Dnmt3a*, *Dnmt3b* and related transcription factors *Sp1* and *Sp3* in resorbed embryos as compared with the viable embryo sired by these BPA-exposed male rats, and suggested that these alterations are the causes for paternal-mediated post-implantation loss induced by BPA exposure (Doshi et al., 2012). Similarly, we also found reduced expression of *dnmt1*, *dnmt3*, *dnmt5* and *sp3* in 5 d old larvae derived from BPA exposed parents (Fig. 7), corroborating their conclusions that BPA may have altered sperm epigenome and leading to increased malformation and mortality found in these larvae. The similarity of findings between zebrafish and rodents suggest that different model species may share common underlying mechanisms of BPA-induced molecular and epigenetic actions.

In summary, our findings revealed that continuous low level BPA exposures for two generations leads to female-biased alteration of sex ratio, reduced sperm counts and quality, and male-mediated reproductive failure as manifested by increased malformation and mortality in F2 offspring. Transcriptional profiling of male gonads indicates mitochondrial dysfunction and an elevated expression of non-canonical Wnt signaling pathways. Though it is unclear whether increased mitochondrial biogenesis is directly modulated by Wnt signaling, these two pathways may cooperatively or independently contribute to decreased sperm counts and sperm quality. Gene expression analysis of larvae derived from F2 parents discovered a reduced expression of DNA methyltransferases and its associated transcription factor, which may contribute to the paternal-specific reproductive failure. Future studies need to further clarify the roles of these different mechanisms in chronic low dose BPA induced male-specific reproductive toxicity.

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