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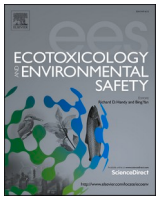

Androgenic overactivation and epigenetic remodeling drive intergenerational toxicity of bisphenol S in zebrafish

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ABSTRACT

Bisphenol S (BPS), a widespread plasticizer and endocrine-disrupting compound, can adversely affect steroidogenesis and the hypothalamic-pituitary-gonadal (HPG) axis. This study exposed adult male zebrafish (*Danio rerio*) to an environmentally relevant BPS concentration (0.5 μg/L) for 14 days (d), assessing its effects on 11-ketotestosterone (11-KT) levels, spermatogenesis, and sperm quality. Additionally, we examined paternal transmission of BPS effects by breeding exposed males with untreated females and evaluating hatching rates, development, survival, and gene expression in offspring. Direct embryonic exposure (0.5 μg/L) was also investigated. BPS exposure increased 11-KT levels in plasma and testes, stimulated meiotic and post-meiotic cysts, and enhanced sperm production. These histomorphometric changes aligned with upregulated expression of *sycp3l* (meiotic marker), *cyp17a1* (androgen synthesis), and genes regulating epigenetic modifications. However, sperm quality was impaired, with reduced motility and fertilization success. In the F1 generation, paternal BPS exposure led to delayed hatching, increased malformations (e.g., absent somites, tail detachment), and higher mortality. In contrast, direct embryonic exposure did not significantly impact development or survival but elevated estrogenic gene expression (*esr1, cyp19a1b, vtg1*). No estrogenic effects were observed in exposed adults or F1 larvae. Our findings uniquely demonstrate that paternal BPS exposure has greater adverse effects on embryo development and survival than direct embryonic exposure. This study highlights the impact of BPS on hormonal regulation, spermatogenesis, sperm quality, and transgenerational viability, providing new insights into its ecological risks.

1. Introduction

Bisphenol S (BPS; 4-hydroxyphenyl sulfone) has become a widely used industrial substitute for bisphenol A (BPA), particularly in the manufacturing of epoxy resins, polycarbonate plastics, thermal paper receipts, canned food linings, food packaging, and reusable water bottles (Tumu et al., 2023; Fu et al., 2024). Like BPA, BPS can migrate from consumer products into the environment, leading to its pervasive detection in various matrices, including human urine and blood (Liao et al., 2012; Fu et al., 2024), sediments, household dust, and surface waters (Qiu et al., 2019). In some aquatic systems, such as the Pearl River (China) and Adyar River (India), BPS concentrations have exceeded those of BPA, with peak values reaching 6.84 μg/L (Yamazaki et al., 2015).

Due to its greater resistance to biodegradation, photodegradation, and thermal decomposition (Fang et al., 2020), BPS exhibits high environmental persistence. As a result, its accumulation may ultimately surpass that of BPA, raising significant concerns regarding ecological and public health impacts. BPS is recognized as an endocrine-disrupting chemical (EDC) because of its capacity to interact with numerous hormone systems, such as sex hormones, pituitary hormones and thyroid hormones, altering endocrine signaling (Zhang et a., 2018; Rubin et al.,

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2022; Cambien et al., 2023).

In vertebrates, including fish, reproduction is orchestrated by the hypothalamic-pituitary-gonadal (HPG) axis, a complex neuroendocrine system regulated by hormonal feedback loops (Ji et al., 2013). Estrogenic or antiandrogenic compounds such as BPS may disrupt this axis by interfering with steroid hormone biosynthesis, ultimately impairing reproductive function (Liu et al., 2010; Chen et al., 2016; Lee et al., 2021). Although BPS has been shown to impair spermatogenesis and reduce sperm quality in mammals (Ullah et al., 2018; Darghouthi et al., 2022; Molangiri et al., 2022), little is known about the mechanisms underlying its reproductive toxicity in aquatic organisms.

Recent research has highlighted the adverse effects of EDCs on gamete quality in fish (Carnevali et al., 2018). Bisphenol analogs, including BPS, have been associated with reductions in sperm density, motility, and fertilization capacity across several fish and mammalian species (Chen et al., 2017; Sahu et al., 2023; Torres-Badia et al., 2023). While mammalian studies report that BPS alters sperm concentration and motility (Sahu et al., 2023; Shi et al., 2017), comparable studies in fish remain limited.

There is growing evidence that environmental contaminants may induce transgenerational effects through epigenetic mechanisms, such as DNA methylation and histone modifications (Hao et al., 2022). These processes are critically involved in spermatogenesis and the establishment of paternal epigenetic marks that can influence offspring phenotype (Hazzouri et al., 2000; Champroux et al., 2018; González-Rojo et al., 2019). The transition from spermatogonial stem cells to spermatozoa thus represents a vulnerable window to epigenotoxic disruption (Pilsner et al., 2017), underscoring the need to investigate the long-term effects of BPS on reproductive integrity and paternal inheritance.

The present study aimed to assess the in vivo effects of BPS on testicular function, sperm production and quality, and intergenerational outcomes following paternal exposure in zebrafish. We examined steroidogenic function by quantifying 11-ketotestosterone (11-KT) levels and assessing the expression of genes involved in germ cell development, steroidogenesis, estrogen signaling, and epigenetic regulation. Sperm quality was evaluated through measurements of count, motility, and swimming behavior. To investigate paternal effects, BPS-exposed males were crossed with untreated females, and we assessed hatching success, survival, development, and gene expression in the F1 generation. These parameters were also evaluated in embryos directly exposed to BPS to compare direct and inherited effects. Together, this comprehensive approach provides new insights into the reproductive and transgenerational toxicity of BPS in an aquatic vertebrate model.

2. Material and methods

2.1. Animal husbandry and ethical compliance

Adult male zebrafish (*Danio rerio*) (4-months old, sexually mature) were reared and maintained under standardized laboratory conditions at the Aquatic Organisms Facility, Department of Structural and Functional Biology, Institute of Biosciences, UNESP (Botucatu, Brazil), and the Department of Biological Sciences, University of Calgary (Calgary, Alberta, Canada). Fish were held in recirculating systems at 28 °C with a controlled photoperiod of 14 h light:10 h dark. Animals were fed twice daily with commercial dry feed (Zeigler®, USA). All procedures adhered to the guidelines established by the National Council for Animal Experimentation Control (CONCEA) and received ethical approval from the Institutional Animal Care and Use Committee at the University of Calgary (Protocol 6605200721-CEUA). Moreover, this study is reported in accordance with ARRIVE guidelines.

2.2. Chemical exposure

Bisphenol S (BPS; 4-hydroxyphenyl sulfone, purity 99.7 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A nominal concentration of 0.5 μg/L was selected based on environmental monitoring data indicating that BPS levels can exceed 1 μg/L in various aquatic systems, such as the Adyar River (India), Buckingham Canal, Cooum River, and Taihu Lake in China (Yamazaki et al., 2015; Qiu et al., 2019). The selected concentration falls within the range considered environmentally relevant (Ji et al., 2013; Wei et al., 2018; Gyimah et al., 2021; Salahinejad et al., 2024). However, the selected dose is representative of possible worst-case exposure scenarios, with the dose reflecting levels detected in highly impacted or restricted environments.

2.3. Experimental design

2.3.1. Parental exposure to BPS

Ninety-six adult male zebrafish were exposed to either BPS (0.5 μg/L) or vehicle control (0.00001 % v/v DMSO) in aquarium water for 14 days, following Wang et al. (2020). This exposure duration corresponds to approximately two full spermatogenic cycles in zebrafish (Leal et al., 2009a). Fish were housed in 10 L tanks (12 fish per tank) within a semi-static exposure system, with complete water renewal and re-dosing every 48 h (Ji et al., 2013; Costa et al., 2025a). Each treatment group included 48 fish distributed across four replicate tanks.

At the conclusion of the exposure period, 84 fish were euthanized using a 1 % benzocaine solution for sample collection. Tissues were processed for hormonal (11-KT) quantification, gene expression profiling, histomorphometric analysis, sperm quantification, and motility assessment using the ImageJ/CASA plugin. To evaluate reproductive capacity, six BPS-exposed males and six DMSO-treated males were mated with untreated females (2:1 male:female ratio) in three replicate tanks per group. Fertilized embryos were collected and individually placed in 24-well plates containing 1 mL of embryo culture medium (Hank's solution, according to ZFIN protocols) and maintained at 28 °C. A total of 100 embryos per group were monitored over 96 h, with daily medium renewal. Embryonic development, hatching rate, and survival were assessed according to a modified Fish Embryo Toxicity (FET) test (OECD Test No. 236). At 96 h post-fertilization (hpf), F1 larvae were pooled in groups of ten (n = 6 pseudoreplicates per group) and frozen at −80 °C for subsequent gene expression analysis.

2.3.2. Direct embryo exposure

Naïve male and female zebrafish were bred (2:1 ratio) in three replicate tanks. Embryos were collected and transferred to 24-well plates (one embryo per well) containing 1 mL of Hank's solution. Embryos were exposed to 0.5 μg/L BPS or vehicle control (DMSO, 0.00001 % v/v) for 96 h (n = 100 embryos per treatment). The culture medium and chemical treatments were renewed every 24 h. Embryonic development, hatching, and survival were evaluated as described above. At 96 hpf, larvae were pooled (10 per pool, n = 6 pseudoreplicates per group) and stored at −80 °C for gene expression analysis.

2.4. Plasma 11-ketotestosterone (11-KT) quantification

Following 14 days of exposure, eight males from each treatment group were euthanized for blood collection via caudal transection using heparinized syringes. Plasma was separated by centrifugation (800 × g, 15 min, 4 °C) and stored at −80 °C until analysis (Costa et al., 2025a). Plasma concentrations of 11-KT were determined using a commercial ELISA kit (Cayman Chemical, Ann Arbor, USA) following the manufacturer's protocol.

2.5. Testicular 11-KT release in ex vivo culture

Testes from euthanized males (n = 8 per group) were excised and processed for *ex vivo* culture based on Leal et al. (2009b). One testis per fish was incubated in Leibovitz's L-15 medium supplemented with recombinant zebrafish follicle-stimulating hormone (rzfFsh, 200 ng/mL; Immunoprecise Antibodies, NL), while the contralateral testis was

incubated in L-15 medium alone. After 48 h, testes were weighed, and the culture medium was collected for 11-KT quantification using the Cayman ELISA kit.

2.6. Histomorphometric analysis of testes

Testes from adult zebrafish (n = 8 per experimental group) were fixed overnight in Karnovsky's fixative (4 % glutaraldehyde, 8 % paraformaldehyde in phosphate buffer, pH 7.2), dehydrated, embedded in Technovit resin (7100; Heraeus Kulzer, Wehrheim, Germany), and sectioned into 3 μm non-serial slices. Sections were stained with 0.1 % toluidine blue for germ cell cyst quantification. Histological images were captured at 100 × magnification using a high-resolution light microscope (Leica DM6000 B, Leica Microsystems, Wetzlar, Germany) equipped with a JVC camera and analyzed using Leica QWIN software. For quantitative analysis, five randomly selected, non-overlapping fields per animal (n = 8 per group) were evaluated using a 540-intersection grid (Costa et al., 2024). The proportions of different germ cell types (type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), spermatocytes (Spc), and spermatids (Spt) were determined based on established criteria such as nuclear size, cyst cell number, and chromatin condensation (Leal et al., 2009a). Results were expressed as fold-change relative to the control group.

Spermatozoa quantification was performed using ImageJ software (NIH, Maryland, USA). For each testis (n = 6 per group), 20 randomly selected, non-overlapping fields per histological slide were analyzed using the Cell Counter plugin in ImageJ (Rodrigues et al., 2022; Camargo-dos-Santos., 2022). The number of spermatozoa per field was averaged across the 20 fields and reported as fold-change relative to the control group.

2.7. Gene expression analysis by quantitative real-time PCR (qPCR)

Total RNA was extracted from testicular tissue (n = 8 per experimental group) using the TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), followed by cDNA synthesis according to the manufacturer's instructions. Quantitative PCR (qPCR) reactions were carried out using SYBR Green Universal Master Mix (Invitrogen, Carlsbad, CA, United States). Transcript levels of spermatogonia markers (*pou5f3, nanog, dazl*) and the meiosis marker *sycp3l* were analyzed. Expression of key steroidogenic enzymes (*cyp19a1a, star, cyp17a1a*), estrogen receptors (*esr1, esr2a*), and genes involved in epigenetic regulation (*dnmt5, tet1, ezh2, kdm6b, kat6a, hdac4*) were also quantified. In F1 larvae and directly exposed embryos (n = 60 per group), transcript levels of developmental genes (*mstn1, vegfa, wnt8*), estrogen-related genes (*esr1, cyp19a1b, vtg1*), and the androgen metabolism gene *hsd11β2* were assessed. Gene expression levels (Ct values) were normalized to the housekeeping gene *β-actin* whose expression remained stable across all samples. Relative expression levels were calculated using the 2ˆ−ΔΔCt method and expressed as fold changes relative to the control group. Primers (listed in Supplementary Table 1) were designed based on zebrafish gene sequences available in GenBank (NCBI).

2.8. Sperm quality analysis

Male zebrafish from the BPS and control groups (n = 10 per group) were euthanized, and their testes were dissected. Testes were immersed in Hank's Balanced Salt Solution (HBSS) to facilitate sperm release (Chen et al., 2017) and maintain sperm in an inactive state (Babiak et al., 2012). To aid dissociation, the tissue was gently fragmented using tweezers. For sperm activation, samples were diluted in Milli-Q water (1 μL sperm: 2 μL water) and immediately loaded into a Neubauer chamber (0.1 mm depth) covered with a 24 × 24 mm glass coverslip (Sanches et al., 2013).

Sperm motility was analyzed using a trinocular light microscope (Solarist Bel) equipped with a 10 × objective and a Basler acA640–120gc camera (Basler, http://www.baslerweb.com), connected to a computer (Intel Core i7, 2.4 GHz, 8 GB RAM, Windows 8). Videos were recorded at 100 frames per second and analyzed using ImageJ with the CASA plugin, as described by Sanches et al. (2013). The following parameters were assessed: total sperm count, motility rate (MOT), curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL).

2.9. Embryo hatching, development, and survival analysis (Adapted FET Test)

Embryos and larvae were monitored for 96 h using an Olympus MCV10 magnifier equipped with X-Cite 120 LED illumination. Morphological assessments were conducted every 24 h to evaluate developmental abnormalities and potential lethality, following an adapted Fish Embryo Acute Toxicity (FET) test protocol (OECD Test No. 236). Assessed parameters included: coagulation of fertilized eggs, absence of somite formation, failure of tail detachment from the yolk sac, absence of heartbeat (from 48 h post-fertilization), spinal deformities, and the presence of yolk sac or pericardial edema.Hatching rate was evaluated from 48 h post-fertilization and calculated as: Hatching Rate (%) = (number of hatched embryos/total number of embryos) × 100. Mortality rate was defined as the cumulative percentage of coagulated embryos or those lacking a heartbeat during the 96 h assessment period.

2.10. Statistical analysis

All data were first tested for normality using the Shapiro-Wilk test, followed by Bartlett's test for variance and homogeneity. Plasma 11-KT levels were compared between groups using an unpaired *t*-test. For 11-KT *ex vivo* release from testes, one-way ANOVA was performed, followed by post-hoc analysis with the Student-Newman-Keuls test. Significant differences in histomorphometry and gene expression were identified using an unpaired *t*-test ($p < 0.05$). Sperm quality parameters obtained from ImageJ/plugin CASA were compared between groups using an unpaired *t*-test. Embryo hatching and development were assessed using the non-parametric Mann-Whitney test. Survival analysis was conducted using Kaplan-Meier curves, and group comparisons were performed with the log-rank test (Mantel-Cox). Data are expressed as mean ± SEM (standard error of the mean). All statistical analyses were performed using GraphPad Prism software (version 4.0; GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Effect of BPS on 11-KT concentration in zebrafish

To evaluate the impact of BPS on androgen production, we measured 11-KT concentrations both *in vivo* (plasma; Fig. 1A) and *ex vivo* (testicular culture medium; Fig. 1B) after 14 d of exposure to BPS. BPS exposure resulted in a significant increase in plasma 11-KT levels, approximately threefold higher than the control group (Fig. 1A). Similarly, *ex vivo* quantification of 11-KT released into the culture medium confirmed elevated androgen secretion from testes of BPS-exposed animals compared to controls (Fig. 1B). Notably, BPS exposure abolished the stimulatory effect of follicle-stimulating hormone (Fsh) on 11-KT release in ex vivo testis cultures, suggesting a disruption in hormonal responsiveness (Fig. 1B).

3.2. Histomorphometric analysis of the effects of BPS on zebrafish spermatogenesis

BPS treatment significantly increased the area occupied by spermatocytes (Spc) and spermatids (Spt), indicating stimulation of the later stages of spermatogenesis (Fig. 2A). Conversely, the area occupied by

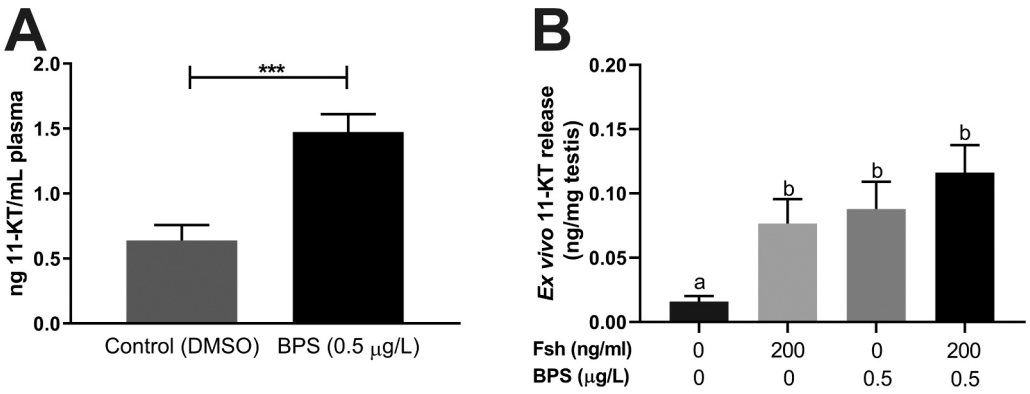

Fig. 1. Quantification of 11-KT following exposure to BPS. (A) Plasma levels of 11-Ketotestosterone (11-KT) (ng/mL of plasma) in male zebrafish after 14 days of exposure to Vehicle (DMSO, control) or BPS (0.5 μg/L). (B) Release of 11-KT by zebrafish testicular explants previously treated with DMSO or BPS (0.5 μg/L) for 14 days. Testes were incubated in culture media (L-15) with or without Fsh (200 ng/mL) for 48 h. Bars represent the mean ± standard error of the mean (SEM) (n = 8). Different letters denote significant differences ($p < 0.05$) between groups. (***) indicates a significant difference ($p < 0.001$).Parte superior do formulário.

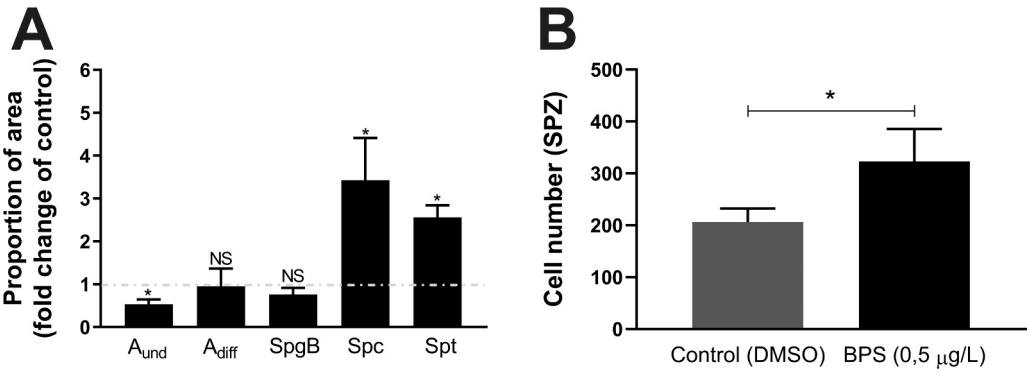

Fig. 2. Histomorphometric analysis of testes following exposure to BPS. (A) Proportion of surface area occupied by spermatogenic cysts after 14 days of exposure to DMSO (Vehicle, control) or BPS (0.5 μg/L). Cysts include undifferentiated type A spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (SpgB), primary spermatocytes (Spc), and spermatids (Spt). Data are presented as fold change relative to the control group. (B) Number of sperm per field obtained using IMAGEJ Software. Results are expressed as mean ± standard error of the mean (SEM) (n = 6). (*) indicates a significant difference observed between the different groups with $p < 0.05$; (NS) indicates no significant difference.

type A undifferentiated spermatogonia (A_{und}) was significantly reduced following BPS exposure, while no significant changes were observed in the areas occupied by type A differentiated spermatogonia (A_{diff}) or type B spermatogonia (SpgB) (Fig. 2A). In addition, quantification of spermatozoa revealed a significant increase in sperm production in the BPS group compared to the control (Fig. 2B).

3.3. Gene Expression analysis of the effects of BPS on zebrafish spermatogenesis

Gene expression analysis revealed that BPS exposure altered testicular mRNA levels after 14 d (Fig. 3A–D). There were no significant differences in the expression of spermatogonial marker genes *pou5f3, nanog,* and *dazl* (Fig. 3A). However, a significant increase was observed in the expression of *sycp3l*, a marker of primary spermatocytes (Fig. 3A). Additionally, transcript levels of the steroidogenic enzymes *star* and *cyp17a1a* were significantly upregulated in the BPS-treated group (Fig. 3B), while no changes were observed in estrogen receptor subtypes *esr1* and *esr2a* (Fig. 3C). BPS exposure also significantly increased the expression of several epigenetic regulators, including *dnmt5, tet1, kat6a,* and *hdac4* (Fig. 3D).

3.4. Effects of BPS on sperm quality in zebrafish

In vivo exposure to BPS significantly increased total sperm count ($p < 0.01$) compared to controls (Supplemental Fig. 1 A). Analysis of sperm kinetic parameters showed that BPS exposure significantly reduced MOT (Supplemental Fig. 1B) and increased VCL (Supplemental Fig. 1 C). No significant differences were found in VAP or VSL between groups (Supplemental Fig. 1D and 1E, respectively).

3.5. Effects of parental male exposure to BPS on hatching, development, and survival of F1 embryos/larvae and directly exposed embryos

F1 embryos derived from males exposed to BPS exhibited delayed and significantly reduced hatching rates, with a final hatching rate of 70 % at 96 h post-fertilization, compared to 95 % in the control group (Fig. 4A). Additionally, a significantly higher incidence of malformations was observed in embryos from the BPS group. These included delayed or absent somite formation (Fig. 4C) and failure of tail detachment from the yolk sac (Fig. 4D), both of which showed significant differences ($p < 0.0001$) at all assessed time points (24, 48, 72, and 96 h). At 96 h, 27 % of embryos in the BPS group displayed these abnormalities, compared to only 2 % in the control. No significant differences were observed between the BPS and control groups for other morphological parameters, including pericardial edema (Fig. 4E), spinal deformities (Fig. 4F), and yolk sac edema. In contrast, direct exposure of embryos to BPS (0.5 μg/L) for 96 h did not significantly affect hatching rates (Fig. 4B), nor did it induce any malformations. All developmental parameters assessed—including somite formation, tail detachment, pericardial and yolk sac edema, and spinal column morphology—were comparable to the control group. Survival analysis further revealed that

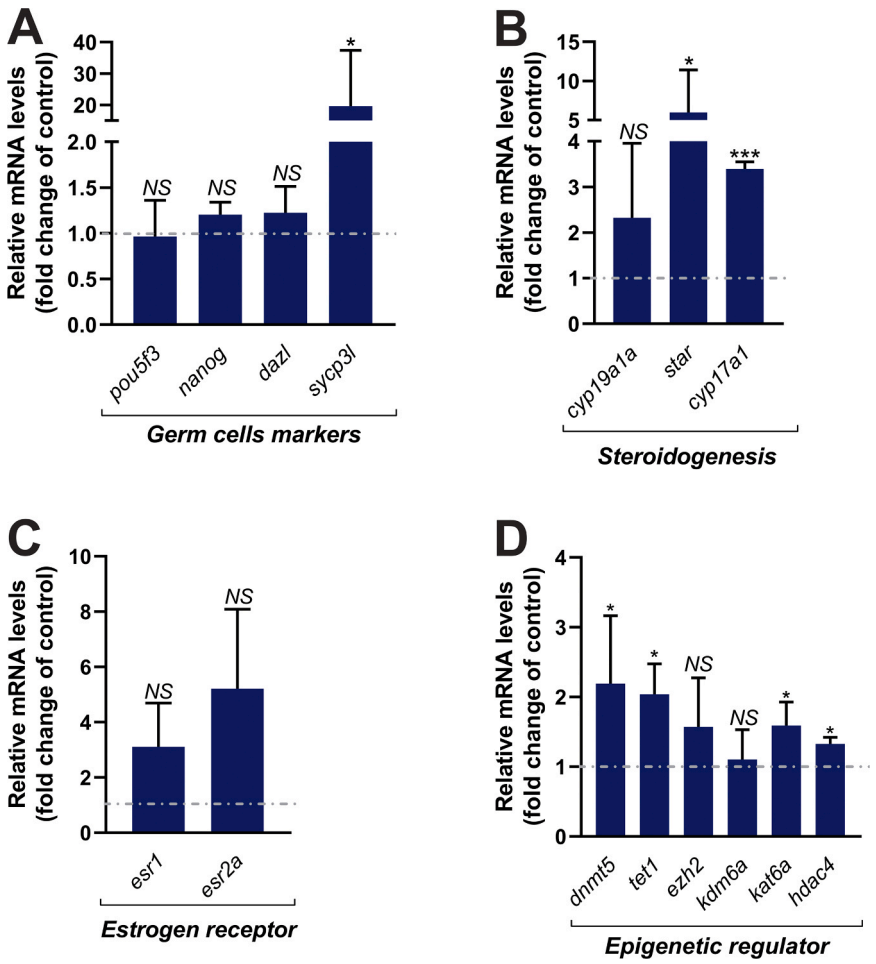

Fig. 3. Gene expression analysis of testes following exposure to BPS. (A) Relative transcript levels of germ cell marker genes (*pou5f3*, *nanog*, *dazl*, and *scyp3l*), steroidogenic enzymes (*cyp19a1a*, *star*, *cyp17a1*), estrogen receptors (*esr1*, *esr2a*), and epigenetic regulation genes (*dnmt5*, *tet1*, *ezh2*, *kdm6b*, *kat6a*, and *hdac4*) in zebrafish testes after 14 days of exposure to Vehicle (DMSO, control) or BPS (0.5 μg/L). Cts were normalized to the reference gene (*β-actin*) and expressed relative to the control group (DMSO). Data are presented as mean ± standard error of the mean (SEM) (n = 8) and as fold induction of the control (DMSO) represented by the dashed line. (*) *and* (**) represent significant differences with $p < 0.05$ and $p < 0.001$, respectively. (NS) indicates no significant difference.

F1 embryos from BPS-exposed males had significantly increased mortality over the 96 h observation period ($p < 0.0001$; Supplemental Fig. 2 A). Peak mortality occurred within the first 48 h, after which it stabilized. Final survival at 96 h was only 32 % in the BPS group, compared to 99 % in the control. In contrast, embryos directly exposed to BPS showed no significant change in mortality relative to controls (Supplemental Fig. 2B).

3.6. Gene expression analysis of F1 and directly exposed larvae to BPS

We analyzed mRNA expression of genes involved in development (*mstn1, vegfa, wnt8*), estrogen signaling (*esr1, cyp19a1b, vgt1*), and androgen regulation (*hsd11β2*) in both F1 larvae (Fig. 5A) and larvae directly exposed to BPS (Fig. 5B). Paternal exposure to BPS significantly altered gene expression in F1 larvae compared to controls. Specifically, transcript levels of the developmental genes mstn1 and vegfa, as well as the androgen-related gene *hsd11β2*, were significantly elevated, while expression of estrogen-related genes (*esr1, cyp19a1b, vgt1*) remained unchanged (Fig. 5A). In contrast, direct exposure of larvae to BPS resulted in a broader transcriptional response. Expression of all assessed developmental genes (*mstn1, vegfa, wnt8*) and estrogen signaling genes (*esr1, cyp19a1b, vgt1*) was significantly upregulated compared to controls (Fig. 5B).

4. Discussion

BPS, an emerging EDC, has been implicated in the disruption of sex hormone regulation, including testosterone and estradiol, across multiple model organisms (Ullah et al., 2018; 2021; Campen et al., 2018; Wang et al., 2023). In male zebrafish, previous studies have primarily reported increased 17β-estradiol and decreased testosterone and 11-KT levels following BPS exposure (Ji et al., 2013; Naderi et al., 2014; Park et al., 2022). However, our findings reveal that BPS significantly increases 11-KT levels both *in vivo* and in *ex vivo* testicular cultures. This contrasts with existing data and the suppressive effects of BPA on androgen production in various teleost fish species (Mandich et al., 2007; Chen et al., 2019; Zhao et al., 2017; Lee et al., 2021; Zhu et al., 2022; Wang et al., 2019; Gonzalez et al., 2021).

Notably, the doses used in our study were over 1,000-fold lower than those reported by Park et al. (2022), suggesting a non-monotonic dose-response characteristic of EDCs (Calabrese et al., 2007; Vandenberg et al., 2014; Costa et al., 2025b). Elevated 11-KT levels persisted in cultured testes from BPS-exposed fish, and Fsh failed to further enhance 11-KT secretion, indicating a potential disruption of Fsh receptor signaling or negative feedback regulation. To our knowledge, this is the first report demonstrating a stimulatory effect of BPS on 11-KT in a vertebrate model.

11-KT plays a central role in spermatogenesis in fish, particularly in promoting progression from meiotic (spermatocytes) to post-meiotic

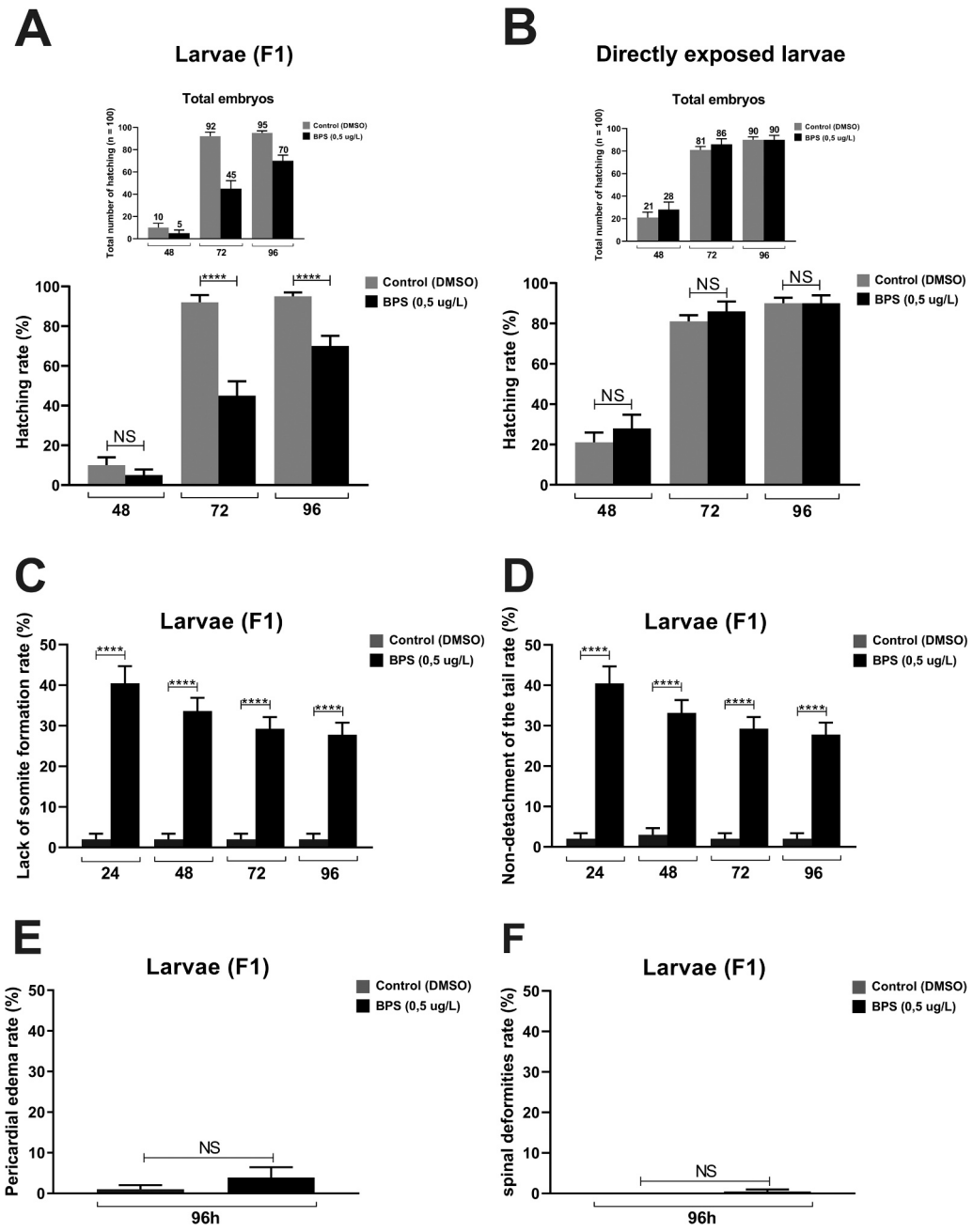

Fig. 4. Effects on hatching and development of zebrafish embryos/larvae. (A) Hatching of larvae (F1) derived from the mating of parental males exposed to DMSO (control) or BPS (0.5 μg/L), maintained in embryonic medium or (B) larvae directly exposed to BPS (0.5 μg/L) after 96 h post-fertilization. (C-F) Development of embryos/larvae (F1) after 96 h in embryonic medium. (C) Absence of somite formation, (D) failure of tail detachment, (E) pericardial edema, (F) spinal deformity. No yolk sac edema was observed in larvae (F1) from both the control (DMSO) and treated (BPS) groups. No developmental changes were identified in directly exposed larvae in either the control (DMSO) or treated (BPS) groups. The following developmental parameters were analyzed: absence of somite formation, failure of tail detachment, pericardial edema, yolk sac edema, and spinal deformity. Results are expressed as mean ± standard error of the mean (SEM) (n = 100). (****) denotes a significant difference with $p < 0.0001$. (NS) indicates no significant difference.Parte superior do formulário.

stages (spermatids and spermatozoa) (Miura et al., 1991). Histological and gene expression analyses in our study support an androgenic effect of BPS, evidenced by increased proportions of spermatocytes and spermatids, upregulation of *sycp3l, star,* and *cyp17a1*. Additionally, the observed decrease in type A_{und} spermatogonia suggests that BPS promotes later-stage differentiation (Spc, Spt, and Spz), reducing proportion of early-stage cysts. These changes indicate stimulation of late-stage spermatogenesis, a pattern contrary to BPA's inhibitory effects reported in mammals and fish (Jin et al., 2013; Liu et al., 2013; Jenardhanan et al., 2016; Sohoni et al., 2001; Lahnsteiner et al., 2005; Batista-Silva et al., 2022). In mammals, BPS exposure has also been shown to suppress spermatogenesis by reducing spermatocytes, spermatids, and overall sperm production (Ullah et al., 2018;2021; Shi et al., 2017; Nawaz et al., 2021; Darghouthi et al., 2022). However, studies on the effects of BPS on fish spermatogenesis remain limited. An earlier study by Hao et al. (2022) reported a reduction in sperm percentage in zebrafish following exposure to 1 and 100 μg/L of BPS from 3 h post-fertilization (hpf) for 120 days. Differences in experimental conditions, particularly the extended exposure window beginning at the embryonic stage, may, in part, explain the contrasting results in sperm production compared to the present study.

Interestingly, although sperm production was increased following

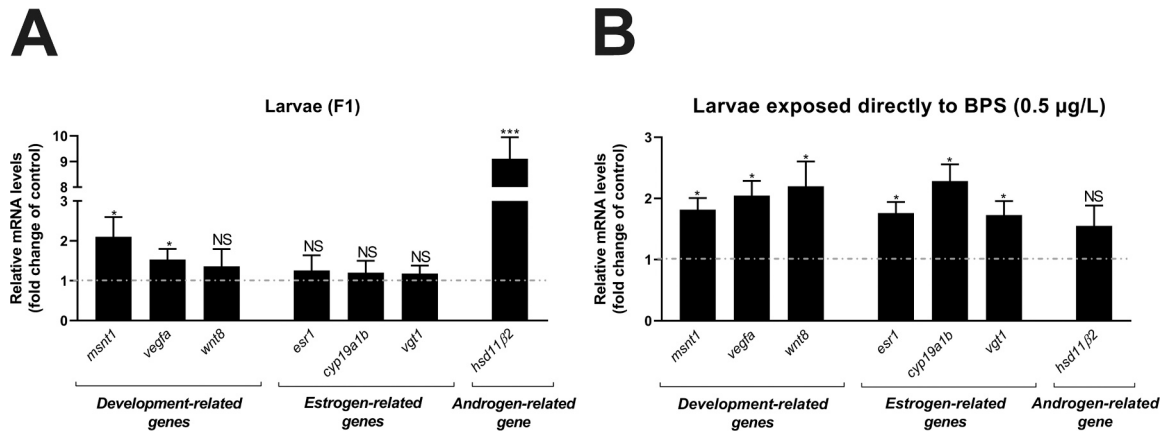

Fig. 5. Gene expression analysis of zebrafish larvae. Relative expression of genes related to development (*mstn1*, *vegfa*, and *wnt8*), estrogen (*esr1*, *cyp19a1b*, and *vgt1*), and androgen (*hsd11β2*) in zebrafish larvae. (A) Larvae (F1) derived from the mating of parental males exposed to BPS (0.5 µg/L) or DMSO (control) after 96 h in embryonic medium. (B) Larvae directly exposed to DMSO (control) or BPS (0.5 µg/L) for 96 h post-fertilization. Cts were normalized to the reference gene (*β-actin*) and expressed relative to the control group (DMSO). Data are presented as mean ± standard error of the mean (SEM) (n = 6 pseudoreplicates of 10 embryos per group) and as fold induction relative to the control (DMSO), indicated by the dashed line. (*) denotes a significant difference with $p < 0.05$. (NS) indicates no significant difference.

BPS exposure, MOT was significantly reduced. The decline in MOT observed in our study is consistent with previous reports in rats (Sahu et al., 2023) and mice (Shi et al., 2017) following BPS treatment. Additionally, BPS exposure altered another key kinetic parameter by increasing VCL, which represents the total distance traveled by sperm per second (Wilson-Leedy and Ingermann, 2007). Such changes in sperm kinetics may interfere with the sperm's ability to locate and fertilize the oocyte (Gallego et al., 2017; Castro et al., 2024). Given that motility and velocity are primary determinants of male fertility in externally fertilizing species, our findings suggest that BPS may impair male zebrafish fertility by compromising sperm quality.

Spermatogenesis involves a series of epigenetic modifications essential for meiosis, which can influence not only the spermatogenic process itself but also the genetic information transmitted to offspring during fertilization (Hazzouri et al., 2000; Champroux et al., 2018; Gonzalez-Rojo et al., 2019). In the present study, we found that BPS stimulated a range of genes involved in epigenetic regulation, leading to increased mRNA levels of genes associated with DNA methylation (*dnmt5*), DNA demethylation (*tet1*), histone acetylation (*kat6a*), and histone deacetylation (*hdac4*), while transcript levels related to histone methylation and demethylation remained unaffected. Alterations in sperm epigenetic profiles can be transmitted to offspring, influencing early development (Miller and Ostermeier, 2006; Lombó et al., 2019). Similarly, Hao et al. (2022) reported testicular methylation changes in zebrafish exposed to BPS, although the impact on offspring was unclear due to concurrent maternal exposure.

Accordingly, our results show that F1 embryos derived from BPS-exposed males exhibited impaired hatching, higher rates of morphological abnormalities (e.g., absence of somites and tail detachment), and markedly increased mortality—phenomena absent in embryos directly exposed to BPS. These findings highlight the importance of paternal contributions to offspring viability via epigenetic inheritance and reinforce the concept that sperm carry regulatory molecules beyond genetic material (Hazzouri et al., 2000; Champroux et al., 2018; Miller and Ostermeier, 2006). Additionally, we assessed the transcript levels of genes related to development, estrogen, and androgen activity in F1 embryos derived from BPS-exposed males or embryos directly exposed to BPS. Development-related genes *vegfa* and *wnt8a* play essential roles in embryogenesis, with *vegfa* critical for vascular development (Chen et al., 2020) and *wnt8a* necessary for dorsal axis formation (Hino et al., 2018). Mstn1 (myostatin), a TGF-β family member, is expressed in various teleost tissues, including muscles, brain, and gonads (Xie et al., 2019). Our findings demonstrate that paternal BPS exposure alters the expression of *vegfa* and *mstn1* in F1 embryos, correlating with developmental changes and reduced survival. Although direct F1 embryos exposure to BPS did not impact hatching, development, or survival, it did alter the expression of development-related genes (*mstn1*, *vegfa,* and *wnt8*). This suggests that gene expression changes may not directly reflect morphological alterations but could result from other unidentified treatment-related conditions. Morphological abnormalities in embryos may arise from imbalances between complex sets of unassessed genes.

EDCs can interfere with zebrafish sexual development, leading to feminization or masculinization (Santos et al., 2017). BPA exposure has been linked to feminization during gonadal development across multiple species (Crain et al., 2007), and BPS similarly shows feminizing tendencies in zebrafish embryos (Naderi et al., 2014). To evaluate BPS's estrogenic effects, we quantified the expression of estrogen-related genes *esr1*, *cyp19a1b*, and *vtg1* (Schiller et al., 2014; Guo et al., 2017). None of these genes were altered in F1 embryos derived from BPS-exposed males, suggesting limited capacity for BPS to promote estrogenic activity across generations via paternal transmission. Notably, *cyp19a1b* and *vtg1*, commonly used biomarkers for estrogenic exposure in male fish (Schiller et al., 2014; Cano-Nicolau et al., 2016), remained unaltered. However, we observed a strong upregulation of *hsd11b2*, a key gene in androgen synthesis (Tovo-Neto et al., 2020; Fang et al., 2022), suggesting an androgenic effect in F1 embryos derived from BPS-exposed males. Nonetheless, it is important to highlight that due to the limited number of replicate parental tanks (n = 3 per treatment group), the results from the F1 generation should be interpreted as preliminary. Additionally, the results derived from larvae - as pseudoreplicates - should be considered with caution. Future studies with larger independent replicates sample sizes need to be conducted to robustly confirm these findings.

Endogenous steroid hormones play critical roles in sexual differentiation during embryonic development, even at low concentrations (Naderi et al., 2014). Previous research has shown that direct embryonic exposure to BPS induces estrogenic effects by elevating 17β-estradiol and vitellogenin and reducing testosterone (Naderi et al., 2014). In our study, direct embryonic BPS exposure upregulated all evaluated estrogen-related genes (*esr1*, *cyp19a1b*, and *vtg1*) while leaving *hsd11b2* unaffected. These findings indicate that direct embryonic exposure to BPS promotes estrogenic activity, unlike the androgenic effects observed in F1 larvae following paternal exposure.

CRediT authorship contribution statement

Eduardo Antonio Sanches: Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis. **Maya Zanardini:** Writing – original draft, Methodology, Investigation, Formal analysis. **Maira da Silva Rodrigues:** Writing – original draft, Methodology, Investigation, Formal analysis. **Ana Regina Seabra de Souza:** Writing – original draft, Methodology, Investigation, Formal analysis. **Daniel Fernandes da Costa:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Rafael Henrique Nóbrega:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis. **Hamid R Habibi:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis. **Adriana Carvalho Natal de Moraes:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rafael Henrique Nobrega reports financial support was provided by State of Sao Paulo Research Foundation. Daniel Fernandes da Costa reports financial support was provided by Coordination of Higher Education Personnel Improvement. Maira da Silva Rodrigues AND Adriana Carvalho Natal de Moraes reports financial support was provided by State of Sao Paulo Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118831.

Data availability

Data will be made available on request.

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