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Life-Cycle Benzo[*a*]pyrene Exposure Induces Sex-Specific Reproductive Impairment, Feminization, and Transgenerational Disruption in Marine Medaka (*Oryzias melastigma*)

Published as part of Environmental Science & Technology special issue "Ocean Health".

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Cite This: *Environ. Sci. Technol.* 2025, 59, 15037−15046

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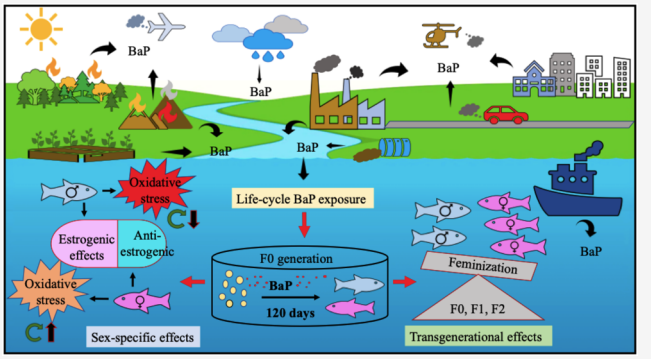
ABSTRACT: Benzo[*a*]pyrene (BaP), a widespread environmental pollutant, has been extensively studied; however, knowledge gaps remain regarding its sex-specific reproductive toxicity and the persistence of its transgenerational effects. Marine medaka (*Oryzias melastigma*) were exposed to environmentally relevant BaP concentrations (1, 4, and 8 μg/L) throughout the F0 generation, with transgenerational effects assessed in F1, F2, and F3 generations reared in clean seawater. BaP exposure significantly affected biometric responses and reproductive parameters, including impaired gametogenesis, reduced fecundity, and decreased fertilization rates. Males were more sensitive to oxidative stress and hormonal imbalances in the gonads and showed delayed recovery during depuration. Genes in the hypothalamus-pituitary-gonad-liver (HPGL) axis were disrupted in a sex-specific manner. A persistent feminization and poor egg quality were observed up to the F2 generation, indicating transgenerational endocrine disruption. Despite recovery initiation in F3, the results reveal persistent sex-specific reproductive toxicity, emphasizing the need to assess sex-specific and transgenerational effects in ecotoxicology.



KEYWORDS: *reproductive toxicity, sex-specific response, feminization, transgenerational effects*

1. INTRODUCTION

Benzo[*a*]pyrene (BaP), a potent carcinogenic polycyclic aromatic hydrocarbon (PAH) and endocrine-disrupting chemical (EDC), has been widely reported to impair reproductive health in aquatic organisms.[1] Its structural similarity to estradiol (E2) allows it to bind estrogen receptors, displacing endogenous estrogens and thereby disrupting endocrine function.[2,3] Numerous studies have demonstrated that BaP exposure alters key steroid hormones including progesterone, 11-ketotestosterone (11-KT), testosterone (T), and estradiol (E2) in fish,[1,4−6] crabs,[7] and bivalves,[8−12] leading to reproductive dysfunction.

In the aquatic environment, fish are particularly vulnerable to xenobiotic endocrine-disrupting effects due to their continuous exposure to these compounds and the sensitivity of their sex determination mechanisms to external influences.[13] EDCs not only interfere with or reverse sex differentiation during critical developmental stages, but also disrupt the steroidogenesis pathway, impair the hypothalamus-pituitary-gonad-liver (HPGL) axis, and adversely affect reproductive processes.[14,15] The regulation of sexual functions, including reproductive organ maturation, is mediated by gonadal steroids such as T and E2.[16,17] These processes are further controlled by gonadotropins, which are secreted by pituitary gonadotrophs in response to hypothalamic gonadotropin-releasing hormone (GnRH) signals. The two primary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), regulate both steroid hormone production (steroidogenesis) and gamete formation (gametogenesis).[18] Steroidogenic pathways rely on key enzymes from the cytochrome P450 family, including those encoded by *cyp11*, *cyp17*, and *cyp19*, as well as hydroxysteroid dehydrogenases like *3βhsd* and *17βhsd*, which are essential for estrogen and androgen synthesis.[19] The steroidogenic acute regulatory protein (*star*) plays a critical role in androgen production, and its disruption can impair spermatogenesis and reduce fertility.[20] These pathways, however, are highly sensitive to chemical interference, which can disrupt hormone biosynthesis and regulation.

Received: April 27, 2025
Revised: July 14, 2025
Accepted: July 15, 2025
Published: July 21, 2025

Biomarkers like vitellogenin (vtg), a yolk precursor protein, are early indicators of endocrine disruption, particularly following estrogenic exposure.[21] Normally, vtg is female-specific, with negligible expression in males; however, xenoestrogen exposure can strongly induce male vtg, signaling endocrine imbalance.[21,22] This induction occurs when elevated estrogen levels bind to hepatic estrogen receptors (*erα*, *erβ*), activating estrogen-responsive genes like *vitellogenins* (*vtg1, vtg2*) and *choriogenins* (*chgl, chgh*).[23] Similarly, aromatase enzymes, primarily encoded by *cyp19a* (ovarian) and *cyp19b* (neural), are crucial for estrogen synthesis.[19,24,25] Dysregulation of these genes can serve as a sex-specific biomarker for assessing endocrine-related reproductive dysfunction in both males and females.

Although the reproductive toxicity of BaP has been extensively investigated, most studies have focused on either male or female organisms in isolation. Even when both sexes were examined, comparative analyses are often lacking, leaving a gap in understanding the sex-specific responses to BaP exposure. Moreover, while the transgenerational effects of BaP have been reported in subsequent generations,[26−29] questions remain regarding the persistence and reversibility of reproductive effects. While our prior studies showed BaP-induced early developmental toxicity, sex-specific hepatotoxicity, and F1 transgenerational effects,[30−32] the sex-specific reproductive outcomes and extended transgenerational consequences remain uncharacterized. This study addresses these gaps by (1) investigating sex-specific reproductive toxicity during full life-cycle exposure to environmentally relevant BaP concentrations using the marine medaka (*Oryzias melastigma*) as a model organism and (2) assessing the persistence of these effects across three subsequent unexposed generations (F1−F3), to evaluate the long-term reproductive and endocrine consequences of parental BaP exposure.

2. MATERIALS AND METHODS

2.1. Chemicals. Benzo[*a*]pyrene (BaP, CAS 50−32−8) and ethyl 3-aminobenzoate methanesulfonate salt (MS-222) were purchased from Sigma-Aldrich (Thermo Scientific). To prepare a concentrated solution, BaP was dissolved in dimethyl sulfoxide (DMSO), which was obtained from Sangon Biotech (Shanghai, China). All solvents used in this study met stringent analytical standards, and the chemicals had a purity of at least 98%.

2.2. Fish Maintenance, Exposure, and Egg Production. The marine medaka stock was maintained in a semistatic system with filtered artificial seawater (30‰ salinity), a temperature of 26 ± 0.5 °C, and a 14 h light:10 h dark photoperiod. Exposure experiments used BaP concentrations of 1, 4, and 8 μg/L, with DMSO as a solvent control (<0.001% v/v). The concentrations chosen for this study come within the environmentally available levels reported in field studies.[33−37]

For the F0 generation, BaP exposure began at the embryonic stage (1-day postfertilization, dpf) and continued until sexual maturity at 120 dpf. Each exposure group included five replicates (n = 5), with three replicates designated for analysis and two as backups or used as needed. Embryos were transferred to 1000 mL of exposure solution after hatching, moved to 4 L containers at 30 dpf, and transferred to 20 L tanks at 60 dpf. A stocking density of 5 individuals/L was maintained throughout the experiment, and the seawater was replaced daily to ensure consistent chemical conditions. All experimental procedures involving fish were conducted in accordance with the guidelines of the Xiamen University Institutional Committee for the Care and Use of Laboratory Animals (XMULAC20190066).

At the end of the 120-day exposure period, the fish were anesthetized using ice water to induce hypothermia. Total body length, weight, and gonadosomatic index (GSI) were measured. Blood, brain, liver, and gonad samples were collected for hormonal, histological, and enzymatic activities and gene expression analysis. All samples were collected in triplicate from each gender (n = 3) and stored at −80 °C for further molecular analysis. The sex ratio in the medaka fish population was determined by observing secondary sexual characteristics and was further confirmed through gonad dissection. To evaluate egg production and fertilization rates in the F0 generation, ten breeding pairs (2 pairs × 5 replicate groups) per treatment were placed in separate tanks for 14 days. Spawns were collected daily and counted, and fertilization rate was calculated as (fertilized eggs/total eggs) × 100. Egg weight, protein, and lipid quantification were measured for egg quality assessment.

To assess recovery and reproductive potential across subsequent generations, the F0 fish were divided into two groups after the 120-day exposure period. One group was transferred to clean seawater for a 2-month depuration phase, while the other group was used to produce the F1 generation. To raise the F1 generation, all of the procedures were consistent with those used for the F0 generation, except for the absence of BaP or DMSO. The same protocol was repeated for the F2 generation, with F3 embryos monitored only until 15 dpf. A comprehensive experimental design (Figure S2) and detailed procedure are provided in Supporting Information (S1).

2.3. BaP Concentrations in Seawater and Fish Body. BaP concentrations were consistently monitored throughout the exposure period, with seawater and chemical solutions replenished daily to ensure stability. The measured BaP concentrations in the exposure water were 0, 0.91, 3.5, and 7.4 μg/L (Table S1). To quantify BaP levels in fish tissues, an enzyme-linked immunosorbent assay (ELISA) kit from Meimian Industry, Jiangsu, China, specifically designed for BaP detection, was utilized. Samples from F0 ovaries, F1 embryos, whole-body F1 adult fish, and F2 embryos were analyzed by using this kit. Detailed methodologies for BaP detection in water and tissue samples are provided in the Supporting Information (S2).

2.4. Histological Analysis. Gonads from both sexes (n = 3/gender) in each group were prepared according to our previously published protocols.[30,31] Images were captured using a Leica DM2500 light-emitting diode (LED) optical upright microscope equipped with a DFC7000 T camera. For quantitative analysis, three images were selected from each section (n = 3/group, totaling nine images/group) for both testes and ovary samples. In the testis, the number of mature spermatozoa and the number of total spermatogenic cells were counted to calculate the percentage of spermatozoa. Similarly, in the ovary, the number of mature oocytes and the number of total follicles were counted to determine the percentage of mature oocytes.

2.5. Oxidative Stress in Gonads. Gonads (testes or ovaries) from 10 fish of the same sex were pooled to create a replicate (n = 3/group). In the case of male fish, more than 10 testes were sometimes pooled, depending on their size, to

https://doi.org/10.1021/acs.est.5c05619
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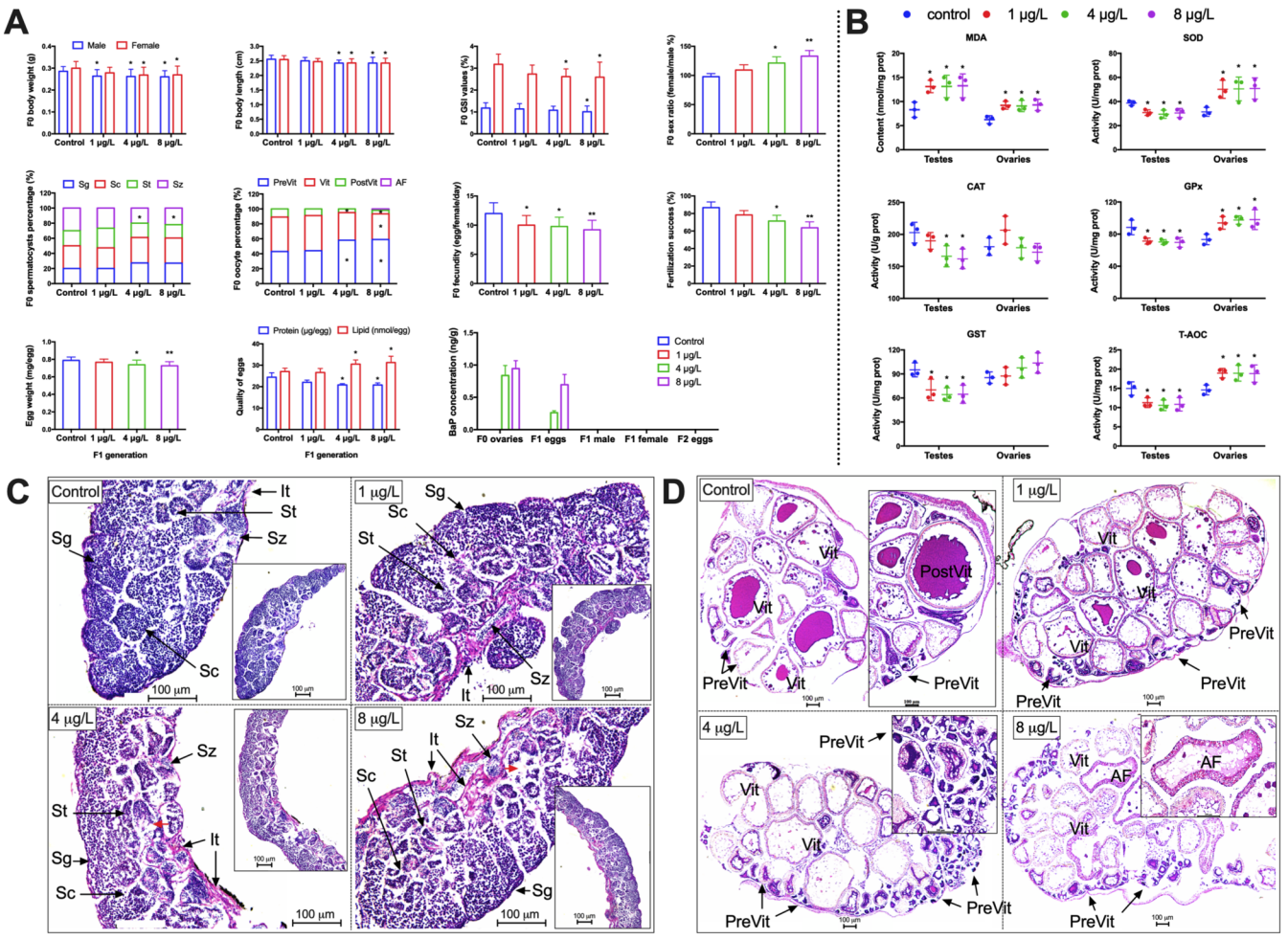

Figure 1. Effects of BaP on F0 marine medaka after life-cycle exposure. Biometric measurements (weight, length, and GSI = weight of the gonad/total body weight × 100), reproductive parameters (sex ratio, spermatocyte/oocyte proportions, fecundity, fertilization rate, F1 egg quality), and BaP levels in F0 ovaries and F1/F2 eggs and adults (A). Oxidative stress markers in gonads (B). Histological changes in testes (C) and ovaries (D). Abbreviations: Sg (spermatogonia), Sc (spermatocytes), St (spermatids), Sz (spermatozoa), It (interstitial tissue); PreVit (previtellogenic), Vit (vitellogenic), PostVit (postvitellogenic) oocytes, and AF (atretic follicles). Scale bars: 100 μm. Significant differences between the control group and BaP exposure groups (1, 4, and 8 μg/L) are indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$. Data: mean ± SD.

ensure adequate sample volume. The pooled gonads were weighed and individually homogenized in 1× phosphate-buffered saline (PBS, pH 7.4) using an electric homogenizer (Tiangen Biotech, China) at 4 °C. Following homogenization, the samples were centrifuged at 3500*g* for 10 min at 4 °C. The resulting supernatant from each tissue was aliquoted for subsequent analysis. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST) levels were measured by using commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) based on the bicinchoninic acid (BCA) method.

2.6. Measurement of Sex Hormonal Levels. Sex steroid hormone levels were measured in plasma samples collected from the fish. Blood from 10 fish of each sex was pooled to create a replicate (n = 3/group). Following tail amputation, 4–10 μL of blood was collected from the caudal vein using heparinized micropipette tips. The blood samples were then pooled and centrifuged at 3000*g* for 10 min at 4 °C to separate the plasma. Estradiol (E2) levels were measured using an ELISA kit from CUSABIO Biotech, while T and 11-KT levels were assessed using kits from Ruixin Biotech (China). The detection limits for E2, T, and 11-KT were 40, 1.6, and 0.5 pg/mL, respectively. Optical density (OD) was measured at 450 nm by using a Tecan Infinite F200 Pro Microplate Reader (Tecan, Switzerland). Hormone concentrations were determined based on standard curves.

2.7. Gene Transcription Analyses. Brains, livers, or gonads from 5 fish of the same sex were pooled to form one replicate (n = 3/group). Total RNA was extracted using the TRIzol reagent (Invitrogen). RNA quality was evaluated by 1.2% agarose gel electrophoresis, and concentrations were determined using a Nanodrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using the PrimeScript RT Reagent Kit with a gDNA Eraser (TaKaRa, China). Real-Time Quantitative PCR (qPCR) was performed using SYBR Green PCR Master Mix (Roche) on an ABI 7500 System, with 18s rRNA as the reference gene. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.[38] Primer sequences are listed in Table S7, and qPCR steps are provided in Supporting Information (S3).

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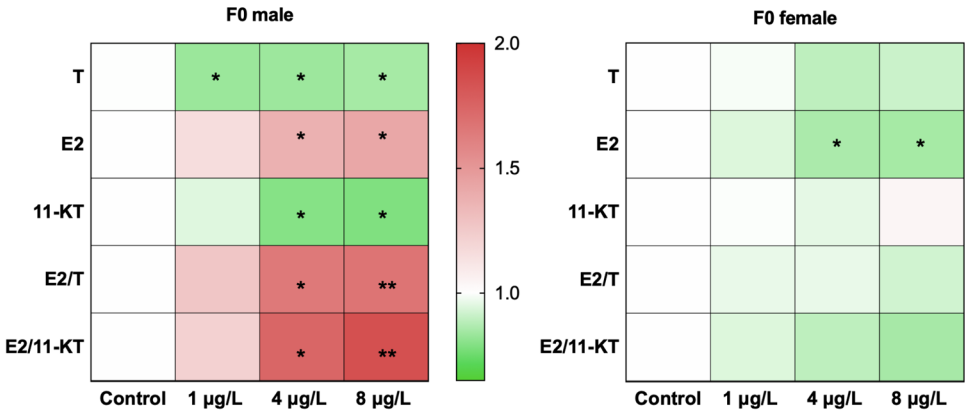

Figure 2. Sex steroid hormone levels in F0 male and female marine medaka following life-cycle BaP exposure. Measured hormones included testosterone (T), estradiol (E2), and 11-ketotestosterone (11-KT), along with E2/T and E2/11-KT ratios. Downregulation is indicated in green; upregulation is shown in red. Significant differences between the control group and BaP exposure groups (1, 4, and 8 μg/L) are indicated by *p < 0.05 and **p < 0.01. Data: mean ± SD.

2.8. Statistical Analysis. Graphs and statistical analyses were performed using GraphPad Prism v. 7.0. Data normality and homogeneity were verified using Shapiro-Wilk and Bartlett's tests, respectively. Sex hormone levels and gene transcription data were expressed as fold changes relative to those of the control group. Significant differences between control and BaP-exposed groups were determined by one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test. Results are presented as mean (M) ± standard deviation (SD), with statistical significance denoted as *p < 0.05, **p < 0.01, and ***p < 0.001.

3. RESULTS

3.1. F0 Life-Cycle Exposure to BaP: Biometric Measurements, Sex Ratio, Fecundity, Fertilization Rates, Histological Observations, and Egg Quality. In a life-cycle study spanning 1−120 dpf, BaP exposure induced sex-specific reproductive impairments. Male medaka exhibited significant body weight reductions at all concentrations (1, 4, and 8 μg/L; p = 0.04, p = 0.02, and p = 0.01, respectively), with body length declining at 4 and 8 μg/L (p = 0.01 for both) and GSI suppressed only at 8 μg/L (p = 0.02). Females exhibited similar trends at both 4 and 8 μg/L, with reduced body weight (p = 0.01), length (p = 0.02), and GSI (p = 0.01), but no significant effects were observed at 1 μg/L (Figure 1A and Table S2). BaP skewed population sex ratios toward females, increasing female-to-male ratios to 109% (1 μg/L), 121% (4 μg/L; p = 0.03), and 133% (8 μg/L; p = 0.003) compared to controls. Further, in F0 marine medaka, egg production declined significantly across all exposure groups, with reductions of 17% (1 μg/L, p = 0.03), 20% (4 μg/L, p = 0.02), and 23.33% (8 μg/L, p = 0.001) compared to controls. Fertilization rates also dropped dose-dependently to 78% (1 μg/L), 71.3% (4 μg/L, p = 0.04), and 63.7% (8 μg/L, p = 0.005), contrasting with the control rate of 86.6% (Figure 1A and Table S3). Transgenerational effects emerged in F1 eggs: those from F0 parents exposed to 4 and 8 μg/L BaP exhibited reduced weight (p = 0.04 and p = 0.009, respectively), lower total protein (p = 0.02), and elevated lipid levels (p = 0.04 and p = 0.02) (Figure 1A and Table S4). Notably, BaP was detected in the F0 ovaries in the 4 and 8 μg/L groups, while it was undetectable in the testes, likely due to their small size. BaP residues were also found in F1 eggs from the 4 and 8 μg/L groups. However, after F1 adults were reared in clean seawater, BaP became undetectable in both mature F1 fish and their F2 offspring, indicating the clearance of the compound across generations (Figure 1A and Table S5). Histological analysis revealed increased testicular interstitial tissue and reduced spermatozoa counts significantly at 4 μg/L (p = 0.02) and 8 μg/L (p = 0.04) relative to controls in F0 males. In females, previtellogenic oocyte proportions significantly increased (4 and 8 μg/L; p = 0.02 and p = 0.01, respectively), while vitellogenic oocytes (8 μg/L; p = 0.01) and postvitellogenic oocytes significantly reduced (4 and 8 μg/L; p = 0.01). Atretic follicles were observed exclusively in ovaries from the 8 μg/L BaP group (Figure 1C,D).

3.2. F0 Oxidative Stress Following Life-Cycle BaP Exposure and Depuration Period. Following a 120-day exposure to BaP in the F0 generation, oxidative stress markers in medaka gonads exhibited distinct sex-specific responses. In males, testes showed elevated MDA levels alongside reduced activity of SOD, CAT (at 4 and 8 μg/L), GPx, GST, and T-AOC across all BaP exposure groups (1, 4, and 8 μg/L). In contrast, female ovaries displayed similarly increased MDA levels but demonstrated compensatory upregulation of SOD, GPx, and T-AOC activity at all exposure concentrations, while CAT and GST levels remained unaffected. These results underscore a clear divergence in oxidative stress regulation between sexes, with male gonads experiencing widespread antioxidant suppression and female gonads mounting adaptive enzymatic responses to BaP exposure (Figure 1B) (statistical details in Table S6). After one month of depuration, female ovaries showed no significant residual effects, indicating full recovery. Male testes, however, retained disrupted antioxidant activity at 4 and 8 μg/L BaP exposures, although the 1 μg/L group recovered fully. By the second month of depuration, antioxidant activity in males normalized across all exposure groups, suggesting a time-dependent restoration of redox balance (Figure S3).

3.3. F0 Hormonal Changes. In this study, essential sex hormones, such as T, E2, and 11-KT, were measured. In male medaka, BaP exposure significantly reduced plasma T at all concentrations (1, 4, and 8 μg/L; p = 0.04, 0.03, 0.03) and suppressed 11-KT at 4 and 8 μg/L (p = 0.02), while E2 levels rose at higher BaP concentrations (4 and 8 μg/L; p = 0.03, 0.02). Conversely, females exhibited reduced levels of E2 at 4

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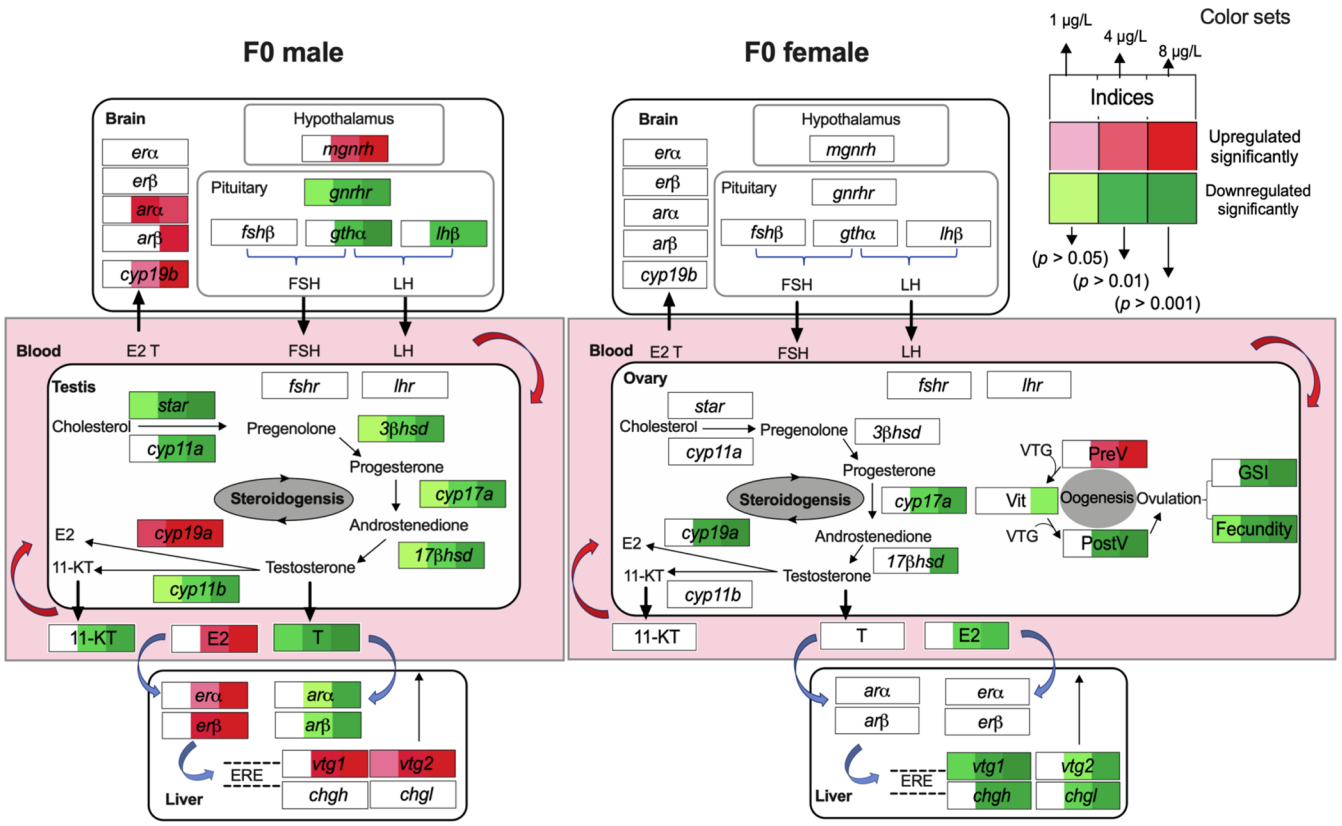

Figure 3. Overview of alterations along the HPGL-axis in male and female marine medaka across multiple levels of biological organization following life-cycle exposure to BaP. The results are presented in three segments corresponding to each concentration: 1 μg/L (left), 4 μg/L (middle), and 8 μg/L (right). Upregulated responses are denoted in red, while downregulated responses are indicated in green, both relative to control values. Statistical significance between the control and BaP-exposed groups is represented by color intensity, with darker shades indicating higher significance levels (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$), and absence of color indicating no statistically significant difference. Gene transcription data are displayed in italic.

and 8 μg/L ($p = 0.03$ and 0.02), though their androgen levels (T and 11-KT) remained unaffected. Hormonal ratios in males shifted markedly: E2/T and E2/11-KT ratios increased significantly at 4 and 8 μg/L ($p = 0.01$ and 0.008), whereas females showed nonsignificant declines in these ratios. Notably, the 1 μg/L BaP-exposed group showed no hormonal perturbations in either sex except for reduced male T (Figure 2).

3.4. F0 Transcription Profiles of the HPGL-Axis Genes. Following a 120-day BaP exposure in the F0 generation, transcriptional profiling of the HPGL-axis revealed sex-specific disruptions across multiple biological tiers integrated into a comprehensive map (Figure 3). In males, BaP induced dose-dependent dysregulation, beginning with suppressed hypothalamic medaka-type GnRH (*mgnrh*) (4 and 8 μg/L) and pituitary *gnrhr*, gonadotropin hormone α subunit (*gthα*), and *lhβ* (4 and 8 μg/L), while *fshβ* remained unaffected. Concurrently, the brain exhibited upregulated androgen receptors (*arα* and *arβ*) and *cyp19b* at higher concentrations (4 and 8 μg/L), though *erα/erβ* levels stayed stable. In testes, steroidogenic genes (*star, 3βhsd, 17βhsd, cyp17a, and cyp11b*) were broadly downregulated across all exposure groups, contrasting sharply with elevated *cyp19a*. Male livers mirrored this imbalance, showing heightened *erα/erβ* and *vtg1/vtg2* expression alongside suppressed *arα/arβ* at 4 and 8 μg/L. In contrast, female medaka displayed no transcriptional changes in hypothalamic or brain genes, maintaining stable levels. Ovarian disruptions were limited to significantly reduced *cyp17a*, *17βhsd*, and *cyp19a* at 4 and 8 μg/L, while other steroidogenic genes remained unaffected. Female livers, however, showed distinct suppression: *erα* declined across all exposures, *erβ* dropped at 8 μg/L, and *vtg1/vtg2* and *chgh/chgl* were inhibited at 4 and 8 μg/L BaP exposure, though *arα/arβ* remained stable. Transcriptional data and statistical significance are detailed in Table S8.

3.5. Transgenerational Biometric Measurements, Sex Ratio, Reproductive Fitness, Hormones, and Egg Quality. Transgenerational effects of parental BaP exposure were assessed across F1−F3 generations. F1 adults largely recovered in body length/weight, except for reduced body weight ($p = 0.03$) in the 8 μg/L lineage. Sex ratios were skewed in F1 offspring from 4 and 8 μg/L parents ($p = 0.03$, 0.02, respectively), while F1 males retained disrupted hormone ratios (elevated E2/T at 4 and 8 μg/L $p = 0.01$, and E2/11-KT at 8 μg/L $p = 0.04$). F1 females from 8 μg/L parents exhibited reduced fecundity ($p = 0.02$), and fertilization rates declined in F1 from 4 and 8 μg/L lineages ($p = 0.04$, 0.02, respectively). Adverse effects persisted in F2 eggs (lower weight $p = 0.04$, elevated lipids $p = 0.02$) from 8 μg/L ancestors (Figure 4A,B). The F2 adults showed no biometric changes. However, F2 sex ratios ($p = 0.04$) and male E2/T ratios ($p = 0.04$) remained disrupted in the 8 μg/L lineage. GSI remained stable in F1 and F2, and protein content in eggs was unaffected across all generations. By the F3 generation, ancestral BaP exposure no longer impacted the egg quality (Figure 4C,D).

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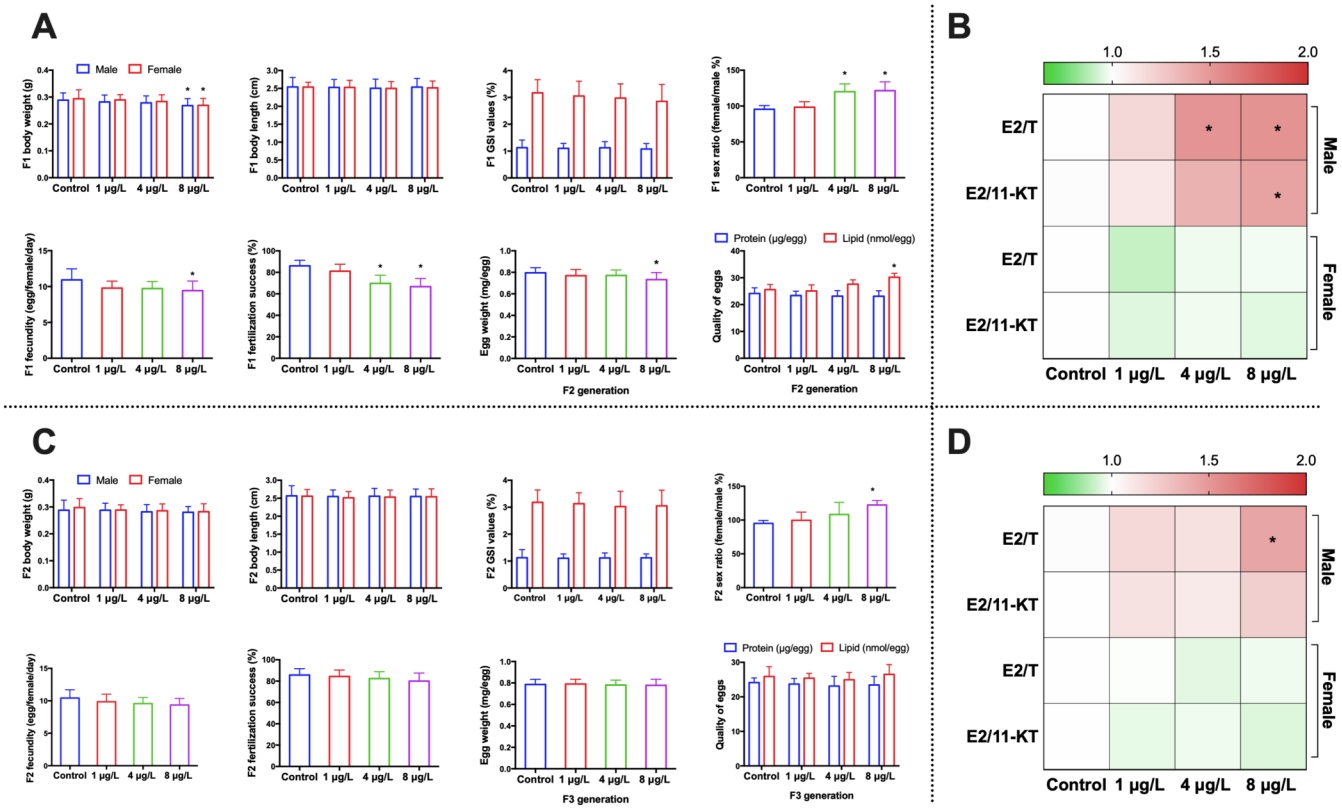

Figure 4. Parental BaP exposure transgenerationally impaired biometric measurements (weight, length, GSI), and reproductive parameters (sex ratio, fecundity, fertilization rate, egg quality), in F1 generation (A), and F2 generation (C). The sex steroid hormone ratio in the F1 generation (B) and F2 generation (D). Significant differences between the control group and BaP exposure groups (1, 4, and 8 μg/L) are indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$. Data: mean ± SD.

4. DISCUSSION

4.1. Sex-Specific Effects. This study demonstrates that life-cycle exposure to environmentally relevant BaP concentrations causes sex-specific endocrine disruption and reproductive impairments across multiple end points in male and female marine medaka. BaP is known to disrupt energy metabolism,[30,39,40] and in response to environmental stressors, organisms often reduce body size to conserve energy for essential functions like future reproduction.[41,42] In this study, the observed morphometric differences are likely linked to the high energy demands of detoxification processes, particularly oxidative stress, which is evident in both sexes. These alterations may reflect adaptive strategies to conserve energy during prolonged BaP exposure, ultimately resulting in trade-offs with reproductive investment. The reduction in GSI values observed in this study was correlated with histological changes in the gonads, including a decline in mature gametes. The low fertilization rates of eggs in this study were likely a result of impaired spermatogenesis in males, consistent with findings in zebrafish,[43] and male scallops (*Chlamys farreri*).[8] In females, the reduction in mature oocytes likely contributed to the observed decrease in fecundity. In addition, the recovery process varied between sexes, with females restoring antioxidant enzyme activity more rapidly, whereas males required a relatively longer period to return to baseline levels. These findings align with our previous study on sex-specific oxidative defenses in the marine medaka liver after BaP exposure.[31] The observed impairments appear to be linked to the high energy demands of detoxification and oxidative stress induced by BaP.

Hormonal disruption further compounded the reproductive impairments observed. Sex steroid hormones (T, 11-KT, and E2) regulate masculinization and feminization in fish,[44] and imbalances, commonly assessed via the E2/T ratio, are indicative of endocrine disruption.[14] In this study, BaP-exposed males showed significantly elevated E2/T and E2/11-KT ratios persisting into the F2 generation, suggesting estrogenic activity and impaired androgen production. This hormonal imbalance likely contributed to the reduced fertilization rates and delayed antioxidant recovery observed in males. The low endogenous E2 in males may partly explain the higher oxidative stress and slower recovery after BaP exposure. Despite BaP elevating the E2 level in males, it failed to confer protection and instead induced feminizing effects, worsened by suppressed androgen levels. The detrimental effect of elevated E2 observed here agrees with findings from a rat study examining E2's role.[45] In females, BaP reduced E2 levels, yet the overall endocrine profile, including the E2/T ratio, remained stable, likely supporting sustained antioxidant responses and recovery due to their naturally higher baseline E2, which appears to enhance antioxidant defenses. These findings align with previous studies showing E2's protective role against oxidative stress.[45−47] Altogether, the interplay between hormonal imbalance and oxidative stress appears to drive the sex-specific impairments observed and may help explain the altered sex ratios in BaP-exposed groups and their F1 and F2 progeny, highlighting the importance of sex-specific considerations in environmental risk assessments.

This study further demonstrated sex-specific disruptions in the HPGL-axis of medaka following BaP exposure, with males

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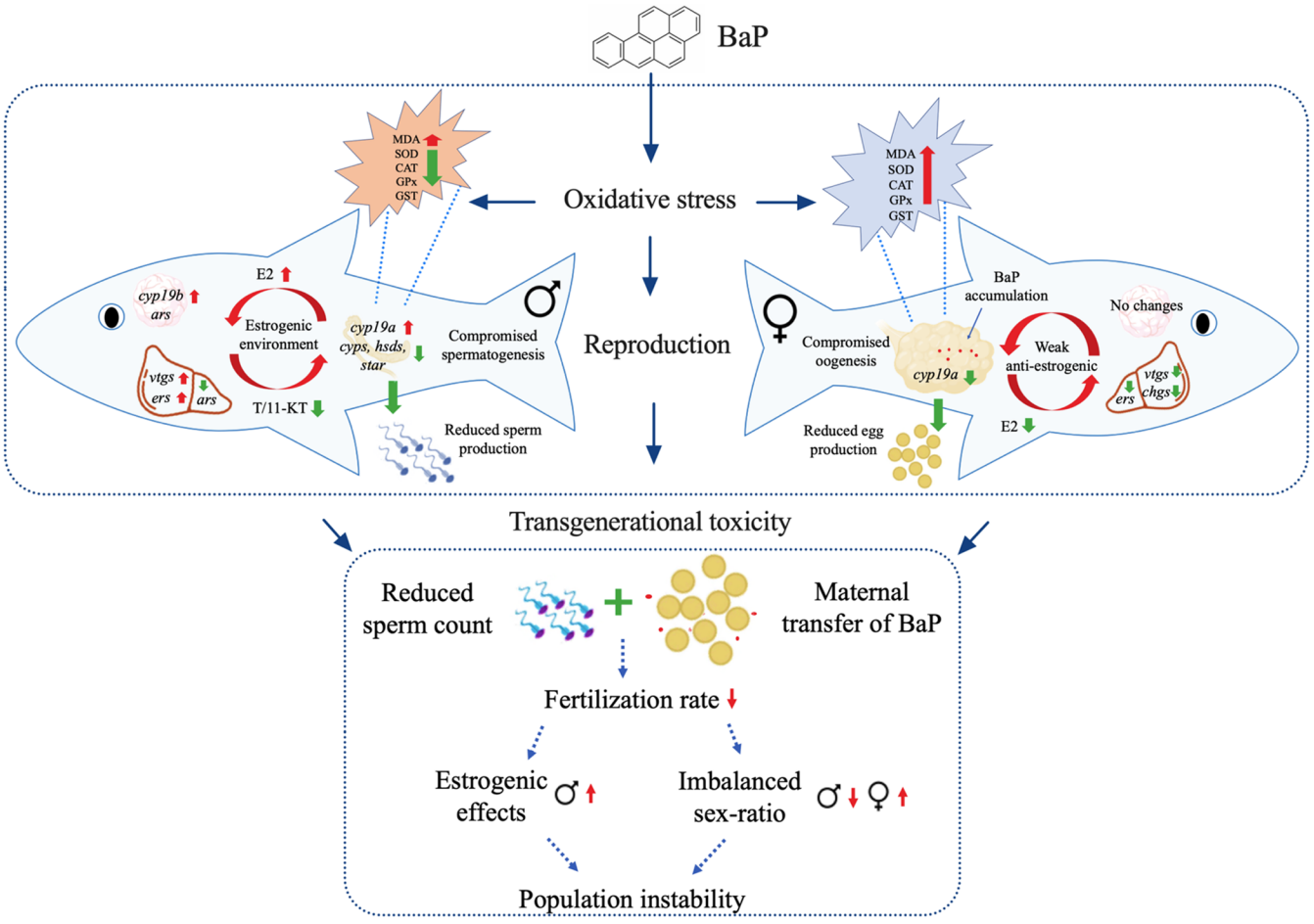

Figure 5. Schematic diagram of BaP-induced sex-specific and transgenerational toxicity in marine medaka.

exhibiting greater sensitivity. In males, BaP exposure significantly altered expressions of *cyp19b*, and *arα/arβ* in the brain, suggesting enhanced local estrogen signaling, consistent with studies showing that BaP can induce brain aromatase activity,[19] and modulate androgen receptors.[48] These changes may result from feedback mechanisms increasing sensitivity to T and E2 or direct effects of BaP on the brain. Such direct action may explain the suppression of key reproductive genes, including *mgnrh*, *gnrhr*, *gthα*, and *lhβ*, with the downregulation of *gthα* and *lhβ* likely contributing to gonadal abnormalities and impaired spermatogenesis. In the testes, the widespread suppression of steroidogenic genes, alongside the upregulation of *cyp19a*, suggests a shift in steroid production toward estrogen synthesis, supporting a feminizing effect. Hepatic elevation of *erα/erβ* and *vtg1/vtg2*, together with lowered *arα/arβ*, supports enhanced estrogenic activity and reduced androgen responsiveness. These gene expression changes align with elevated plasma E2 and decreased T and 11-KT levels, and the increase in *vtgs* in males further suggests a link to disrupted spermatogenesis. Together, these patterns indicate that male fish exhibited a more pronounced estrogenic response, reflecting BaP's estrogen-mimicking properties, consistent with evidence that BaP and its monohydroxy metabolites can interact with estrogen receptors and act as ER agonists, contributing to estrogenic effects.[49,50] Conversely, in females, the absence of gene expression changes in the brain suggests limited disruption at the central level of the HPGL-axis. However, suppression of *cyp19a*, *cyp17a*, and *17β-hsd* in the ovaries indicates reduced steroidogenesis, including impaired aromatase activity and decreased E2 synthesis, as supported by decreased plasma E2 levels. In the liver, decreased expression of *erα/erβ*, *vtg1/vtg2*, and *chgh/chgl* reflects impaired hepatic estrogen response, likely due to diminished circulating E2 or disrupted ER signaling, while the suppression of *vtgs* and *chgs* also explains the observed impairment in oocyte maturation, thereby reducing egg production. Collectively, these ovarian and hepatic gene changes represent an antiestrogenic response in female medaka. These findings align with reports showing BaP and other PAHs exert antiestrogenic effects, thereby reducing E2 synthesis, primarily through activation of the xenobiotic-sensing receptor, oxidative stress, and disruption of hormone receptor signaling.[5,19,27,51] The present findings highlight the dual endocrine-disrupting actions of BaP, exhibiting both estrogenic and antiestrogenic effects in a sex-specific manner: in males, BaP mimics estrogenic activity by upregulating aromatase and ER targets and suppressing androgen pathways, while in females, it acts through antiestrogenic mechanisms by disrupting steroidogenesis and hepatic estrogen signaling. This duality is consistent with previous reports,[2,8,9,19] and underscores the complexity of BaP's endocrine-disrupting potential in fish.

4.2. Transgenerational Effects. In the transgenerational analysis of this study, BaP was detected in F1 embryos of marine medaka, suggesting maternal transfer as the primary route, supported by its accumulation in the ovaries, consistent

https://doi.org/10.1021/acs.est.5c05619
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with findings in killifish (*Fundulus heteroclitus*).[52] However, this contrasts with zebrafish (*Danio rerio*) studies, where dietary BaP exposure did not lead to F1 detection,[28] possibly due to shorter exposure durations that limited maternal tissue accumulation. Additionally, parental BaP exposure significantly reduced F1 fertilization rates, likely due to male reproductive impairment and poor sperm quality, with previous studies also emphasizing paternal contributions to transgenerational effects.[26,32] In the present study, parental BaP exposure reduced both the quantity and the quality of F1 eggs. However, when the F1 generation was reared through a full life cycle in clean water, partial recovery in egg quality was observed in F2, with further improvement seen in F3. These findings suggest that although BaP-induced impairments persisted through F1 and F2, the recovery observed in F3 may indicate compensatory mechanisms that mitigate long-term reproductive toxicity.

Another notable direct and transgenerational effect observed in this study was the feminization of the medaka population. A female-skewed sex ratio was consistently recorded from the F0 to F2 generations following life-cycle BaP exposure. Since nearly all fish survived beyond the juvenile stage, male-biased mortality is unlikely to explain this pattern. Instead, disrupted E2/T ratios and altered expression of sex-steroid-related genes suggest that BaP disrupted hormonal pathways. These findings align with reports that xenoestrogens interfere with endocrine signaling and induce feminization through estrogen−androgen imbalance.[53−56] The resulting skewed sex ratio raises ecological concerns for species dependent on balanced sex ratios.[46,57,58] While previous reports of BaP-induced feminization were limited to a single generation, as seen in marine polychaetes,[46] and killifish,[1] our study extended this investigation across three generations in marine medaka. These findings highlight BaP's potential to cause persistent feminization, with implications for long-term reproductive dynamics in exposed populations.

Overall, this study demonstrates that life-cycle BaP exposure induces sex-specific endocrine disruption with transgenerational consequences. The observed divergent responses in males and females, including alterations in oxidative stress markers, hormonal profiles, and gene expression patterns, resulted in a persistent female-biased population shift, as illustrated in the mechanistic framework (Figure 5). These BaP-mediated effects underscore the importance of incorporating sex-specific analyses in toxicological assessments to better understand differential vulnerabilities. The transgenerational persistence of these effects further emphasizes the need to consider the long-term impacts of chemical exposures. While apparent female resilience may temporarily mitigate reproductive decline, sustained feminization poses potential risks to genetic diversity and population viability. Future research directions could include applying this sex- and transgenerational-focused approach to other environmental contaminants, employing multiomics technologies to elucidate underlying epigenetic mechanisms, and developing sex-specific biomarkers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.5c05619.

BaP measurement methods (S1); detailed exposure procedures (S2); quantitative PCR steps (S3); lateral views of male and female marine medaka (Figure S1); experimental design (Figure S2); oxidative stress indices following depuration period (Figure S3); BaP concentrations in water (Table S1); F0 growth metrics (Table S2); F0 fecundity, fertilization rate, and sex ratio (Table S3); health of the eggs (Table S4); BaP concentrations in F0 fish ovaries and F1 embryos (Table S5); oxidative stress statistical values (Table S6); list of primer (Table S7); gene transcription changes along the HPGL-axis (Table S8) (PDF)

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Author Contributions

R.Z.: conceptualization, methodology, data analysis, and writing original draft; X.Y.: methodology and analysis; F.C.: review and editing; J.B.: analysis and review and editing; K.-J.W.: conceptualization, funding, and supervision. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was funded by the National Natural Science Foundation of China (Nos. 41977211, 42377278), the Marine

Biotechnology Economic Integration Service Platform (Fujian Association for Science and Technology, China), the Xiamen Ocean Development Bureau (Grant No. 22CZP002HJ08), and the Pingtan Research Institute of Xiamen University, China (Grant No. Z20220743). We extend our thanks to Engineer Zhiyong Lin for technical assistance with experimental equipment and Engineer Ming Xiong for maintaining the fish aquariums.

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