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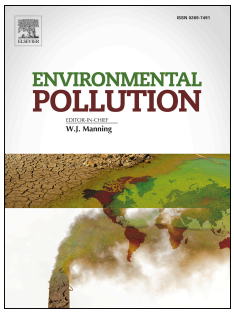

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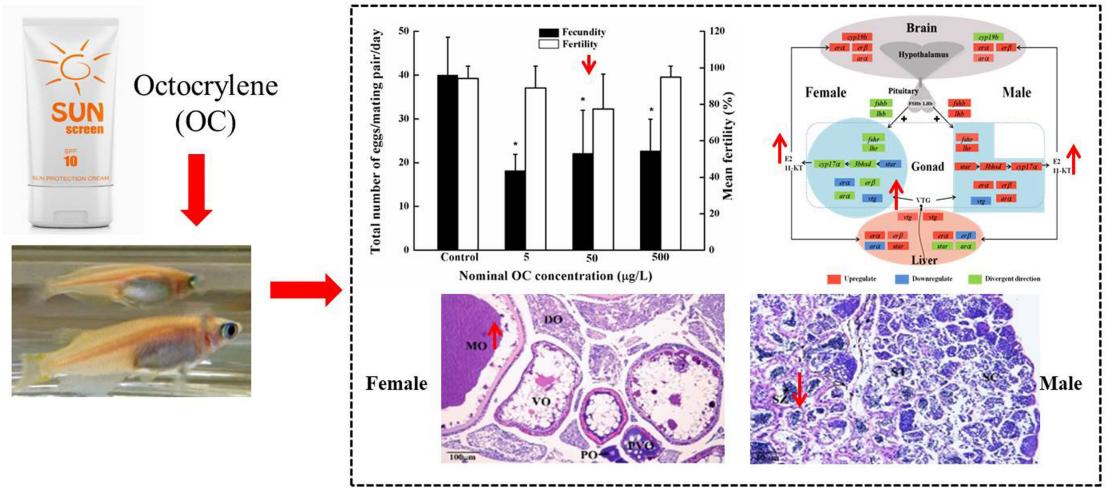

Reproductive toxicity and estrogen activity in Japanese medaka (*Oryzias latipes*) exposed to environmentally relevant concentrations of Octocrylene

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Abstract: The growing use of octocrylene (OC) in sunscreens has posed a great threat to aquatic organisms. In the present study, to assess its reproductive toxicity and mechanism, paired Japanese medaka (*Oryzias latipes)* (F0) were exposed to OC at nominal concentrations of 5, 50, and 500 μg/L for 28 d. Significant increases were observed in the gonadosomatic index (GSI) and hepatosomatic index (HSI) of F0 medaka at 500 μg/L OC ($p < 0.05$) without significant differences in fecundity. The fertility was significantly decreased at all treatments ($p < 0.05$). Significant increases in the percent of mature oocytes were observed at 5 and 500 μg/L OC, in which contrary to the percent of spermatozoa ($p < 0.05$). The plasma sex hormones and vitellogenin levels significantly increased in males at all treatments and in females at 50 and 500 μg/L OC ($p < 0.05$). In addition, the levels of *fshβ* and *lhβ* in the brains and the levels of *fshr*, *lhr* and *cyp17α* in the gonads were significantly upregulated in males at all treatments ($p < 0.05$), in line with those of *ar*, *erα*, *erβ* and *cyp19β* in the brains of male and female. The upregulation of *vtg* in male and female livers was observed only at 500 μg/L OC and upregulation of *star* and *hsd3β* was observed in testis at all treatments ($p < 0.05$). Continued exposure to OC significantly induced increases in the time to hatching, morphological abnormality rates, and cumulative death rates of F1 embryos, inconsistent with body length of F1 larvae ($p < 0.05$). Therefore, the responses of the exposed fish at the biochemical and molecular levels indicated reproductive toxicity and estrogenic activity of OC, providing insights into the mechanism of OC.

Capsule: OC induced reproductive toxicity and exhibited estrogen activity in

45 Japanese medaka (*Oryzias latipes*) at relevant environmental concentrations.

46 **Keywords**: Octocrylene; Japanese medaka (*Oryzias latipes*); Embryonic development;

47 Endocrine disrupting; Histopathology

48

1. Introduction

With the increased public awareness of excessive sun radiation hazards, UV filters (UVFs) have been promoted for extensive use in personal care products, cosmetics, and other products for protection from UV irradiation (Langford et al., 2015; Sunyer et al., 2019). Therefore, large amounts of UVFs enter the aquatic environment directly or indirectly (Santos et al., 2012). Due to their stability in aquatic environments, most UVFs are difficult to completely remove in a wastewater treatment plant, which causes UVFs (e.g., octocrylene) to be frequently detected in aquatic ecosystems (Brausch and Rand, 2011; Gago-Ferrero et al., 2013; Ebele et al., 2017), and some UV filters were found at concentrations up to 40 μg/L in river waters and swimming pools (Ekpeghere et al., 2016). With the high detected concentrations of UVFs, their impacts on aquatic ecosystems have received ongoing attention in invertebrates.

Octocrylene (OC) is the most commonly used UV filter in many countries because of its high absorptive efficiency for UVA and UVB light, and it is frequently detected in aquatic environments due to its high lipophilicity, stability, and resistance to sunlight degradation, which has attracted significant attention in the environmental protection field (Gago-Ferrero et al., 2013). Previous studies have reported that OC can easily accumulate in aquatic organisms due to its high lipophilicity ($\log_{Kow}$ = 6.9), at a level of 2400 ng/g lipid weight (l.w.) in brown trout (Buser et al., 2006), 89~782 ng/g (l.w.) in Franciscana dolphins (Gago-Ferrero et al., 2013), and 25~11,875 ng/g body weight in cod (Langford et al., 2015). The detected concentrations of OC range

from ng/L to μg/L (Apel et al., 2018), such as at levels from 103 to 6812 ng/L in seawater from Hong Kong (Tsui et al., 2015), the high concentration of 167 ng/L in tap water (Díaz-Cruz et al., 2012), an average of 38 μg/L in (household) gray water (Hernández-Leal et al., 2010) and up to 12 μg/L in wastewater (Magi et al., 2013). In addition, Zhang et al. (2016) reported 1.8 μg/L OC in the effluent of domestic wastewater treatment plants. Therefore, the incomplete removal of UVFs in wastewater treatment plants leads to their continuous discharge to environmental systems, which poses great threats to aquatic organisms (Hopkins et al., 2017).

Although frequent detection in the aquatic environment occurs, unfortunately, little information on the toxic effects of OC have been reported in aquatic species (Blüthgen et al., 2014). OC can trigger the production of potentially harmful free radicals (reactive oxygen species) when it releases its absorbed energy (Gago-Ferrero et al., 2015). Previous studies have reported that some UVFs have oxidative hepatic toxicity (Liu et al., 2015), hormonal activities (Zhang et al., 2016), and bio-accumulation (Gago-Ferrero et al., 2015) and can affect nuclear receptors, transcripts of responsive genes, levels of steroidogenesis (Zucchi et al., 2011; Christen et al., 2011; Kim et al., 2014) and reproduction in fish (Weisbrod et al., 2007; Grabicova et al., 2013). In addition, Stien et al. (2019) reported that OC could accumulate in the tissues of *Pocillopora damicornis* in the form of fatty acid conjugates and induce cell mitochondrial dysfunction by metabolomics. Although previous studies have reported the potential toxicity of OC, its adverse effects and modes of action are still poorly understood. Fish reproduction and development play

93 important roles in predicting ecosystem population variation (Overturf et al., 2015).
94 Therefore, the potential risks associated with OC may need to be concentrated on its
95 possible reproductive and development effects in fish once it accumulates in the
96 bodies of aquatic animals.

97 Japanese medaka (*Oryzias latipes*) have been used as a model for many years in
98 systems to evaluate the chemicals in water due to its advantages (Zhang et al., 2008;
99 Papoulias et al., 2014). Therefore, our objective was to assess reproductive toxicity of
100 OC in Japanese medaka at environmentally relevant concentrations (5, 50, 500 μg/L).
101 Many endpoints were achieved, including histological changes, plasma steroid
102 hormones and VTG, and transcripts of HPG axis-related genes. Finally, the
103 underlying mechanism of OC in fish was preliminary illustrated based on these
104 endpoints.

105 **2. Materials and methods**

106 *2.1. Chemicals*

107 OC (CAS No. 6197-30-4, purity > 99%) was purchased from J&K Chemical Ltd.
108 (Beijing, China). Stock solutions were prepared in ethanol (100%) and the final
109 concentration of ethanol was less than 0.01% in the exposure solutions.

110 *2.2. Maintenance of medaka and exposure*

111 The Japanese medaka (d-rR) stock used in the present study was provided by the
112 Laboratory of Freshwater Fish, Bioscience Center of Nagoya University in Japan. The
113 broodstock was cultured in dechlorinated tap water (pH: 7.2-7.6; 44.0-61.0 mg
114 $CaCO_3$/L; temperature: 25 ± 2 °C) with flow-through systems in a photoperiod of

115 16:8 h (light: dark). Fish were fed twice daily with a combination of commercial flake
116 fish food (Trea, Germany) and newly hatched brine shrimp (*Artemia nauplii*).

117 Japanese medaka (approximately 3-4 months old) were chosen to breed in pairs
118 in 18 L glass aquaria and acclimated for 2 weeks. The conditions were the same as the
119 brood stock. Then, 300 breeding pairs of mature medaka with similar and high levels
120 fecundity and fertility were selected to carry out the reproductive test and were
121 randomly divided into five groups equally, including solvent control, negative control
122 and three exposure groups (5, 50 and 500 μg/L). Each group contained 20 breeding
123 pairs of mature medaka, fifteen pairs of which were in an 18 L glass aquarium and 5
124 pairs of which were in five 1-L beakers. Each group contained three replicate aquaria.
125 The exposure solutions were changed daily to clean the feces and food particles
126 during 28 d exposure. The eggs were quickly collected in each beaker within 4 h after
127 fertilization at the 21st exposure and counted to calculate fertilization rate. Then, the
128 fertilized eggs were equally divided into two parts to assess the developmental
129 toxicity of OC. One-half of the fertilized eggs were hatched in dechlorinated tap water,
130 and the other half continued their exposure to OC with the same concentration as their
131 parents.

132 After 28 d exposure, 20 breeding pairs of mature medaka in each group were
133 sacrificed in a 200 mg/L MS-222. The tissue samples (brain, liver and gonad) and the
134 blood from each fish were obtained for further analysis. The development indices of
135 the embryos and larvae were recorded or measured at 14 d post hatching (dph),
136 including hatchability, time to hatching, morphological abnormality rates, body length

and cumulative death rates. The solvent control is not significantly different from the negative control and the data of solvent control are not shown in the results. The present study was conducted according to the Guide for the Care and Use of Laboratory Animals.

2.3. Chemical analysis

According to Zhang et al. (2016), the concentration of OC in exposure water was measured by high-performance liquid chromatography (HPLC) with a Hypersil BDS-C18 column (4.6 mm×250 mm, 5 μm particle size) coupled to a UV detector at 290 nm. Each treatment group was taken 200 mL of exposure water at the beginning (0 h) and prior to water renewal (24 h). Three water sample replications from each group were made. Water samples were condensed by solid-phase extraction with Agilent C18 (500 mg and 3 cc) cartridges and dried with N_2. The extraction was then resuspended in 1 mL of methanol and stored in the dark at 4 °C. 20 μL of each extracted sample was injected in duplicate and finished in 12 min.

2.4. Gonadosomatic index and hepatosomatic index

The gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated according to Liang et al. (2014) and the formula is as follows.

GSI (%) = (gonad weight (g) / body weight (g)) ×100

HSI (%) = (liver weight (g) / body weight (g)) ×100

2.5. Histological analysis

Before analyzed, the gonad tissues from 9 mature medaka of each sex were sampled, fixed and paraffin-embedded (Yan et al., 2018). Then, tissues were

159 dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95%,
160 100%) and embedded in paraffin. Paraffin sections (4-5 μm) were cut and stained with
161 hematoxylin and eosin (H&E). Photos of the sections from three randomly selected
162 fields of vision were taken with a BX53 optical microscope (Olympus, Tokyo, Japan)
163 and analyzed with cellSens Standard imaging software (Olympus, Tokyo, Japan).

164 *2.6. Measurement of plasma vitellogenin and sex steroid hormones levels*

165 The blood from 20 mature fish of each sex was collected and determined as
166 described by Chen et al. (2016). Plasma vitellogenin (VTG), estradiol (E2) and
167 11-ketotestosterone (11-KT) levels were determined by a fish VTG enzyme-linked
168 immunosorbent assay (ELISA) kit (Cusabio, Wuhan, Hubei, China), fish E2 ELISA
169 kit (Cusabio, Wuhan, Hubei, China) and fish 11-KT ELISA kit (Nanjing Jiancheng,
170 China), respectively, following the manufacturer's recommendations. The optical
171 density of the reaction solution was measured at 450 nm.

172 *2.7. Real-time PCR analysis*

173 Tissue samples (brain, liver and gonad) of 10 mature medaka (female:male=1:1)
174 from each treatment were stored in TRIzol reagent (Life Technology) to extract the
175 total RNA according to Yan et al. (2018). 2 μg of total RNA from each sample was
176 reverse transcribed to cDNA in line with the manufacturer's instructions (Promega)
177 and stored at -80 °C for RT-qPCR analysis. RT-qPCR analysis was performed with a
178 three-step profile according to the method of Yan et al. (2018). 20 μL reaction solution
179 contained SYBR Green Master Mix (Vazyme Biotech, Nanjing, China), Rox
180 Reference Dye 2, forward and reverse primer and was reacted in a 7500 real-time

PCR system (Applied Biosystems, California, USA). The primer pairs used for real-time PCR are shown in Table S1. Ribosomal protein L7 (RPL7) RNA was used as a reference gene for normalization (Zhu et al., 2015) and the results of the mRNA expression were calculated by the previously described method of $2^{-\Delta\Delta ct}$ (Livak and Schmittgen, 2001).

2.8. Data analysis and statistics

Data were analyzed with SPSS 19.0 (SPSS, Chicago, IL, USA) and illustrated graphically by OriginPro 8.0 (OriginLab, Northampton, MA, USA). Results are expressed as the mean ± standard error of the mean (S.E.M.). After checked normality and homogeneity of variance, all data were analyzed by one-way analysis of variance (ANOVA) with Turkey's HSD and Duncan's multiple comparisons test. Differences were considered statistically significant at the level of $p < 0.05$.

3. Results

3.1. Analysis of OC in exposure water

OC concentrations were not detected in the negative control and solvent control samples. The actual concentrations (mean ± standard deviation, % before/after renewal of the exposure solution) of OC were 2.71±0.32 (54%), 14.29±1.53 (29%) and 232.62±7.51 (47%) μg/L at nominal concentrations of 5 μg/L, 50 μg/L and 500 μg/L, respectively.

3.2. Reproductive effects

Significant decreases in the fecundity of adult Japanese medaka were observed in all OC treatment groups ($p < 0.05$). However, the fertility showed no significant

changes at all treatment levels compared with that of the controls (Fig. 1). The results of the F1 embryo development in dechlorinated tap water (Table 1) or in the same concentrations of OC as their parents (Table 2) were recorded. No significant changes were observed in hatchability, time to hatching, and morphological abnormality rates of F1 embryos compared with control groups, similar to the F1 larvae body length results (14 dph) (Table 1). However, cumulative death rates of F1 embryos in dechlorinated tap water with parental exposure to 500 μg/L OC were significantly increased ($p < 0.05$, Table 1). Meanwhile, a decreasing trend with no significant changes in hatchability of the F1 embryos was observed with increasing concentrations of OC (Table 2). Significant increases in the time to hatching and morphological abnormality rates of F1 embryos were observed at 50 and 500 μg/L OC, similar to the cumulative death rates of F1 embryos at all treatment concentrations ($p < 0.05$, Table 2). The body lengths of F1 larvae at 14 dph were significantly reduced at all treatment concentrations ($p < 0.05$, Table 2).

3.3. Growth and development of adult medaka

The body lengths and body weights in both male and female medaka were not significantly different at all treatment concentrations, which is similar to the GSI and HSI in male medaka at all treatments (Table S2). The GSI and HSI of female medaka did not significantly increase at 5 and 50 μg/L OC, whereas they significantly increased at 500 μg/L OC ($p < 0.05$, Table S2).

3.4. Histopathology

Different stage oocytes in ovarian tissues, such as primary oocytes (POs),

previtellogenic oocytes (PVOs), vitellogenic oocytes (VOs), mature oocytes (MOs) and degenerating vitellogenic oocytes (DOs), were observed in all treatment groups (Fig. 2A-D). Significant decreases in the percent of MOs and POs were observed at 500 μg/L OC ($p < 0.05$, Fig. 2E), similar to those of VOs at 5 μg/L OC ($p < 0.05$, Fig. 2E). In addition, the testes of adult medaka mainly included spermatogonia (SG), spermatocytes (SC), spermatids (ST) and spermatozoa (SZ), whose histopathology is shown in Fig. 2F-J. No significant changes in the percent of SG and ST were observed at all treatments (Fig. 2J). Significant increases were observed in the percent of both SC at 500 μg/L OC and SZ at 5 μg/L OC ($p < 0.05$, Fig. 2J). However, the percent SZ at 500 μg/L OC significantly decreased ($p < 0.05$, Fig. 2J).

3.5. Plasma steroid hormones and vitellogenin levels

The levels of VTG and E2 were significantly increased in males at all treatments and in females at 50 and 500 μg/L OC ($p < 0.05$, Fig. 3). Significantly increase in 11-KT level was found in females at all treatments and in males at 50 and 500 μg/L OC. No significant changes were observed in the VTG and E2 levels of females at 5 μg/L OC (Fig. 3). The levels of 11-KT were significant decreased in males at 5 μg/L OC ($p < 0.05$, Fig. 3).

3.6. Transcripts of HPG axis-related genes

In the brains, the androgen receptor (*arα*), estrogen receptors (*erα* and *erβ*) and cytochrome P450 aromatase 19β (*cyp19β*) mRNA levels were significantly increased in females at all treatments, similar to the expressions of *arα*, *erα* and *erβ* in males at all treatments ($p < 0.05$, Fig. 4 and Table S3). The expressions of follicle stimulating

hormone β (*fshβ*) and luteinizing hormone β (*lhβ*) in females were significantly decreased at 50 μg/L OC, contrary to those at 5 and 500 μg/L OC ($p < 0.05$, Fig. 4 and Table S3). The expressions of *fshβ* and *lhβ* in males were significantly increased at 5 and 50 μg/L OC ($p < 0.05$, Fig. 4 and Table S3).

In the livers, significant increases in females were observed in the levels of *erα*, *vtg* and steroidogenic acute regulator (*star*) at all treatments ($p < 0.05$, Fig. 4 and Table S3), similar to those of *erβ* at 5 and 500 μg/L OC and contrary to those of *arα* at all treatments ($p < 0.05$, Fig. 4 and Table S3). Significant increases in males were observed in the levels of *star* at 5 and 50 μg/L OC and *erα* at 50 and 500 μg/L treatments, similar to those of *arα* at 50 μg/L OC and *vtg* at 500 μg/L OC ($p < 0.05$, Fig. 4 and Table S3). Significant decreases in males were found in the levels of *arα* at 5 and 500 μg/L OC and in *erβ* at all treatments, similar to that of *star* at 500 μg/L OC ($p < 0.05$, Fig. 4 and Table S3).

In the gonads, significant decreases in the ovary were observed in the expressions of *arα*, *erα*, *erβ*, *cyp17α*, follicle stimulating hormone receptor (*fshr*) and luteinizing hormone receptor (*lhr*) at 50 and 500 μg/L OC, similar to those of *3βhsd* at 50 μg/L OC and *star* at 5 and 50 μg/L OC ($p < 0.05$, Fig. 4 and Table S3). Similarly, the expressions of *vtg* at all treatments significantly decreased in the ovary and testis ($p < 0.05$, Fig. 4 and Table S3). Significant increases in the expressions of *arα*, *erβ*, *cyp17α*, *3βhsd, fshr* and *lhr* were observed in the ovary at 5 μg/L OC ($p < 0.05$, Fig. 4 and Table S3), similar to those of *fshr* at 50 and 500 μg/L OC, and those of *arα*, *erα*, *erβ*, *cyp17α*, *3βhsd*, *star* and *lhr* at all treatments in the testis. ($p < 0.05$, Fig. 4 and

Table S3).

4. Discussion

OC has been reported as an endocrine disrupting chemical (EDC) (Wang et al., 2016); however, its toxicity and related mechanism of action on Japanese medaka at relevant environmental concentrations are still unknown. In the current study, OC can induce the reproductive toxicity and estrogen activity in Japanese medaka according to multiple measured endpoints.

4.1. Chemical Analysis of OC in exposure water

In the present study, the OC was significantly decreased compared with the nominal concentrations during the exposure. Zhang et al. (2016) have indicated the similar results on the decreased OC levels were only found in the exposure water with zebrafish. In addition, Gomez et al. (2012) has reported that OC is rapid uptake by organism, but is followed by elimination within 24 h and excreted to some extent. Therefore, this result can be induced by uptake into zebrafish or adsorption to surplus food or faeces (Zhang et al., 2016) and metabolism and/or excretion in vivo (Gago-Ferrero et al., 2015).

4.2.Reproductive effects

The fecundity of fish is important to predict the population variation (Overturf et al., 2015) and many environmental factors can mediate the overall reproductive fitness of organisms. As reported by Overturf et al. (2015), hormonally active UVFs have impaired fish reproduction. In the current study, OC had reproductive toxicity due to the decreased fecundity and significantly decreased fertility. Similar results

have also been reported in medaka exposed to benzophenone-3 (BP-3) for 28 d (Kim et al., 2014) and in fathead minnows following 74 and 285 μg/L 3-benzylidene camphor treatments for 21 d (Kunz et al. 2006), as did benzophenone-2 (> 1.2 mg/L) for 15 d (Weisbrod et al. 2007). In the present study, OC significantly induced increases in the time to hatching, morphological abnormality rates and cumulative death rates of F1 embryos (p<0.05). Similarly, Torres et al. (2016) reported that 3-(4-Methylbenzylidene) camphor (4-MBC) induced malformation development and extended the hatching time of zebrafish embryo exposed to 5 mg/L or higher concentrations of 4-MBC. Moreover, 4-MBC also induced developmental malformations in frog *Pelophylax perezi* eggs (Martins et al., 2017). In addition, 4-MBC significantly inhibited the lengths of zebrafish larvae at concentrations higher than 2 μg/L (Torres et al., 2016), which is similar to our results. In addition, endocrine disrupting chemicals (EDCs) can alter the reproductive capabilities of nontarget organisms, specifically fish, at environmentally relevant concentrations and adversely affect the population of aquatic organisms (Overturf et al., 2015). Therefore, our findings reflected that OC exhibited reproductive and developmental toxicity to medaka that are severe enough to potentially affect fish populations.

4.2. Histopathology

Histopathology is an important method for environmental and ecological risk assessment by examining early changes in morphology (Guardiola et al., 2013a) and has been used to evaluate the toxic effects of various contaminants on tissue organs (Velma and Tchounwou, 2010; Guardiola et al., 2013b). In the present study,

significant decreases in the percent of MOs and POs at 500 μg/L OC or VOs at 5 μg/L OC indicated that OC impaired ovary composition, similar to the results of 394 μg/L EHMC in female fathead minnows (Christen et al., 2011). Similar to OC, inhibition of testicular development was observed in EE2-exposed fish (Pawlowski et al., 2004; Jackson et al., 2019), BP-3-exposed fish (Christen et al., 2011) and 4-nonylphenol-exposed fish, which reduced the fecundity of fish (Harries et al., 2000). Similarly, alterations have been reported in different fish species from contaminated environments, which might result from environmental chemicals in their living environments (Ameur et al., 2012). Therefore, OC may exhibit an estrogenic or antiandrogenic effect, which could induce the reproductive toxicity.

4.3. Plasma sex steroid hormones and vitellogenin levels

Sex steroid hormones (such as testosterone (T) and 17b-estradiol (E2)) could be used to evaluate reproductive effects in fish (Lubzens et al., 2010; Wang et al., 2013), and affect fish gonadal development (Shang et al., 2006) by directly regulating fish spermatogenesis (Liu et al., 2018), which is largely regulated by androgens (e.g., 11-ketotestosterone (11-KT)) (Miura et al., 1991). Many environmental toxicants have been reported to have adverse effects on the synthesis and release of T and E2 in adult zebrafish (Liang et al., 2014; Zhao et al., 2015). Similar to OC, 90 μg/L BP-3 induced reproductive effects on medaka after 28 d by affecting plasma sex steroid hormones and VTG levels (Kim et al., 2014). Plasma VTG is synthesized in fish livers during exposure to estrogen and plays an important role in ovarian development, oocyte maturation and yolk biosynthesis (Yuan et al., 2013; Yu et al., 2014). Our results

suggest that an increase in VTG levels in females help to promote ovarian development, while increased VTG levels in males can result from estrogenic or antiandrogenic chemicals, which suppresses testicular development and sperm maturation (Barucca et al., 2006). Similarly, 9.91, 99.1 and 991 mg/L 4-MBC increased plasma VTG levels in male medaka (Inui et al., 2003), as did 268 μg/L BP-3 in adult male zebrafish during 12 d exposure (Kinnberg et al., 2015). E2 is related to VTG expression, which is regulated by ER signaling pathways (Yan et al., 2018), which could illustrate our results of E2 and VTG levels in female and male medaka. Therefore, the reproductive effects of OC on Japanese medaka may result from the significantly increased 11-KT levels in females, leading to decreased fecundity at all treatments. In addition, OC increased levels of sex hormones and exhibited estrogenic activity in medaka, which caused the significantly reduced number of spermatozoa and arrested fish spermatogenesis (Liu et al., 2018; Yin et al., 2017).

4.4. Transcripts of the HPG axis-related genes

Reproductive activity could be regulated by the hypothalamus-hypophysis (pituitary gland)-gonadal (HPG) axis, which coordinates the action of gonadotropin-releasing hormone from hypothalamus and two gonadotropins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) in the pituitary (Wang et al., 2019). LH and FSH play vital roles in steroidogenesis and gametogenesis of the gonads by combining FSHR and LHR (Yan et al., 2018), which may lead to the increased 11-KT levels in the present study (Ji et al., 2013). This

result could be explained by the increased plasma E2 levels in females and males according to the upregulated levels of *fshβ*, *lhβ*, *fshr* and *lhr* in the present study. Similar to our results, Zucchi et al. (2011) reported significantly upregulated *vtg1* expression in adult male zebrafish livers exposed to 2.2 and 890 μg/L EHMC for 14 d. Therefore, exposure to OC induced alterations in the upstream genes involved in the HPG axis, leading to the response of downstream genes.

Involved in the reproduction process, *er* and *ar* are located in the cytoplasm and cell nucleus and combine with the relative hormone gland to regulate the levels of target genes in the ER/AR signaling pathways (Li et al., 2016), which also affect the reproductive and developmental toxicity (Yan et al., 2018). In the current study, the expressions of *erα* and *erβ* in the testis were significantly increased at all treatments, consistent with the results of OC in zebrafish (Zhang et al., 2016) and is partially responsible for the histological changes in the testes of the exposed fish. Exposure to OC could affect the expression of the genes involved in fish steroidogenesis; for example, *star* and *hsd3β*. StAR is involved in regulating steroidogenesis by transferring cholesterol across the mitochondrial membrane (Yan et al., 2018), and *hsd3β* can further catalyze pregnenolone to synthesize progesterone, leading to the synthesis of 11-KT and E2 (Liang et al., 2014). The upregulation of *star* and *hsd3β* expression in males can explain the increase in 11-KT and E2. *Cyp19β* is known to be a sensitive signal of estrogens in aquatic environment (Diotel et al., 2010), and previous studies have reported that estrogens can promote *cyp19β* expression in adult zebrafish brains (Mouriec et al., 2009; Callard et al., 2001). In addition, *cyp19β* also

plays important roles in regulating the reproductive processes in teleosts (Kazeto et al., 2001; Kuhl et al., 2005) and can convert testosterone to estradiol in the gonads. The synthesis of testosterone is responsible for *cyp17α* (Martinez-Arguelles et al., 2013). The increased E2 and 11-KT levels in females and males in the present study can be explained by the upregulation of *cyp19β* and *cyp17α* and can be interpreted as an estrogenic response (Zhang et al., 2016). Therefore, these results in the HPG axis further demonstrate that OC has estrogenic activity and reproductive toxicity.

5. Conclusion

The present study reveals that OC inhibited SZ synthesis and promoted MO maturation by histopathology, increased plasma sex steroid hormone (E2 and 11-KT) and VTG levels, and upregulated the expression of HPG-axis genes related to steroidogenesis and reproduction both in female and male medaka, which shows that OC poses a great threat to the reproductive system of medaka and adversely affects their development.

Acknowledgments

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Supplementary material

The detailed materials and methods are shown in part one of Supplementary material and supplemental tables were in part two; Detail information of primer sequences and product size used for Japanese medaka (*Oryzias latipes*) (Table S1);

401 Growth and development results of F0 adult medaka exposed to 5, 50, and 500 μg/L
402 OC for 28 d (Table S2); Relative gene expression levels in the brains, livers and
403 gonads of F0 adult medaka after exposure to OC for 28 d (Table S3).
404

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Figure and Table Captions

Fig. 1. The fecundity and fertility of paired mature medaka exposed to different concentrations of OC for 28 d. Data are presented as the mean ± S.E.M (n=3). The asterisk symbol (*) indicates that $p < 0.05$ is considered a significant difference compared to the control.

Fig. 2. Histopathology of ovarian tissues (A-E) and testicular tissues (F-J) in mature medaka exposed to OC, stained with hematoxylin and eosin. A: control in females; B: 5 μg/L; C: 50 μg/L; D: 500 μg/L. E: The relative distribution of different stage follicles in the control and exposure groups (*$p < 0.05$; Data are presented as the mean ± S.E.M. (n=3)). PO, primary oocyte; PVO, previtellogenic oocyte; VO, vitellogenic oocyte; MO, mature oocyte; DO, degenerating vitellogenic oocyte. F: control in males; G: 5 μg/L; H: 50 μg/L; I: 500 μg/L; J: The ratio of different stages of sperm in the control and exposure groups (*$p < 0.05$; Data are presented as the mean ± S.E.M. (n=3)). SG, spermatogonia; SC, spermatocyte; ST, spermatid; SZ, spermatozoa.

Fig. 3. Levels of plasma steroid hormones and vitellogenin in adult medaka exposed to OC. Data are expressed as the mean ± S.E.M. (n=3). Different letters (a, b, c, d) indicate significant differences compared to the control group at levels of $p < 0.05$.

Fig. 4. The heat map of genes involved in the HPG axis of medaka in response to environmental concentrations of OC. Data are expressed as the mean ± S.E.M. (n=3).

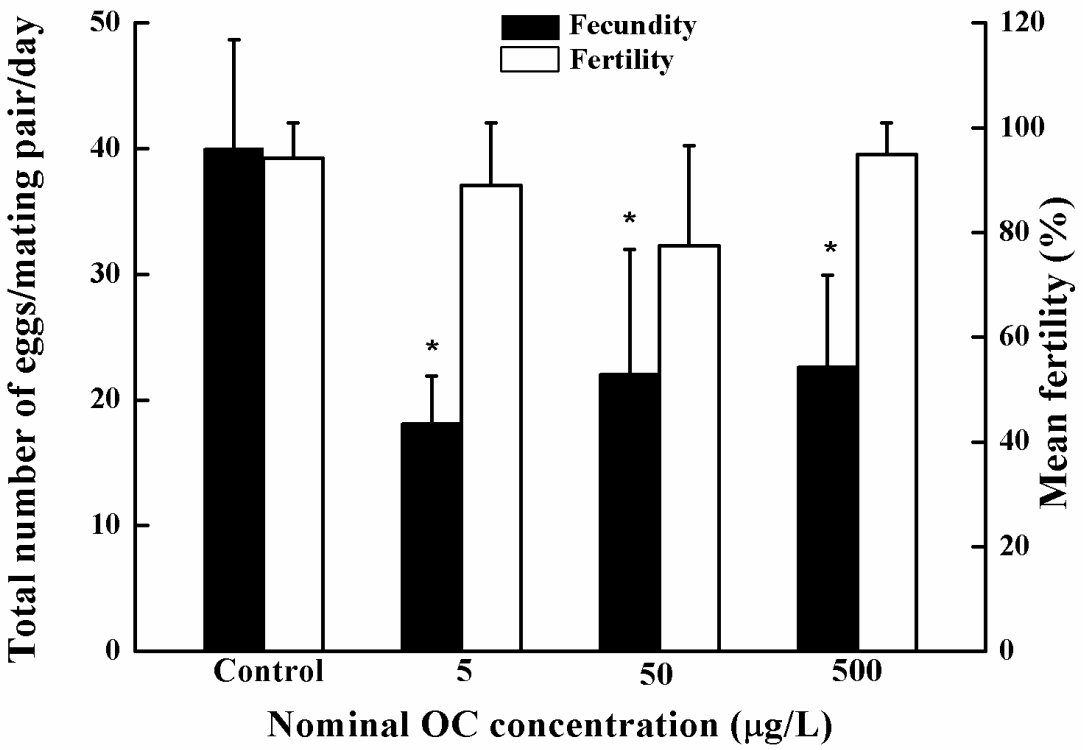
Table 1 Development of F1 generation fertilized eggs in dechlorinated tape water and continued exposed to OC with the same concentration as their parents. F1 generation embryos were from F0 generation exposed to 5, 50 and 500 μg/L OC during the last week exposure.

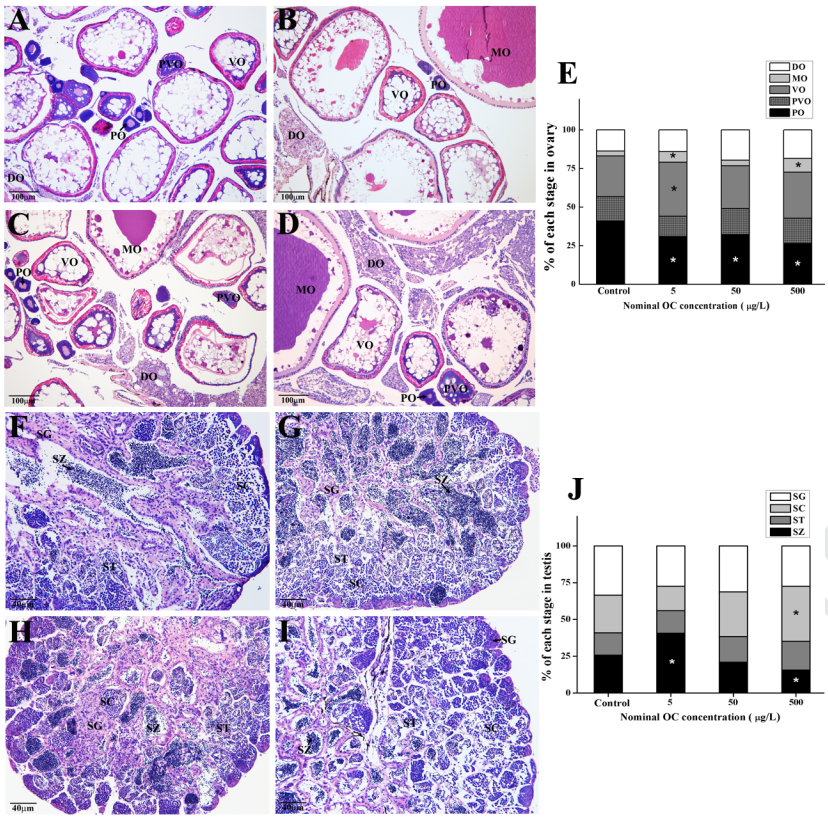
Table 1 Development of F1 generation fertilized eggs in dechlorinated tape water and continued exposed to OC with the s

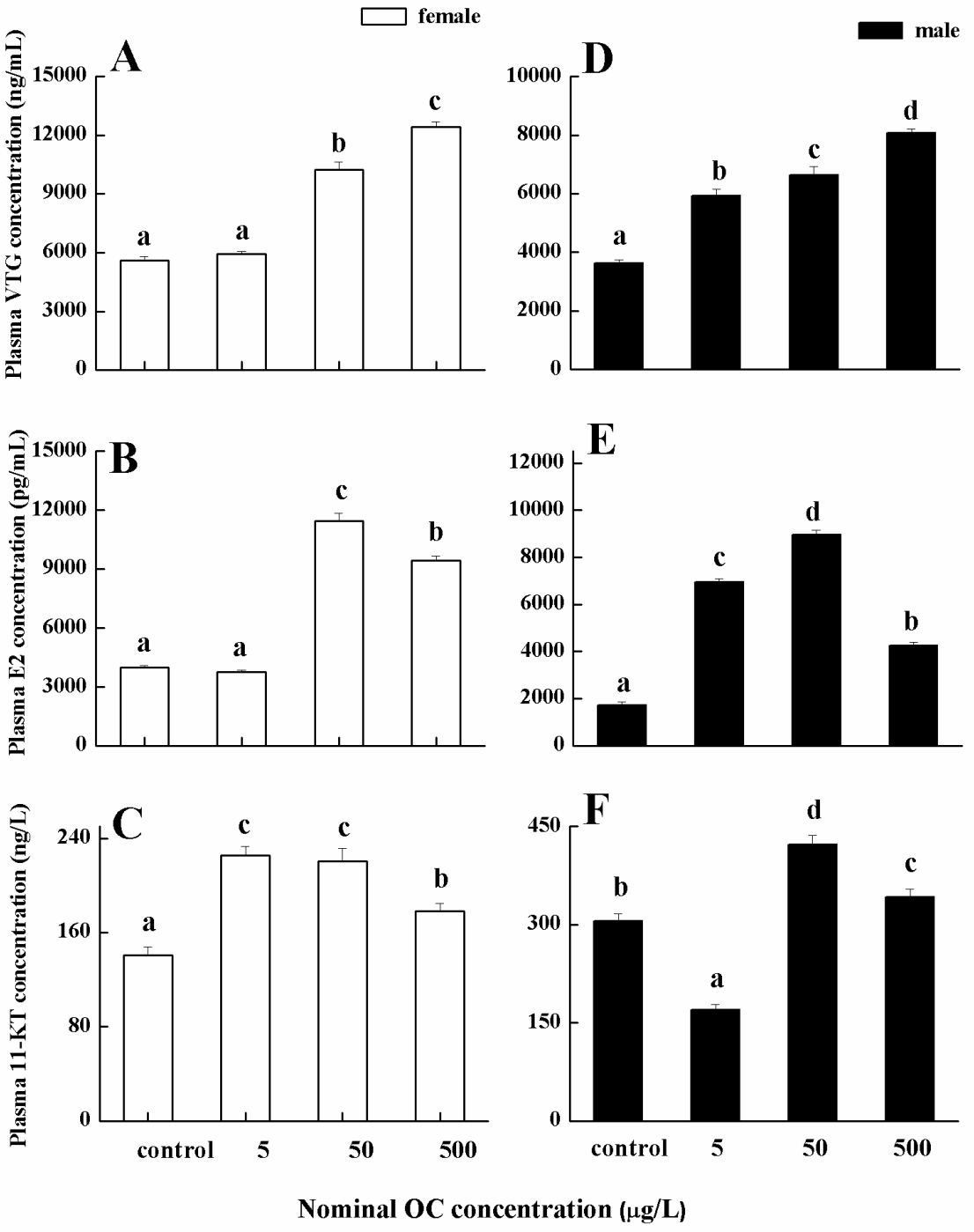
parents. F1 generation embryos were from F0 generation exposed to 5, 50 and 500 μg/L OC during the last week exposure.

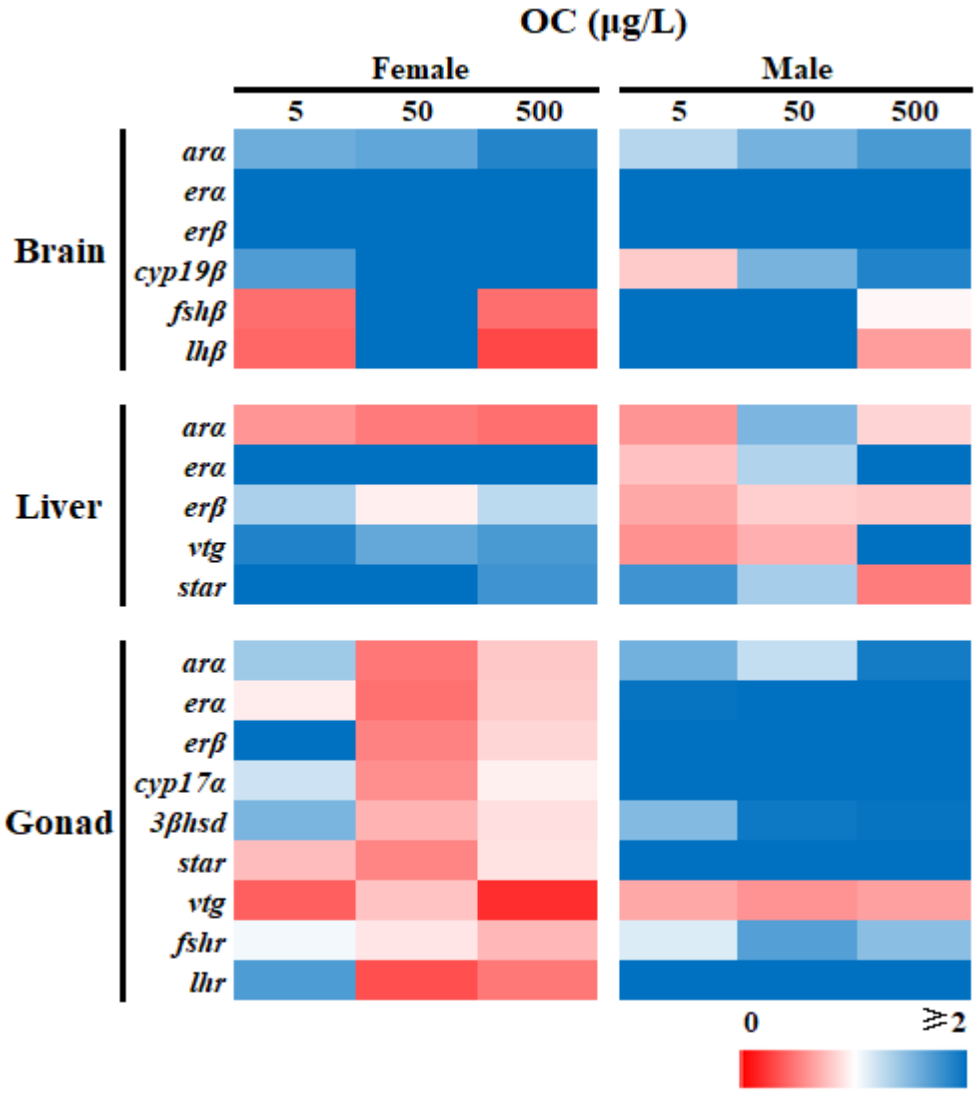
Concentration (μg/L)		Hatchability (%)	Time to hatching (days)	Morphological abnormality rates (%)	Accumulative death rates (%)
F0	F1				
Control	0	93.9 ± 4.6	12.5 ± 0.5	8.5 ± 2.9	14.9 ± 5.2
5	0	87.4 ± 8.3	13.6 ± 0.6	21.2 ± 10.9	30.0 ± 11.5
50	0	91.8 ± 6.3	13.5 ± 1.0	17.0 ± 9.4	34.1 ± 18.9
500	0	85.1 ± 6.1	15.2 ± 2.1	15.3 ± 8.5	49.0 ± 12.9*
Control	0	94.6 ± 1.5	11.1 ± 1.7	6.4 ± 2.0	15.3 ± 2.9
5	5	91.6 ± 6.6	16.6 ± 4.3	26.4 ± 10.2*	51.4 ± 9.2*
50	50	84.5 ± 9.2	22.0 ± 2.9*	29.3 ± 7.8*	52.8 ± 11.3*
500	500	88.5 ± 8.1	22.6 ± 1.1*	29.1 ± 8.6*	62.2 ± 13.4*

Data expressed as mean ± S.E.M. of each treatment (n=3). The asterisk (*) indicates statistically significant difference from


Fecundity
Fertility
Total number of eggs/mating pair/day
Mean fertility (%)
0
10
20
30
40
50
0
20
40
60
80
100
120
*
*
*
Control
5
50
500
Nominal OC concentration (μg/L)




female
male
A
B
C
D
E
F
Plasma VTG concentration (ng/mL)
Plasma E2 concentration (pg/mL)
Plasma 11-KT concentration (ng/L)
control
5
50
500
Nominal OC concentration (μg/L)


OC (μg/L)
Female
Male
5
50
500
5
50
500
Brain
arα
erα
erβ
cyp19β
fshβ
lhβ
Liver
arα
erα
erβ
vtg
star
Gonad
arα
erα
erβ
cyp17α
3βhsd
star
vtg
fshr
lhr
0
≥2

Highlights

- OC induced reproductive toxicity and estrogenic toxicity in medaka.
- OC significantly increased plasma VTG and sex steroid hormones levels.
- OC significantly upregulated the expressions of genes related to the HPG-axis.
- Histopathological observation reflected OC inhibited spermatogenesis.

Author statement

Yan Saihong: Investigation, Formal analysis, Writing-Review & Editing

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Conflict of Interest

The authors declare no conflict of interest.