



# New insights into the metabolism and toxicity of bisphenol A on marine fish under long-term exposure<sup>☆</sup>

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## ABSTRACT

Bisphenol A (BPA) exposure receives great ecotoxicological concern. However, gaps in knowledge, such as metabolism of BPA and inconsistent reports on reproductive toxicity, still exist. In this study, a marine fish model (*Oryzias melastigma*) was exposed to serial concentrations of BPA throughout its whole life cycle. The level of BPA-glucuronide (BPAG) dramatically increased throughout the embryonic stage since 4 dpf. Accordingly, the mRNA level and enzymatic activity of UDP-glucuronosyltransferases (UGTs) increased across the embryonic stage. The mRNA level of UGT2 subtype rather than UGT1 or UGT5 showed a concentration dependent response to BPA exposure. BPA exposure led to the morphological disruption of the chorion and villi as shown by scanning electron microscopy; however, the hatchability was not significantly influenced after exposure. Newly hatching larvae were continuously exposed to BPA for 120 days. Lower mRNA levels of hormone metabolism-related genes, decreased ratio of E2/T, slower ovary development and decreased egg production confirmed the inhibitory effect of BPA on reproduction. Overall, our results showed the conjugation of BPA into BPAG by UGT2 at the embryonic stage and convinced the reproductive toxicity from multiple levels after whole life exposure to BPA.

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## 1. Introduction

Bisphenol A (BPA) is a high-production-volume chemical with a variety of applications. It has been widely detected in environmental matrices and organisms. For example, its levels ranged from 4.4 to 8000 ng/L in water samples from North America, Europe, and Asia (Jonkers et al., 2010; Staples et al., 2000, 2018; Xu et al., 2018). The range is 14–2358 ng/L in industrial and municipal wastewater in the area of Thessaloniki, Northern Greece (Arditsoglou and Voutsas, 2010). BPA was also detected in water organism. Marine fish caught in the Gulf of Naples and off the Latium coast of Italy had body burdens of 0.5–6 mg/kg BPA (Mita et al., 2011), and levels ranged from 0.19 to 25.2 µg/kg BPA in fish sampled from the sea around Taiwan (Lee et al., 2015). Similarly, the level range is 0.83–19.25 µg/kg in marine fish from local market in Hongkong (Wong et al., 2017). As the existence of BPA in water and aquatic

organisms, exposure to BPA is of great concern ecotoxicologically (Im and Löffler, 2016; Tisler et al., 2016; Villeneuve et al., 2012).

BPA can be metabolized after entering the organisms. The metabolism of BPA has been widely studied in mammals. In rats, the primary metabolite of BPA is BPA-glucuronide (BPAG) as 65% of the BPA absorbed in the liver was glucuronidated into BPAG within 1 h after administration (Inoue et al., 2001). BPAG rather than BPA could migrate to a rat fetus, where unidirectional deconjugation from BPAG to free BPA occurred (Nishikawa et al., 2010). Reversible metabolism between BPA and BPAG also occurred in the fetal compartment of sheep, and this conjugation - deconjugation cycling contributed to a 43% increase of the overall fetal exposure to free BPA (Gauderat et al., 2016). In contrast, the knowledge on BPA metabolism were relatively few in fish. In a previous study, BPA conjugation was found to occur in the early embryonic stage of brown trout (Bjerregaard et al., 2008). UDP-glucuronosyltransferases (UGTs) play key roles in the conjugation reaction. UGTs are membrane proteins located on endoplasmic reticulum and nuclear envelope. They can catalyze the transfer of the glucuronic acid component of UDP-glucuronic acid to BPA. UGT gene family has various kinds of homologous genes and enzymes encoded by these homologous genes show different

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enzymatic activities in different tissues and developmental stages in mammals (Hanioka et al., 2008; Yabusaki et al., 2015). In contrast, the roles of UGT family members remain unclear in fish.

BPA has been regarded as an endocrine disruptor. Due to the structural similarity, BPA can mimic or antagonize the function of estradiol. This led to one of the major concerns that BPA can affect the embryonic development and reproduction (Peretz et al., 2014). At the embryonic stage of *O. melastigma*, our previous study showed that exposure to BPA disrupted the cardiac development (Huang et al., 2011). At the juvenile stage, exposure to BPA inhibited the growth of ovary and led to the decreased expression of luteinizing hormone/choriogonadotropin receptor (LHCGR) in the ovary of zebrafish (Chen et al., 2017b). Adverse effects were also observed across the generation as malformation and mortality increased in F1 offspring from parent fish exposed to BPA compared to control fish (Chen et al., 2017a). However, results have been inconsistent, to which the use of various exposure doses and periods with different models have contributed (Tamschick et al., 2016). For example, waterborne exposure to BPA for 15 days promoted the egg production of breeding zebrafish (Laing et al., 2016). In contrast, egg production and hatchability in *Pimephales promelas* was both inhibited upon BPA exposure (Sohoni et al., 2001). The effects can differ even within the same species. No significant impacts of BPA at similar levels on egg production and hatchability were observed in another study also using the same fish species – *P. promelas* (Mihaich et al., 2012). Waterborne BPA exposure also affected the hatching of fish embryo (Mu et al., 2018). Hatching success relies on the normal expression of hatching enzymes, including HCE (high choriolytic enzyme, choriolysin H) and LCE (low choriolytic enzyme, choriolysin L). These two enzymes act synergistically to catalyze the degradation of chorion, thus facilitate the hatching of embryos (Yasumasu et al., 2010). Our previous study has confirmed the abnormal mRNA expression and enzymatic activity of both HCE and LCE after embryonic exposure to another organic pollutants – perfluorooctane sulphonate (PFOS) (Wu et al., 2012). In contrast, the impacts of BPA on hatching enzymes are still unclear.

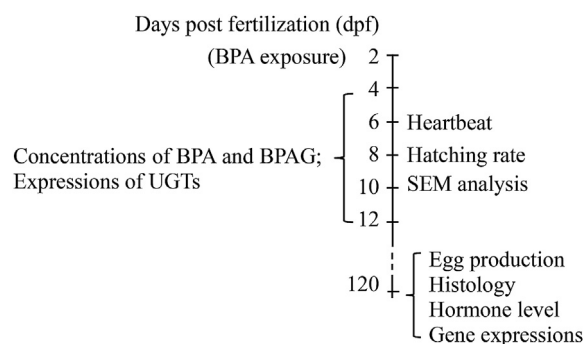
In all, to get a more realistic understanding on the ecotoxicology of BPA in the environment, long-term exposure and multiple ways of toxicity assessment are needed. In this study, *O. melastigma* was exposed to BPA from embryonic stage to adult. The metabolism of BPA and its responsible genes in fish embryos were systematically studied. Integrated methods from molecular to individual levels were recruited together to study the effects of BPA on the development and reproduction of *O. melastigma*.

## 2. Materials and methods

### 2.1. Fish culture and treatment

*O. melastigma* was maintained in artificial seawater with a salinity of 30‰. The temperature was at  $28 \pm 1$  °C and the photoperiod was at a constant 14 h light/10 h dark. BPA (97% purity, Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) before use. Fertilized eggs at 2 dpf (days post fertilization) were collected and randomly distributed to control (0.1% DMSO) and BPA-treated groups. Nominal concentrations of BPA included 0, 50, 200, 800, and 1600 µg/L. Each group contained three replicates. Embryos were cultured in 90 mm petri dishes containing 20 mL of artificial seawater (30‰). The exposure solution was renewed every two days without changing respective nominal concentration of BPA. At 6 dpf, four embryos from each dish were randomly collected for heartbeat measurement. Heart rate was counted under microscope (XTL-340, Changfang Optical Instrument Co. Ltd. Shanghai, China PR) equipped with a CCD camera (CF-2098, Changfang Optical Instrument Co. Ltd. Shanghai, China PR). The

heartbeats were recorded as beating times in a minute. At 8 dpf, the hatching rate was calculated. At 10 dpf, embryos are randomly selected and fixed for scanning electron microscopy (SEM) detection. The hatching larvae were transferred to a new tank containing the exposure solution with the same nominal concentrations of BPA to that at the embryonic stage. The exposure solution was also renewed every two days without changing exposure concentrations. After a period of 120 days, the weight of female fish body and gonads were both measured. Another ten pairs of female/male fish were randomly selected and transferred to a new tank without changing the BPA level. The number of produced eggs from these ten pairs was calculated every day for one week. Egg production is presented as the average number per day. Then, female fish were sacrificed and the ovary was dissected for examination. All the samples were stored in  $-80$  °C before use unless otherwise specified. All fish-use protocols were carried out ethically in accordance with the Animal Welfare Act and approved by Animal Ethics Committee of Institute of Urban Environment, Chinese Academy of Sciences (IUELAC2016.09.02). The exposure procedure was summarized as follows,



### 2.2. Measurement of BPA and BPAG

Embryos were exposed to BPA at the concentration of 200 µg/L since 2 dpf. The concentrations of BPA and its metabolite - BPAG were both measured every two days from 4 to 12 dpf. HPLC (Shimadzu Prominence LC-20A, Kyoto, Japan) and MS/MS (Applied Biosystems 3200, Foster City, CA) were used for chemical analysis. To eliminate potential BPA contamination, all the containers were carefully cleaned and then calcined at 500 °C for 4 h before use. Ten embryos were randomly gathered from each group and washed three times with clean seawater. Then, the embryos were homogenized in ethyl acetate containing  $^{13}\text{C}$ -BPA (Cambridge isotope laboratories Ins, MA) as an internal standard. The organic extract was obtained by centrifugation at  $3000g \times 15$  min and then evaporated to dryness under nitrogen gas. To improve the detection sensitivity, samples were derivatized by incubating the sample with acetone, dansyl chloride and  $\text{Na}_2\text{CO}_3$  buffer (0.1 mol/L, pH = 10.5) at 50 °C for 30 min in an ultrasonic bath. The organic extract was obtained as described above and then concentrated under nitrogen gas. Samples were re-dissolved in methanol for HPLC-MS/MS analysis. To measure the levels of BPAG, samples were treated as that for BPA analysis but without derivatization. After treatment, samples were separated on a Kinetex 2.6 µm C18 100A column (100 mm × 4.6 mm, 2.5 µm particle size, Phenomenex, Torrance, CA). Multiple reaction monitoring was applied for the transitions of BPA (695.0–171.2),  $^{13}\text{C}$ -BPA (707.0–171.0), and BPAG (403.0–113.0). The calibration curves were linear over a concentration range from 0 to 100 ng/mL, with a correlation coefficient ( $r^2$ ) that was greater than 0.99. The range of detection limits were 0.04–0.31 ng/mL BPA and 0.15–0.96 ng/mL BPAG. The recovery parameter was listed in

**Table 1.** The analytical variability (<10%) did not significantly contribute to the detected levels of BPA or BPAG.

### 2.3. Scanning electron microscopy (SEM) of embryos

At 10 dpf, embryos were randomly collected and fixed in a 2.5% glutaraldehyde/PBS solution (pH = 7.4) for 24 h. Then, they were rinsed twice with PBS and then fixed in osmium for 2 h. Embryos were dehydrated using a gradient of ethanol solutions. The ethanol was replaced by isoamyl acetate through centrifugation. The samples were successively frozen at  $-20$ ,  $-40$ ,  $-80$  °C for 12 h before dryness. After coating, the embryos were observed using SEM. Energy dispersive spectrometer (EDS) analysis was also performed on these samples.

### 2.4. Real-time RT-PCR

The relative expression levels of UGTs family in embryos were measured every two days from 2 to 12 dpf. The levels of hatching enzymes were detected in embryos at 8 dpf. The mRNA levels of aromatase CYP19 and Choriogenin H (ChgH) were examined in fish after 120 days' exposure. Total RNA was isolated from embryos or ovaries. Then, the quality and quantity of RNA was determined using gel electrophoresis and nanophotometer (Implen, GmbH, Germany), respectively. First-strand cDNA was synthesized from RNA using a PrimeScript RT-PCR Kit (TaKaRa, Dalian, China). Then, SYBR real-time RT-PCR was performed using a Roche 480 platform with a SYBR Premix Ex Taq Kit (TaKaRa). Primer sequences were available from our previous study (Fang et al., 2012) and [Supplementary Table S1](#). The PCR conditions were as follows: 30 s of pre-incubation at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s, and then applied to a final melting curve analysis. Sole product was confirmed by the peak of melting curve. Three replicates of RT-PCR were performed for each sample. The relative fold change of the tested genes was analyzed using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001). Ribosomal protein L7 (RPL7) was selected as the reference gene.

### 2.5. Determination of the enzymatic activity of UGT

Embryos were homogenized and then applied to the detection. The protein concentration of the homogenate was assayed by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The enzymatic activity of UGTs was detected by a commercial UGT Activity Assay/Ligand Screening Kit according to the instruction (Catalog number K692-100, Biovision, Milpitas, CA). Briefly, the reaction solution was composed of sample, substrate, diclofenac, assay buffer and test ligand. After adding UDPGA to start the reaction, the fluorescence was immediately monitored at Ex/Em = 415/502 nm in kinetic mode for 30–40 min at 37 °C. The fluorescence lost due to substrate glucuronidation was then calculated. One unit of UGT activity is the amount of enzyme that glucuronidates 1  $\mu$ mole of fluorescent substrate per min.

**Table 1**  
Recovery of BPA and BPAG determined by HPLC-MS/MS.

	Recovery (%)					
	low		median		high	
	average	RSD	average	RSD	average	RSD
BPA	106.6	8.2	120.6	7.6	110.5	8.5
BPAG	97.2	7.2	81.7	8.6	83.9	9.2

BPA: low (0.5 ng/mL), median (5 ng/mL), high (50 ng/mL).  
BPAG: low (10 ng/mL), median (50 ng/mL), high (100 ng/mL).

### 2.6. Construction of phylogenetic tree

Phylogenetic tree was constructed to analyze the homology of UGT family between *O. melastigma* and human beings. Protein sequences of *O. melastigma* UGT family genes were obtained from our established transcriptome database (Huang et al., 2012). Protein sequences of human UGT family genes were downloaded from Ensembl database (Aken et al., 2016). Sequence alignment was done by MUSCLE and phylogenetic tree was then constructed by MEGA4.0 software.

### 2.7. Histology of the sex organs

At 120 days post hatching, gonadosomatic index (GSI) of female fish from each group (0–1600  $\mu$ g/L) was calculated using the formula (ovary weight/total body weight \* 100%). The isolated gonads were fixed in Bouin's fluid and subsequently embedded in paraffin wax. Hematoxylin-eosin (H&E) staining was performed to examine the histological structure of the organs. The cell types of the ovary were determined under an Olympus CKX41 inverted microscope (Olympus, Tokyo, Japan). Three sections in each group were randomly selected to count the number of oocytes at various developmental stages.

### 2.8. Hormone determination by enzyme linked immunosorbent assay (ELISA)

Whole body of adult female fish were used to detect the hormone concentrations after 120 days' exposure according to a previous study (Bjerregaard et al., 2008). Samples were extracted with ethyl acetate and concentrated to dryness under nitrogen gas. Then, ELISA was adopted to measure the concentrations of both 17 $\beta$ -estradiol (E2) and testosterone (T) following the instructions of the ELISA kit (Cayman Chemical, Ann Arbor, MI).

### 2.9. Statistical methods

Data are shown as mean  $\pm$  SD. Significant differences within various groups were evaluated by one-way ANOVA using SPSS16.0, \* $p$  < 0.05, \*\* $p$  < 0.01, and \*\*\* $p$  < 0.001.

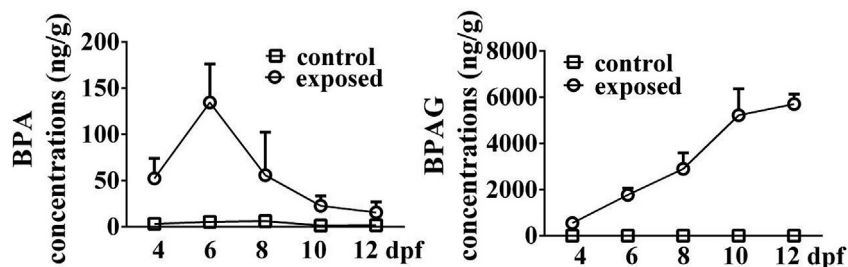
## 3. Results

### 3.1. Metabolism of BPA in the embryos of *O. melastigma*

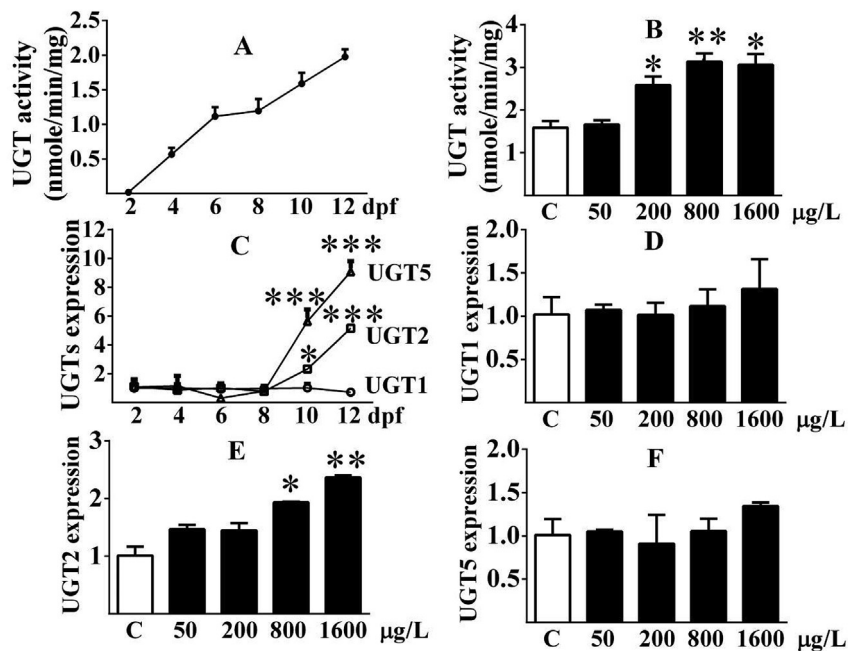
The BPA levels in embryos increased up from 4 to 6 dpf and then the level fell back after 6 dpf (Fig. 1). The BPA burden ranged from 15.6 to 134.5 ng/g (wet weight) with an average of 56.3 ng/g which is similar to that was detected in wildlife. The uptake rate was  $0.21 \times 10^{-3} \pm 0.00$  L/g/d at 4–6 dpf. The level of BPAG dramatically increased from 4 dpf (550.5 ng/g) to 12 dpf (5700.0 ng/g). The ratio of BPAG/BPA increased from 12.6 to 635.4.

### 3.2. Expressions of genes related to BPA metabolism

The UGT family plays crucial roles in the conjugation reaction of BPA into BPAG. The total enzymatic activity of UGTs increased with the development of embryos (Fig. 2A). BPA exposure significantly promoted the enzymatic activity of UGTs (Fig. 2B). The contribution of various subtypes was then studied in UGT family. The mRNA levels of UGT1 remained stable through the embryonic stage from 2 to 12 dpf (Fig. 2C). In contrast, the mRNA expression of UGT2 and UGT5 both dramatically increased following the embryonic development. The expression levels of these two subtypes were both significantly higher at 10 and 12 dpf than that at earlier stages. The



**Fig. 1.** Metabolism of BPA in *O. melastigma* embryos. Embryos were exposed to 200  $\mu\text{g/L}$  BPA at 2 dpf, and the burden of BPA and BPAG were both detected by HPLC-MS/MS every two days until 12 dpf.



**Fig. 2.** Expressions of UGT family genes in *O. melastigma* embryos. (A) Enzymatic activity of UGTs at various embryonic stages. (B) Effects of BPA exposure on the enzymatic activity of UGTs at 10 dpf, one way ANOVA,  $^*p < 0.05$  and  $^{**}p < 0.01$ , vs. the control. (C) Gene expression pattern of UGT1, 2 and 5 through the developmental stages,  $^*p < 0.05$  and  $^{***}p < 0.001$  vs. the expression levels at 2 dpf; circles indicate UGT1, squares indicate UGT2, triangles indicate UGT5. (D) Effects of BPA exposure on the expression of UGT1 at 10 dpf. (E) Effects of BPA exposure on the expression of UGT2 at 10 dpf,  $^*p < 0.05$  and  $^{**}p < 0.01$ , vs. the control. (F) Effects of BPA exposure on the mRNA expression of UGT5 at 10 dpf.

mRNA expressions of these three UGT isoforms were also detected upon exposure to serial concentrations of BPA (0, 50, 200, 800, and 1600  $\mu\text{g/L}$ ) at 10 dpf. The mRNA expression of UGT2 was concentration-dependently up-regulated after exposure to BPA (Fig. 2E). In contrast, neither the expressions of UGT1 nor UGT5 was significantly changed upon exposure (Fig. 2D and F). Phylogenetic analysis showed that UGT2 was the orthologue of UGT2B15 which possessed the highest enzymatic activity in human (Supplementary Fig. 1).

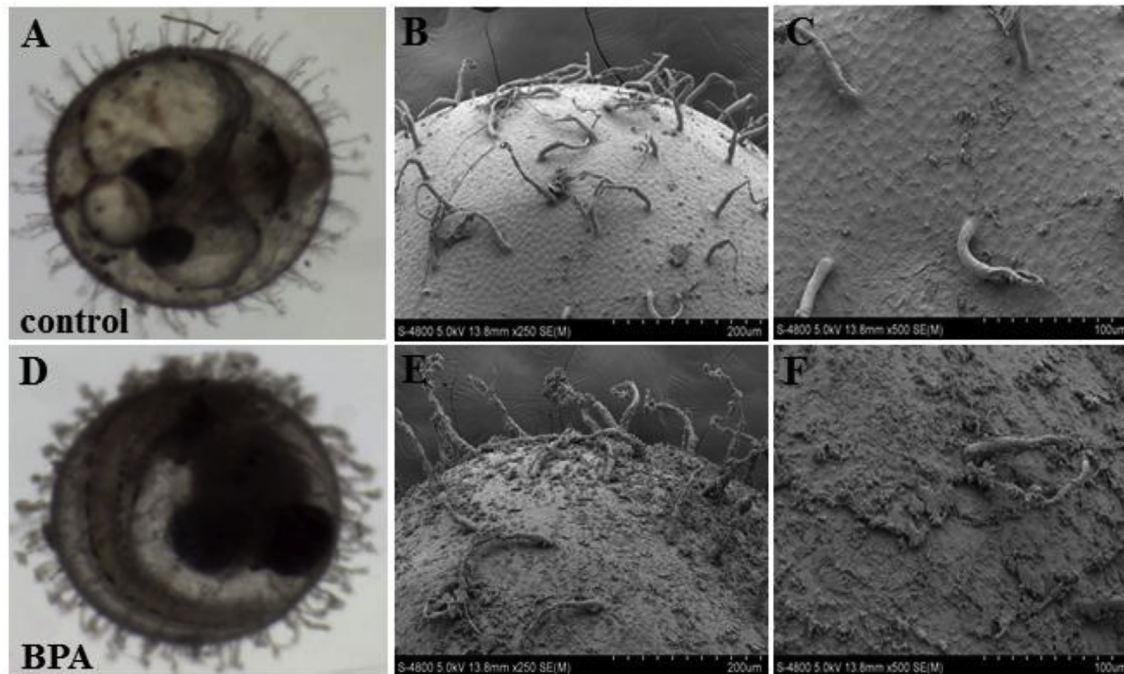
### 3.3. Toxicity of BPA towards the morphology and development of *O. melastigma* embryos

The morphology of the embryos was examined under light microscopy and SEM after exposure to 200  $\mu\text{g/L}$  BPA at 10 dpf (Fig. 3). The morphology of villi on the embryo envelope was different between control and BPA-exposed group. The villi was attached by materials and the chorion surface became rough after exposure to BPA. The components of the materials attached to chorion were further compared between control and BPA exposed group by EDS analysis. The major elements were both carbon and

oxygen in both groups (Supplementary Fig. 2). The heartbeat of the embryos significantly increased upon exposure to serial concentrations of BPA (Supplementary Fig. 3). In contrast, neither hatching rate nor hatching period was significantly affected after exposure (Supplementary Fig. 4). Neither of the mRNA expressions of hatching enzymes (high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE) was significantly altered after exposure (Supplementary Fig. 5).

### 3.4. Reproductive behavior of adult fish experiencing embryonic exposure to BPA

After a period of 120 days' exposure, their reproductive ability was assessed. Histological analysis showed that the ovary development was inhibited after BPA exposure (Fig. 4A). The major cell types in control ovary were mature spawning follicles (M) and early vitellogenic oocytes (E). The existence of post-ovulatory follicles (F) indicated recent spawning. In the groups treated with lower BPA concentrations (50 and 200  $\mu\text{g/L}$ ), the number of mature spawning follicles (M) decreased, and the major cell type in ovary switched to early vitellogenic oocytes (E), cortical alveolar oocytes (C) and



**Fig. 3.** Morphology of *O. melastigma* embryos upon exposure to BPA from 2 to 10 dpf. (A, D) Light microscopy photograph of embryos without or with BPA exposure. (B, C, E, F) SEM photograph of embryos without or with BPA exposure.

perinucleolar phase oocytes (P). In the groups treated with higher BPA concentrations (800 and 1600  $\mu\text{g/L}$ ), few mature spawning follicles (M) or early vitellogenic oocytes (E) were observed, whereas follicular atresia (FA) appeared (Fig. 4B). The larvae hatching from embryos with BPA exposure were continuously treated with the same concentrations of BPA as that at the embryonic stage. Female fish gonadosomatic index (GSI) significantly decreased upon BPA exposure (Fig. 5A). The E2/T ratio was significantly lower in BPA-treated groups than that in the control fish (Fig. 5B). The mRNA expression of aromatase CYP19A, which catalyzes the transition of testosterone to estradiol, was significantly inhibited (Fig. 5C). The mRNA level of E2 responsible gene - ChgH correspondingly decreased upon exposure (Fig. 5D). The number of produced eggs was significantly reduced after exposure (Fig. 5E). No eggs were produced by fish exposed to the highest concentration (1600  $\mu\text{g/L}$ ).

#### 4. Discussion

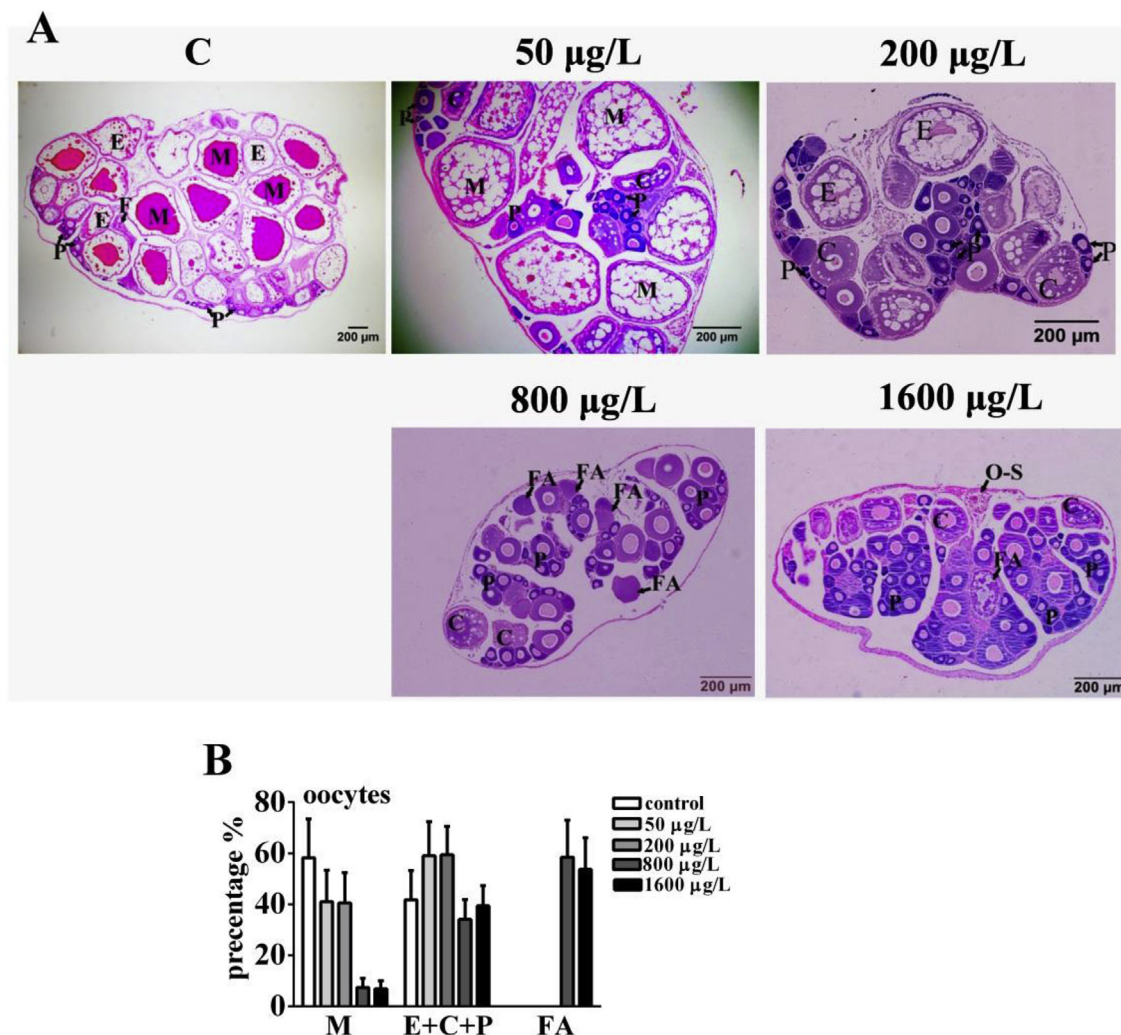
Exposure concentrations and period can significantly affect the outcome upon exposure to contaminants. Long-term exposure is able to mimic the real exposure existing in the environment. In our study, *O. melastigma* was exposed to BPA throughout the whole life period from embryonic stage to adults for four months. Combined phenotypes including GSI, histology of ovary, egg production, and estrogen levels were detected to assess the reproductive function of fish after exposure to BPA. Results showed that BPA can disrupt the reproductive function in female fish. This could help to understand the reproductive toxicity of BPA.

Serial concentrations of BPA was adopted in the study. The concentration of 200  $\mu\text{g/L}$  BPA has been widely regarded as a low dose for aquatic organisms (Inagaki et al., 2016; Ramakrishnan and Wayne, 2008). In the experiment about the metabolism of BPA, after exposing the embryos of *O. melastigma* to 200  $\mu\text{g/L}$  BPA in water, the accumulated BPA levels in embryos were within the

range of concentrations which are detected in wild fish. For example, wild fish from marine and estuarine locations in the Netherlands contained 1–75 ng/g BPA (Belfroid et al., 2002), and the concentrations in marine fish from Hongkong market ranged between 0.83 and 19.25 ng/g w.w (Wong et al., 2017). And the level range was 0.2–13 000 ng/g in wildlife as summarized in a review (Corrales et al., 2015).

Understanding the metabolism of BPA is key to assess its risk potential to fish embryos. In our study, BPA burden increased after exposure until 6 dpf, then the burden decreased. Similar trend was observed in trout as the peak concentration occurred in the middle of the developmental stage (Birceanu et al., 2015; Bjerregaard et al., 2008). BPAG was detectable in the initial developmental stage, and the level continuously increased following embryonic development. BPAG was the major metabolite and its level reached 12.6  $\mu\text{g/g}$  in adult zebrafish after exposure to 100  $\mu\text{g/L}$  BPA for 24 h (Lindholm et al., 2003). In a larger fish, brown trout, BPAG was also detectable in the early embryonic stage, and its level increased linearly from 1 to 22  $\mu\text{g/g}$  through the embryonic exposure period (Bjerregaard et al., 2008).

UGTs are key to the transformation from BPA to BPAG. The conjugation rate increased as the mRNA levels and enzymatic activity of UGTs dramatically increased in the later embryonic developmental stage. In addition, BPA exposure could also enhance the conjugation of BPAG. An *in vitro* study with a zebrafish liver cell line also demonstrated the impact of BPA on phase II metabolism enzymes, including UGT family (Yang and Chan, 2015). In our study, the subtype of UGTs was further determined as BPA exposure stimulated the up-regulation of UGT2 expression rather than UGT1 or UGT5. Furthermore, *O. melastigma* UGT2 is the orthologue of UGT2 in human (named as UGT2B15), which showed the highest enzymatic activity among the UGT family (Hanioka et al., 2008). These results suggested that BPA was likely to be mainly glucuronidated by UGT2 rather than UGT1 or UGT5 in marine fish.



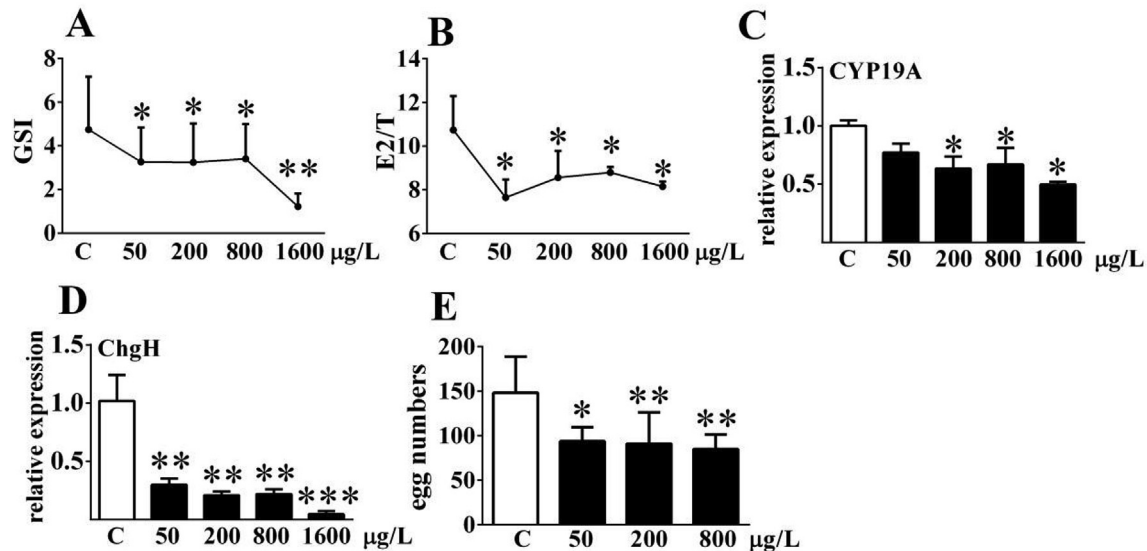
**Fig. 4.** Impacts of BPA exposure on the ovary histology of adult female *O. melastigma*. **(A)** H&E histology of ovaries after BPA exposure. The letters in the figures represent the following: C, cortical alveolar oocytes; E, early vitellogenic oocytes; F, post-ovulatory follicles; M, mature spawning follicles; P, perinucleolar phase oocytes; FA, follicular atresia. **(B)** Percentage of oocytes at various developmental stages. M, mature spawning follicles; E+C+P, early vitellogenic oocytes + cortical alveolar oocytes + perinucleolar phase oocytes; FA, follicular atresia. The data are expressed as mean ± SD (n = 3).

BPA is an uncharged, relatively small, and moderately hydrophobic molecule which favors passive diffusion. Water BPA level was recovered every two days in our exposure system. It seems that BPA could be re-absorbed into embryos when the inner BPA level decreased due to the transformation into BPAG. However, the BPA level in embryos decreased after 6 dpf. This may partly be explained by the changing of chorion surface induced by BPA. The chorion surface became rough and thickened after exposure. Since the chorion acted as the first major barrier to keep the embryos from environmental stimulation (de Koning et al., 2015), its morphological change may block the passive influx of BPA from water at the later developmental stage. In contrast, BPAG is a much larger hydrophilic anion at physiological pH levels and requires mediated transport to cross cellular membranes (Mazur et al., 2012). The efflux of BPAG outside the embryo was unlikely to occur. In mammals, BPAG concentration in fetus plasma is higher compared to that in the mother due to the inability of BPAG to cross the placental barrier (Gauderat et al., 2016). Continuous increasing of BPAG burden in embryos was likely to due to the inability of BPAG to cross the chorion. The morphological change of embryo chorion is also likely to affect the uptake of other water pollutants. This will

provide a new way to address synergistical effect between various water pollutants.

Previous studies reported the reproductive toxicity of BPA from individual level, gene level, tissue level, or combination of several levels. For example, individual level (GSI and egg reproduction) is highlighted in a study with a freshwater fish - Fathead Minnow (Sohoni et al., 2001). Another study with the same fish contained three related parameters (GSI, egg reproduction and ovary histology) (Mihaich et al., 2012). Embryo hatching and GSI was included in the study with *O. latipes* (Ramakrishnan and Wayne, 2008). In our study, toxic effects from molecular level to individual level were all assayed. Consistently inhibitory effects of BPA were observed, as indicated by slowed oocyte development, decreased egg production, lower levels of estrogen and decreased expression of estrogen-responsive gene. This complete evidence chain could increase the reliability of the toxic data and give a more comprehensive understanding of BPA toxicity.

In summary, our study demonstrated the continuous conversion of BPA to BPAG catalyzed by UGT2 in the embryos of marine fish upon BPA exposure. BPA exposure disrupted the morphology of embryo chorion, but did not affect the hatchability of *O. melastigma*.



**Fig. 5.** Impacts of BPA exposure on the reproduction of adult female *O. melastigma*. (A) GSI of female fish after exposure for 120 days. (B) Estradiol: testosterone ratio (E2/T) after BPA exposure in female fish. (C) Relative expression levels of CYP19A after BPA exposure. (D) Relative expression level of E2 marker gene ChgH. (E) Average egg production per day from ten pairs of parent fish after BPA exposure; \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  vs. control.

However, long-term exposure from embryos to adults impaired the reproductive ability as confirmed by a series of data from molecular to individual levels. These will help to understand the ecotoxicology of BPA.

## Main findings

The conjugation of BPA into BPAG by UGT2 occurred at the fish embryonic stage. BPA can disrupt fish chorion and reproduction upon whole-life low-concentration exposure.

## Conflicts of interest

All the authors have no actual or potential competing financial interests.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.envpol.2018.07.048>.

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