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# Effects of UV filters 2-ethylhexyl 4-methoxycinnamate and benzophenone-3 on *in vitro* steroidogenesis during oocyte maturation of the longchin goby, *Chasmichthys dolichognathus*

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We investigated the estrogenic effects of 2-ethylhexyl 4-methoxycinnamate (EHMC) and benzophenone-3 (BP-3) on steroidogenesis in maturing oocytes of the longchin goby. Oocytes with 0.77-, 0.82-, and 0.87-mm diameters were incubated with EHMC (0.5–500 ng/mL) or BP-3 (0.5–500 ng/mL) for 24 h with [<sup>3</sup>H]17 $\alpha$ -hydroxyprogesterone as a precursor. The major steroid metabolites were separated and identified as androstenedione, testosterone (T), estradiol-17 $\beta$  (E<sub>2</sub>), estrone, and 17,20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ 20 $\beta$ P). The E<sub>2</sub> metabolite was significantly increased at 5 and 0.5 ng/mL of EHMC in 0.82- and 0.87-mm-diameter oocytes, respectively. Furthermore, it was increased significantly at 5 ng/mL of BP-3 in the oocytes of 0.87 mm. In addition, 5, 50, and 500 ng/mL of EHMC increased the ratio of E<sub>2</sub>/17 $\alpha$ 20 $\beta$ P in the oocytes of 0.82 mm. 0.5 ng/mL of BP-3 increased the ratio of E<sub>2</sub>/T in the oocytes of 0.87 mm. These results suggest that EHMC and BP-3 have a potential estrogenic activity in the steroidogenic shift process, vitellogenic stage to final maturation stage.

## KEYWORDS

benzophenone-3, estrogenic effects, longchin goby, maturing oocytes, steroidogenesis, 2-ethylhexyl 4-methoxycinnamate

## 1 Introduction

UV filters are a group of compounds invented to protect the skin from UV radiation and are used in sunscreens, a wide range of cosmetics and personal care products (Lebarone, 2022; Zhang et al., 2024). Additionally, it is used in plastics, cartons, and transparent packing materials to minimize UV-induced damage to products (Manová et al., 2013; Ramos et al., 2015). UV filters can be released into the aquatic environment via two pathways: either indirectly via wastewater treatment plant effluent or directly from swimming and other recreational activities, as well as

industrial wastewater discharge (Pal et al., 2014; Ramos et al., 2015). The most common UV filter ingredients are 2-ethylhexyl-4-methoxycinnamate (EHMC) and 2-hydroxy-4-methoxybenzophenone (BP-3). According to previous research indicating their potential for bioaccumulation, EHMC and BP-3 have been observed in aquatic biota. BP-3 concentrations of up to 6.52 ng/g have been found in the muscles of lionfish (*Pterois volitans*) from Grenada, West Indies (Horricks et al., 2019). EHMC and BP-3 levels as high as 241.7 ng/g body weight (BW) and 24.3 ng/g BW, respectively, were detected in Andalusian barbel fish (*Luciobarbus sclateri*) collected from the Guadalquivir River, southern Spain (Gago-Ferrero et al., 2013).

In teleosts, oocyte maturation is regulated by sex steroid hormones synthesized from follicle cells, which are controlled by the hypothalamus–pituitary–gonad axis (Nagahama and Yamashita, 2008). In female fish, estradiol-17 $\beta$  (E<sub>2</sub>), as a major sex steroid, is transported to the liver and produces vitellogenin (VTG) during vitellogenesis. Following vitellogenesis, progestogens, including 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ ,20 $\beta$ P) and/or 17 $\alpha$ ,20 $\beta$ ,21-trihydroxy-4-pregnen-3-one (17 $\alpha$ ,20 $\beta$ ,21P), induce final oocyte maturation and result in ovulation (Patiño et al., 2001). Multiple *in vitro* and *in vivo* studies in teleosts have reported adverse effects of EHMC and BP-3 on VTG levels. EHMC increased plasma VTG levels in male fathead minnow, whereas VTG gene expression profiles showed antiestrogenic activity in fathead minnow and zebrafish (Christen et al., 2011; Zhou et al., 2019a). BP-3 induces VTG expression in zebrafish, rainbow trout, male medaka, and male California halibut (Coronado et al., 2008; Schlenk et al., 2005; Rodríguez-Fuentes et al., 2015). Although the effects of EHMC and BP-3 on the reproductive endocrine system have been investigated, their effects on steroidogenesis, including progesterone production, during oocyte maturation have not been reported.

The longchin goby, *Chasmichthys dolichognathus*, is a small marine fish of the family Gobiidae that inhabits the coastal waters and tide pools of Korea and Japan (Kim et al., 1986). Gobiid fish are appropriate subjects for investigating the effects of various chemicals due to their small size, ease of handling through *in vitro* and *in vivo* study, and strong tolerance with environmental changes (Robinson et al., 2007). This species has been used in our previous studies to test reproductive endocrinology with different endocrine-disrupting chemicals (Baek et al., 2007; Hwang and Baek, 2011). Herein, we investigated the potential estrogenic effects of EHMC and BP-3 on *in vitro* steroid metabolism in maturing oocytes of the longchin goby. In particular, we focused on the effects of these chemicals during the critical period of the steroidogenic shift from estrogens to progestogens.

## 2 Materials and methods

### 2.1 Chemicals

EHMC (CAS number: 5466-77-3) and BP-3 (CAS number: 131-57-7) were purchased from Sigma–Aldrich. (St. Louis, MO, USA). They were dissolved in ethanol to obtain a concentrated stock

solution. Steroid hormones, 17 $\alpha$ -hydroxyprogesterone (17 $\alpha$ P), androstenedione (A<sub>4</sub>), testosterone (T), E<sub>2</sub>, estrone (E<sub>1</sub>), 17,20 $\alpha$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ ,20 $\alpha$ P), 17 $\alpha$ ,20 $\beta$ P, and 17 $\alpha$ ,20 $\beta$ ,21P were purchased from Sigma–Aldrich or Steraloids (Wilton, NH, USA). The radioactive steroid, [<sup>3</sup>H]17 $\alpha$ P, was obtained from Amersham Biosciences (London, England).

### 2.2 Experimental fish and histological analysis of ovarian follicles

We selected the oocytes of 0.75–0.90 mm in average diameter according to our previous studies (Baek et al., 2007; Kim et al., 2023); the developmental stage of these oocytes is from fully vitellogenic stage to final maturation stage. Female longchin gobies were collected from tide pools in Cheongsapo, Busan, Korea, between February and March 2020. For histological analysis, fish were anesthetized, and ovarian fragments from each oocyte diameter, 0.77, 0.82, and 0.87 mm, were fixed in 10% neutral formalin for 24 h and subsequently embedded in paraffin. The paraffin-embedded tissues were prepared in 5–6- $\mu$ m-thick sections. Sections were stained with Mayer's hematoxylin and eosin and observed under a light microscope (BX50; Olympus, Tokyo, Japan).

### 2.3 *In vitro* oocyte incubation

Ovaries were isolated from seven fish. Individual oocytes were manually dissected from ovaries. The oocytes were immediately transferred into an ice-cold balanced salt solution (132.96 mM NaCl, 3.09 mM KCl, 0.28 mM MgSO<sub>4</sub> 7H<sub>2</sub>O, 0.98 mM MgCl<sub>2</sub> 6H<sub>2</sub>O, 3.40 mM CaCl<sub>2</sub> 6H<sub>2</sub>O, and 3.65 mM HEPES) under sterile conditions. Subsequently, the oocytes were separated in accordance with the average oocyte diameter of 0.77, 0.82, and 0.87 mm and were selected for the *in vitro* incubation. There were 20 oocytes of each diameter that were incubated in 24-well plates (conducted in triplicates) containing 1 mL Leibovitz L15 medium (Gibco, Grand Island, NY, USA).

To evaluate the estrogenic potency of EHMC and BP-3 on the steroidogenic metabolism of oocytes, we quantified steroid metabolites after EHMC and BP-3 exposure in the presence of a precursor. We incubated two oocyte groups of 0.77 and 0.87 mm with 0.5, 5, 50, and 500 ng/mL EHMC and BP-3 in the presence of [<sup>3</sup>H]-17 $\alpha$ P (55 kBq) as a precursor in two separate experiments. Exposure concentrations were considered and selected with relevant concentrations from coastal waters, from sediments, and even in ecotoxicological research according to previous studies (Watkins and Sallach, 2021; Bordalo et al., 2025). Additionally, we incubated the oocytes of 0.82 mm with 5, 50, and 500 ng/mL EHMC in the presence of the precursor. Ethanol as a solvent for EHMC, BP-3, and [<sup>3</sup>H]-17 $\alpha$ P was evaporated by nitrogen gas and re-dissolved in incubation medium.

Culture plates were incubated for 24 h at 18°C with gentle shaking. The media's pH and osmolarity were adjusted to plasma

values of 7.7 and 300 mOsm, respectively. At the end of the incubation, the medium and oocytes were collected and stored at  $-76^{\circ}\text{C}$  until analyses.

## 2.4 Analysis of the steroid metabolites

Steroid metabolites were analyzed as previously described (Hwang and Baek, 2023). Briefly, the metabolites were extracted, concentrated, and subjected to thin-layer chromatography (TLC). Each separated metabolite was eluted and identified using reverse-phase high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) (QP5050A, Shimadzu, Japan). The details are demonstrated in Supplementary Materials. The metabolic rate (%) of each isolated metabolite was calculated based on the percentage of the total steroid-recovered radioactivity from the initial precursor radioactivity.

## 2.5 Data and statistical analyses

Data were checked using Kolmogorov–Smirnov and Levene's tests to verify whether the variance fulfilled the conditions of normality and homogeneity, respectively. Differences between groups were analyzed using the two-sample t-test or one-way analysis of variance (ANOVA), followed by Scheffe's test. The Kruskal–Wallis test was used if data were not normally distributed, followed by the Mann–Whitney U test with Bonferroni correction. Results are indicated as the means  $\pm$  standard error of the mean, and a value of  $p < 0.05$  was considered statistically significant using SPSS ver. 21.0 (IBM, Armonk, NY, USA). The EHMC and BP-3 effects on each steroid metabolite were analyzed and visualized by heatmap analysis using the pheatmap package ver. 1.10.12 in R project ver. 4.0.2.

## 3 Results

### 3.1 Histological observations of the oocytes

Numerous yolk granules (Yg) were spread over the ooplasm, and the nucleus (N) was located in the vicinity of the center of oocytes in oocytes of 0.77 mm in average diameter (Figure 1A). Yg continued to accumulate in the ooplasm, and N began to migrate in oocytes of 0.82 mm in average diameter (Figure 1B). In oocytes of 0.87 mm in average diameter, N migration was clearly observed compared with 0.77 and 0.82 mm oocytes (Figure 1C).

### 3.2 Identification of the major steroid metabolites

Steroid metabolites converted from [ $^3\text{H}$ ]17 $\alpha$ P using the maturing oocytes (0.77, 0.82, and 0.87 mm average diameter) were observed via

TLC (Figure 2). Estrogen metabolites ( $\text{E}_1$  and  $\text{E}_2$ ) (colorless spots developed by iodine vapor) were separated via HPLC and identified using GC/MS (Figure 3).

### 3.3 Effects of EHMC on steroid metabolites during the oocyte maturation process

In the oocytes of 0.77-mm diameter, EHMC did not substantially modulate any metabolites (Table 1). In the oocytes of 0.82- and 0.87-mm diameters, 5 and 0.5 ng/mL of EHMC increased the  $\text{E}_2$  metabolites compared with the controls, respectively ( $P < 0.05$ ). The levels of the other metabolites did not substantially increase or decrease. The heatmap analysis of each metabolite also supported these findings (Figure 4). Additionally, the alterations in the 17 $\alpha$ 20 $\beta$ P metabolite stood out from the other metabolites in the oocytes of 0.87 mm from the heatmap analysis, although the differences were insignificant.

In the  $\text{E}_2/\text{T}$  and  $\text{E}_2/17\alpha20\beta\text{P}$  ratios, as sensitive biomarkers of estrogenicity (Bevans et al., 1996; Folmar et al., 1996), 5, 50, and 500 ng/mL EHMC significantly increased the  $\text{E}_2/17\alpha20\beta\text{P}$  ratio compared with the controls in the oocytes of 0.82 mm diameter ( $P < 0.05$ , Figure 5). In the oocytes of 0.87 mm diameter, no significant effects were observed on either ratio, although these ratios peaked at the lowest dose.

### 3.4 Effects of BP-3 on steroid metabolites during the oocyte maturation process

In the oocytes of 0.77 mm diameter, BP-3 did not markedly modulate any metabolites (Table 2). In the oocytes of 0.87 mm diameter, 5 ng/mL BP-3 increased the  $\text{E}_2$  metabolites compared with the controls ( $P < 0.05$ ). The heatmap analysis supported these findings for each metabolite (Figure 6). The alterations in the 17 $\alpha$ 20 $\beta$ P metabolite were obvious in the oocytes of 0.87-mm diameter as observed in the heatmap analysis, although the difference was insignificant.

In the oocytes of 0.77-mm diameter, no obvious effect of BP-3 on the  $\text{E}_2/\text{T}$  or  $\text{E}_2/17\alpha20\beta\text{P}$  ratios was observed (Figure 7). However, 0.5 ng/mL BP-3 significantly increased the  $\text{E}_2/\text{T}$  ratio compared with the controls in the oocytes of 0.87-mm diameter ( $P < 0.05$ ). Additionally, the lowest BP-3 dose increased the  $\text{E}_2/17\alpha20\beta\text{P}$  ratio, although that peak was not considerably different.

## 4 Discussion

The endocrine-disrupting profiles of UV filters, including EHMC and BP-3, have been reported in mammals, amphibians, and freshwater fish (Wang et al., 2016). Although EHMC and BP-3 are widespread in aquatic biota, information on their effects on sex steroid production, especially in marine fish species, is scarce. Herein, we aimed to show the potential estrogenic effects of EHMC and BP-3 on the *in vitro* oocyte maturation process in

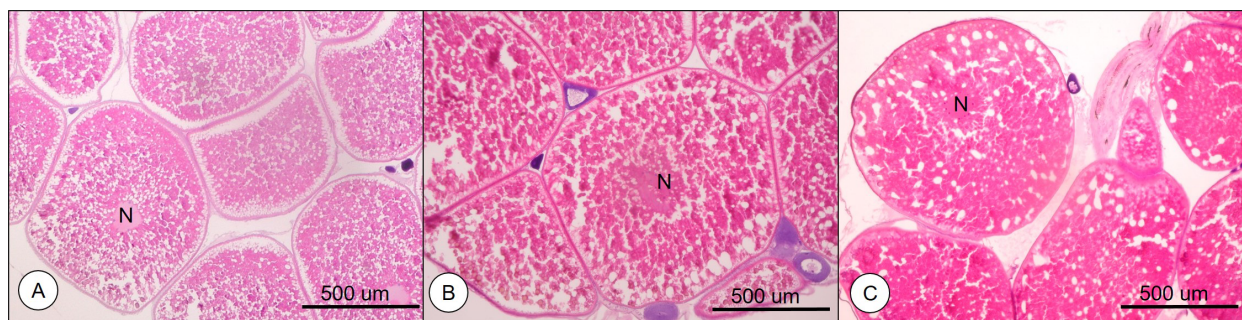


FIGURE 1

Histological observation of the oocytes from longchin goby. (A) oocytes of 0.77 mm in diameter; (B) oocytes of 0.82 mm in diameter; (C) oocytes of 0.87 mm in diameter. N, nucleus.

marine fish species during steroidogenic shifts from estrogens to progestogens.

In steroid metabolites,  $E_2$  metabolic rates decreased and  $17\alpha20\beta P$  metabolic rates increased with an increase in the oocyte diameter. In teleosts,  $E_2$  is produced in the oocyte follicle cell layer and transported to the liver during the vitellogenic phase. The liver subsequently produces vitellogenin, the yolk protein precursor. After vitellogenesis,  $E_2$  decreases, and progestogens such as  $17\alpha20\beta P$  increase, inducing final maturation (Patiño et al., 2001).

This is in good agreement with the histological finding of the present study that oocytes with average diameters of 0.77 mm during the fully vitellogenic stage, and 0.82 mm and 0.87 mm during the germinal vesicle migration stage, were transitioning from vitellogenesis to maturation.

Our results indicate that EHMC and BP-3 demonstrate estrogenic potency in steroidogenesis during oocyte maturation. In particular, both chemicals exhibited a weak estrogenic effect on  $E_2$  metabolites in fully vitellogenic oocytes (0.77 mm in oocyte

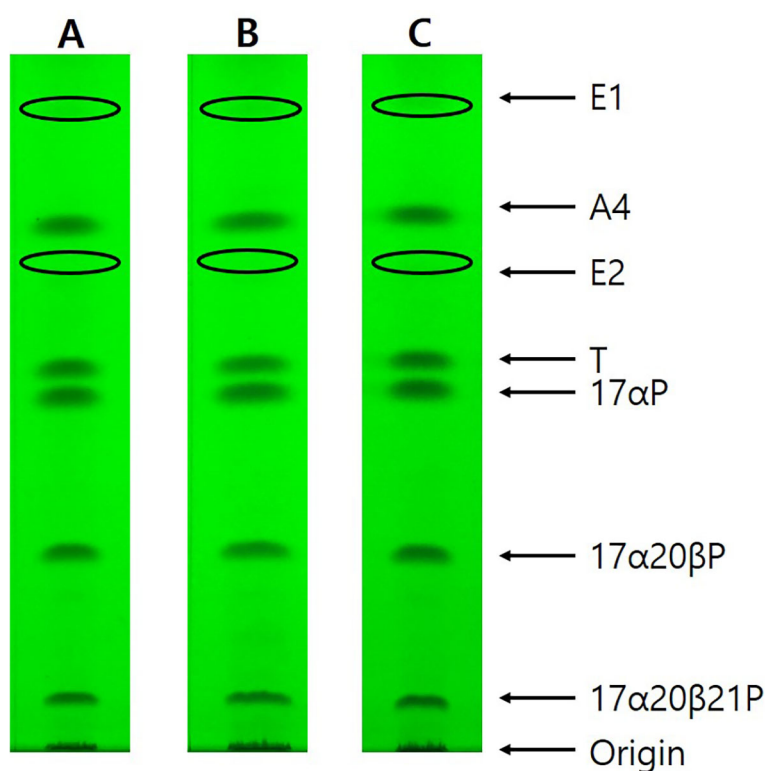


FIGURE 2

Thin layer chromatography under UV 254 nm of steroid metabolites incubated with  $[^3H]17\alpha P$  from longchin goby oocytes at two different diameters. Seven metabolites were separated by TLC developed with a benzene: acetone = 4: 1 (v:v) and benzene: ethyl acetate = 4: 1 (v:v) solvent system.  $E_1$  and  $E_2$  bands were observed after exposure to iodine vapors. (A) oocytes of 0.77-mm average diameter; (B) oocytes of 0.82 mm average diameter; (C) oocytes of 0.87 mm average diameter.



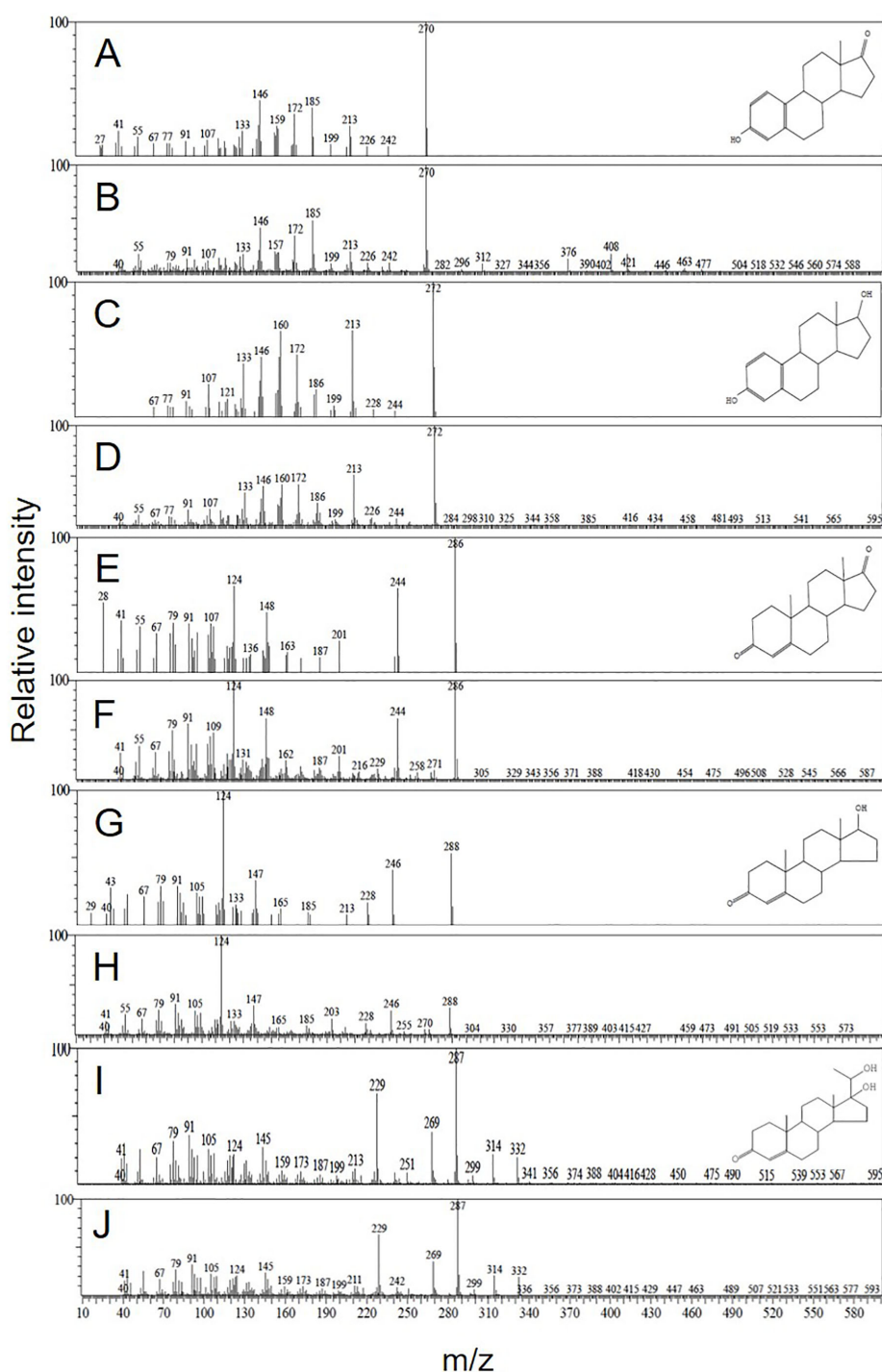


FIGURE 3

Mass spectra of steroid metabolites identified in longchin goby oocytes. (A) authentic  $E_1$ ; (B) metabolized  $E_1$ ; (C) authentic  $E_2$ ; (D) metabolized  $E_2$ ; (E) authentic A4; (F) metabolized A4; (G) authentic T; (H) metabolized T; (I) authentic  $17\alpha 20\beta P$ ; (J) metabolized  $17\alpha 20\beta P$ .

diameter), although the  $E_2$  metabolite values increased. In the oocytes of 0.82- and 0.87-mm diameters, EHMC or BP-3 substantially increased  $E_2$  metabolite and weakly decreased  $17\alpha 20\beta P$  metabolite, although the difference was insignificant. These findings suggest that EHMC and BP-3 have potential estrogenic effects on oocyte maturation following vitellogenesis. In

a previous study, EHMC showed estrogenic effects that increased vitellogenin levels and gene expression, although its antiestrogenic effects have also been reported (Inui et al., 2003; Christen et al., 2011; Zucchi et al., 2011; Lee et al., 2019; Zhou et al., 2019a, b). Additionally, EHMC demonstrated androgenic effects with estrogenic effect inhibition including a decrease in the expression

TABLE 1 Effects of EHMC on steroid metabolism rate from [ $^3$ H]17 $\alpha$ P of longchin goby oocytes (average diameter= 0.77, 0.82, and 0.87 mm) after 24 h of incubation.

Oocyte diameter (mm)	EHMC (ng/mL)	Metabolic rate (%)				
		A4	T	E <sub>1</sub>	E <sub>2</sub>	17 $\alpha$ 20 $\beta$ P
0.77	0	1.07 $\pm$ 0.17	1.57 $\pm$ 0.08	10.06 $\pm$ 2.78	14.99 $\pm$ 2.20	2.48 $\pm$ 0.44
	0.5	1.16 $\pm$ 0.07	1.77 $\pm$ 0.01	8.04 $\pm$ 1.75	18.17 $\pm$ 3.46	2.83 $\pm$ 0.32
	5	1.04 $\pm$ 0.13	1.56 $\pm$ 0.78	9.37 $\pm$ 1.96	16.73 $\pm$ 3.73	2.65 $\pm$ 0.60
	50	1.19 $\pm$ 0.28	1.65 $\pm$ 0.51	8.81 $\pm$ 2.58	17.43 $\pm$ 3.33	2.87 $\pm$ 0.74
	500	1.21 $\pm$ 0.08	1.68 $\pm$ 0.04	8.63 $\pm$ 1.24	17.75 $\pm$ 6.63	2.77 $\pm$ 0.78
0.82	0	1.34 $\pm$ 0.39	2.57 $\pm$ 0.19	4.81 $\pm$ 0.57	10.55 $\pm$ 0.66	3.23 $\pm$ 0.34
	5	1.96 $\pm$ 0.09	2.89 $\pm$ 0.33	7.44 $\pm$ 0.41	<b>13.99 <math>\pm</math> 0.79*</b>	3.11 $\pm$ 0.23
	50	2.07 $\pm$ 0.71	3.16 $\pm$ 0.21	6.93 $\pm$ 0.45	11.26 $\pm$ 0.53	2.75 $\pm$ 0.22
	500	1.84 $\pm$ 0.62	2.75 $\pm$ 0.23	6.99 $\pm$ 0.50	12.52 $\pm$ 0.37	2.93 $\pm$ 0.20
0.87	0	1.72 $\pm$ 0.01	1.85 $\pm$ 0.13	7.21 $\pm$ 0.78	10.93 $\pm$ 0.91	3.85 $\pm$ 0.51
	0.5	1.22 $\pm$ 0.06	1.94 $\pm$ 0.28	9.43 $\pm$ 0.89	<b>14.77 <math>\pm</math> 0.54*</b>	3.13 $\pm$ 0.70
	5	1.57 $\pm$ 0.14	2.16 $\pm$ 0.22	7.94 $\pm$ 2.16	12.97 $\pm$ 0.41	3.18 $\pm$ 0.35
	50	1.12 $\pm$ 0.01	1.95 $\pm$ 0.14	7.60 $\pm$ 1.03	10.34 $\pm$ 0.15	2.84 $\pm$ 0.06
	500	1.34 $\pm$ 0.99	1.78 $\pm$ 0.03	8.54 $\pm$ 1.54	11.40 $\pm$ 0.25	2.94 $\pm$ 0.44

The percentage of radioactivity associated with each isolated steroid was calculated to the percentage of total steroid recovered from initial TLC. Values are mean  $\pm$  SEM of the rate of each steroid in two or three replicate wells with 20 oocytes/well. Asterisks indicate significant difference compared with controls ( $p < 0.05$ ).

The bold values were for emphasizing the statistically significant difference with asterisk.

of E<sub>2</sub>, VTG, estrogen receptor (ER), progesterone receptor, aromatase, and 17 $\beta$ -HSD (Zhou et al., 2019b).

In a previous study, BP-3 demonstrated estrogenic effects with ER agonism and the VTG gene upregulation, although adverse or unclear effects have also been reported (Coronado et al., 2008;

Molina-Molina et al., 2008; Kerdivel et al., 2013; Rodríguez-Fuentes et al., 2015; Mustieles et al., 2023). Moreover, Kunz and Fent (2006) reported the estrogenic and anti-estrogenic effects of EHMC and BP-3 as multiple steroidal responses. Additionally, they reported on whether multiple hormonal activities are the general characteristics

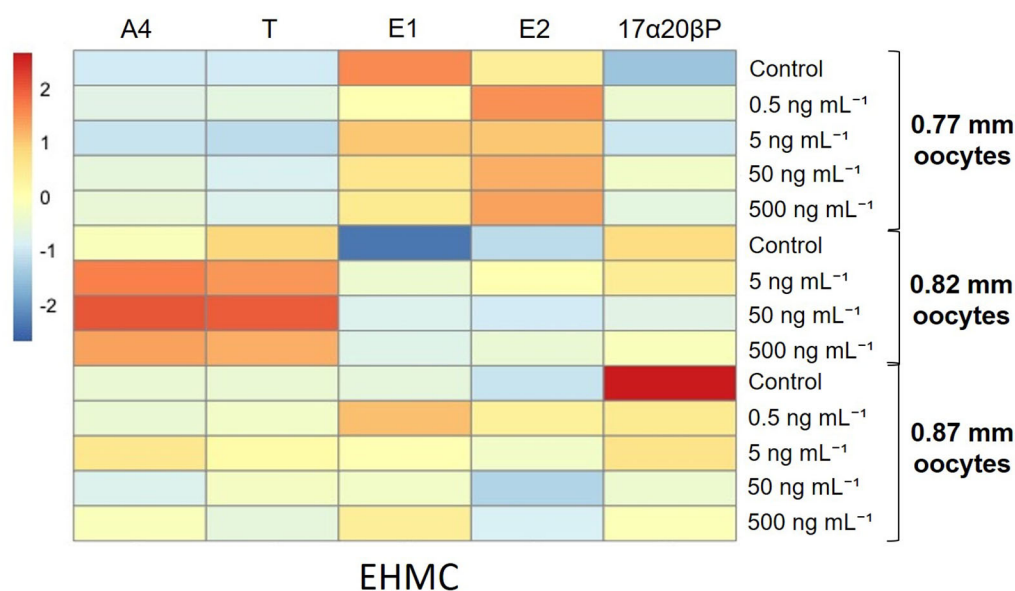


FIGURE 4

Heatmap analysis representing different levels of steroid metabolic rates of longchin goby oocytes exposed to EHMC. The lower relative rates are represented by blue color, and the higher relative rates are represented by red color. EHMC dosages were represented in the columns. Each steroid metabolite was represented in the row and scaled in the row direction (Z-scores).

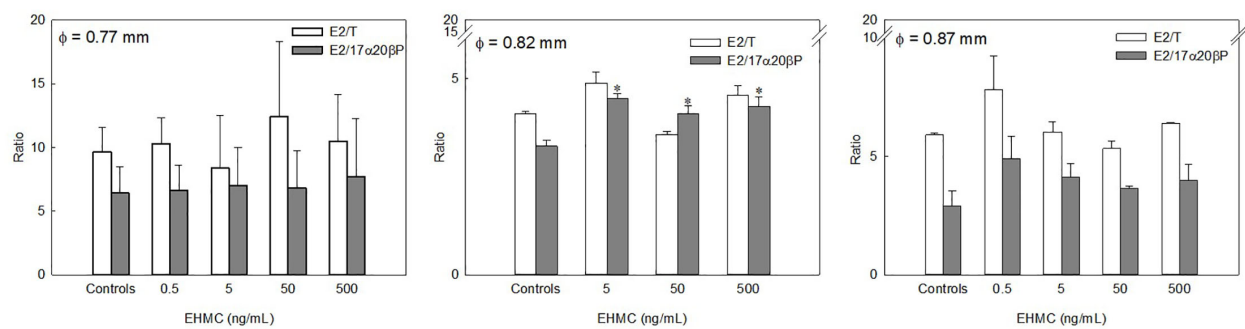


FIGURE 5

Effects of EHMC on the ratio of  $E_2/T$  and  $E_2/17\alpha20\beta P$  calculated from each steroid metabolite from [ $^3H$ ]17 $\alpha P$  of longchin goby oocytes (average diameter = 0.77, 0.82, and 0.87 mm) after 24 h of incubation. Values are mean  $\pm$  SEM in two or three replicate wells with 20 oocytes/well. Asterisks indicate the significant differences between each treatment and controls ( $P < 0.05$ ).

of certain chemicals is unclear, although most endocrine-disrupting chemicals have not been completely analyzed. The reasons for these contradictory results remain elusive. A possible hypothesis is that the sensitivity of the specimens differs depending on the chemical. This includes various fish species, exposure methods, and oocyte developmental stages (Baek et al., 2007; Lee et al., 2019). Another hypothesis is restricted VTG responses, a representative index of estrogenicity in the endocrine activity of fish. A recent study reported that VTG responses do not always detect endocrine activity and that it is rare for substances without endocrine activity *in vitro* to cause a concentration-dependent VTG response in fish (Brown et al., 2023). In this regard, we comparatively analyzed vitellogenic oocytes and mature oocytes for consecutive steroid hormonal shifts during the maturation process induced by EHMC and BP-3 exposure.

As mentioned above, 17 $\alpha20\beta P$  is known as a maturation-inducing steroid synthesized and secreted from the ovarian

follicle layers and plays a crucial role in the final maturation and ovulation process of oocytes (Nagahama and Yamashita, 2008). The decrease in 17 $\alpha20\beta P$  in the matured oocytes by EHMC or BP-3 exposure was intuitive on the results of heatmap analysis; however, no significant difference was observed compared with the controls. Contrastingly, in the oocytes of 0.82-mm diameter, EHMC markedly increased the  $E_2/17\alpha20\beta P$  ratio, which is thought to have been combined with the results of the decrease in 17 $\alpha20\beta P$  along with the increase in  $E_2$ . Additionally, BP-3 markedly increased the  $E_2/T$  ratio, which was the combined result of the decrease in T along with an increase in  $E_2$ . We were unable to obtain sufficient amounts of 0.82-mm oocytes with limitations for collecting the appropriate developmental stage of oocytes from matured females; therefore, we could not conduct BP-3 exposure experiments with them. Despite the insufficient data, we carefully presume that BP-3 may act analogously to EHMC in 0.82-mm oocytes since the results of BP-3 exposure in the other two oocyte

TABLE 2 Effects of BP-3 on *in vitro* steroid metabolism rate from [ $^3H$ ]17 $\alpha P$  of longchin goby oocytes (average diameter = 0.77 and 0.87 mm) after 24 h of incubation.

Oocyte diameter (mm)	BP-3 (ng/mL)	Metabolic rate (%)				
		A4	T	$E_1$	$E_2$	17 $\alpha20\beta P$
0.77	0	1.07 $\pm$ 0.17	1.57 $\pm$ 0.08	10.06 $\pm$ 2.78	14.99 $\pm$ 2.20	2.48 $\pm$ 0.44
	0.5	1.33 $\pm$ 0.02	1.84 $\pm$ 0.02	7.66 $\pm$ 2.40	18.55 $\pm$ 2.89	2.76 $\pm$ 0.15
	5	1.06 $\pm$ 0.20	1.86 $\pm$ 0.05	8.38 $\pm$ 2.73	15.63 $\pm$ 2.15	3.04 $\pm$ 0.45
	50	1.45 $\pm$ 0.21	1.76 $\pm$ 0.18	8.68 $\pm$ 1.61	17.87 $\pm$ 2.99	2.88 $\pm$ 0.22
	500	1.25 $\pm$ 0.32	1.69 $\pm$ 0.04	7.01 $\pm$ 1.62	18.15 $\pm$ 4.80	2.45 $\pm$ 0.05
0.87	0	1.72 $\pm$ 0.01	1.85 $\pm$ 0.13	7.21 $\pm$ 0.78	10.93 $\pm$ 0.91	3.85 $\pm$ 0.51
	0.5	1.37 $\pm$ 0.10	1.72 $\pm$ 0.01	8.74 $\pm$ 1.11	15.25 $\pm$ 0.57	2.67 $\pm$ 1.08
	5	1.22 $\pm$ 0.03	2.05 $\pm$ 1.03	7.83 $\pm$ 0.86	<b>17.08 <math>\pm</math> 1.07*</b>	2.69 $\pm$ 0.58
	50	1.35 $\pm$ 0.02	2.29 $\pm$ 0.24	9.31 $\pm$ 0.75	12.33 $\pm$ 1.30	2.73 $\pm$ 0.38
	500	1.28 $\pm$ 0.02	2.06 $\pm$ 0.43	8.26 $\pm$ 2.03	10.51 $\pm$ 1.50	2.74 $\pm$ 0.41

The percentage of radioactivity associated with each isolated steroid was calculated to the percentage of total steroid recovered from initial TLC. Values are mean  $\pm$  SEM of the rate of each steroid in two or three replicate wells with 20 oocytes/well. Asterisk indicates significant difference compared with controls ( $p < 0.05$ ).

The bold values were for emphasizing the statistically significant difference with asterisk.

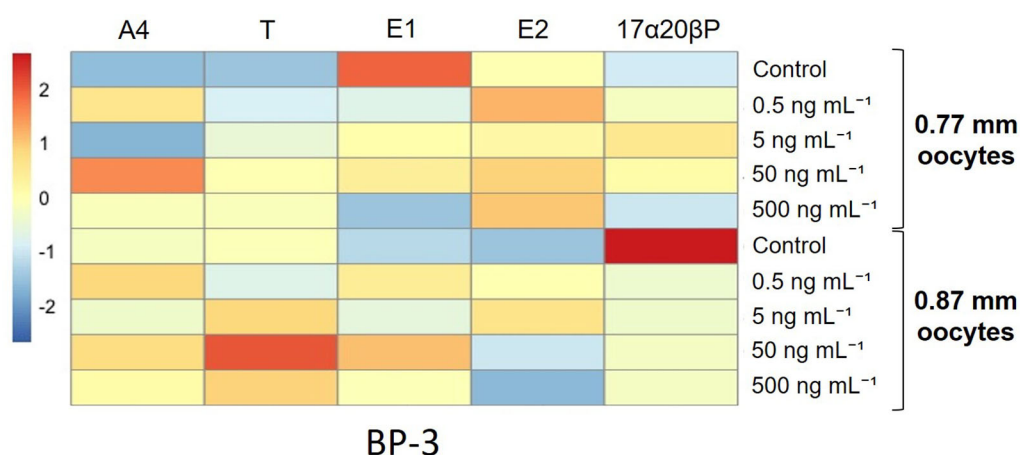


FIGURE 6

Heatmap analysis representing different levels of steroid metabolic rates of longchin goby oocytes exposed to BP-3. The lower relative rates are represented by blue color, and the higher relative rates are represented by red color. BP-3 dosages were represented in the columns. Each steroid metabolite was represented in the row and scaled in row direction (Z-scores).

groups were analogous to the results of EHMC exposure. In the oocytes of 0.87-mm diameters showing that the metabolic rate of 17α20βP was the highest among three groups of oocytes, BP-3 increased significantly the E<sub>2</sub> metabolite and E<sub>2</sub>/T ratio. In general, progesterone acts as a maturation-inducing steroid after vitellogenesis (Lubzens et al., 2010) and increased estrogenic effects by BP-3 in that stage would be mentioned as an estrogenic potency of BP-3. This finding is consistent with a recent review by Mustieles et al. (2023) that 20 references demonstrated estrogenic effects of BP-3.

To date, only a few studies have investigated the effects of these chemicals on oocyte maturation. Schreurs et al. (2005) reported that EHMC acts as a weak ER agonist and potent progesterone receptor antagonist. BP-3 inhibits gonad maturation in females and males and induces feminization in zebrafish. Recently, Jin et al. (2021) showed BP-3 to inhibit the release of the first polar body and disrupt

spindle assembly during oocyte maturation in mice. The authors did not mention BP-3's estrogenic activity, and the specific link between estrogenic activity and BP-3 during oocyte meiosis should be studied in future research. In the progesterone profiling disruption by EHMC or BP-3 exposure, EHMC and BP-3 showed antiprogesterone activity in the CALUX assay; however, BP-3 did not exhibit antiprogesterone activity in the gonads of the common carp (Amankwah et al., 2024). Additionally, the authors reported that EHMC was not detected in the fish tissues from the collection site and suggested that the values indicating no effect of specific chemicals should be established. We strongly agree with this suggestion and hope that our findings will help designate a relevant index for progesterone evaluation by these chemicals.

Disruption of the oocyte maturation process by these chemicals may pose ecological risks in aquatic ecosystems because reproduction is directly associated with progeny production.

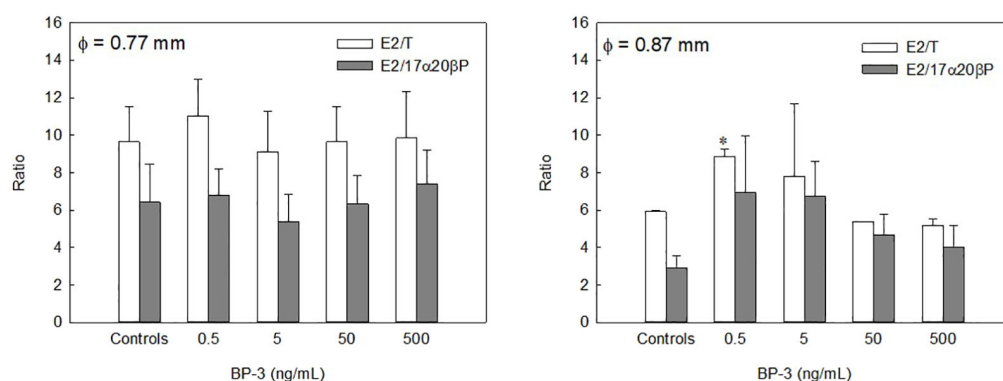


FIGURE 7

Effects of BP-3 on the ratio of E<sub>2</sub>/T and E<sub>2</sub>/17α20βP calculated from each steroid metabolite from [<sup>3</sup>H]17αP of longchin goby oocytes (average diameter = 0.77 and 0.87 mm) after 24 h of incubation. Values are mean ± SEM in two or three replicate wells with 20 oocytes/well. Asterisk indicates the significant differences between each treatment and controls (P < 0.05).



Recently, Xu et al. (2021) reported possible transgenerational effects of BP-3 in zebrafish, where BP-3 exposure induced parental feminization and upregulated the expression of estrogen-related genes, such as ER and VTG. Moreover, the progeny hatching rate from the exposed adults decreased by half. Previous reports are in agreement with this finding. EHMC and BP-3 reduced egg production in exposed adult medaka and zebrafish, although the hatching rates of the progeny were not affected (Kim et al., 2014; Lee et al., 2019). Additionally, the thyroid hormone levels, which play a major role in growth in the juvenile stage, decreased in the progeny of EHMC-exposed adult medaka; however, survival did not decrease (Lee et al., 2019). Contrastingly, although the hatching rates of both F0 and F1 decreased, EHMC showed anti-estrogenic effects in the exposed adults (Zhou et al., 2019b). Furthermore, the progeny exhibited stronger biochemical responses and oxidative damage by EHMC than their parents. Comprehensively, future studies with *in vivo* exposure to extend the production of F1 generation from exposed F0 will enhance our understanding of the endocrine disruption mechanism despite our findings showing that EHMC and BP-3 demonstrated estrogenic potency, followed by progesterone inhibition during the oocyte maturation stage.

## 5 Conclusion

EHMC and BP-3 showed estrogenic potency during the oocyte maturation process, with a steroidal shift in the longchin goby. Additionally, our study underscores the progesterone metabolism inhibition and estrogen metabolism induction in the maturing oocytes. Despite the absence of reliable indices for precisely assessing estrogenicity or anti-progesterogenicity, as mentioned by Amankwah et al. (2024), our findings of *in vitro* altered steroid production by EHMC and BP-3 in marine fish species would provide fundamental information for understanding the impact of these chemicals on marine fish reproduction. Future research on the gene expression of estrogen and/or progesterone receptors and *in vivo* experiments, including the influences of parental transfer of these chemicals, should be performed.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was approved by Animal Ethics Committee of Pukyong National University (PKNU; Regulation No. 554). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

HW: Visualization, Formal analysis, Investigation, Software, Writing – original draft. HB: Supervision, Conceptualization, Writing – review & editing, Project administration, Funding acquisition. IH: Funding acquisition, Writing – original draft, Formal analysis, Visualization, Validation, Data curation, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2025.1642233/full#supplementary-material>

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