

## ***In Vitro* Steroidogenesis on Oocyte Development in the Starry Flounder, *Platichthys stellatus***

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**ABSTRACT** : In this study, oocyte steroidogenesis are investigated in relation to oocyte development in the starry flounder, *Platichthys stellatus*, a marine multiple spawner. Vitellogenic (0.52 and 0.55 mm oocyte diameter) and mature oocytes (0.63, 0.66 and 0.71 mm oocyte diameter) were incubated *in vitro* in the presence of [<sup>3</sup>H]17 $\alpha$ -hydroxyprogesterone ([<sup>3</sup>H]17 $\alpha$ -OHP) as a precursor. Steroid metabolites were extracted from the incubated media and oocytes, the extracts were separated and identified by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). The major metabolites produced from [<sup>3</sup>H]17 $\alpha$ -OHP were androgens [androstenedione (A<sub>4</sub>) and testosterone (T)] and estrogens [17 $\beta$ -estradiol (E<sub>2</sub>) and estrone (E<sub>1</sub>)] and progestins [17 $\alpha$ ,20 $\alpha$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ 20 $\alpha$ P) and 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ 20 $\beta$ P)] in vitellogenic and mature oocytes. The results from this study suggest the potential roles of E<sub>1</sub> in the oocytes with diameter 0.52-0.71 mm, 17 $\alpha$ 20 $\alpha$ P and 17 $\alpha$ 20 $\beta$ P at the oocytes of 0.63, 0.66 and 0.71 mm.

**Key words** : Starry flounder, Maturation inducing steroid, Steroid metabolite, Oocyte maturation, vitellogenesis

### **INTRODUCTION**

Oocyte developmental process in teleosts is under the influence of sex steroid hormone that is generated from the follicle layer, and the sex hormone is regulated by the pituitary gonadotropin (GtH) (Nagahama et al., 1994; Planas & Swanson, 2008). In female fish, estradiol-17 $\beta$  (E<sub>2</sub>), testosterone (T) and progestins are the main gonadal steroids. During vitellogenesis, E<sub>2</sub> is the major sex steroid, synthesized at follicle cell layer and transported to liver. Then stimulated liver produced vitellogenin, the precursor of yolk protein. After vitellogenesis, progestins such as 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)

act as maturation inducing steroid (MIS) and induce final oocyte maturation including germinal vesicle breakdown (GVBD) and ovulation (Patiño & Sullivan, 2002; Baek, 2008; Nagahama & Yamashita, 2008). Androgens, T and 11-ketotestosterone (11-KT), are generally considered playing a role in regulating testis differentiation and spermatogenesis (Borg, 1994). However, it was reported in several species of fish including those of the Anguillidae family, that 11-KT was also identified as the major androgens in the blood of females (Lokman et al., 2002; Setiawan et al., 2012). The role of these sex steroids in fish reproductive process is still unclear, due to the high diversity of reproductive strategies. This may be different in fish species with synchronous or asynchronous ovarian development

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(Tyler & Sumpter, 1996).

The starry flounder (*Platichthys stellatus*), which is a regional specific species in East Sea of Korea, exhibits multiple spawning and asynchronous ovarian development (Lim et al., 2007; Hwang et al., 2012). Since it is getting hard to catching the natural starry flounder, we are planning to recover fisheries resource and increase fisheries stocks by releasing artificial fish seed; however, few studies have investigated concerning its reproductive characteristics. Understanding steroidogenesis in starry flounder could elucidate the endocrine control of reproductive cycle. Therefore, in this study, we analyzed *in vitro* oocyte steroidogenesis and identified steroid metabolites from vitellogenic and post-vitellogenic stages (mature oocytes) of development in the starry flounder.

## MATERIALS AND METHODS

### 1. Experimental fish and oocyte incubation *in vitro*

The starry flounder used in this study were captured in coastal waters of Pohang, Korea during the breeding season (December-March). Oocytes were separated into groups using fine forceps. Oocytes with average diameters of 0.52-0.71 mm were used for incubation. After separating the ovaries into small pieces in 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), approximately 30 follicle-enclosed oocytes were incubated in the presence of 55 kBq of [<sup>3</sup>H]-17 $\alpha$ OHP as the radiolabeled precursor in each well of 24-well culture plates containing 1 ml of Leibovitz L15 medium (Gibco). The pH and osmolarity of the media were adjusted to 7.9 and 300 mOsm, respectively. The plates were incubated for 24 h at 12°C with constant gentle shaking. Some pieces of ovary from each individual were fixed in Bouin's solution for 24 hours for histological observations of oocytes. The fixed samples were washed, dehydrated, and embedded in paraffin. Serial sections of 4-6  $\mu$ m thickness were prepared; the slides were stained in Mayer's hematoxylin and 0.5% eosin and mounted with malinol.

Histological samples were observed through a light microscope (BX50, Olympus, Japan).

### 2. Steroid extraction and analysis of steroid metabolism

At the end of the incubation, steroids were extracted three times from the media and oocytes using 4 ml dichloromethane. The extracts were concentrated and applied to a thin-layer chromatography (TLC) plate (60F254; Merck, Darmstadt, Germany) with non-radioactive standard steroids as carrier steroids, and developed in a mixture of benzene: acetone (4:1) and benzene: ethyl acetate (4:1). Radioactive steroid metabolites were analyzed using a BAS 1500 bio-imaging analyzer (Fuji Film, Tokyo, Japan), and estrone (E<sub>1</sub>) and E<sub>2</sub> standards were visualized by exposure to iodine vapor. Other standard steroids were detected by UV absorption at 254 nm.

The migration zones corresponding to the carrier steroids were eluted twice from the silica plate bands with 5 ml of dichloromethane: methanol (90:10). Following centrifugation at 1,000 $\times$ g for 10 min, the supernatants were vacuum dried before finally being dissolved in 20  $\mu$ l of acetonitrile. Extracts were then analyzed by reverse-phase high-performance liquid chromatography (HPLC). Briefly, a Waters Alliance HPLC system (Waters, Milford, MA, USA) equipped with a binary pump (515 HPLC pump, Waters) and UV detector (2487 multi-wavelength absorbance detector, Waters) was used. Chromatographic separation was performed on a Sunfire C18 analytical column (4.6 $\times$ 150-mm I.D., 3.5- $\mu$ m particle size; Waters Sunfire). The flow rate and mobile phase were 0.6 ml/min with 20% methanol and 0.4 ml/min with absolute acetonitrile.

The steroid metabolites were identified by gas chromatography-mass spectrometry (GC-MS). Steroids were analyzed on a DB-5MS column (60 m $\times$ 0.25 mm O.D. $\times$ 0.25  $\mu$ m I.D.) using a Shimadzu GCMS-QP2010 mass spectrometer interfaced to GC-2010 gas chromatograph. The following conditions were used: splitless injection at 80°C (injector temperature of 280°C), followed by a 1-min delay before maximal temperature 20°C/min to program starting temperature of 250°C. Thereafter, the temperature was programmed at

5°C/min to maximum 295°C.

## RESULTS

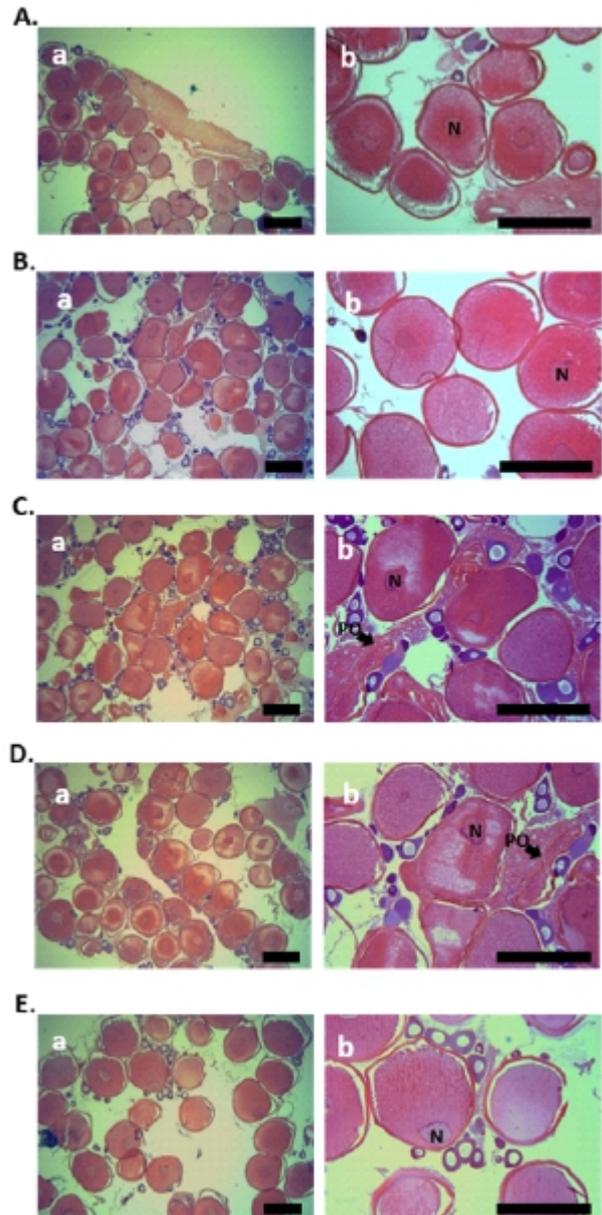
### 1. Histological observation of oocytes

Histological analysis of fish ovary was performed using the samples, collected at five each sampling during the breeding season (January to February). In both the 0.52 and 0.55 mm diameter oocytes of starry flounder, yolk granules were spread throughout the ooplasm, and nucleus (N) was located near the center of oocytes (Fig. 1A and B). In 0.63 and 0.66 mm diameter oocytes of starry flounder, migration of N was observed (Fig. 1C and D). Later, nucleus was gradually displaced toward the animal pole in 0.71 mm diameter oocytes of starry flounder; yolk granules were increased and combined together (Fig. 1E).

### 2. Steroid metabolism

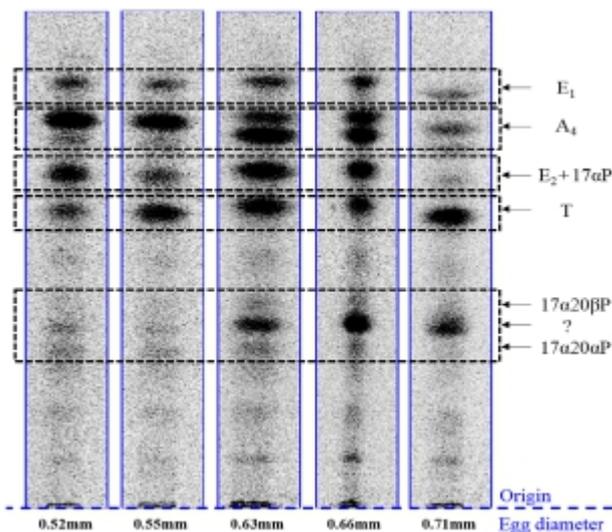
When vitellogenic oocytes (0.52 and 0.55 mm in diameter) were incubated with [<sup>3</sup>H]-17 $\alpha$ P, the four major separated metabolites were A<sub>4</sub>, T, E<sub>1</sub> and E<sub>2</sub> (Fig. 2); E<sub>2</sub> and 17 $\alpha$ P were overlapped slightly by TLC, and E<sub>2</sub> was further identified by HPLC. In 0.63, 0.66 and 0.71 mm diameter oocytes of starry flounder, undergoing germinal vesicle migration, unknown metabolites (?), predicted to progesterone between 17 $\alpha$ 20 $\beta$ P and 17 $\alpha$ 20 $\alpha$ P were also detected. Moreover, the presence of a small quantity of estrogens was observed in the oocytes of 0.71 mm. The five zones of metabolites (converted from [<sup>3</sup>H]-17 $\alpha$ P using the oocytes of 0.52-0.71 mm) corresponding to the carrier steroid in TLC were eluted and identified by HPLC (data not shown). From the oocytes of 0.63 and 0.66 mm it looks like there were two separated spots of A<sub>4</sub>, but it turned out to be only the presence of A<sub>4</sub>. The GC-MS analysis was performed for the identification of 17 $\alpha$ 20 $\beta$ P, 17 $\alpha$ 20 $\alpha$ P and E<sub>1</sub> (Fig. 3). The GC-MS analysis of unknown metabolites (?) in the oocytes of 0.63, 0.66 and 0.71 mm revealed the presence of 17 $\alpha$ 20 $\beta$ P and 17 $\alpha$ 20 $\alpha$ P.

## DISCUSSIONS

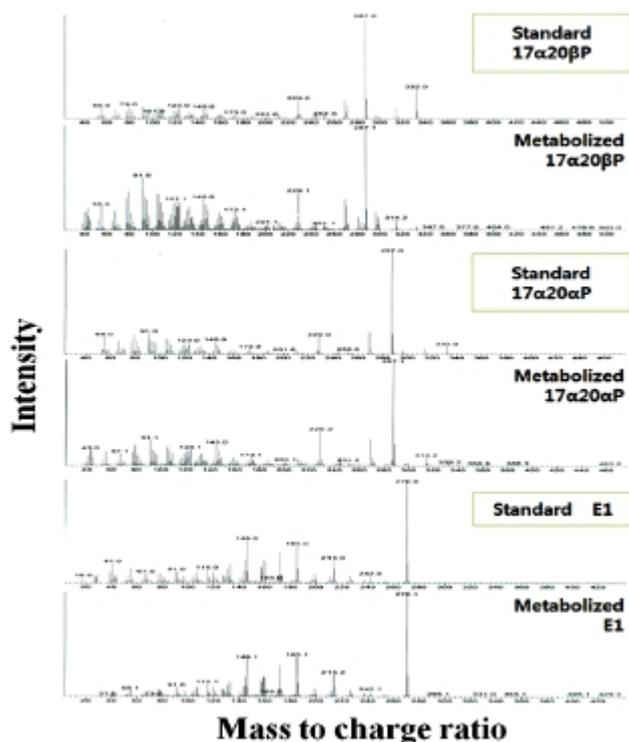


**Fig. 1. Histological observation of ovary in starry flounder, *Platichthys stellatus*.** A, Ovary with oocytes of 0.52 mm diameter; B, Ovary with oocytes of 0.55 mm diameter; C, Ovary with oocytes of 0.63 mm diameter; D, Ovary with oocytes of 0.66 mm diameter; E, Ovary with oocytes of 0.71 mm diameter. a: 40 $\times$ , b:100 $\times$ . Scale bar 500 $\mu$ m. N, nucleus; PO, post ovulatory follicles.

Steroid hormones play important roles in the reproduction of vertebrates. In fish, steroid hormones are critical to maintain hypothalamus-pituitary-gonad axis function, and feedback controls on the system are achieved largely through alterations in steroid production (Ankley et al., 2009). In



**Fig. 2.** Autoradiograms of steroid metabolites produced from [ $^3\text{H}$ ]17 $\alpha$ -hydroxyprogesterone after 24 hrs incubation with isolated oocytes from starry flounder. Steroid metabolites from radiolabeled precursor were separated by thin layer chromatography developed with a benzene:acetone (4:1) and benzene:ethyl acetate (4:1) solvent system. Androstenedione ( $\text{A}_4$ ), testosterone (T), estrone ( $\text{E}_1$ ), estradiol-17 $\beta$  ( $\text{E}_2$ ), 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ 20 $\beta$ P), 17 $\alpha$ ,20 $\alpha$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ 20 $\alpha$ P).



**Fig. 3.** Mass spectra of steroid metabolized by starry flounder oocytes identified as 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ 20 $\beta$ P), 17 $\alpha$ ,20 $\alpha$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ 20 $\alpha$ P) and estrone ( $\text{E}_1$ ).

female fish, estradiol-17 $\beta$  ( $\text{E}_2$ ), testosterone (T) and progestins are the main gonadal steroids.  $\text{E}_2$  is the major sex steroid during vitellogenesis, and progestin such as 17 $\alpha$ 20 $\beta$ P and 17 $\alpha$ 20 $\beta$ 21P is important during oocyte final maturation leading to GVBD (Patiño & Sullivan, 2002; Baek, 2008; Nagahama & Yamashita, 2008).

In the present study, we verified the steroid metabolites from vitellogenic (0.52 and 0.55 mm in diameter) and mature oocytes (0.63, 0.66 and 0.71 mm oocyte diameter) with [ $^3\text{H}$ ]-17 $\alpha$ P as a precursor. The major metabolites produced from the oocytes of starry flounder were  $\text{A}_4$ , T,  $\text{E}_2$ ,  $\text{E}_1$ , 17 $\alpha$ 20 $\beta$ P and 17 $\alpha$ 20 $\alpha$ P. From these steroids, we were interested in  $\text{E}_1$  and 17 $\alpha$ 20 $\alpha$ P.

In the most of species, the main estrogen is  $\text{E}_2$ , but vitellogenic oocytes of starry flounder also synthesized  $\text{E}_1$  as a major steroid metabolite. This metabolite was produced high through the entire period of maturation. From just prior to GVBD (in the oocytes of 0.71 mm), trace amounts of estrogens was detected.  $\text{E}_1$  has about one fifth activity of  $\text{E}_2$  in inducing trout vitellogenin synthesis (Routledge et al., 1998). Similarly, Specker & Sullivan (1994) reported that  $\text{E}_1$  may have a minor vitellogenic role in some species. In red seabream, *in vitro* conversion of  $\text{E}_1$  into  $\text{E}_2$  was 16-fold greater than T conversion into  $\text{E}_2$ , suggesting that  $\text{E}_2$  is synthesized mainly via  $\text{E}_1$  rather than through T (Ohta et al., 2002). The steroidogenic pathways for estrogen production are different among species. Further studies should be performed physiological roles for intermediate products other than T in the  $\text{E}_2$  synthetic pathway.

When starry flounder oocytes (0.63, 0.66 and 0.71 mm oocyte diameter) were incubated with [ $^3\text{H}$ ]-17 $\alpha$ P, two additional metabolites were appeared, 17 $\alpha$ 20 $\beta$ P and 17 $\alpha$ 20 $\alpha$ P. The activity of 17 $\alpha$ 20 $\alpha$ P, an isomer of 17 $\alpha$ 20 $\beta$ P, was higher than 17 $\alpha$ 20 $\beta$ P in the migratory oocytes (data not shown). But 17 $\alpha$ 20 $\beta$ 21P was not detected. In this species, two progestins, 17 $\alpha$ 20 $\beta$ P and 17 $\alpha$ 20 $\alpha$ P, likely act as MIS.

A large amount of 17 $\alpha$ 20 $\alpha$ P has been found in the blood and ovarian incubates of mature flatfish species

(Canario & Scott, 1990; Scott & Canario, 1990), and cyprinids (Kime et al., 1992). These authors suggested that it is not a potent inducer of oocyte maturation. The role of this steroid is still unknown.

Until now, there are two major MIS identified in fish,  $17\alpha,20\beta$ P and  $17\alpha,20\beta,21$ P. They both probably act as MIS in most fishes, but normally one of them is the predominant MIS for a given species. The  $17\alpha,20\beta$ P is the major MIS in several salmonid and non salmonid species, while  $17\alpha,20\beta,21$ P is the major MIS in Atlantic croaker, spotted sea trout, striped bass and black porgy (Yueh et al., 2005).

Data presented in this study suggest that metabolite  $17\alpha,20\alpha$ P may have some roles during oocyte maturation process.

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