

In Vitro Sex Steroid Metabolism in Red Spotted Grouper, *Epinephelus akaara* during Oocyte Maturation

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Conflict of interests

The authors declare no potential conflict of interest.

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Authors' contributions

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Writing-review & editing: Hwang IJ, Baek HJ

Abstract

We studied steroid metabolites produced from red-spotted grouper ovarian follicles during maturation. Oocytes with 350–500 μm diameter were *in vitro* incubated in the presence of [³H] 17 α -hydroxyprogesterone as a precursor. Steroid metabolites were extracted from incubated media and oocytes. The extracts were separated and identified using thin layer chromatography, high performance liquid chromatography and gas chromatography-mass spectrometry. The identified metabolites were androstenedione (A_4), testosterone (T) and estrone (E_1). The metabolites of A_4 was dominant in all size of oocytes and it was the highest in 480 μm diameter oocytes. The metabolites of two progestins, 17 α ,20 β -dihydroxy-4-pregnen-3-one and 17 α ,20 α -dihydroxy-4-pregnen-3-one were detected in the oocytes less than 480 μm diameter although they were not identified definitely. In the oocytes of 480 μm diameter, metabolite of progestin was the highest, and germinal vesicle (GV) was still in the middle of cytoplasm. In the oocytes of 500 μm diameter, GV was began to migrate and the major metabolites were A_4 and E_1 . The metabolite of E_1 was detected in all size of oocytes and it was higher than that of E_2 . These results suggest that oocytes of 480 μm diameter are the transitional stage involving steroidogenic shift to final oocyte maturation and potential function of E_1 during maturation process.

Keywords: Red spotted grouper, Steroid metabolite, Oocyte, Maturation

INTRODUCTION

Oocyte maturation process in teleosts is under the control or feedback of sex steroids, synthesized from the follicle cell, and they are regulated by the hypothalamus-pituitary-gonad axis (Nagahama & Yamashita, 2008; Planas & Swanson, 2008). In female fish, it is well known that estradiol-17 β (E_2) is the major sex steroid, and transported to liver during vitellogenesis, the period of vitellogenin production in liver. Subsequently, progestins function as such as maturation inducing steroid (MIS), 17 α ,20 β -dihydroxy-4-pregnen-3-one (17 α ,20 β P) and/or 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one (17 α ,20 β ,21P) induce final oocyte maturation, i.e., germinal vesicle breakdown (GVBD) and ovulation (Patiño & Sullivan, 2002; Baek, 2008; Nagahama & Yamashita, 2008). Although the actions and role of these sex steroids had been studied in many fish species, the profiling of various sex steroids in fish reproduction is still insufficient because of not only diverse fish species but their diverse

Ethics approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

reproductive characteristics (Frisch A, 2004; Tokarz et al., 2015).

The red-spotted grouper, *Epinephelus akaara* is a marine multiple spawner and protogynous, as a Serranid fish distributed mainly in Korea, southern Japan, southern China and East China Sea (Craig et al., 2011; Lee et al., 2020). This species is economically important in aquaculture industry and it is interested in artificial control of its reproduction such as induction of maturation and masculinization (Li et al., 2006; Lee et al., 2014; Oh et al., 2018; Lee et al., 2020). However, there is little information about sex steroid synthesis related to oocyte maturation of this species. In the present study, we investigated the *in vitro* profiling of sex steroid metabolites from ovarian follicles of red spotted grouper.

MATERIALS AND METHODS

1. Chemicals

Authentic steroids were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Steraloids (Wilton, NH, USA). Mother solutions (mg/mL) were prepared by dissolving the authentic steroids in pure ethanol. These were further diluted in incubation media. The ethanol concentration in the incubation medium was maintained at less than 0.1%. Radioactive [³H]-17 α -hydroxyprogesterone ([³H]-17 α P) was obtained from Amersham Life Science (London, UK).

2. Preparation of oocytes for incubation

The experimental fish were reared and adjusted to 14L:10D and 25 °C in recirculating aquaria (500 L) from Marine Science Institute, Jeju National University, Korea. Oocytes were obtained by cannulation after anesthetization of eight mature females. The cannulated oocytes were put into cold balanced salt solution (BSS; 132.96 mM NaCl, 3.09 mM KCl, 0.28 mM MgSO₄ · 7H₂O, 0.98 mM MgCl₂ · 6H₂O, 3.40 mM CaCl₂ · 6H₂O, 3.65 mM HEPES). Oocytes were separated into groups with the largest diameter using fine forceps from each mature fish. Oocytes with average diameters of 350, 400, 420, 440, 480, and 500 μ m were used for the *in vitro* incubation. Thirty follicle-enclosed oocytes were incubated in each well of triplicates.

We incubated isolated oocytes in 1 mL of Leibovitz L15 medium (Gibco, Grand Island, NY, USA) with 55 kBq [³H]-17 α P a radiolabeled precursor for separation of metabolites. The pH and osmolality of the media were adjusted to 7.7 and 360 mOsm, respectively. The plates were incubated for 24 h at 18 °C.

Some ovarian fragment from each fish were fixed, washed, dehydrated and paraffin-embedded. Sections of 4–5 μ m were HE stained and observed through a light microscope.

3. Analysis of steroid metabolism

At the end of the incubation, steroids extraction, separation and identification of metabolites were followed by our previous studies (Hwang et al., 2012; Baek et al., 2013; Lee & Baek, 2015). Steroid metabolites produced from [³H]-17 α P were compared with the values were expressed as photo-stimulated luminescence (PSL) – background (BG) per mm² of each fraction area from autoradiography.

RESULTS

1. Oocyte developmental stage

In histological observation of each diameter of oocytes, oocytes of 350–480 μ m diameter were

vitellogenic stage; yolk granules were spread throughout the cytoplasm and oil droplets (Od) were accumulated near the nucleus (N) as oocyte diameter increased (Fig. 1A–E). In particular, nucleus was observed in the middle of cytoplasm in these size of oocytes. In the oocytes of 500 μm diameter, migration of N was observed and many Ods were observed near migrating N (Fig. 1F).

2. Identification of steroid metabolites

After incubation of oocytes with [^3H]-17 α P, 7 major fractions appeared on thin layer chromatography (TLC). Fraction 1, 2, 4, 5, and 6 were as the same retention factor (RF) of standard estrone (E_1), androstenedione (A_4), 17 α P, T, and 17 α 20 β P, respectively (Fig. 2). Fraction 3 and 7 were overlapped with E_2 +unknown metabolite and 17 α , 20 α -dihydroxy-4-pregnen-3-one (17 α 20 α P)+unknown metabolite, respectively. Among these 7 metabolites, fraction 1, 2, and 5 were identified as E_1 , A_4 , and T through further analysis with high performance liquid chromatography and GC/MS (Fig. 3). The other metabolites were not identified due to their low measured activities.

3. Metabolites profiling during maturation process

We compared each metabolite with PSL value from TLC autoradiography followed each oocyte diameter for steroid metabolites profiling during oocyte maturation (Fig. 4). The A_4 metabolite was dominant [15.47–92.41 (PSL-BG)/ mm^2] in all size of oocytes and it was the highest in 480 μm diameter oocytes. T metabolite increased gradually [4.55–13.76 (PSL-BG)/ mm^2] as oocyte diameter increased although it was not detected at 350 and 500 μm diameter oocytes in the autoradiography. E_2 overlapped with unknown metabolite was detected only in 350 μm diameter oocytes [4.27 (PSL-BG)/ mm^2]. E_1 metabolite [3.08–9.24 (PSL-BG)/ mm^2] was detected in all size of oocytes and it was the highest in 420 μm diameter oocytes. 17 α 20 β P metabolite was detected in 350–420 μm diameter oocytes [2.27–4.64 (PSL-BG)/ mm^2]. 17 α 20 α P overlapped with unknown metabolite was detected 350, 420–480 μm diameter oocytes [3.37–10.82 (PSL-BG)/

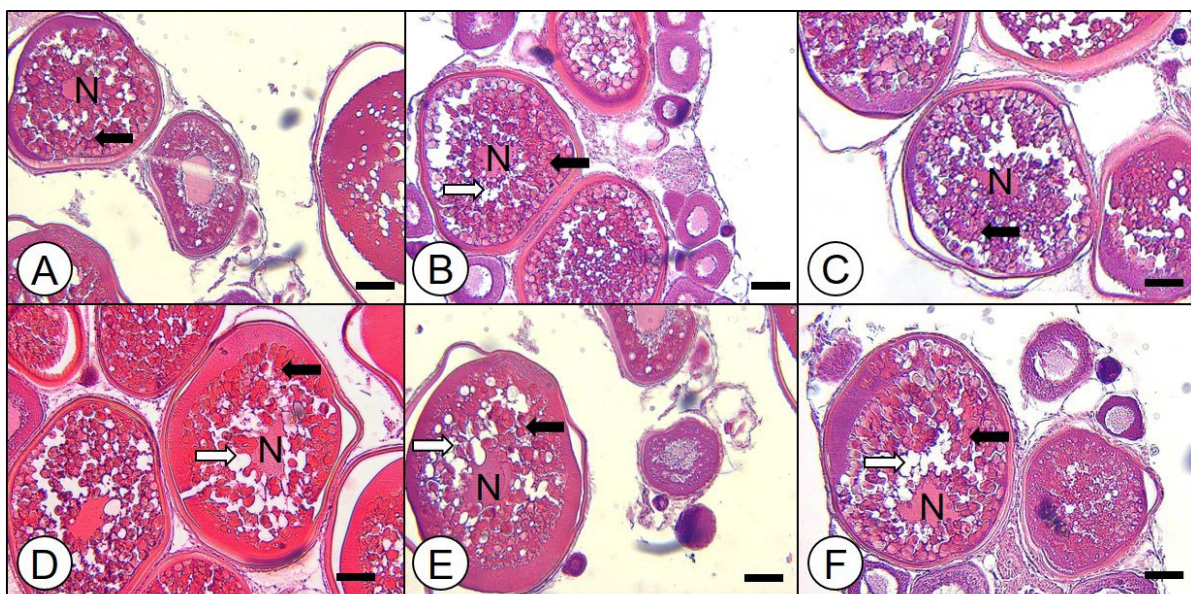


Fig. 1. Histological observation of ovarian fragment from mature red spotted grouper. (A) oocytes of 350 μm , (B) oocytes of 400 μm , (C) oocytes of 420 μm , (D) oocytes of 440 μm , (E) oocytes of 480 μm , (F) oocytes of 500 μm . Scale bars=100 μm , stained with H-E. N, nucleus; blank arrow, oil droplet; solid arrow, yolk granule.

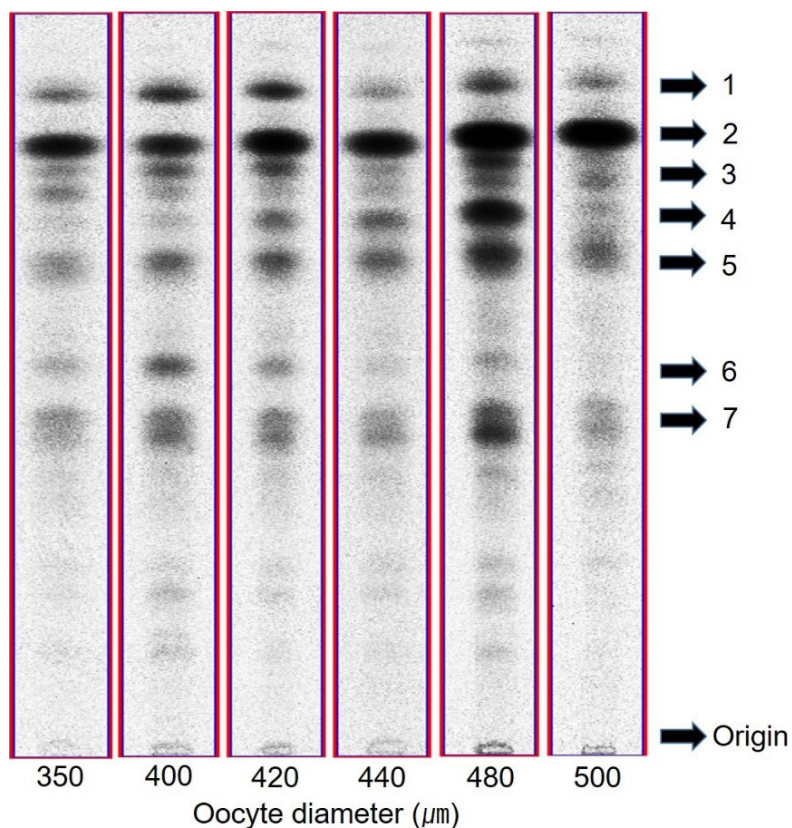


Fig. 2. Autoradiograms of steroid metabolites produced from [^3H]17 α -hydroxyprogesterone after 24 h incubation with isolated oocytes from red spotted grouper. Steroid metabolites from radiolabeled precursor were separated by thin layer chromatography developed with a benzene:acetone (4:1) and benzene:ethyl acetate (4:1) solvent system. 1, estrone (E_1); 2, androstenedione (A_4); 3, estradiol-17 β (E_2)+unknown metabolite; 4, 17 α P; 5, testosterone (T); 6, 17 α ,20 β -dihydroxy-4-pregnen-3-one (17 α 20 β P); 7, 17 α ,20 α -dihydroxy-4-pregnen-3-one (17 α 20 α P)+unknown metabolite.

mm 2] with the highest in 480 μm diameter oocytes. 17 α 20 α P or 17 α 20 β P metabolite was not detected in 500 μm diameter oocytes.

DISCUSSION

In general, E_2 , the principle estrogen is dominant in the early stage of maturation, i.e. oocyte growing and MIS such as 17 α 20 β P or 17 α 20 β 21P dominant in the late stage of maturation, i.e. GVBD and ovulation (Tokarz et al., 2015). In histological observation of oocytes, 350–480 μm diameter oocytes were vitellogenic stage and 500 μm diameter oocytes were final maturation stage with migration of germinal vesicle (GV). We consider the 480 μm diameter oocytes would be fully vitellogenic stage; GV was still in the middle of cytoplasm and prior to GVM stage.

We identified the major steroid metabolites and investigate their profiling from vitellogenic to germinal vesicle migration oocytes of red spotted grouper. The major metabolites synthesized from the oocytes were A_4 , E_1 , T, E_2 and two progestins. Among these metabolites, A_4 , E_1 , and T were identified through GC/MS, but the others were not identified due to their low activities. Although we could not identify 2 progestins, progestin 1 and 2 co-migrated with 17 α 20 α P and 17 α 20 β P in TLC system. To date, MIS of groupers had not been identified yet although 17 α 20 β P

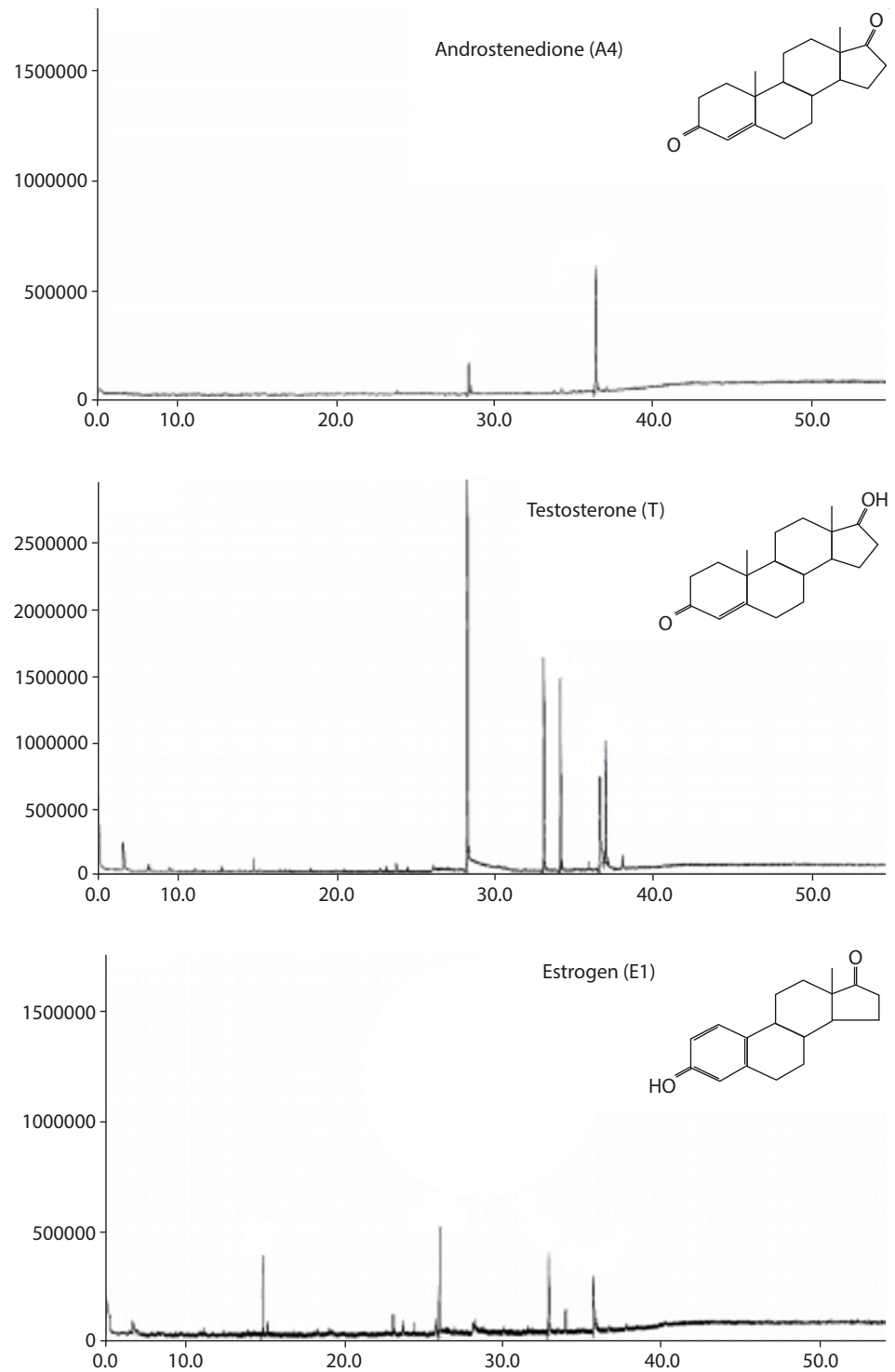


Fig. 3. Mass spectra of steroid metabolites identified as androstenedione, testosterone and estrone from oocytes of red spotted grouper.

is well-known MIS in many fish species and was measured from plasma of *Epinephelus morio* (Johnson et al., 1998; Shein et al., 2004; Nagahama & Yamashita, 2008). Interestingly, progestin 1 metabolite, suspected $17\alpha 20\alpha P$ increased gradually in 420–480 μm diameter oocytes. Previous studies reported that $17\alpha 20\alpha P$ may act as a MIS of flatfish, *Limanda limanda*, *Verasper variegates*

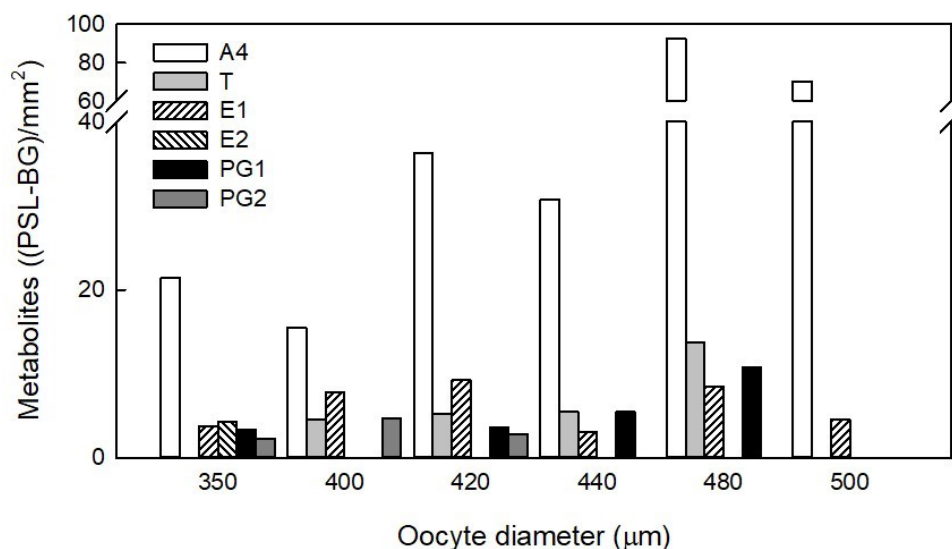


Fig. 4. Steroid metabolites from radioactive precursor during oocyte maturation. The values indicate digital photo-luminescence (PSL) by autoradiography and were expressed as PSL-background (BG) per mm² of each fraction area. A₄, androstenedione; T, testosterone; E₁, estrone; E₂, estradiol-17β; PG₁, 17α,20α-dihydroxy-4-pregnen-3-one; PG₂, 17α,20β-dihydroxy-4-pregnen-3-one.

and gobiid fish, *Chasmichthys dolichognathus* (Canario & Scott, 1990; Baek, 2001, 2008). In this regard, 17α20αP metabolite would be related to maturation process with its increase until fully vitellogenic stage and then decrease at GVM stage although its role is not clear. Further study that identification of MIS and its maturation inducing capacity should be conducted.

Among the metabolites, A₄ was predominant in all size of oocytes even in the GVM stage (500 µm oocyte diameter). In our previous study, A₄ was also produced dominantly in vitellogenic oocytes from red lip mullet, *Chelon haematocheilus*, blacktip grouper, *Epinephelus fasciatus*, and blackfin flounder, *Glyptocephalus stelleri* (Baek et al., 2011; Hwang et al., 2012; Lee & Baek, 2015). A few studies reported that A₄ is related to sex change in protogynous hermaphrodite, *Monopterus albus* and *Sparisoma viride* (Yeung & Chan, 1987; Cardwell & Liley, 1991; Yeung et al., 1993). Moreover, Montero et al. (1995) reported that androgen could act as a substrate for estrogen production in vitellogenesis of European eel, *Anguilla anguilla*. In the present study, we suspect that A₄ may regulate oocyte maturation process rather than sex change of red spotted grouper since there was no testicular tissue in gonad and it's the highest value with progestin production at 480 µm diameter oocytes, the transitional stage into final maturation.

We demonstrated that E₁ metabolite was also detected in all size of oocytes and its detected value was higher than that of E₂ metabolite. In a majority of fishes, it is well known that E₂ is converted from T by aromatase (Fukada et al., 1996; Chang et al., 1997; Aggarwal et al., 2014). However, E₁ may have a minor vitellogenic role in some species (Specker & Sullivan, 1994; Routledge et al., 1998) and act as a precursor of E₂ synthesis (Kazeto et al., 2000; Ohta et al., 2001; Ohta et al., 2002; Mindnich et al., 2004; Zhou et al., 2005). Our results also suggest that E₁ may regulate vitellogenesis with E₂ and also has a certain role in oocyte maturation process despite insufficient data. Future study such as exogenous E₁ treatment on oocyte maturation would provide more definite information about steroidogenic profiling. In conclusion, the present study provides *in vitro* steroid metabolites profiling that identification of major metabolites and its changes during oocytes maturation of red spotted grouper. We also suggest that 17α20βP and/or 17α20αP may act as a MIS and the potential role of E₁ in maturation process.

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