

Stimulation of gonad maturation in mullet fish *Mugil dussumieri* using MT, E2, hCG, and Ovaprim hormone

Stimulasi pematangan gonad ikan belanak *Mugil dussumieri* menggunakan hormon MT, E2, hCG, dan Ovaprim

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ABSTRACT

The present study is a preliminary research for producing mullet fry. The research aimed to evaluate stimulation of gonad maturation in mullet using hormones. The method used for research was completely randomized design consist of three treatments and each individual replication was repeated three times. Two experiments were conducted separately. First experiment used 9-14.7 cm body length of fish treated with different hormone injections i.e. 4 mg/kg 17 α -methyltestosterone (MT), 0.07 mg/kg estradiol-17 β (E2), and 0.5 ml/kg 0.9% physiological solution as control. Second experiment used 10-31 cm body length fish treated with 750 IU/kg chorionic gonadotropin (hCG), 0.5 ml/kg Ovaprim, and 0.9% physiological solution as control. Gonadosomatic index (GSI) value of the first experiment on day 60 showed that GSI of E2 treatment was higher than both MT treatment and control. On the contrary, the second experiment on day 60 showed that GSI of hCG treatment was higher than both Ovaprim treatment and control. Egg diameter frequency distribution for control in the first experiment on day 30 ranged from 9–144 μ m. Egg diameter for E2 and MT treatments on day 60 ranged from 9–243 μ m and were higher than control. In the second experiment, egg diameter on day 30 for control ranged from 9–144 μ m, hCG treatment ranged from 9–441 μ m, while Ovaprim ranged from 9–111 μ m. Blood glucose, blood cholesterol, testosterone and estradiol hormone level in the first and second experiment showed no significant differences. The results showed that estradiol-17 β and 17 α -methyltestosterone induction in 9-14.7 cm body length mullet increased gonad maturity to stage II while hCG induction in 10–31 cm body length mullet increased gonad maturity to stage III.

Keywords: estradiol-17 β , hCG, 17 α -methyltestosterone, *Mugil dussumieri*, Ovaprim

ABSTRAK

Penelitian ini merupakan rintisan untuk menghasilkan benih ikan belanak. Penelitian ini bertujuan mengevaluasi pematangan gonad ikan belanak dengan hormon. Penelitian dilakukan dengan rancangan acak lengkap yang terdiri dari tiga perlakuan dan tiga kali ulangan. Terdapat dua percobaan berbeda dengan hormon berbeda. Percobaan pertama menggunakan ikan berukuran 9–14,7 cm dengan hormon 17 α -metiltestosteron (MT) 4 mg/kg, estradiol-17 β (E2) 0,07 mg/kg dan kontrol larutan fisiologis 0,9% 0,5 ml/kg. Percobaan kedua menggunakan ikan berukuran 10–31 cm dengan *human chorionic gonadotropin* (hCG) 750 IU/kg, Ovaprim 0,5 ml/kg dan kontrol larutan fisiologis 0,9 % 0,5 ml/kg. Nilai *gonadosomatic index* (GSI) percobaan pertama pada hari ke-60 menunjukkan perlakuan E2 lebih tinggi dibandingkan MT dan kontrol. Hasil percobaan kedua pada hari ke-60 nilai GSI menunjukkan bahwa pemberian hCG lebih tinggi dibandingkan Ovaprim dan kontrol. Sebaran frekuensi diameter telur pada percobaan pertama untuk kontrol hari ke-30 menunjukkan kisaran 9–144 μ m. Sebaran jumlah diameter telur untuk ES dan MT hari ke-60 menunjukkan kisaran yang lebih banyak dibandingkan kontrol. Sebaran frekuensi diameter telur pada percobaan kedua untuk kontrol hari ke-30 menunjukkan kisaran 9–144 μ m, hCG 9–441 μ m sedangkan Ovaprim 9–111 μ m. Kadar glukosa darah, kolesterol darah, hormon testosteron dan estradiol pada percobaan pertama dan kedua tidak menunjukkan perbedaan nyata. Hasil penelitian menunjukkan induksi hormon estradiol-17 β dan 17 α -metiltestosteron pada ikan berukuran 9–14,7 cm dapat meningkatkan kematangan gonad mencapai TKG II sedangkan induksi hormon hCG pada ikan berukuran 10–31 cm dapat meningkatkan kematangan gonad mencapai TKG III.

Kata kunci : estradiol-17 β , hCG, 17 α -metiltestosteron, *Mugil dussumieri*, Ovaprim

INTRODUCTION

Mullet (*Mugil dussumieri*) from the mugilidae family has the prospect of being cultivated in the tropics. Mulletts have widely geographical distribution and catadromous, adaptability in a variety of salinity, temperature, various foods, habitats and have rapid growth and are resistant to diseases (FAO, 1999). Mullet has adaptability in various environments and is suitable to the domestication of species to fresh water, brackish water and marine water (Rostami *et al.*, 2016). Mullet is euryhaline fish that widely culture in brackish water and freshwater ponds in semi-intensive culture (CMFRI, 2014; Anil *et al.*, 2010). Mullet is a detritus feeder that efficiently obtains food on detritus and organic matter, whilst playing an important role in reducing the content of organic matter from water and pond sediments to improve water quality parameters. The feeding habits of mullet can also indirectly affect the abundance of external opportunistic parasites in shrimp such as *Zoothamnium* sp. and *Epistylis* sp., which depends on organic waste (Aghuzbeni *et al.*, 2015; Aghuzbeni *et al.*, 2016).

Some research has been achieved in the use of induction hormones for gonad maturation. The results of Zeinab *et al.* (2007) reported that GSI at a dose of 5000 IU hCG hormone induction was $0.57 \pm 0.08\%$ while 10000 IU hCG was $1.60 \pm 0.45\%$, when compared to the GSI control of $0.22 \pm 0.07\%$, the two treatments produced a higher GSI than control. The results of Meseda *et al.* (2006) the dose of hCG 10000 IU at the first injection resulted in an egg diameter ranged from 510–610 μm . According to Shehadeh *et al.* (1973) induction of hCG at a dose of 6900 IU resulted egg diameter ranged from 520–750 μm and 440–740 μm , while a dose of 8000 IU egg diameter ranged from 620–790 μm . According to Sukendi *et al.* (2016) Ovaprim injection treatment with a dose of 0.60 ml/kg increased egg diameter 0.1925 mm, egg maturity level 20% and GSI value of 14.75% in pawas fish (*Osteochillus hasselti* CV). Ovaprim is a premix product consisting of a mixture of salmon gonadotropin-releasing hormone analogue (sGnRH-a) D-Arg6-Pro9-NetsGnRH-a with dopamine antagonist from domperidone type. Ovaprim is used to stimulate gonad maturation and spawning on broodstock. GnRH-a contained in Ovaprim acts to stimulate the pituitary to release gonadotropin while hCG contains FSH and LH, hCG activity is more directed to LH than FSH which can reduce aromatase activity, therefore the production

of estradiol-17 β decreased and testosterone production increased, high testosterone level can stimulating oocyte maturation, in addition to the increase in LH will increase the activity of 20 β -HSD to stimulate increased production of 17 α -20 β dihydroxy progesterone as a result of gonad maturation followed by oocyte ovulation (Reading & Sullivan, 2017).

Aquaculture in Indonesia is dominated by carnivorous (shrimp and grouper) and omnivorous (tilapia and mas) fish. At present there is no detritivorous fish culture to utilize feed remnants and waste from carnivorous and omnivorous fish culture. Mullet are detritivorous fish that have the potential to be cultivated but it has not been domesticated and the fry cannot be obtained from hatchery, even though these fish can be used as environmental cleaners and sources of protein, therefore mullet has the potential to expose be a new commodity for aquaculture. The research aimed to evaluated stimulation of gonad maturation in mullet (*Mugil dussumieri*) using hormones.

MATERIALS AND METHODS

The experiment design

The method used for research was completely randomized design consisting of three treatments and each individual replication was repeated three times. Two experiments were conducted separately with different treatments. The first experiment treatment used the following mullet length 9–14.7 cm with 17 α -methyltestosterone (MT) 4 mg/kg, estradiol-17 β (E2) 0.07 mg/kg and control physiological solution 0.9% 0.5 ml/kg, the hormone treatment usually used for stimulation of gonad maturation in fish. The second experiment treatment used the following mullet length 10–31 cm with human chorionic gonadotropin (hCG) 750 IU/kg, Ovaprim 0.5 ml/kg and control physiological solution 0.9% 0.5 ml/kg, the hormone treatment usually used for stimulation of gonad maturation, ovulation and spawning in fish broodstock.

The experiment implementation

The experiment period and location

The research was conducted from March to August 2018 in UPTD PAPLWU Sungai Buntu Karawang, West Java, Fish Reproduction and Genetics Laboratory Department of Aquaculture, FPIK IPB and Laboratory of Research Institute for Ornamental Fish Culture, Depok, West Java.

Fish rearing

The fish used were obtained from inlet and outlet drainage ponds in UPTD PAPLWU Sungai Buntu Karawang, West Java. The brackish water pond used are 1000 m² with depth 110–120 cm which are equipped with six unit nets 2×1 meter. Each net contains 13 fish acclimatized to culture for 10 days. Feed given with a formulation of 32% protein content. Percentage of daily feed 1% of the body weight. The feeding frequency given twice a day at 11:00 and 17:00. Mullet cultured for 60 days. Water quality during culture is appropriate and harmless to fish (Table 1).

Table 1. Water quality measurement

Parameters	Range
pH	8.00–9.50
Temperature (°C)	23.30–35.00
Salinity (g/L)	20.00
Dissolved oxygen (mg/L)	6.60–9.50
NO ₂ ⁻ (mg/L)	0.10–0.57
NO ₃ ⁻ (mg/L)	1.20–5.30
NH ₄ ⁺ (mg/L)	0.10–0.60
NH ₃ (mg/L)	0.002–0.02
Total alkalinity (mg/L)	185.00–280.00
Total organic matter (mg/L)	20.20–56.90
Total plankton(cell/ml)	3.50–4.83

Hormone induction

The first step is anesthesia, the fish is given an anesthetic using a fish stabilizer at a dose of 0.3 ml/L, while the fainting fish were measured by the length and body weight. The second step is injection was carried out by determining the dose of the hormone according to the weight of the fish using a 10–100 µL micropipette then injected

intramuscularly into the dorsal muscle according to the treatment dose on day 0 using 1 ml syringe. The third step is recovery, where the injected fish was put into a container with strong aeration for 10 minutes, after the fish has been recovered, put back into the nets culture.

Parameter measurement

Gonadosomatic index (GSI) value

Gonadosomatic index (GSI) is the calculation of the gonad mass as a proportion of the total body mass. The GSI propose is as an alternative way to estimate the gonad maturity. It is presented by the formula.

$$GSI (\%) = \frac{GW}{BW} \times 100$$

Annotation :

GW=gonad weight (g)

BW= body weight (g)

Hepatosomatic index (HSI) value

Hepatosomatic index (HSI) is the calculation of the liver mass as a proportion of the total body mass. It is presented by the formula.

$$HSI (\%) = \frac{LW}{BW} \times 100$$

Annotation :

LW= liver weight (g)

BW= body weight (g)

Egg diameter measurement

Egg diameter is determined from female fish that have stage I–III. Two hundred eggs were taken from the subgonads, which were anterior, middle,

Table 2. Gonad maturity stage in mullet (*M. dussumieri*)

Stage	Male	Female
I	The testicles are like threads, shorter and visible in the abdominal cavity, transparent.	The ovaries are like threads, long to the front of the abdominal cavity, transparent, slippery surfaces.
II	The size of testis is larger, the color white like milk, the shape is clearer than stage I.	The size of ovary is larger, the color is yellowish, the eggs are not clearly visible with the eyes.
III	The surface of the testicles is jagged, the colors are whiter and bigger. In the circumstances preserved the testicles easily break.	Ovaries are yellow, the eggs are clearly visible to the eye.
IV	Like stage III the testicles appear clearer and more dense.	Ovaries grow, eggs are yellow, easily separated, oil grains are not visible. Ovaries fill half to two per third of the abdominal cavity and intestines are pressed.
V	The anterior of the test is deflated and the near part of the discharge contains	Ovaries are wrinkled, thick walls, remaining eggs are located near the release, many eggs like stage II

Table 3. GSI and HSI first experiment

Parameter	Measurement	Control	estradiol-17 β	17 α -methyltestosterone
GSI (%)	Day 0	0.00 \pm 0.00 ^a	0.36 \pm 0.20 ^{ab}	0.43 \pm 0.22 ^{ab}
	Day 30	0.19 \pm 0.19 ^{ab}	0.11 \pm 0.11 ^{ab}	0.17 \pm 0.03 ^{ab}
	Day 60	0.54 \pm 0.20 ^{ab}	1.31 \pm 0.94 ^b	1.00 \pm 0.51 ^{ab}
HSI (%)	Day 0	2.74 \pm 0.10 ^c	2.38 \pm 0.32 ^c	2.12 \pm 0.33 ^{bc}
	Day 30	0.97 \pm 0.21 ^a	0.76 \pm 0.19 ^a	1.13 \pm 0.20 ^a
	Day 60	0.60 \pm 0.28 ^a	1.27 \pm 0.58 ^{ab}	1.24 \pm 0.24 ^{ab}

Note : Different letters on the same line show significant difference affects ($P < 0.05$). The values listed are mean \pm SE.

Table 4. GSI and HSI second experiment

Parameter	Measurement	Control	hCG	Ovaprim
GSI (%)	Day 0	0.42 \pm 0.11 ^a	1.63 \pm 1.16 ^a	0.74 \pm 0.07 ^a
	Day 30	0.28 \pm 0.17 ^a	2.53 \pm 2.07 ^a	0.88 \pm 0.33 ^a
	Day 60	6.72 \pm 0.32 ^{bc}	7.18 \pm 0.59 ^c	3.29 \pm 2.66 ^{ab}
HSI (%)	Day 0	1.27 \pm 0.58 ^a	1.49 \pm 0.50 ^a	1.45 \pm 0.79 ^a
	Day 30	0.67 \pm 0.08 ^a	0.75 \pm 0.23 ^a	0.82 \pm 0.12 ^a
	Day 60	1.22 \pm 0.20 ^a	1.41 \pm 0.10 ^a	1.18 \pm 0.21 ^a

Note : Different letters on the same line show significant difference affects ($P < 0.05$). The values listed are mean \pm SE.

and posterior respectively. Egg sampling was carried out on the day 0, 30 and 60. Measurement of egg diameter using a microscope was equipped with an ocular micrometer that has been tuned in advance with an objective micrometer 10 \times 10 magnification, it was measured using IMAGE-J v1.48 software (National Institute of Health, USA).

Gonad maturity stage analysis

Gonad maturity stage is a grouping of gonad maturity of fish based on changes that occur in the gonads. Gonad maturity stage in mullet is shown in Table 2.

Gonad and liver histology

Testing the effect of hormonal stimulation on gonad and liver cell development was carried out through histological observations. Gonad and liver samples was carried out on the day 0, 30, and 60. The gonads and livers were fixed using a 10% buffer neutral formalin (BNF) fixation solution for 1–2 days. Histological preparation was carried out through stages of fixation, dehydration, clearing, embedding, deparaffination, staining of preparations, and dehydration. The gonads and livers were trimmed treatment and put into plastic tapes to make wax blocks. The wax blocks formed were cut using a microtome machine and placed in a glass object then *hematoxylin eosin*

(HE) staining was done. Histology observation using a microscope was equipped with an ocular micrometer that has been tuned in advance with an objective micrometer 10 \times 40 magnification.

Blood glucose level and blood cholesterol level measurement

Blood glucose and blood cholesterol levels were analyzed using easy-touch kit, by way of paper strips inserted into the easy-touch kit hole, wait a while until the monitor screen displays a choice of blood unit components (mmol/L or mg/dL), taking blood for 3 samples of test fish in each treatment, on the day 0, 30 and 60. Blood was dropped on a paper strip that has been installed on the easy-touch kit, waiting until the monitor displays the results according to the unit selected. According to Bartoňková *et al.* (2016), measuring using a kit was easier and faster with results whose accuracy was not much different from conventional methods, such as spectrophotometers.

Testosterone and estradiol hormone level measurement

Blood plasma was collected from 3 samples of fish in each treatment. Blood plasma was carried out on the day 0, 30 and 60. Anesthetically fish using a fish stabilizer at a dose of 0.3 ml/L then blood was taken from the arteries at the base of the

tail as much as 1 ml using a 3 ml volume syringe that has been given anticoagulants (solution of citrate phosphate dextrose), then centrifuged at 5000 rpm for 15 minutes, blood plasma was taken and stored in a freezer at -20°C and then measured using the Vidas ELISA kit for estradiol (EIA-2693) and testosterone (EIA-1559), then data was analyzed using program *Gen 5* (BioTek® Instruments, Inc.).

Data analysis

The egg diameter, gonad maturity stage, gonad, and liver histology were explained descriptively. Parameters measurement include gonadosomatic index (GSI) value, hepatosomatic index (HSI) value, blood glucose level, blood cholesterol level, testosterone and estradiol hormone level were analyzed statistically using analysis of variance (ANOVA) using the SPSS version 16 program with Duncan's test.

RESULTS AND DISCUSSIONS

Gonadosomatic index (GSI) value and hepatosomatic index (HSI) value

GSI value of the first experiment on day 0 and 30 showed no significant difference, however on day 60 GSI of estradiol-17 β treatment ($1.31 \pm 0.94\%$) was higher than 17 α -methyltestosterone ($1.00 \pm 0.51\%$) and control ($0.54 \pm 0.20\%$). Overall the GSI value in all treatments showed oocyte development from day 0 to day 60. The HSI value of the first experiment on day 30 and 60 showed no significant difference, however HSI value for day 0 was higher than day 30 and 60, result is shown in Table 3.

In the second experiment on day 0 and 30 GSI value showed no significant difference, however on day 60 GSI value of hCG treatment ($7.18 \pm 0.59\%$) was higher than Ovaprim ($3.29 \pm 2.66\%$) and control ($6.72 \pm 0.32\%$). Overall the GSI value in all treatments showed oocyte development from day 0 to day 60. In the second experiment, HSI value for day 0, 30 and 60 showed no significant difference, however HSI value on day 30 decreased and then on the day 60 increased again, result is shown in Table 4. hCG, 17 α -methyltestosterone and estradiol-17 β hormone can increase level of estradiol-17 β hormone in the blood due to the work activity of the hormone FSH (GtH-1). According to Reading and Sullivan (2017) follicle stimulating hormone (FSH) will stimulate theca cells to release testosterone hormone which in turn stimulates granulosa cells to produce estradiol-

17 β hormone. The theca cell layer is affected by gonadotropin to produce testosterone. In granulosa cells, testosterone is converted to estradiol-17 β by aromatase enzyme. The resulting estradiol-17 β is released into the blood then stimulates the liver to carry out vitellogenin synthesis. Vitellogenin is then released back into the blood and will be selectively absorbed by oocytes. The results of vitellogenesis will result in the development of egg diameter and gonad. Vitellogenesis is affected by endogenous and environmental factors such as food, seasonal changes and temperature. The results showed that the GSI and HSI values in the first experiment and the second experiment showed an inverse relationship, if the HSI value increases the GSI value decreases. The HSI value increases because the dose in the treatment is the most appropriate amount to increase the content of gonadotropins secreted by the pituitary, then gonadotropin will stimulate the theca cell layer to produce testosterone. Inside the granulosa layer the testosterone hormone is converted by the aromatase enzyme to estradiol-17 β . The increased level of estradiol-17 β in the granulosa layer will stimulate liver to increase vitellogenin synthesis. This activity will increase the value of the HSI (Reading & Sullivan 2017; Mujtahidah *et al.*, 2016). At the growth stage of hepatocyte cytoplasm, female fish accumulates by granulosa. Nucleus is found in the middle of hepatocytes. Sinusoid endothelium appears between hepatocytes. During the maturation period, the yolk deposition stage occurs in hepatocytes characterized by enlarged size of hepatocytes and increased basophilic cytoplasmic granules, and nucleoli undergo hypertrophy. Hepatocyte binucleate was found to increase and sinusoids with well vascularization (Chakrabarti & Chatterjee, 2014; Rajeshkumar, 2015, Abdallah *et al.*, 2013; Pinto *et al.*, 2010; Tayel *et al.*, 2014; Yilmaz *et al.*, 2016). GSI and HSI in Mullet fish has fluctuative value based on spesies and location (Abdalhafid & El-Mor, 2014; El-Halfawy *et al.*, 2007; El-Boray *et al.*, 2012; Ghaninejad *et al.*, 2010; Hashemi *et al.*, 2012; Karim, 2017; Maryam *et al.*, 2011; Nahdi *et al.*, 2008; Sulistiono *et al.*, 2001; Mokhtar *et al.*, 2015; Saoudi & Aun., 2014; Wigati & Syafei., 2013; Yones *et al.*, 2016).

Egg diameter, gonad maturity stage, gonad and liver histology

Egg diameter, gonad maturity stage, gonad and liver histology in first experiment are presented in Figure 1, Figure 3 and Table 5 while in second

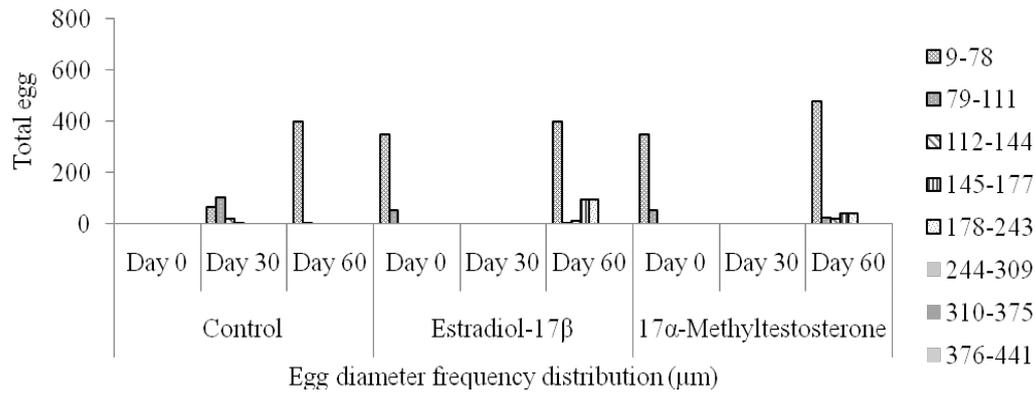


Figure 1. Egg diameter frequency distribution first experiment

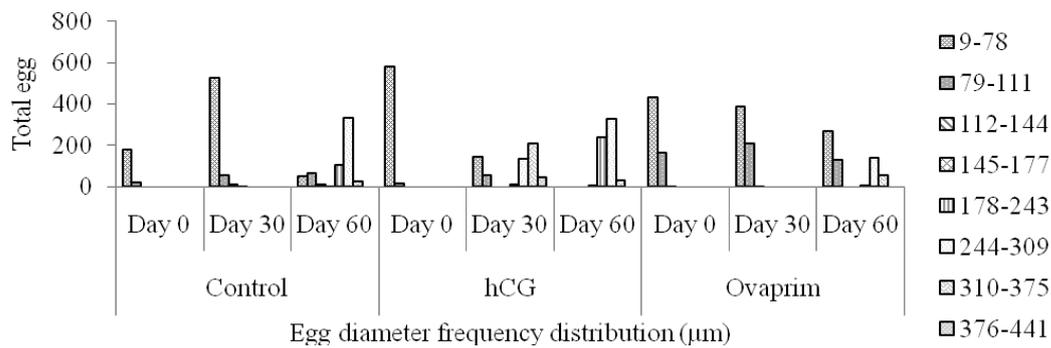


Figure 2. Egg diameter frequency distribution second experiment

Table 5. Gonad maturity stage and oocyte development first experiment

TKG	Oocyte development (%)	Control			Estradiol-17β			17α-Methyltestosterone		
		Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
I	Oogonia	100	100	100	33.33	100	33.33	33.33	100	NF
II	Early perinucleolus	NF	NF	NF	66.66	NF	33.33	66.66	NF	66.66
	Late perinucleolus	NF	NF	NF	NF	NF	33.33	NF	NF	33.33
III	Vacuolization	NF	NF	NF	NF	NF	NF	NF	NF	NF
	Vitelogenic	NF	NF	NF	NF	NF	NF	NF	NF	NF
IV	Germinal vesicle migration	NF	NF	NF	NF	NF	NF	NF	NF	NF
V	Mature yolk	NF	NF	NF	NF	NF	NF	NF	NF	NF

Note : NF (Not Found).

experiment are presented in Figure 2, Figure 4 and Table 6.

In the first experiment egg diameter on day 30 for control showed ranged from 9–144 µm while on day 60 decreased. Egg diameter in estradiol-17β and 17α-methyltestosterone treatment on day 60 showed ranged from 9–243 µm. estradiol-17β treatment on day 60 had larger size than 17α-methyltestosterone and control. In the second experiment egg diameter for hCG treatment on

day 30 showed ranged from 9–441 µm while Ovaprim on day 30 showed ranged from 9–111 µm. Control on day 30 showed ranged from 9–144 µm. hCG treatment had larger size than Ovaprim. hCG hormone can increase egg diameter. hCG contains the estradiol-17β hormone which can stimulate vitelogenesis. Vitelogenesis is the process of induction and synthesis of vitelogenin in the liver in response to estradiol-17β hormone. Vitelogenin produced by liver is released into the

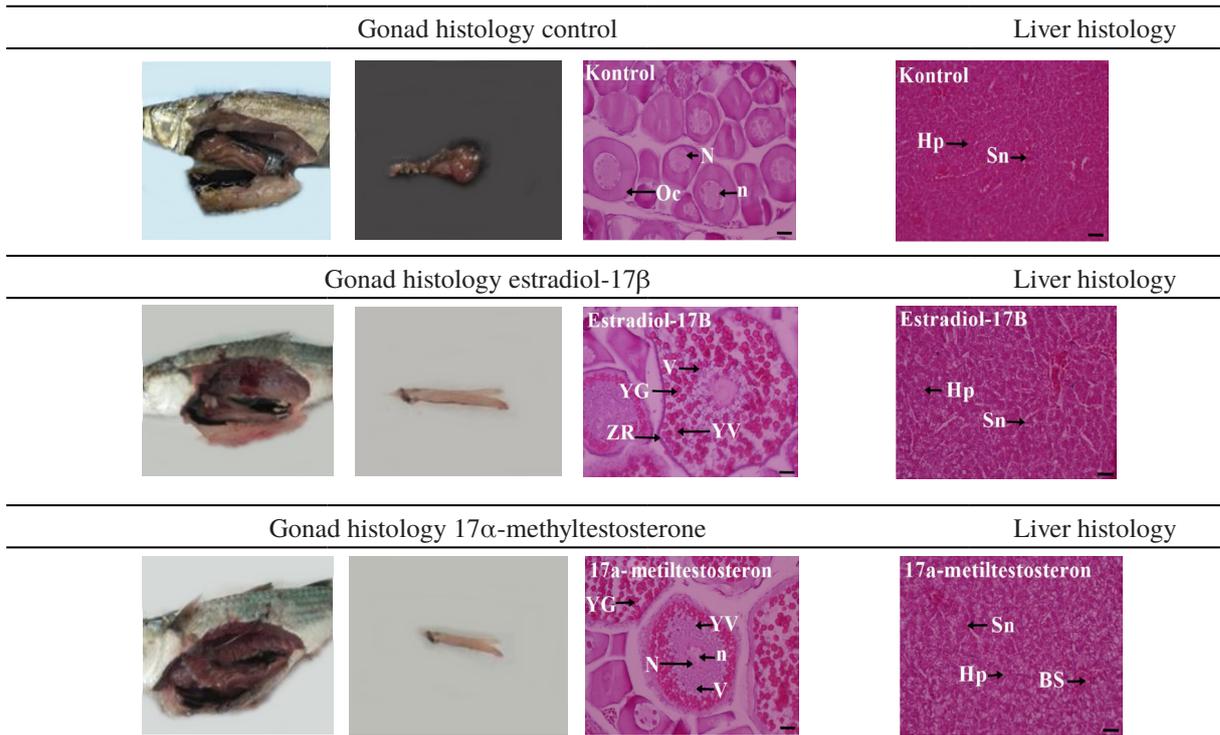


Figure 3. Gonad and liver histology in first experiment on day 60. Gonad histology : oogonia (Og), oocyte (Oc), basofilic cytoplasm (BC), nucleus (N), nucleolei (n), vesicle (V), yolk vesicle (YV), yolk globule (YG), radiate zone (ZR) and epitel folikel (EF). Liver histology : hepatocyte (Hp), sinusoid (Sn), blood sinus (BS). (HE) x 400.

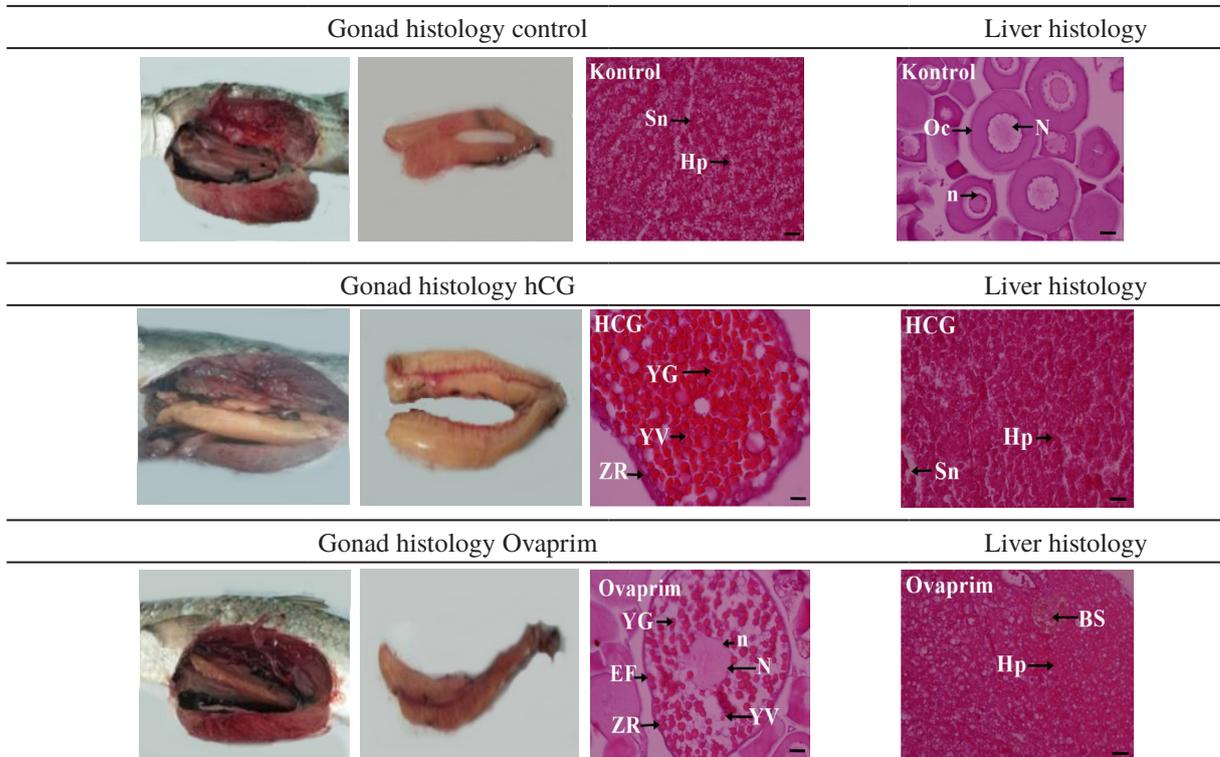


Figure 4. Gonad and liver histology in second experiment on day 30. Gonad histology : oogonia (Og), oocyte (Oc), basofilic cytoplasm (BC), nucleus (N), nucleolei (n), vesicle (V), yolk vesicle (YV), yolk globule (YG), radiate zone (ZR) and epitel folikel (EF). Liver histology : hepatocyte (Hp), sinusoid (Sn), blood sinus (BS). (HE) x 400.

Table 6. Gonad maturity stage and oocyte development second experiment

TKG	Oocyte development (%)	Control			hCG			Ovaprim		
		Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
I	Oogonia	66.66	NF	NF	NF	NF	NF	NF	NF	NF
II	Early perinucleolus	33.33	66.66	NF	100	33.33	NF	100	100	66.66
	Late perinucleolus	NF	33.33	NF	NF	NF	NF	NF	NF	NF
III	Vacuolization	NF	NF	NF	NF	NF	NF	NF	NF	NF
	Vitellogenic	NF	NF	100	NF	66.66	100	NF	NF	33.33
IV	Germinal vesicle migration	NF	NF	NF	NF	NF	NF	NF	NF	NF
V	Mature yolk	NF	NF	NF	NF	NF	NF	NF	NF	NF

Note : NF (Not Found).

Table 7. First experiment

Parameter	Measurement	Control	estradiol-17 β	17 α -methyltestosterone
Blood glucose (mmol/L)	Day 0	1.10 \pm 0.00 ^a	1.10 \pm 0.00 ^a	1.10 \pm 0.00 ^a
	Day 30	1.10 \pm 0.00 ^a	1.25 \pm 0.15 ^a	1.39 \pm 0.29 ^a
	Day 60	1.40 \pm 0.30 ^a	1.10 \pm 0.00 ^a	1.10 \pm 0.00 ^a
Blood cholesterol (mmol/L)	Day 0	18.17 \pm 0.43 ^d	13.39 \pm 2.25 ^c	7.16 \pm 2.92 ^b
	Day 30	2.50 \pm 0.00 ^a	2.50 \pm 0.00 ^a	2.50 \pm 0.00 ^a
	Day 60	2.50 \pm 0.00 ^a	2.50 \pm 0.00 ^a	2.50 \pm 0.00 ^a
Testosterone hormone (pg/ml)	Day 0	593 \pm 61.33 ^a	699 \pm 46.91 ^a	581 \pm 70.82 ^a
	Day 30	593 \pm 37.60 ^a	568 \pm 51.38 ^a	636 \pm 93.99 ^a
	Day 60	532 \pm 63.51 ^a	554 \pm 35.93 ^a	633 \pm 111.95 ^a
Estradiol hormone (pg/ml)	Day 0	67.89 \pm 2.70 ^a	75.19 \pm 10.83 ^a	68.08 \pm 17.23 ^a
	Day 30	52.46 \pm 5.31 ^a	48.87 \pm 5.67 ^a	58.31 \pm 8.00 ^a
	Day 60	51.17 \pm 4.17 ^a	58.72 \pm 11.14 ^a	63.46 \pm 7.33 ^a

Note : Different letters on the same line show significant difference affects ($P < 0.05$). The values listed are mean \pm SE.

Table 8. Second experiment

Parameter	Measurement	Control	hCG	Ovaprim
Blood glucose (mmol/L)	Day 0	1.10 \pm 0.00 ^a	1.10 \pm 0.00 ^a	1.10 \pm 0.00 ^a
	Day 30	1.16 \pm 0.06 ^{ab}	1.42 \pm 0.29 ^{ab}	2.13 \pm 0.13 ^c
	Day 60	1.75 \pm 0.33 ^{bc}	1.23 \pm 0.13 ^{ab}	1.73 \pm 0.32 ^{abc}
Blood cholesterol (mmol/L)	Day 0	7.62 \pm 5.12 ^a	7.44 \pm 4.94 ^a	4.79 \pm 2.29 ^a
	Day 30	2.50 \pm 0.00 ^a	4.09 \pm 1.59 ^a	4.09 \pm 1.59 ^a
	Day 60	5.68 \pm 1.62 ^a	3.72 \pm 1.22 ^a	5.63 \pm 1.62 ^a
Testosterone hormone (pg/ml)	Day 0	619 \pm 97.49 ^{ab}	622 \pm 37.17 ^{ab}	701 \pm 49.62 ^c
	Day 30	580 \pm 38.95 ^{ab}	425 \pm 89.79 ^{ab}	382 \pm 38.91 ^a
	Day 60	684 \pm 113.17 ^{ab}	616 \pm 83.69 ^{ab}	679 \pm 177.91 ^{ab}
Estradiol hormone (pg/ml)	Day 0	48.30 \pm 7.35 ^a	32.35 \pm 12.71 ^a	35.85 \pm 9.36 ^a
	Day 30	42.91 \pm 7.02 ^a	47.79 \pm 21.26 ^a	58.40 \pm 11.59 ^a
	Day 60	611.02 \pm 71.07 ^b	129.03 \pm 72.58 ^a	83.13 \pm 21.95 ^a

Note : Different letters on the same line show significant difference affects ($P < 0.05$). The values listed are mean \pm SE.

circulatory system, then selectively absorbed by oocyte to accumulate into the yolk in the form of lipovitelin and fosvitin. Vitelogenin absorption activity by oocytes causes the egg diameter to increase (Reading & Sullivan, 2017; Isriansyah, 2011; Nuraini *et al.*, 2012; Suriansyah *et al.*, 2011; Yousefian *et al.*, 2009; Putra & Razai, 2017). The results of measurement egg diameter in the Ovaprim treatment increased but when compared to hCG and control was lower. According to Sukendi *et al.* (2016); Adebiyi *et al.* (2013); Ali *et al.* (2015); Aziz & Kaleseran (2017); Jhaharia (2011); Marimuthu *et al.* (2015); Mousavi & Yousefian (2012); Muchlisin *et al.* (2014); Okere *et al.* (2015); Rachimi *et al.* (2015); Rehman *et al.* (2015); Ghanemi & Khodadadi (2017); Rawat *et al.* (2017); Acharjee *et al.* (2017); Achionye-Nzeh & Obaroh (2012) Ovaprim treatment can produce larger diameter eggs. Increased egg diameter is thought to occur because the FSH content increases so that the follicle develops and egg diameter increases.

The stage of oocyte development includes the oogonia stage characterized by small round cells with relatively cytoplasmic clear zone. Oogonia was occurred either solitary or small nests with cell diameter ranged from 10–15 μm . It was weakly basophilic cells with a size larger than the nucleus, nucleus diameter varying from 8–13 μm . The early perinucleolus stage was mostly polygonal oocytes and sizes from 17–38 μm . The nucleus diameter is 12–32 μm . Homogeneous and strongly basophilic cytoplasm is clearly seen. The oocyte membrane was not yet differentiated. Nuclei were arranged in the periphery of the nucleus and varyinacharjeeg number from 2–5. The late perinucleolus stage is the last stage in the immaturation period, the oocyte diameter reached to 150 μm . The nucleus size reached to 50 μm with a numerous number of nucleolei 7–11. The nucleolei arranged in the periphery of the nucleus. The maturation period includes four stages. The first stage is vaculization, characterized by yolk vesicles in the cytoplasm. The vesicle structure appeared empty. Oocyte reached to 150 μm . Nuclear diameter ranged from 60–90 μm . At the end of this stage the yolk vesicle diameter reached to 23 μm . The yolk vesicles were few in number and appeared in the periphery of the cytoplasm. The oocytes were surrounded by zone radiate of about 3 μm in thickness coated with a follicular epithelial layer of 1 μm . The second stage is vitellogenic, this stage is a accumulation proceeded rapidly of yolk and oocyte diameter

ranged to 400 μm and the nucleus appeared with diameter 98 μm in average. The yolk granules were densely packed and occupied mostly the total volume of the cytoplasm of oocyte. The zone radiate increased in thickness varying from 4–9 μm and follicular epithelial layer was 4.5 μm . Vacuoles diameter ranged from 2–10 μm (El-Halfawy *et al.*, 2007; Al bieri & Araújo, 2010; Vazirzadeh & Ezhdehakoshpour, 2014; Mousa, 2010). The third stage germinal vesicle migration and fourth stage of mature yolk were not found in the experiment.

Blood glucose level, blood cholesterol level, testosterone and estradiol hormone level

Blood glucose level and blood cholesterol level, testosterone and estradiol hormone level on day 0, 30 and 60 in first experiment are shown in Table 7.

Blood glucose level and blood cholesterol level, testosterone and estradiol hormone level on day 0, 30 and 60 in second experiment are shown in Table 8.

In the first experiment, glucose level of estradiol-17 β and 17 α -methyltestosterone treatment on day 0, 30 and 60 showed no significant difference. However glucose level on day 30 increased then on day 60 decreased. In the second experiment, glucose level of hCG and Ovaprim on day 0 showed no significant difference. Glucose level on day 30 in Ovaprim treatment was higher than hCG treatment and control. Glucose level on day 60 in Ovaprim treatment decreased. Increased glucose level is indicated release of glucose into bloodstream which will be used to high energy requirements for oocyte development in gonad. Decreasing glucose level is a signal to synthesize and secrete glucagon. Glucose level according to El-Shafei (2017) for 7 days (17.25 ± 2.08 mg/dL), 14 days (17.6 ± 18 mg/dL) and 21 days (17.4 ± 18 mg/dL) while according to Rakovac *et al.* (2008) mean 7.86 mmol/L ranged from 3.23–13.17 mmol/L. According to Martínez-Porchas *et al.* (2009); Hastuti *et al.* (2003); Svoboda *et al.* (2001) increased glucose level caused by increase in glucocorticoids from the release of corticosteroids and catecholamines to overcome high energy requirements during stress.

In the first experiment, cholesterol level of estradiol-17 β and 17 α -methyltestosterone treatment on day 30 and 60 showed no significant difference, however on day 0 cholesterol level was higher than day 30 and 60. In the second experiment, cholesterol level of hCG and

Ovaprim treatment on day 0, 30 and 60 showed no significant difference. Cholesterol level on day 30 decreased then on day 60 increased again. According to El-Shafei (2017) cholesterol level on 7 days culture (54.06 ± 4.50 mg/dL), 14 days (55.06 ± 6.40 mg/dL) and 21 days (56.06 ± 3.52 mg/dL) while according to Rakovac *et al.* (2008) mean 2.66 mmol/L ranged from 1.23–4.22 mmol/L. Cholesterol is a major component of forming steroid hormones, such as progesterone, testosterone and estradiol, which act as the main components of the basic constituent of cell membranes and serve to maintain the permeability of cell membranes and energy reserves for reproductive activity. Cholesterol acts as a precursor to synthesis of steroid hormones in ovarian follicles. Steroid hormones play a role in vitellogenesis and maturation. The addition of the size of the egg diameter caused decrease in cholesterol level at vitellogenesis, but during the maturation process cholesterol level increased, this fluctuation occur because of use cholesterol in steroidogenesis (Reading & Sullivan, 2017). According to Kocaman *et al.* (2005) cholesterol level in *Oncorhynchus mykiss* fish during reproductive period decrease in pre-maturation stage to maturation stage. This showed that there is use of energy reserves for reproductive activities. In the first experiment, testosterone hormone level of estradiol-17 β and 17 α -methyltestosterone treatment on day 0, 30 and 60 showed no significant difference. In the second experiment, testosterone hormone level of hCG and Ovaprim treatment on day 60 showed no significant difference. However, on day 0 testosterone hormone level in Ovaprim treatment was higher than hCG treatment and control, then on day 30 decreased and on day 60 increased again. According to Hefny *et al.* (2016) testosterone hormone level was ranged from 29750 ± 4922 pg/ml to 2690 ± 2807 pg/ml with the lowest 17400 ± 1054 pg/ml and the highest 30500 ± 2037 pg/ml. According to Kumar *et al.* (2015) 938 ± 7.87 pg/ml while according to Yelghi *et al.* (2012) 11 pg/ml, 70 pg/ml and 370 pg/ml. According to Reading and Sullivan (2017) increased testosterone hormone level can be affected by FSH which acts on the theca cell layer as the main regulator of estradiol production. The induction of treatment hormones is thought to stabilize the aromatization of testosterone to estradiol due to this process an increase in the level of estradiol in blood plasma and available in sufficient quantities can stimulate the liver to synthesize vitellogenin

for oocyte growth to maximum size. In the first experiment, estradiol hormone level of estradiol-17 β and 17 α -methyltestosterone treatment on day 0, 30 and 60 showed no significant difference. In the second experiment, estradiol hormone level of hCG and Ovaprim treatment on day 0, 30 and 60 showed no significant difference, however on day 60 estradiol hormone level in control was higher than hCG and Ovaprim treatment. Estradiol hormone level in the treatment and control increased from day 0 to day 60 with the highest increased in control. According to Hefny *et al.* (2016) estradiol hormone level was 199 ± 87 pg/ml and 102 ± 16 pg/ml, Kumar *et al.* (2015) 323 ± 13 pg/ml, Chang *et al.* (1995) 547.3 ± 19.3 pg/ml, 533.1 ± 54.1 pg/ml and 1354.1 ± 297.0 pg/ml and Yelghi *et al.* (2012) 1800-3600 pg/ml. Increased estradiol hormone level result in stimulation of vitellogenesis which accelerates gonad development (Zahri *et al.* 2018; Sinjai *et al.*, 2014).

CONCLUSION

The results showed that estradiol-17 β and 17 α -methyltestosterone induction in mullet in size 9–14.7 cm increased gonad maturity to stage II while hCG induction in mullet in size 10–31 cm increased gonad maturity to stage III.

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