Analysis of Male Urine as Pheromone to Increase Reproduction in Female Tiger Grouper (*Epinephelus fuscoquattatus*)

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Abstract. The aim of this study was to identify the steroid hormone compound in the urine of male tiger grouper, and the role of steroid hormone suspected as pheromone of the male fish to induce gonad maturity of female tiger grouper. The methods employed in this study included broodstock maintenance, the collection of male urine samples, pheromone analysis of male urine, and application test of urine for gonad maturity of female tiger grouper. The analysis of pheromone content in urine was performed by using LC-MS/MS and FTIR methods. Urine was applied for several females placed in concrete tanks with a volume of 12 tons, 4 females for each tank. The parameters tested were male urine dose: 1 ml, 2 ml, and 3 ml. The results of pheromone analysis using LC-MS/MS method in urine and sperm of male tiger grouper indicated a peak of ion percussion of 255 ms and 273 ms, while the ion products showed 159 F and 255F. Based on the result of FTIR test, it could be seen that the resulted bands were infrared absorption which had certain character patterns, in which urine had six infrared spectra; this steroid was classified in alcohol group β-sitosterol. The role of urine as a pheromone carrier administered to female was observed through the concentrations of estradiol-17β hormone and testosterone in blood plasma. The results showed that the concentration of estradiol-17β was much higher when compared with testosterone, either before or after treatment. The high concentration of estradiol was related to the stages of egg development in the female before the spawning season. From the results of this study, it could be concluded that male urine of tiger grouper was a pheromone that could increase the stimulation and gonad maturity of female broodstock.

Keywords: Tiger grouper, urine, pheromone

1. Introduction

Tiger grouper is reproductively matured firstly as female, then turned into a functional male fish [1]. This fish is spawned in groups. A number of male and female fish excrete sperm and eggs concurrently in a suitable environment. A large number of eggs is allowed to drift in the open water, carried away and floated by the current turbulence.

Fish uses olfaction to find a partner for spawning. Odor stimuli promote maturation of gonad and induce the spawning process. Sexual aroma is widely used by male fish as a chemical marker [2,3]. The male goldfish will increase the activity of swimming when they find the smell of female estrogen [4].
In response, mature males will emit odors from androstenedione (an androgenic steroid secreted in large quantities by other males), resulted in high aggressive characters [5].

At the time of the process prior to and during spawning, teleost fish will respond to the odor signal of the sender by the increase in the development of gonad and the changes of hormonal that induce gamete maturation [6,7]. Natural chemical compounds in the odor are steroid gonads, prostaglandins, or precursors and their metabolites, which are a type of hormonal pheromone [7].

Pheromones can affect the social behavior of fish, to distinguish between two fish of the same species and the pattern of fish behavior when there is a competition [8]. Maruska and Fernald [9] suggested that in the dominant A. burtoni fish, there is a function of pheromone in its social behavior, in which pheromone is in its urine. This suggests that the release of urine from dominant males serves to suppress other nearby dominant males. The dominant male O. mossambicus is also proven to store urine and then release it to actively demonstrate their dominance status to show the rival male in an area. Chemical signals of urine are used by males against other males. This suggests that, in addition to visual signals such as discoloration (for example, darkened eye lines during competition), males also take advantage of chemical communications to convey social, territory, or dominance levels.

The importance of the pheromone function in the reproductive cycle of fish is inseparable from the olfactory system function of the fish. The olfactory system of the fish is able to detect and distinguish between thousands of odorants because the smell receptors are functioned in a combinatorial way. The fish detects the presence of an odor receptor in the form of a chemical stimulus. The stimulus through the nostril is changed in the form of electrical signals derived from the movement of cilia which then passes an olfactory lamella in the form of a rosette. The signals produced on the lamella olfactory are passed in the olfactory bulb and olfactory tract which are then translated to the telencephalon brain. The olfactory receptor cell is the main sensitive cell that is the nerve cell (sensory neuron). The receptor cells have thick dendrites, derived from the nuclear part of the neuron and reach the epithelial surface. The apical part of the dendrite ends at the rounded end (knob) that projects the epithelial surface [10].

Female fish that are ready to spawn usually will release pheromone so it can attract the presence of male fish. This mechanism is used by fish to defend its territory from foreign fish. In goldfish, sex pheromone is also used by male fish to distinguish the mature female with the immature female. They showed an increase in motion activity and increased the concentration of gonadotropin II in plasma in response to the presence of sex pheromone 17alpha, 20beta-P in the waters. However, the fish swimming speed did not change. Furthermore, it is said that the concentration of 17alpha, 20beta-P pheromone sex released by goldfish decreases shortly before ovulation [11].

Preovulated pheromones of the female goldfish are a mixture of androstenedione (AD), 17α, 20β-dihydroxy-4-pregnene-3-one (17, 20β-P), and 17, 20β-P-20β-sulfate (17, 20β-PS), in which the ratio of steroids changed dramatically during luteinizing hormone pre-ovulation (LH). In ovulation (12 hours after the increase in LH), the pre-ovulatory pheromone drops dramatically, and the oviductal oocytes stimulate prostaglandin F2α synthesis (PGF2α) which acts as hormones to trigger female sexual behavior. PGF2α and 15-keto-PGF2α are excreted in the form of urine [7,12, 13].

On the basis of some of the above descriptions, the purpose of this study was to identify the steroid hormone compound in the male urine of tiger grouper, and the role of steroid hormone suspected as pheromone of male fish to induce gonad maturity of female tiger grouper.

2. Methods

2.1 Analysis of pheromone content in urine using LC-MS/MS method

Isolation of pheromones refers to Yambe et al. [14] with modifications; ±10 ml of urine was collected from several tested fishes. Then, it was added with distilled water at the same volume and soaked with diethyl ether or ethyl acetol n-hexane with the ratio (1 : 1). Ten microliters of dissolved urine sample were injected into Pro C18 RS YMC-Pack column (150 × 2.0 mm, particle size 5 m, Japan). The samples were delivered at a flow rate of 200 ml/min. The mobile phase comprises 0.1% formic acid in distilled
water and 2 mM ammonium acetate with methanol : acetonitrile (2 : 1 v/v). The separation of LC was done with a linear solvent gradient program with LC running time of 15 minutes.

The LC system was connected directly to a quadrupole stage of three TSQ Quantum Ultra Mass spectrometers (Thermo Scientific, USA) equipped with ionizing-electro-spray ionization (H-ESI) probe. The mass spectrometer run in negative ion mode ([MH]-) and was used as a means of ionization with a 350°C capillary temperature, a 5.0 kV spray voltage, an evaporating temperature of 250°C, a gas-sheathing pressure of 60 psi, and an additional gas pressure of 40 psi.

2.2 Analysis of urine using FTIR method

Urine was sterilized using a 0.2 μm Milipore tool (Balerica, MA), rinsed first using 20 ml of ethanol and 60 ml of distilled water then passed to a polystyrene resin column (1 g TSK-G3000S; Tohs Chemical, Tokyo, Japan) 30 ml/min. The columns were rinsed using 20 ml of ethanol 50%, 80% and absolute sequentially. Next, it was extracted on the Sephadex LH-20 column (10 x 500 mm; Amersham, Piscataway, NJ) with 50% ethanol followed by 70% ethanol of 0.5 ml/min. Subsequently, the samples were placed into 5C18-AR-II chromatography columns (4.5 x 250 mm) Waters’ Sep-Pak® cartridges, Waters Corporation, Milford, MA, USA) which were fed 0.2 ml/min methanol 28%.

Analysis using Fourier Transform Infrared was done with modification of the research that had been done by Schutze [15], every 1 ml of the urine sample of the test fish was placed in a microtube and centrifuged at 3000 rpm for 2 minutes. The supernatant was discarded, while the pellet obtained was added with 2 μl PBS. The sample was placed in an attenuated total reflection (ATR). Prior to channeling to the infrared ray pathway, the sample was treated by automatic evaporation of CO2/H2O for 10 minutes. Further, the spectrum was measured using FTIR spectrometer at wavelength 400-4000 cm-1. To analyze and display infrared spectrum, Perkin-Elmer software (PerkinElmer, Norwalk, CT, USA), and Omnic (Thermo-Nicolet, Madison, WI, USA) were used. Observations were made on the appearance of the top of the graph and then the image was stored.

2.3 Application of urine and sperm for gonad maturation of female

Urine was applied to some female broodstocks, by spraying it into the rearing water. The gonadal maturity of the female was observed first, by way of cannulation. The female broodstocks were placed in a concrete tank with a volume of 12 tons. Each tank was stocked with 4 female broodstocks. The sizes of the broodstocks used as the test fish were 55 – 59 cm in length and 4 – 4.5 kg in weight. The parameters tested were male urine dose of 1 ml, 2 ml, and 3 ml. The male urine spraying was done at night (19:00 pm). During 6 hours of urine administration, the rearing water was recirculated, with the aim that urine was not wasted. Observations were done for female activities and gonad maturity. These observations were carried out for 6 days before the dark moon until the peak of the dark moon. Experimental design in this research was a completely randomized design (CRD).

2.4 Observation of hormone profile in blood plasma

The measurement of hormone concentration in blood was done at the beginning and the end of treatment. The fishes were first anesthetized using anesthesia MS222 to collect the blood sample. The blood was taken from an unconscious fish at the base of the tail as much as 1 ml using a 3 ml syringe capacity that had been added with anti-coagulant (citrate-phosphate-dextrose solution), then, put in microtube volume of 1.5 ml and stored in a cool box. The blood that was accommodated in the microtube was centrifuged at 10,000 rpm for 10-50 minutes. A blood plasma supernatant was taken and moved into a new microtube and stored in a freezer at a temperature of minus 4 °C [16].

The measurement of hormones was carried out using ELISA method [17]. The measurement of blood hormone levels was done using Vidas ELISA kit for 17-estradiol (REF 30 330) and testosterone (REF 30-406) (BioMarieux, Inc., France). Blood steroid measurements were performed using ELISA reader at 405 nm wavelength for estradiol17β and 450 nm for 11-KT testosterone. The principle of the western blot method used the method of Towbin et al. [18] using the Protein DetectorTM kit (KPL, USA).
Furthermore, the resulted gel sheet of SDSPAGE containing the protein bands was transferred to the nitrocellulose paper using a semi-dry blotter.

2.5 Observations on gonadal maturity stages of female tiger grouper
Egg sampling was carried out by cannulation. The samples were collected before and after the treatment of urine and male sperm. The eggs obtained were stored with bouin solution which was then processed for histological examinations. The results of histological examinations were used as parameters to determine the level of gonadal maturity.

2.6 Histological examination
The egg samples obtained from the cannulation were fixed in neutral buffered formaldehyde solution for 48 h, then were dehydrated in a 70%, subsequently to an absolute alcohol solution, alcoholized in xylol, infiltrated in xylol-paraffin mixture and embedded in paraplast (Sigma p3858). Then, sectioned transversally with 3 μm thickness and colored with hematoxylin and eosin solution. A histological observation was done for egg’s diameter using a microscope [16].

3. Results and Discussion

3.1 Analysis of urine content
The results of analysis using LC-MS/MS in urine of male tiger grouper showed some peaks in the curve (Figure 1). These results showed the ion percussion 255 ms and 273 ms. Meanwhile, the ion products showed 159F and 255F. Based on mass bank database, these products showed similarity with literature, based on Hauser et al. [19], that were 17β-Estradiol compounds, Androstenediol, Épiandrosterone, Épietiocholanolone, Etiocholanolone, and androsterone.

| Class of analytes | Analyte                | Retention time (min) | Precursor ion (m/z) | Product ion (F) |
|-------------------|------------------------|----------------------|---------------------|-----------------|
| Oestrogens        | 17 β-Estradiol         | 2.93                 | 255                 | 159             |
|                   | Androstenediol         | 4.42                 | 273                 | 255             |
|                   | Épiandrosterone        | 4.42                 | 273                 | 255             |
| Androgens         | Épietiocholanolone     | 4.42                 | 273                 | 255             |
|                   | Etiocholanolone        | 4.42                 | 273                 | 255             |
|                   | Androsterone           | 4.42                 | 273                 | 255             |

The results of steroid hormone compound analysis contained only two groups of Estrogen and Androgens, each with an abundance of 4369 and 43739, with respectively a resistance time of 2.93 minutes and 4.42 minutes (Table 1). The molecular structure of the hormones present in the urine can be seen in Fig. 2.
Figure 1. The results of analysis on the urine of male tiger grouper using LC-MS/MS method

Figure 2. Chemical compounds found in the urine and sperm of male tiger grouper
3.2 The results of visual observation on female behavioral during the administration of urine and male sperm

Visual observations on female tiger grouper behavior administered with urine and sperm of male tiger grouper can be seen in the table below. The female tiger grouper treated with male urine and sperm showed different behavior when compared with control (Table 2).

Table 2. Female behavior during the administration of male urine and sperm

| Female behavior                  | Urine administration | Control |
|----------------------------------|----------------------|---------|
| More aggressive                  | √                    | -       |
| Irregular swimming patterns      | √                    | -       |
| Decreased appetite               | √                    | -       |
| Enlarged genital hole            | √                    | -       |

The behavior of female fish on two days after the administration of urine and sperm began to show response, that was more aggressive movement and irregular swimming patterns. Observations on the fourth day showed that the appetite of female was decreased, and on the sixth day, there was a visible appearance of the enlarged genital hole.

3.3 Results of hormone analysis in blood plasma after urine administration

3.3.1 Estradiol-17β

Based on blood plasma test in female fish, it was known that the urine administration could increase the concentration of hormone estradiol-17β. This can be seen in the blood plasma test results before and after treatment (Table 3).

Table 3. The concentrations of estradiol-17β (pg/ml) hormone in the blood of female tiger grouper treated with male urine

| Treatment | Replication | Before treatment | After treatment | Enhancement |
|-----------|-------------|------------------|-----------------|-------------|
| Control   | 1           | 97.031           | 130.433         | 33.402      |
|           | 2           | 99.864           | 122.509         | 22.645      |
|           | 3           | 105.683          | 137.887         | 32.204      |
|           | 4           | 75.877           | 100.555         | 24.678      |
| A         | 1           | 85.08            | 183.298         | 98.218      |
|           | 2           | 98.871           | 152.071         | 53.20       |
|           | 3           | 100.77           | 169.276         | 68.506      |
|           | 4           | 110.256          | 201.28          | 91.024      |
| B         | 1           | 148.892          | 225.552         | 76.66       |
|           | 2           | 95.745           | 197.906         | 102.161     |
|           | 3           | 108.792          | 205.922         | 97.13       |
|           | 4           | 86.565           | 198.842         | 112.277     |
| C         | 1           | 122.576          | 232.025         | 109.449     |
|           | 2           | 92.698           | 215.54          | 122.842     |
|           | 3           | 119.755          | 224.465         | 104.71      |
|           | 4           | 87.071           | 158.077         | 71.006      |

The results showed that in controlling the fish, there was an increase in hormone of 28.232 (pg/ml) with a range of 22.645-33.402 (pg/ml) while with 1 ml of urine administration, it was found an increase 77.737 (pg/ml) or 2.75 times compared to the controls with a range of 53.2-98.218 (pg/ml). The treatment of urine as much as 2 ml had the ability to increase hormone 97.057 (pg/ml) or 3.48 times from the control with the range of 97.13-112.277 (pg/ml).
The treatment with urine administration of 3 ml could increase the hormone 102.002 (pg/ml) or 3.61 times from the control with the range of 71.006-122.842 (pg/ml). Based on the blood plasma test of each treatment, it was found that administration of 3 ml of urine had the highest ability to increase the concentration of estradiol-17β hormone and the lowest yield resulted from the control treatment. Based on the analysis of variance, the potency of urine administration in increasing estradiol-17β hormone in female fish showed a very significant different effect (highly significant) between treatments. The result of ANOVA showed that urine administration had a highly significant effect on the concentration of estradiol-17β hormone in female fish.

Based on the least significant difference test results, the control treatment had the lowest increase in hormone concentration. Then, between treatments did not have a noticeable difference in triggering an increase in estradiol-17β hormone concentration. To know the relationship between treatment of urine in the rearing water and the concentration of hormone estradiol-17β, regression analysis was used. The regression form used was linear because the difference is very real and the equation \( y = 240.63x + 401.63 \).

In the regression analysis, there were two commonly used coefficients namely determination (R²) and correlation (r). In this study, the coefficient of determination obtained (R²) = 0.68 means that 68% increase in estradiol-17β hormone concentration in female blood was affected by the dose of urine sprayed into the rearing water. In this study, the obtained correlation coefficient was 0.83 (close to 1), means that the concentration of hormone estradiol-17β had a high correlation with the dose of urine. The highest increase in estradiol-17β hormone in the female blood was found in the treatment of urine of 3 ml into 4 tons of rearing water (Fig. 3).

![Figure 3](image_url)  
**Figure 3.** The relationship between doses of urine administration in rearing water with increased concentrations of an estradiol-17β hormone

### 3.3.2 Testosterone

Testosterone hormone of the control fish increased by 0.1737 (pg/µl) in the range of 0.90-0.356 (pg/µl) whereas with 1 ml of urine administration, there was an increase in hormone 0.1662 (pg/µl) or 0.96 times compared to controls in the range 0.77-0.339 (pg/µl) (Table 4). Treatment of urine as much as 2 ml had the ability to increase hormone 0.1417 (pg/µ) or 0.82 times from control with the range 0.61-0.374 (pg/µ). Treatment with 3 ml of urine was able to increase hormone 0.1477 (pg/µ) or 0.85 times from control with the range of 0340-0.393 (pg/µ). Based on blood plasma test from each treatment, it
was known that control fish had the highest ability in increasing testosterone hormone concentration and the lowest result from treatment of 2 ml urine.

Table 4. The concentration of testosterone (pg/μl) hormone in the blood of female tiger grouper after male urine administration

| Treatment | Replication | Before treatment | After treatment | Enhancement |
|-----------|-------------|------------------|----------------|-------------|
| Control   | 1           | 0.328            | 0.482          | 0.154       |
|           | 2           | 0.411            | 0.420          | 0.90        |
|           | 3           | 0.390            | 0.566          | 0.176       |
|           | 4           | 0.266            | 0.622          | 0.356       |
| A         | 1           | 0.342            | 0.438          | 0.96        |
|           | 2           | 0.290            | 0.629          | 0.339       |
|           | 3           | 0.502            | 0.579          | 0.77        |
|           | 4           | 0.394            | 0.547          | 0.153       |
| B         | 1           | 0.421            | 0.487          | 0.66        |
|           | 2           | 0.255            | 0.629          | 0.374       |
|           | 3           | 0.322            | 0.388          | 0.66        |
|           | 4           | 0.521            | 0.582          | 0.61        |
| C         | 1           | 0.278            | 0.671          | 0.393       |
|           | 2           | 0.437            | 0.549          | 0.112       |
|           | 3           | 0.439            | 0.521          | 0.82        |
|           | 4           | 0.348            | 0.388          | 0.40        |

Based on the analysis of variance, the potential of urine to increase the hormone testosterone in female fish did not show significant different effects between treatments, as well as in the control. This was indicated by the value of F arithmetic which was smaller than the value of F table with the degrees of freedom 12 and probability 5% or 1%. Consequently, there was no need for further tests to find the least significant difference, since all treatments had almost the same effect.

3.4 Observation on the egg of tiger grouper

Table 5. shows that the overall eggs of the tested fish had increased in diameter by the dark moon. This occurred in controlled fish and in the absence of urine; the diameter of the egg samples were originally 441.375 μm but they were enlarged to 547.625 μm when observed at day 6. Then, 6 days post urine administration of 1 ml (treatment A) into the rearing media, the fish egg diameter increased to 746.375 μm from the initial size of 444 μm. The female broodstocks treated with 2 ml of urine (B) had an increase in egg diameter from 441.375 μm to 752.625 μm. The fish with the addition of 3 ml of urine (C) in the rearing media also had the same response that the egg diameter increased from 438.875 μm to 765.125 μm.

The result of ANOVA test showed that control treatment was significantly different from the treatment A, B, and C. Treatment A was significantly different from treatment B and C. The results of this test also indicated that treatment B and C gave a better result from treatment A or control.
Table 5. The diameter of the egg before and after treatment with the male urine of tiger grouper

| Treatment | Replication | Urine administration | Enhancement (μm) |
|-----------|-------------|-----------------------|-----------------|
|           |             | Before (μm) | After (μm) |             |
| Control   | 1           | 515         | 620         | 105          |
|           | 2           | 420         | 525         | 105          |
|           | 3           | 420         | 460.5       | 40.5         |
|           | 4           | 410.5       | 585         | 174.5        |
| Average   |             | 441.375     | 547.625     | 106.25       |
| A         | 1           | 515.5       | 755         | 239.5        |
|           | 2           | 420         | 760         | 340          |
|           | 3           | 435         | 710.5       | 275.5        |
|           | 4           | 405.5       | 760         | 354.5        |
| Average   |             | 444         | 746.375     | 302.375      |
| B         | 1           | 520         | 780         | 260          |
|           | 2           | 410         | 720.5       | 310.5        |
|           | 3           | 425.5       | 765         | 339.5        |
|           | 4           | 410         | 745         | 335          |
| Average   |             | 441.375     | 752.625     | 311.25       |
| C         | 1           | 510.5       | 770         | 259.5        |
|           | 2           | 420         | 755.5       | 335.5        |
|           | 3           | 410         | 795         | 385          |
|           | 4           | 415         | 740         | 325          |
| Average   |             | 438.875     | 765.125     | 326.25       |

The results of histological examination on the egg after urine treatment can be seen in Figure 4.

![Figure 4. Histological observation on the egg of tiger grouper](image-url)
The response of the female mother given urine and sperm was immediately visible, where the female parent tried to find the source of urine given. This showed that the released compound was sensed by the Vomeronasal organ (VNO) and then this signal would be forwarded to the hypothalamus to provide a response/responses. The time needed to respond to the response was only a tenth of a thousand seconds, then there would be a response from the brain through psychological changes, such as an increase in the hormone gland and the action of the production of the hormone testosterone (in males) or the estrogen hormone (in females).

Pheromone that is a composite of certain chemical compounds naturally produced by individuals can induce specific and adaptive responses for other individuals. The suspected pheromone compounds in urine and sperm of male tiger grouper were a combination of several compounds namely 17β-Estradiol, Androstenediol, Epiandrosterone, Epieiotcholanolone, Etoiocholanolone, and Androsterone. The stimuli used by minnow fish are hypoxanthine-3-N-oxide, although the explanation of olfactory activity of these compounds as well as their presence in fish has not been demonstrated that the hypoxanthine-3-N-oxide compound is one of the complex components associated with the response to stimuli. The response of 17, 20β-P in carp female is by increasing ovulation.

In tilapia, the male is known to release urine more frequently before the spawning process. The dominant male bladder is larger and more muscular than other males, allowing for greater storage of urine volume. Keller-Costa et al. have identified 5β-pregnane-3α, 17α, 20β-triol-3α-glukuronida and 5β-pregnane-3α, 17α, 20α-triol-3α-glucuronide in male urine, and its concentration depends on the social status of the fish and which also acts as a strong pheromone in females.

The excreted urine of male generated an increase in 17,20β-P in the maturation of female oocytes, whereas the prevention of urine effluents was fewer in the confluence of male fish with other males. Colombo et al. reported that the etiocholanolone glucuronide has a function as a male pheromone in black goby fish (Gobius niger or Gobius jozo) and van den Hurk and Lambert stated that steroid glucuronides compounds serve as female sex pheromones in zebrafish. Higher steroid levels in urine (11-ketotestosterone, 11-KT) follow social interactions in some species of cichlid fish. Glucuronides steroids have been shown to be pheromones that play a role in the reproduction of several species of fish.

Six days before the dark moon were selected as the time of research to know the regulation of reproduction hormone found in grouper fish. Groupers breed when the moon does not shine brightly between the 25th and 5th days of the following month. As revealed by Garcia et al. that the formation of steroid hormones runs more actively when entering the spawning season compared to the normal days. The role of urine as a pheromone carrier administered to female broodstock was observed through the concentrations of estradiol-17β hormones and testosterone in blood plasma. The results showed that estradiol-17β hormone was obtained in a much higher concentration when compared with testosterone, either before or after treatment. The high estradiol hormone was related to the stages of egg formation in female fish before the spawning season.

Based on the above information, the high concentration of estradiol-17β hormone until the end of the study or 6 days before the dark moon indicated that female broodstock was undergoing vitellogenesis. This was also supported by the increase in diameter of the egg with the cytoplasm that appeared filled with egg yolk grains (Figure 17). Vitellogenesis is the longest stage in oogenesis associated with the formation of protein precursors by liver organ after obtaining stimulation of estradiol-17β carried through the bloodstream. Furthermore, this protein will be carried by the bloodstream and internalized into oocytes through specific receptors or micropinocytosis. In oocytes, vitellogenin is further processed into smaller yolk proteins that will be used as food reserves for the embryo.

The development of diameter in the treatment of urine showed that the pheromone contained therein gave a positive effect on the gonadal maturity level of the female. The results of the relationship between estradiol-17β hormone levels and the development of eggshell diameter had a linear response pattern and positive correlation. The increased estradiol-17β hormone in the blood occurred along with the increase in egg diameter. The association between estradiol17β hormone concentrations in blood plasma
with the changes in the gonadal development is an important indicator in understanding the role of hormones in the process of fish reproduction [40].

4. Conclusion
The pheromone compounds found in the male urine of tiger grouper were 17β-Estradiol, Androstenediol, Epiandrosterone, Epieiocholanolone, Etochoanalolone, and Androsterone. The response of the female tiger grouper to the male urine was an increase in the hormone content of 17β-Estradiol in the blood plasma during 6 days of research at the time of the dark moon. Pheromones found in male urine and male sperm could increase gonadal maturity level of female broodstock characterized by an increase in egg diameter

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