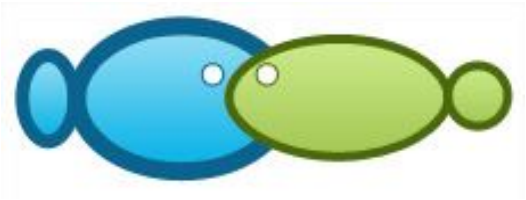

The application of thyroxine hormone and *Melastoma malabathricum* leaf extract as stimulators in gonadal maturation of *Scylla serrata* in traditional ponds

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Abstract. This study aims to evaluate the application of thyroxine hormone and leaf extracts of *Melastoma malabathricum* injection on gonadal maturation of female *Scylla serrata* broodstock reared in traditional ponds. This study used mangrove crab (*S. serrata*), the weight of female mangrove crabs used was ± 200 g / individual, which has entered the second ovary maturity level. The number of female mangrove crabs used was 36 individuals. The experiment consisted of three treatments in triplicates, i.e. group A (control), group B (thyroxine hormone injection at 0.1 µg g^{-1} body weight), and group C (*M. malabathricum* leaf extract injection at 0.01 mg g^{-1} body weight). The results showed that the administration of thyroxine hormone and *M. malabathricum* leaf extract significantly accelerated gonadal maturation process of *S. serrata* compared to the control. Thyroxine supplementation provides faster ovarian development than other treatments. The highest GSI value was obtained in the thyroxine hormone treatment, 20.5±1.53, then in the injection of *M. malabathricum* leaf extract, 18.1±1.00, and the lowest was the control treatment which was 15.2±0.58. In conclusion thyroxine hormone and *M. malabathricum* leaf extract injection can be used as an alternative strategy to accelerate gonadal maturation of *S. serrata.*

Key Words: steroids, phytosteroids, maturation, pond, ovaries.

Introduction. Mud crab is a coastal fisheries commodity that has a high economical value in North Kalimantan, Indonesia. To present, the demand of mud crab for both national and international market is mostly fulfilled by fishing of wild specimens. The massive exploitation of mud crab has threathened the sustainability of its resources. Generally, fishing activity of mud crab caught all sizes of crab including egg-bearing females. As the demand of mature broodstock is higher, the catch of mud crab at this size from the wild has been done intensively causing significant reduction in the wild population (Iromo et al 2018). One of the strategies to alleviate this problem is by aquaculture of mud crab. However, mud crab aquaculture also still relies on mature broodstock obtained from fishing activity. Furthermore, the availability of mature mud crab broodstock in the wild can be limited depending on the season (Farizah et al 2017). Sufficient supply of mature broodstock is an essential key in hatchery production. Gonadal maturation in cultured crabs has done by using three methods that are nutritional approach, hormonal approach, and environmental approach.

Hormonal approach has been commonly applied in gonadal maturation in crustaceans (Pamuru 2019). One of the hormones that play a role in the reproductive process is thyroxine. This hormone has an essential role in early development of ovary. Thyroxine can be found in female and male mud crabs, and its concentration increases with the maturity level of ovary (Iromo et al 2014). This hormone has been considered to have significant roles in egg yolk absorption, feed efficiency and growth rate

improvement. The application of this hormone has been demonstrated to accelerate gonadal maturation and the survival of mud crabs (Iromo et al 2015). Besides thyroxine hormone, previous studies showed that natural steroids such as those originating from plants could accelerate gonadal maturation process in fish and crustaceans (Dhas et al 2017; Farizah et al 2017; Alam et al 2019). For instance, *Melastoma malabathricum* leaf extract significantly affected the acceleration of ovary maturation in *Scylla olivacea* (Farizah et al 2017). Based on previous studies, it can be hypothesized that both thyroxine and *Melastoma* leaves could accelerate ovarian maturation process in crustacean. In this context, the present study aimed to evaluate the effect of thyroxine and *M. malabathricum* leaf extracts injections on ovarian maturation process of *Scylla serrata* reared in traditional ponds.

Material and Method. The research was conducted for 20 days on February 2020 in traditional ponds, located in Lingkas Ujung, Tarakan, North Borneo, Indonesia (Figure 1).

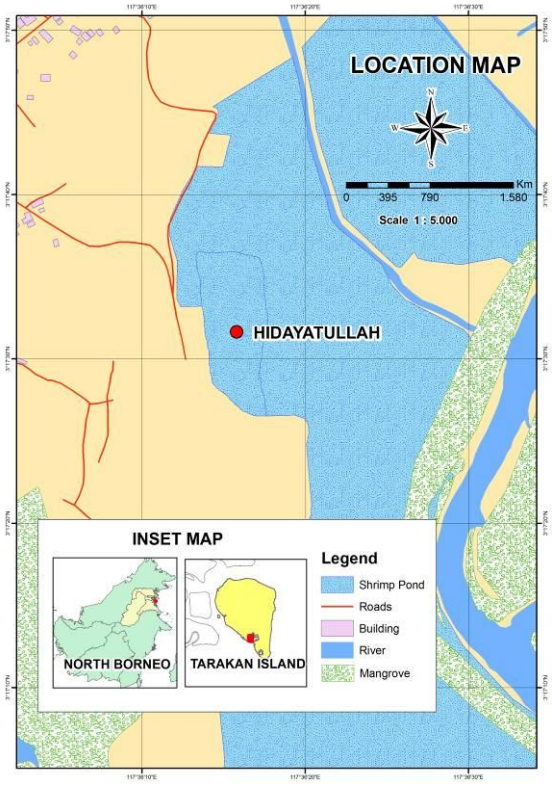

Figure 1. The location of traditional ponds.

Ovarian development. Ovarian development was observed at Aquaculture Laboratory, Faculty of Fisheries and Marine Sciences, University of Borneo Tarakan. Water quality parameters were observed both *in-situ* and *ex-situ.* Water quality analysis was performed at the Water Quality Laboratory, Faculty of Fisheries and Marine Sciences, University of Borneo Tarakan. The observation of ovarian maturity level (OML) was carried out every five days (5, 10, 15 and 20), following the procedures described in Islam et al (2010), Farizah et al (2017) and Iromo et al (2015). The observation of ovarian morphology was carried out by observing the changes in the color and volume of ovary. The levels of ovarian maturity are: OML I, the color of ovary is light white, and the size is still small; OML II, the color of ovary is yellow; OML III, the ovary is orange in color and covers most of the body cavity, appearing solid (Iromo et al 2015).

The cultivation media. The experiment was done in a large wooden cage within which three units of net with a size of 66 x 100 x 170 cm^3 were installed to house the mud crab with different experimental treatments. Some wooden board was added at the bottom of the cage, and sand was added as a substrate at about 10 cm depth.

The preparation of thyroxine hormone. The procedure for thyroxine hormone preparation refers to Iromo et al (2015). Thyroxine hormone used in this study was a commercial hormone with an active concentration of 100 µg tablet^{-1}, namely Levothyroxine sodium or Thyrax tablets (N.V.organon, Oss, The Netherlands). Thyrax was ground according to the dosage and dissolved in a physiological solvent for 24 hours. The injection was given once, according to the doses, at the internode of the mud crab swimming leg.

The preparation of Melastoma malabathricum leaf extract. The leaves of *M. malabathricum* were subjected to the maceration process, as defined by Farizah et al (2018). Briefly, the extraction method employed a maceration technique with ethanol solvent concentration of 80% (1:5). The maceration was carried out with 100 g of leaf powder on a chamber glass and 500 mL of 80% ethanol as a solvent. The maceration process was performed three times (3 x 24 hours) at room temperature in a dark atmosphere before filtering the extract. The ethanol extract was concentrated in a rotary evaporator at a temperature of 40ºC.

Experimental animals. The tested animals were selected based on their body weight and length, completeness of body parts, which were also active and showed aggressive behaviour. The crabs were obtained from the local catchers. Thirty six female mud crab broodstocks at an average body weight of about 200 g and early OML II maturity stage were used in this experiment. The selected female broodstock were subsequently acclimized to the experimental condition for 2-3 days prior to experimentation.

Experimental design. This study consisted of three treatments with three replications (36 female mud crab):

- group A: mud crab received 100 µL distilled water injection (control);
- group B: mud crab received 100 µL thyroxine hormone injection at 0.1 µg g^{-1} body weight (Iromo et al 2015);
- group C: mud crab received 100 µL *M. malabathricum* leaf ethanol extract injection at a dose of 0.01 mg g^{-1} body weight (Farizah et al 2017).

Injection treatments were performed on day 5, 10, 15, and 20 by using a 1 mL syringe with a 27G needle at the internode of the mud crab swimming leg. Each crab was injected, with a concentration of 0.1 mL kg^{-1} of body weight (Fujaya et al 2011). Following injection, the mud crabs were placed back into the bamboo cage. The mud crab was fed with trash fish twice a day at a level of 5% of body weight for 20 days of culture period. Sampling was done every five days to measure gonadosomatic index (GSI) and hepatosomatic index (HIS).

The measurement of gonadosomatic index (GSI) and hepatosomatic index (HSI). GSI and HSI were calculated based on the weight of ovary and hepatopancreas. The ovaries and hepatopancreas were dissected out from the crab on each sampling period. GSI and HSI were calculated as follow:

$$GSI = \text{gonad weight} / \text{body weight} \times 100$$

$$HSI = \text{hepatopancreas weight} / \text{body weight} \times 100$$

The measurement of survival. Mud crab survival was calculated using using the formula of Huyn & Fotedar (2004) formula:

$$SR = (n_1/n_0) \times 100$$

where: n_1 is the number of crabs at time t and n_0 is the number of crabs at the commencement (36 crabs).

Water quality. The water quality parameters observed included dissolved oxygen, pH, salinity, and temperature monitored from the pond throughout the experimental period. To measure all the water quality we used a Hanna HI 9828 device (manufactured in the USA). The measurements were carried out in situ.

Data analysis. The data were analyzed statistically using one way ANOVA followed by Tukey post hoc test. Statistical analysis was performed using SPSS software (version 23.0). The differences in ovarian morphology between the treatments were analyzed descriptively by scoring method.

Results. The results of *in vivo* assays were observed by mud crab ovarian maturation stages that were determined according to ovarian morphology, GSI and HSI.

The macroscopic development of ovaries. The macroscopic development of crab ovaries in each treatment is shown in Figure 2.

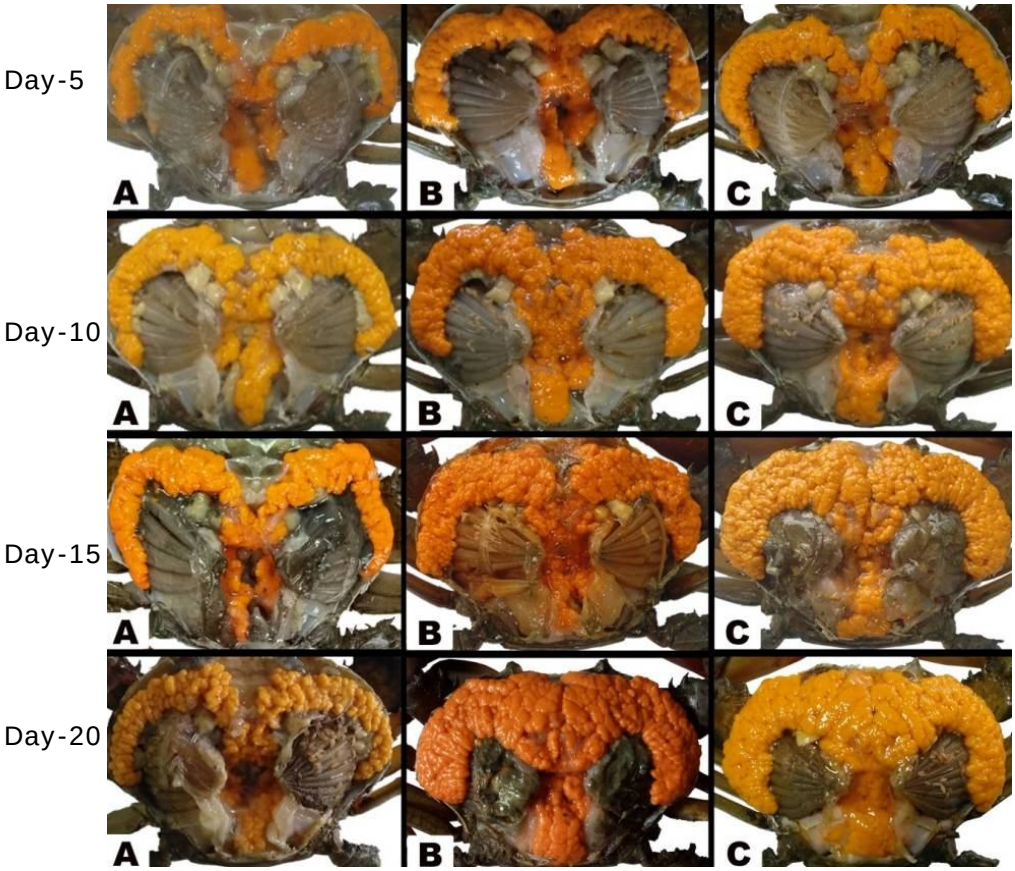

Figure 2. The morphology of *Scylla serrata* ovary on day 5, 10, 15 and 20 after thyroxine hormone and *Melastoma malabathricum* leaf extract injections (Group A: control, Group B: thyroxine hormone, 0.1 µg g^{-1} body weight, and Group C: *Melastoma malabathricum* leaf extract, 0.01 mg g^{-1} body weight).

Figure 2 demonstrated that the volume and color intensity of the mud crab ovaries in each treatment increased with the increase of the culture period. The observation on day 5 showed that ovarian development for thyroxine hormone treatment was higher than those of other treatments. The color of the ovaries of mud crab in this treatment was more intense and darker, and the volume of ovary was more massive than those of other treatments.

Similar results were also observed on the 10th day of sampling where the crab in the thyroxine hormone treatment showed darker yellow to orange ovary color. The mud crab in the control showed a bright yellow and smaller volume of ovary than those of the thyroxine and *Melastoma* leaf extract treatments. Based on the color and the volume of the ovaries, the gonadal maturity stage of the mud crab in all treatment on the first 10 days of culture was at ovarian maturity stage II (OML II), as indicated by the change of ovarian color from yellow to an orange and ovarian volume which was bigger than OML I.

The results of sampling on day 15 showed that the crab ovaries in the control treatment was still small and yellow in color, and eggs granules were still unclear, which indicate that the crab in this treatment was still on stage OML II. On the other hand, the crab in the thyroxine treatment showed higher volume of ovaries that covered most of the hepatopancreas. The ovaries were light orange and appeared dense and compact; eggs granules were starting to be visible. The ovarian morphological appearance indicates that the crabs in thyroxine treatment were in late OML III stage. Although not as advanced as thyroxine hormone treatment, similar trend was observed in the treatment of *M. malabathricum* leaf extract, where the crabs demonstrated ovarian maturity stage of early OML III, with larger size of ovarian volume than that of the control, that covered considerable part of the abdomen so that the hepatopancreas was almost not visible. However, despite the same size, the colour of the crab's ovary in *M. malabathricum* leaf extract was not as intense as the crab in the thyroxine treatment.

On day 20, the control treatment showed ovarian morphology that was still at OML II maturity stage, ovarian volume had increased in size, but the color was still pale yellow and egg granules were not clearly visible. In contrast, for thyroxine hormone treatment, ovarian development had reached late OML III maturity stage, which was characterized by the increased size of ovary that completely covered the hepatopancreas, the bright orange ovarian color, and the clearly visible egg granules.The mud crab with *M. malabathricum* leaf extract injection showed increasing size of ovary and color change from yellow to orange. The ovary completely covered the hepatopancreas and eggs granules were clearly visible. The maturity level of the crabs in treatment of *M. malabathricum* leaf extract was still at OML III towards the end of secondary vitellogenesis.

Significant increase in GSI values along with the culture period was observed in all treatments (Table 1). There were no significant differences ($p > 0.05$) in GSI between the thyroxine hormone and *M. malabathricum* extracts treatments in all sampling time, except on day 20. However, the GSI in these treatments were consistently higher than that of the control ($p < 0.05$). On day 20, the GSI in mud crabs in thyroxine hormone treatment was the highest amongst other treatments ($p < 0.05$).

Table 1

Gonadosomatic index (GSI) of *Scylla serrata* female broodstock on day 5, 10, 15 and 20 after thyroxine hormone and *Melastoma malabathricum* leaf extract injections

Treatment	*Day-5*	*Day-10*	*Day-15*	*Day-20*
Control	3.1 ± 1.53^{a}	7.7 ± 2.00^{a}	11.2 ± 2.00^{a}	15.2 ± 0.58^{a}
Thyroxine hormone	5.3 ± 2.08^{b}	12.0 ± 2.52^{b}	16.9 ± 2.65^{b}	20.5 ± 1.53^{c}
Melastoma extract	4.2 ± 1.00^{b}	10.0 ± 1.53^{b}	15.5 ± 1.53^{b}	18.1 ± 1.00^{b}

Different letters following mean values (±standard deviation) in the same column indicate significant differences ($p < 0.05$).

The hepatosomatic index (HSI). The increase in GSI was inversely related to the HSI. The HSI value of *S. serrata* during the 20 days of the research is shown in Table 2. HSI value of the mud crabs in all treatments was decreasing as the culture progressed.

Table 2

Hepatosomatic index (HSI) of *Scylla serrata* female broodstock on day 5, 10, 15 and 20 after thyroxine hormone and *Melastoma malabathricum* leaf extract injections

Treatment	*Day-5*	*Day-10*	*Day-15*	*Day-20*
Control	7.0±1.73	5.6±1.53	3.4±1.00	2.6±1.53
Thyroxine hormone	4.9±1.00	3.8±0.58	2.1±1.53	1.3±0.58
Melastoma extract	5.4±1.00	4.3±1.00	2.5±1.53	2.3±1.73

Different letters following mean values (±standard deviation) in the same column indicate significant differences ($p < 0.05$).

The survival. The mud crab survival was 100% for all treatments, and there were no significant differences between treatments (Table 3).

Table 3

The survival of *Scylla serrata* female broodstock on day 5, 10, 15 and 20 after thyroxine hormone and *Melastoma malabathricum* leaf extract injections

Treatment	*Survival (%)*			
	Day-5	*Day-10*	*Day-15*	*Day-20*
Control	100	100	100	100
Thyroxine hormone	100	100	100	100
Melastoma extract	100	100	100	100

Water quality parameters. Water quality parameters measured during the study were temperature, salinity, pH, and dissolved oxygen (Table 4). Water quality parameters during the experimental period were within optimum range for mud crab maintenance activities.

Table 4

The range of water quality parameters during 20 days of *Scylla serrata* broodstock culture

Parameter	*This experiment*	*Threshold value*	*References*
Temperature (^oC)	27-33	22-36	Iromo et al (2018)
Salinity (ppt)	15-18	10-20	Iromo et al (2018)
pH	6.76-8.25	6.0-8.0	Iromo et al (2018)
Dissolved oxygen (mg L^{-1})	6.4-7.8	> 4	Shelley & Lovatelli (2011)

Discussion. The application of thyroxine hormone and *M. malabathricum* leaf extract as an ovary maturation stimulator had significant effects on the development and the acceleration of gonadal maturation in *S. serrata* reared in traditional ponds. The observations carried out on days 5, 10, 15 and 20, showed that the maturity stage (OML) of *S. serrata* females treated with thyroxine hormone and *M. malabathricum* leaf extract were consistently higher and faster than those of treatment control (Figure 2). Meanwhile, the range of GSI values indicated that the control treatment had a lower range than the thyroxine and extract treatment (Table 1).

The reduction in HSI value could be due to the transfer of vitellin from the hepatopancreas to the ovaries resulting higher GSI value (Table 2). The process of gonadal development and maturation in crustacean was characterized by an increase in GSI, followed by a decrease in HSI. In the developmental stage of ovary maturation, the hepatopancreas actively synthesizes the vitellin protein (raw material for egg yolk), which will be transferred through the hemolymph to the ovaries as the main target organ. Vitellin deposition leads to the increase of ovarian volume and egg cell diameter, and has a vital role in the embryogenesis processes (Boulangé-Lecomte et al 2017; Farizah et al 2017).

Thyroxine hormone treatment showed a fastest acceleration of the ovary maturation process than that of other treatments. These results confirmed previous study by Iromo et al (2015) reporting that the application of thyroxine hormone could increase the acceleration of gonadal maturity and the survival of *S. serrata*. Thyroxine hormone plays a vital role in metabolic processes and tissue growth so that it indirectly affects the reproductive process (Turner & Bagnara 1976). Thyroxine stimulated the rate of the oxidation process, consumption of oxygen, and could act as an agent to promote growth and metamorphosis process. Thyroxine hormone indirectly helps the absorption of egg yolks that provide the energy that is required for larval development. In general, the energy from the consumed feed that is used for reproductive development comes from fat and protein. The application of thyroxine hormone in fish reproduction was reported to improve survival, development, egg absorption during early larvae stage, and larval growth. The presence of thyroxine hormone has been observed in eggs and larvae of fish

(Ayson & Lam 1993). Administration of thyroxine hormone by injection in Nile tilapia (*Oreochromis niloticus*) broodstock could increase larval growth by two times compared to the control (Khalil et al 2011). The presence of thyroxine hormone in female mud crabs has been reported previously. Its concentration in the ovaries increased with the increase of maturity level of the ovaries, indicating the role of this hormone in ovarian maturity in mud crabs (Iromo et al 2014).

Besides thyroxine hormone administration, *M. malabathricum* leaf extract administration also provided a positive response on the acceleration of *S. serrata* ovaries maturation. The mud crab in the *M. malabathricum* extract treatment also demonstrated faster and higher gonadal development process than that of the control. These results strongly suggested that *M. malabathricum* herbs could improve the reproductive performance of *S. serrata.* Previous studies showed that plant bioactive substances could have physiological effects similar to hormones or steroids present in animals. Steroid or steroid-like compounds such as; phytosterols, phytoecdisteroids, and plant-derived phytoestrogens have been widely studied in humans, mammals, and crustaceans, particularly in biological activities such as growth and development (Dinan 2001). The use of plant-derived compounds in aquaculture has been widely used. For instance, Aslamsyah & Fujaya (2010) demonstrated that *Amaranthus tricolor* L. was able to stimulate moulting in mud crabs (*Scylla* sp.). Dietary supplementation of bee pollen and paprika could increase sperm concentration and prevent the melanization of sperm in shrimp *Farfantepenaeus paulensis* (Braga et al 2013). Mulberry leaf extract (*Morus alba*) could be used to resolve the failure of moulting process in *Portunus pelagicus* (Fujaya et al 2014). Indeed, many studies have been done to observe the effect of bioactive compounds from herbs in aquaculture activities (Citarasu 2010).

M. malabathricum has been widely reported as a herb that plays a role in improving reproductive performance and as a tonic for fertility (Koay 2008; Joffry et al 2012). The ethanolic extract of *M. malabathricum* leaves could increase the concentration and motility of spermatozoa in albino rats (Balamurugan et al 2013). The use of ethanolic extract of *M. malabathricum* leaves could stimulate the acceleration of ovarian maturity, and to control the reproductive and spawning processes in crustaceans (Farizah et al 2017, 2018; Alam et al 2019; Awaluddin et al 2020). This study confirmed the positive effects of *M. malabathricum* leaf extract on *S. serrata* gonadal maturation. This could be seen from the sampling results on day 5, 10, 15, and 20, where the mud crab ovarian development was faster and higher than that of the control treatment, although still slower than that of the thyroxine hormone treatment. The results of this study also confirmed previous research by Farizah et al (2017) demonstrating that *M. malabathricum* leaves could be potential stimulator in the acceleration of ovary maturation in *Scylla olivacea*.

The application of thyroxine hormone and *M. malabathricum* leaf extract as a stimulator in the maturation process was safe for *Scylla serrata* (Table 3). The high survival in this study was strongly determined by the initial process broodstock in broodstock selection, where high quality and healthy broodstock was used for the experiment.

Conclusions. The application of thyroxine hormone and *M. malabathricum* leaf extract significantly improved the development and acceleration of maturation process of *Scylla serrata* ovaries raised in traditional ponds. Thyroxine hormone treatment resulted in the fastest maturation process of mud crab ovary. Thyroxine hormone and *M. malabathricum* leaf extract could be used as alternative hormone sources in accelerating the maturation of *Scylla serrata* ovaries cultivated in traditional ponds.

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Received: 04 April 2021. Accepted: 02 May 2021. Published online: 29 June 2021.
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How to cite this article:
Iromo H., Farizah N., Puryono, 2021 The application of thyroxine hormone and *Melastoma malabathricum* leaf extract as stimulators in gonadal maturation of *Scylla serrata* in traditional ponds. AACL Bioflux 14(3):1769-1777.