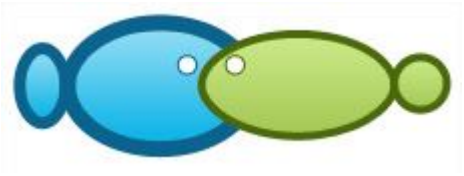

Accelerated ovarian maturation of mud crab (*Scylla olivacea*) using ethanol extract of *Melastoma malabathricum* leaf

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Abstract. Herb has been widely used in aquaculture as bioactive agents to increase the aquaculture production. Herbs could act as a precursor to increase the growth rate of cultivant, the tonic for immunity, the appetizer stimulator, the gonadal maturation inducer, the antimicrobials and the antistress agents. One of the herbs that has a potential as a stimulant in gonadal maturation is *Melastoma malabathricum* L. The research was conducted to assay the potential of *M. malabathricum* herb on gonadal maturation of mud crab (*Scylla olivacea*). Females of mud crab were injected with ethanol extract of *M. malabathricum* leaf at three dosages, consisting of: group A (control, no ethanol added); group B (0.25 mg g^{-1}); group C (0.5 mg g^{-1}) and group D (1 mg g^{-1}) of the body weight. The result showed that group D have significantly ($p < 0.05$) increased relative weight of the ovary compared to group A (control). This treatment (group D), showed the highest mean of gonadosomatic index (GSI) (14.06±0.41%), compared with the other treatments. Macroscopic changes in ovaries of mud crab for groups B, C and D were indicated by the bright orange colour and an increase of ovarian volume from 0 to 20 days after injection compared with group A (control). Based on histological examination, the group D had the highest mean of oocyte diameter (138.34±4.68), with many fully grown oocytes. Thus, it could be concluded that *M. malabrathicum* extract could stimulate gonadal maturation in mud crab.
Key Words: herb, histology, *Melastoma malabathricum* mud crab, gonad maturation.

Introduction. Mud crab aquaculture has been intensively conducted in Indonesia. The activities still rely on seeds which were wildy-caught and have a limited amount. As a consequence, the seed stock depends on the climate season. Seed stock production through hatchery had been done but survival rate of juvenile crabs were still low (Le Vay et al 2008). Supposedly, the broodstock quality in breeding process has not yet been standardized and information about mature female with high quality for breeding purpose is limited. Basically, enhancing gonadal development in crustacean could be stimulated by hormonal, nutrition, and environmental approaches. Currently, the most usable approaches to induce the ovarian maturation in shrimp and crab are hormonal manipulation. Hormonal manipulation in aquaculture is common for the past few years. Hormonal is a practical alternative way in inducing gonad development that has less handling and it gives lesser stress (Ikhwanuddin et al 2013). Manipulation of the crustacean endocrine system to accelerate reproductive development could reduce the overall ripening time (Nagaraju 2011). The common method used for ovarian maturation process in crustacean is eyestalk ablation method (ESA), by removing the endogenous gonad inhibiting hormones (Brown & Jones 1949), but the eggs do not develop properly following ESA (Anilkumar & Adiyodi 1985).

The addition of exogenous hormones through vertebrate steroid or extract from an organ or tissue has biological effect like estrogen on gonad maturation (Nagaraju 2011). Steroid hormone testing from mammalian had been done. It showed that 17α-hydroxyprogesterone and 17β-estradiole significantly affected gonad development (Gunamalai et al 2006). Organ extraction such as eye stalk and brain in *Paratya compressa* (Takayanagi et al 1986), thoracic ganglia in *Scylla olivacea* (Fujaya 2004) significantly affected maturation of ovary.

The potential for using steroid or steroid-like compound from plant which has physiological act similar with steroid hormone has been studied. Using a plant compound for testing in some biological activities on Crustacean have been reported, e.g. adding pollen and paprika through diet in *Farfantepenaeus paulensis* showing the enhancement of sperm concentration and preventing melanization (Braga et al 2013), adding mulberry extract (*Morus alba* Linn) for stimulating molting process in *Portunus pelagicus* L. (Fujaya et al 2014). Using herbs in aquaculture have been done. More than 50 herbs have biological effect as growth promoters, immunostimulants, antistress, antibacterial, antifungal, antivirus, appetizer stimulator and aphrodisiac (Citarasu 2010). The herbal content is consisted of several compounds, such as phenol, polyphenol, alkaloid, quinone, triterpenoid, steroid, lectin, and polypeptide. The advantages of using herbs are environmentally safe and are not hazardous for cultivated organisms.

One of the herbs which potentially improves reproductive performance of mud crab is *Melastoma malabathricum* L. This plant is used to treat reproductive problems, e.g. enhanced fertility and strengthen the uterus (Koay 2008), increasing the concentration and motility of spermatozoa, and also testosterone level in rats (Balamurugan et al 2013). Several researchers (Faravani 2009; Zakaria et al 2006) reported *M. malabathricum* contents, e.g. flavonoid, tannin, saponin, steroid, and triterpenoid. Steroid and triterpenoid from plant also have the structure and function similar with steroid hormone which has estrogenic activity or estrogen-like. This work was conducted to examine the effect of the ethanol extract of *M. malabathricum* on gonad maturation in mud crab, *S. olivacea*.

Material and Method. The study was conducted from January to April 2016, on the research pond of the Faculty of Marine Science and Fishery, Hasanuddin University, Makassar, Indonesia.

Plant material. The materials were used are fresh leaves of *M. malabathricum* and they were collected from North Samarinda, East Borneo, Indonesia. The plants used in this study were identified by Indonesian Institute of Sciences (LIPI) Bogor.

Preparation of ethanol extracts of Melastoma malabathricum. The leaf extraction was conducted based on Balamurugan et al (2013) with modification procedure. The extraction used maceration technique with 80% ethanol solvent (1:5). The maceration was conducted with 100 g leaf powder on chamber glass and 500 mL 80% ethanol as a solvent. The maceration procedure was repeated for three times (3x24 hours) at room temperature with dark environment, then the extract was filtered. The ethanol extract was concentrated in a rotary evaporator at a temperature of 40ºC. The ethanol extract was used for terpenoid or steroid compound testing based on Goad & Akihisa (1997) method.

Experimental animals. The immature female mud crabs were used for in vivo assay. The body weight of female mud crabs were between 200-250 g with carapace length between 100-130 mm. Mud crab adaptations was performed in bamboo cages with the depth of the water about 80 cm, for three days. Each bamboo cage consisted of one female mud crab, with three replicates.

Experimental design. The female mud crabs were divided into 4 groups consisting of 3 animals:

- group A: mud crab received 100 µL aquadest soluble for 5, 10, 15 and 20 days (control), by injection;
- group B: mud crab received 100 µL ethanol extract of leaf of *M. malabathricum* L. at the dose of 0.25 mg g^{-1} of the body weight for 5, 10, 15 and 20 days, by injection;
- group C: mud crab received 100 µL ethanol extract of leaf of *M. malabathricum* L. at the dose of 0.5 mg g^{-1} of the body weight for 5, 10, 15 and 20 days, by injection;
- group D: mud crab received 100 µL ethanol extract of leaf of *M. malabathricum* L. at the dose of 1 mg g^{-1} of the body weight for 5, 10, 15 and 20 days, by injection.

The injections were performed using 1 mL syringe with 27G needle at the internode of swimming leg of crab. Each crab was injected, with concentration of 1/10 mg kg^{-1} of the body weight (Fujaya et al 2011). After that, the mud crabs were placed back into the bamboo cage after receiving the injection.

Female mud crabs were monitored for 20 days in the bamboo cage. During the culture period, the mud crabs were fed with fish and squid approximately 10% of crab body weight for two times per day. Water quality measurements were consisted of: dissolved oxygen (DO), pH, salinity and temperature.

In vivo assays were observed by mud crab ovarian maturation stages that were described based on ovarian morphology, gonadosomatic index (GSI), hepatosomatic index (HSI), oocyte diameter size, hormonal assay and ovary histology evaluation.

Ovarian maturation stages. The observation of ovarian maturation stages was conducted morphologically (macroscopic). Data was collected every 5 days of treatment for ovarian maturation stages observation. This step followed the procedure of Islam et al (2010). After the observation, ovarian tissue was dissected in formaldehyde 4% for histology preparation.

Gonadosomatic index (GSI) and hepatosomatic index (HSI) measurement. GSI and HSI were calculated based on ovary and hepatopancreas weight. The ovaries and hepatopancreas were dissected out from the crab. GSI and HSI were calculated as followings:

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

Hormone levels measurement. Estradiol 17β hormone levels were analyzed from hemolymph sample with ELISA method (Estradiol 17β-ELISA test kit) according to the manufactured instruction. Hemolymph samples were taken from coxa (the forth walking leg of crab) through internode. About 1 mL hemolymph samples were taken using 1 mL syringe which contains anticoagulant. Then, serum was separated by centrifugation at 2500 rpm for 15 min and stored at -18 to -20ºC (Pattiasina et al 2010). Standard range for making standard curve is from 1-25 µg dL^{-1}. For concentration accuracy of estradiol hormone, each sample was analyzed with duplicate.

Histology preparation. Microscopic observation was performed from histology slide from each treatment. Sample preparations followed standard laboratory protocols for Haematoxylin-Eosin (HE) staining (Kiernan 1981).

Oocyte diameter size measurement. The mean oocyte diameter of mud crabs were conducted on 100 oocyte cell/replicate for each group. The oocyte with nuclei that showed in histological assessments will be used for measurement. Oocyte diameter sizes are measured using ImageJ software. The normality of oocyte data was tested with Kolmogorov-Smirnov normality testing using SPSS ver. 16.

Statistical analysis. Data was expressed as mean±SEM. The collected data was calculated using Ms. Excel 2010. The data was examined statistically by analysis of variance (ANOVA) using SPSS 16 at $p < 0.05$ (95%). Statistically significance was determined by Duncan's multiple range test. Macroscopic observation and histological analysis were determined descriptively.

Results. The results of in vivo assays were observed by mud crab ovarian maturation stages that were described based on ovarian morphology, GSI, HSI, hormonal assay, oocyte diameter size, and ovary histology assessments.

Ovarian morphological development stages. Figure 1 shows the macroscopic morphological developmental stages in mud crab ovary. The results showed that the volume and coloration in mud crab ovary had changed every 5 days along 20 days of observations. Ovarian maturation stages assessment based on Islam et al (2010) procedure showed that ovarian stage possessed early maturing stage on day 5 in all treatment groups. In this stage, primary vitellogenesis process had begun and showed the ovarian color was changed from creamy white to yellow with 3-7 mm of tissues thickness and taken 10-20% from cardiac stomach. Ovarian development was increased along the observation days in all treatment groups (A, B, C, and D). The most rapid ovarian development was the group D treatment.

In the treatment of group D, the crab ovarian maturation increased gradually in 20 days compared to the group A (control). When ovarian entered the maturation stages (also known as secondary vitellogenesis stage) it showed the yolk globules and started to appear macroscopically, the ovary was orange to deep orange with 10-20 mm of tissues thickness and eventually covered the cardiac stomach (> 75%).

Figure 1. Dissected female mud crab *Scylla olivacea* displaying. Ovarian morphological development for all treatment groups A (control), B (0.25 mg g^{-1}), C (0.5 mg g^{-1}) and D (1 mg g^{-1}). Notes: 5, 10, 15, 20 (gonad sampling days), ♦= ovary, ● = hepatopancreas, and = ➔cardiac stomach; bar▬ = (1 cm).

Gonadosomatic index (GSI). GSI and HSI are two of the indicators of ovarian development. The GSI of crabs in the treatment group B, C, and D during the treatment was significantly different compared to the treatment group A (control) ($p < 0.05$),

showing an accelerated ovarian maturation. Table 1 shows the mean GSI in every treatment group. Comparatively, the treatment group D had the highest mean of GSI (14.06±0.41%), compared to the other treatments.

Table 1

Mean of gonadosomatic index (GSI) of mud crab with ethanol extract of *Melastoma malabathricum* leaf on day 5, 10, 15, and 20 of cultured periods

Treatment groups	*Day-5*	*Day-10*	*Day-15*	*Day-20*
A (Control)	1.67 ± 0.25^{a}	4.40 ± 0.76^{a}	6.34 ± 0.27^{a}	9.01 ± 0.77^{a}
B (0.25 mg g^{-1})	2.58 ± 0.18^{b}	5.46 ± 0.38^{ab}	7.32 ± 0.31^{a}	11.97 ± 0.62^{b}
C (0.5 mg g^{-1})	3.56 ± 0.21^{b}	6.10 ± 0.52^{bc}	10.53 ± 1.00^{b}	12.25 ± 0.61^{b}
D (1 mg g^{-1})	4.14 ± 0.17^{c}	7.09 ± 0.81^{c}	11.54 ± 0.47^{c}	14.06 ± 0.41^{c}

Values are mean±SD, superscript letter showed statistically significant value ($p < 0.05$).

Hepatosomatic index (HSI). Table 2 shows the ovarian maturation stage of crab based on HSI indicator throughout the observation. During cultivation periods, the HSI was increasing by day 5 and day 10, afterwards the HSI value was decreasing on day 15 and day 20. The increased HSI values on day 5 and day 10 of observation were positive and strongly correlated with GSI values. On the other hand, the decreased HSI values on day 15 and day 20 of observations also showed GSI values was higher in day 15 and day 20 of observations.

Table 2

Mean of hepatosomatic index (HSI) of mud crab that gave ethanol extract of *Melastoma malabathricum* leaf on day 5, 10, 15, and 20 of cultured periods

Treatment groups	*Day-5*	*Day-10*	*Day-15*	*Day-20*
A (Control)	5.54 ± 0.17^{a}	5.78 ± 0.07^{a}	4.72 ± 0.12^{a}	5.01 ± 0.07^{a}
B (0.25 mg g^{-1})	5.81 ± 0.25^{a}	6.72 ± 0.18^{a}	5.30 ± 0.06^{a}	5.05 ± 0.27^{a}
C (0.5 mg g^{-1})	5.88 ± 0.23^{a}	6.75 ± 0.20^{a}	5.46 ± 0.04^{a}	4.55 ± 0.49^{a}
D (1 mg g^{-1})	5.92 ± 0.16^{a}	7.39 ± 0.43^{a}	4.25 ± 0.52^{a}	4.29 ± 0.16^{a}

Values are mean±SD, superscript letter showed statistically significant value ($p < 0.05$).

Hormonal assay. The standard used in the measurement of estradiol is presented in Table 3. Expected concentrations and measured concentrations of the standard estradiol were almost the same. The recovery ranged from 97.6 to 104.8%. The concentrations of estradiol in the hemolymph for all treatments are presented in Table 4. The concentration of estradiol in hemolymph for group D treatment is higher than the other treatments (A, B and C). It was about 46.80±0.85% compared with the treatment of group A (control), group B (0.25 mg kg^{-1}) and group C (0.5 mg kg^{-1}). The group D was significantly different ($p < 0.05$), showing the level of estradiol 17ß concentration higher than treatments of groups A and B, while for treatment of group C was not significantly different.

Table 3

Expected concentrations and measured concentrations of standard of DRG estradiol and their recoveries

Standard	*Expected conc. (pg mL^{-1})*	*Measured absorbance*	*Measured conc. (pg mL^{-1})*	*Recovery (%)*
1	25	2.095	24.6	98.3
2	50	1.993	49.8	99.7
3	100	1.775	104.8	104.8
4	250	1.4	254.7	101.9
5	500	1.052	497.5	99.5
6	1000	0.706	975.6	97.6
7	2000	0.402	2044.0	102.2

Table 4

The mean concentrations of estradiol (pg mL^{-1}) in the hemolymph of female mud crab on 20 days of cultured periods

Treatment groups	*Day-20*
A (Control)	25.50 ± 5.09^{a}
B (0.25 mg g^{-1})	32.10 ± 0.99^{a}
C (0.5 mg g^{-1})	43.15 ± 0.21^{b}
D (1 mg g^{-1})	46.80 ± 0.85^{b}

Values are mean±SD, superscript letter showed statistically significant value ($p < 0.05$).

Oocyte diameter size. The oocyte development was evident by the measurement of oocyte diameter. Gradual increase was observed in the oocyte diameter throughout the maturation stages. The mean oocyte diameter of crabs in each treatment was shown in Table 5. The results showed the mean oocyte diameter increased in all the treatment groups (A, B, C, and D) throughout the treatment period. The largest oocyte diameter was in treatment group D (138.34±4.68) compared to other treatment groups. The enhancement of oocyte diameter size was positively correlated with the mean GSI value in each treatment.

Table 5

Mean of oocyte diameter size of mud crab that gave ethanol extract of *Melastoma malabathricum* leaf on day 5, 10, 15 and 20 of cultured periods

Treatment groups	*Day-5*	*Day-10*	*Day-15*	*Day-20*
A (Control)	46.58 ± 1.49^{a}	86.07 ± 3.35^{b}	126.96 ± 3.56^{b}	130.03 ± 2.36^{b}
B (0.25 mg g^{-1})	60.46 ± 2.18^{b}	104.78 ± 2.48^{c}	128.02 ± 3.61^{b}	131.39 ± 3.85^{b}
C (0.5 mg g^{-1})	70.60 ± 3.33^{c}	112.63 ± 3.34^{d}	129.00 ± 3.17^{b}	132.60 ± 3.07^{bc}
D (1 mg g^{-1})	84.30 ± 4.12^{d}	113.00 ± 3.42^{d}	136.60 ± 3.88^{c}	138.34 ± 4.68^{c}

Values are mean±SD, superscript letter showed statistically significant value ($p < 0.05$).

Histology of ovarian tissue. Histological observation in mud crab ovary in all treatment groups showed the development of oocyte which taken on day 5 and day 20 (Figure 2).

Discussion. *M. malabathricum* had a potential as stimulator for gonad maturation process in mud crab, and significance ($p < 0.05$) occurred in accelerated ovarian maturation compared to the control treatment (without extract addition) (Figure 1 and Table 1). This result showed that *M. malabathricum* had a potential for gonad maturation induction. Balamurugan et al (2013) have been reported that ethanol extract of *M. malabathricum* leaf was able to enhance the concentration and spermatozoa motility, and also the testosterone level in rats. After injected with ethanol extract of *M. malabathricum* leaf through in vivo in crab, the GSI was significantly different between treatment group D and treatment groups A, B, and C on day 5, 10, 15 and 20 of observations (Table 5). The GSI of treatment group D was higher than the other treatment groups.

M. malabathricum leaf content was consisted of α-tocopherol, sitosterol, and squalene that expected to act on accelerated ovarian maturation in crab (Farizah et al 2016). Bioactive compounds of *M. malabathricum* particulary on leaves and root consisted of melastomic acid and β-sitosterol (Manzoor-I-Khuda et al 1981), β-sitosterol, α-amyrin, uvaol, sitosterol-3-O-β-D-glucopyranoside, quercitrin, rutin (Nuresti et al 2003), and vitamin E (Susanti et al 2008).

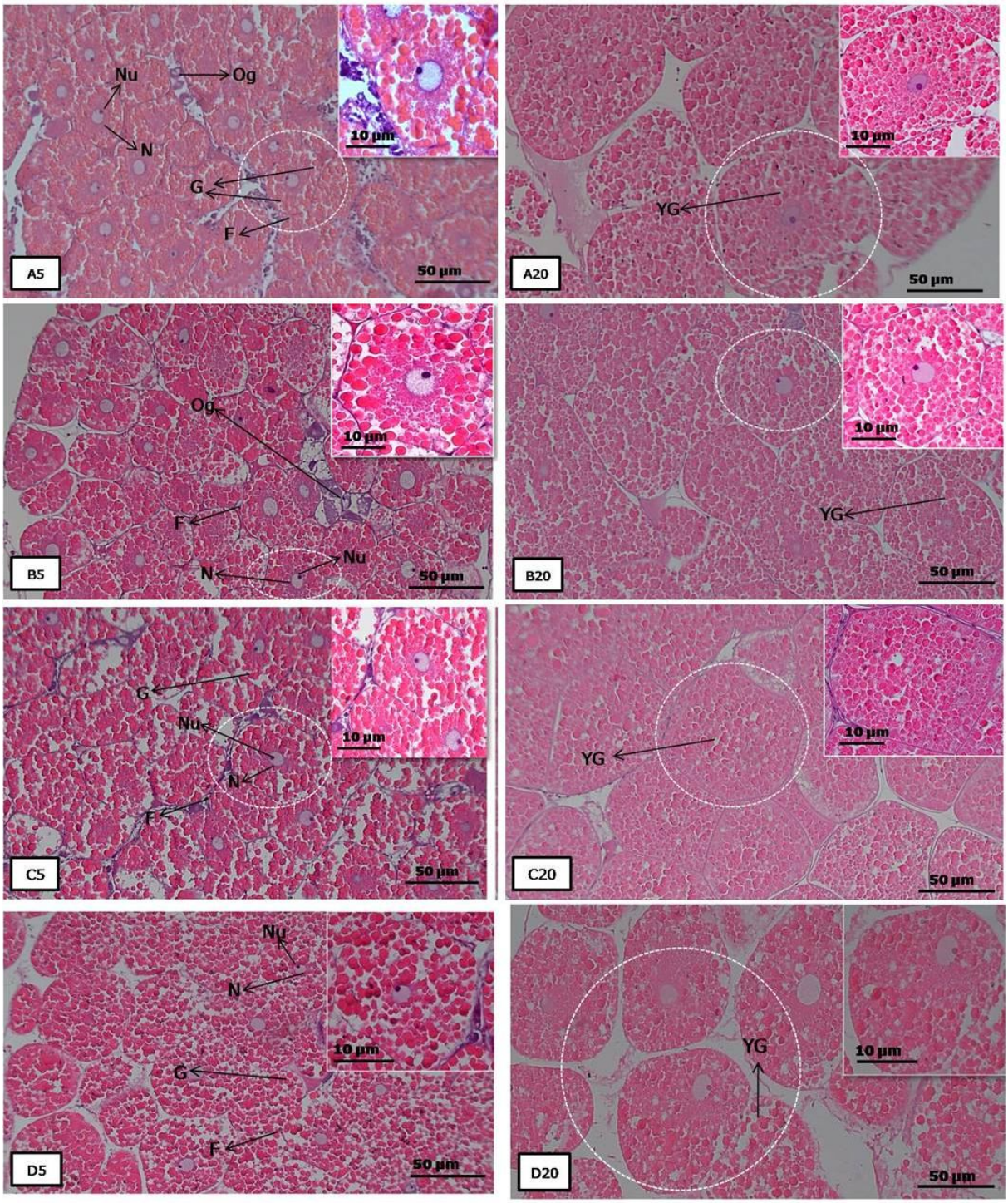

Figure 2. Transverse section of ovaries of all treatment collected from mud crab: A (control), B (0.25 mg g^{-1}), C (0.5 mg g^{-1}) and D (1 mg g^{-1}). Notes: 5 and 20 (gonad sampling days). All samples were stained with haematoxylin-eosin. F: follicle cell; N: nuclei; Nu: Nucleoli; G: Granule; Og: oogonium; Yg: yolk globule.

All crustacean species (crab and shrimp) are unable to synthesize the cholesterol through de novo. Cholesterol plays essential role for life, including reproductive hormone biosynthesis (estrogen and progesterone). Organisms can obtain the cholesterol from food which contains cholesterol. Cholesterol is the main sterol which is commonly consumed by carnivores. On the other hand, herbivore organisms consume sterol 24-alkylate, such as: β-sitosterol, campesterol, and stigmasterol (Peliserro & Sumpter 1992). Wouters et al (2001) has reported that vitamin E form was like α-tocopherol in

crustacean. Biological role of vitamin E acted as the lipophilic antioxidant to protect the cell membrane and cell organelles from free radicals. α-tocopherol has important role during reproductive process, embryo and larva development in *Macrobrachium rosenbergii* (Cavalli et al 2003). Wouters et al (2001) has detected the concentration enhancement of α-tocopherol concentration in ovarian before sexual maturation.

The enhancement of HSI value occurred on day 5 to day 10 in each treatment groups (A, B, C and D). On this stage, ovary entered vitellogenesis stage which affected the HSI enhancement. Vitellogenesis is hoarding process and yolk accumulation which causes oocyte size development. Induction process and vitellogenin synthesis were occurred on liver by estradiol 17β hormone, then released onto blood vessel to oocyte (Peliserro & Sumpter 1992).

Ovarian maturation processes were correlated to estradiol 17β levels. Based on Table 4, the group D, it showed the highest estradiol 17ß level in hemolymp compared with the group A (control). High concentrations of estradiol 17ß hormone in the group D, were associated with ovarian maturation stages, that are faster than in other treatments. Vitellogenin serum levels during different ovarian maturation stages show fluctuations with estradiol 17ß and progesterone levels in hemolymph of *Penaeus monodon* (Quinitio et al 1994). Ovary synthesizes estradiol and releases it into the hemolymph from where it reaches the hepatopancreas to stimulate vitellogenin synthesis. The fluctuation of vitellogenin levels during ovarian maturation is correlated with estradiol-17β (E2) levels in *P. monodon* (Quinitio et al 1994). The vitellogenin was increasing when ovary fragments were incubated with E2 in *Litopenaeus vannamei* (Quackenbush 1992). Estrogen exposure in crustaceans can induce Vtg expression (Atienzar et al 2002; Vandenbergh et al 2003).

The vitellogenin absorption by oocyte affected the increase of oocyte diameter size. The diameter of the oocyte increased as the ovarian developmental stage progressed, resulting in an increase in the volume of the ovary as shown by both the GSI and external ovary. The higher mean of oocyte diameter was group D. The deposition of yolk material in the oocytes when vitelogenesis occurred, that resulted in a rapid increase in oocyte diameter (Sagi et al 1995; Tsukimura 2001). The oocytes and the ovary became increased in size and volume as ovarian developmental stage progressed (Islam et al 2010). Determination of oocyte diameter provided basic information on classification of ovarian development (Peixoto et al 2005).

The oogenesis in brachyuran crabs can be divided into two phases. Firstly, the previtellogenic phase, which contained essentially primary oocytes, and second, the vitellogenic phase, the oocytes increasing in size as yolk globules appear in the cytoplasm. Developmental stages of oocyte were identified on day 5 to day 20 in all treatment groups through histological observation (Figure 2). Ovarian developmental stages were primary vitellogenic phase on day 5 of observation. Based on histological observation, noticeable large vocuolated globules in oocyte were observed and indicated that the vacuolated globules were oil globules. Yolk accumulation initiated during primary vitellogenesis, when basophilic small yolk globules came into sight at the cortical cytoplasmic region. Vacuolated globules were visible in the cytoplasm of oocytes before the initiation of vitellogenesis, which proven oil globules in other crustaceans (Kodama et al 2004; Quinitio et al 2007). Yano (1988) suggested that oil globules were functionally related to the accumulation of yolk protein in prawns and crabs. In brachyuran crabs, cytoplasm changes from basophilic to acidophilic after oil globules move to the periphery of the oocyte and diffuse (Minagawa et al 1993).

On the day 20, the oocyte membrane became more flimsy and the diameter size of oocyte became more enlarged. It caused by accumulated of the yolk protein (vitellogenin). The ovary got into the secondary vitellogenic phase, that showed the size of oocyte increased, the wall thickness reduced and its yolk content increased. The macroscopy of the size ovaries become larger covering the abdomen and the digestive gland (hepatopancreas) was not visible from the dorsal side (Figure 1), the oocytes grew rapidly (120-200 mm). Yolk globules occupied the entire cytoplasm with larger globular inclusions towards the periphery, while follicle cells were hardly visible. These changes also showed on ovarian macroscopic observation. The enhancement of ovarian volume

was shown by the increase of GSI and the coloration of ovary from yellow to deep orange. The changes of the ovary specific colour related to maturation stage, it has a correlation with carotenoid components (Arculeo et al 1995). This result was similar with other research reports (Quinitio et al 2007; Islam et al 2010) which reported that the ovarian coloration and volume enhancement were occurred in *Scylla paramamosain* and *Scylla serrata* that showed the ovarian maturation stage.

Conclusions. The present results clearly explained that the administration of ethanol extract of *Melastoma malabathricum* leaf on mud crab (*Scylla olivacea*) accelerated gonad maturation compared to the control treatment. This acceleration of ovarian maturation was presented in external morphology, GSI, HSI, concentration of 17β estradiol hormone, mean of oocyte diameter size, and ovarian histological observation.

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