Development of ovarian maturations in orange mud crab, *Scylla olivacea* (Herbst, 1796) through induction of eyestalk ablation and methyl farnesoate

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ABSTRACT

Many crustacean species including mud crab, genus *Scylla* are incapable of natural maturation under captivity, thus putting high pressure on the wild catch. Therefore, to increase the availability of mature broodstocks in captivity, this study determined the effect of Eyestalk Ablation (EA) and Methyl Farnesoate (MF) administration on ovarian maturation stages of the orange mud crab, *Scylla olivacea*. The study was conducted using a control group (T1) consisting of 95% alcohol (widely used as a chemical solvent), and three treatment groups consisting of: 5 ml/g oral administrated MF in the diet (T2), EA (T3) and a combination of both treatments of MF and EA (T4). The highest percentage of ovarian maturation Stage 4 was found in the T4 treatment (20.8%) compared to the other treatments which were T1 (0%), T2 (8.33%) and T3 (12.5%). Ovarian development of the treated groups (T2, T3, and T4) was significantly different compared to the control group (T1) (p < 0.05). However, there was no significant difference observed in the mean Gonadosomatic Index (GSI) in orally administrated MF (T2) compared to the EA group (T3) (p > 0.05), but it was significantly different when compared to the combination group of MF and EA (T4) (p < 0.05). It can be concluded that the combination method of oral administration of MF through diet and EA (T4) is the best technique for producing mature ovaries.

ARTICLE HISTORY

Received 28 March 2018
Revised 7 February 2019
Accepted 23 February 2019

KEYWORDS

Eyestalk ablation; methyl farnesoate; orange mud crab; ovarian maturation stages; *Scylla olivacea*

1. Introduction

Mud crab (genus *Scylla* De Haan 1833; Crustacea: Decapoda: Brachyura: Portunidae) is a potential aquaculture species due to its high nutritional value and good market acceptability (Amin-Safwan, Muhd-Farouk, Mardhiyyah, Nadirah, & Ikhwanuddin, 2018; Sagi, Homola, & Laufer, 1991). According to Cholik (1997), the development of mud crab aquaculture and production, both in domestic and international markets, can create significant opportunities for farmers and rural area development. However, the rare availability of mature females year round make it not possible to rely on natural resources alone. Therefore, production of mature females in captivity is needed where methods for inducing the maturation of a female's ovary are crucial to ensure a sufficient seed supply for the mud crab aquaculture industry. Broodstock maturation technique plays a significant role in the success of commercially important cultured species including crustaceans (Azra & Ikhwanuddin, 2016). The most challenging problem in the aquaculture field is to obtain mature gonads, as most of the crustacean species are incapable of maturing naturally in captivity (Amin-Safwan, Muhd-Farouk, Mardhiyyah, Nadirah, & Ikhwanuddin, 2018; Sagi, Homola, & Laufer, 1991).

Size at sexual maturity and fecundity are important aspects of the reproductive biology of crab (Rasheed & Mustaquim, 2010). Usually, there are two types of criteria applied to determine the size at which sexual maturity is attained; one is reproductive criteria (or physiological maturity), in which developmental stages of the gonad are examined. Within the genus *Scylla*, there are four to five stages of ovarian maturation that have been identified, namely immature, early maturing, late maturing and fully mature; or immature, early maturing, late maturing, fully mature and spent stage based on both external (ovarian colouration and Gonadosomatic Index) and histological studies.
(oocytes diameter and structure) (Amin-Safwan et al., 2018; Muhd-Farouk, Jasmani, & Ikhwanuddin, 2016; Ikhwanuddin et al., 2014; Islam, Kodama, & Kurokora, 2010; Quinitio, De Pedro, & Parado-Estepa, 2007). Meanwhile, secondary criteria are the study of relative growth of abdominal width (abdomen) with respect to body size. Sexual maturity determined by the second criterion is also known as functional maturity (Rasheed & Mustaquim, 2010), and is defined as the capacity to mate effectively. On the other hand, fecundity can be defined as the number of ova shed during a particular spawning season (Pillay, 1964) or the number of eggs produced in a single batch of spawning (Potter, Chrystal, & Loneragan, 1983). Both of these aspect are crucial especially for broodstocks selection for the production of mature females in captivity.

Eyestalk Ablation (EA) technique has been used by many aquaculture farms to stimulate gonad development such as on shrimps, Penaeus spp. (Sainz-Hernandez, Racotta, Dumas, & Hernandez, 2008). The structure and organs correlated with the crustacean eyestalk play a vital role in the endocrinological control of growth, reproduction, pigmentation, metabolism, morphogenesis, osmoregulation, feeding and food conversion efficiency (Allayie, Ravichandran, & Bhat, 2011; Millamaena & Quinitio, 2000; Kaplus and Hulata, 1995; Castillo and Negre-Sadargues, 1991; Charmantier, Charmantier-Daures, & Aiiken, 1988; Mcwinnie & Mohrherr, 1970). Santiago (1977) stated that crustacean eyestalks are known to be the distribution centre of the vitellogenin and Moult Inhibiting Hormones (MIH). Adiyodi and Adiyodi (1970) have shown that precocious moulting or gonad development produced by eyestalk removal depends on the relative interaction of other ambient environmental factors and age of the treated animal.

Methyl Farnesoate (MF) (Methyl-(2E,6E,10E)-3,7,11-trimethylododecatri-2,6,10-eneoate), a sesquiterpenoid compound secreted by the mandibular organ (MO) has been identified by Lafer, Landau, Homola, and Borst (1987). MF has been found in a large number of crustacean species including crabs, shrimps, prawns, lobsters and crayfish. Over the past three decades, both direct and indirect researches have emerged for the role of MF as a hormone in crustacean species. Gunawardene et al. (2002) stated that MF was synthesized de novo by a ductless gland and secreted at nanomolar levels in the hemolymph. MF can also be metabolized in crustaceans through the hepatopancreas and peripheral tissue. MF released by the MO in hemolymph is transported to the target tissue by a protective lipoprotein known as Methyl Farnesoate–Binding Protein (MF–BP). Several MF–BPs from crustaceans’ hemolymph have been characterized (Tamone, Prestwich, & Chang, 1997; Takac, Laferu, & Prestwich, 1993; Li & Borst, 1991). Takac et al. (1993) have found MF–BPs in the ovaries, testes, accessory glands and hemolymph of the spider crab, Libinia emarginata. This finding strongly supports that MF plays an important role in crustacean reproduction.

Hormone administration and EA combination could be the best solution to overcome the problem of producing mature female mud crabs in captivity. Removing the eyestalk will change the hormonal regulation in mud crab, which is a vital part for coordinating complex physiological processes in crustaceans such as growth, metamorphosis, metabolism, reproduction, development and behaviour (Allayie et al., 2011). Therefore, it is important to understand the physiological effect of EA on the endocrine system of the mud crab.

Identification of an ovarian stimulation substance would stimulate female S. olivacea to reproduce in captivity (Sagi et al., 1991). By applying these two techniques, EA and MF (administered in its diet), perhaps the production of continuous mature mud crab stock year round on a commercial scale would be possible. The data reported from the present study could be useful in the establishment of efficient mud crab broodstocks management and could provide baseline data to be used in mud crab aquaculture industry. The present study is also expected to produce a useful method in stimulating ovarian maturation in mud crab to ensure the sustainability of mud crab seed production. Literature reviews show that no studies have been made on the effect of EA and MF roles in mud crabs. Thus, the main objective of the present study is to determine the effect of EA and MF induction on the ovarian maturation of the orange mud crab, Scylla olivacea.

2. Materials and methods

2.1. Samples collection

A total of 200 newly matured female S. olivacea (Stage 1 ovary; Body Weight (BW): 80–90 g; Carapace Width (CW): > 90.0 mm; intermediate and pale abdominal flap) (Amin-Safwan et al., 2018; Amin-Safwan et al., 2016; Ikhwanuddin et al., 2014) were obtained from Setiu Wetlands, Terengganu, Malaysia (5°31’23.1"N 102°55’56.1"E) in July 2015 (sampling time: 1600 h–0800 h; duration: three times a week; using a collapsible trap; bait: chicken head; moulting stage: postmoult). S. olivacea identification was based on the morphological description by Keenan, Davie, and Mann (1998). A previous study by Ikhwanuddin, Bachok, Hilmi, Azmie, and Zakaria (2010) stated that the sizes at maturity (CW50)
recorded for female *S. olivacea* from the same sampling site used in this study were 90.6 mm.

Setiu Wetlands is a common fishing ground and no licensing was required for acquisition of mud crabs (Amin-Safwan et al., 2018). We adhered to the ASAB (2012) “Guidelines for the treatment of animals in behavioural research and teaching” published in Animal Behaviour 83: 301–309. None of the work involved endangered or protected species. All experimental procedures and crab handling were according to “Malaysian code of practice for the care and use of animals for scientific purposes” outlined by the Laboratory Animal Science Association of Malaysia.

All crab samples were transported to the marine hatchery of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Terengganu, Malaysia. They were acclimatized for five days in tanks containing filtered sea water before been transferred into an individual aquarium with water temperature of 26 ± 1 °C, water salinity of 32 ± 1 ppt and ambient photoperiod of 12h:12d. Out of 200 crabs, only 96 that were in good condition were selected for the experiment. The crab was defined as in good condition when it had complete appendages (chelipeds, pleopods, and perieopods), good appetite and active behaviour (reacted when being approached).

### 2.2. Experimental design

A total of 96 newly matured female *S. olivacea* were assigned to four groups. Treatment 1 (T1) was the control group, while the other three groups were treatment groups; Treatment 2 (T2) was MF orally administered in the diet, Treatment 3 (T3) was for EA, and Treatment 4 (T4) refers to the combination of MF in the diet and EA. Every seven days, three crabs from each treatment were sacrificed to determine the external morphological and histological characteristics of the ovaries. The external morphological features of the ovary were determined by analysing the ovary colouration and Gonadosomatic Index (GSI), while the histological characteristics were identified by examining the oocyte structure and diameter size (µm).

### 2.3. Broodstock management

Each mud crab was kept individually in an aquarium (20 L capacity) containing 15 L of filtered seawater. Water quality parameters were maintained daily at a temperature of 26 ± 1 °C, salinity of 32 ± 1 ppt and the water quality parameters were monitored daily at 0800 hour using the portable water parameter quality meter (YSI) Model 556 Multiparameter. Water culture medium was thoroughly aerated at 5.0–7.2 mg/L oxygen and daily water exchange was conducted at 80% of the water volume. Any leftover food or faeces at the bottom of the tank was siphoned before the water exchange. The crabs were fed with fresh blood cockle, *Anadara granosa* (10% of the crab BW) twice daily in the morning and evening.

### 2.4. Eyestalk ablation technique

There are two techniques of EA that have been practised in shrimp aquaculture which are unilateral (one eyestalk removed) and bilateral (both eyestalks removed). However, only unilateral EA was conducted during the present study. First, in order to reduce stress during ablation, the crabs were held in pre-cooled water (20 °C) as the low temperature slowed down the metabolism of the crab (Hesni, Shabanipour, Atabati, & Bitaraf, 2008). Then, continued by eyestalk removal using sterile surgical scissors, the wound was cauterized by placing a hot rod over it (Caillouet, 1973). The crabs then were kept individually and feed was not given for 24 hours in order to reduce stress.

### 2.5. Methyl farnesoate (MF) hormone preparation

MF was prepared by dissolving the MF hormone in 95% ethanol, as a solvent vehicle (Muhd-Farouk et al., 2016). The concentration used was 5.0 µg dose per crab every day, which was the dose based on previous studies by Laufer, Demir, Pan, Stuart, and Ahl (2005) on crayfish, *Procambarus clarkii* and Tsukimura, Nelson, and Linder (2006) on tadpole shrimp, *Triops longicaudatus*.

### 2.6. Diet preparation

*A. granosa* were injected with MF dissolved in 95% ethanol by using a 27-gauge needle syringe. The control diet was prepared by injecting only 95% ethanol to the *A. granosa* (Laufer, Biggers, & Ahl, 1998). All the injected *A. granosa* were then air dried for 30 min for vaporization of the ethanol residual.

### 2.7. Ovarian maturation stage characteristics

The ovarian maturation stages were classified through external morphological and histological characteristics. These characteristics were determined by examining the colour changes in the ovary and the ovary mass size through the GSI due to yolk accumulation (Amin-Safwan et al., 2016;
The formula used to calculate the GSI is as follows:

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\text{Gonadosomatic Index (GSI)} = \frac{\text{Ovary weight}}{\text{Body weight}} \times 100\%
\]

For histology procedure, samples of the dissected ovarian lobes were fixed in Davidson's solution for 24 hours, dehydrated in 70% ethanol for less than 24 hours and lastly placed in the tissue processor for 18 ± 1 hours at 60°C. The medium involved during the tissue processing was a series of ethanol, xylene and paraffin wax. After processing, samples were mounted onto their cassettes using paraffin wax, sectioned into 5-μm thin films using a microtome (Leica RM835), transferred into a water bath (between 40 and 45°C) for expansion before mounting onto slides. The slides were dried using a hot plate (40°C) overnight.

The staining procedure used in this study was based on the method of haematoxylin and eosin staining which was modified for staining the ovary of mud crabs, *S. olivacea* and mud spiny lobster, *Panulirus polyphagus* by Muhd-Farouk et al. (2016) and Fatihah, Muhd-Farouk, Amin-Safwan, Mahsol, and Ikhwanuddin (2017), respectively. The dried slides were immersed in a series of xylene, dehydrated in a series of ethanol, stained with a haematoxylin solution, decolourized in 1% acid alcohol, re-stained in 0.5% aqueous eosin and finally, mounted with dibutyl phthalate polystyrene xylene (DPX). The slides then were observed under an advance microscope (Leica Microsystem, Wetzlar GmbH, DM LB 2, Germany). During observations, 100 oocyte diameters for each crab were measured, and the oocyte diameter was recorded.

### 2.8. Statistical analysis

Data collected included ovary colouration, GSI, oocyte structure and oocyte diameter. All the collected data were analysed descriptively using one-way ANOVA, followed by Duncan’s Multiple Range Test, a modification of the Newman–Keuls method test using Minitab Statistical version 16.0 Software at 5% level of significant difference and presented as the mean ± standard deviation (SD).

### 3. Results

#### 3.1. External morphological characteristics

**3.1.1. Ovary colouration**

Figure 1 shows the four ovarian maturation stages that were recorded and used to distinguish the ovary stages based on ovary colouration. At the end of the experiment (Table 1), control treatment (T1) appeared to have the highest number of crabs maintained in Stage 1 maturity (95.83%), where the ovaries had a white-translucent/creamy-white colour with ribbon-like structure. Stage 2 (light yellow) maturity only occurred for the control group at 49 days (4.17%). Regarding T2 (MF treatment), the ovaries were observed having 16.67% occurrence of Stage 2 maturity with a light yellow or yellow colour. There was also some occurrence of Stage 3 (light orange) and Stage 4 (reddish orange) maturity which were 12.5% and 8.33%, respectively. For T3 (EA treatment), 12.5% of the crabs had Stage 4 ovary diameters.
maturity, followed by 25% at Stage 3, 16.67% at Stage 2, while 45.83% were maintained at Stage 1. With regard to T4 (MF and EA treatment), it was observed that the ovary maturity stages were 33.33% at Stage 1, 20.87% at Stage 2 and 25% at Stage 3. This treatment produced the highest Stage 4 maturity with 20.8% compared to other treatments. In the fully ripened ovary of Stage 4, the lobules shapes were clearly developed in the carapace and upper digestive gland with individual eggs seen clearly by the naked eye.

3.1.2. Gonadosomatic index (GSI)
The GSI of mud crab (Figure 2) showed that there was no significant development at any point of the data collected every seven days for the whole 56 days for T1 (control). In T2 (MF treatment), it took 35 days for crabs to obtain significant GSI development. T2 results showed an increasing trend whereby the GSI value kept increasing until it reached the highest GSI at 8.2% on the fifty-sixth day of the culture period. T3 (EA treatment) showed no significant development of the GSI from the start of the experiment up to the twenty-first day. By the twenty-eighth day, there was some increment in the GSI, but no significant development was observed. Similarly to the T2 (MF treatment), the crabs in T3 (EA treatment) only showed significant development starting on day 35. However, it was only able to reach the highest GSI at 7.9%. T4 (MF and EA treatment) also showed the GSI increased; however, it had a significant GSI development much earlier, which was on day 21. It also had the highest value of GSI of 9.0%. Other than T1 (control), all the other three treatments were able to produce significant ($p < 0.05$) development of the GSI within the culture period.

3.2. Histological assessments
3.2.1. Oocyte’s morphology and diameter size
Oocyte morphological structure characteristics were determined on the ovary through histology for every week of each treatment throughout 56 days (eight weeks) of the experimental culture period; 95.83% of ovary maturity stage in T1 (control) was observed to be in Stage 1 where it was full of oogonia, primary oocytes with large nuclei and follicle cells spotted around the primary oocytes (Figure 3A). All treatments showed similar histological characteristic of Stage 1 maturity at the beginning of the experiment. No yolk globules were observed at this stage of maturity. The follicle cells were small and distributed mostly at the periphery of the ovary where they were surrounded by oogonia and primary oocytes. The smallest oocytes remained at the centre of the ovaries whereas the larger ones appeared to be more peripherally located. At this stage, primary oocytes have a distinct nucleus with prominent nucleoli. No significant changes on morphology characteristics of the ovary were observed during ovary maturity Stage 2 (Figure 3B). An abundance of primary oocytes was observed with the follicle cells surrounding oocytes and the nucleus was visible in the larger oocytes. During this stage, the formation of yolk globules started to appear in the cytoplasm of the oocytes. The oocytes were oval and rounded in shape. These characteristics of Stage 2 can be seen in all treatments while oocytes in Stage 3 maturity or late-maturing stage showed no primary oocytes visible where it was filled with significantly larger oocytes (Figure 3C). At this stage of maturity, large and distinct yolk globules were seen in the cytoplasm and the follicle cells started to flatten and could hardly be seen. The nucleus was shrunken and decreased in size. Stage 4 maturity or matured ovary showed an incredibly larger size of oocytes (average: 237.51 ± 15.65 μm) compared to the other stages where they were filled with yolk globules and fused to each other. The oocytes were irregular in shape while follicle cells were hardly visible and formed a single line surrounding the oocytes. The nucleus was flattened, small, and barely visible (Figure 3D).

All three treatments (MF, EA and combination of MF and EA) showed positive results on ovarian maturation by producing matured ovaries of Stage 2, 3, and 4, which were determined by the oocyte structure characteristics. This result indicated that MF and

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### Table 1. Percentage (%) of ovarian maturation stage frequency through external morphology based on ovarian colouration throughout a 56-day culture period for the ovarian maturation of *S. olivacea* of control (T1), treatment of Methyl Farnesoate (T2), eyestalk ablation (T3), and combination of Methyl Farnesoate and eyestalk ablation (T4).

| Treatment                                      | Immature Stage Ovary | Mature Stage Ovary |
|------------------------------------------------|----------------------|--------------------|
| T1 Control 95% alcohol μl/g                    | 95.83%               | 4.17%              |
| T2 Methyl Farnesoate 5.0 μl/g                  | 62.50%               | 16.67%             |
| T3 Eyestalk Ablation                           | 45.83%               | 16.67%             |
| T4 Methyl Farnesoate and Eyestalk Ablation     | 33.33%               | 20.87%             |
|                                                 | Yellow Stage 2        | Orange Stage 3     |
|                                                 | 12.50%               | 25.00%             |
|                                                 | 8.33%                | 22.00%             |
|                                                 | Red Orange Stage 4    | 20.80%             |
EA were able to produce a mature ovary within 56 days of the study period.

Figure 4 shows the increase of the mean diameter of oocytes during the 56 days. T1 (control) showed no significant growth of the mean diameter of oocyte size and the highest diameter size was recorded on day 35 throughout the whole culture period. The increase of T2 (MF treatment) mean diameter of oocytes was also not significant up to the forty-second day. Only on day 49, the T2 (MF treatment) showed significant development of the oocyte diameter size. In T2, the highest mean diameter of oocytes was observed on day 56 with 118.5 ± 50.7 μm. For T3 (EA treatment) and T4 (MF and EA treatment), the mean oocyte diameter increased significantly on day 35 and day 28, respectively. Both T3 (EA treatment) and T4 (MF and EA treatment) had the highest mean oocyte diameter on day 56, which was 209 ± 50.7 μm and 234.5 ± 15.1 μm, respectively.
The statistical analysis using one-way ANOVA, followed by Duncan’s Multiple Range Test showed that the mean oocyte diameter for all treatments (T1, T2, T3, and T4) was significantly different ($\rho < 0.05$). Although the time needed for them to mature was distinct from each treatment, the oocyte’s morphology and size were similar to each other as shown in Table 2.

4. Discussion

All treatments showed a positive effect on ovarian maturation as they were able to produce a mature ovary. A similar result was obtained by Laufer et al. (1998) where the ovarian maturation of crayfish, *Procambarus clarkii* was stimulated by MF enrichment food. The evidence in the present study supports that orally administered MF could boost the production of the mature ovary in *S. olivacea*. A previous study by Hall, Mastro, and Prestwich (1999) also showed that 5.5 $\mu$g of MF mixed with pellets increased the fecundity, egg fertility and hatching rate in eye-ablated Giant Tiger Prawn, *Penaeus monodon*. These results indicate that MF can play a significant role in the ovarian maturation of *S. olivacea* due to the presence of an MF receptor or MF binding protein in ovarian tissue (Takac et al., 1993). The colouration of the ovaries in each stage was consistent in all individual crabs. The colouration might be due to the fact that they were being fed with the same type of feed, which was *A. granosa*. The reason *A. granosa* was chosen as a food in the present study was mainly due to its ability to retain the hormones and high protein contents (Haslaniza, Maskat, Wan Aida, & Mamot, 2014). The ovary colour depends on the diet that would influence the carotenoid content (Lee & Puppione, 1988). The same ovary colour depends on the stage, showed that administration of MF and EA haveno side-effects on the ovaries’ colouration in the present study.

Although all treatments were able to produce mature ovaries, the value of GSI from the present study was much lower than previous research by Quinitio et al. (2007) on the ovary of mud crab, *S.*
steroid hormones were given to induce the maturation of the ovary, the GSI of *S. olivacea* did not increase as much as in the wild. It was shown that although the ovary stage was induced by these steroid hormones, the number of oocytes or the eggs may not be as many as when they were produced naturally.

The methods used in present study involved the removal of sinus glands at the eye-stalk and also removed the site production of neuropeptide hormones: Gonad Inhibitory Hormone (GIH) which is also known as Vitellogenesis Inhibiting Hormone (VIH) and Moulting-Inhibiting Hormone (MIH) (Nagaraju, 2011; Yano & Wyban, 1992) which is responsible for the inhibition of ovary maturations. An increase in GSI was observed after EA in *S. olivacea* which indicated that increase in reproductive activity was due to the removal of the GIH present in the X-organ of the eyestalk causing acceleration of gonad development. This result is supported by Borst and Tsukimura (1992) on their experiment where MF concentration level in the hemolymph was undetected (<0.4 ng/ml) in intact crabs, but the MF level increased after EA was performed, which reached variable levels (2.0–31.2 ng/ml) and dropped back to undetectable level when the sinus gland extract was injected. These results showed that sinus gland negatively regulated the MF through the hemolymph. Also, hypertrophy of the MO can be observed in response to EA, and the amount of MF levels in the MO also increased (Nagaraju, Reddy, & Reddy, 2004). Similar experiments have been carried out on other species of freshwater prawn, *Macrobrachium nipponense*, the unilateral and bilateral EA showing an accelerated ovarian growth by observing the GSI (Han & Kim, 1993). A study by Varalakshmi and Reddy (2010) showed that the control group took 25 days while unilateral and bilateral EA group took only 12 days to reach a high ovarian index on freshwater prawn, *Macrobrachium lanchesteri*.

Similar observation was seen in this study where the ablated crabs (T3 and T4) had different GSI and achieved Stage 4 maturity compared to intact crabs treated with MF only (T2) at the end of the experiment. Removal of the eyestalk caused the level of MF in the hemolymph to increase by reducing the MIH and GIH secretion by the X-organ, which enhanced the precocious ovarian maturation of ablated crabs. It appeared that the higher concentration of MF caused the rate of ovarian maturation to increase, which reflected in higher GSI's. EA elevated MF concentration level in the hemolymph in several species (Nagaraju et al., 2004; Lauffer et al., 1987). Previous studies have shown positive effects of MF (Lauffer et al., 1987, 1998, 2005), however, for *S. olivacea*, it seemed that using MF alone achieved ineffective result in comparison with the other two treatments (excluding the control group) as only 8.33% reached mature ovary Stage 4 and this was significantly lower compared to T4 which have the treatment of both EA and MF administration.

Since MF is a regulating hormone between moult- ing and reproduction, insufficient dosage given may result in reproduction failure in the non-breeding season. To obtain the actual levels and sufficient amount of MF in the reproduction process, hormone levels must be measured on every ovarian maturation stage of the sexually mature females obtained from the wild.

The present study showed that crabs treated with MF alone tended to grow rather than to reproduce. X-sinus gland complex controls moultling in crustaceans by secreting peptide hormone, known as MIH where it inhibits a pair of Y-organs situated in the cephalothorax to synthesize and secret ecdysone. Ecdysone is a moulting hormone and will be converted into 20-hydroxyecdysone (20-HE), which is an active moulting hormone in the peripheral tissue. The MF also induces stimulation of ecdysteroid secretion from the Y-organ. A study by Tamone and Chang (1993) observed that secretion of ecdysteroid from the Y-organ was increased as the MF concentration increased. A low level of MF (0.1 µM) did not trigger a response after 48 hour while stimulation of ecdysteroid secretion can be observed at the higher concentration of MF (10 µM). In the present study, only 8.33% achieved a mature ovary (Stage 4) while 62.5% remained in Stage 1 in the MF treatment (T2). Although the MF diet administered had a slower reaction, it does show a positive effect by enhancing the ovarian maturation stage of *S. olivacea*.

The combination of the MF diet administered and EA showed the fastest reaction by producing Stage 2 maturity on day 21 (Week 3). This treatment (T4) induced the increment of oocyte diameter of *S. olivacea* and it was consistent until the end of the experiment. The mean oocyte diameter of this treatment was also the highest compared to the other treatments. Hagedorn and Kunkel (1979) reported that the development of oocytes was associated with yolk. In the present study, the oocyte diameter size increment corresponded to GSI and maturation stages due to yolk accumulation. Oocyte diameter also increased with the maturation stage. Though the results might have been better if a bilateral ablation technique had been used, according to Santiago (1977) it would also increase the risk of mortality of the mud crabs. Hence, that is the reason why this study used the unilateral eyestalk ablation technique.
5. Conclusion

This present study showed that treatment with the combination of MF administration and EA gave the best results. T4 (MF and EA treatments) recorded the highest number of ovaries Stage 4 and the highest GSI. The study also proved that MF administration and EA were able to produce mature ovaries from newly matured females S. olivacea, thus signifying that both techniques (MF and EA) induced vitellogenesis of S. olivacea. However, further study is needed for crucial understanding of their mechanism correlated with the vitellogenesis. Therefore, we highly recommend the use of both combinations of MF administration and EA for S. olivacea aquaculture in the future.

Acknowledgements

This study was funded by Malaysia’s Ministry of Higher Education under Niche Research Grant Scheme (NRGS) of Vote No. 53131. The authors would like to thank the late Assoc. Prof. Dr. Safiah Jasmani for her endless support and guidance, and also to the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Terengganu, Malaysia for providing the necessary equipment and space. Gratitude is sincerely expressed to all people involved directly or indirectly in this study.

Disclosure statement

There are no conflicts of interest among the authors. This material has not been published in whole or part elsewhere. The manuscript also is not currently being considered for publication in another journal. All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

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