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Artificial Crablets Production of Orange Mud Crab, *Scylla olivacea* (Herbst, 1796) Through *in-vitro* Fertilization Technique

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ABSTRACT

Crablets production in mud crab, genus *Scylla* is highly depends on the depleted wild crablets supply. Artificial crablets production through *in-vitro* fertilization technique can be an alternative for the crablets production of this mud crab. Therefore, this study was conducted to explore the artificial crablets production through *in-vitro* fertilization technique on orange mud crab, *Scylla olivacea* through the determination of (1) Fertilization rate and (2) Embryonic developments from the manipulation of sperm mass with Stage 4 ovary. The sperm mass were collected from the spermathecae of mature females, as well as from the vas deferens of males and allowed to fertilize the Stage 4 ovary in the Ca-F saline medium. Fertilized ovary were incubated in artificial seawater with controlled salinity (28-30 ppt) and temperature (28-30°C in water bath). Observation on fertilization rate and embryonic development were observed daily. Fertilization Membrane (FM) was developed when the ovary was fertilized. However, the embryos could only develop until Day 5 at most. Subsequently, FM could not be maintained and started to deteriorate resulting the embryos to lose their form. Further development could not be observed after Day 5. Manipulation of sperm from vas deferens (male) shows lower fertilization rate than spermathecae (female). Present study has proven that artificial crablets production *S. olivacea* through *in-vitro* fertilization technique from the manipulation of sperm mass with Stage 4 ovary was a success.

Key words: Artificial production, crablets, *in-vitro* fertilization, orange mud crab, *Scylla olivacea*

INTRODUCTION

Orange mud crab, *S. olivacea* is an important and the most abundance mud crab species in the Malaysia (Ikhwanuddin *et al.*, 2010a, b, 2011). To date, the crablets supplies for *S. olivacea* aquaculture are mainly dependent on natural stocks because successful hatchery crablets production is yet to be developed. In addition, obtaining the berried females mud crab for the production of hatchery-reared crablets appears to be difficult. Whilst obtaining the mud crab crablets through natural mating in captivity might be possible (Baiduri *et al.*, 2014) with artificial crablets production can be an alternative to overcome the crablets supply problems for stocking in the farm and natural resources sustainability.

Artificial crablets production in mangrove crab, *Perisesarma bidens* has been reported by Sarker *et al.* (2009) through *in-vitro* technique in which the gametes were allowed to fertilize in

culture dish. Although, *in-vitro* fertilization technique have been applied in other aquatic invertebrates such as in rock shrimp, *Rhynchocinetes typus* (Dupre and Barros, 2011), squid, *Loligo paeleii* (Crawford, 2002) and marine snail, *Thais haemastoma* (Belisle and Byrd, 1980), these attempts were not yet commercially practiced and entirely in the laboratory stage. Embryonic development is one of the important components in order to verify the success of *in-vitro* fertilization (Sarker *et al.*, 2009; Rojas and Alfaro, 2007).

Although, Sarker *et al.* (2009) has reported that *in-vitro* fertilization in mangrove crab, *P. bidens* could occur, *in-vitro* fertilization in mud crabs, genus *Scylla* has not yet been reported. Thus, the aim of this study is to investigate the possibilities of artificial crablets production of orange mud crab, *S. olivacea* through *in-vitro* fertilization technique by determining the (1) Fertilization rate and (2) Embryonic developments from the manipulation of sperm mass with Stage 4 ovary.

METHODOLOGY

Both mature female and male crabs of *S. olivacea* used were collected from Setiu Wetlands, Terengganu coastal water, ranged from 9-13 cm Carapace Width (CW), the size at the onset of sexual maturity (Ikhwanuddin *et al.*, 2010a, b). Crab samples were cold-anesthetized and dissected for gamete collection. Stage 4 ovary with reddish orange colour (Ikhwanuddin *et al.*, 2014; Quintio *et al.*, 2007) were collected from the abdominal cavity (Fig. 1), placed into Petri dishes provided with Ca-F saline medium and cut into small pieces to obtain ovary mass. Ca-F saline medium was prepared according to Memon *et al.* (2012) and Noor-Hidayati *et al.* (2014). Mature sperms of *S. olivacea* were extracted from both left and right spermathecae (ST) of the same females for Trial 1 (T-ST) and also from the Vas Deferens (VD) of males for Trial 2 (T-VD). The ST and VD were cut and homogenized in Ca-F saline to obtain sperm suspension. Female ST is whitish in colour and attached to the ovary of newly mated female (Fig. 2) and mature male VD is also white in colour and swollen containing spermatophores (Fig. 3) (Noorbaiduri *et al.*, 2014).

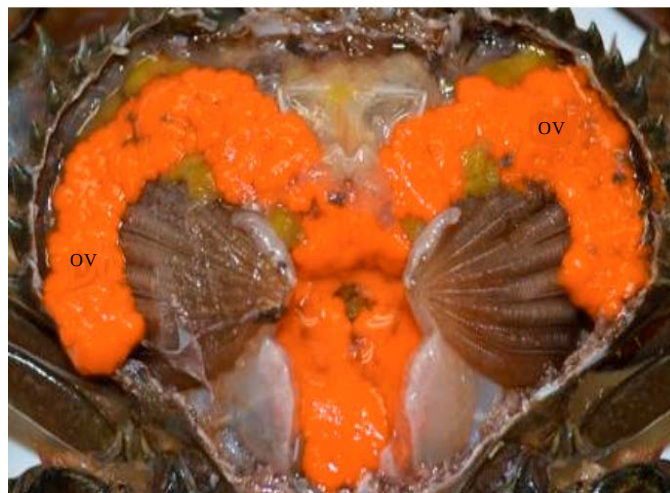


Fig. 1: Stage 4 ovary of *S. olivacea* with reddish orange colour filling up the abdominal cavity, OV: Ovary

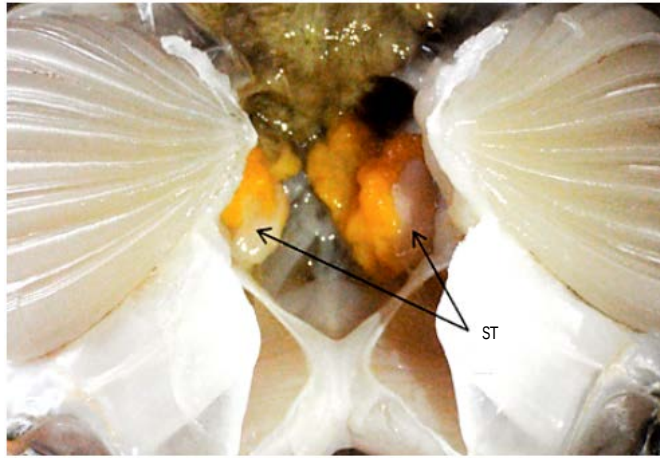


Fig. 2: Female spermathecae (whitish colour) attached to the ovary of newly mated female *S. olivacea*, ST: Spermathecae

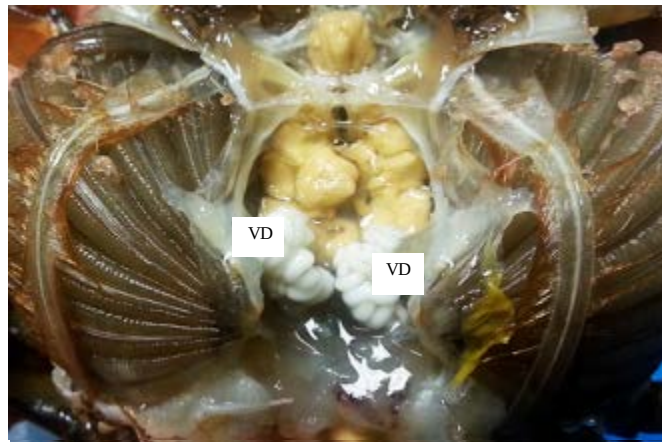


Fig. 3: Reproductive system of mature male *S. olivacea*, VD: Vas Deferens

ST and VD sperm suspension were added into Stage 4 ovary mass of *S. olivacea* and agitated for 2-3 min for gametes attachment and allowed to fertilize in 1-2 min at room temperature before excess sperm were removed by consecutive washes with Artificial Seawater (ASW). Then, the gametes were transferred into culture container containing 100 mL ASW with adequate aeration at salinity of 28-30 ppt. Temperature was control at 28-30°C using water bath. Fertilization was observed within 1-2 h after the introduction of gametes and embryonic development was continuously observed for the following days.

Subsequently, fertilized gametes were termed as embryo once the Fertilization Membrane (FM) was observed. Embryonic development were described and determined according to Ates *et al.* (2012) and Sarker *et al.* (2009). Observation of 30 embryos development was done under the Advanced Research Microscope ECLIPSE 80i daily. Image of embryos were taken and the

diameters were measured to the nearest 0.01 μm using NIS-Elements imaging software. The embryos incubation period was determined as the number of days from fertilization. Fertilization Rate (FR) and embryonic development rate (ER) was calculated by the following equation:

$$\text{FR (\%)} = \frac{\text{No. of fertilized embryos}}{\text{Total embryos counted}} \times 100$$

$$\text{ER (\%)} = \frac{\text{No. of developing embryos}}{\text{Total embryos counted}} \times 100$$

Statistical analysis: Data were presented as Mean $\pm$ Standard deviation. All data were analysed with ANOVA for significant level at $p < 0.05$.

RESULTS

In-vitro fertilization trials was done by utilization of sperm from two different sources which were from mature female's spermathecae (T-ST) and male's vas deferens (T-VD) to fertilize the Stage 4 ovary. Fertilization Membrane (FM) will be developed when the ovary was fertilized. Embryos with presence of Fertilization Membrane (FM) were observed in both trials, T-ST and T-VD (Fig. 4). Higher fertilization rate was observed in T-ST with 55.00% as compared to T-VD with only 33.30% (Fig. 5).

Following fertilization, embryonic development of *S. olivacea* through *in-vitro* fertilization was observed from Day 1-5 (Fig. 6). Embryos were observed up to 16-cell stage at Day 1. Blastula stages were observed at between 1-2 Day. As the cleavage continued, the yolk cells increased in number and thus, fill up the Perivitelline Space (PS). Gastrula stages were observed at 1-3 Day. Tissue Cup (TC) started to appear. Embryos gradually develop from 2-5 Day and egg yolk start to take form leaving the Yolk-Free Space (YFS) inside the embryos. As the developments continue, YFS gets widen. However, the embryos could only develop until Day 5 at most. Subsequently, FM could not be maintained and started to deteriorate resulting the embryos to lose their form due to the released of yolk substance (Fig. 7). Further development could not be observed after 5 Days.

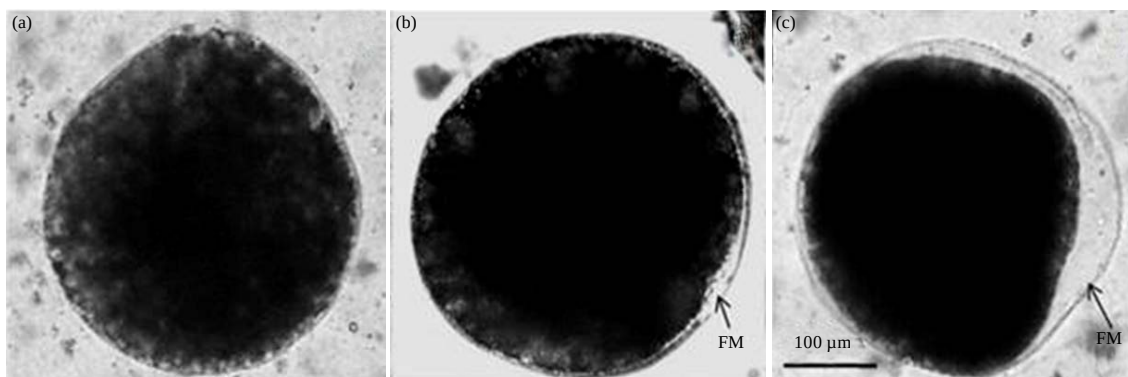


Fig. 4(a-c): Fertilization of *S. olivacea* gametes through *in-vitro* fertilization. (a) Unfertilized ovary, without FM formation and (b-c) Fertilized ovary with FM formation, FM: Fertilization Membrane

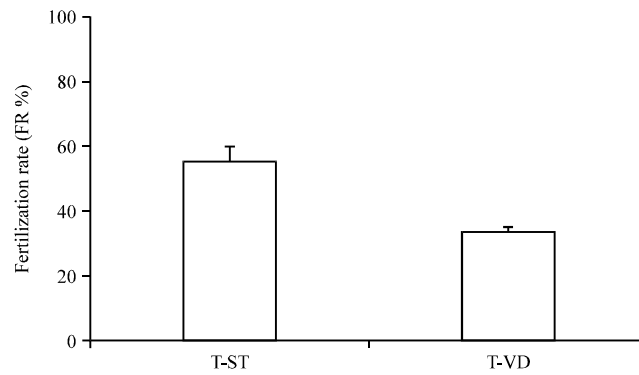


Fig. 5: Fertilization rate of *S. olivacea* through *in-vitro* fertilization by utilization of sperm from two different sources, T-ST: Sperm from female's spermathecae, T-VD: Sperm from male's vas deferens, N = 30

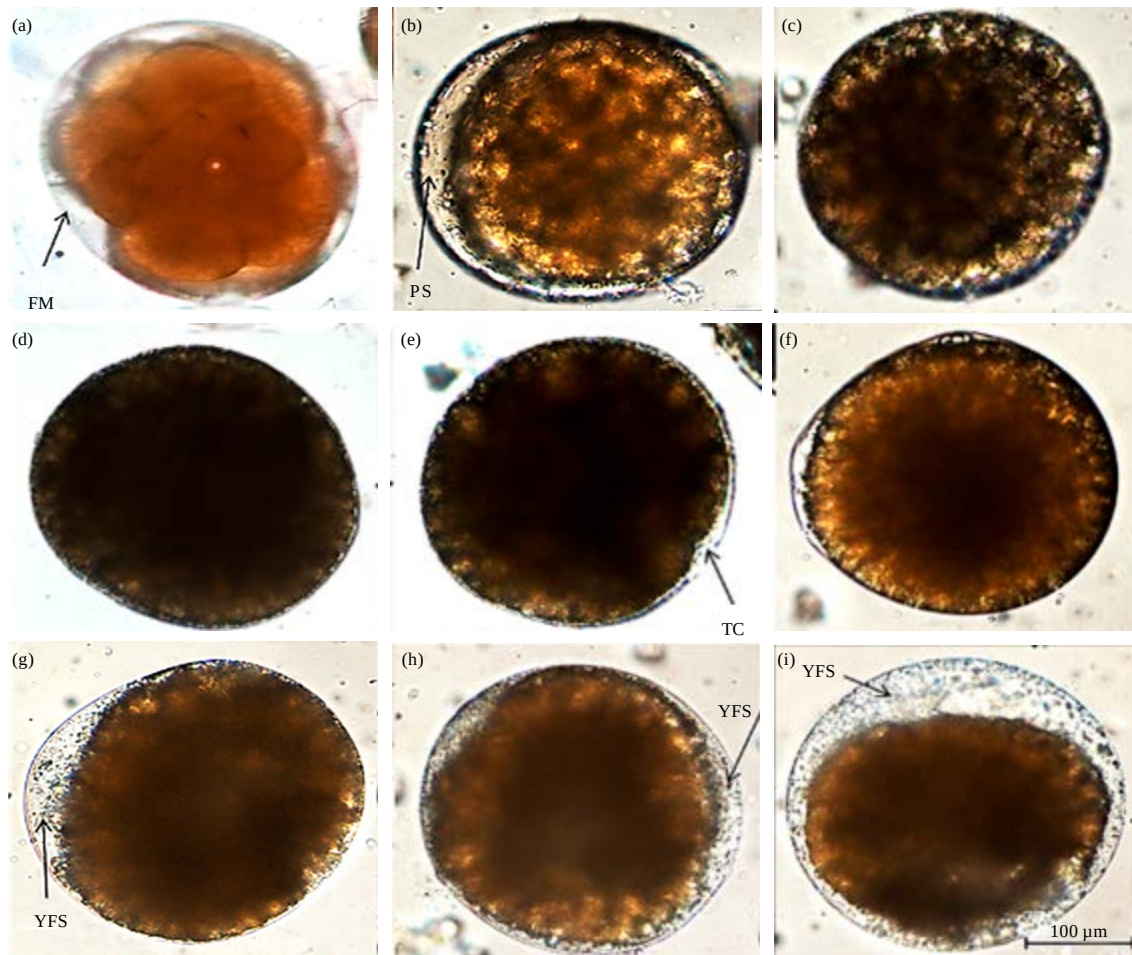


Fig. 6(a-i): Early embryonic development of *S. olivacea* through *in-vitro* fertilization technique (a) 16-cell stage, Day 1, (b-c) Blastula, Day 1-2, (d-f) Gastrula, Day 1-3, (g-i) Embryo, Day 2-5, FM: Fertilization Membrane, PS: Perivitelline space, TC: Tissue Cup, YFS: Yolk-Free Space

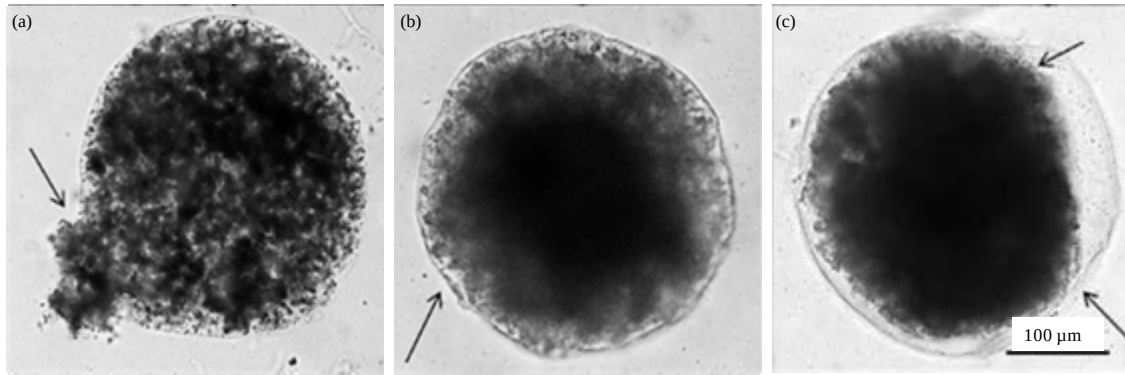


Fig. 7(a-c): Deterioration of embryos through *in-vitro* fertilization of *S. olivacea*. (a) Embryos with deterioration of FM releasing yolk substance and (b-c) Embryos with deteriorating FM, resulting abnormal shape, (a-c) Arrows, sign of deterioration of FM

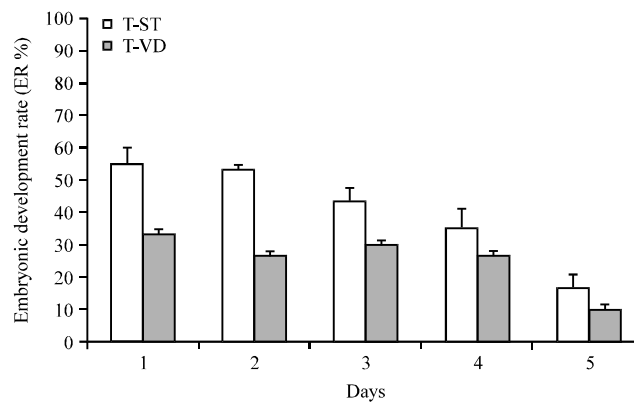


Fig. 8: Embryonic development rate (ER) of *S. olivacea* through *in-vitro* fertilization by utilization of sperm from different sources, T-ST: Sperm from female's spermathecae, T-VD: Sperm from male's vas deferens, N = 30

Figure 8 shows the embryonic development rate (ER) of *S. olivacea* through *in-vitro* fertilization by utilization of sperm from ST and VD. T-ST show higher ER as compared to T-VD throughout the 5 days trial period. However, both trials T-ST and T-VD show the declining of ER as the number of days increased. After Day 3, deterioration of FM were observed on the cultured embryos which resulting the decrease of ER. The *in-vitro* fertilization culture seems to facilitate the growth of microorganisms throughout the culture period. Microorganism may feed on and caused damage to the FM and the whole embryo subsequently. Thus, prevent further development of the embryos.

Figure 9 shows the mean diameter of *S. olivacea* embryos through *in-vitro* fertilization. The mean size of embryos diameter was range from 145.94+9.71-185.93+41.00 µm. There was no significant difference of embryos size from Day 1-5 in both *in-vitro* fertilization trials, T-ST and T-VD. In addition, the mean diameters were fluctuated and show neither size increment nor declining throughout the culture period.

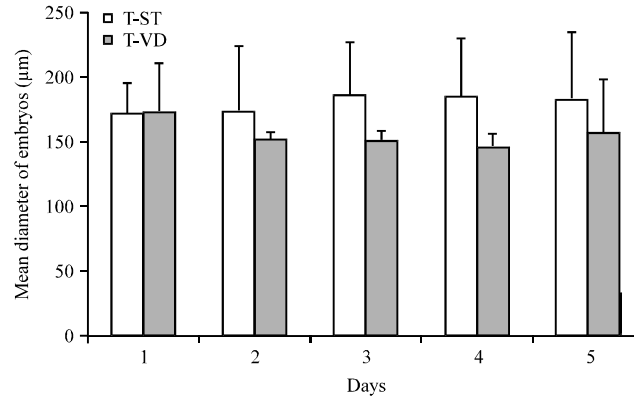


Fig. 9: Mean diameter of *S. olivacea* embryos through *in-vitro* fertilization by utilization of sperm from different sources, T-ST: Sperm from female's spermathecae, T-VD: Sperm from male's vas deferens, N = 30

DISCUSSION

Present study shows the possibilities of *S. olivacea* artificial crablets production through *in-vitro* fertilization technique. Although hatching larvae could not be obtained throughout the trials, considerably high fertilization rate was observed. T-ST show higher fertilization rate as compared to T-VD. VD-sperm might not fully mature and need further maturation before fertilization (Rojas and Alfaro, 2007). In addition, Noorbaiduri *et al.* (2014) stated that ST-sperm possess more advance acrosomal activation compared to VD-sperm which allow higher fertilization rate.

Although, study by Sarker *et al.* (2009) revealed 95.00% fertilization rate and 65.00% hatching rate, the present study shows only 55.00% fertilization rate without hatching. Such difference was due to that the present study was utilizing the Stage 4 ovary instead of unfertilized embryos for the *in-vitro* fertilization. Therefore, indicates that fertilization can still occur between sperms and final stage ovary. Although, study by Rojas and Alfaro (2007) in shrimp, *Litopenaeus* sp. also utilized the matured ovary, fertilization rate was not reported.

Hatching could not be obtained through out the present study and this might due to the unfavourable incubation condition. *Scylla olivacea* usually incubate their embryos attached within the abdominal flaps from spawning until hatching (Ates *et al.*, 2012) which might provide essential protections and oxygen supplies. In addition, provided aeration in the present study has caused continuous water agitation which result in unsettlement of individual embryos. Therefore, some modification on the incubation condition needs to be done to permit complete development or hatching.

Embryos size in the present *in-vitro* fertilization study range from 145.94±9.71-185.93±41.00 µm which are moderately smaller than embryos diameter from natural fertilization which range between 275-343 µm (Ates *et al.*, 2012). During the incubation period, some embryos were deteriorating and microorganisms were presence in the culture medium overnight. These microorganisms may feed on the embryos and thus, inhibit further embryo development. Following Caceci *et al.* (1996), scheduled treatments for *in-vitro* fertilization incubation involving formaldehyde, hydrogen peroxide, antifungal and antibiotic treatments should be done for better results. However, deleterious effects of these treatments on the embryo development should also be taken into consideration.

In fact, the present study has proven that the *in-vitro* fertilization technique from manipulation of sperm mass with Stage 4 ovary of *S. olivacea* was a success. These achievements can be an initiative for a selective breeding program to facilitate large scale crablets production in mud crab farming in the near future. Obtaining berried females *S. olivacea* can be much harder as compared to the females with Stage 4 ovary. Furthermore, gamete collection was simple and fertilization could be done in desirable time without the requirement of pre-spawning condition (Sarker *et al.*, 2009). However, necessary adjustment and modification on the culture medium and culture condition are very crucial in order to complete this *in-vitro* fertilization technique in the near future.

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