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Research Article
Determine the Sperm Quantity and Histological Characteristics on the Testes of Male Mud Crab, *Scylla tranquebarica*

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Abstract

Background and Objective: Mud crab, *Scylla tranquebarica* has been reported to be present in Sabah coastal waters and this specie is one of commercially important crustaceans in the trade markets as demand that has been reported to be increased every year. However, high demands had caused exploitation to this species in Asia and there is still lack of in-depth study about this species mainly on sperm quantity and sexual maturity size. The present study was carried out to investigate the sperm quantity and sex maturation stages based on histology examination. **Materials and Methods:** Twenty of *S. tranquebarica*'s male were dissected and the testes were dissected out and divided into two parts where ¾ was used for determination of sperm quantity and ¼ was used for histological examination. The sperm were observed by using microscope under 40x magnification and the quantity were count by using haemacytometer. For histological observation, the testes were fixed in Bouin's solution for less than 24 h, dehydrated pass through a series of increasing ethanol concentration, cleared with xylene, immersed in paraffin and formed a tissue block. The samples were stained by pass through a series of xylene, decreasing ethanol concentration, hematoxylin and Eosin Y. **Results:** There was a significant difference between Body Weight (BW), Carapace Width (CW) and sperm quantity with range of body weight for male *S. tranquebarica*. The highest sperm quantity was recorded on *S. tranquebarica* with the body weight; (401-500 g). **Conclusion:** The study concluded that the maturity size of *S. tranquebarica* was at 246.04 g in BW and 10.6 cm in CW.

Key words: Mud crab, *Scylla tranquebarica*, sperm quantity, maturation stages, histological

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mud crab, *S. tranquebarica* has been reported to be present in Sabah coastal waters[1]. Presently, mud crab farming has great attention in Malaysia. Furthermore, mud crabs (genus *Scylla*) are very demand as a quality food owing to their size, meat content and delicate[2] and thus, it has a high prices in domestic and international markets[3,4]. However, these farming activities were only for fattening and the production of soft-shelled crabs from the wild[4,5]. Furthermore, increasing the human population and market demand led to the constraint supply of seed stocks caused by over exploitation[6]. According to Phelan and Grubert[7], the female mud crab have a wider and globular abdomen, while the male mud crab have a narrow and straight abdomen which looks like T-shaped abdomen[8]. There are some previous studies that had touch on the male gonad of genus *Scylla* such as Ikhwanuddin *et al.*[9], Waiho *et al.*[10] and Ikhwanuddin *et al.*[11].

Based on Islam and Kurokura[12], there are three common methods to determine the sex maturity on male crustaceans. First by morphometric method where the change of allometric relationship between sizes of body parts are identified[13]. Second by the histological method where the histological examination of the gonad are conducted to see if sperm are present in the testes[14] and the third method by functional maturity where this method conduct a detection on the scars on the sternum or forward walking legs which are produced by abrasion with the female during the precopulatory embrace[15]. Besides, one of the main constraints with supplies from wild caught seed is sperm quality and quantity[3]. This study was conducted to determine the sperm quantity and testes maturation stages, male gonad is the main target to be identified. Male gonad of mud crab is known as testes. Testes are located next to the hepatopancreas which place under the carapace of the mud crab. Testes are connected to a vas deferens which looks like a thin white coiled tube[16]. Identification of testes make able for the determination of sperm quantity and maturation stages on testes of the mud crab. Thus, histological examination was used to determine the maturity stages of the target species. Therefore, this study was conducted to provide new guidelines on the size of *S. tranquebarica* species that are appropriate for commercial capture to avoid overexploitation of this species.

MATERIALS AND METHODS

Study site and sample of male mud crab, *Scylla tranquebarica*: The research study was done from January-March, 2018 in Shrimp Hatchery and laboratory at Borneo Marine Research Institute, Universiti Malaysia Sabah. In this study, the male mud crab, *S. tranquebarica* were used as a target species. The species was bought from Kota Kinabalu Central Wet Market, Kota Kinabalu, Sabah. A total of 20 males *S. tranquebarica* were used as the samples and choose randomly based on 5 groups of Body Weight (BW) classification. Each group were represented by 5 males of *S. tranquebarica* (Table 1).

Measurement of Body Weight (BW) and Carapace Width (CW): The samples were labelled by using pen marker. The BW and CW of each samples were taken and recorded where the BW were collected by using digital electronic balance weight and the measurement of the CW were taken by using vernier callipers in unit of centimetre (cm).

Collection of sperm: The samples were sacrificed and after the samples were sacrificed, dissections of the samples were conducted where the carapace was opened by using scissors. This procedure was conducted with careful to avoid organs that located under the carapace from become damaged. The locations of the male gonad were identified and the testes were detected. By using a forceps, the testes were removed from the whole body and put into a clean petri dish. Then, the testes was divided into 2 parts which the 1st part is ¾ of the testes that used for determination of the sperm quantity and 2nd part is ¼ of the testes that used for the histological

Table 1: Classification for 20 males of *Scylla tranquebarica* with the information of their Body Weight (BW) and Carapace Width (CW)

Classification of crabs (g)	No. of crabs	BW (g)	CW (cm)
101-200	1	174.97	9.3
	2	176.05	9.4
	3	189.77	9.7
	4	143.58	9.1
	5	188.03	10.3
201-300	1	264.98	11.4
	2	246.04	10.6
	3	277.47	11.6
	4	214.01	10.4
	5	226.95	10.5
301-400	1	359.39	11.8
	2	309.39	10.8
	3	352.81	11.5
	4	333.71	11.2
	5	372.2	11.8
401-500	1	453.97	13.2
	2	471.06	13.1
	3	413.75	12.0
	4	417.87	12.5
	5	406.79	12.0

examination. Testes that collected for histological examination were placed in fixation solution which is Bouin's solution and testes that collected for sperm quantity determination were placed quickly on the mortar to conduct the next step in this study.

Sperm quantity assessment: By using the mortar and pestle, the testes were homogenized manually with 200 µL of Ca-F saline to form a sperm suspension. Then, stain solutions (eosin-nigrosin solution) were prepared with 0.5 g of eosin and 10.0 g of nigrosin were dissolved in 100 mL of distilled water for each stain solution[9]. After that, 10 µL of sperm suspension was transferred to a clean Petri dish and mixed with 5 µL of eosin-nigrosin for sperm viability. The stained sperm suspension was transferred to a clean glass slide. By using light microscope under 40x magnifications, the condition of the sperm was observed. The observations of the sperm quantity were conducted in replicate for each sample. For counting the sperm quantity, one petri dish was prepared, where 10 µL of sperm suspension and eosin-nigrosin were mixed until homogenized. The stained of sperm suspension then was transferred to a clean haemocytometer. By using light microscope under 40x magnifications, the sperm quantity of *S. tranquebarica* was counted and the data was taken and recorded.

Histological of testes in *Scylla tranquebarica*: Histological examinations were conducted for the all of 20 males mud crab. A first step in histological examination is fixation. In this study, Bouin's solution that made up by 100% picric acid, 37% formalin and 100% of acetic acid with the ratio of 15:5:1 was used as the fixatives solution[17]. Each ¼ of the testes that have been separated for histological examination were preserved in the Bouin's solution for less than 24 h.

A next step in histological examination is dehydration. The samples were transferred into 70% of isopropyl alcohol (IPA). The IPA that had been used in this study was ethanol. After less than 24 h in Bouin's solution, the samples were transferred into 70% ethanol for 24 h. The samples can be immersed in the 70% ethanol for a long time. Next, the samples were transferred pass through a series of increasing ethanol concentration, where samples were immersed for about 30 sec in each ethanol series. After dehydration, a clearing process was conducted. During the clearing process, each sample was immersed twice in xylenes which are xylene I and xylene II for 30 min, respectively. Then, the samples were placed inside the paraffin for a twice where paraffin I contain the mixture of paraffin and xylene, while paraffin II contain only the paraffin. Each samples was placed inside both paraffin for 60 min, respectively and this work was conducted under the control of oven with temperature of 57-60°C to prevent the paraffin from become solid. Then, the samples were embedded by using mold and were left for overnight to form the embedded samples. The next day, all of the embedded samples were placed on a wooden block for blocking purposes. The samples then were cut and trimmed into a cube shape. By using rotary microtome, each sample was cut to 5 µm of cutting thickness which produced a cross section ribbons[18]. Each ribbons were placed in a water bath with temperature 35-37°C to produce inflorescence and non-wrinkle ribbons. Then, each ribbon were placed on the glass slides and left overnight on the slide warmer with temperature 20°C.

The next day, the samples were stained by following the stains method. The samples were deparaffinised by using xylene, then rehydrated through a series of descending ethanol solutions (Ethanol 100, Ethanol 95, Ethanol 80 and Ethanol 70) to demineralized water. Then, the samples were stained with Hematoxylin by immersed it for 8-15 min. After that, the samples were placed in distilled water until dye is no longer being released from the samples. The samples next were counterstained with one of the contrasting solution which is Eosin Y. The samples were immersed in Eosin Y for 8-15 min also. After that, the samples were dehydrated by immersed it into two exchanges of 95% ethanol solution and three exchanges of 100% ethanol solution. The final steps in staining where the samples were cleared by immersed it in two exchanges of xylene. As the last procedure in histology, the slides that contain the samples were fixed with appropriate medium like Canada balsam and then were covered with cover glass. All the samples then were observed under light microscope for histological examination.

Statistical analysis: Data analysis was collected on the BW (g) and CW (cm) of *S. tranquebarica*. Data was analyzed by using Microsoft Excel 2013. Besides, data were presented as mean±standard deviation. Statistical analyses was conducted by using one-way ANOVA ($p<0.05$) through the application of SPSS software version 24.0.

RESULTS

Relationship between Body Weight (BW), Carapace Width (CW) and sperm quantity of *Scylla tranquebarica*: The mean BW and CW in 20 males *S. tranquebarica* were

identified and divided into 5 range of BW (101-200, 201-300, 301-400 and 401-500 g). Figure 1 and 2 showed the mean BW and CW were increased when the range of BW were increased. There were significant different between the mean BW and the range of BW samples ($p<0.05$). However, there were no significant different between the CW of *S. tranquebarica* with range of BW; 201-300 and 301-400 g. For the sperm quantity, Fig. 3 showed the sperm quantity was increased when the range of BW were increased. However, there were no significant different between the sperm quantity on samples with range of BW; 101-200, 201-300 and 301-400 g.

Testes maturation stages of *Scylla tranquebarica*: A total of 20 male crabs were assessed to observe the gonadal condition. The testes maturation stages were determined by histological observation on the testes of *S. tranquebarica*. Findings on the histological observation found that there were three stages of testicular maturation stage on *S. tranquebarica* such as immature stage, maturing stage and mature stage (Table 2, Fig. 4). In the present study, there were 35% in immature, 40% in maturing and 25% in mature. The findings in the present study showed the smallest mature male was found at 246.04 g in BW and 10.6 cm in CW, which was classified in 201-300 g range of BW (Table 3). Based on Table 3, at 201-300 g of BW, the histological observation found that each stage (Immature, maturing and mature) were presented in this range of BW. Besides, the present study also found that immature stage can be found at 101-400 g in BW while immature stage was absent at 401-500 g in BW. From 20 males of *S. tranquebarica*, 7, 8 and 5 samples were categorized as immature, maturing and mature and the maturing stage can found at all ranges of BW (101-500 g). For mature stage, the present study found that mature stages were presented at 201-300 and 401-500 g in BW.

Table 2: Stages of physiological sexual maturity of male *Scylla tranquebarica* and their composition in respective stage

Characteristics	Remarks	Individuals (%)
Stage I		
Testes not visible to the naked eye	Immature	35
Vas deferens resemble translucent filament		
Presence of spermatogonia		
Stage II		
Small testes	Maturing	40
Thin vas deferens		
Formation of primary and secondary spermatocytes		
Stage III		
Testes swollen	Mature	25
Opaque and white		
Vas deferens swollen and pink		
Containing spermatophores		

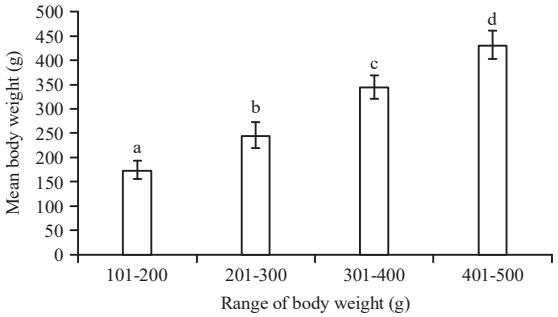

Fig. 1: Relationship between mean body weight and range of body weight (n = 5)

a, b, c and d indicates the differences between mean body weight on each range of body weight, error bars indicate standard deviation

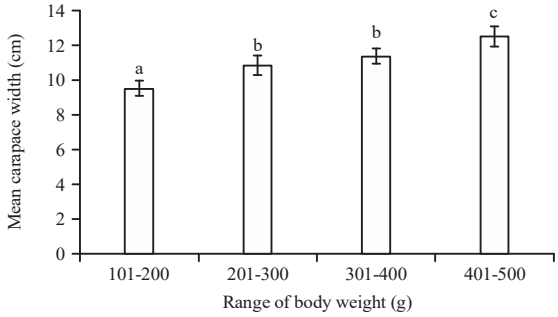

Fig. 2: Relationship between mean carapace width and range of body weight (n = 5)

a, b and c indicates the differences between mean carapace width on each range of body weight, error bars indicate standard deviation

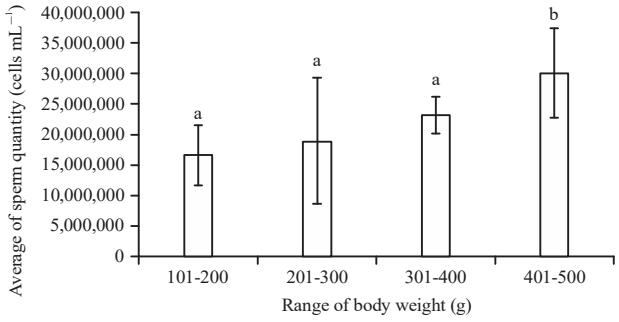

Fig. 3: Relationship between average of sperm quantity and range of body weight (n = 5)

a and b indicates the differences between average of sperm quantity on each ranged of body weight, error bars indicate standard deviation

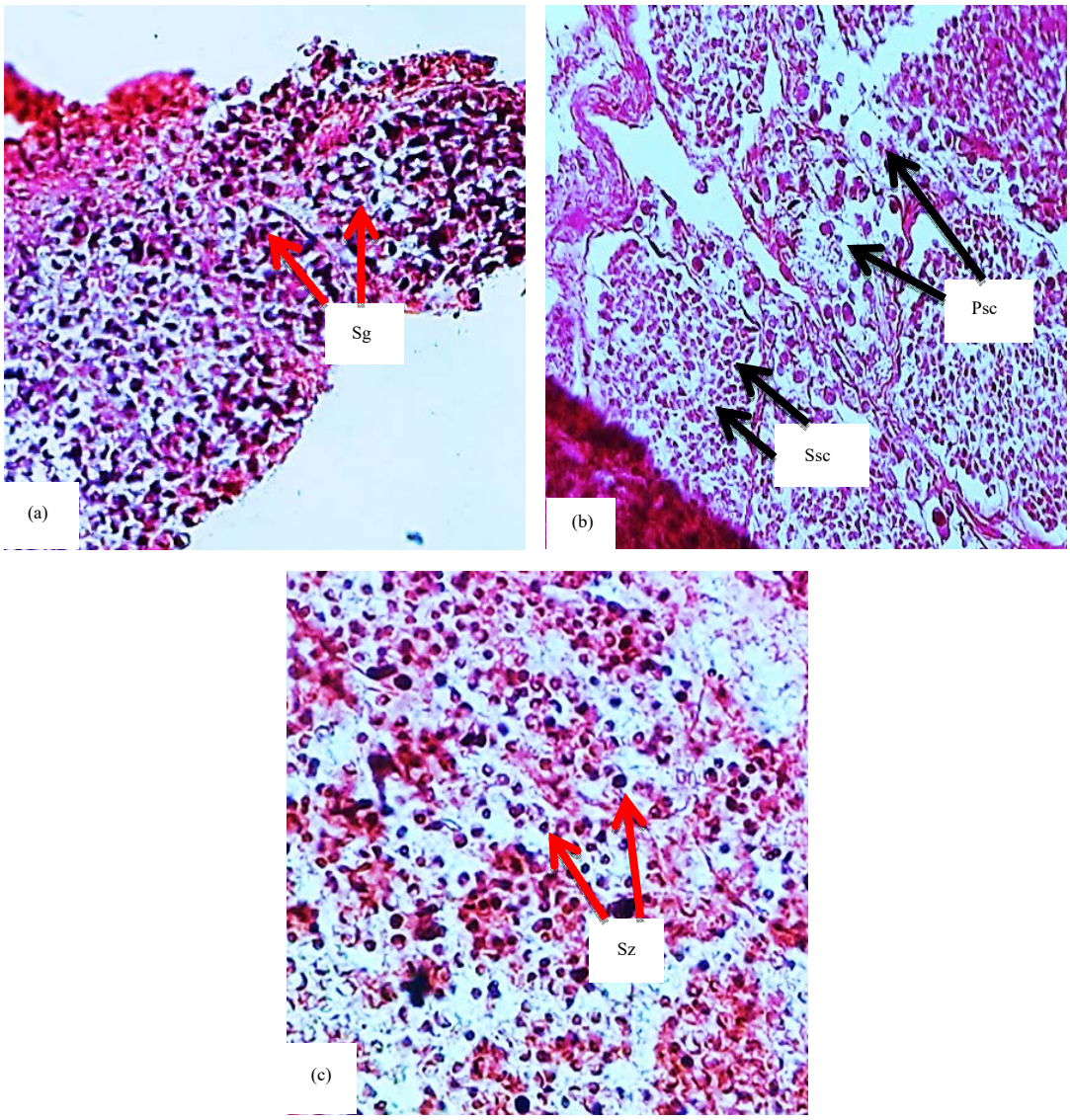

Fig. 4(a-c): Testes development stages based on histological observation, (a) Immature, (b) Maturing and (c) Mature
Sg: Spermatogonia, Psc: Primary spermatocytes, Ssc: Secondary spermatocytes, Sz: Spermatozoa

Table 3: Testes maturation stages for each range of body weight

No. of samples	Range of body weight (g)			
	101-200	201-300	301-400	401-500
1	Immature	Maturing	Maturing	Maturing
2	Immature	Immature	Maturing	Mature
3	Maturing	Mature	Immature	Maturing
4	Maturing	Immature	Maturing	Mature
5	Immature	Mature	Immature	Mature

Figure 4 showed three stages of testes maturation stages were identified using histological examination. The immature stages were classified as spermatogonia which identified as it forms incomplete and larger circle. Besides, the maturing stage showed the primary and secondary spermatocytes which identified as it forms two types of circle. The mature stage showed the spermatozoa which identified as it forms a complete and small circle.

DISCUSSION

The larger male *S. tranquebarica* at 401-500 g (BW) has the higher sperm quantity in the testes rather than small males and this is same with the findings of Sato *et al.*[19], which they found that in anomurans (decapod crustaceans). From the present study, the sperm quantity was increased

according to the increase of the body weight. From previous study, sperm quantity was increased based on the increased of gonad size and the build-up of seminal fluids as the weight of testes was also increased dramatically during this period[10]. Therefore, these findings showed *S. tranquebarica* with high BW has a high sperm quantity than mud crab with low BW. Less sperm quantity may due to the size of testes were still small and immature stage was only found on range of BW (101-200, 201-300 and 301-400 g) but not in 401-500 g.

From previous studies, Robertson and Kruger[20] described the presence of sperm on testes of *S. serrata* while, Waiho *et al.*[10] also described the formation and development of sperm in *S. olivacea.* Besides, three gonad development stages were observed by histological examination and appear to be equivalent in *S. olivacea* and *S. paramamosain*, respectively[13,21]. The stage I (immature) and II (maturing) defined as the males with undifferentiated testes and males with differentiated testes but no spermatophores or spermatids, while stage III (mature) characterized as males with prominent and convoluted testes that containing spermatophores. Based on the present study, 35% in immature, 40% in maturing and 25% in mature was identified from 20 males of *S. tranquebarica*. The smallest mature male *S. tranquebarica* was recorded at 246.04 g (BW) and 10.6 cm (CW). Previous study by Islam and Kurokura[13,21] showed the estimation in mature size for male *S. olivacea* and *S. paramamosain* were at 8.4 and 11.0 cm (CW), respectively. Thus, the maturation size in male *S. tranquebarica* was larger than *S. olivacea* but almost similar to *S. paramamosain*.

CONCLUSION

Males *S. tranquebarica* at 401-500 g (BW) contained the high sperm quantity and at 246.04 g (BW) and 10.6 cm (CW) or more were considered as a mature testes that ready for fertilization. Hopefully, the data from the present study would able to contribute for the future biological studies and give contribution for aquaculture and fisheries management for mud crab sustainability.

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