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# Research Article
# Histological Characteristics on the Testes of Mud Spiny Lobster, *Panulirus polyphagus* (Herbst, 1793)

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## Abstract

**Background and Objective:** Mud spiny lobsters, *Panulirus polyphagus* (*P. polyphagus*) are one of the most important fisheries resources now-a-days due to quality that it's possessed. However, there is still lack of in-depth study about this species mainly on males testicular characteristics and sexual maturity size. Therefore, the present study was carried out to investigate the histological characteristics on the testes and sexual maturity sizes of mud spiny lobster, *Panulirus polyphagus*. **Materials and Methods:** The testes were dissected out and fixed in 10% buffered formalin solution for 11 h, dehydrated in 70% alcohol and lastly placed in tissue processor for 18±1 h at 60°C. The tissues blocks were cut at the thickness of 4 µm on a rotary microtome. Stained tissues were taken under Advance Microscope (Nikon Eclipse 80i Nomarski DIC). Collected data were analyzed using Microsoft Excel 2013. Data were presented as mean±standard deviation. Statistical analyses were done using one-way ANOVA using SPSS (Version 22). **Results:** These lobules of mature *P. polyphagus* were formed via different germinative lineage cells such as spermatogonia, spermatocytes, spermatids and spermatozoa. The histological characteristics of testes showed that the process of spermatogenesis went through the stages of four testes maturation which were spermatogonia I and II, spermatocytes I and II, spermatids and spermatozoa stages within different body weight of *P. polyphagus*. It was found that there were significant difference between body weight and carapace length to the testicular maturation stages (one-way ANOVA and $p = 0.000$). **Conclusion:** The results of this experiment indicated that males *P. polyphagus* have four stages of testes maturation and can be considered to have fully mature testes that ready for fertilization at 452 g body weight (BW) and 107 mm carapace length (CL) or more.

Key words: Mud spiny lobster, *Panulirus polyphagus*, testes, testicular histological assessment, sexual maturity, spermatogenesis process

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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

*Panulirus polyphagus* is usually known as mud spiny lobster or previously known as long-whiskered rock lobster[1,2]. In Malaysia, the culture of spiny lobsters is still in its early years with activities based in the State of Sabah mostly concentrated in the Darvel Bay (Lahad Datu and Semporna), Kudat and Kota Kinabalu[3]. Besides, similar other palinurid lobster, *P. polyphagus* is also a marketable and expensive fisheries commodity in Indonesia[4]. The major problem of mud spiny lobster, *P. polyphagus* is in breeding technology with a low survival rate of the larvae stages and difficult to maintain from the pueruli stage until adult[5-10].

The reproductive system of mud spiny lobster is extended outside the cephalothorax, lies on the hepatopancreas and elongates longitudinally at both sides of the median body plane. The male reproductive system comprises of paired testes, vas deferentia and ejaculatory ducts[11]. The testes are tubular organs in the anterior region of the body that extend along the sides of the stomach and are commonly united by a medial commissure between the posterior end of the stomach and the anterior region of the heart[12,13]. The lobster testes have been revealed as long, highly convoluted tubes and connected medially by a transversal commissure resembling an H-shape in histology studies[14]. It is very difficult to assess male lobster maturity in the field[15] than in female lobster. In some male spiny lobsters, the development of the second and third walking legs enables them to attract/remove suitable female lobsters from their den[16]. Their size of maturity (SOM) is detected by plotting the size of walking legs against carapace length (CL) for both, mature and immature lobsters[17].

The significances of the study are to give an overview of the reproductive biology in male *P. polyphagus* which include the testes characteristics and sexual maturity size. Thus, the aim of this study was to describe the testes histological characteristics at every testes maturation stage and to identify the sexual maturity size of male *P. polyphagus*.

## MATERIALS AND METHODS

**Sample of *Panulirus polyphagus*:** The experiment was conducted at Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Terengganu, Malaysia. The broodstocks with 140 adult males of *P. polyphagus* were sampled from Tanjung Sedili, Johor coastal waters, Malaysia (1°51′ N and 104°09′ E) with randomly sizes on June, 2013-June, 2014. *P. polyphagus* were rearing at the hatchery and were fed daily at 10% biomass with fresh squid (*Loligo* sp.) before the samples were sacrificed by cold treatment (10 min at -20°C) in the laboratory.

**Histology of testes in *Panulirus polyphagus*:** After removal of the carapace of *P. polyphagus*, the testes were dissected out for the histological examination. Sample preparations followed the modified standard laboratory protocols for haematoxylin and eosin staining correspondingly to Muhd-Farouk *et al.*[18]. The testes were fixed in 10% buffered formalin solution for 11 h, dehydrated in 70% alcohol for less than 24 h and lastly placed in tissue processor (Leica TP1020) for 18±1 h at 60°C[18]. After processing, samples were mounted onto cassettes using paraffin wax, sectioned into 4 µm films using rotary microtome (Leica RM2125RT), transferred into water bath (between 40-45°C) for expansion before mounting onto slides. The slides were dried using a hot plate (40°C) overnight. Then, the slides were immersed into a series of xylenes (100 mL-product No. D16D662922, EMD Millipore Corporation, USA), dehydrated in a series of ethanol (100 mL-product No. 20821.321, VWR Chemicals, France), stained in a haematoxylin solution (100 mL), decolorized in 1% acid alcohol (100 mL), re-stained in 0.5% aqueous eosin (100 mL) and lastly, mounted with DPX and top with a cover slip[18]. The slides with samples were then placed on a hot plate (40°C) for overnight. The slides were observed on the next day and photographs were taken under an Advance Microscope (Nikon Eclipse 80i Nomarski DIC, Japan).

**Data analysis:** Collected data (body weight, carapace length and testicular maturation stages) were analyzed using Microsoft Excel 2013. Data were presented as mean±standard deviation. Statistical analyses were done using one-way ANOVA ($p<0.05$) through the application of Social Sciences (SPSS) software (version 22.0 for Windows; SPSS Inc., Armonk, NY, USA: IBM Corp.)[19].

## RESULTS

**External morphology and histological characteristics of testes of *P. polyphagus*:** The mean body weight (BW) and carapace length (CL) of 140 adult males *P. polyphagus* were identified and they were divided into five class of BW. The testes lie around the cephalothorax on top of the hepatopancreas under the carapace (Fig. 1). The testes are also paired structures (left and right) that were located medially between the two vas deferens of *P. polyphagus*. The matured testes were slightly yellowish organ and forming elongated lobes which were much small, while the immature testes were transparent and short lobes. The interstitial gap between the fibrous mantle and tubules was overflowing by the connective tissues composed of several elements such as an intercellular substance containing distribute fibers, fibroblast, spread hemocytes and vessels with hemolymph (Fig. 2).

Table 1: Mean body weight±standard deviation, mean carapace length±standard deviation and testicular lobule containing in male *P. polyphagus* (n = 140)

| Range of body weight sampled (g) | Mean body weight (g) | Mean carapace length (mm) | Testicular lobule containing |
|---|---|---|---|
| 135-220 | 174.36±26.59 | 81.83±6.57 | Spermatogonia |
| 221-358 | 293.12±36.58 | 96.88±3.16 | Spermatocytes |
| 359-451 | 395.35±24.39 | 105.44±2.31 | Spermatids |
| 452-471 | 460.96±7.64 | 107.55±1.46 | Spermatids and spermatozoa |
| 472-485 | 478.42±4.60 | 107.72±1.65 | Spermatozoa |

Table 2: Histological characteristics of testes during the follicular development of testes maturation stages of *P. polyphagus* (n = 140)

| Testis stages | Histological characteristics |
|---|---|
| Spermatogonia I and II | Close to the follicular fibrous, the spermatogonia appeared. Type I spermatogonia had large, round nuclei measuring 6.3082±0.8364 μm and displaying small knots of condensed chromatin scattered all over the nucleus (Fig. 3a) |
| Spermatocytes I and II | Spermatocytes I and II may only distinguished by a difference in size. The nuclei of type II spermatocytes measuring 4.1138±0.604 μm. Some type I spermatocytes may not mature into type II and eventually degenerate presenting signs of nuclear and cytoplasmatic regression (Fig. 3b) |
| Spermatids | The spermatids were small cells through peripheral and in an even way stained nuclei measuring 2.44±0.6248 μm (Fig. 3c) |
| Spermatozoa | The spermatozoa resemble spermatids, but were rather smaller. The nucleus measured 1.6283±0.2882 μm and was strongly basophilic and rather large compared to the cytoplasm, which appeared like a barely discernible peripheral ring (Fig. 3d) |

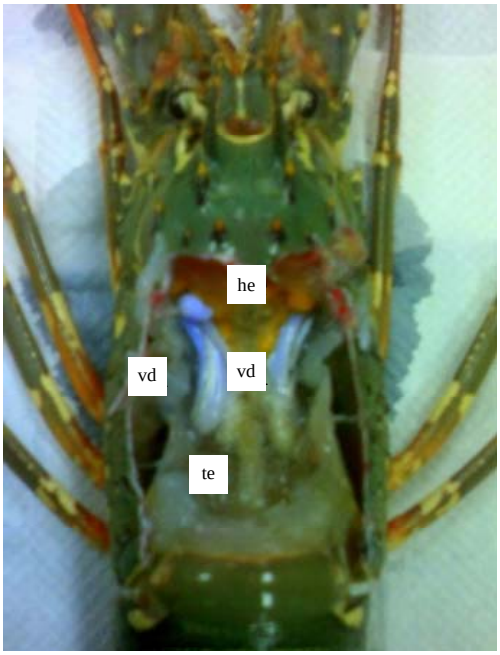


Fig. 1: Dorsal view of cephalothorax showing the localization of reproductive tract:testes (te), vas deferens (vd) and hepatopancreas (he) of male *P. polyphagus*

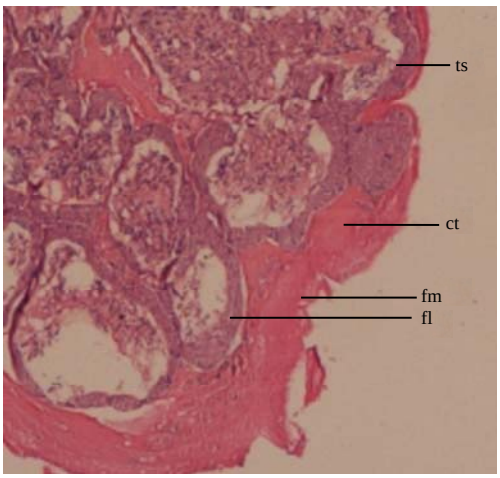


Fig. 2: Photomicrograph showing tubules (ts), connective tissues (ct), fibrous mantle (fm) and fibrous layer (fl) of *P. polyphagus* testes (magnification of 20×)

These lobules of mature *P. polyphagus* were formed via different germinative lineage cells such as spermatogonia, spermatocytes, spermatids and spermatozoa (Table 1, 2). From Table 1, the range of body weight, 135-451 g comprised of spermatogonia, spermatocytes and spermatids. While, the range of BW, 452-485 g comprised of spermatids and spermatozoa. There were four stages of the follicular development in mature *P. polyphagus*: (a) The majority of spermatogonia I and II, (b) Ever-increasing numbers of spermatocytes I and II, (c) Spermatids were widespread and (d) Spermatozoa and spherical bodies are also widespread (Fig. 3). Each stage of *P. polyphagus* has different histological characteristics. The histology of the testes indicated that spermatogenesis occurred in all the specimens studied. The majority of the lobes contain spermatids or spermatozoa as well as many non-germinal, sertoli cells in these mature males.

**Relationship between body weight and carapace length on testes maturation stages of *P. polyphagus*:** The results from the present study show that mean BW and mean CL increased as the testes maturation stages of *P. polyphagus* advanced to another stage. The relationship between BW with testes maturation stages was shown in Fig. 4, while Fig. 5 shows the relationship between CL with testis maturation stages of *P. polyphagus* in the present study.

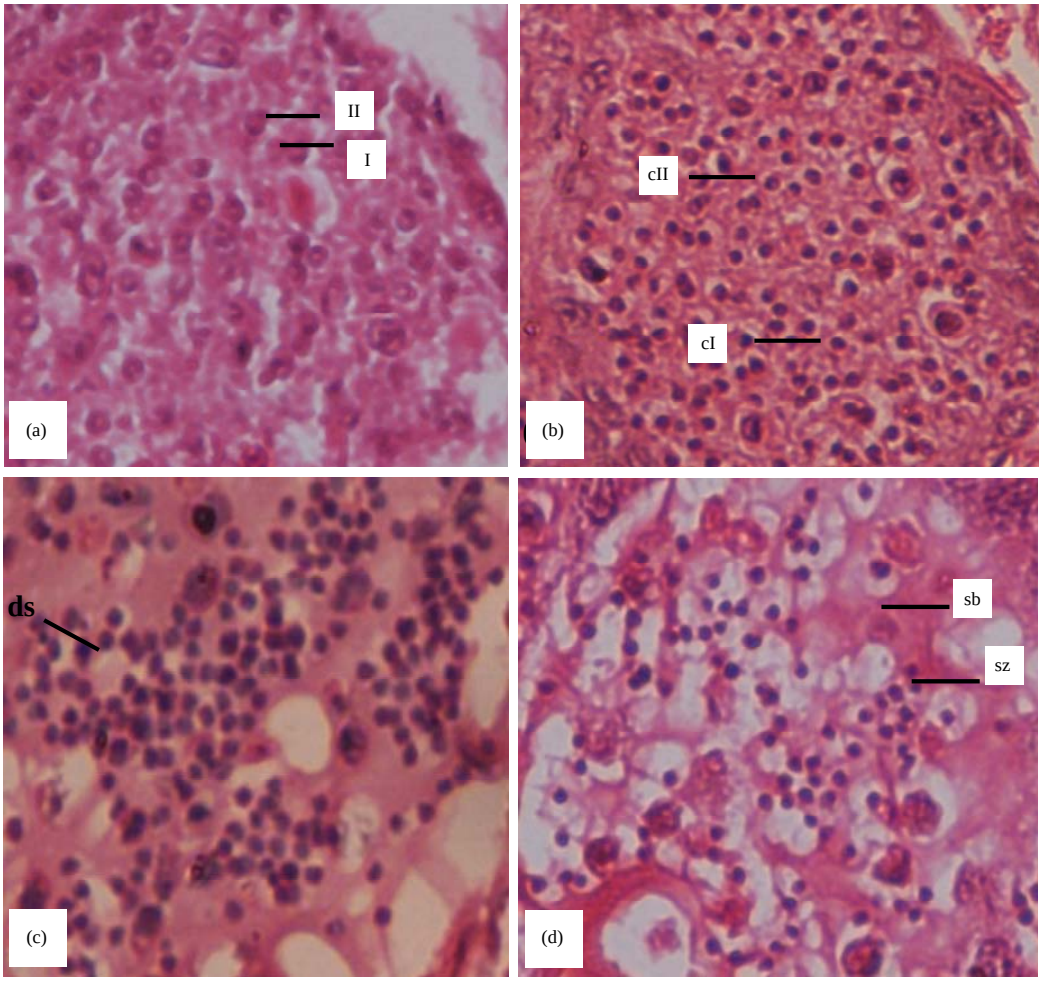


Fig. 3(a-d): Histological section of testis of *P. polyphagus*. Testis of *P. polyphagus* in the process of spermatogenesis and stained with haematoxylin and eosin (a) Photomicrograph showing testicular lobule containing Type I spermatogonia (I) and Type II spermatogonia (II), (b) Type I spermatocytes (cI) and Type II spermatocytes (cII), (c) spermatids (ds) and (d) spermatozoa (sz) and spherical bodies (sb) (magnification of 40×)

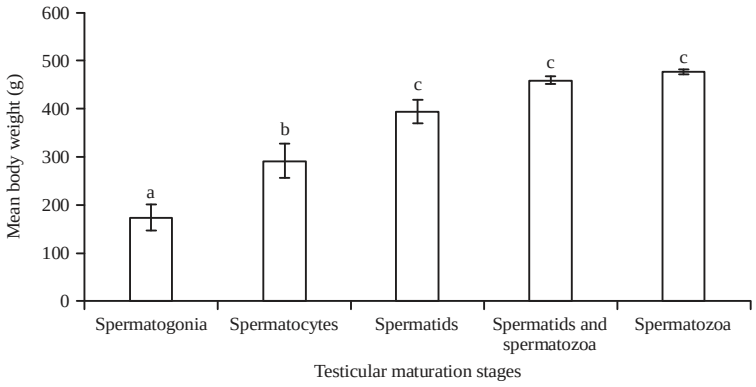


Fig. 4: Relationship between mean body weight and testes maturation stages (n = 140), a, b and c indicates the differences between mean body weight on each testicular maturation stages, error bars indicate standard deviation

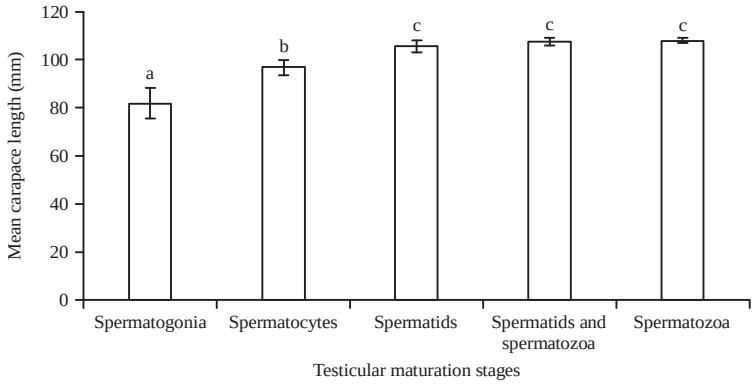


Fig. 5: Relationship between mean carapace length and testes maturation stages (n = 140), a, b and c indicates the differences between mean carapace length on each testicular maturation stages, error bars indicate standard deviation

As for the relationship between BW and testes maturation stages, there were significant difference between BW with the two stages earlier; spermatogonia and spermatocytes ($p<0.05$). However, there were no significant difference between BW with the spermatids, spermatids and spermatozoa and spermatozoa stages.

Meanwhile, the relationship between CL and testes maturation stages also shows the same pattern that has been showed by BW. Statistical analysis shows there was a significant difference between CL with the spermatogonia and spermatocytes stages ($p<0.05$), however, there were no significant difference between CL with the spermatids, spermatids and spermatozoa and spermatozoa stages. Overall data shows that there was a significant difference between BW and CL with the testes maturation stages (one-way ANOVA, $p = 0.000$).

## DISCUSSION

In the present study, the large BW of male *P. polyphagus* has a long length of testes while the samples with smaller BW possessed a shorter length of testes. A previous study stated that the length of testes was variable based on the BW of lobster[14]. Besides, the existence of paired testes and vas deferens were observed in the present study and similar findings were reported in other decapods crustaceans[11]. The findings of the present study showed that the size of paired testes and vas deferens depend on the BW of *P. polyphagus* compared to the size of CL. The study for the red-clawed mangrove tree crab, *Goniopsis cruentata*[13] and *P. laevicauda*[14] was corroborated with the description of the testes of *P. polyphagus*.

In *P. polyphagus*, testicular lobules are containing such as spermatogonia, spermatocytes, spermatids and spermatozoa. Testicular lobules were filled with a large number of spermatocytes in diverse stages of development[12,20]. In the testicular lobules, there were stages of the follicular development. *P. polyphagus* of 135-220 g BW, group has totally different stages of follicular development (spermatogonia) than the BW of *P. polyphagus*, 452-471 g (spermatozoa) and 472-485 g. There were different testicular lobules present in the immature of *P. polyphagus*. Testicular lobules in the immature samples consist of spermatogonia, spermatocytes and spermatids while, in the mature *P. polyphagus*, testicular lobule consists only of spermatozoa or a few of spermatids and most of the spermatozoa. The different histological section of *P. polyphagus* mature and immature were also highly related to the different BW of *P. polyphagus*.

In the present study, there were anterior, intermediate and posterior regions of testes. The process of spermatogenesis was observed in all *P. polyphagus* samples in the present study when the histological observations were done. In some cases, testes were also reported to consist of anterior, intermediate and posterior regions based on histological observation[13].

This present study proved that males *P. polyphagus* that has 452 g BW and 107 mm CL or more can be considered to have fully mature testes that are ready for fertilization. However, based on statistical analysis that has been done, there was a significant difference between BW and CL with the earlier stages of testicular maturation (spermatogonia and spermatocytes) only, but no significant results for the spermatids, spermatids and spermatozoa and spermatozoa stages. This meant that the BW and CL can be referred to the determination of both earlier stages (spermatogonia and spermatocytes); however for the advanced stages, the BW and CL parameters cannot be taken for determination precisely. Therefore, histological assessment is needed as it gives more accurate results[21].

As a recommendation, gonad somatic index (GSI) can be taken as maturation stages determination factors[20] and can be seriously noted for future study of this species. Besides, a higher number of samples is needed for confirmation of testicular maturation stages in the future as the present study was carried out with only 140 samples. Therefore, there is an urgency for further study related to the sexual maturity as the external morphology such as BW and CL cannot determine precisely the testicular stage of male *P. polyphagus* for advanced stages (spermatids, spermatid and spermatozoa and spermatozoa stages). Results from the presents study hopefully would contribute important information to researchers for future testes maturation stages determination and biological information of *P. polyphagus*, besides give better options for mud spiny lobster breeders regarding broodstocks selection for breeding purposes, fishery conservation and also management practice in the future.

Furthermore, the histological characteristics on the testes of *P. polyphagus* are very important in the production of male gametes. The results from the present study showed that male *P. polyphagus* testes presented the same pattern of the other lobster species. Thus, there is hope that the present study will help to understand the reproductive biology of male *P. polyphagus* and so that effective strategies can be formulated for the management of hatcheries and to improve the production of *P. polyphagus*.

## CONCLUSION AND FUTURE RECOMMENDATIONS

The results of this study indicated that males *P. polyphagus* at 452 g BW and 107 mm CL or more can be considered to have fully mature testes that ready for fertilization. Histologically, there were four stages of spermatogenesis process occurred during the testes maturation stages: Spermatogonia I and II, spermatocytes I and II, spermatids and spermatozoa (sperm). However, there are still lacks of in-depth study about testes maturation stages of male *P. polyphagus*.

Hopefully, the data from the present study would able to contribute for the future biological studies through breeding technology practices, fishery conservation and management of *P. polyphagus* especially in Malaysia and also for worldwide practices.

## SIGNIFICANCE STATEMENT

The present study discovers the full mature spermatozoa of mud spiny lobster, *Panulirus polyphagus* started with 452 g of body weight (BW) and carapace length (CL) of 107 mm. There are no sound studies that have been performed on testicular characteristics and sexual maturity sizes of this species. Therefore, proper management practices can be plotted in the aquaculture industry by focusing on the testes biology of this species. The present study will contribute the knowledge on testicular histological characteristic and sexual maturity sizes of *P. polyphagus* for the future biological studies through breeding technology practices, fishery conservation and management.

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