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Morphology of Fresh and Cryopreserved Sperms of Banana Shrimp *Penaeus merguiensis* (De Man, 1888)

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Abstract: The spermatophore morphology of the *P. merguiensis* from Kedah, Malaysia is described. About 10 cryopreserved groups as 6, 12 and 24 h, 7, 30, 60, 90, 120, 150 and 180 days and fresh as control was examined from each of three replication were evaluated for sperm gross morphology evaluations. A fully mature male's broodstock of *P. merguiensis* was taken with fresh spermatophores and evaluated for sperm morphologically. Cryopreserved spermatophore after the thawing 27°C/2 min (fresh and frozen) individually transferred into glass homogenizer (High speed variable speed reversible, Glas-col, Terre Havte In USA) with 200 μL of Ca-F saline. Fixation, dehydration by series of alcohol, Critical Point Dry (CPD) and mount specimens on to stubs using or carbon dots as well as using Auto Fine Coater and Sputter Coater moreover scanning by Model JEOL 6360LA scanning electron microscope. The cryopreserved spermatophore shows similarities with those of fresh, there were no significant differences ($p>0.05$) between the freshly spermatophore and spermatophorestored up to 90 days at -196°C liquid nitrogen.

Key words: Banana shrimp, *Penaeus merguiensis*, spermatophore, cryopreservation morphology, Malaysia

INTRODUCTION

Microscopic examinations of the external morphology of sperms are very crucial in successful artificial insemination process (Dall *et al*., 1990; Griffin *et al*., 1987). Number of studies were attempted to determine morphological and anatomical variations in the fresh spermatophore and sperms of various shrimps species such as *Penaeus aztecus*, *P. setiferus* and *P. duorarum* (Raymond, 1986) and fresh water giant prawn *Macrobrachium rosenbergii* (John *et al*., 1983). However, the study on the morphological variation in the matured sperm due to various cryopreservation techniques is still scanty. It is well understood that the complete sperm (fully matured) with acrosome and tail has greater ability to fertilize the eggs more efficiently than the immature and defective sperms (Nishioka *et al*., 1989). Study has proven that an acrosome reaction during the time of sperm's approach to the egg may allow the sperm to recognize and bind to the vitelline layer of the immature egg which eventually would lead to cell fusion between the inner acrosomal membrane of the reacted sperm and the egg plasma membrane (Nishioka *et al*., 1989). In contrast, fertilization in the animal kingdom involving non-motile male gametes is poorly understood, particularly in the decapod crustaceans. Sperm of the decapods are considered atypical, non motile gametes and are separated into the multistellate sperm of the reptantians (crabs, crayfish and lobsters) and the unistellate sperm of the natantians (shrimp) (Wilson, 1928; Lu, 1976; Talbot and Summers, 1978). Hence, it is necessary to determine the morphology of matured sperm (fresh and time elapsed sperm) under high powered microscope in order to find the morphological abnormalities caused due to an elapsing time.

Sperm cell's external morphology evaluations have been developed by a number of researchers to distinguish reproductive potential in penaeid shrimp using a technique developed by Leung-Trujillo and Lawrence (1987a). This technique or close adaptations of it has been used earlier *Penaeus setiferus* (Leung-Trujillo and Lawrence, 1987a, b, 1991; Rosas *et al*., 1993), *L. vannamei* (Leung-Trujillo and Lawrence, 1985, 1991; Alfaro and Lozano, 1993; Heitzmann *et al*., 1993; Wang *et al*., 1995) and *P. monodon* (Gomes and Honculada-Primavera, 1993; Pratoomchaat *et al*., 1993). The sequence of the

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morphological events leads ultimately to gamete fusion that has been well documented in several vertebrate and invertebrate species (Boving, 1969; Epel and Vacquier, 1978).

However, this study has focused on external morphological structure of sperm using Scanning Electron Microscope (SEM). Artificial Insemination (AI) of penaeid shrimp has been proficient (Bray *et al*., 1982; Sandifer *et al*., 1984) and analysis of sperm quality is important in shrimp mariculture currently. It is worthy to note that the study on the morphological characterization of *P. merguiensis* sperms is still scanty and thus present study was aimed to addressed the comparison between external morphology of spermatophore and describe structural changes in spermatophores prior and after the cryopreservation. The study also focuses to assess practically the reproductive potential of fresh and frozen sperm of males of *P. merguiensis*. Determining whether, the hatchery technician could also be used as AI.

MATERIALS AND METHODS

In this study, determination of mature sperm morphology and ultra-structure of *P. merguiensis* was adopted by scanning electron microscope technique and compared them with the fresh and cryopreserved sperms.

Source of animals: Sexually matured *P. merguiensis* male specimens were collected from Kota Kuala Muda, Palau Sayak, Kedah, Malaysia (5°39"N; 100°19"E) with mean Body Weight (BW) of 24.2±3.84 g and mean Total Length (TL) of 14.4±0.5 cm were used in this study. They immediately transported to the marine hatchery at the Institute of Tropical Aquaculture, University Malaysia Terengganu (UMT) in an aerated condition. Precautions were taken to reduce the external stress to the brood stocks by providing ambient environmental conditions during transportation.

Spermatophore collection: Specimens were weighed and selected with sign of a clear white swelling around the coxae at the base of the 5th walking leg (pereiopods). Left and right spermatophores did not differ in weight (0.048-0.092 g). Only non-melanized spermatophores were selected for preservation studies as reported by (Dougherty and Dougherty, 1990). Slight pressure was applied with the thumb between the abdomen and the base of the 5th walking leg to eject out the spermatophores. The protruded spermatophores were pulled out with a pair of sterile forceps and each spermatophore was weighed.

Cryopreservation of spermatophore: Spermatophores were subsequently transferred to 0.5 mL of cryoprotectants solution (15% of $MgCl_2$ in Ca-F saline) inside 1.8 mL cryovial. After 15 min equilibration in room temprature (25°C) and exposure with cooling points selected as; 10 min exposure in each step stage 1st; 25, 20, 16, 4 and 2°C in stage 2nd; -20, -80°C in 3rd stage; -100 to -150°C (liquid nitrogen vapor) and immediately stored in liquid nitrogen -196°C for 6, 12 and 24 h, 7, 30, 60, 90, 120, 150 and 180 days. Frozen spermatophore were thawed at 27°C for 2 min and evaluated by using vital stains up to 180 days storage in liquid nitrogen. Cryopreserved spermatophores were stored in liquid nitrogen to evaluate over time sperm morphology during different periods of storage. Freshly collected spermatophores were examined by morphology and served as controls.

General processing protocol for SEM: The sperm were fixed following modified Ro *et al*. (1990) method. Fixation was carried out for 1-2 h using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 sample were rinsed; 3×15 and 45 min 0.1 M sodium cacodylate buffer, pH 7.2 after that post-fix 1-2 h 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 sample were rinsed; 3×5 and 15 min 0.1 M sodium cacodylate buffer, pH 7.2, after that dehydrate 35, 50, 60, 70, 80, 90, 95 and 100% of ethanol series and Critical Point Dried (CPD). Samples were mounted on to stubs, using carbon dots. Samples were then sputter-coating with, gold for the scanning electron microscope observation (scanning electron microscopy of a JEOL 6360 LA Tokyo, Japan). For SEM analysis, a specimen was normally required to be completely dry, since the specimen chamber was at high vacuum. Living cells, tissues and whole soft-bodied organisms usually required chemical fixation to preserve and stabilize their structure.

Statistical analysis: Data ware analyzed as factorial CRD (2 factors or more). Analyses of Variance (ANOVA) were analysis using MSTAT C program. The factors involved were CRD; different durations of cryopreservation spermatophores. Means for individually factor were test by LSD ($p>0.05$) and the interaction were test by Duncan. Parameters means were support by Pearson correlation (2 tailed).

RESULTS AND DISCUSSION

Microscopic observation: The sperms were in different length and consisted of a cup-shaped main body and a spike extending from the convex surface of the main body. The main body had a nucleus and a cup-shaped base. The

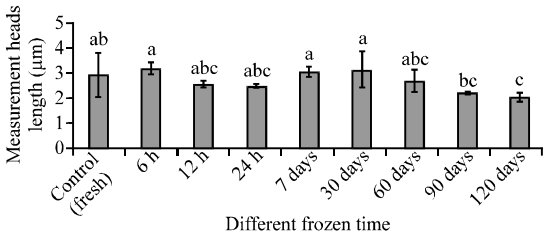

Fig. 1: Means compression effect of different frozen time on head length of sperm. Values are mean±SD (n = 20 referred 40 spermatophore). Different letters indicate significant difference among groups (p>0.05)

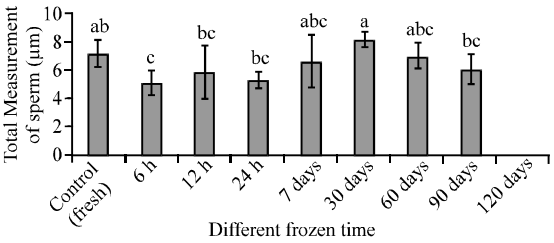

Fig. 3: Means compression effect of different frozen time on total length of sperm. Values are mean±SD (n = 20 referred 40 spermatophore). Different letters indicate significant difference among groups (p>0.05)

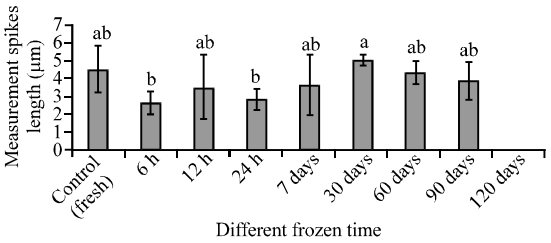

Fig. 2: Means compression effect of different frozen time on spike length of sperm. Values are mean±SD (n = 20 referred 40 spermatophore). Different letters indicate significant difference among groups (p>0.05)

heads were in different length. The spike also, observed in different length, consisted of the central core filled with electron-lucent materials and the electron-dense wall containing a number of tubular-like structures that were aligned along the longitudinal spike. The sperm structure of *P. merguiensis* particularly in the spike possessing tubular-like structures.

Figure 1-3 show the fresh (control) sperms mean of heads were recorded as 2.91±0.86 µm in length. The mean of spikes were noted as 4.48±1.31 µm in length as well as the mean of Total Length (TL) of fresh sperms was recorded as 7.13±0.96 µm in length. In the 6 h frozen sperms, mean of heads was recorded as 3.16±0.22 µm in length. The mean of spikes was noted as 2.63±0.66 µm in length as well as the mean of Total Length (TL) of 6 h frozen sperms was recorded as 5.11±0.88 µm in length. The 12 h frozen sperms mean of heads was recorded as 2.54±0.14 µm in length. The mean of spikes was noted as 3.49±1.82 µm in length as well as the mean of Total Length (TL) of 12 h frozen sperms was recorded as 5.87±1.91 µm in length. The 24 h frozen sperms mean of heads was recorded as 2.47±0.08 µm in length. The mean of spikes was noted as 2.83±0.59 µm in length as well as the mean of Total Length (TL) of 24 h frozen sperms was recorded as 5.29±0.57 µm in length. The 7 days frozen sperms mean of heads were recorded as 3.03±0.22 µm in length. The mean of spikes was noted as 3.61±1.69 µm in length as well as the mean of Total Length (TL) of 7 days frozen sperms was recorded as 6.64±1.87 µm in length. The 30 days frozen sperms mean of heads was recorded as 3.13±0.73 µm in length. The mean of spikes was noted as 5.01±0.29 µm in length as well as the mean of Total Length (TL) of 30 days frozen sperms was recorded as 8.14±0.53 µm in length. The 60 days frozen sperms mean of heads was recorded as 2.69±0.43 µm in length. The mean of spikes was noted as 4.29±0.65 µm in length as well as the mean of Total Length (TL) of 60 days frozen sperms was recorded as 6.98±0.95 µm in length. The 90 days frozen sperms mean of heads was recorded as 2.22±0.02 µm in length. The mean of spikes were noted as 3.85±1.08 µm in length as well as the mean of Total Length (TL) of 90 days frozen sperms was recorded as 6.07±1.09 µm in length. The 120 days frozen sperms mean of heads was recorded as 2.04±0.18 µm in length. There was no spike found in this treatment.

The means length of heads 6 h to 90 days were not different ($p>0.05$) from that for controls and were higher ($p<0.05$) than that in 120 days frozen treatments. The means length of spikes 6 h to 90 days were not different ($p>0.05$). The means of TL 30 and 60 days were not different ($p>0.05$) from that for controls and were higher ($p<0.05$) than that in 6, 12 and 24 h and 7, 90 and 120 days of frozen treatments. At the 120 days treatment was found only few heads. Generally complete sperm cell death recorded after the 120 days treatment.

This study demonstrated the external morphology of sperm before and after cryopreservation using n = 20 and 40 spermatophores. External morphology of fresh and cryopreserved sperms was done at 6, 12 and 24 h and 7, 30, 60, 90, 120, 150 and 180 days. The sperms which kept up to 90 days in liquid nitrogen, resulted same external morphological structure as compare to fresh sperms.

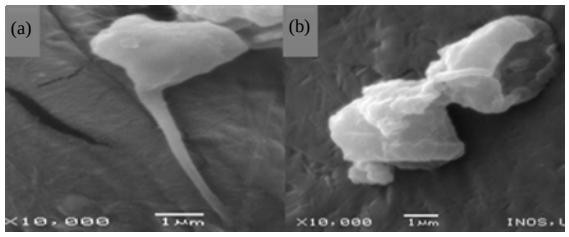

Fig. 4: a) Normal sperms of the *P. merguiensis* had spherical body and an elongate spike. The spike is active the sperm base retains a marked cupped shape. Bar = 1 µm and b) Mature abnormal sperm of the *P. merguiensis*. They had spike lost. Bar = 1 µm

Over all up to 90 days sperm with a spherical body and an elongated spike (stellate appendages) were found and considered as normal which were similar as reported by Talbot *et al*. (1989). Also during scanning, the thumb tack or inverted shaped sperms were observed and found with spike as reported earlier by Chow (1982) and John *et al*. (1983). The fresh sperm mean body length was 7.13±0.96 µm and the mean of spike length about 4.48±1.31 µm (scale bar was 1 µm) was not different from cryopreserved sperm size. The mean length of thumb tack (head) was 2.91±0.86 µm. In present study, it observed that an abnormal sperm in 120, 150 and 180 days (frozen) and morphologically abnormal sperm had spike lost which is same as reported by Wang *et al*. (1995) and Leung-Trujillo and Lawrence (1987a). On the other hand, normal sperms were achieved from 90 days by storing in liquid nitrogen which resulted same external morphological structure as compare to fresh sperms. Normal sperms had spherical body and an elongated spike which were similar as reported by Chow (1982), John *et al*. (1983) and Talbot *et al*. (1989) (Fig. 4a and b). As compare to this study, Toshihiro *et al*. (1998) also got success regarding morphology, motility and viability of frozen spermatozoa of Southern Minke Whales, *B. acutorostrata* by using scanning electron microscope. John *et al*. (1983) carried out morphological examination of sperm egg-interaction in fresh water prawn, *Macrobrachium rosenbergii*. Anchordoguy *et al*. (1988) also done work on sperm from a marine shrimp, *Sicyonia ingentis* frozen to 196°C using a variety of cooling rates and cryoprotectants then sperm viability was assessed using scanning electron micrographs. Aungsuchawan *et al*. (2011) used scanning electron microscopic for *L. vannamei* to observe morphological changes associated with a capacitation process, the morphological and biochemical changes. Moreover, study on spermatophore morphology of different parts of the hermit crab *Isocheles sawayai* was done by Mantelatto *et al*. (2009) and the scanning electron microscope was used. Previously stated by Ceballos-Vazqueza *et al*. (2003) that analysis of sperm quality is important in shrimp aquaculture. The output of this study would support the breeding technology and AI practice should be started routinely in *P. merguiensis* because this practice in penaeid shrimp has been proficient (Bray *et al*., 1982; Sandifer *et al*., 1984). It is worthy to note that the present study is the file time documentation on the comparison of fresh and cryopreserved sperm's morphology.

CONCLUSION

This study concludes that the morphology of fresh and cryopreserved sperms does not show any significant differences. However, the cryopreservation of sperm up to 90th day gave similar results as like fresh sperm and its redundancy was reducing with elapsing days of preservation.

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