

Preliminary Studies on the Sperm Characteristics and Sperm Quality of *Macrobrachium rosenbergii* (De Man, 1879)

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ABSTRACT: Upsurge of economical, technical tools and well-established assay kits provide spermatologists with several devices for objective and quantifying assessments of sperm quality. The present paper reports quality assessment of prawn sperm using both traditional and modern methods. Sperm characteristics and quality of *Macrobrachium rosenbergii* was investigated using mature male broodstocks weighing about 45.30 ± 3.74 g. For a randomly selected group of mature blue clawed male giant freshwater prawn, the Gonado Somatic Index (GSI) was 0.63 ± 1.56 (%). The spermatophores weighted about 0.091 ± 2.50 g. The sperm count was recorded as $6.23 \pm 4.02 \times 10^8$ cells/ml. Sperm morphology using light and scanning electron microscopy was investigated. Feasibility of dual staining protocol and flow cytometry for sperm viability analysis was compared. In conclusion, GSI, spermatophore weight, sperm count, sperm viability and morphology were supposed as suitable parameters for predicting sperm quality in *M. rosenbergii*.

KEYWORDS: Sperm quality, Sperm viability, Spermatophore, *Macrobrachium rosenbergii*, Electroejaculation, Scanning Electron Microscopy,

I. INTRODUCTION

Increasing demands and rising prices for seafood are raising the profile of freshwater prawn as an important aquaculture commodity. The giant freshwater prawn *Macrobrachium rosenbergii* is a species with high commercial value in the aquaculture industry. *M. rosenbergii* is one of the world's largest freshwater crustacean and an important aquaculture species in many tropical countries, particularly in Asia [1]. To date research on shellfish gametes is relatively scarce, and only about 30 species of aquatic invertebrates have been studied. There is a paucity of information on assessment of sperm quality, viability, sperm morphology and ultrastructure of *M. rosenbergii*. Optimal sperm quality is a fundamental feature for management of shrimp broodstock. Besides understanding of sperm quality is useful to select potential male broodstock. Interest in controlled production of captive shrimp stocks for reproduction has been increasing worldwide, and the goal is a consistent production of high quality seed with improved culture potential, known health status, and disease resistance [2]. The need of high quality gametes has necessitated research into various ways of enhancing gamete quality to meet the growing demands [3] The development of new methods for preservation of valuable genetic resources has tremendously exponentiated the analysis of gamete quality. Hence there is a need for a reliable measure of sperm quality. Sperm quality in Penaeid shrimp was first evaluated by [4] by

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determining sperm count and percentage of live and abnormal sperm. The same parameters have been used to study sperm quality under different conditions [5,6,7,8,9,10,11].

Morphological abnormalities of sperm have a detrimental impact upon fertilization and subsequent embryonic development. Generally vertebrate semen used for commercial artificial insemination are selected to a certain degree on the basis of a low incidence of morphologically abnormal spermatozoa, although some evidence for a relationship between sperm morphology and fertility in bulls has been presented. Hence a complete morphological examination is, therefore, recommended [12, 13]. The consensus is that the reproductive potential of males plays an important role in the productivity of broodstock of captive penaeids [11]. Several factors such as lack of spermatophore adhesiveness, disease of the male reproductive tract, time of confinement, temperature, and electrical stimulation are known to affect the reproductive potential of male penaeid shrimp [14,15,16, 17]. These factors among others, contribute to unstable and non-optimum productivity in hatcheries throughout the world [7]. This study is of major interest because optimal sperm quality is important for effective broodstock management, and a major criterion in the selection of male broodstocks. For this purpose, the present study analyzed the sperm quality in *M. rosenbergii* males, using spermatophore weight, sperm count, sperm viability and morphology as indicators using traditional and modern techniques.

II. MATERIAL AND METHODS

Animal collection and maintenance:

Freshwater prawn *Macrobrachium rosenbergii* were collected from Kattur near Meenjur, Chennai, Tamil Nadu. The collected prawns were brought to the laboratory in aerated bags along with habitat water. Healthy prawns having intact appendages without any injury were selected and conditioned in 10,000 L circular cement tanks, filled with freshwater up to a depth of 40 cm at $29 \pm 1^\circ\text{C}$. Commercial shrimp-feed pellets were provided ad libitum twice a day.

Gonadosomatic Index (GSI):

The testis of each fish were dissected out from the body cavity and weighed using an electronic balance. The gonadosomatic index (GSI) for each male was measured as described by [18].

Electroejaculation:

Spermatophores were obtained from mature males by electrical stimulation technique. A pair of electrical probes was placed on each side at the base of the coxa of the fifth pereopod just below the opening of the gonopore. An electrical stimulus of 9 Volts was applied for three to four times. Extruded Spermatophores were gently picked up by forceps and stored in 2ml cryogenic vials [19].

Sperm quality assessment:

Sperm quality was assessed by sperm count, viability and morphology. Sperm count was determined using a Neubauer haemocytometer, according to the method described by [4], by homogenizing the spermatophore in a calcium-free saline solution (composition per 1-l solution: 21.63g NaCl, 1.12g KCl; 0.53g H₃BO₃; 0:19g NaOH; 4:93g MgSO₄; 7H₂O and pH adjusted to 7:4 with 1NHCl). To determine the percentage of live and dead spermatozoa, cells were stained with a solution of 0.4% trypan blue and 10% eosin and 0.5% nigrosin (SIGMA, St. Louis, USA) prepared in calcium-free saline solution that was mixed with sperm suspension [4]. After 10 to 15 minutes, dead sperm was counted under a Leica 2500 microscope (Germany). The percentage of live sperm was scored based on their staining characteristics from 100 random counts. In addition, gross morphology of live sperm was examined under optical microscope. Sperm with a convex cup shaped body and an elongate spike were considered normal [20].

Scanning Electron Microscopy:

For scanning electron microscopic study, spermatophores were fixed in 6% glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7.3) for 4 hrs. Spermatophores were sliced transversely into 1 mm segments while in fixative. They were then rinsed in several changes of cold 0.1 M cacodylate buffer, post-fixed in cold 1% OsO₄ for 1 - 1.5 hr.

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Spermatophore segments were dehydrated in ethanol series and subjected to critical point drying. The samples were mounted on aluminum stubs and coated with gold. The samples were viewed in a JEOL JSM-35C scanning electron microscope and photomicrographs were taken at various magnifications by accelerating the voltage.

Flow cytometry:

Sperm viability was evaluated using the flow cytometer as described by [21]. The sperm samples were incubated in fluorescent dyes propidium iodide (PI). Propidium Iodide (PI) was added to a final concentration of 12 mM and placed in the dark for about 10 min. Flow cytometric analysis were performed using the flow cytometer (FAC Sort Plus Analyzer, Becton Dickinson, USA) The fluorescence emitted by PI was red fluorescence and detected using a 610 nm filter. The flow cytometer was equipped with standard optics and an Argon ion laser (Innova 90, Coherent, USA), with CellQuest software for Macintosh (version 3.0, Becton Dickinson, USA) for acquisition and analysis and with FacComp software (version 1.0) for daily setup and quality control. The evaluation was carried out for all samples in triplicates.

Statistical analysis:

The data were subjected to analysis of variance (ANOVA) followed by post-hoc test (Duncan's multiple range test) to determine the level of significance. Software statistical package 10.0 was used for statistical analysis

III. RESULTS AND DISCUSSION

Spermatophores are defined as a complex, comprised of spermatozoa and male secretions which wrap or embed the sperm. They play an important role in sperm transfer, sperm storage, and fertilization, for a wide range of animals [22]. The origin, morphology, function and/or histology of the spermatophore have been reported for over 50 species of decapod crustaceans [23]. The macrophotograph (Fig.1) of the Electroejaculated spermatophores of *Macrobrachium rosenbergii* below shows the milky white appearance of the spermatophores. The spermatophores are spindle or pod shaped and measured about 5-10 mm in length, 2-3 mm in diameter. The Spermatophores are usually more pointed at one end than at the other and bellied in the centre. The macrophotograph below clearly shows the spermatophores exhibiting a pod shape and milky white appearance. The spermatophores tend to lose their adhesive property when preserved in saline for further experimentation.

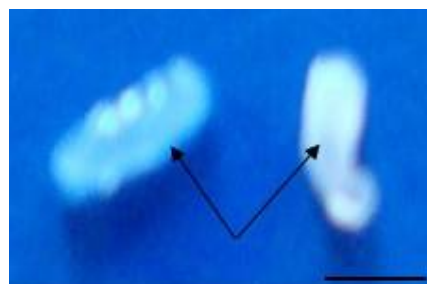


Fig. 1 Macrophotograph of a pair of electroejaculated spermatophores. Each spermatophore exhibits a bean or pod shape (arrows). Bar = 1 mm

Spermatophores become strongly adhesive soon after discharge to ensure adherence and fixation to the female sternum. [24] Reported such structural similarities in *M.rosenbergii*, *Macrobrachium nipponense*, *Macrobrachium latidactylus* and *Palaemon paucidens* spermatophores whilst the adhesive substance is highly porous in *M. latidactylus*. To assess the reproductive competence of the selected animal, the reproductive indices such as animal weight, animal length, GSI, spermatophore weight, sperm count and sperm viability were analyzed. Significant correlations were observed among the parameters at ($P < 0.9$). Similar results were obtained for *Penaeus monodon* [8] and *Litopenaeus vannamei* [9]. In *Penaeus setiferus* maximum value of 45.37 million of normal sperm/spermatophore (asymptotic value)

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for males over 36 g was registered ^[17]. It is notable that higher sperm count corresponded to a high proportion of normal sperm and lower proportions of abnormal and dead sperm. In conflict, to the present observations, negative correlation between percentage of spermatophore weight and sperm count for *Penaeus stylirostris* was reported ^[5]. The figure below compares the various reproductive indices to identify the reproductive performance of the selected brood stock. The correlation ($P < 0.9$) among the various parameters are evident and clearly illustrated in the graph below (Fig.2).

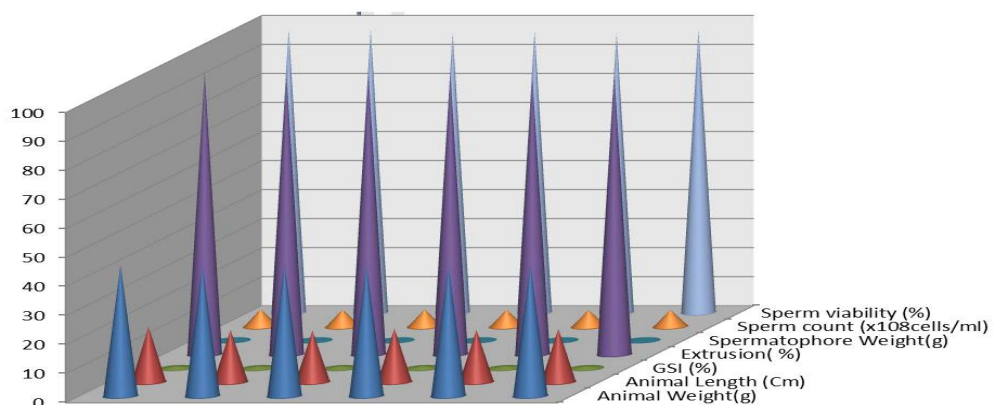


Fig. 2. Graph showing the characteristic sperm quality variables of Animal weight (g), Animal length (g), GSI (%) , Spermatophore weight (g) Extrusion (%), Sperm count ($\times 10^8$ cells/ml) and Sperm Viability. All correlations were significant at $P < 0.9$.

Further light microscopical studies showed that the sperm of *M. rosenbergii* is shaped like an alpin or everted umbrella. The disk-shaped head or basal plate has a slight constriction in the center and cross section appears Y-shaped. The diameter of the head division is 8-10 microns and length up to the tail or spike measured about 12 microns. The edge of the spikes base plate has a ruffled appearance consisting of many sac like structures called the acrosome. Mature sperm taken from the spermatophores appeared similar to that of the testicular sperm. The putative acrosomal sacs at the edge of the spike are prominent (Fig.3). The figure below clearly shows the sperm containing a distinct head or basal late and spike. Results obtained on the sperm structure associated with that of ^[24].



Fig.3. Photomicrograph of mature sperm obtained from spermatophores by electroejaculation showing distinct head or basal plate (Bp) and spike.(S) (Bar: 40X)

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SEM images of the spermatophore revealed that the median mucus consisted of anastomosing strands of extracellular material which was continuous with the inner surface of the capsule. The sperm masses consisted of sperm embedded in extracellular matrix. Further, scanning electron micrographic studies of the mature sperm showed an everted-umbrella shape with a very long spike measuring about 12 to 14 μm projecting from its convex surface. Numerous epaulette like acrosomal sacs are clearly observed at the edges of the spikes basal plate. The basal plate of the sperm is supported by 14 to 20 radial ridges or fibrils that stretch across from the ruffled border of the base plate to the proximal end of the spike are shown clearly in the (Fig.4).

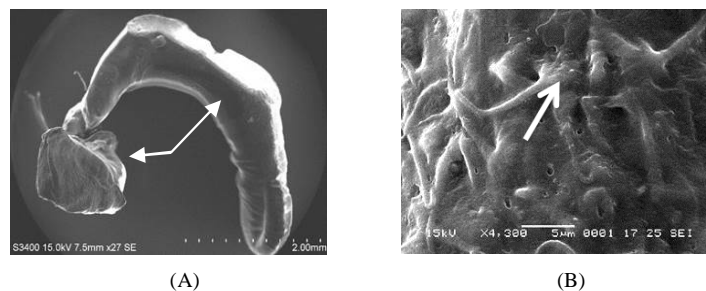


Fig.4 A) A scanning electron micrograph of *Macrobrachium rosenbergii* spermatophore with extruded mass as a whole (arrows). B) A scanning electron micrograph more clearly illustrating the anastomosing radial fibrils of the base (arrow).

Although some fine structural differences are present, the sperm of *M. rosenbergii* conform to the basic morphology reported in other caridean shrimp. The mature sperm are non-motile and comprise a cup-shaped base housing the nucleus and a single spike which projects from the convex surface of the base. In the natantians, the spike has been suggested to be part of the acrosome^[25,26,27,28] a role also suggested for the cross striated structures associated with the isopod and schizopod sperm. The spike of *Sicyonia ingentis* and *Penaeus aztecus* sperm has been demonstrated to be a part of an elaborate acrosomal complex based on observations both in vitro and during the fertilization process and may represent the normal function of the spike in the penaeid shrimp sperm. The structure of the spike in *M. rosenbergii* is dissimilar to the structure observed in *P.aztecus* and *S. ingentis*, however, and its possible role as an acrosome is still uncertain since an acrosomal reaction has not been observed in the macrobrachium cell, although a dramatic spike bending process is observed during fertilization. Cross-striations similar to those observed in the spikes of macrobrachium sperm have also been reported in the sperm of *Penaeus elegans* and *Palaemonetes paludosus*^[25, 26, 27, 28, 29, 30, 31, 32, 33].

The viability of the sperm smear sample was determined using a choice of vital dyes such as eosin-nigrosin and trypan blue. The results correlated significantly ($P < 0.05$) with that of animal body weight. Live cell excludes the stain while dead cell incorporated the stain. The viability percentages of sperm cells were recorded as $97.32 \pm 6.54\%$ with eosin nigrosin and $96.11 \pm 3.36\%$ with trypan blue. (Fig.5), the viability was also assessed using fluorescent dyes such as Propidium iodine and Rhodamine B. The figure clearly shows that the dead cells absorb the stain while the live cells exclude the stain. The dead cells tend to incorporate the stain due to a break in the plasma membrane. Likewise, Propidium iodine binds to and stains the DNA of sperm. The percentage of dead cells recorded using propidium iodine was 91.76 ± 6.72 in sperm samples. Rhodamine B is yet another fluorescent dye that binds to live cells rather than dead cells unlike eosin nigrosin and Propidium iodine. The percentage of live cells recorded with Rhodamine B was about 96 ± 4.56 for semen smear samples.

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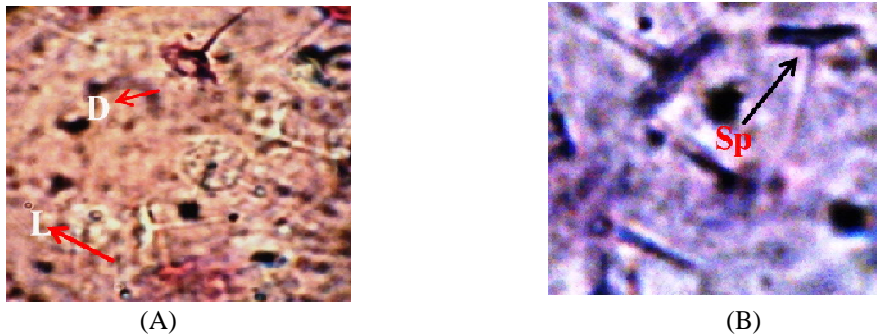


Fig. 5. A). Photomicrograph showing viable i.e. live sperm (LS) and non-viable sperm i.e. dead sperm (DS) stained using eosin- nigrosin. B). Photomicrograph of non-viable sperm i.e. dead sperm (DS) stained with trypan blue. Note that the live sperm (LS) excludes the stain while dead sperm (DS incorporates the stain. (Bar: 10µm)

This study reports the use of flow cytometry for the first time for analysing viability in *M.rosenbergii* sperm. The sperm cells were stained with PI (deteriorated cells fluoresce red; living cells are not labelled with PI). For the population of sperm incubated with PI, approximately 6,000 sperm cells were measured at a rate of 500 cells /second. The stained cells were excited at 610nm. Every single fluorescent intensity was digitized and the number of cells in the sample with particular red fluorescence intensity was stored. A considerable amount of non-sperm particles ran through the flow cytometer. The non-sperm particles scatter properties differed from those of the sperm cells. Each analysis was completed within a 2-5 minute period of staining. *M.rosenbergii* sperm exhibited the highest viability by morphotype analysis (97%) as well as flow cytometry (94%) (Fig.6). Significant differences were testified between the methodologies used. The results obtained were in accordance to that of [21]. The present study has thus reported that identification of cell viability by flow cytometry is more sensitive and less prone to manual errors when compared to light microscopy [18]. The results obtained have shown a significant positive correlation ($P < 0.06$) in the cell viability determined by optical microscopic observations and flow cytometry. These findings indicated that it is feasible to perform an initial screening using morphotype analysis, a simpler and faster technique, and to reserve flow cytometry for confirmation of results as described in *Littopenaeus vannamei* [34]

Sperm viability percentages obtained from optical microscopy and Flow cytometry (FCM) methods were compared. The Box plot below clearly indicates the viability variations amongst the two methods. The figure identifies the efficiency of flow cytometry as a better technique as against optical microscopy considering the time, efficiency and no of events analysed.

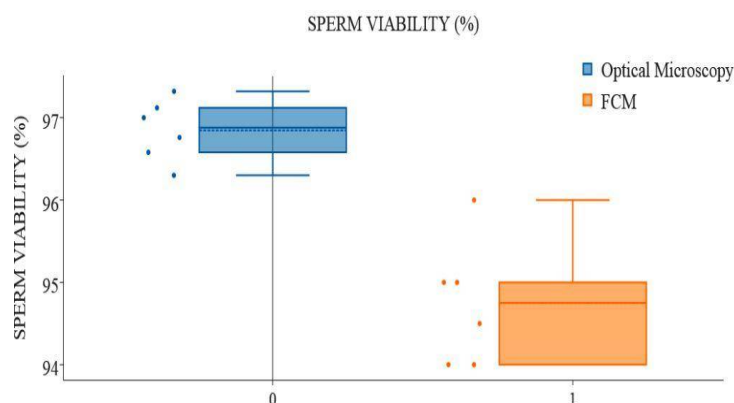


Fig.6. Comparison of sperm viability percentages obtained from optical microscopy and Flow cytometry (FCM) methods among the brood stock population using box plots.

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Elementary research on gamete characteristics and quality are considered effective in developing appropriate quality evaluation methodologies and early biomarkers of reproductive success, with potential future industrial applications for early prediction of gamete quality. Such quality evaluation may be relevant in order to better understand the mechanisms by which sperm are affected and to control some of the factors influencing overall gamete quality. Nevertheless the great potential of these techniques in other species, together with the preliminary results that have been reported, would help us to imagine a great applicability of these techniques in the field of aquaculture in the near future.

IV. CONCLUSION

In conclusion, sperm characteristics and quality assessment methods discussed in the present study should be effective for broodstock management for the freshwater prawn *M.rosenbergii*. Such investigations serve as major criterions in the selection of superior quality male broodstocks for further studies like Cryopreservation and Aquaculture practices.

V. ACKNOWLEDGEMENT

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