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# Assessment of Sperm Viability, Determination Fertilization Capacity and Hatching Rate by Artificial Insemination of Banana Shrimp, *Penaeus merguiensis* (De Man, 1888)

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**Abstract:** The study was conducted to every hour assess the sperm viability of banana shrimp (*Penaeus merguiensis*) collected from Kedah water, West Malaysia (5°39'N; 100°19'E). Evaluations were done on three ways: group A: whole fresh specimens maintained at 2°C prior to extraction, group B: whole spermatophore maintained at 2°C and C: sperm suspension maintained at 2°C. Spermatophores counts were determined by sperm suspension using modified eosin-nigrosin staining method. Percentage of mean sperm viability for the fresh sample in group A at time zero was 93.96±3.74%, B was 98.8±0.7% and C was 99.02±0.7%. In group A, viability of the sperm considerably decreased after the first 60 min was 76.4±5.96% but in group B and C was gradually decreased at 91.4±0.9 and 97.6±1.2%. These were 67.9±5.13 and 62.5±5.1% in group A, 82.3±1.4% and 75.9±2.2% in group B and in group C was 93.3±1.9 and 88.2±3.1%, respectively after 1st-2nd h times elapsed. At 7th h in all groups the viability decreased significantly to <60% ($p<0.05$). Following, mean sperm viability were considerably decreased to group A was 50.7±4.79 and 37±6.52%, group B was 51.2±1.6 and 31.6±1.4% and group C was 56.2±1.9 and 41.5±2.3%, respectively after 7th and 9th h. However, for spermatophores in group A after 7th h fertilization and hatching rate was 44.3 and 64.4% in group B was 61.9 and 67.7% and group C was 42.9 and 61.3%, respectively at rates comparable to control 88.2 and 76.2%, respectively. There was no significant relationship was observed between biomass of the spermatophore to the body weight of the shrimp ($p>0.05$). The present study also revealed that specimens, spermatophore or sperm suspension maintained at 2°C could be utilized for fishery management through artificial insemination process till 7th h.

**Key words:** Banana shrimp, *Penaeus merguiensis* sperm viability, artificial insemination, fertilization, spermatophore, shrimps

## INTRODUCTION

*Penaeus merguiensis* (De Man, 1888), commonly known as Banana shrimp is among the most important penaeid species in Malaysia. This species also inhabits the inshore waters of Indo-West Pacific region from the Persian Gulf to Thailand, Hong Kong, the Philippines and Indonesia to New Guinea, New Caledonia and Northern Australia (Grey *et al*., 1983). Due to its commercial value this species was also introduced into aquaculture field in Malaysia but not cultured as widely as *Penaeus monodon* or *Penaeus vannamei* due to its inefficiency in growing to a marketable size in higher stocking density (Othman, 2006). Being an indigenous species, it is of advantage to the countries in South East Asia and Asia Pacific region especially for Malaysia where the government is encouraging farmers to produce more of high value aquaculture species such as *P. merguiensis* (Othman, 2006). Besides this, it will also be an effective choice of shrimp species for aquaculture may reduce the disease outbreak that is an unsolved problem with *Penaeus monodon* culture and be a good alternative for the competitive market of *Penaeus vannamei*. At present, obtaining quality brood stock is one of the utmost important. Experience in this respect showed that individual mating is always hard to success (Othman, 2006). Thus sperm injection seems to be a practical choice. The ability to prolong the sperm activity after being accumulated from good quality male partners is prerequisite to ensure the apparent success. It is evident

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from the recent research that preservation of spermatophore for long periods would be a greater advantage for future breeding programs (Nimrat *et al*., 2005). Chilled storage is a useful technique which has been employed to extend the life of extracted spermatophore of freshwater shrimp, *Macrobrachium rosenbergii* (Chow, 1982) and lobster, *Homarus americanus* (Ishida *et al*., 1986). Development of a sperm viability assessment technique for spermatophore could provide a more reliable supply of high quality spermatophore, ease short-term spermatophore banking and artificial insemination activities, improve efficiencies in hatchery management and allow easy transportation of male gametes in specialized breeding programs (Grout *et al*., 1992). The classic method of assessing the viability of sperm is to determine the percentage of progressively motile cells by using a microscope reported by Motoh (1981). Different staining combination has been shown to be a rapid and reliable means for determining the proportions of living and dead sperm in several species across taxonomic lines reported by Adams *et al*. (2003). Sperm viability and fertilizing capability such as trypan blue stain exclusion reported by Thomas *et al*. (1998), eosin-nigrosin stain reported by Bhavanishankar and Subramoniam (1997). However, report on sperm viability assessment in *P. merguiensis* is still scanty. The investigation will uncover the change in viable sperm percentage of *P. merguiensis* for long time as well as role of that sperm in fertilization and hatching rate and their relationship with the body and spermatophore weight of the animal.

## MATERIALS AND METHODS

**Specimen source and maintenance:** Sexually matured males and female specimens of *P. merguiensis* were collected from Kota Kula Muda, Pulau Sayak, Kedah, Malaysia (5°39'N; 100°19'E) and transported to marine hatchery at the Institute of Tropical Aquaculture, University Malaysia Terengganu (UMT). On arrival, each specimen was treated with 3 ppm potassium permanganate ($KMnO_4$), solution for 10-15 min to eradicate unwanted microorganisms (Nimrat *et al*., 2005). The shrimp specimens male and female were transferred separately to holding tank at a density of three individuals per $m^2$ in a 5 $m^2$ rectangular tanks with water depth of 80 cm. Samples were kept in sea water at 30 ppt. The male specimens were maintained in hatchery condition under normal local photoperiod cycle of about 12 h light: 12 h dark and female specimens under dark room. Feeding were commenced twice daily, morning and afternoon with mixed fresh squid and mussels at a rate of 30% of the biomass. Following, leftover foods were siphoned out and water exchanges were done at about 20% daily.

**Spermatophore collection:** Specimen was selected with sign of a clear white swelling around the coxae at the base of the fifth walking leg (pereiopods). Only spermatophores that were not melanized were selected for preservation studies (Dougherty and Dougherty, 1990). Upon observation, the identified males were then removed and wrapped in a wet cloth. Following, soft pressure was applied with the thumb between the abdomen and the base of the fifth walking leg to eject out the spermatophores. The protruded spermatophores were pulled out with a pair of sterile forceps and each spermatophore was weighed and transferred into glass homogenizer (High speed variable speed reversible, Glas-col, Terre Havte In USA) with 200 μL of Ca-F saline.

**Spermatophore assessment study**

**Sperm sample preparation:** Sperm viability assessments were done in every hour (within range of time 0-9 h) For group A the live specimens were sacrificed and maintained freshness at 2°C (Jeyalectumie and Subramoniam, 1989) in group B only spermatophores were extracted and in group C after the spermatophores extraction they were immediately homogenized. The time zero was referred to the collection of sperm immediately after the shrimp was sacrificed/used. Spermatophores of three male's specimens in every group were sampled out in every hour. The spermatophore from each study group was then divided equally to produce six replications to produce a final of 18 analyzed samples.

**Sperm viability assessment:** Sperm viability was determined by a modified eosin-nigrosin staining protocol method (Nimrat *et al*., 2005). Initially, a known spermatophore specimens were weighed and transferred to a glass homogenizer with 200 μL of Ca-F saline and gently ground to form a sperm suspension. The 50 μL of sperm suspension was transferred to a clean glass slide and mixed with 50 μL of eosin and nigrosin (dissolving 0.5 g of eosin in 100 mL distilled water and the nigrosin solution was prepared by dissolving 0.10 g of nigrosin also in 100 mL distilled water) and air-dried prior to microscopic observation. Viability spermatophores were subsequently evaluated under an Advanced Research Microscope (EPI-Fluorescence) Japan. Live sperm was unstained against the blue background of nigrosin whereas dead sperm appeared pink. Percentage of viable sperm was evaluated from a minimum of 300 sperm cells from each slide.

**Artificial insemination, fertilization capacity and hatching rate assessment:** Artificial insemination was performed to determine the fertilization and hatching capacity of 7th h spermatophores. Eyestalk ablation was performed on females to induce sexual maturation (AQUACOP, 1979). Spermatophores implanted into the thelycum of newly molted females between of 29-33 g and total 12 no's of females were used. Spermatophores kept in 2°C for 7 h were selected to evaluate their fertilization and hatching capacity. In the control group, wild mated specimens were used. After AI, each female was allowed to spawn in a 300 L fiberglass tank. The fertilization capacity in each replication was observed in 200 eggs after the spawning. Fertilization rate was estimated 2-3 h by comparing the number of fertilized eggs in gastrula stage with the 200 of inseminated eggs. Hatching rate was assessed 13-15 h after spawning at 26-28°C. Fertilization and hatching rates were evaluated in three specimens in each group with three replicates.

**Data analysis:** Data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). One way ANOVA test was performed to check the significance value ($p<0.05$). Pearson correlation (2 tailed) was calculated to determine the influence of sample weight and spermatophore weight over the percentage of viable sperm count at different times. Percentages of viable sperm were calculated by following formula:

$$\text{Percentage of live sperm} = \frac{\text{Observed no. of live sperm}}{\text{Total no. of sperms observed}} \times 100$$

## RESULTS

Mean sperm viability of the fresh samples in all groups ware 93.96±3.74, 98.8±0.7 and 99.1±0.7% at initial time (time zero). Viability of the sperm decreases in the first 60 min 76.4±5.96, 91.4±0.9 and 97.6±1.2%. There was no much fluctuation in the viability of sperm observed when the time elapses next 120 min. At 5 h in the specimens maintained at 2°C, the viability of sperm was decreased to <70% showing that the exposure of viable sperm to the low temperature for >5 h could mitigate the viability of the sperm considerably. It was also observed that spermatophore retained above 50% of viable till 7 h. Significant decline in the viable sperm count was observed at 8 h with mean percentage of viable sperm in all groups were 43.6±5.85, 35.4±2.2 and 49.2±2.1% showing that sperm viability of the specimens maintained at 2°C decreases with elapsing time (Table 1). There was no significant relationship was observed between biomass of the spermatophore to the body weight of the shrimp ($p>0.05$) (Table 2).

Table 1: Variation in the viability of the sperm in all groups (A-C) over the time (Every hour) (N = 90)

| Time (h) | A | B | C |
|---|---|---|---|
| 0 | 93.9 | 98.9 | 99.1 |
| 1st | 76.4 | 91.4 | 97.6 |
| 2nd | 67.9 | 82.3 | 93.3 |
| 3rd | 62.5 | 75.9 | 88.2 |
| 4th | 59.4 | 69.5 | 80.7 |
| 5th | 57.7 | 63.6 | 71.7 |
| 6th | 56.6 | 56.6 | 62.7 |
| 7th | 50.7 | 51.2 | 56.2 |
| 8th | 43.6 | 35.4 | 49.2 |
| 9th | 37.0 | 31.6 | 41.5 |

Table 2: Relationship between weight of spermatophore to the body weight of the shrimp in all groups (A-C) (N = 90)

| Time | A | | B | | C | |
|---|---|---|---|---|---|---|
| (h) | B/weight | Sp/weight | B/weight | Sp/weight | B/weight | Sp/weight |
| 0 | 26.5 | 0.077 | 25.8 | 0.068 | 26.5 | 0.058 |
| 1st | 26.4 | 0.071 | 27.2 | 0.069 | 26.8 | 0.051 |
| 2nd | 26.5 | 0.074 | 26.5 | 0.055 | 27.4 | 0.064 |
| 3rd | 26.8 | 0.074 | 26.5 | 0.072 | 26.7 | 0.064 |
| 4th | 27.3 | 0.079 | 27.1 | 0.075 | 26.4 | 0.069 |
| 5th | 26.2 | 0.068 | 26.8 | 0.076 | 27.3 | 0.079 |
| 6th | 26.5 | 0.079 | 27.5 | 0.073 | 27.2 | 0.074 |
| 7th | 24.1 | 0.071 | 26.3 | 0.070 | 27.7 | 0.074 |
| 8th | 25.1 | 0.067 | 26.2 | 0.065 | 26.8 | 0.068 |
| 9th | 24.9 | 0.070 | 27.1 | 0.061 | 27.1 | 0.068 |

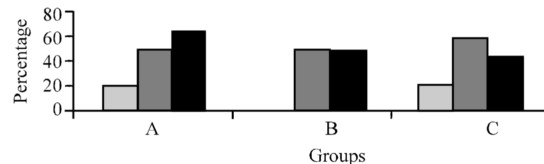


Fig. 1: Variation in the percentage of the accepted sperm in all groups (N = 9)

**Artificial insemination, fertilization capacity and hatching rate assessment:** In all three groups newly molted female were used, artificial insemination in *P. merguiensis* was challenging task previously no any successful result available in literature however, researchers done artificial insemination by using a device. Because of device there get reasonable result in all three groups female shrimp accepted sperms, percentage as shown in Fig. 1.

After the 5th h insemination eye stalk ablation were done and for control group wild mated animals were used. There was no significant difference ($p>0.05$) in the fertilization capacity and hatching rate of different groups, compared to control. Average fertilization rate was 44.3 in group A, group B 61.9 and group C was 42.9%, respectively at rates comparable to control 88.2%, respectively. Hatching rates of fertilized eggs were 64.4, 67.7, 61.3% for 7th h which were not different ($p>0.05$) from those of the control group 76.2%, respectively (Table 3).

Table 3: Variation in the fertilization and hatching in all groups (A-C) including control (N = 24 M/F)

| Groups | Fertilization | ±SD | Hatching | ±SD |
|---|---|---|---|---|
| Control | 88.2 | 8.3 | 76.2 | 8.4 |
| A | 44.3 | 18.1 | 64.4 | 14.1 |
| B | 61.9 | 6.7 | 67.7 | 7.2 |
| C | 42.9 | 16.2 | 61.3 | 15.4 |

## DISCUSSION

Mean sperm viability of the fresh sample was 93.96±3.74%, 98.8±0.7, 99.1±0.7 at initial time (time zero). This observation might be due to the hatchery holding time or quality of the wild male shrimp used in analyzing the sperm viability of the species. Similar observation was documented by earlier study where the quality of sperm/spermatophores in mature males was often related directly to hatchery holding time (Aiken and Waddy, 1980) in this study, expected mean sperm viability of 100% at initial (time zero). On the other hand, average sperm viability of 97.8% was documented in fresh *P. monodon* at zero time (Vuthiphandchai *et al*., 2007) and prefreezes evaluation of spermatophore viability were >98% in *Scylla serrata* crab (Jeyalectumie and Subramoniam, 1989).

Viability of the sperm considerably decreased in the first 60 min in all groups A, B and C 76.4±5.96%, 91.4±0.9, 97.6±1.2. Similar observation was reported by (Thomas *et al*., 1998) where highest sperm survival of 56% was observed when sperms of marine shrimp *Sicyonia ingentis* were kept at 1°C. There was no much fluctuation in the viability of sperm observed when the time elapsed next 120 min. This might be due to the tolerable capacity for sperms to with stand the gradually decreasing/ maintaining temperature to the considerable amount of time. At 5 h viability of sperm decreased to <70% showed that the exposure of viable sperm to the low temperature for >5 h could mitigate the viability of the sperm considerably. Significant decline in the viable sperm count was observed at 8 h of specimens maintained at 2°C 43.6±5.85%, 35.4±2.2 and 49.2±2.1. The results showed that sperm viability of the specimens maintained at 2°C decreases with elapsing time. Similar observation was reported in *P. monodon* when the viable sperms were stored for longer duration in liquid nitrogen (>60 days) significantly decreased sperm viability ($p<0.05$) (Vuthiphandchai *et al*., 2007). However, after 7th h for spermatophores in group A fertilization and hatching rate was 44.3 and 64.4% group B 61.9 and 67.7%, group C was 42.9 and 61.3%, respectively at rates comparable to control 88.2 and 76.2%, respectively. There was no significant relationship was observed between biomass of the spermatophore to the body weight of the shrimp ($p>0.05$) (Table 2). Similarly there was no relationship observed among the weights of the sample, spermatophore weight with their corresponding viable sperm count (Table 4-6).

Table 4: Relationship among the weights of the sample, spermatophore weight in group A with their viable sperm count

| Time (h) | B/weight | Sp/weight | Viability (%) |
|---|---|---|---|
| 0 | 26.50 | 0.078 | 93.9 |
| 1st | 26.40 | 0.072 | 76.4 |
| 2nd | 26.50 | 0.074 | 67.9 |
| 3rd | 26.80 | 0.075 | 62.5 |
| 4th | 27.30 | 0.079 | 59.4 |
| 5th | 26.20 | 0.068 | 57.7 |
| 6th | 26.50 | 0.079 | 56.6 |
| 7th | 24.10 | 0.075 | 50.7 |
| 8th | 25.13 | 0.068 | 43.6 |
| 9th | 24.90 | 0.071 | 37.0 |

Table 5: Relationship among the weights of the sample, spermatophore weight in group B with their viable sperm count

| Time (h) | B/weight | Sp/weight | Viability (%) |
|---|---|---|---|
| 0 | 25.8 | 0.068 | 98.8 |
| 1st | 27.2 | 0.069 | 91.4 |
| 2nd | 26.5 | 0.055 | 82.3 |
| 3rd | 26.5 | 0.072 | 75.9 |
| 4th | 27.1 | 0.075 | 69.5 |
| 5th | 26.8 | 0.076 | 63.6 |
| 6th | 27.5 | 0.073 | 56.6 |
| 7th | 26.3 | 0.070 | 51.2 |
| 8th | 26.2 | 0.065 | 35.4 |
| 9th | 27.1 | 0.061 | 31.6 |

Table 6: Relationship among the weights of the sample, spermatophore weight in group C with their viable sperm count

| Time (h) | B/weight | Sp/weight | Viab (%) |
|---|---|---|---|
| 0 | 26.46 | 0.058 | 99.0 |
| 1st | 26.76 | 0.051 | 97.6 |
| 2nd | 27.35 | 0.064 | 93.3 |
| 3rd | 26.70 | 0.064 | 88.2 |
| 4th | 26.37 | 0.069 | 80.7 |
| 5th | 27.26 | 0.079 | 71.7 |
| 6th | 27.23 | 0.074 | 62.7 |
| 7th | 27.65 | 0.074 | 56.2 |
| 8th | 26.78 | 0.068 | 49.2 |
| 9th | 27.08 | 0.068 | 41.5 |

## CONCLUSION

The study proves that elapsing time has significant influence on the percentage of viable sperm count. The specimens that were maintained at 2°C had spermatophore which ultimately reduces the percentage of live sperm count but retains the considerable amount of viable sperm (>50%) until 7th h specimens. These observations provide apparent evidence that the specimens, spermatophore or sperm suspension maintained at 2°C could be utilized for fishery management through artificial insemination process until 7th h.

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