http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 17 (9): 1069-1073, 2014
ISSN 1028-8880 / DOI: 10.3923/pjbs.2014.1069.1073

Sperm Viability Assessment over Elapsing Time Maintained at 2°C of Orange Mud Crab, *Scylla olivacea* (Herbst, 1796)

[1]M. Ikhwanuddin, [1]H. Muhd-Farouk, [1,3]A.J. Memon, [2]W. Wendy and [2]A.B. Abol-Munafi
[1]Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Kuala Terengganu, 21030, Terengganu, Malaysia
[2]Faculty of Fisheries and Aqua-Industry, Universiti Malaysia Terengganu, Malaysia
[3]Directorate of Fisheries Inland Hyderabad, Government of Live Stock and Fisheries of Sindh, Pakistan

Abstract: The aim of this study was to evaluate how long the fresh sperm maintained at 2°C would be utilized for fishery management. The study was conducted every 2 h to assess the sperm viability of orange mud crab *Scylla olivacea*. Evaluations were conducted as 3 treatments; T1, T2 and T3. In T1, the live specimens were sacrificed; for T2, only spermatophores were extracted and for T3 spermatophore extraction followed by homogenization to create a sperm suspension. All samples were stored with ice in an insulated box was keep fresh longer at 2°C. The time '0' referred the immediate collection of sperm after the specimen was sacrificed. Spermatophore viability was determined using the sperm suspension by eosin-nigrosin staining method. Sperm viability for the fresh sample at time zero was 97.36±0.53%. Viability of the sperm significantly decreased in the 2nd h in all treatments, T1 was 44.66±0.54 to 4.2 ±0.22% at 16 and 18th h, T2 was 36.56±0.5 to 2.69±0.06% at the 12 and 14th h and T3 was 33.69±1.26 to 6.4±0.29% at 8 and 10th h. In comparison, T1 showed significantly higher than other treatments ($p<0.05$). Extremely low viability percentages were recorded in T3. This study also proved that the time elapse had significant impact on the percentage of viable sperm count.

Key words: Sperm, viability, orange mud crab, *Scylla olivacea*

INTRODUCTION

In recent years the demand for seafood, particularly lobster, shrimp and crab, for export has been increasing quite considerably. There are also growing local requirements. The crab fishery in Malaysia is yet to be recognized as a major fishery, despite the abundant occurrence of food crab all along the Malaysia coastal water (Ikhwanuddin and Oakley, 1999; Say and Ikhwanuddin, 1999). Among the edible species, *Scylla olivacea*, commonly called the orange mud crab, is important. It is popular because of its size, meat quality, high price and export potential. The increasing market price of mud crabs in Malaysia has encouraged many coastal fishing communities to initiate culture trials in floating cages shallow earth ponds, also pen enclosure in mangrove forests. Allan and Fiedler (2004) have expressed concerns that further expansion of mud crab aquaculture will need an alternative source of supply, as the maximum sustainable yield from wild stocks has been reached or even exceeded in some location. Currently, aquaculture production relies on wild-caught seed for stocking ponds, as larval rearing at a commercial scale is still difficult (Keenan, 1999).

In order to advance development of these mud crab industries, an improved supply of good quality seed is required. Good quality sperm via spermatophore preservation would provide an alternative source of reliable and high quality spermatophores.

Sperm viability assessment of fresh sperm maintained at 2°C, also provides an extra reliable supply of high quality spermatophore and could ease short-term spermatophore supply of orange mud crab *S. olivacea* (Memon *et al*., 2011, 2012). This method also improves breeding efficiencies in hatchery and allows easy transportation of male gametes in specialized breeding programs (Grout *et al*., 1992; Memon *et al*., 2011, 2012). For this study, modified eosin-nigrosin staining method was used to assess viability (Bart *et al*., 2006; Vuthiphandchai *et al*., 2007; Memon *et al*., 2011, 2012).

The application of sperm maintained at 2°C would be advantageous for hybridization, selection breeding, gynogenesis, domestication and conservation of endangered species (Memon *et al*., 2011, 2012). Sperm maintained at 2°C help in maintenance of threatened genetic strains, benefits in aquaculture research and guaranteed the supplies of sperm without seasonal limitation (Memon *et al*., 2011, 2012). One problem with

Corresponding Author: M. Ikhwanuddin, Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Kuala Terengganu, 21030, Terengganu, Malaysia

spawning crab in culture is the uncertainty of individuals spawning at the required time. Off season spawning can be induced in cultured species but the techniques are cost intensive. When sperm preservation is available, manipulation of spawning season could be restricted to female only (Suquet *et al*., 2000). This can help in simplifying brood stock maintenance by reducing the number of males needed in hatchery operations. Little labour and space is needed to maintain the adult broodstocks, thus decreasing the high cost maintenance (Gwo, 2000). In addition, sperm maintained at 2°C can help in transportation of gametes to be much easier. It is useful when male and female gametes are collected in different locations. This will enable the introduction of new genes from the wild into hatchery stocks (Memon *et al*., 2011, 2012).

It is noteworthy to declare that at present there is no report available in the literature on sperm viability assessment of *S. olivacea*. Only few works had been done on the spermatozoa research of mud crab *S. serrata* (Jeyalectumie and Subramoniam, 1989). Hence, the objective of this study was done to determine the sperm viability percentage over elapsing time maintained at 2°C of orange mud crab, *S. olivacea*.

MATERIALS AND METHODS

Specimen source and maintenance: Adult, malebrood stocks mud crabs were caught from Pulau Sayak, Kedah, Malaysia (5°39”N; 100°19”E) and transported to Institute of Tropical Aquaculture (AKUATROP) hatchery, University Malaysia Terengganu (UMT). The male crab specimens were reared in 6 holdings tanks at a density of 2 individual m^{-2} in a 3 m^2 rectangular tanks with water depth of 30 cm. Daily water temperatures were recorded as the mean of maximum and minimum temperatures for each 24 h period. Salinity was maintained at 28 to 30 ppt and pH did not vary outside the range 7.2-7.8. A natural light/dark sequence of 12/12 h approximately was used throughout the study. Crabs were fed with cockles twice a day at rate of 20% of the biomass. Uneaten food was removed each morning. Approximately 50% of the volume of each tank was flushed daily.

Materials: A 1,000 L capacity tank was used for each brooder. Dissolve oxygen, temperature, salinity and pH measured daily using YSI Model 85. Spermatophores were extracted and maintained at 2°C using ice box before the assessment (Ozogul *et al*., 2005; Memon *et al*., 2011). The micro-centrifuge of 1.5 mL size was used to keep the spermatophore and sperm suspension. Haemacytometer was used in order to count the live and dead sperm cells. Calcium-Free Saline (Ca-F Saline) was used as a dilution for the sperm cells. Eosin and nigrosin was used as staining to assessment of the sperm viability (Bart *et al*., 2006; Vuthiphandchai *et al*., 2007; Memon *et al*., 2011, 2012).

Spermatophore collection: After the crabs were sacrificed, the carapaces were split open and separated from the whole body where the white in color vas deferens can be seen clearly in the middle of the body cavity (Fig. 1). The vas deferens containing seminal plasma and vesiculates permatophores were removed using forceps and placed into a micro-centrifuge tube (1.5 mL) that already been filled with calcium-free saline (Ca-F saline).

The preparation Ca-F saline in the present study following protocols of (Bart *et al*., 2006; Vuthiphandchai *et al*., 2007; Memon *et al*., 2011, 2012). Preparation of the Ca-F saline used the following chemicals in 1 L sterilized distilled water (adjusted to pH 7.4 with 1N HCl): NaCl = 21.63, KCl = 1.12, H_3BO_3 = 0.53, NaOH = 0.19, $MgSO_4$. $7H_2O$ = 4.93 g.

The micro-centrifuged tube containing the vas deferens were then placed in the Styrofoam box filled with crushed ice which will keep the spermatophores in good condition.

Sperm sample preparation: Sperm viability was assessed every 2 h (within the time range of 0-22 h). Then, viability assessment was conducted in 3 treatments; T1, T2 and T3. In T1, the live specimens were sacrificed and placed in a ice box at 2°C. In T2, only spermatophores were extracted and held in micro-centrifuge tube (1.5 mL) at 2°C. In T3, spermatophore extraction was conducted followed by transfer to a glass homogenizer with Ca-F saline to homogenize and create sperm suspension before

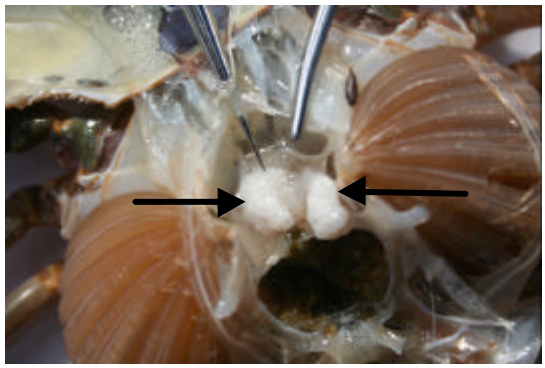

Fig. 1: Area of spermatophore collected. Arrows shows the vas deferens containing seminal plasma and vesiculate spermatophores

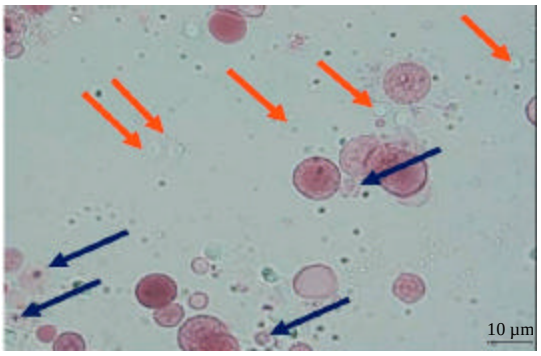

Fig. 2: Red arrows show the live unstained sperm cells and blue arrows show dead sperm cells stained with the eosin pink colour that was observed under the 400x magnification of compound light microscope

placed in the freezer at 2°C. In this time, '0' referred the immediately collection of sperm after the crab were sacrificed.

Spermatophores of three males in every treatment were sampled every 2nd h and then divided equally to produce 4 replications.

Sperm viability assessment: Sperm viability was demonstrated by a modified eosin-nigrosin staining protocol method (Nimrat *et al*., 2005; Bart *et al*., 2006; Vuthiphandchai *et al*., 2007; Memon *et al*., 2011, 2012). Initially, known spermatophore specimens were weighed and transferred to a glass homogenizer or manually grounded using mortar and pestle with 200 µL of Ca-F saline to form a sperm suspension. The eosin solution was prepared by dissolving 0.5 g of eosin in 100 mL distilled water. Nigrosin solution was prepared by dissolving 10.0 g of nigrosin in 100 mL distilled water. The 10 µL of sperm suspension was transferred to a clean glass slide and mixed with 10 µL of eosin-nigrosin prior air-drying. The viability of spermatophores was subsequently evaluated under an Advanced Research Microscope (EPI-Fluorescence, Japan 400X magnifications). Live sperm was observed unstained against the blue background of nigrosin whereas dead sperm showed pink color (Fig. 2).

Statistical analysis: Data are expressed as means±SD. Sperm viability among different groups in different time was analyzed using 2-way ANOVA. The significant values were cross examined using Tukey's *post-hoc* test at $p<0.05$.

RESULTS

Table 1 illustrates high mean viability percentage recorded in all treatments at the time '0' was

Table 1: Mean sperm viability percentage (%) of *Scylla olivacea* over elapsing time for all treatments (T1, T2, T3)

Time (h)	T1	T2	T3
0	97.36±0.53^a	97.36±0.53^a	97.36±0.53^a
2	74.50±0.57^a	72.15±0.45^a	75.89±0.99^a
4	72.35±0.90^a	70.67±1.13^a	72.49±0.15^a
6	71.60±0.37^a	67.58±0.82^b	50.71±0.55^c
8	71.03±0.35^a	64.10±0.14^b	35.73±0.46^c
10	70.54±0.48^a	38.50±0.47^b	33.69±1.26^c
12	60.26±0.37^a	36.56±0.50^b	6.40±0.29^c
14	48.20±0.70^a	2.69±0.06^b	1.22±0.19^b
16	44.66±0.54^a	1.42±0.27^b	0.00^c
18	4.20±0.22^a	0.00^b	0.00^b
20	2.96±0.13^a	0.00^b	0.00^b
22	1.14±0.33^a	0.00^b	0.00^b

T1: Specimen with spermatophores maintained freshness at 2°C, T2: Spermatophores were extracted and maintained freshness at 2°C, T3: Spermatophores extraction followed by immediate homogenization and maintained freshness at 2°C, Data are expressed as Mean±SD, n = 33, Data with same letters do not differ significantly, $p<0.05$)

97.36±0.53%. When compared, T1 showed high value. Extremely low viability percentages were recorded at the 14th h exposure in T3, at 16th T1 and at 22nd h T2 with values of 1.22±0.19, 1.42±0.27 and 1.14±0.33%, respectively ($p<0.05$). Intermediary viability percentages were recorded in T1, T2 and T3 at 10th h exposure obtaining values of 70.54±0.48, 38.50±0.47 and 33.69±1.26%, respectively. By the interaction between T1, T2 and T3, the T1 treatment showed higher viability at 12th h 60.26±0.37% as compared to T2 (36.56±0.50%) and T3 (6.40±0.29%) ($p<0.05$).

Mean viability of exposure in treatments T1, T2 and T3 showed maximum viability at time '0' (97.36±0.53%) and the minimum was recorded at the 22nd, 16 and 14th h. In comparison, T1 showed 60.26±0.37% viability at the 12th h of exposure, which was much higher than other groups ($p<0.05$). There was less variation observed in viability of sperm at the 2nd h. By the 8th h, the viability of sperm had decreased to between 71.03±0.35% in T1, 64.10±0.14% in T2 and 35.73±0.46 in T3. Besides that, the spermatophore retained viability above 50% in T1 at the 12 h, T2 at 8 h and T3 at 6th h of exposure. This showed that sperm viability of the specimens maintained at 2°C decreased with the time elapse.

DISCUSSION

In this study, mean sperm viabilities of the fresh sample at time '0' was significantly greater than 97% for all treatments. This was influenced by the hatchery holding time or quality of the wild male crab used as the similar was documented (Vuthiphandchai *et al*., 2007; Memon *et al*., 2011). It was observed that mean sperm viability of 97.8% was for *P. monodon* and *P. merguiensis* at time '0' and pre-freeze evaluation of spermatophore viability was greater than 98% in mud crab, *Scylla serrata* (Jeyalectumie and Subramoniam, 1989). Therefore, the quality of sperm in matured males was often related directly to the hatchery holding time (Aiken and Waddy,

1980). Viability of the sperm significantly decreased in the 2nd h for all treatments 74.50±0.57, 72.15±0.45 and 75.89±0.99%, respectively. Similar observation was reported by Anchordoguy *et al*. (1988) with highest sperm survival of 56% when sperms of the marine shrimp, *Sicyoniaingentis* which were stored at 1°C.

The viability was significantly higher in T1, T2 and T3 and not much fluctuation was observed when the time elapsed to the 6th h. This is due to the tolerable capacity for sperms to withstand the gradually decreasing temperature with time. At the 12th h of exposure, mean viability of sperm in all treatments decreased which showed that the exposure of sperm to the low temperature could reduce the viability of the sperm significantly. The declination of sperm viability may be caused by the process of extracting the spermatophore and its form into sperm suspension, the heat due to homogenizing the spermatophore. Significant declination of sperm viability was observed at the 14th h in all treatments. With this, the results showed that sperm viability maintained at 2°C decreases with time. In addition, sperms of *P. monodon* stored for longer period in liquid nitrogen for more than 60 days and *P. merguiensis* showed similar results as the current findings (Vuthiphandchai *et al*., 2007; Memon *et al*., 2011).

CONCLUSION

The present study concludes that the time has a significant impact on the percentage of sperm viability. These observations provide apparent evidence that the specimens, spermatophore or sperm suspension maintained at 2°C may be utilized for fishery management.

ACKNOWLEDGMENT

This study had been funded by Malaysia's Ministry of Education under Fundamental Research Grant Scheme (FRGS) of Vot. No. 59267. Our great appreciation to Institute of Tropical Aquaculture, Universiti Malaysia Terengganu and to all people who involved directly or indirectly during this study.

REFERENCES

Aiken, D. and S. Waddy, 1980. Reproductive Biology. In: The Biology and Management of the American Lobster, Cobb, S. and B. Phillips (Eds.). Vol. 1, Academic Press, New York, pp: 215-276.

Allan, G. and D. Fielder, 2004. Mud crab aquaculture in Australia and Southeast Asia. ACIAR Working Paper No. 54. http://www.aciar.gov.au/ublication/WP54.

Anchordoguy, T., J.H. Crowe, F.J. Griffin and W.H. J.R. Clark, 1988. Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis*. Cryobiology, 25: 238-243.

Bart, A.N., S. Choosuk and D.P. Thakur, 2006. Spermatophore cryopreservation and artificial insemination of black tiger shrimp, *Penaeus monodon* (Fabricius). Aquaculture Res., 37: 523-528.

Grout, B.W.W., G.J. Morris and M.R McLellan, 1992. Cryopreservation of gametes and embryos of aquatic orgamsms. Symp. Zool. Soc. London, 64: 63-71.

Gwo, J., 2000. Cryopreservation of aquatic invertebrate semen: A review. Aquacult. Res., 31: 259-271.

Ikhwanuddin, A.M. and S. Oakley, 1999. Culture of mud crabs in mangrove areas: The Sarawak experience. Proceedings of the TCE-Project 3rd Workshop on Integrated Management of Mangrove/Coastal Ecosystems for Sustainable Aquaculture Development, March 23-25, 1999, Kuching, Sarawak, Malaysia.

Jeyalectumie, C. and T. Subramoniam, 1989. Cryopreservation of spermatophores and seminal plasma of the edible crab *Scylla serrata*. Biol. Bull., 177: 247-253.

Keenan, C.P., 1999. Aquaculture of the Mud Crab, Genus *Scylla*-Past Present and Future. In: Mud Crab Aquaculture and Biology, Keenan, C.P. and A. Blackshaw (Eds.). Australian Centre for International Agricultural Research, Brisbane, Australia, pp: 9-13.

Memon, A.J., M. Ikhwanuddin, A.D. Talpur, M.I. Khan, M.O. Fariddudin, J. Safiah and A.B. Abol-Munafi, 2011. Assessment of sperm viability, determination fertilization capacity and hatching rate by artificial insemination of banana shrimp, *Penaeus merguiensis* (De Man, 1888). Res. J. Applied Sci., 6: 174-178.

Memon, A.J.A. F., M. Ikhwanuddin Bin Abdullah and A.M.B.A. Bolong, 2012. Spermatophore Cryopreservation of Banana Shrimp, Penaeus Merguiensis: Effects of Food Type on the Quality of Spermatophores and Subsequent Cryopreservation and Fertilization in Banana Shrimp. LAMBERT Academic Publishing, Saarbrucken, Germany, ISBN: 9783659274701, Pages: 236.

Nimrat, S., T. Sangnawakij and V. Vuthiphandchai, 2005. Preservation of black tiger shrimp *Penaeus monodon* spermatophores by chilled storage. J. World Aquacult. Soc., 36: 76-86.

Ozogul, Y., G. Ozyurt, F. Ozogul, E. Kuley and A. Polat, 2005. Freshness assessment of European eel (*Anguilla anguilla*) by sensory, chemical and microbiological methods. Food Chem., 92: 745-751.

Say, W.C.W. and A.M. Ikhwanuddin, 1999. Pen culture of mud crabs, genus scylla in the mangrove ecosystems of Sarawak, East Malaysia. Proceedings of the International Scientific Forum, April 21-24, 1997, Darwin, Australia, pp: 83-88.

Suquet, M., C. Dreanno, C. Fauvel, J. Cosson and R. Billard, 2000. Cryopreservation of sperm in marine fish. Aquacult. Res., 31: 231-243.

Vuthiphandchai, V., S. Nimrat, S. Kotcharat and A.N. Bart, 2007. Development of a cryopreservation protocol for long-term storage of black tiger shrimp (*Penaeus monodon*) spermatophores. Theriogenology, 68: 1192-1199.