ARTIFICIAL INSEMINATION STUDIES IN *MACROBRACHIUM IDELLA* (HILGENDORF, 1898)

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Abstract: A simple method for artificial insemination of *Macrobrachium idella* has been developed. Observations on virility in different size groups of males have been reported. The insemination procedure involved extrusion of spermatophores by electrical stimulus and attachment of sperm mass at the receptive area of newly moulted normal and eye ablated females. Manually placed spermatophores were retained through spawning. Fertilization was obtained and eggs fertilized in this way, hatched to normal healthy larvae. The exact time for placement of spermatophores after the pre-spawning moult of female has been worked out. Further, successful spawning could be observed for more than one female simultaneously by using whole sperm mass of a single male. Freshly extruded spermatophores and those stored in refrigerated condition (6°C) for different time duration have also been used in inseminating females artificially with good success.

INTRODUCTION

Establishment of sperm bank and artificial insemination are widely practised in animal husbandry and controlled breeding programmes (Leverage *et. al,* 1972). Such attempts among crustaceans aimed to improve gamete quality and propogation of species in culture, specially in commercially important groups like crabs, lobsters and prawns are of much practival value. The attempts in this direction were made by Uno and Fujita (1972), Clarke et al. (1973) and Sandifer and Smith (1979). The initial attempts involved mechanical extrustion of spermatophore, a process which often injured the males. The electroejaculation technique of extruding spermatophore from males simplified artificial insemination technique in crustaceans to a certain extent. This technique was adopted by different researchers in penaeid prawns (Laubier-Bonichon and Ponticelli, 1981; Lumare, 1981 and Muthu and Laxminarayana, 1984) as well as palaemonid prawns (Sandifer and Lynn, 1980). Tave and Brown (1981) further refined the technique by use of gill irrigator and restraining device for the receptive females. The importance of stockpiling and exchanging the selected male genetic material in Crustacea, have led some researchers for evolving methods for preservation of spermatophore for different durations. Artificial insemination using cryopreserved spermatophore had been undertaken in the lobster, *Homarus americanus* by Kooda Cisco and Talbot (1983) and in the prawn *Macrobrachium rosenbergii* by Chow (1982) and Chow *et al.* (1985).

In the present work, artificial insemination using electroejaculation technique has been attempted in the freshwater prawn *Macrobrachium idella.* The best possible permutations and combinations to get maximum viable yield of larvae have been explored. The possibility of using refrigerated spermatophore for artificial insemination has also been tapped.

MATERIAL AND METHODS

Animal collection and maintenance

Specimens of *M. idella* were collected from Vembanad lake at Panavally village near Cochin, Kerala. All the animals were maintained in clean filtered water (S = 6‰) with continuous aeration. The animals were fed *ad libitum* with clam meat and boiled, chopped poultry egg white. They were maintained individually in containers of 10 to 35 litre capacity or communally in tanks of upto 1000 litre capacity. Details of the design of experiment and rearing facility of experimental animal are shown in Fig. 1. Considering the pugnacious nature and cannibalistic tendency, the males were maintained individually in 10 l capacity plastic troughs.

In all the experiments receptive females of 71 to 75 mm (total length) were used for the artificial insemination trials. A receptive female was distinguished by the fully developed ovary noticeable through the trans-

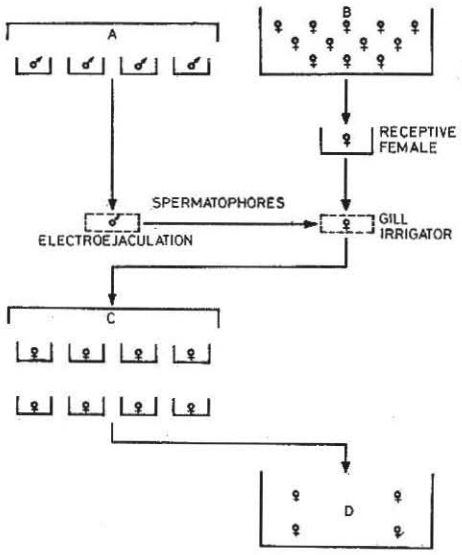

Fig. 1. Diagramatic representation of the artificial insemination facility

A. Male (to be used for spermatophore extrusion) maintenance facility. B. Female maintenance facility. C. Maintenance facility for artificially inseminated ovigerous females. D. Hatching tank.

lucent exoskeleton; she also undergoes a prespawning moult. Such receptive females were transferred from the communal tank to the 35 litre perspex tanks and were housed individually in these tanks prior to artificial insemination. The artificially inseminated ovigerous females with brown eggs were transferred to hatchery tank.

Technique of artificial insemination

The apparatus used for extrusion of spermatophore by electrical stimulus is shown in Plate 1. The apparatus is similar to the one used by Sandifer and Lynn (1980) for electroejaculation of *M. rosenbergii* males. The gill irrigator (Plate I.1) in conjunction with a restraining device (Tave and Brown, 1981 model) was used to reduce the stress on receptive females during spermatophore transfer. Artificial insemination of female *M. idella* involved two steps viz. extrusion of spermatophore employing electroejaculation technique and placement of the extruded spermatophore on the seminal receptacle of the receptive female.

Electroejaculation technique

The male was held securely, so that the ventral surface was exposed. The pair of electrical probes were positioned near the fifth pereiopods where the gonopore opens (Plate I.2). The apparatus was switched on and the voltage increased to 4.5 volts within 15 seconds time. Upon stimulation, the membranous flap of gonopore was lifted and a sperm cord extruded from both the sides (Plate I.3). The two sperm cords formed a complete spermatophore. Depending on experiment, the spermatophore was either used immediately for artificial insemination or preserved in Ringer's solution* for further refrigeration.

Placement of spermatophore

The receptive female was placed in the cradle of the restraining device, ventral side up and securely positioned with the rubber bands (Plate I.4). The delivery tubes were placed in each branchial cavity and a continuous flow of water was maintained during the placement of spermatophore (Plate 4). Depending on the experiment, the electroejaculated spermatophore (either full or part of it) retrieved from the male prawn was placed securely on the sperm receptacle area of the female (Plate I.5 and I.6). The artificially inseminated female was then freed from the restraining device and released in the trough. The complete process of artificial insemination normally takes 3 to 5 minutes.

Two days after the artificial insemination act, the individual female was tagged using plastic tags.

Observations and interpretation of results

In all the experiments, fertilization in the artificially inseminated females was confirmed by observing cleavage, two or three days after oviposition. The larvae were counted by aliquot sampling and records of larval yield from individual experimental females were maintained. The results obtained in experiment 1 and 2 were utilized in experiment 3 and 4 for achieving higher success in larval yield through artificial insemination.

* ($NaCl$ = 1.35g, KCl = 0.06g, $NaHCO_3$ = 0.02 g, $CaCl_2$ = 0.025 g and $MgCl_2$ = 0.035 g in 100 ml distilled water).

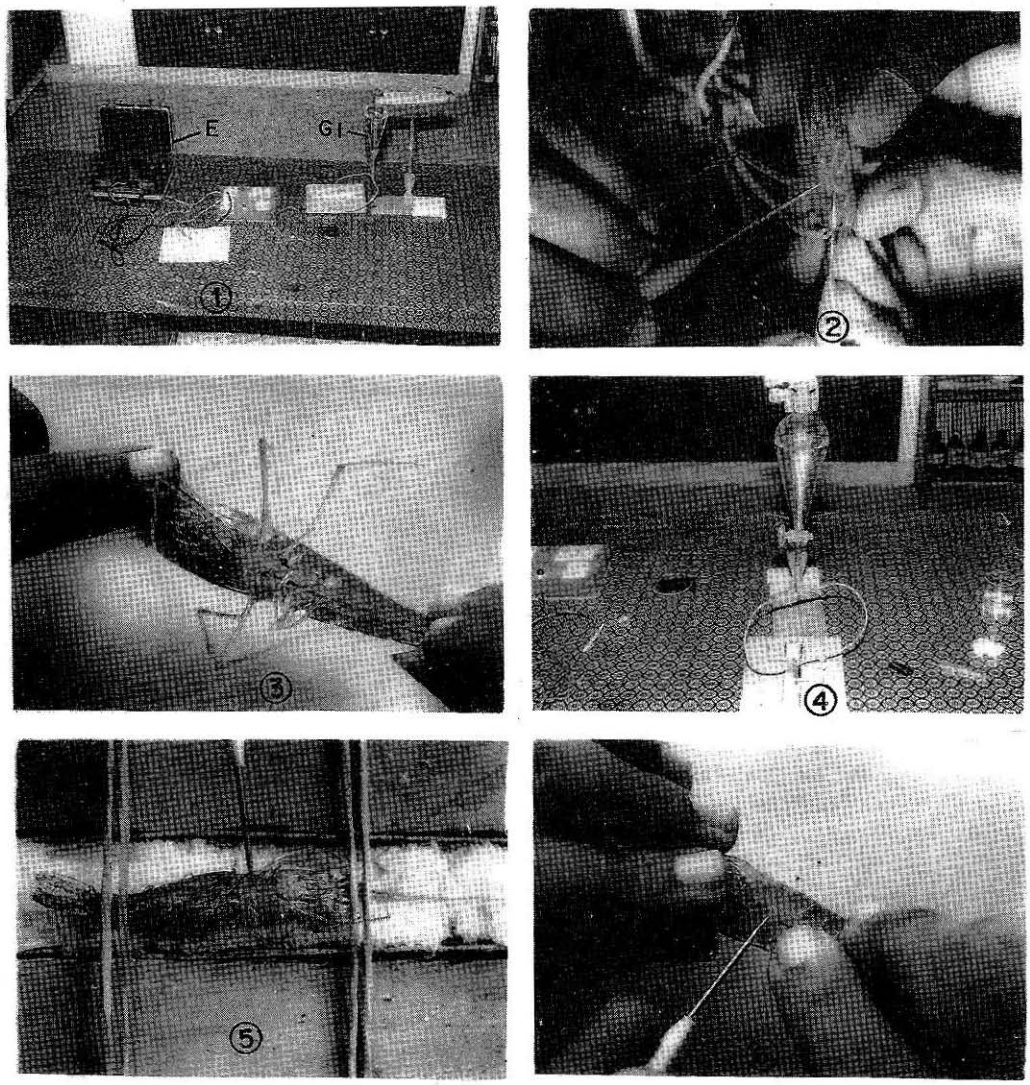

Plate I.1. Apparatus for electroejaculation (E) of spermatophore and gill irrigator (GI).
Plate I.2. The act of electrical stimulus: The pair of electrical probes positioned at the coxae of fifth pereopods, near to the gonopore opening.
Plate I.3. The spermatophore extruded from a male specimen after electrical stimulus (shown by arrow).
Plate I.4. The gill irrigator with restraining device. The receptive female placed in the cradle of the restraining device, and delivery tubes placed in each of the branchial cavity.
Plate I.5. The receptive female being placed in the cradle of the restraining device and the sperm mass deposited on the sperm receptacle (shown by pointer).
Plate I.6. Artifically placed spermatophore on the receptive area of female (shown by pointer).

RESULTS AND DISCUSSION

Experiment 1: **Testing virility in males using electroejaculation technique -**

The results of the experiment are presented in Table 1. It is noted that the spermatophore extruding capacity of a male is directly related to its size. The largest male in the size range of 91 to 95mm total length were found to be the most suitable for electroejaculation and retrieval of sperm mass, since males in this size range extruded spermatophores 13 times on an average within a period of 15 days. Therefore, in the subsequent experiments, males of this size group were only used. It was noticed that during subsequent electroejaculation trials, the ejaculated quantity appeared to diminish slightly, however, no mortality or any other ill effects could be seen in the males due to electroejaculation.

Experiment 2: **Assessing of correct time for placement of spermatophore after pre-spawning moult:**

The most appropriate timing for deposition of spermatophore on receptive female, that would result in successful artificial insemination was worked out by this experiment. The results are shown in Table 2. It is inferred from this experiment that placement of spermatophore 2.5 to 4 hrs after the pre-spawning moult leads to successful artificial insemination, thus this period is considered to be most suitable to carry out spermatophore placement.

The findings of experiment 1 and 2 i.e. the ideal size range of male for electroejaculation and the ideal timing for placement of spermatophore were used in experiment 3 and 4 for better results.

Experiment 3: **Artificial insemination trials with fresh spermatophore**

Freshly extruded spermatophores from the males were used to inseminate one/two/four receptive wild females simultaneously. Similarly the eyestalk ablated, captive mature females were also inseminated artificially. The results are given in Table 3.

Experiment A: Of the 52 trials, 34 trials turned to be successful in releasing healthy and normal larvae after an incubation period of 12 to 13 days (Table 3). Of the 18 unsuccessful trials, in 6 trials fertilized eggs were observed but the berry got detached from the abdominal pouch within 1 to 2 days after oviposition and in 12 trials the spermatophore got dislodged from the seminal receptacle of the female resulting in failure of artificial insemination.

Experiment B: In this, 66 trials were conducted and in each trial the wild receptive female was artificially inseminated using one sperm cord which is half of the normal spermatophore. Spermatophore of one male was thus used to inseminate two females simultaneously. Of the 66 trials, 14 trials were successful as evidenced by hatching out of healthy larvae (Table 3).

Table 1. Virility in different size groups of *M idel.a*

Sl No.	Size group of males (mm)	Number of replicates	Average number of times spermatophore extruded
1.	71-75	5	7
2.	76-80	5	8
3.	81-85	5	9
4.	86-90	5	11
5.	91-95	5	13

Experimental period : 15 days

Experimental condition : Salinity : 6 ‰ , temperature :27-29°C, PH : 8-8.3

Electroejaclation : Electrical stimulus (4.5 volts) applied near the base of each 5th pereiopod of male. Each male was given electrical stimulus daily once and the extrusion of spermatophores were observed for 15 consecutive days.

Table 2. Assessing proper time duration for spermatophore placement in Female *M.idella*

Trial No.	No. of replicate	Time interval between pre-spawning moult and placement of spermatophore (hrs)	Result	Remarks
1	1	0.5	Unsuccessful	Female died within 2 hrs after spermatophore placement
2	1	1.0	Unsuccessful	Female too soft. Spermatophore dislodged. Female died next day.
3	1	1.5	Unsuccessful	Female still soft. Spermatophore dislodged and female oviposited unfertilized eggs 5 hrs after pre-spawning moult.
4	1	2.0	Unsuccessful	Spermatophore dislodged. No oviposition. Ovary got resorbed.
5	1	2.5	Successful	Spermatophore retained. Oviposition 5.5 hrs after pre-spawning moult.
6	3	3.0	Successful	All females oviposited 6 hrs after pre-spawning moult.
7	1	3.5	Successful	Female oviposited fertilized eggs, 5 hrs after pre-spawning moult.
8	1	4.0	Successful	Female oviposited fertilized eggs 5.5 hrs after pre-spawning moult.
9	1	4.5	Unsuccessful	Female too hard. Spermatophore dislodged. Female died next day.
10	1	5.0	Unsuccessful	Female oviposited unfertilized eggs before artificial insemination.
11	1	5.5	Unsuccessful	Female oviposited unfertilized eggs before artificial insemination.
12	1	6.0	Unsuccessful	Female oviposited unfertilized eggs before artificial insemination.

Experimental Conditions : Salinity : 6‰, Temperature : 28-29°C, pH: 8-8.2

Successful : Female spawned fertilized eggs, embryo development was normal and healthy normal larvae hatched out after an incubation period of 11-13 days.

Experiment C: Attempts were made to artificially inseminate four wild receptive females simultaneously with spermatophore retrieved from single male, each female thus receiving only a quarter of normal spermatophore. Here out of the 12 trials, 2 trials turned to be successful in producing normal and healthy larvae. However, the average larval yield was found to be much less being only 2352 numbers.

Experiment D: In this experiment, the experimental females were matured in captivity by adopting unilateral eyestalk ablation technique. Each such female was inseminated artificially using one complete spermatophore. Of the 10 trials attempted, 6 were found to be successful as indicated by release of healthy larvae averaging 4365 numbers per female.

Experiment 4: **Artificial insemination trials with refrigerated spermatophore:**

In this experiment, the effect of refrigeration (6°C) on the fertilizability of the spermatophore was studied. The fertilizability was expressed as number of larvae hatched out by artificially inseminated females. The results of the experiment are presented in Table 4.

Among 8 trials using refrigerated (24 hrs) spermatophore, 5 trials were successful. Of the three trials which failed, in one, fertilized eggs were observed after artificial insemination. However, the eggs dropped off from the female before completion of embryonic development.

Among 8 trials using refrigerated spermatophore (storage time 48 hrs) 2 trials were successful. Fertilized eggs were observed in 2 others and in the remaining 4 it totally failed as the spermatophore lost their stickiness and got dislodged from the female.

Among 6 trials using 72 hrs refrigerated spermatophores, only 1 was a success. Fertilized eggs were observed in another but in 4 others the spermatophore got dislodged from the female.

Among 5 trials using spermatophore that were refrigerated for 96 hrs only 1 trial was successful. In the 4 failures, the spermatophore was not sticky at all and hence got dislodged from the seminal receptacle.

Among the 5 trials with spermatophore stored for 120 hrs in refrigerator, none succeeded.

With increase in the refrigeration time, chances of success in artificial insemination declined successively. Moreover, the larval yield in such artificially inseminated females also exhibited an inverse relationship with the duration of refrigeration. When the spermatophores were refrigerated for 24 hrs, the average yield of larvae was 3948. When the storage time increased to 48 hrs the average yield of larvae decreased to 3110. Increasing the storage time further to 72 hrs and 96 hrs, the

Table 3. Artificial insemination trials with fresh spermatophores in *M idella.*

Experiment No.	Female Source	Details of Experiment	No. of trials+	No. of successes++	No. of failures		Average larval yield
					A*	B**	
3A	Wild unablated	Spermatophore from one male used to inseminate one female (i.e. both sperm cord used)	52	34	6	12	3950
3B	Wild unablated	Spermatophore from one male used to inseminate two females simulatenously. (Each female receiving single sperm cord)	66	14	8	44	3180
3C	Wild unablated	Spermatophore from one male used to inseminate four females simulatneously. (Each female receiving half sperm cord)	12	2	2	8	2352
3D	Matured in captivity by unilateral eyestalk ablation	Spermatophore from one male used to inseminate one female	10	6	1	3	4365

Experimental conditions : Salinity: 6‰ , Temperature: 28-31°C, pH: 7.8-8.2

Trials+ : Attempts made on artificial insemination of a sexually receptive female.

Success++ : Releasing of healthy zoea larvae after successful completion of incubation period.

Failure : A : eggs were fertilized by the artificially placed spermatophore but not viable.

B : Eggs were not fertilized by the artificially placed spermatophore.

larval yield further declined to 2860 and 2320 respectively.

During mating and insemination in the wild there is no control over the mating pair and so over their progeny. On the contrary, by adopting artificial insemination technique, selective breeding could be achieved. In the present study using such technique *M. idella* female was impregnated by artificial means, using spermatophore ejaculated from males. The electroejaculation of spermatophore has been reported as the most effective and simple method of spermatophore retrieval in *Macrobrachium* prawns (Sandifer and Lynn, 1980). In the present work an electrical stimulus of 4.5 volts was found to be sufficient for extrusion of spermatophore in male *M. idella.* In fact a stimulus of 4.5 volts was without any ill effect, even when the electroejaculation was attempted once in 24 hrs. When electrical stimulus of more than 5 volts was applied, terminal ampoules became blackish in colour probably due to tissue lysis. The electrical stimulus needed for electroejaculation is reported to vary from species to species. For example 2 volts in Palaemonetes, 5-6 volts in *M. rosenbergii* (Sandifer and Lynn, 1980), and 5 volts in P. japonicus (Lumare, 1981). However, in the lobster, *H. americanus* (Kooda-Cisco and Talbot, 1983) and the sand lobster *Thenus orientalis* (Silas and Subramoniam, 1987) electrical stimulus of 12 volts have been reported to be essential for electroejaculation of spermatophores.

A few reports describing the virility of male prawn and lobster are available. Sandifer and Lynn (1980) have reported in *M. rosenbergii* that six males were electroejaculated on 12 consecutive days without any ill effects and each time a male could extrude spermatophore after 24 hrs recovery period. In *T. orientalis* a 12 hrs recovery period has been reported (Silas and Subramoniam, 1987). In the present investigation in male *M. idella,* the capacity to extrude spermatophore on consecutive days was found to be directly related to size and robustness of the male. The largest males of size group 91-95 mm were found to be most virile, extruding spermatophore on an average 13 times in 15 days period. Such reports depicting the virility of male prawn in relation to body size is not available.

It was observed that spawning in *M. idella* takes place roughly within 4 to 6 hrs after the pre-spawning moult. It was further observed that after the pre-spawning moult, spawning leading to oviposition takes place, irrespective of availability of male and the mating act. These results agree with the observations of Chow et al. (1982) for *M. rosenbergii.* Artificial insemination attempt was successful only when placement of spermatophore was conducted 2.5 to 4 hrs after the pre-spawning

Table 4. Artificial insemination trails with refrigerated spermatophores in *M idella*

Sl. No.	Duration of spermato phore refrigeration (hrs)	No. of trials+	No. of sucess++	No. of failures		Average larval yield
				A*	B**	
1	24	8	5	1	2	3948
2	48	8	2	2	4	3110
3	72	6	1	1	4	2860
4	96	5	1	0	4	2320
5	120	5	0	0	5	-

Experimental conditions : salinity: 6‰, Temperature: 28-31°C, pH : 8 to 8.3 Spermatophores stored in refrigerator at 6°C.

Trails+ : Attempts made on artificial insemination of a sexully receptive female

Success++ : Releasing of healthy zoea larvae after successful completion of incubation period.

Failure : A* : Eggs fertilized by the artifically placed spermatophore were not viable.

B** : Eggs were not fertilized by the artficially placed spermatophores.

moult. Placement of spermatophore immediately after pre-spawning moult led to failure may be that the female was too soft to withstand the stress of handling, resulting in death. It was also observed that delay (more than 4 hrs) for placement of spermatophore, after pre-spawning moult also resulted in failure of artificial insemination, probably due to hardening of moulted female and dislodging of the spermatophore.

Of the 52 artificial insemination trials on *M. idella,* using complete spermatophore, 34 were successful. Failure in 18 trials may be due to the stress developed during handling. Another reason for failure was small size of receptive females, which were delicate to handle. Sandifer and Lynn (1980) working on identical lines reported success in fertilizing females in 11 out of the total 18 trials. It was observed in the present study that frequent handling of females during artificial insemination act resulted in delayed spawning and loss of manually placed spermatophore leading to failure in artificial insemination. Identical observations were recorded by Sandifer and Lynn (1980) for *M. rosenbergii.*

The gill irrigator used ensured continuous supply of water to the branchial cavity of female and provided better chances of success in artificial insemination. Tave and Brown (1981) have reported that after using the gill irrigator and restraining device during spermatophore transfer, 88% of the females that received spermatophore have spawned and finally released healthy larvae. Lumare (1981) on the other hand performed the artificial insemination without any device and could achieve only limited success.

From the earlier attempts on artificial insemination by different workers (Sandifer and Smith, 1979; Sandifer and Lynn, 1980; Lumare, 1981; Bray et al., 1982; Lin and Ting, 1984 and Silas and Subramoniam, 1987) and the present attempts in *M. idella,* it was evident that discovery of electroejaculation technique to extrude spermatophores in the males have simplified the artificial insemination to a great extent.

Attempts to inseminate 2 females with spermatophore from single male, yielded a marginal success. The larval yield after such inseminations was also low, compared to larval yield from a female inseminated with complete spermatophore. Similar attempts to fertilize two females with the sperm mass from one male were undertaken by Sandifer and Lynn (1980) in *M. rosenbergii,* achieving success in 50% of the trials. Compared to this, in the present study the success was only 21.21%.

When 4 females of *M. idella* were inseminated simultaneously, using spermatophore from single male only a marginal success could be achieved. The larval yield was also very low. Difficulty experienced in these trials was non-adhering of the piece of sperm cord on the seminal receptacle of the female. The half sperm cord, lost its stickiness within a short period and consequently the sperm mass got dislodged from the spterm receptacle before spawning occurred. Earlier, Sandifer and Smith (1979) examined the possibility of inseminating 4 females simultaneously with sperm mass from single male and encountered similar problems. Though the attempts to inseminate 2 and/or 4 females simultaneously with the sperm mass of single male could yield only marginal success in *M. idella,* after some refinements, this technique could prove highly useful, when maintenance of males in captivity is problematic. In the present investigation, *M. idella* males were noted to be more aggressive and more prone to diseases, compared to females. Sandifer and Lynn (1980) also observed that the males of *M. rosenbergii* were aggressive and pugnacious and generally experienced higher mortality in laboratory holding tanks.

In the present investigation the captive females (matured after unilateral eyestalk ablation) were also artificially inseminated and 60% of such attempts were successful. No reports of such attempts are available in *Macrobrachium* spp., although among penaeids such reports of artificial insemination of unilaterally ablated females are available. In the prawn *P. monodon,* Lin and Ting (1984) and Muthu and Laxminarayana (1984) have reported successful artificial insemination of unilaterally ablated and matured females.

Attempts on artificial insemination using refrigerated spermatophore showed that the spermatophore could remain as active as freshly extruded ones when refrigerated for 24 hrs at 6°C, but with further storage the fertilizability as well as viability were found to

decrease. The larval yield also declined. Working on identical line in *M. rosenbergii* Sandifer and Lynn (1980) concluded that the spermatophore could be stored under refrigeration for 24 hrs without losing their activity.Chow (1982) preserved the spermatophore of *M. rosenbergii* in Ringer's solution in refrigerator at 2°C and concluded that spermatophores retained their viability upto 4 days. In the present investigation, the spermatophores refrigerated at 6°C retained their viability for 72 hrs. However, the larval yield declined with increasing storage period. Chow (1982) reported that spermatophores, when preserved for longer time lost the protective and adhesive matrix and, were subjected to damage and propagation of bacilli resulting in fast degeneration of sperm mass. In the present study the damage and bacilli propagation were not observed, but the spermatophore lost its stickiness and changed its consistency making it difficult to pick up and place on the seminal receptacle of the female.

The technique of artificial insemination tried here in *M. idella* being simple and reliable might prove to be a good tool to biologists, culturists and genetic engineers.

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