CHAPTER IV

ARTIFICIAL INSEMINATION AND CRYOPRESERVATION STUDIES
INTRODUCTION

Major constraint in the hatchery production of crustaceans is inability to get spawners in nature at desired time. The eyestalk ablation experiments leading to induced maturation of prawns by different workers (Caillouet, 1972; Aquacop, 1975; Muthu and Laxminarayana, 1984 and Ruangpanit et al., 1984) all over the world solved the problem of getting gravid females to a considerable extent. But, mating of penaeid prawns in captivity is still not a feasible proposition. There is no scope for selection of parents when individuals mate in the wild. Artificial insemination technique in captivity using fresh and cryopreserved spermatophores can however, be thought of as feasible solution to ensure seed stock availability in crustacean aquaculture (Bray, et al., 1982).

Sperm bank and artificial insemination have been widely practised in animal husbandry and controlled research programmes (Leverage et al., 1972). However, it is rarely adopted to improve gamete quality and propagation of species in culture, specially in commercially important groups of crustaceans like crabs, lobsters and prawns.
At present, our knowledge is limited to reproductive behaviour and natural mating habit in different penaeid and palaemonid prawns. In this line Ling (1969a) has furnished an excellent description of breeding behaviour in *Macrobrachium rosenbergii*. An elegant account of pre-mating, mating and spawning behaviour of *M. rosenbergii* has been given by Sandifer and Smith (1979) and Chow et al. (1982). The breeding and mating behaviour of other species of *Macrobrachium* have been discussed by few other workers (Pillai and Mohamed, 1973; Ruello et al., 1973; Mashiko, 1981). Among penaeids the natural mating behaviour of different species of shrimps has been reported by various researchers namely Aquacop (1977a), Primavera (1979), Lumare (1981) and Emmerson (1983).

The principal obstruction in adopting artificial insemination was, obtaining spermatophore from mature males without harming them. In the initial phase of artificial insemination technique, the process involved picking up of freshly extruded spermatophore from the seminal receptacle of the naturally mated female and manual placement of it on the seminal receptacle of another receptive female. This simple technique was attempted by few workers viz. Uno and Fujita (1972) in *Macrobrachium* spp., Sandifer and Smith (1979) in *M. rosenbergii* and *M. acanthurus*; Sandifer and Lynn (1980) in *Palaemonetes pugio* and *P. vulgaris*. 
Applying the technique of artificial insemination, Clark et al. (1973) attempted in vitro fertilization of *Peneaus aztecs*. Persyn (1977) patented a method for artificial insemination of open thelycum shrimp which involved dissection of male, taking out spermatophore and placement of it on the receptive area of the ripe female. Sandifer and Smith (1979) used similar technique in *M. rosenbergii*.

The next phase in development of artificial insemination technique in crustaceans was the use of certain crude methods such as squeezing or pressing the gonopore region of males and getting the spermatophore extruded. The technique was also attempted in *M. rosenbergii* by Sandifer and Smith (1979). However, this method too was of little practical use, since it involved the risk of injury and mortality of the males.

The electroejaculation score of spermatophore extrusion and after effects of electroejaculation on the mature males have been studied in the prawn *M. rosenbergii* and the lobster *Homarus americanus* by Sandifer and Lynn (1980) and Kooda-Cisco and Talbot (1983) respectively. Silas and Subramoniam (1987) have worked out similar aspect in the sand lobster, *Thenus orientalis*. The electroejaculation technique of extruding spermatophore from males simplified the artificial insemination technique in crustaceans to a certain extent and therefore, this was adopted in different penaeid prawns like *Penaeus vanamei* and *P. setiferus* (Laubier-Bonichon and Ponticelli, 1981), *P. japonicus* (Lumare, 1981) and *P. monodon* (Lin and Ting, 1984, as reviewed by Primavera, 1984) and (Muthu and Laxminarayana 1984).

Tave and Brown (1981) further refined the artificial insemination technique by use of gill irrigator and restraining device to reduce the stress on the female prawn during spermatophore transfer by artificial means. In female *P. setiferus* Bray et al. (1982) and Bray and Lawrence (1984) employed artificial insemination technique to overcome unmated condition. Lin and Ting (1984) endeavoured artificial insemination in *P. monodon* using electroejaculated spermatophore on the ripe females in which gonadal development was induced by eyestalk ablation.
The technique of electroejaculation of spermatophore and artificial insemination have simplified the selective breeding studies (Sandifer and Smith, 1979). Malecha (1977) during his selective breeding studies in M. rosenbergii encountered difficulties owing to non-availability of sufficient number of mature male prawns in captivity. To overcome this problem, Sandifer and Smith (1979) during their studies on M. rosenbergii proposed that artificial insemination could simplify heritability analyses with shrimp, since up to four females could be inseminated simultaneously with sperm from a single male. Sandifer and Lynn (1980) attempted fertilizing as many as four females of M. rosenbergii simultaneously with the sperm mass electroejaculated by single male.

Recently the importance of stock-piling and exchanging the selected male genetic material in Crustacea have prompted scientists to look for the preservation of spermatophores for shorter or longer period and to use this stored spermatophores for intra-specific hybridization (Sandifer and Lynn, 1980). Attempts to test the fertilizibility of refrigerated spermatophores were done by Sandifer and Lynn (1980) and Chow (1982) in M. rosenbergii. Of late, cryopreservation of spermatophore and establishing sperm bank for crustaceans have attracted the attention of many researchers. (Subramoniam, personal communication). The work in this direction has been

A general survey of the literature regarding the artificial insemination in prawns reveals that all the work is of recent origin and that most of the work is concentrated on a single prawn species i.e. M. rosenbergii, while other species have remained unexplored. In India, though it has been attempted on the penaeid prawn, P. monodon by Muthu and Laxminarayana (1984) no attempts were made to explore the possibility of applying this technique to the Macrobrachium prawns.

Thus, in view of lack of information on this crucial aspect and realising the great potential for aquaculture of Macrobrachium in the country, an attempt has been made here to study artificial insemination in the freshwater prawn, Macrobrachium idella. The various aspects in the present work included studies on the electro-ejaculatory capacity of males and the optimal time after the prespawning moult for placement of spermatophore on the receptive females, artificial insemination using freshly extruded as well as refrigerated spermatophore, artificial insemination on the wild mature females and artificial insemination involving insemination of one/two/four females simultaneously with sperm mass from single male. Final
results obtained are in terms of larval yields and it has been compared with the available literature.

MATERIAL AND METHODS

ANIMAL COLLECTION AND MAINTENANCE

Specimens of *Macrobrachium idella* required for the study were collected from Vembanad Lake at Panavally village near Cochin. All the animals were maintained in clean filtered water of salinity 6%, with continuous aeration. The water temperature during the experiments was maintained between 27 to 31°C and the pH between 7.8 to 8.3. The animals were fed *ad libidum* 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 up to 1,000 litre capacity. Details on the design of experiment and maintenance facility are given in a diagrammatic presentation (Fig. 1).

MALE PRAWNS

The males were maintained individually in 10 litre capacity
Fig. 1. Diagrammatic 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.
Fig 1.
FEMALE PRAWNS

In all the experiments, ripe and receptive females of size group 71-75 mm in total length were used for artificial insemination trials. Depending on the reproductive state of experimental females and the state of embryo development, the females were maintained in either of the rearing media described below -

Prior to initiation of experiment, the females were maintained communally in 1,000 litre capacity fibreglass tanks. Every morning and evening the females from fibreglass tank were examined to locate receptive females. A receptive female was distinguished by the fully developed ovary observed through the translucent exoskeleton. She also undergoes a pre-spawning moult. Such receptive females were transferred from the fibreglass tank and transferred to the 35 litre capacity perspex tanks. The females were housed individually in these tanks prior to artificial insemination.

After artificial insemination trial, the ovigerous females bearing fertilized eggs on her abdominal pouch were maintained individually in small 10 litre plastic troughs, till the embryonic
development was complete and the eggs were about to hatch, which was evident by brown colour of the berry.

The ovigerous females with brown embryos later were transferred to 1,000 litre capacity fibreglass tanks, which was used as hatching tank.

**SET UP TO CONDUCT EXPERIMENT ON ARTIFICIAL INSEMINATION**

The gadgets of the experiment comprised of an electroejaculation apparatus and the gill irrigator with restraining device.

**Electroejaculation apparatus:**

The apparatus used for extrusion of spermatophore by electrical stimulus is shown in Plate 1. The electrical stimulator consisted of an electrical transformer to reduce the voltage, a rheostat and two electrodes made from blunt dissecting needles. An electrical stimulus of 4 to 5 volts was found to be sufficient for spermatophore extrusion.

**Gill irrigator:**

To reduce the stress on receptive females during spermatophore transfer, a continuous gill irrigator in conjunction with a restraining device was used. The gill irrigator model of Tave and Brown (1981) was adopted (Plate 6).
Plate 1. Apparatus for electroejaculation of spermatophore.

Plate 2. Ventral surface of the male exposed to show the position of gonopores. (indicated by arrow).
PROCEDURE FOR ARTIFICIAL INSEMINATION

During the present study, artificial insemination of female *M. idella* involved two steps viz. extrusion of spermatophore from the male employing electroejaculation technique, and placement of the extruded spermatophore on the seminal receptacle of receptive female.

Electroejaculation technique:

The male was removed from the rearing container and held securely up side down so that the ventral surface is exposed (Plate 2). The pair of electrical probes were positioned near the base of fifth pereiopods where the gonopore opens (Plate 3). A gradual increase in voltage (up to 4 to 5 volts) was delivered. Upon stimulation, the membranous flap of the gonopore was lifted and a spermatophore was extruded from both the sides (Plate 4 and Fig.2C). The sticky spermatophore was retrieved from the male. Depending on the experiment the spermatophore was either used as a fresh for artificial insemination or preserved in Ringer's Solution, (NaCl = 1.35 g, KCl = 0.06 g, NaHCO$_3$ = 0.02 g, CaCl$_2$ = 0.025 g and MgCl$_2$ = 0.035 g in 100 ml distilled water) for further refrigeration.

Placement of spermatophores:

Before electroejaculating a male, the receptive female was
Fig. 2.  

A. Ventral view of cephalothorax of male showing male gonopore position, covered by flaps.

B. Ventral view of cephalothorax of female showing sperm receptacle area between the third and fifth pereiopods and gonopores at the base of coxae of third pereiopods.

C. Ventral view of cephalothorax of male showing extrusion of spermatophores after electric stimulus.

D. Ventral view of cephalothorax of female showing artificially placed spermatophore on the sperm receptacle area.
Fig. 2
Plate 3. The act of electrical stimulus: The pair of electrical probes positioned at the coxae of fifth pereiopods, near to the gonopore opening.

Plate 4. The spermatophore extruded from a male specimen after electrical stimulus. (shown by arrow).
Plate 5. A female specimen placed ventral side up, showing the sperm receptacle area (shown with pointer).

Plate 6. Gill irrigator with restraining device.
Plate 7. The receptive female, being placed in the cradle of the restraining device and delivery tubes placed in each of the branchial cavity.

Plate 8. Artificially placed spermatophore on the receptive area of female (shown by arrow).
placed in the cradle of the restraining device, ventral side up and securely positioned with the rubber bands (Plate 7). The delivery tubes were placed in each branchial cavity and a continuous flow of water was maintained during the placement of spermatophore. The water flow bathed each set of gills and reduced stress on the female. The electroejaculated spermatophore retrieved from the male was placed securely on the sperm receptacle area of the female, (Plate 8 and Fig.2D). The artificially inseminated female was now freed from the restraining device and held in the plastic trough, still ventral side up in such a way that the branchial cavity gets immersed in water. After holding the female in this position for two minutes, the female was slowly released in the trough.

The complete process of artificial insemination, normally took 3 to 5 minutes. When the gill irrigator, with restraining device was used the female did not experience any stress and no mortality was observed.

ARTIFICIAL INSEMINATION EXPERIMENTS

EXPERIMENT 1: TESTING VIRILITY IN MALES USING ELECTROEJACULATION TECHNIQUE.

In this experiment the virility of males was tested in five different size groups of 71-75, 76-80, 81-85, 86-90 and 91-95 mm
total length. Each size group comprised of five males and the experiment period was 15 days. During experiment each male was electroejaculated once in a day in the morning. After electrical stimulus, whether the male extrudes spermatophores or not was recorded. The average number of times the ejaculation of spermatophores took place in each size group was recorded and from this the most virile male size group was identified.

EXPERIMENT 2: ASSESSING CORRECT TIME FOR ARTIFICIAL INSEMINATION AFTER THE PRE-SPAWNING MOULT.

Among Macrobrachium prawns the female invariably moult before mating and the moult is referred as pre-mating moult or pre-spawning moult. For successful artificial insemination of the newly moulted receptive female it is essential to assess the correct time for implantation of spermatophore. Keeping this in view the experiment was set up. A total number of 14 female prawns were used and 12 artificial insemination trials were attempted during this experiment (Table 2). The time of pre-spawning moult for individual receptive female was recorded and the artificial placement of spermatophore was conducted at different durations after the pre-spawning moult. The duration ranged from 0.5 to 6 hours.
EXPERIMENT 3: ARTIFICIAL INSEMINATION TRIALS USING FRESHLY EXTRUDED SPERMATOPHORES.

Experiment A: In this experiment one complete spermatophore (comprised of two sperm cords) from one male was used for inseminating one female. Fifty two such trials were carried out, in which 52 spermatophores from 52 males were used for artificially inseminating 52 wild receptive females.

Experiment B: In this experiment one complete spermatophore from one male was used to inseminate two receptive wild females simultaneously. Thus here 66 such trials were carried out in which 66 receptive females were inseminated artificially using spermatophores from 33 males.

Experiment C: In this experiment one spermatophore from one male was used to inseminate four receptive females simultaneously. For this purpose the male was electroejaculated selectively only on one of the gonopores, so that one sperm cord was extruded at a time. This sperm cord was cut into two pieces with the help of sharp razor blade, and this half sperm cord was immediately transplanted on the seminal receptacle of receptive female, thus four females were fertilized simultaneously from sperms of a single male. In this experiment, 12 such trials were carried out in which
12 wild females were inseminated artificially using spermatophores from 3 males.

**Experiment D:** In each such trial one spermatophore from one male was used to inseminate one ripe and receptive captive female which is matured in captivity, using unilateral eyestalk ablation technique.

In all these 4 experiments sperm transfers were carried out 3-4 hrs after the pre-spawning moult of female (3-4 hrs time was assessed to be the most ideal time for sperm transfer for greater success).

**EXPERIMENT 4: ARTIFICIAL INSEMINATION TRIALS USING REFRIGERATED SPERMATOPHORES.**

A total number of 32 receptive females were used during this experiment. Spermatophore from a single male was used for inseminating a single female at a time. The spermatophores were retrieved from males adopting electroejaculation technique, and refrigerated following Chow's technique (Chow, 1982) in which the retrieved spermatophores were stored in Ringer's solution prior to refrigeration. The refrigerated spermatophores were used for artificial insemination after 24, 48, 72, 96 and 120 hrs of refrigeration.
at 6°C: The artificial insemination was conducted employing the method already described.

METHODS OF OBSERVATIONS AND INTERPRETATION OF RESULTS

During artificial insemination with fresh/preserved spermatophore fertilization was confirmed by observing cleavage, two to three days after oviposition. The larvae hatched out after successful artificial insemination were counted by aliquot sampling and records of larval yield from individual experimental females were maintained.

The success of experiment 3 and 4 was interpreted in terms of quantity of larval hatching of artificially inseminated females.

RESULTS

OBSERVATIONS ON MATING BEHAVIOUR AND COPULATION OF ANIMAL

Before initiating artificial insemination experiments the mating behaviour and copulation of the animal was studied carefully. When a mature male was introduced in the tank containing a receptive
female that has recently undergone a pre-spawning moult, the courting behaviour was seen immediately. The male started lifting its head, waving its feelers and raising and extending its long and powerful chelate legs in an embracing gesture. This display continued for 10 to 15 minutes before the female is won over. Now the female responded and preferred to be in the cheliped region of the male, and this type of behaviour of male and female is identified as "pair formation". It was observed that this pair formation existed for 2 to 6 hrs, during which the soft female was protected from other males and nonovigerous hard females by the strong male. The mating act was found to last for a few seconds, during which the female was observed to be ventral side up, while the male pressed down from above, bringing its genital pores (Fig. 2A) in close contact with the ventral side of female and with sudden vigorous vibrations of the pleopods and trembling of the body the sperm mass was ejected. It was observed that the ejected spermatophore (i.e. two sperm cords) was deposited in a gelatinous mass between the third to fifth pereiopod region medioventrally on the female's body. The region is identified as sperm receptacle area (Plate 5 and Fig. 2B). Immediately after copulation the spermatophore appeared as two short fused cords of a white sticky material that lay parallel to the long body axis, between the female's last three pairs of pereiopods.
EXPERIMENT 1: TESTING VIRILITY IN MALES USING ELECTROEJACULATION TECHNIQUE.

In this experiment spermatophore extrusion score of male _M. idella_ was observed in different size groups. The results of the experiment have been presented in Table 1. It was noted that the spermatophore extruding capacity of a male is directly related to it's size. The largest males group in the size range of 91 to 95 mm total length, extruded spermatophores 13 times on average in a period of 15 days. One male in this group was found to extrude sperm mass on all 15 days consecutively. On the contrary the smallest size group (total length 71-75 mm) extruded spermatophore only 7 times in 15 days period. 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 males due to electroejaculation.

It was inferred from this experiment that the large male in the size range of 91-95 mm total length were the most suitable males for electroejaculation and retrieval of sperm mass. Therefore, in the subsequent experiments, males of this size group were only used.
### Table - 1. Studies on the assessment of virility in different size groups of *M. idella*.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Size group of males (mm)</th>
<th>Number of replicates</th>
<th>Average number of times spermatophore extruded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71-75</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>76-80</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>81-85</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>86-90</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>91-95</td>
<td>5</td>
<td>13</td>
</tr>
</tbody>
</table>

Experimental period : 15 days  
Electroejaculation : 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.
EXPERIMENT 2: ASSESSING OF CORRECT TIME FOR ARTIFICIAL INSEMINATION AFTER THE PRE-SPAWNING MOULT.

The most appropriate timing of deposition of spermatophore on receptive female, that would result in successful artificial insemination was worked out in this experiment. The results are shown in Table 2. The artificial insemination attempts were carried out, as and when the receptive females are available. The results of the experiment are as described below:

Placement of spermatophore that was conducted 0.5 hrs after the pre-spawning moult resulted in failure in artificial insemination and caused mortality of the experimental female.

Placement of spermatophore 1.5 hrs after the pre-spawning moult resulted in retention of the spermatophore for a short duration, but these were soon dislodged, leading to failure in artificially inseminating the female.

Placement of spermatophore 2 hrs after the pre-spawning moult also turned to be a failure, wherein, the experimental female did not oviposit, and the ripe ovary was resorbed soon.

Placement of spermatophores 2.5, 3, 3.5 and 4 hrs after
Table - 2. Assessing proper time duration for spermatophore placement in Female M. idella.

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

Experimental conditions: Salinity: 6%, Temperature: 28-29°C, pH: 8-8.2

Trial Successful: Attempts made on artificial insemination of a sexually receptive female

Successful: Female spawned fertilized eggs, embryo development was normal and healthy normal larvae hatched out after an incubation period of 11-13 days.
the pre-spawning moult led to successful artificial insemination, which was evident by retention of the spermatophore by the females, fertilization of eggs and hatching of healthy and normal larvae, after completion of incubation period.

Placement of spermatophore 4.5 hrs after pre-spawning moult, led to failure in artificial insemination. The spermatophore was not retained by the receptive female, may be because of the hardening of the female by this time. Moreover, the female died one day after the artificial insemination attempt.

It was, however, planned to carry out placement of spermatophore 5, 5.5 and 6 hrs after pre-spawning moult, but in all the three cases the experimental females were observed to oviposit unfertilized eggs before the stipulated time of artificial insemination.

It was noticed that irrespective of the availability of male, the receptive female oviposited between 5 to 6 hrs after the pre-spawning moult, but such female freed itself of these unfertilized eggs within 2 to 3 days.

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: In this, 52 artificial insemination trials were made. In each trial electroejaculated spermatophore from a male was used to artificially inseminate a wild receptive female. Of the 52 artificial insemination trials, 34 trials turned to be successful 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 1–2 days after oviposition and in 12 trials the spermatophore was dislodged from the seminal receptacle of the female resulting in failure of artificial insemination.
### Table - 3. Artificial insemination trials with fresh spermatophores in M. idella.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Female Source</th>
<th>Details of Experiment</th>
<th>No. of trials</th>
<th>No. of ++Successes</th>
<th>No. of failures A*</th>
<th>B**</th>
<th>Average larval yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A</td>
<td>Wild unablated</td>
<td>Spermatophore from one male used to inseminate one female i.e. both sperm cord used</td>
<td>52</td>
<td>34</td>
<td>6</td>
<td>12</td>
<td>3950</td>
</tr>
<tr>
<td>3B</td>
<td>Wild unablated</td>
<td>Spermatophore from one male used to inseminate two females simultaneously. (Each female receiving single sperm cord)</td>
<td>66</td>
<td>14</td>
<td>8</td>
<td>44</td>
<td>380</td>
</tr>
<tr>
<td>3C</td>
<td>Wild unablated</td>
<td>Spermatophore from one male used to inseminate four females simultaneously. (Each female receiving half sperm cord)</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>2352</td>
</tr>
<tr>
<td>3D</td>
<td>Matured in captivity by unilateral eyestalk ablation</td>
<td>Spermatophore from one male used to inseminate one female</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>4365</td>
</tr>
</tbody>
</table>

Fresh spermatophores: Spermatophores immediately after extrusion from electroejaculated male.

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.
Experiment B: In this, 66 trials of artificial insemination were made 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 found to be successful as evidenced by hatching out of healthy larvae.

The average yield of larvae in experiment 'A' was 3950 and it was considerably high when compared to 3180 numbers of experiment 'B' (Table 3).

Experiment C: Here the attempts were made to artificially inseminate four wild receptive females simultaneously with spermatophore retrieved from a single male, each female thus receiving only a quarter of one normal spermatophore. Here 12 trials were made of which, 2 trials turned to be successful producing normal and healthy larvae. However, the average larval yield was found to be much less at 2352 numbers. Of the remaining 10 unsuccessful trials, fertilized eggs were observed in 2 but the berry got dislodged afterwards. In the remaining 8 trials that totally failed the sperm mass (quarter spermatophore) did not stick to the seminal receptacle of the female and got dislodged.
**Experiment D:** In this experiment females utilized were matured in captivity by adopting unilateral eyestalk ablated technique. Each of these females was inseminated artificially using one complete spermatophore electroejaculated from a male. Of the 10 trials attempted, 6 were found to be successful as indicated by release of healthy larvae averaging 4365 numbers per female. Attempts in the remaining 4 trials were total failure, though in one, fertilized eggs could be seen.

**EXPERIMENT 4: ARTIFICIAL INSEMINATION TRIALS WITH REFRIGERATED SPERMATOPHORE.**

Here the refrigerated spermatophores were used for artificial insemination and the effect of refrigeration on the fertilizability of the spermatophores was studied. The fertilizability was expressed as number of larvae hatched out by artificially inseminated females. The results of the experiment are given in the Table 4.

Among 8 trials of artificial insemination using refrigerated spermatophore, that were stored for 24 hrs, 5 trials were observed to be successful. Of the 3 trials which failed, fertilized eggs were observed after artificial insemination in one. However, the berry dropped off from the female before completion of embryonic development.
### Table - 4. Artificial insemination trials with refrigerated spermatophores in *M. idella*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Duration of spermatophore refrigeration (hrs)</th>
<th>No. of +trials</th>
<th>No. of ++Success A*</th>
<th>No. of ++Success B**</th>
<th>Average larval yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>96</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

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

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 not viable
         B: Eggs were not fertilized by the artificially placed spermatophores
Among 8 trials using refrigerated spermatophores that were stored for 48 hrs, 2 were found to be successful. Fertilized eggs were observed in 2 others and in the remaining 4 it thoroughly failed as the spermatophores 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 spermatophores 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 spermatophores 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 spermatophores refrigerated for 120 hrs, nothing was successful. With increase in the time of refrigeration chances of success in artificial insemination thus declined
successively. Moreover, the number of larval yield in such artificial insemination also exhibited an inverse relationship with the duration of refrigeration. When the time of refrigeration was 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. When the storage time was raised to 72 hrs and 96 hrs the larval yield further declined to 2860 and 2320 respectively.

**DISCUSSION**

The pre-mating behaviour and act of mating observed during the present investigation in *M. idella* were found to exhibit close similarity with that of *M. rosenbergii* (Ling, 1969a; Sandifer and Smith, 1979). It was observed in the present study that immediately after the pre-spawning moult, the male gets attracted to the receptive female and thereafter protects her prior to spawning. Ling (1969a) reported similar behaviour for male *M. rosenbergii* and he further stated that immediately after the pre-spawning moult, the female might be secreting some substance which strongly attracts the male. Beth et al., 1984 while working on mating behaviour of *Palaemon pugio* described that the receptive female may be secreting some pheromone, which strongly attracts the male.
During mating and insemination in the wild no one has control over the mating pair and so over their progeny. On the contrary, adopting artificial insemination technique, selective breeding could be achieved. Considering the increasing importance of artificial insemination, attempts were made in the present study to impregnate M. idella female by artificial means, using spermatophores ejaculated from males applying electrical stimulation. 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. When electrical stimulus of more than 5 volts was applied, terminal ampoules became blackish in colour probably due to tissue lysis. In fact a stimulus of 4.5 volts was without any ill effect, even when the electroejaculation was attempted once in 24 hrs. Working on the lobster T. orientalis Silas and Subramoniam (1987) have reported that repeated electrical stimulation of the male resulted in infection of the stimulated gonopores, leading to failure of any further extrusion of spermatophores. The electrical stimulus needed for electroejaculation was found to vary from species to species. For example 2 volts in Palaemonetes and 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 T. 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 in literature. 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 a 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. No other report depicting the virility of male prawn in relation to its body size is available.

In the present study it was observed that spawning in *M. idella* takes place roughly within 4 to 6 hrs after the pre-spawning moult. Similarly Ling (1969a) has also noticed that spawning occurs roughly within 24 hrs after the pre-spawning moult. It was further observed in the current studies 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) in M. rosenbergii. Sandifer and Smith (1979) in their work on M. rosenbergii have not mentioned about the exact time for placement of spermatophore after pre-spawning moult. In the present study it was observed that artificial insemination attempt was successful only when placement of spermatophore was conducted 2.5 to 4.0 hrs after the pre-spawning moult. It was also noted in the present work that placement of spermatophore immediately after pre-spawning moult led to failure in artificial insemination, because just immediately after the pre-spawning moult the female was too soft to withstand stress of handling during placement of spermatophore, resulting in consequent death of animal. 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. Thus it was inferred in the present work, that knowledge about the exact time of placement of spermatophore on seminal receptacle of the moulted female M. idella is of crucial importance in achieving success in artificial insemination. No earlier reports depicting this type of work are available. However, while working out the natural mating and spawning behaviour in M. rosenbergii, Chow et al. (1982) have observed that the time interval between the pre-spawning moult and oviposition ranges from 16:45 to 29:00 hrs.
In 52 artificial insemination trials on *M. idella*, using complete spermatophores, 34 were successful. Failure in 18 trials was believed to be due to the stress developed during handling. Another reason for failure was small size of receptive females, which were delicate and difficult to handle. Sandifer and Lynn (1980) worked on identical lines and reported success in fertilizing females in 11 out of the total 18 trials. However, the principal difference in the expression of results in the present work and that of Sandifer and Lynn (1980) is that in the present study success in artificial insemination, implied, release of normal healthy larvae by the artificially inseminated females, while in Sandifer and Lynn's (1980) work on *M. rosenbergii*, success implied, successful production of fertilized eggs only. Sandifer and Lynn (1980) further stated that frequent handling of females during artificial insemination act resulted in delayed spawning. This delay in spawning he opined due to loss of manually placed spermatophore, leading to failure in artificial insemination. In the present work also it was felt that frequent handling of experimental female might have led to either resorption of ovary and so non-spawning or late spawning (i.e. 10-12 hrs after the pre-spawning moult).

To avoid failure in artificial insemination due to handling and stress, precautions were taken, firstly by setting up the experiment in such a way that females were least disturbed and secondly by
using the gill irrigator and restraining device model (Tave and Brown, 1981) to reduce the strain. The gill irrigator 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 spermatophores have spawned and finally released healthy larvae. Lumare (1981) performed the artificial insemination in *P. japonicus* without using any device and could achieve only limited success. On the other hand in *P. setiferus* out of the 51 trials of artificial insemination that were conducted during the sourcing cruises without any device, 41 trials were found to be successful (Bray et al., 1982). The reason for such a high success in this experiment may be, that the animals used in the trials were freshly collected from the sea and artificial insemination was conducted on the boat itself.

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 the discovery of electroejaculation technique to extrude spermatophores in the male prawns have simplified the artificial
insemination to a great extent.

In the present study in *M. idella*, the attempts to inseminate, 2 females with spermatophore from a single male, yielded a limited success (Table 3). The larval yield after such insemination was also low, compared to the larval yield from a female inseminated with complete spermatophore. It could be therefore, inferred that a single spermatophore (consisting of two sperm cords) is essential to yield better success in artificial insemination. 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%.

During further experiments on artificial insemination, 4 females of *M. idella* were inseminated simultaneously, using spermatophore from a single male. A limited success could be achieved in these experiment. In the successful trial, the larval yield was very low. Difficulty experienced in this was non-adhering of the pieces of sperm cord on the seminal receptacle of the female. The half sperm cord, lost its stickiness in a short duration, and consequent dislodging of the sperm mass, from the sperm receptacle before spawning resulted. Earlier Sandifer and Smith (1979) tried
to inseminate 4 females simultaneously with the sperm mass from one male and encountered similar problems, but achieved marginal success. It was concluded from such experiment in *M. idella* that it was necessary to refine the technique of cutting the sperm cord into pieces and transferring the same quickly on the sperm receptacle of the female, without losing the sperm mass. Though the attempts to inseminate 2 and 4 females simultaneously with the sperm mass of a single male could yield only marginal success in *M. idella*, this technique has substantial potential in heritability analysis. Sandifer and Smith (1979) have emphasised this point, stating that artificial insemination technique could solve problem in maintenance of males since up to 4 females could be inseminated simultaneously. This is essential in the case of *M. idella* since maintenance of males compared to female was found to be more difficult in captive condition. Sandifer and Lynn (1980) also observed that males of *M. rosenbergii* were aggressive and pugnacious and generally experienced higher mortality rates in laboratory holding tanks.

In the present investigation the captive females which matured after unilateral eyestalk ablation, were also artificially inseminated using electroejaculated spermatophores and 60% of such attempts were successful. No reports of such attempts are available in *Macrobachium*. However, among penaeid prawns reports depicting
artificial insemination of unilaterally ablated females are available. Muthu and Laxminarayana (1984) reported that 3 such females of *P. monodon* were artificially inseminated 10 times and every time they matured and spawned, and yielded healthy larvae. Working on similar line, Lin and Ting (1984) reported successful artificial insemination of unilaterally ablated and matured females of *P. monodon*.

In the present study attempts on artificial insemination using refrigerated spermatophores were undertaken. It may be inferred from these experiments that 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 was found to decrease. The larval yield was also found to decline. It may be inferred from the decreasing number of larvae with increase of time of refrigeration of spermatophore, that as storage time increased, the viability decreased, potentially fertilizing less number of eggs and consequently yielding less number of larvae.

Sandifer and Lynn (1980) working on identical line for *M. rosenbergii* 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 spermatophore retained their viability up to 4 days. In the present investigation on *M. idella*, the spermatophore refrigerated at 6°C retained their viability for 72 hrs. However, the larval yield declined with increasing storage period. Chow (1982) further reported that spermatophore, when preserved for longer time lost the protective and adhesive matrix and were subjected to damage and propagation of bacilli and degenerated fast. In the present study the damage and propagation of bacilli 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 artificial insemination attempts, simple and reliable as applied presently in *M. idella* might prove a good tool in future to biologist, shrimp culturist and genetic engineers to increase the production of quality prawns.

**SUMMARY**

The artificial insemination technique is well established in mammals since long and is being fairly used in improving quality
of the cattle. In fishes also such attempts are being made. In this arena, research is of recent origin for prawns and has begun only in eighties. Though work in this field is scanty it has proved beyond doubt that artificial insemination is potentially useful to increase larval production. Moreover, it promises genetic manipulation. On account of these the present endeavour was made and its salient findings are given below.

To assess the virility of male *M. idella*, they were electro-ejaculated once in 24 hrs. The virility test in different size groups of male revealed that it was related directly to the size of male prawn. Larger males were more virile, extruding spermatophore more often, as compared to the less virile smaller males, which extruded spermatophore less often in a unit period. Thus large males in the size range of 91-95 mm were found to be most virile and so most suitable for use in artificial insemination attempts.

For successful artificial insemination it was essential to locate the suitable time for placement of spermatophore on the seminal receptacle of the female. Experiment in this direction revealed that placement of spermatophore, when carried out 0.5-2.0 hrs and 4.5-6.0 hrs after pre-spawning moult led to failure since the receptive female was too soft and too hard respectively. The
placement of spermatophore on a soft female sometimes resulted in mortality of female and placement of same on a hard female resulted in dislodging of spermatophore from seminal receptacle of female, leading to failure in the experiment. The period 2.5-4.0 hrs after pre-mating moult was judged to be the most suitable time for placement of spermatophore, since this ensured fair chances of success.

The freshly electroejaculated spermatophore from a single male was used to artificially inseminate 1/2/4 females simultaneously. In these the chances of success were noted to be highest when a complete spermatophore was used to inseminate one female. On the other hand, chances of success decreased when a spermatophore was used to inseminate 2 or 4 females simultaneously. Further in such attempts, the larval yield was recorded to be highest when a complete spermatophore was used, while it decreased successively when a single spermatophore used to inseminate 2 or 4 females simultaneously. However, multiple insemination will ease the burden of maintaining more males in the culture systems.

The females were matured in captivity adopting unilateral eyestalk ablation technique and such females were inseminated artificially using a complete spermatophore. A fair degree of success could be achieved in these trials. The larval yield in these trials
was noted to be higher compared to that of artificially inseminated unablated females.

The artificial insemination trials using refrigerated spermatophore were also attempted. The spermatophore refrigerated 24 to 120 hrs used to inseminate the females indicated that as freezing time of sperm increased chances of success declined successively. It appears that fertilizability of refrigerated spermatophore declines with freezing time. This was evident, since the larval yield in such attempts decreased with increase in refrigeration time of the spermatophore.

These different attempts form a guideline for future workers in this field.