Aquat. Living Resour., 1993, **6**, 139-144

The effect of electrical stimulation on spermatophore regeneration in white shrimp *Penaeus setiferus*

Carlos Rosas [1], Adolfo Sanchez [1], M. A. Eugenia Chimal [1], G. Saldaña [1], Laida Ramos [2] and Luis A. Soto [3]

[1] *Laboratorio de Ecofisiología, Depto. Biología, Fac. de Ciencias, UNAM, México 04510, D.F., México.*
[2] *Centro de Investigaciones Marinas, Universidad de la Habana, Cuba.*
[3] *Laboratorio de Ecología del Bentos, Instituto de Ciencias del Mar y Limnología, UNAM, México 04510, D.F., México.*

Received November 12, 1992; accepted February 22, 1993.

Rosas C., A. Sanchez, M. A. E. Chimal, G. Saldaña, L. Ramos, L. A. Soto, *Aquat. Living Resour.*, 1993, **6**, 139-144.

Abstract

The effect of electrical stimulation on the quality of *P. setiferus* sperm cells was studied during the regeneration of the spermatophore. The proportion of the animals that responded to electroejaculation was quantified and the number of live, abnormal and dead cells, as well as the relationship between the number of cells and body weight, were taken into consideration as indicators of the effect of manipulation during stimulation. It was found that electrical stimulation caused some degree of spermatophore expulsion in 78% of the cases. Total expulsion of the spermatophores occurred in only 8.3% of the cases. A logistic curve was adjusted to the relationship between the number of sperm cells and the weight of the shrimp. After regeneration no curve was adjusted to the relationship. Eighty per cent of the stimulated shrimp regenerated the spermatophore in a maximum of 168 h. Fifty per cent regeneration was obtained in 96 h after stimulation. A decrease in the number of live cells from 26.8 to 7.6 million and an increase in abnormal (from 0.02 to 3.92 million) and dead (from no cells dead to 4.6 million) respectively, were registered for regenerated animals. Necrotic areas in 60% of the ampulae of regenerated shrimps were also observed. Bacteria associated with the necrotic ampulae was observed when counting sperm. As the spermatophore of this species is complex, it is necessary to search for a way to reduce the stress produced by the handling of the ampulae so as to avoid altering the reproductive capacity of the shrimp.

Keywords: *Penaeus setiferus*, spermatophore, regeneration, ampulla terminal, electrical stimulation.

Efecto de la estimulación electrica en la regeneración del espermatóforo del camaron blanco Penaeus setiferus.

Resumen

Se investigó el efecto de la estimulación electrica en la calidad de las células espermáticas de *P. setiferus* durante la regeneración del espermatóforo. Se cuantificó la proporción de animales que respondieron a la electroeyaculación y se consideró el número de células vivas, anormales, muertas y la relación entre el número de células y el peso corporal, como indicadores del efecto de la manipulación durante la estimulación. Se encontró que la estimulación eléctrica causó algun grado de expulsión del espermatóforo en el 78% de los casos aunque solo el 8.3% respondió con la expulsión total de los espermatóforos. Una curva logística se ajustó a la relación entre el número de células espermáticas y el peso de los camarones. Después de la regeneración no se pudo ajustar ningún tipo de curva a esa relación. Se encontró que el 80% de los camarones estimulados regeneraron el espermatóforo en un

tiempo máximo de 168 h. El tiempo medio de regeneración se obtuvo a las 96 h de la estimulación. Los animales regenerados presentaron una disminución en el número de células vivas de 26.8 a 7.6 millones de células/espermatóforo/animal. También se observó un aumento en las células anormales de 0.02 a 3.92 millones de células/espermatóforo/animal y en las células muertas de cero a 4.6 millones de células/espermatóforo/animal en camarones regenerados. Asi mismo se observaron áreas necrosadas en el 60% de las ámpulas de los camarones regenerados. Una gran cantidad de bacterias asociadas a las ámpulas necrosadas fué observada en los conteos de los espermatozoides de los animales regenerados. Debido a la complejidad del espermatóforo de esta especie es necesario buscar la manera de reducir el estrés producido por la manipulación del ámpula para no alterar la capacidad reproductiva de los camarones.

Palabras claves : *Penaeus setiferus*, espermatóforo, regeneración, estimulación electrica.

INTRODUCTION

Although shrimp species can be bred in captivity, there are many problems associated with maturation and insemination that have not been solved. For this reason many producers of postlarvae depend on the capture of fertilized females from natural environments. The loss of the spermatophore as a result of the stress caused by capture and transportation is a frequent problem, especially in species with an open thelycum such as *Penaeus setiferus*, *P. stylirostris*, *P. schmittii* and *P. vannamei*.

In order to solve this, some researchers have proposed a method of artificial fertilization of mature females (AQUACOP, 1983). Sandifer *et al.* (1984), suggested that the use of electrical stimulation for obtaining spermatophores is an adequate method for penaeid shrimp. This technique has been successfully used with *Macrobrachium rosenbergii*, *Palaemonetes pugio*, *Penaeus vulgaris* and *Sicyionia ingentis* (Harris and Sandifer, 1986; Sandifer and Lynn, 1980), and with *Homarus americanus* (Kooda-Cisco and Talbot, 1983). Notwithstanding that the technique is adequate, there has been only one report where the time for the regeneration of the spermatophore in shrimps has been evaluated (Leung-Trujillo and Lawrence, 1991) and no reports of the quality of the sperm of penaeid shrimp after regeneration of spermatophore.

The white shrimp *P. setiferus* is a promising species for shrimp culture in the American Atlantic. For this reason, the purpose of this study was to establish the regeneration time of the spermatophore after electrical stimulation of terminal ampulae and the effects of this technique on the quality and quantity of sperm cells, as well as the general state of the terminal ampulae of *P. setiferus*.

MATERIAL AND METHODS

Adult *Penaeus setiferus* males were collected in the coastal area of the North West of the Gulf of Mexico, specifically to the north of the state of Veracruz, Mexico. Sixty adult males were selected with wet weights of 16 to 47 g and placed in a 2 500 l fibreglass tank for 48 h for acclimation. During this time sea water was partially changed (50%) and kept at $28 \pm 2°C$, $34‰$, pH 8.2 ± 0.5, $N\text{-}NH_3 < 0.02$ mg/l and a concentration of oxygen greater than 5 mg/l. Sea water was filtered through a 20 µm cartdrige filter. The shrimp were fed a mixture of fresh shrimp, squid and oyster twice a day, up to 10% of their body weight during the acclimation and experimental periods. A 100% survival was registered during this time.

Once the acclimation period was over the shrimp were electrically stimulated. None of the animals showed melanization of the terminal ampulae (Chamberlain *et al.*, 1983). A system similar to that described by Sandifer *et al.*, 1984) was used for electrical stimulation. It consisted of a low voltage source connected to two electrodes. These were placed on the base of the fifth pair of pereiopods and a single stimulus of 4.5 volts was provided for 1 second. The spermatophores were removed with forceps after muscular contraction. The animals were then marked on the cephalothorax and the spermatophores were kept in a calcium-free saline solution for one hour at most (Leung-Trujillo and Lawrence, 1987). Shrimp were placed in groups of ten with sea water of the same quality as that used during the acclimation period.

The quality of the sperm cells obtained before and after regeneration was evaluated through sperm cell counts as well as the viability of the cells. The latter was obtained through the quantification of the number of sperm cells stained with Trypan blue.

The number of normal and abnormal cells was also recorded. Abnormal cells were recognized through malformations of the head or the absence of the spike (Leung-Trujillo and Lawrence, 1987).

Shrimp were checked on the outside daily to verify regeneration. Stimulation of shrimp in which a new spermatophore could be seen was repeated. These shrimp were again placed in the tank of a second

regeneration. Since all these animals died, the results presented here refer only to the first stimulation-regeneration.

The regenerated spermatophore was analysed and compared to those obtained through the first stimulation. Macroscopic observations of the terminal ampulae were also carried out to detect changes that could indicate damage to the organ. Some macroscopic characteristics of the male reproductive tract degenerative syndrome (MRTDS) were taken into account (Chamberlain *et al.*, 1983).

A relationship was obtained between the number of normal cells and shrimp weight before and after regeneration. The logistic model (Brower and Zar, 1977):

$$C = \frac{K}{1 + e^{\alpha - m(w)}}$$

was used, where C is the number of cells ($\times 10^6$/spermatophore/animal) K is the maximum average of sperm cells when the curve becomes asymptotic, m is the slope, e is the base of the natural logarithms, w is the live shrimp weight and α is a constant.

The average number of sperm cells of shrimp heavier than 36 g wet weight was used to calculate K. The value for m was obtained from the linear relationship between log $[(K - N w)/N w]$ and weight (w). A least squared analysis was carried out to prove the predictability of the model (Zar, 1974). Student t tests were applied to compare the number of cells before and after stimulation. An arc-sine transformation was used before the analysis of the percentage values (Zar, 1974).

RESULTS

All 60 shrimps used in this study had mature spermatophores. This could be verified as each spermatophore consisted of the dorsal plate with the spermatic mass, the wing, and the adhesive mass. No organisms were observed with a melanized spermatophore.

Five of the 60 stimulated males expelled the spermatophores totally (8.3%). The rest (75%) partially expelled the content of both terminal ampulae or of only one (3.3%). The remaining males (13.3%) did not respond to the stimulus. A second response and the content of the ampulae was extracted by manual pressure. As these males died before 72 h, they were not included in the regeneration results.

The relationship between the number of normal sperm cells and body weight was fitted to a logistic curve (*fig.* 1 *a*). A maximum value of 45.37 million cells/spermatophore (K) and m of 0.24 were obtained from the data for shrimp heavier than 36 g. A correlation coefficient (r^2) of 0.89 and a $p < 0.0001$ were obtained for this model. No mortality of shrimp was observed after the first electrical stimulation and during the 7 days of observation.

Regeneration was observed 72 h after stimulation in 20% of the shrimp (*table* 1). Eighty per cent regeneration was observed after 168 h and 50% regeneration was observed 96 h after stimulation. The last 20% were the shrimp that regenerated the content of one terminal ampulae or did not regenerate.

A decrease in the number of normal cells associated with an increase in abnormal and dead cells was observed in all regenerated animals with respect to initial values (*table* 1). Normal cells decreased by 71% with a range of 64 to 77% which mean a decrease

Table I. – Values of spermatic cell counting for *P. setiferus* before and after electrical stimulation. R: percentage of regenerated animals, A: cells $\times 10^6$/spermatophore/animal, B: mean ± standard error (%).

Time (hours)	R (%)	Spermatic cell viability profile						
		Normal		Abnormal		Dead		Total
		A	B	A	B	A	B	A
0		26.8*	99.9*	0.02*	0.1*	–		26.82*
± s.e.		9.3		–				5.60
72	20	6.0	37.5	5.3	33.1	4.7	29.4	16.0
± s.e.		1.7		2.7		0.8		2.6
96	50	6.8	40.0	4.9	28.8	5.3	31.2	17.0
± s.e.		2.6		1.4		0.8		1.9
120	57	9.6	62.7	1.8	11.8	3.9	25.5	15.3
± s.e.		1.6		0.8		0.7		1.4
144	71	7.9	52.3	3.0	19.9	4.2	27.8	15.1
± s.e.		2.8		0.4		0.5		2.3
168	80	7.3	43.7	4.6	27.5	4.8	28.7	16.7
± s.e.		0.9		0.7		0.5		2.6
Regenerated cells, mean		7.5	48.1	3.9	23.4	4.6	28.5	16.0
± s.e.		1.4		1.4		0.5		0.8

* Statistically different from regenerated animals, $p < 0.05$.

from 26.8 to 7.6 million cells/spermatophore in regenerated animals (*table* 1). The amount of abnormal cells increased from 0.02 to 3.8 million cells/spermatophore after electrical stimulation, and the amount of dead cells increased from 0 to 4.6 million in regenerated animals (*table* 1). It was also observed that the amount of normal cells in regenerated animals was apparently not related to body weight (*fig.* 1).

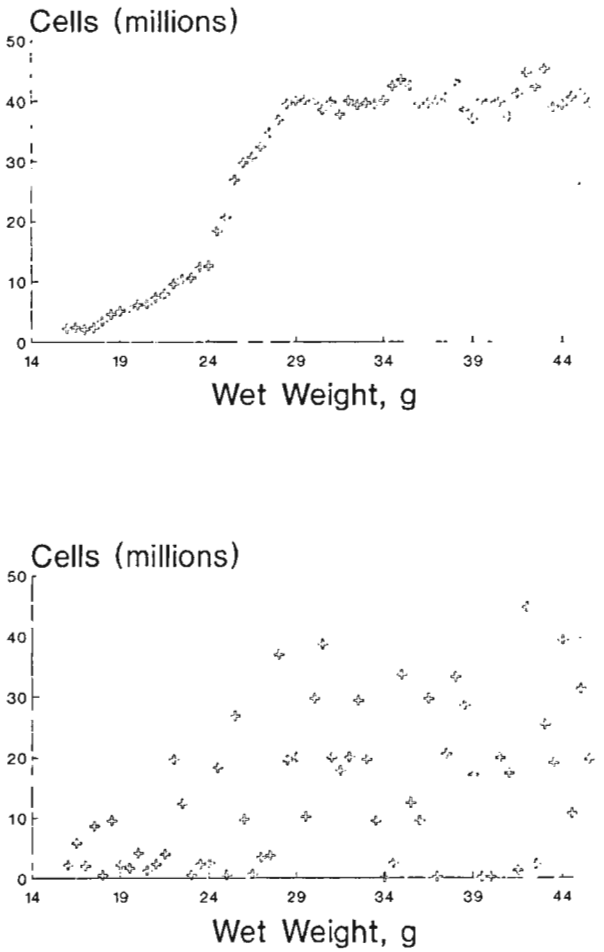

Figure 1. – Spermatic cells and wet weight relationship of adult males of *P. setiferus*. *a*) From natural populations. *b*) From regenerated animals.

Among the macroscopic characteristics of the ampulae of regenerated shrimp were necrotic areas in 60% of the terminal ampulae that were analysed. Necrosis was more common in the gonopore opening than in the internal regions of the ampulae. A great amount of bacteria associated with necrotic ampulae was observed in the spermatic mass, although it was not quantified. When the ampulae of the animals that regenerated partially were dissected, a change in colour and turgescence was observed. These were less white and flaccid compared to those of the animals before regeneration. The ampulae that did not regenerate contained wing fragments and bits of the adhesive matrix mixed with the spermatic mass which was identified by the presence of a few spermatic cells. Necrotic areas were observed inside these ampulae associated with a great amount of bacteria. Ampulae of non-regenerated shrimp were similar to those of partially regenerated animals, although in these the bits of spermatic cells and spermatophore structure were observed in both ampulae. Necrosis of the ampulae was also more evident in these animals.

DISCUSSION

The *P. setiferus* males used for this study were in good condition and had an average sperm count similar to that reported by other authors for this and other species. Leung-Trujillo and Lawrence (1985, 1987) reported 31.9 million cells/spermatophore for *P. vannamei* adult males and between 39.7 and 54.4 million cells/spermatophore for *P. setiferus* adult males. The relationship between body weight for the number of spermatic cells and body weight for *P. setiferus* is documented here for the first time. As can be seen, the sigmoidal behaviour of the relationship shows that the number of sperm cells reaches a maximum of 45 million cells/spermatophore for animals between 16 and 48 g. Thus, animals heavier than 36 g have the greatest reproductive potential for the production of sperm cells fit for the fertilization of females of this species. These values were significantly greater than those previously estimated for the species by Leung-Trujillo and Lawrence (1991) of 28.7 ± 4.76 million cells/spermatophore.

In this study nearly 80% of the individuals electrostimulated displayed spermatophore regeneration in 7 days; early regeneration was observed in 20% of the population during the third day. Leung-Trujillo and Lawrence (1991) recorded in *P. setiferus* a period of full regeneration from 5 to 7 days, although the process also starts 3 days after electrical stimulation. Nevertheless, there are morphological indications that sperm cells are being produced during stage III of maturation (2 to 3th day of stimulation), the fragile nature of the spermatophore (Ro *et al.*, 1990; Chow *et al.*, 1991) prevents its liberation by induced muscular contraction. Therefore, the above 20% regeneration obtained in this study corresponds to mature individuals in phase IV that take from 3 to 7 days to become fertile again. The slight discrepancy between the maturation intervals here recorded and those given by Leung-Trujillo and Lawrence (1991) for *P. setiferus* can be attributed to either population differences (Physiological race), or to the experimental procedure employed.

Electrical stimulation was observed here to favour the liberation of the contents of the terminal ampulae without the total liberation of the spermatophore.

This implies that it is necessary to manipulate the ampula and use forceps to extract manually the spermatophore. A light pressure has to be applied at the base of the ampulae to ease the extraction of the contents. This combined procedure warrants nearly 75% of spermatophore release in *P. setiferus* as indicated also by other authors (Sandifer *et al.*, 1984; Leung-Trujillo and Lawrence, 1991).

The terminal ampulae of *P. setiferus* is very big compared to the size of the opening of the gonopore, and the spermatophore is big and complex (King, 1948; Perez-Farfante, 1978, Chow *et al.*, 1991). These anatomical characteristics make it difficult for the shrimp to completely expel the spermatophore with a muscular contraction provoked by electrostimulation. The use of a stronger electrical shock or of a greater number of stimuli as alternative to obtain the spermatophore has killed the shrimp in this and other studies (Sandifer *et al.*, 1984). There was no mortality among the stimulated animals that responded in one way or another. This results coincide with those of Chow (1982) for *Macrobrachium rosenbergii* and Sandifer *et al.* (1984) for *P. setiferus.*

However, there was a significant decrease in the quantity and quality of spermatic cells in the regenerated animals which reduced the reproductive potential of the animals to 71% (*table* 1). These results support those of Leung-Trujillo and Lawrence (1991) in *P. setiferus* where more new spermatophores were produced in males mating naturally than those males whose spermatophores were removed electrically. The presence of bacteria in the spermatic mass of the spermatophores in 60% of the cases could be the cause of the reduction in the number of viable cells (Bray *et al.*, 1985). The entry of bacteria through the gonopore could be related to an opportunistic conduct associated with the general stress that is produced during electrical stimulation (Talbot *et al.*, 1989). The manipulation of the ampulae for the extraction of the spermatophore could be the first cause of the infection. The application of the stimulus and the brushing of the electrodes on the ampulae at the time of muscular contraction, as well as the local injury caused by the forceps to the opening of the gonopore during extraction, are the procedures that are directly involved in the entry of the bacteria into the ampulae. A progressive darkening from the gonopore towards the interior of the ampulae could be interpreted as proof of bacterial growth in the ampulae.

Although the direct causes of death of the spermatic cells have not been established, the development of bacteria in the extracellular matrix of the spermatic mass could cause the reduction of viable cells, and the increase in the number of abnormal and dead cells in the spermatophores of the regenerated shrimp. The results obtained from the relationship between the number of spermatic cells and the weight of the regenerated shrimp proved that the effect of electrical stimulation is not related to the weight or to the state of health of the animals (Bolger and Connolly, 1989), but to the stress to which each animal is subjected during the regeneration time. The death of all shrimp after the second stimulation highlighted the stress on these animals when subjected to this sort of stimulation.

Although electrical stimulation may be a viable alternative to obtain spermatophores in shrimp species like *P. vannamei* (Sandifer *et al.*, 1984; Leung-Trujillo and Lawrence, 1985), it is necessary to look for a better method of insemination without manipulation for shrimps like *P. setiferus* that do not regenerate easily after electroejaculation. Regeneration may produce negative characteristics (such as MRTDS) during reproduction in captivity. Undoubtedly a significant research effort must be applied in studying shrimp reproductive behaviour, particularly coupling mechanisms. This should secure a healthy reproductive stock that, as in the case of *P. setiferus*, offer a great potential for aquaculture development in Mexico.

Acknowledgements

We appreciate the support given by the Comisión Federal de Electricidad through the Central Termoelectrica "Adolfo López Mateos". This research is part of the FC-109 project that is financied by the Programa de Atención a Estudiantes de Posgrado from UNAM, by the División de Investigacón y Posgrado from Departamento de Biología of Facultad de Ciencias, UNAM and by Dirección de Atención al Personal Académico from UNAM with project IN-201292 To Dr. Luis A. Soto and Dr. Carlos Rosas.

REFERENCES

AQUACOP, 1983. Panaeid larval rearing in the Centre Oceanologique de Pacifique. *In*: Crustacean Aquaculture, CRC Handbook of Mariculture, Vol. I, J. P. McVey ed., CRC Press, Boca Raton, Florida, 123-128.

Bolger T., P. L. Connolly, 1989. The selection of suitable indices for the measurement and analysis of fish condition. *J. Fish. Biol.*, 171-182.

Bray W. A., J. R. Leung-Trujillo, A. L. Lawrence, S. M. Roberts, 1985. Preliminary investigation of the effects of temperature, bacterial inoculation, and EDTA on sperm

quality in captive *Penaeus setiferus*. *J. World Maricult. Soc.*, **15**, 250-257.

Brower J. E., J. H. Zar, 1977. Field and laboratory methods for general ecology. WM. C. Brown Co. Pub., 194 p.

Chamberlain G., G. X. Johnson, D. L. Lewis, 1983. Swelling and melanization of the male reproductive system of captive adult penaeid shrimp. *J. World Maricult. Soc.*, **14**, 135-136.

Chow S., 1982. Artifitial insemination using preserved spermatophores in the palemonid shrimp *Macrobrachium rosenbergii*. *Bull. Jpn. Soc. Sci. Fish.*, **48**, 1693-1695.

Chow S., M. M. Dougherty, W. J. Dougherty, P. A. Sandifer, 1991. Spermatophore formation in the white shrimp *Penaeus setiferus* and *P. vannamei*. *J. Crust. Biol.*, **11**, 201-216.

Harris E. G. S., P. Sandifer, 1986. Sperm production and the effects of the electrically induced spermatophore expulsion in the prawn *Macrobrachium rosenbergii*. *J. Crust. Biol.*, **6**, 633-647.

King J. E., 1948. A study of reproductive organs of the common marine shrimp, *Penaeus setiferus* (Linnaeus). *Biol. Bull. Woods Hole (Mass)*, **94**, 244-262.

Kooda-Cisco M. J., P. Talbot, 1983. A technique for electrically stimulating extrusion of spermatophores from lobster, *Homarus americanus*. *Aquaculture*, **33**, 221-227.

Leung-Trujillo J. R., A. L. Lawrence, 1985. The effect of eyestalk ablation on spermatophore and sperm quality in *Penaeus vannamei*. *J. World Maricult. Soc.*, **16**, 258-266.

Leung-Trujillo J. R., A. L. Lawrence, 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. *Aquaculture*, **65**, 363-370.

Leung-Trujillo J. R., A. L. Lawrence, 1991. Spermatophore generation times in *Penaeus setiferus*, *P. vannamei* and *P. stylirostris*. *J. World. Acuacult. Soc.*, **22**, 244-251.

Pérez-Farfante I., 1975. Spermatophores and thelica of the American white shrimp *Penaeus*, subgenus *Litopenaeus*. *Fish. Bull.*, **73**, 463-486.

Ro S., P. Talbot, J. Trujillo, A. Lawrence, 1990. Structure and function of the vas deferense in the shrimp *Penaeus setiferus*: segments 1-3. *J. Crust. Biol.*, **10**, 455-468.

Sandifer P. A., A. L. Lawrence, S. G. Harris, G. H. Chamberlain, A. D. Stokes, W. A. Bray, 1984. Electrical stimulation of spermatophore expulsion in marine shrimp, *Penaeus* spp. *Aquaculture*, **41**, 181-187.

Sandifer P. A., J. W. Lynn, 1980. Artifitial insemination of caridean shrimp. *In*: Advances in invertebrates reproduction, W. H. Clark Jr., T. S. Adams eds., Elsevier, North-Holland, Amsterdam, 271-288.

Talbot P., D. Howard, J. Leung-Trujillo, T. W. Lee, W.-Y. Li, A. L. Lawrence, 1989. Characterization of male reproductive tract degenerative syndrome in captive penaeid shrimp (*Penaeus setiferus*). *Aquaculture*, **78**, 365-377.

Zar J. H., 1974. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, N.J.