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Induction of sperm activation in open and closed thelycum penaeoid shrimps

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Abstract

A modified egg water (EW) technique for in vitro induction of sperm activation was applied to *Trachypenaeus byrdi*, *Xiphopenaeus riveti* (closed thelycum shrimps), and *Litopenaeus occidentalis* (open thelycum) from a tropical estuary, Golfo de Nicoya, Costa Rica. The study was designed to investigate the changes that occur in the sperm following contact with egg water, and to determine the potential of the technique for the assessment of differences in quality between sperm from spermatophores and sperm taken from the seminal receptacles. The modified technique induced activation of sperm removed from females' seminal receptacles, and demonstrated that sperm from males of *T. byrdi* and *X. riveti* do not react against conspecific EW, indicating that further maturation is required in seminal receptacles. Sperm from wild males of *L. occidentalis* reacted against conspecific EW, but at a low rate, suggesting that further maturation may be required in the external surface of the thelycum. Activation rates were low or variable between individuals in each species despite the expected high sperm quality from wild shrimp, indicating that the technique is not yet an useful sperm quality assay for the captive reproduction industry. The interspecific interaction between *T. byrdi* sperm (seminal receptacles) and EW from *X. riveti* and *L. occidentalis* generated no acrosome reaction, which may be an indication that molecular recognition is missing.

Keywords: Sperm; Acrosome reaction; Shrimp; Dendrobranchiata; Penaeoid

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1. Introduction

The present understanding of gamete interaction amongst the Dendrobranchiata is restricted to selected species of penaeoid shrimps (Clark et al., 1984; Shigekawa and Clark, 1986; Pillai et al., 1988). Sperm of penaeoid shrimp are unistellate, have a non-motile "spike", and undergo acrosome reaction during sperm–egg interaction (Clark et al., 1981; Griffin et al., 1988).

In *Sicyonia ingentis*, sperm mature in the female's thelycum, where further ultrastructural development within the cells takes place (Clark et al., 1984; Shigekawa and Clark, 1986). The acrosome reaction in *S. ingentis* is a bi-phasic event. The first phase is the depolymerization of the spike followed by exocytosis of an acrosomal vesicle exposing two glycoprotein acrosomal components. The second phase is the formation of an acrosomal filament (Clark et al., 1981, 1984). In *Farfantepenaeus aztecus*, a closed thelycum species, and in *Litopenaeus setiferus*, an open thelycum shrimp, an acrosome reaction is induced in sperm when incubated with conspecific egg water. However, the characteristic acrosomal filament of *S. ingentis* is not observed (Clark et al., 1980; Clark and Griffin, personal communication). In *Litopenaeus vannamei* the acrosome reaction was described as a loss of the spike followed by an eversion of the cell contents, and a forward movement of several microns (Wang et al., 1995).

It has been stated that induction of the acrosome reaction is the only definitive criterion for determining sperm viability in penaeoid shrimp (Griffin and Clark, 1987). The egg water technique for in vitro induction of the acrosome reaction in *S. ingentis* was first described by Griffin et al. (1987), generating 75% reactive sperm by 5 min exposure. The technique uses the jelly precursor released by eggs during spawning in artificial seawater, contained in beakers. The penaeoid jelly precursor contains 75% protein and 25–30% carbohydrate; proteases, antibacterial agents, and acrosome reaction inducers are suggested as constituents (Lynn and Clark, 1987).

Recently, the technique was used for sperm analysis in *Penaeus monodon* (Pratoomchat et al., 1993) and *L. vannamei* (Wang et al., 1995). The first study reported 4.2% reactive sperm for wild-caught males and the second one reported 37.4% reactive sperm for pond-grown males.

To date, few observations on sperm activation in penaeoid shrimp have been published. Knowledge on the induction of the acrosome reaction in penaeoid shrimps is required for implementing a practical and reliable sperm quality assay as well as improving the knowledge on sperm maturation and possible hybridization barriers. In this study, a modified egg water technique for inducing sperm activation was evaluated, using batches of egg water of different protein concentration and sperm of two closed thelycum shrimps (*Trachypenaeus byrdi* and *Xiphopenaeus riveti*) and one open thelycum species (*Litopenaeus occidentalis*) from a tropical estuary, Golfo de Nicoya, Costa Rica. The aim was to investigate the changes that occur in the sperm following contact with egg water, and to determine the potential of the technique for the assessment of differences in quality between sperm from spermatophores and sperm taken from seminal receptacles.

2. Material and methods

2.1. Animals

Adult males and females (in different stages of ovarian maturation) of *T. byrdi* and *X. riveti*, were captured by trawling off Golfo de Nicoya, on the Pacific coast of Costa Rica. *L. occidentalis* were collected with gill nets from the same estuary. Animals were transported in containers with constant aeration to the Estación de Biología Marina, Puntarenas.

2.2. Modified egg water (EW) collection

The protocol followed for EW isolation is based on the original technique developed by Griffin et al. (1987), with the following modifications: Female spawning in glass beakers with chilled seawater was avoided because some species do not spawn completely under these stressful conditions. Centrifugation of EW as a purification step was eliminated.

Females with turgid, broad and green ovaries (*T. byrdi* and *X. riveti*) or dark red ovaries (*L. occidentalis*) were isolated in dark containers with 1.0 l (*T. byrdi* and *X. riveti*) or 10 l (*L. occidentalis*) of aged and 1-μm filtered natural seawater (NSW) at 31–35 ppt and 27–28 °C. Females were checked every hour; after detecting spawning activity, females were removed and the egg suspension was left to settle for 5 min, then the volume was reduced to 100 ml. The eggs were resuspended and left to settle for another 5 min. The supernatant seawater (=EW) was then removed by pipette and 1-ml aliquots were stored in liquid nitrogen if not used immediately. The frozen EW was thawed at ambient temperature before use. The protein concentration in each batch of EW (derived from a single spawn) was determined by the Bradford (1976) technique. In brief, a comassie-blue stain for total protein was applied to each EW batch, using serum albumin as spectrophotometric standard.

2.3. Sperm activation by EW

Spermatophores from *X. riveti* and *L. occidentalis* males were removed by artificial ejaculation following a modified procedure of that described by King (1948) in which the spermatophore was partially ejected with gentle pressure and then completely expelled employing disinfected fine tweezers. For *T. byrdi*, terminal ampoules were dissected to remove spermatophores. For the closed thelycum species *T. byrdi* and *X. riveti*, the thelyca of females were dissected to remove the sperm mass from seminal receptacles.

Spermatophores or sperm masses were homogenized in a glass tissue grinder with 3.0 ml of NSW, then one drop of sperm suspension was mixed with five drops of EW in a test tube. Another drop of sperm suspension in NSW was monitored as a negative control. Microscopical observations were performed every 30 min over a microscope slide, cataloging the number of reacted and nonreacted cells for at least 100 sperm (three replications per trial). The percentages of reacted sperm were calculated as percentage of total number of observed cells.

2.4. Scanning electron microscopy

Spermatophores and suspensions of reacted sperm were fixed in a solution reported by Ro et al. (1990) for marine shrimp reproductive systems, which consists of paraformaldehyde (2.0%), glutaraldehyde (2.5%), and sucrose (5%) in 0.1 M sodium cacodylate buffer at pH 7.4. In the laboratory, samples were rinsed three times in the same buffer during 10 min each; centrifugation at 2000 rpm was applied to sperm suspensions at each rinse. Post-fixation was done in 1% osmium tetroxide for 1 h. Samples were rinsed four times with buffer, and dehydrated in a graded ethanol series, and critical point dried. Samples were then coated with 30 nm of platinum and examined on a Hitachi S-2360 N scanning electron microscope.

2.5. Experimental design and statistics

Sperm activation over time was evaluated by incubating sperm from spermatophores (*L. occidentalis*) or sperm from seminal receptacles (*T. byrdi* and *X. riveti*) with a batch of conspecific EW during 120 min. Samples were taken every 30 min. For each species, this experiment was repeated three times, each time with sperm from a different male or female, but with the same EW batch.

Based on the time course obtained in the first series of experiments, a second series of sperm exposures was executed. Sperm from three *L. occidentalis* males were incubated with conspecific EW for 90 min. This experiment was repeated three times, each time with different males and different EW batches. For *T. byrdi* and *X. riveti*, sperm from spermatophores were compared with sperm from seminal receptacles of females. Sperm from three males and sperm taken from three females were incubated with conspecific EW for 90 min. This experiment was repeated three times, each time with a different EW batch. No design for statistical comparison between protein concentrations was included in the study.

Percentages of sperm activation at 90 min from EW exposure were transformed using arcsine of squared root (Ott, 1984; Bray et al., 1990a), to make the variance independent of the mean; one-way classification analysis of variance (ANOVA) and Tukey's W procedure (Ott, 1984) were applied to each EW batch comparing activation rates (%) between sperm from seminal receptacles and spermatophores exposed to EW and NSW; alpha levels for all tests were set at 0.05. Untransformed data are presented as mean ± standard deviation.

3. Results

Fig. 1 presents the morphology of sperm before and after activation for *T. byrdi*, *X. riveti*, and *L. occidentalis*. Sperm in *T. byrdi* are packed in many 100–160 μm-spherical capsules (spermatophores), the spike is deflected 80° from the body axis, the sperm body length is 6 μm, and the spike length is 3.5 μm. *X. riveti* sperm are also packed in spherical capsules (80 μm), the body length is around 11–12 μm, and the spike length is 4 μm. *L. occidentalis* sperm are spherical with a body diameter of 4 μm and a spike of 3 μm in length; spermatophores are 0.30 g in weight. The activation is characterized by a loss of

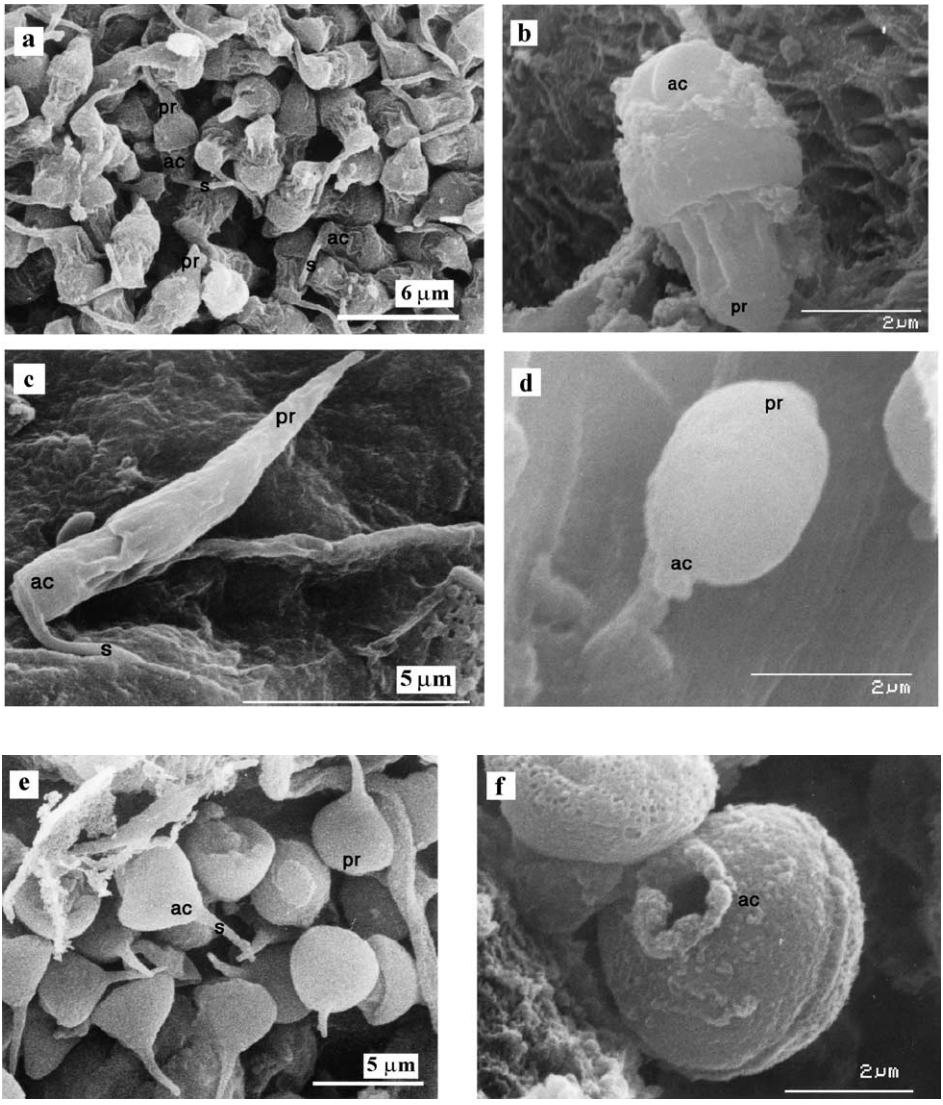

Fig. 1. Scanning electron micrographs of unreacted sperm and an EW reacted sperm of *T. byrdi* (a, b), *X. riveti* (c, d), and *L. occidentalis* (e, f). Unreacted sperm show three sections: acrosomal region (ac) which includes the spike (s), and the posterior region (pr) which contains the nucleus. Reacted sperm have lost their spike.

the spike followed by a morphological change into a spherical shape (*T. byrdi* and *X. riveti*), and the visible eversion of the cell contents (*T. byrdi* and *L. occidentalis*). In *X. riveti* no visible eversion of cell contents was observed.

Fig. 2 presents the timing for sperm activation under in vitro induction for wild *T. byrdi*, *X. riveti*, and *L. occidentalis*. The response is similar for the three species; the pattern observed in this figure indicates a progressive activation of cells immediately after EW

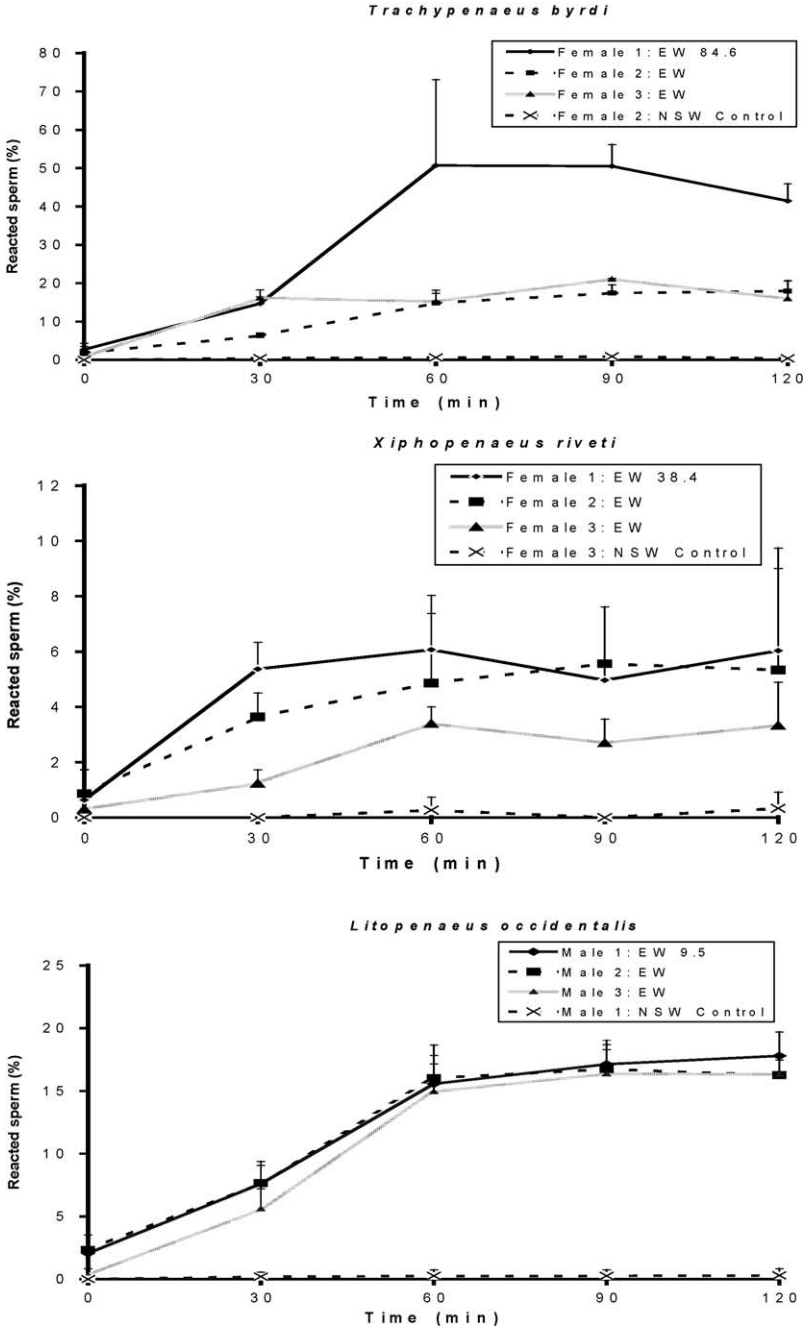

Fig. 2. Sperm activation (%) over time from individuals of *T. byrdi*, *X. riveti* (seminal receptacles), and *L. occidentalis* (spermatophores) exposed to conspecific egg water (EW; protein concentration in μg ml^{-1}). Controls exposed to natural seawater (NSW) gave negligible activation; therefore only one control is illustrated for comparison.

exposure, until reaching a stable percentage between 60 and 90 min from exposure. Comparatively, a negligible activation is observed in each control test, under NSW exposure, during 120 min of observation.

Table 1 shows the percentages of sperm activation induced with different EW batches for the closed thelycum shrimp, *T. byrdi*. Sperm removed from females' seminal receptacles generated a statistically significant increase in activation compared to controls ($P<0.05$); on the other hand, sperm removed from males' spermatophores did not experience activation when exposed to conspecific EW ($P>0.05$).

Three EW batches, with different protein concentrations, were evaluated. It seems that the activation percentage increases at higher concentrations; however, the experimental design was not intended for statistical comparisons on this variable. Sperm from seminal receptacles gave a variable response in sperm activation within replicates, as indicated by the high measured standard deviation.

Sperm activation was also induced in sperm removed from the seminal receptacles of *X. riveti* (Table 1), with the three EW batches tested ($P<0.05$). Sperm removed from males' spermatophores were activated at a low rate for EW batch 1 ($P<0.05$); male sperm evaluated with batches 2 and 3 were not activated as compared to controls ($P>0.05$). The degree of activation in *X. riveti* sperm was remarkably lower than in *T. byrdi* sperm. The

Table 1
Evaluation of sperm activation (%) from seminal receptacles (females) and spermatophores (males) of *T. byrdi*, *X. riveti*, and *L. occidentalis*, incubated for 90 min in different EW batches

Species	EW batch (μg ml^{-1} protein)	Sperm activation (%)[1]			
		Seminal receptacle sperm		Male sperm	
		EW[2]	NSW[3]	EW[2]	NSW[3]
T. byrdi[4]	1 (84.6)	29.67[a] ± 18.15	0.25[b] ± 0.23	0.25[b] ± 0.09	0.19[b] ± 0.16
	2 (56.4)	15.20[a] ± 16.30	0.16[b] ± 0.28	0.22[b] ± 0.18	0.20[b] ± 0.17
	3 (14.1)	5.78[a] ± 4.85	0.26[b] ± 0.44	0.51[b] ± 0.17	0.18[b] ± 0.16
X. riveti[4]	1 (38.4)	4.41[a] ± 1.50	0.0[c] ± 0.0	1.17[b] ± 0.65	0.0[c] ± 0.0
	2 (22.8)	3.17[a] ± 0.60	0.43[b] ± 0.36	1.11[ab] ± 1.12	0.32[b] ± 0.45
	3 (7.6)	5.67[a] ± 2.61	0.55[b] ± 0.51	0.52[b] ± 0.17	0.42[b] ± 0.19
L. occidentalis[4]	1 (48.0)	–	–	4.24[a] ± 2.13	0.41[b] ± 0.74
	2 (37.0)	–	–	2.96[a] ± 0.95	0.35[b] ± 0.60
	3 (9.5)	–	–	16.75[a] ± 0.41	0.29[b] ± 0.02

[1] n = three replicates per treatment.
[2] EW: egg water.
[3] Control: incubated in natural seawater (NSW).
[4] Different letters within row indicate statistically significant differences ($P<0.05$).

highest mean value was measured for seminal receptacle sperm at the lowest protein concentration (7.6 μg ml^{-1}; 5.67%).

The response in sperm activation for *L. occidentalis* spermatophores is shown in Table 1. In general, every male gave a positive activation compared to controls ($P < 0.05$), regardless of EW batch; however, the percentages of sperm activation were lower with the EW batches containing more protein. EW at 9.5 μg ml^{-1} induced a uniform response in sperm activation in the three males evaluated (16.75 ± 0.41%).

Sperm cells removed from the thelycum of *T. byrdi* did not react against EW isolated from the other two species (results not shown).

4. Discussion

The modified EW technique did induce activation of sperm in two closed thelycum shrimps, *T. byrdi* and *X. riveti*, and one open thelycum species, *L. occidentalis*. The morphological changes experienced by sperm were similar to the acrosome reaction described in *L. stylirostris* (Clark and Griffin, personal communication) and *L. vannamei* (Wang et al., 1995); to our knowledge these are the first descriptions of sperm activation from these species. In general, the activation is characterized by spike depolymerization followed by a morphological change of the sperm body into a spherical shape, for the elongated cell types (*T. byrdi* and *X. riveti*); then visible eversion of the acrosomal contents occurs (*T. byrdi* and *L. occidentalis*).

X. riveti did not experience a visible eversion of the acrosomal contents, indicating that this may be a feature for this species. However, the percentage of activation was very low, compared to *T. byrdi* and *L. occidentalis*. This may be an indication that EW collected from *X. riveti* was of low quality as acrosome reaction inducer.

The apparent differences between EW batches may be related to variability in the EW isolation technique and associated differences in final protein concentrations. Previous reports have been performed with EW at 50 μg ml^{-1} of protein (Griffin et al., 1987; Wang et al., 1995). In this study, EW batches around 50 μg ml^{-1} were included in the evaluation of the three species.

Besides the original findings from wild *S. ingentis*, in which high rates of sperm activation were obtained (75–50%; Griffin et al., 1987; Anchordoguy et al., 1988), the recent reports on penaeoideans of aquaculture importance (*P. monodon* and *L. vannamei*) gave variable results with no relationship with other quality parameters like gross sperm morphology.

Wang et al. (1995) found that spermatophores of pond-grown males (*L. vannamei*) exhibited high rates (over 80%) of normal gross morphology sperm, but the acrosome reaction was variable from 7.4% to 87% (mean value = 37.4 ± 18.5%) This high variability between individuals was also found in the present study, particularly for *T. byrdi*, indicating that the induction is affected by factors other than the chemical composition of each EW batch.

This study was based on wild populations of three penaeoid species from a tropical estuary, Golfo de Nicoya, Costa Rica. Gametes from wild brooders are of optimum quality, as indicated by hatching rates of *T. byrdi* from 70% to 99% (Alfaro et al., 2001), and

spermatophore parameters (sperm count = 49.52 million, abnormalities = 22%) from wild *L. occidentalis* (Alfaro et al., 1993). The laboratory is routinely spawning and producing nauplii from these wild broodstocks. Therefore, the low and variable sperm activation, regardless of EW batch, seems to indicate that unknown factors are interfering with the normal activation of cells.

In open thelycum shrimps, it has been assumed that spermatophores within terminal ampoules contain fully matured sperm, but no scientific observations have been published to improve our knowledge on this crucial topic. In vitro fertilization has yielded negative or very low fertilization rates. Alfaro et al. (1993) got no acrosome reaction nor fertilization for wild *L. occidentalis* using cones and beakers as fertilization containers, Misamore and Browdy (1997) obtained 2.48% fertilization for *L. setiferus* and 3.88% for *L. vannamei* using an original devise. On the other hand, artificial insemination in open thelycum species normally generates acceptable fertilization and hatching rates (Bray and Lawrence, 1992). After natural or artificial insemination, sperm will remain attached to the supporting matrix on the external surface of the thelycum for a few hours until spawning.

Based on these facts and the low sperm activation obtained with the EW technique applied to sperm removed from spermatophores, it is proposed that final sperm maturation on the external surface of the thelycum may be required for fertilization. To further explore this hypothesis ultrastructural observations of the final steps for sperm maturation comparing sperm from spermatophores and from female's thelycum before spawning, are needed.

In closed thelycum species sperm must be matured in the seminal receptacles before fertilization. In *S. ingentis* electron microscopy has revealed that ultrastructural changes in the subacrosomal region take place subsequent to the transfer and storage of sperm within seminal receptacles (Shigekawa and Clark, 1986). This study also demonstrated that activation is not induced in sperm removed from *T. byrdi* and *X. riveti* males. The low activation (4.2%) reported by Pratoomchat et al. (1993) for spermatophores removed from *P. monodon* males, seems to indicate that this closed thelycum species also requires the maturation of sperm in the seminal receptacles, to be capable of fertilization. However, the authors did not evaluate sperm from females' thelycum.

Sperm from seminal receptacles did experience activation when exposed to conspecific EW; however, the response was variable between individual females. This variability may be the result of different stages in the process of final maturation. From the moment of natural insemination, sperm will start to modify their ultrastructure until reaching full maturation, before spawning. This subject requires further attention in penaeoidean reproductive physiology.

The EW technique, at its present stage of development, is a practical tool to explore sperm maturation in open and closed thelycum shrimps, as demonstrated in this document. The technique can also be used to explore hybridization barriers, in order to define recognition between EW and sperm from different species.

Attempts at hybridization in penaeoid shrimps have been limited. Hybridization has only been achieved through artificial insemination (Misamore and Browdy, 1997). The main obstacle to hybrid production lies in the successful fertilization and hatching of eggs (Benzie et al., 2001). The low hatch rates so far reported for different penaeoid hybrids may be related to sperm–egg incompatibility, resulting in an absence of the acrosome

reaction. Benzie et al. (2001) reported 1.3% hatch rate for *P. monodon* × *Penaeus esculentus*, Bray et al. (1990b) obtained 0.2% for *L. setiferus* × *Litopenaeus schmitti*, and Lin et al. (1988) reported 30% for *P. monodon* × *Penaeus penicillatus*. Our findings on interspecific sperm: EW interaction between sperm from *T. byrdi* and EW from *X. riveti* and *L. occidentalis* seems to indicate that acrosome reaction inducers are species-specific within penaeoids. Further research is required to define the degree of specificity and the molecular structure of such inducers from different species.

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