Microfilament and DNA localization in lobster and crayfish spermatozoa

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*Although decapod spermatozoa are not motile they do have some components of the cytoskeleton within the cytoplasmic body and also in projections that radiate from the sperm body. Crayfish spermatozoon (**Orconectes propincus**) isolated from the vas deferens presents a round and slightly flattened body with 5-6 folded arms that extend and radiate when the sperm are placed in water. The body is formed by a ring-shaped structure that contains actin filaments and a posterior region with DNA that extends to the base of arms. Arms are composed of clustered filaments that extend from the center of the body. Spiny lobster (**Jasus frontalis**) mature spermatozoa are conformed to a round body from which 3-4 filamentous arms radiate. The body contains a lens-shaped vesicle and nucleus-cytoplasmic material behind it. The vesicle contains actin filaments. They are concentrated in a gear-shaped structure as revealed by rhodamine-phalloidin dye and confocal microscopy. The remainder of the body is composed of DNA as revealed by Hoechst 33258 dye.*

Key words: *Acrosome, crayfish, lobster, spermatozoon.*

INTRODUCTION

The spermatozoa of Decapod crustaceans are atypical, nonflagellated and nonmotile. They are constituted by a body containing a nucleus with no condensed chromatin (Anderson and Ellis, 1967; Pochon-Masson, 1969; Hinsch, 1986), and one or multiple filiform appendages radiating from the body. Two models of spermatozoa can be differentiated: the *Reptantia* spermatozoa formed by a spherical or elongated body with multiple appendages emerging from it, and the *Natantia* spermatozoa which are formed by a tack-shaped body (*Penaeidea*: Clark *et al*, 1973; 1981a; Brown *et al*, 1976; Yudin *et al*, 1979; 1980) or bell-shaped body (*Caridea*: Pochon-Masson, 1969; Köheler, 1979; Sandifer and Lynn, 1981; Lynn and Clark, 1983; Dupré and Barros, 1983) with a single appendage emerging from its vertex. Both models of spermatozoa have different acrosomes, the former has a round-shape or elongated acrosomal vesicle immersed in the body and the latter has a rigid spike emerging from the body.

The acrosomes of both, the *Reptantia* (Chevaillier and Maillet, 1965; Anderson and Ellis, 1967; Hinsch, 1969) and the *Natantia* (Kleve *et al*, 1980; Clark *et al*, 1981a,b), have been described and the acrosome reaction has been examined during sperm-egg interaction in crabs (Brown, 1966; Goudeau, 1982) and in penaeid shrimps (Clark *et al*, 1981b; Griffin *et al*, 1988; Yudin *et al*, 1979) and during the induction with ionophore in lobster (Brown *et al*, 1977; Talbot and Chanmanon, 1980). However, the mechanism that triggers this response has not yet been determined.

Although decapod spermatozoa are not motile, they do have some components of the

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cytoskeleton. Microtubules are found in the sperm body of shrimps (Pochon-Masson, 1965), crabs (Anderson and Ellis, 1967; Langreth, 1969; Hinsch, 1969; 1971; 1986; Pérez *et al*, 1986) and crayfish (Moses, 1961); actin and tubulin-like proteins are present in the radial arms of the sperm of shrimp (Pérez *et al*, 1991), crabs (Moses, 1966; Anderson and Ellis, 1967; Hinsch, 1969; 1971; Pérez *et al*, 1986) and crayfish (Moses, 1961). Actin filaments have been found in the spike of a penaeid shrimp sperm (Brown *et al*, 1976; Kleve *et al*, 1980; Clark *et al*, 1981a).

The actual participation and role of actin filaments in the decapod crustaceans sperm during fertilization is not fully understood, although actin has been reported that could play an active role in drawing the penaeid shrimp sperm closer to the egg (Clark *et al*, 1981a).

The present study examines the morphology of the spermatozoa with low voltage scanning electron microscopy and presents evidence of the localization of actin filaments and DNA in the sperm of a spiny lobster and a crayfish by fluorescent probes.

MATERIALS AND METHODS

Sexually mature males of both the spiny lobster *Jasus frontalis* and the crayfish *Orconectes propincus* were obtained from commercial catches in the Robinson Crusoe island, and in the Wisconsin river, respectively.

Spermatophores of *J. frontalis* containing mature spermatozoa were removed from dissected vas deferens and fixed in 0.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M phosphate buffer at pH 7.6. Spermatozoa were suspended in the same buffer and washed twice by centrifugation. They were incubated in the dark, at room temperature, for 15-25 min in PBS solution containing 0.5 µg/ml rhodamine-phalloidin and 0.1% lysolecithin (modified from Cline and Schatten, 1986). Spermatozoa were then affixed on polylysine-coated slides (Sawada and Schatten, 1989), washed three times with PBS and dyed with a drop of 5 µM Hoechst 33258 for 20-30 min (Holy and Schatten, 1991a). The slides were immersed in three changes of PBS, and mounted in 90% glycerol medium as described by Holy and Schatten (1991b). The spermatozoa were immediately examined by means of fluorescence, using a Zeiss Axiophot epifluorescence microscope or a Bio-Rad MRC-600 laser scanning confocal microscope (LSCM). The setting of LSCM was as described by Holy and Schatten (1991b). In control preparations, spermatozoa were incubated in PBS without rhodamine-phalloidin or Hoechst 33258 and observed with a rhodamine or fluorescein filters.

The crayfish spermatozoa, removed from dissected vas deferens, were fixed for 1 h in 0.5% glutaraldehyde, 2.5% paraformaldehyde in PBS containing 0.5 µg/ml lysolecithin and 50 nM rhodamine-phalloidin (modified from Cline and Schatten, 1986). The spermatozoa were washed three times in PBS by centrifugation and then treated as described above for lobster spermatozoa.

For scanning electron microscopy, freshly isolated *O. propincus* spermatozoa were washed in 0.1 M phosphate buffer three times and fixed for 1-2 h in 2.5% glutaraldehyde, 0.5% paraformaldehyde and 1% tannic acid in 0.1 M phosphate buffer. The specimens were dropped on a polylysine-fomvar-coated grid, dehydrated in ethanol and critically point dried in CO_2. The platinum-carbon coated samples were examined in a field emission Hitachi S-900 Low Voltage Scanning Electron Microscope (LVSEM).

The designation of anterior and posterior region of the gamete follows that established for *Astacus astacus* (López-Camps *et al*, 1981) where the acrosome complex that includes a ring-like formation is considered the anterior part and the nuclear region the posterior part of the gamete.

RESULTS

Orconectes propincus spermatozoa

Crayfish spermatozoa from the vas deferens display a round and slightly flattened body with 5 or 6 arms (22 µm long) folded around the body (Fig 1a) that extend and radiate when they are placed in PBS (Fig 1b). The

arms are composed of clustered filaments that extend from the nuclear region of the body (Fig 2). The body is formed by: 1) a ring-like structure of 4.3-4.6 μm in diameter (Fig 3) that occupies more than half of the sperm body, and 2) the nuclear region. In a polar view, Hoechst 33258 stained spermatozoa lightly compressed with a coverslip show the ring-like structure with a circular outline and a central depression (Fig 4a) of 1.6-1.8 μm in diameter that opens to the nuclear region laying underneath when viewed with LVSEM (Fig 3). The Hoechst 33258 fluorescence is located between the ring-like structure and the external border of the body, but not in the center of the ring-like structure (Fig 4b).

In a polar view of a whole mounted spermatozoa (Fig 5a) stained with Hoechst 33258 (Fig 5b), the fluorescence is present in the entire body including the base of radial arms.

When the spermatozoa are stained with rhodamine-phalloidin and viewed laterally the ring-like structure appears as a flattened crescent-shaped structure (Fig 6a) with the concave inner area in contact with the nuclear region. Rhodamine fluorescence is located only in the area below and enclosed by the crescent-shaped structure (Fig 6b). The rest of the body does not fluoresce. No fluorescence was observed in the control preparation incubated in the absence of the rhodamine-phalloidin viewed with rhodamine filter.

Jasus frontalis spermatozoa

Mature spermatozoa are formed by a spherical body from which 3 or 4 filamentous arms radiate. The body contains: 1) a lens-shaped acrosome vesicle, and 2) a nuclear region (Fig 7a). Filamentous arms of vas deferens spermatozoa encircle the body and extend when placed in sea water.

Hoechst 33258 labelled spermatozoa reveal intense fluorescence below the acrosome vesicle corresponding to the nuclear region; there is no fluorescence inside the vesicle zone (Fig 7c).

Rhodamine-phalloidin treated spermatozoa show fluorescence in the lens-shaped acrosome vesicle region (Fig 7b, 8b) and within the base of the radial arms (Fig 8b). More precise localization of the rhodamine fluorescence was determined by confocal microscopy (Fig 8a, 8b). A transversal optical section of the center of the acrosome, performed under confocal microscopy, shows a highly fluorescent gear-shaped formation denoting a different concentration of actin when compared with the remainder of the acrosomal vesicle content (Fig 8a). Each of the 6 radius of the gear-like formation extends to the periphery of the vesicle and continues to the anterior top of this vesicle as 6 rod-like formations as determined by confocal microscopy.

The localization of actin and DNA of the spiny lobster and crayfish sperm is summarized in the diagrams of Figure 9.

DISCUSSION

Results obtained in this study show that the eccentrically located lens-shaped vesicle inside of the body of lobster spermatozoa contain actin filaments in all the vesicle, but they are concentrated in a gear-like shape located at the base of the vesicle which extends peripherally in 6 rod-like formation from the widest to the minor diameter in the anterior part of the vesicle.

Because of the similarity of the general structure of this spermatozoa (Dupré, unpublished observations) with *Panulirus argus* (Talbot and Summers, 1978), it is feasible to suggest that the lens-shaped vesicle corresponds to the acrosome vesicle of *P. argus*. Furthermore, the acrosome of the latter species presents a periacrosomal filamentous material dispersed near the base of the acrosome. As this material passes over the acrosome, filaments converge forming electron-dense bundles, between which anastomosing filaments exist. In frontal sections of the acrosome, the filament bundles appear as electron-dense core in pockets formed by the plasma membrane and the acrosomal membrane. Those structures resemble the gear-like and the rod-like formation of *J. frontalis* evidenced in the present study and correspond presumably to a different concentration of actin filaments. Actin filaments have also been found in the acrosomes of other crustaceans like *Limulus*

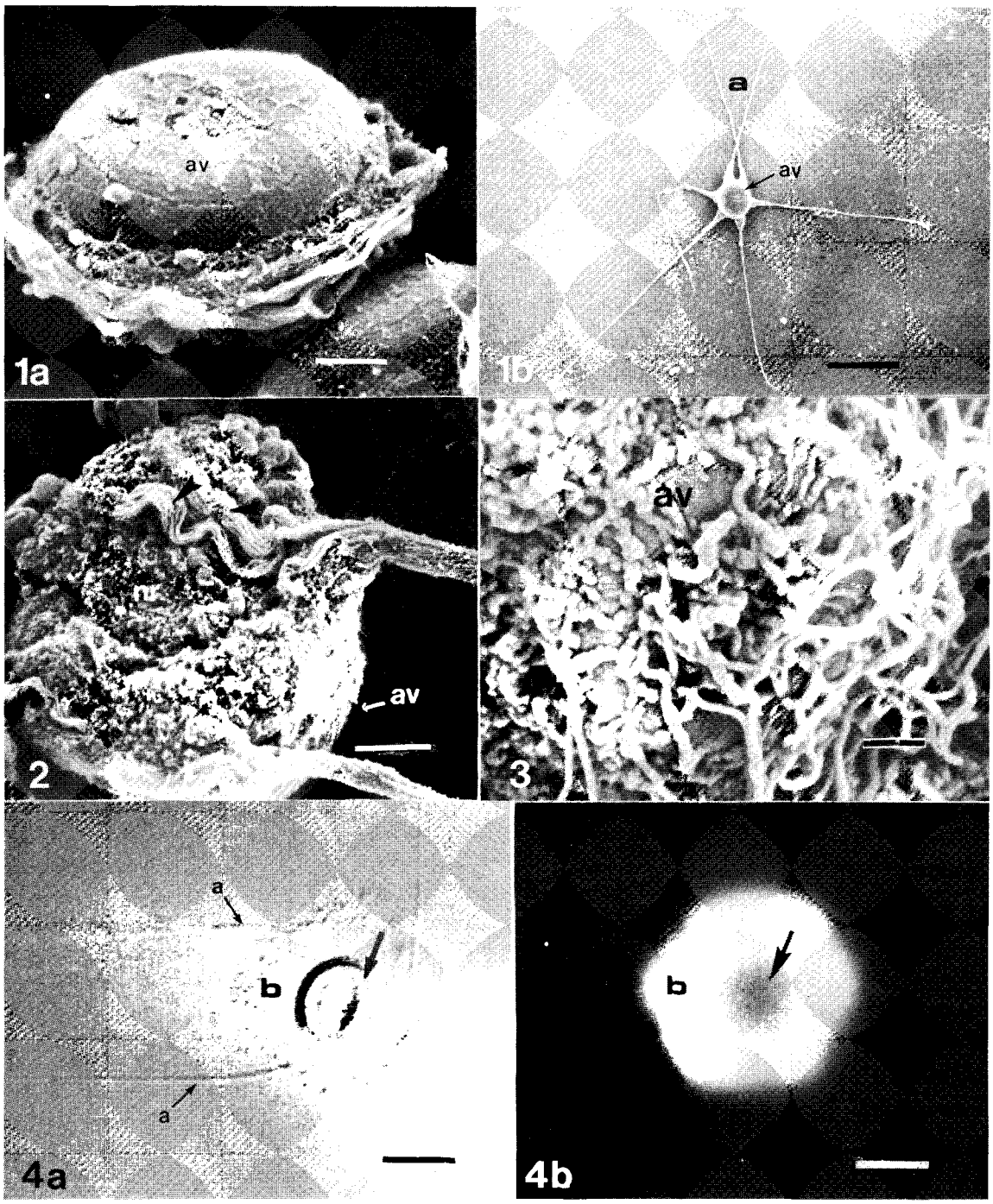

Fig 1. Low voltage SEM of mature spermatozoa. a) Spermatozoon of *O. propincus* within the spermatophore. Note the circular furrow on the anterior surface of the acrosomal vesicle (av). The arms are wrapped around the body. Bar = 1 μm. b) Spermatozoon of *O. propincus*. Acrosomal vesicle (av), radial arms (a). Bar = 10 μm.

Fig 2. Low voltage SEM of posterior region of spermatozoon of *O. propincus*. Plasma membrane was removed. Note the clustered filaments (arrow head) in the nuclear region (nr) and inside the radial arms. Acrosomal vesicle (av). Bar = 1 μm.

Fig 3. Low voltage SEM of the acrosome vesicle of *O. propincus* sperm from a posterior view. The plasma membrane was removed. Note the doughnut-shaped acrosome vesicle (av) with its central canal opened to the nuclear region. Bar = 1 μm.

Fig 4. Mature compressed spermatozoon of *O. propincus* labelled with Hoechst 33258. a) With Nomarski illumination. Note the nuclear region (b), the arms (a) and the ring-shaped acrosome vesicle (arrow). b) With epifluorescence illumination. Intense fluorescence is present in the nuclear region but not in the center of ring-shaped acrosome vesicle (arrow). Bar = 5 μm.

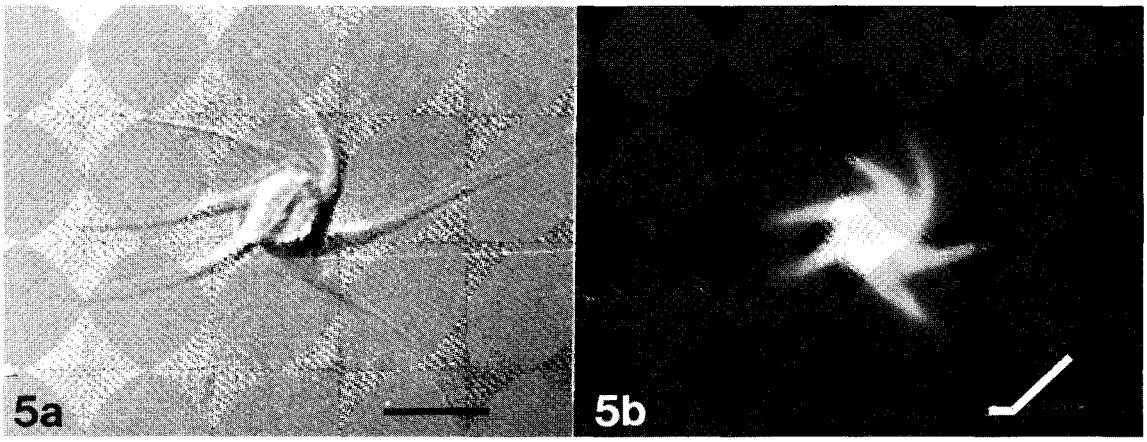

Fig 5. Intact whole mounted sperm of *O. propincus* labelled with Hoechst 33258. a) With Nomarski illumination. b) Viewed with epifluorescence microscope. Observe intense fluorescence in the body and within the base of radial arms. Bars = 5.4 μm.

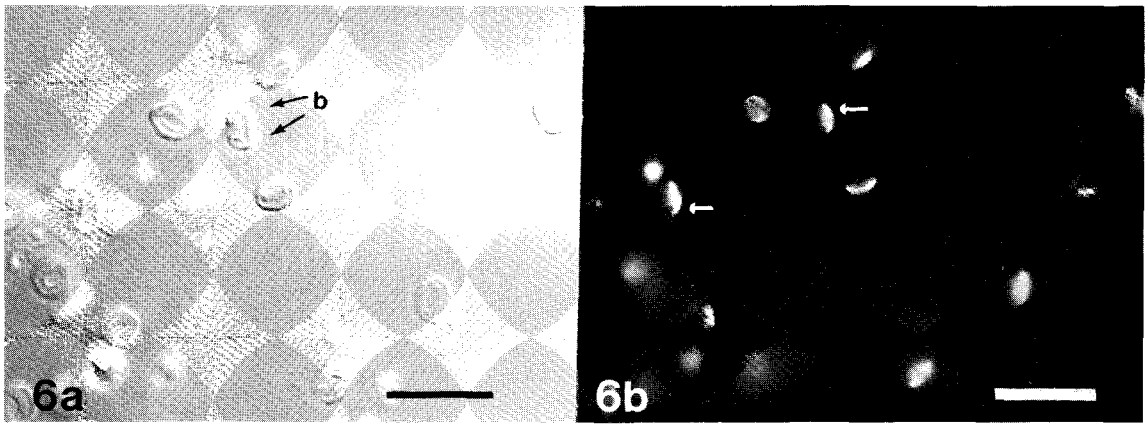

Fig 6. Spermatozoon of *O. propincus* labelled with rhodamine-phalloidin. a) With Nomarski illumination. Note the body's edge (b) of a spermatozoon containing a conspicuous acrosomal vesicle shaped as a flattened crescent when viewed laterally. b) Viewed under epifluorescence microscope. Intense fluorescence is present inside the area below and enclosed by the crescent-shaped structure mainly in the lateral zone (arrow). Bars = 10 μm.

polifemus (Tilney, 1985; Mabuchi and Nonomura, 1986) and in the penaeid shrimp *Sicyonia ingentis* (Brown *et al*, 1976).

Detailed description of the morphology of *O. propincus* has not been reported; however, previous studies in other freshwater crayfish spermatozoa (Anderson and Ellis, 1967; Moses, 1961; López-Camps *et al*, 1981) have been particularly significant in establishing the general morphology of the present species studied, *O. propincus*. The ring-like structure or, when laterally viewed, the crescent-shaped structure of the studied spermatozoa can be assumed to correspond to the acrosome complex of *A. astacus* (López-Camps *et al*, 1981). In spite of the fact that actin filaments have been found in the arms of other *Natantia* as *Rhynchocinetes typus* (Pérez *et al*, 1991), actin filaments in *O. propincus* were detected in the center and in the lateral area enclosed by the ring-shaped structure or acrosome vesicle, but not in the radial arms.

López-Camps *et al* (1981) showed that the acrosome central canal of the crayfish *Astacus astacus* can be differentiated into two areas by the texture of its fibres. The most peripheral part, which borders the concave surface of the ring-like structure as well as the most distal part of the vesicle, presents more dense fibres than the other more internal area that occupies the center of the vesicle as well as its ventral opening. The former areas coincide with the fluorescence

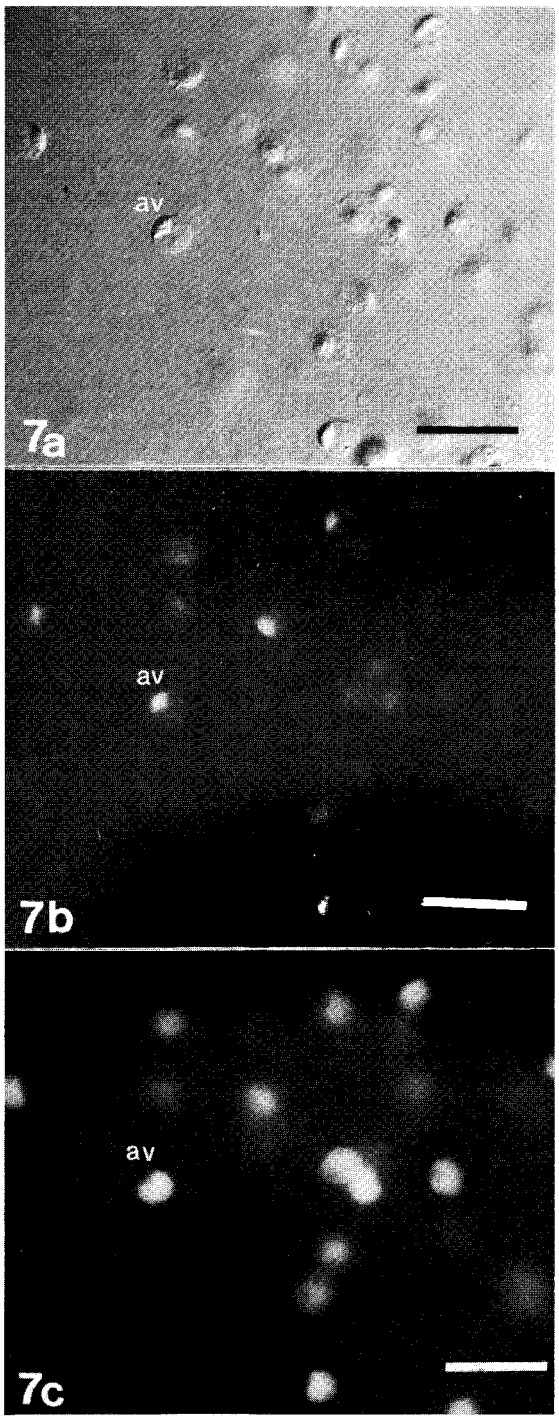

Fig 7. Three micrographs of mature spermatozoa of *Jasus frontalis* labelled with Hoechst 33258 and rhodamine-phalloidin. **a)** Under Nomarsky illumination. Note the body with an acrosomal vesicle (av) and a nuclear region. **b)** With rhodamine filter the spermatozoa show intense fluorescence in the acrosomal vesicle (av) but not in the nuclear region. **c)** With fluorescein filter only the nuclear region fluoresces. Note the acrosomal vesicle area (av) without fluorescence. Bars = 13 μm.

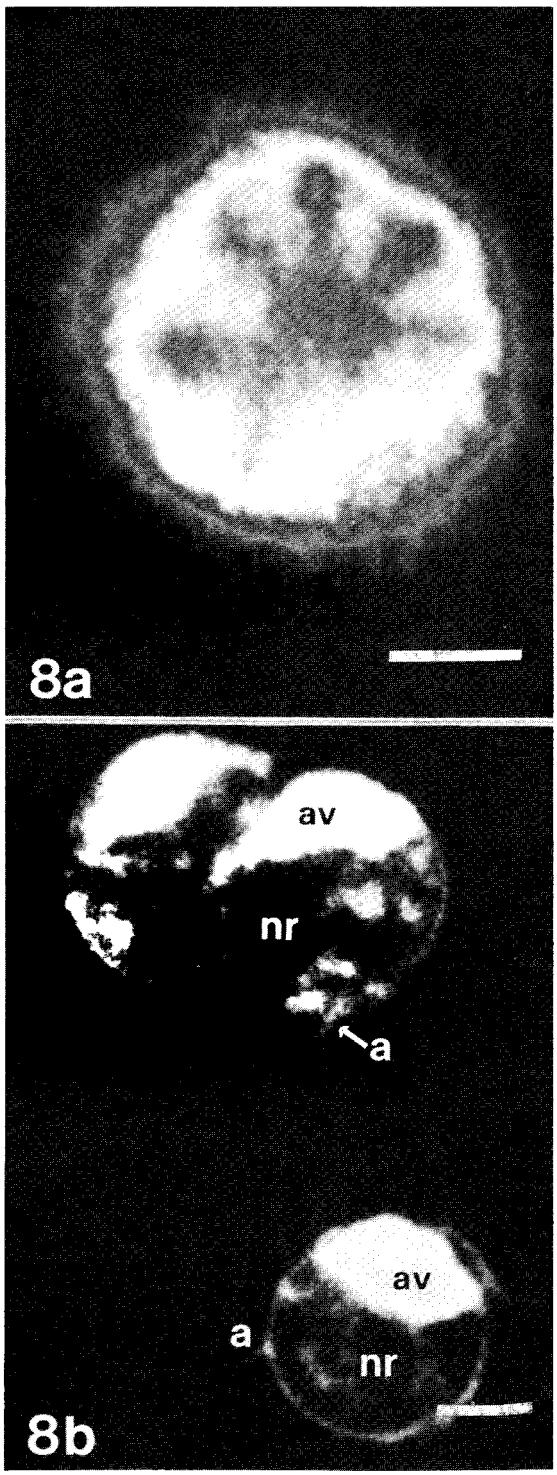

Fig 8. Confocal scanning micrographs of *Jasus frontalis* spermatozoon labelled with rhodamine-phalloidin. **a)** Transversal optical section through the acrosomal vesicle. It shows intense fluorescence in the gear-like structure. Bar = 1 μm. **b)** Anterior-posterior optical section through the acrosomal vesicle (av) and the nuclear region (nr). Note the fluorescence in the base of radial arm (a). Bar = 2 μm.

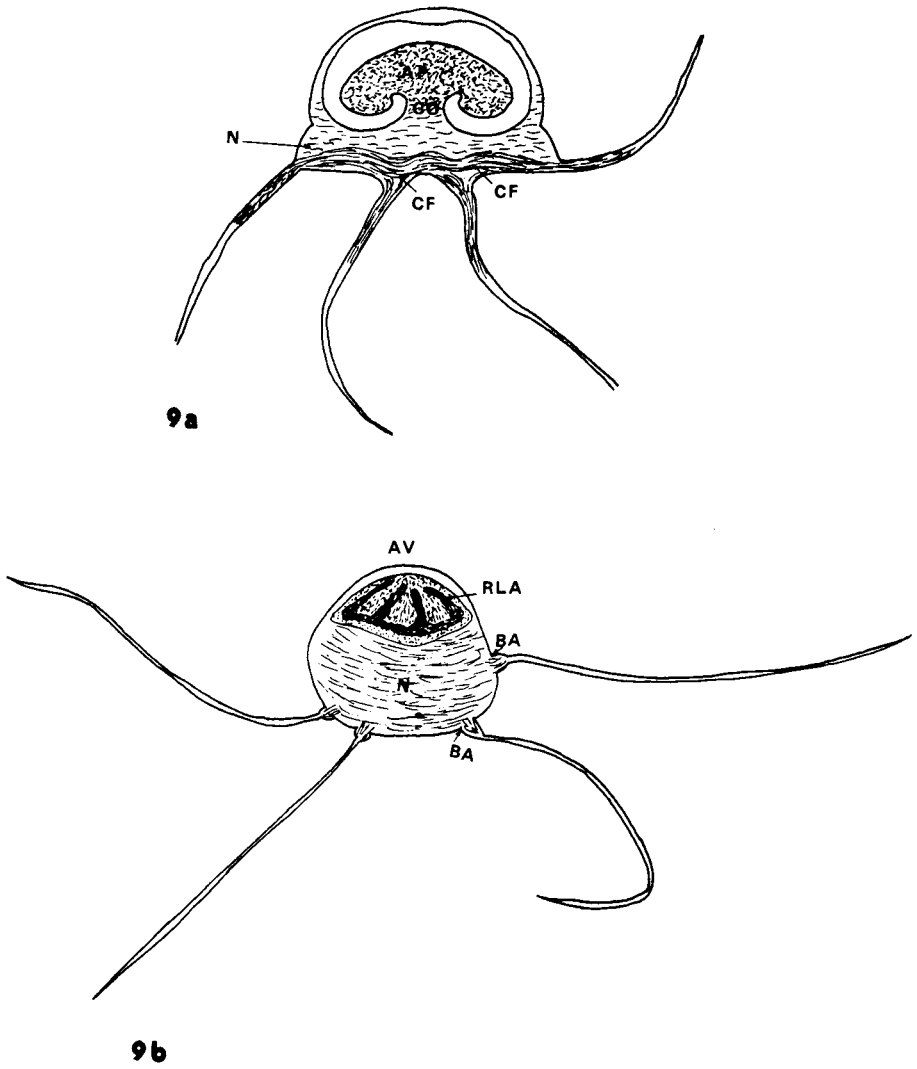

Fig 9. Proposed diagrammatic section of: a) *O. propincus* crayfish sperm. The length of the arms is not proportional to the diameter of the body. The actual length is 5 times longer. b) *J. frontalis* lobster sperm. (AF) actin filaments, (AC) crescent-shaped acrosomal vesicle, (AV) acrosomal vesicle, (BA) base of radial arm, (CF) cluster filaments, (CO) central opening, nucleus (N). Concentrated actin filament in a rod-like structures (RLA).

that appears in the central canal located in the area below and enclosed by the crescent-shaped structure or acrosome vesicle of *O. propincus* sperm.

In the crab *Libinia emarginata* sperm, actin filaments were detected along the periphery of the cell, at the base of the sperm and within the three radial processes in a close spatial relationship with the myosin components, suggesting a functional or contractile activity (Pérez *et al*, 1986). Nevertheless, in lobster sperm, *J. frontalis*, only a weak fluorescence reveals actin filaments in the small area at the base of the arms and no fluorescence revealing actin appears within the arms of *O. propincus*. In both spermatozoa studied, actin filaments were detected in the acrosome vesicle.

Anderson and Ellis (1967), in *Cambarus sp*, described intranuclear tubules of greater diameter (300 A) than the microtubules present in the cytoplasm of interstitial cells. They extended from the pericentriolar material and were randomly distributed among the filamentous chromatin material. Similar distribution was detected in *O. propincus*

where clustered filaments that extend into the radial arms are located within the area that is occupied by DNA.

The function of sperm actin is unknown, but the localization within the acrosome vesicle of both spermatozoa suggests an important participation in the evagination of this vesicle in order to form the acrosome tube as described for lobster sperm (Talbot and Chanmanon, 1980), or the acrosome filament in the penaeid shrimp *Sicyonia ingentis* (Griffin *et al*, 1988).

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