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Presence of F-actin in sperm head of *Armadillidium peraccae* (Isopoda, Oniscidea)

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ABSTRACT

Sperm of *Armadillidium peraccae* have been examined with cytochemical and immunocytochemical methods for fluorescence and electron microscopic visualization of cytoskeleton components. Sperm incubation in an antibody anti-β-tubulin shows only the presence of two centrioles located in the cytoplasmic region above the nucleus; no other microtubules are present in the sperm head. Instead, fluorescence microscopy of sperm incubated in FITC-phalloidin allowed to detect the presence of a large amount of F-actin in the apical region of the sperm head. The incubation of ultrathin sections of sperm embedded in Lowicryl K4M with a phalloidin–gold complex allowed a more precise localization of F-actin in the amorphous part of the acrosome and in the cytoplasmic region between acrosome and nucleus; F-actin is also present in the thin cytoplasmic layer between plasma membrane and nuclear envelope at the apical portion of the nucleus. Although the sperm was always found completely devoid of motility, the discovery of the presence of an actin cytoskeleton leads us to hypothesize a possible acquisition of motility by the sperm at the time of its interaction with the female gamete. Such a hypothesis is supported by what is known for ostracods whose aflagellate sperm implement a type of amoeboid movement only at the time of their interaction with the female gamete.

1. Introduction

Like other orders of peracarids – as mysids, cumaceans and amphipods – isopods have a particular type of aflagellate, immotile spermatozoon, made up of two linear associated components: the cell body containing a long ribbon-like nucleus, above which an acrosomal complex is present, and a long flexible tail with a paracrystalline structure (Reger et al., 1970, 1979; Fain-Maurel et al., 1975a,b; Cotelli et al., 1976). No cytoskeleton component has been noted in the sperm and the only microtubular structures present are represented by two peculiar centrioles, made up of nine doublets (Cotelli et al., 1975).

The loss of sperm motility is an event so widespread to have been established in 36 different taxa, from the red algae to fishes (Morrow, 2004). In the monoandrous taxa, the lack of sperm competition results in a significant reduction of the selective pressure directed to the production of motile sperm by the males; aflagellate sperm production is less costly, in terms of energy and time, compared with the production of motile sperm (Morrow, 2004).

On the other hand, the loss of sperm motility raises the question how the interaction between sperm and egg, and then fertilization, can take place.

These considerations can be fully related to isopods about which, moreover, nothing is known in the literature with regard to egg surface organization, site of the gamete interaction and morphological and molecular aspects of gamete interaction.

A recent research of Longo and Trovato (2008) has shown that sperm of *Armadillidium granulatum* can be stored for long time after mating into the female genital system without loosing the fertilizing ability. Moreover, within the female genital system, sperm of oniscideans also undergo separation of the tail from the sperm head; while the tail is frequently captured by the epithelial cells of seminal receptacle or of ovarian wall to be digested as a result of an spermiophagic activity (Longo et al., 1998; Longo and Trovato, 2008; Longo and Trovato, unpublished observations), the sperm head becomes probably the only protagonist in the interaction with the surface of the egg cytoplasm and in the egg fertilization.

But the sperm head, in the course of its interaction with the female gamete, remains immotile or may undergo a transitory and targeted motility? This latter hypothesis is supported by knowledge of what is known for ostracods, whose giant and aflagellate sperm acquire, in the presence of eggs, an amoeboid motility that will enable them to reach and fertilize the eggs (Reger, 1970; Matzke-Karasz, 2005).

With the aim to test the validity of this hypothesis, we have carried out a research on sperm of *Armadillidium peraccae* Tua 1900 based on the use of cytochemical and immunocytochemical methods to assess the presence and location in the sperm head

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of cytoskeleton components, particularly actin, responsible for its eventual motility.

2. Materials and methods

The research was carried out on sexually mature males of *A. peraccae*, kept in isolation for some days from the females and then sacrificed by decapitation and immersed in Ringer's saline solution (RSS) modified for terrestrial isopods by Legrand (Besse, 1976); the seminal vesicles were exposed following incision of abdominal cavity in order to collect the spermatophores contained in their lumen.

2.1. Anti-tubulin labeling

Spermatophores collected from the seminal vesicles were washed three times in RSS, fixed in 3.7% formaldehyde in RSS for 30 min and again repeatedly washed.

Sperm were permeabilized in 0.2% Triton X-100 in RSS for 5 min and washed 3 times in RSS. Spermatophores were then immersed in 6% bovine serum albumin (BSA) in RSS for 30 min to block non-specific sites, washed in RSS and incubated in 1 μg/1 ml antibody anti-tubulin diluted 1:2000 (monoclonal anti α-tubulin, clone B-5-1-2, from Sigma–Aldrich) for 90 min. After repeated washing in 1% BSA in RSS, spermatophores were incubated in 200 μl of component A (2 mg/ml Alexa Fluor 488, rabbit anti-mouse IgG, from Molecular Probes) at 1:200 dilution for 30 min in the dark followed by washing in 1% BSA–RSS and incubation in 200 μl of component B (2 mg/ml Alexa Fluor 488, goat anti-rabbit IgG) at 1:200 dilution for 30 min in the dark. After a further wash in 1% BSA–RSS and then in distilled water, the spermatophores were mounted on glass slide with a mounting medium (Sigma–Aldrich) and photographed with an Olympus BX50 fluorescence microscope equipped with a Leica DC500 digital color camera.

2.2. FITC-phalloidin labeling

Collected spermatophores were washed repeatedly in Ringer's saline solution then fixed in 3.7% formaldehyde–2.0% saccharose in RSS for 5 min at room temperature and then rinsed three times in RSS; subsequently the sperm were permeabilized in a solution containing 20 mmol/L Hepes, pH 7.4, 300 mmol/L saccharose, 50 mmol/L NaCl, 3 mmol/L $MgCl_2 \cdot 6H_2O$, 0.5% Triton X-100 for 4 min at 4 °C and then rinsed in RSS. After, the spermatophores were incubated in a solution of 2 μg/ml FITC-phalloidin diluted 1:5 for 30 min at 37 °C in CO_2 incubator, washed two times in RSS, dried in an oven at 40 °C for 20 min, mounted on a microscope slide in mounting medium Sigma and photographed with an Olympus BX50 fluorescence microscope equipped with a Leica DC500 digital color camera.

Some spermatophores, after incubation in FITC-phalloidin and subsequent washing, were incubated in a solution of propidium iodide (50 μg/ml in 0.1 mol/L phosphate buffer, pH 7.2) for 15 min at room temperature in the dark and repeatedly washed in the same buffer. Subsequently, the spermatophores were mounted on microscope slide with a mounting medium (Sigma–Aldrich), and then observed and photographed using a confocal microscope Zeiss LSM 510 META.

2.3. Sperm and spermatophores ultrastructure

Spermatophores fixation was carried out in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2 for 4 h at room temperature; after repeated washing in the same buffer the specimens were post fixed in 1% OsO_4, in the same buffer, for 1 h at room temperature.

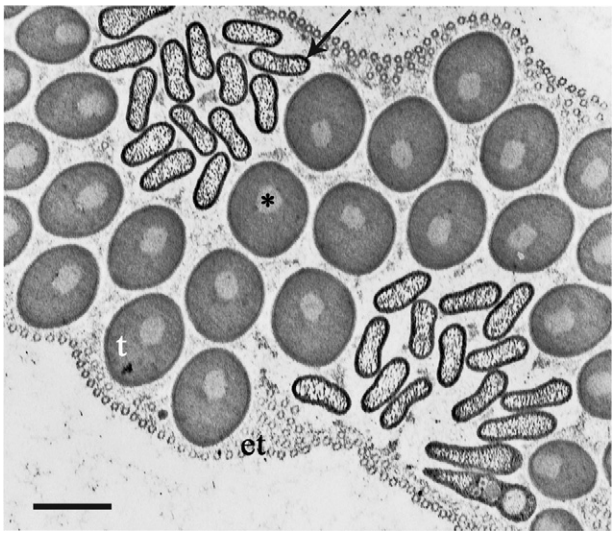

Fig. 1. Cross section of spermatophore. Sperm heads (arrow) and tails (t) are enveloped by a continuous layer of extracellular tubules (et) and embedded in a granular matrix. The tail has a paracrystalline structure and presents a central region (*) less dense and amorphous. Scale bar = 1 μm.

The samples were dehydrated in ethanol followed by propylene oxide and embedded in Embed 812 (EMS). Thin sections were cut on an Ultracut Leica ultramicrotome using a Diatome diamond knife, placed on uncoated Cu/Rh grids, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined with an electron microscope Philips CM 10.

Some sperms were fixed in 2.5% glutaraldehyde for 2 h at room temperature, rinsed in buffer and post-fixed in buffered 1% OsO_4, for 1 h at room temperature, dehydrated in ethanol, dried in a Emscope CPD 750 critical point dryer, coated with gold in a Sputter Coater, Polaron SC7640 and examined in a Field Emission Scanning Electron Microscope – Hitachi S4000.

2.4. Ultrastructural localization of F-actin by phalloidin–gold labeling

Spermatophores were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2 over night at 4 °C and three times briefly washed in the same buffer. After dehydration in alcohol at −35 °C, the spermatophores were embedded in Lowicryl K4M and then UV cured for 16 h at −40 °C and after for 2 days in condition of natural light and at room temperature.

Ultrathin sections (100 nm) were transferred to 200 meshes nickel grids coated with formvar and processed for labeling as follows.

- Preparation of the phalloidin–gold complex

Purified phalloidin from *Amanita phalloides* and colloidal gold (10 nm particles in solution) were supplied by Sigma–Aldrich. For the preparation of the complex, 250 μg of the toxin were dissolved in 100 μl of distilled H_2O and mixed with 10 ml of a solution of colloidal gold as suggested by De Roe et al. (1987) and by Lachapelle and Aldrich (1988).

After centrifugation (31,300 × *g*, 40 min at 4 °C) the pellet was re-suspended in 0.5 ml of 0.1 mol/L phosphate buffer, pH 7.2 containing 0.02% PEG (polyethylene glycol, MW 20,000) and finally diluted 1:5 in 0.1 mol/L phosphate buffer, pH 7.2.

- Phalloidin–gold labeling

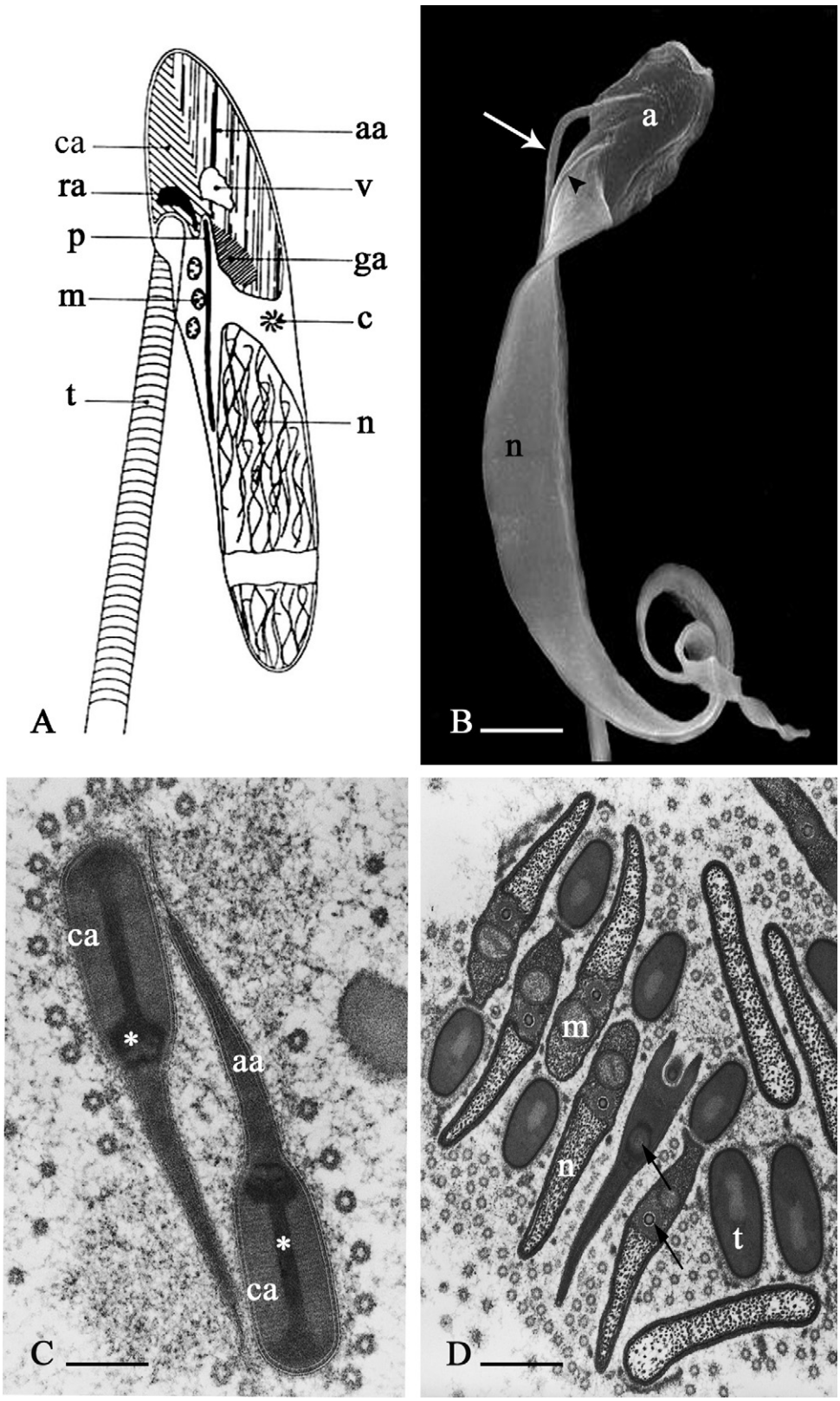

Fig. 2. (A) Schematic drawing of apical portion of the spermatozoon of oniscidean isopods (modified from Cotelli et al., 1976 and Wägele, 1992); aa, amorphous part of acrosome; c, centrioles; ca, crystalline part of acrosome; ga, granular part of acrosome; m, mitochondrion; n, nucleus; p, perforatorium; ra, reniform part of acrosome; t, tail; v, vesicle. (B) SEM micrograph of spermatozoon of *Armadillidium peraccae*. a, acrosome; arrow, tail; arrowhead, perforatorium; n, nucleus. Scale bar = 2.5 μm. (C) Cross section of a spermatophore showing the apical portion of the acrosoma of two sperm; the crystalline (ca) and amorphous part (aa) of acrosome are visible; *, dumbbell-shaped denser core of the crystalline acrosome. Scale bar = 0.3 μm. (D) Cross section of spermatozoa at different levels: it is possible to observe the nucleus (n), containing a finely fibrillar chromatin, mitochondria (m) and a thin perforatorium (arrow). Scale bar = 0.5 μm.

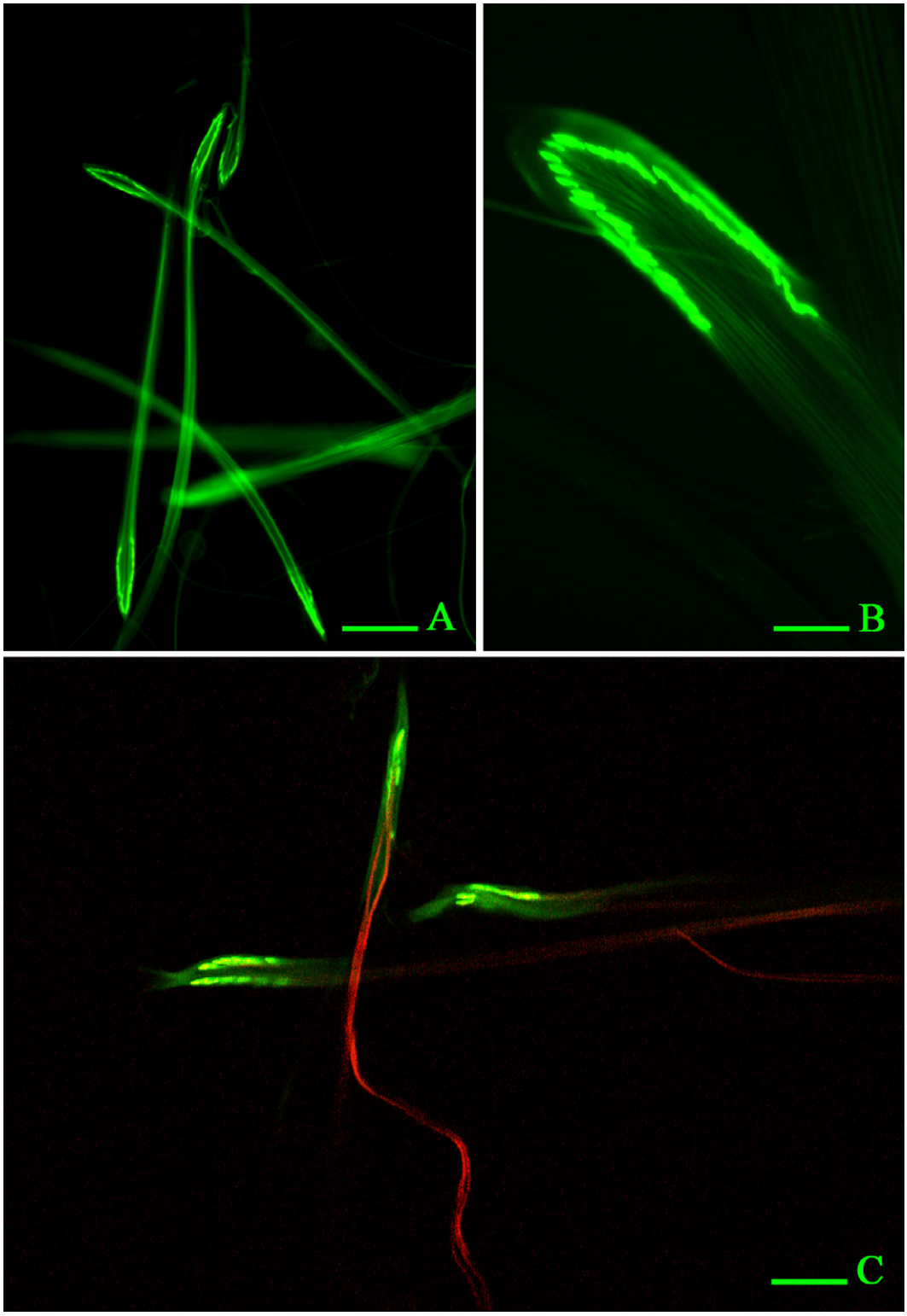

Fig. 3. (A and B) Spermatophores incubated in FITC-phalloidin. Sperm exhibit a strong fluorescence at the apical portion of their head. (C) Spermatophores incubated in FITC-phalloidin and propidium iodide and observed at confocal microscope; F-actin is clearly localized in the acrosome and in the cytoplasm above the apical portion of the nucleus. Scale bar: A = 60 μm; B = 10 μm; C = 30 μm.

Ultrathin sections were incubated on a drop of 0.5% BSA in 0.1 mol/L phosphate buffer, pH 7.2 for 10 min in a moist chamber at room temperature and then placed on a drop of phalloidin–gold diluted 1:5 with 0.1 mol/L phosphate buffer–0.1% Tween for 60 min. Afterwards the sections were rinsed 3 times in 0.1 mol/L phosphate buffer–0.1% Tween for total 30 min and then in distilled H_2O. Sections were contrasted with 2.0% uranyl acetate for 8 min and 1.0% lead citrate for 3 min at room temperature. The specimens were examined with an electron microscope Philips CM 10.

The specificity of the phalloidin–gold labeling, was verified by the following control experiments:

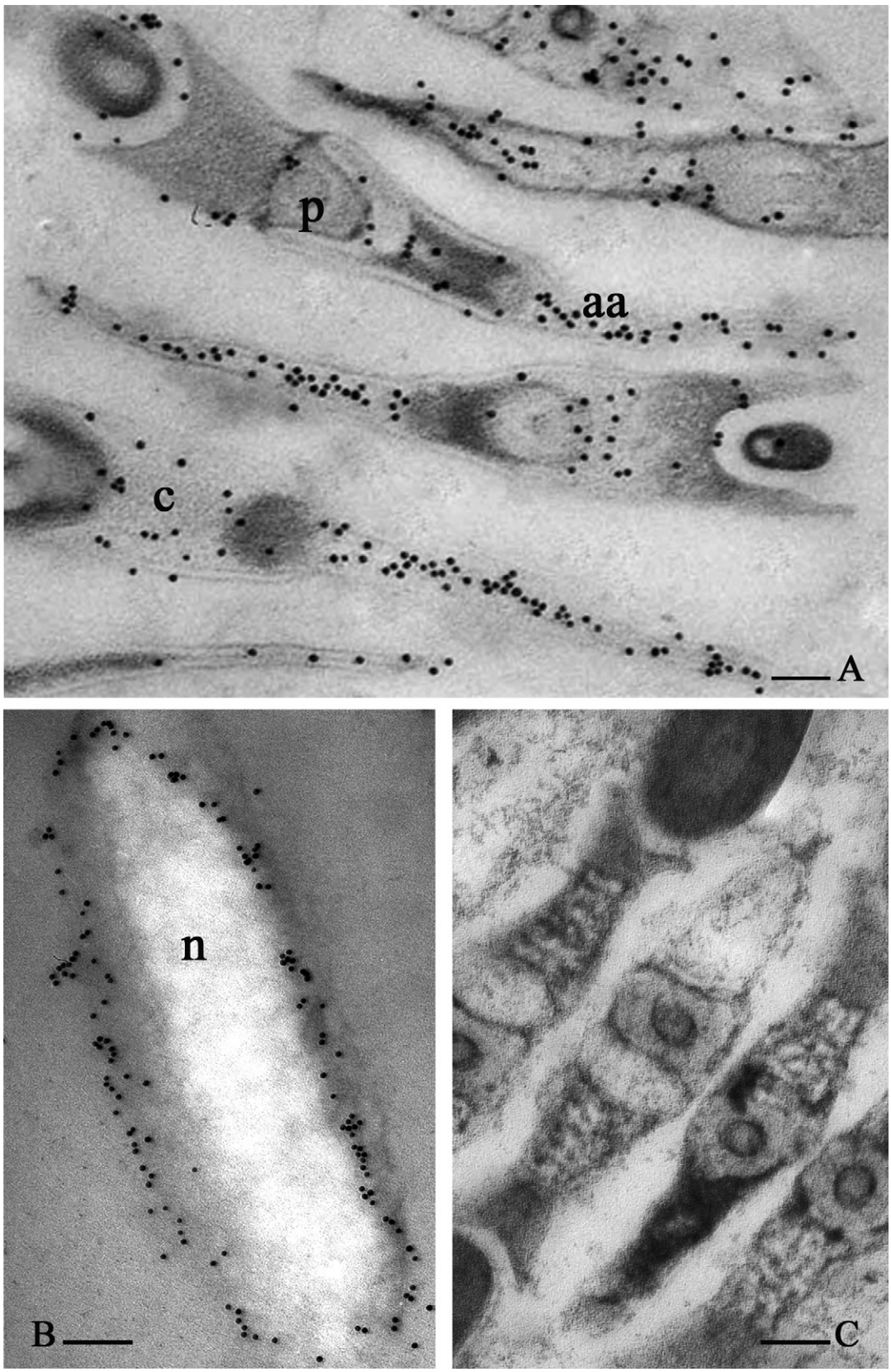

Fig. 4. Phalloidin–gold labeling of sperm embedded in Lowicryl K4M. (A) Most of the gold particles are found in correspondence of the amorphous part of acrosome (aa), around the perforatorium (p) and in the cytoplasm (c) above the apical end of nucleus. Occasionally, a non-specific binding of gold particles with material present in extracellular space was observed. (B) Numerous gold particles are present in the thin layer of cytoplasm surrounding the apical portion of nucleus (n). (C) In the specimens incubated with phalloidin before labeling with the phalloidin–gold complex or incubated in the phalloidin–gold complex in presence of F-actin, no gold particles labeling was observed. Scale bars from all the images = 0.25 μm.

(1) incubation of the sections in diluted phalloidin–gold complex in the presence of F-actin (1 mg/1 ml) (Cytoskeleton Inc, Denver);
(2) incubation of the sections in phalloidin aqueous solution (1 mg/1 ml) for 2 h at room temperature, followed by incubation in diluted phalloidin–gold complex.

3. Results

Morphology and ultrastructure of sperm and spermatophores of *A. peraccae* appear to be quite similar to those already known in literature for most species of oniscideans (Fain-Maurel, 1966, 1970; Reger and Fain-Maurel, 1973; Cotelli et al., 1976; Itaya, 1979; Reger et al., 1979; Longo and Musmeci, 2002). The spermatophore is made up of a bundle of spermatozoa – whose number in this species ranges between 30 and 40 – enclosed in a finely granular matrix and surrounded by a cone-shaped assembly of longitudinally aligned extracellular tubular structures (Fig. 1). The mature spermatozoon is an elongated cell made up of two linear components joined together: a sacciform head and a long immotile tail (Fig. 2A and B). The sperm head contains a long ribbon-like nucleus (Fig. 2A) and a wedge-shaped acrosome with a heterogeneous structure (Fig. 2A–C); between acrosoma and nucleus there is a cytoplasmic area containing some small mitochondria (Fig. 2D) and two centrioles. A thin perforatorium with a paracrystalline structure runs along the apical portion of the nucleus to reach the acrosoma (Fig. 2B and D).

The sperm tail is a long rod with a paracrystalline structure, round or slightly ovoidal in cross section of its central portion (Figs. 1 and 2D).

The goal of this research was to show, for the first time, the eventual presence of cytoskeleton components within the sperm head.

Sperm incubation in anti-tubulin antibody resulted in the appearance of a very weak fluorescence only at the cytoplasmic area between acrosome and nucleus where the pair of centrioles is located; therefore, no other microtubular component is present in the sperm head.

Instead, after incubation in FITC-phalloidin, sperm of *A. peraccae* exhibit an intense fluorescence exclusively localized in the apical portion of their head (Fig. 3A and B); the observations carried out with the confocal laser microscope, after double incubation in FITC-phalloidin and propidium iodide, corroborate the presence of a consistent lot of F-actin in the region of the sperm head above the nucleus (Fig. 3C).

To confirm the presence and the exact localization of F-actin, some ultrathin sections of sperm embedded in Lowicryl K4M were incubated in a phalloidin–gold complex. The labeling signal was strong and clearly localized both at the amorphous part of the acrosome and at the cytoplasmic area between acrosoma and nucleus (Fig. 4A); furthermore, numerous gold particles were observed in the thin cytoplasmic layer between plasma membrane and nuclear envelope at the apical portion of the nucleus (Fig. 4B).

Only very few colloidal gold particles were occasionally found without a specific binding on the ultrathin sections observed.

The specificity of the binding of the complex was assessed by the control experiments based on the preliminary incubations of the ultrathin sections in F-actin or in phalloidin–gold complex in presence of abundant F-actin; in all the sections observed the presence of gold particles was never detected (Fig. 4C).

4. Discussion

Since the first description by Gilson (1886), who depicted it like a whip in which the whip is the sperm head and the stick is its long tail, the spermatozoon of isopods, as well as that of other peracarids, has been always regarded as entirely devoid of motility (Blanchard et al., 1961; Fain-Maurel, 1970; Cotelli et al., 1976; Fain-Maurel et al., 1975a; Pochon-Masson, 1978).

The lack of sperm motility *in vitro* does not necessarily exclude the possibility of their motility *in vivo*, as observed for the peculiar giant sperm of ostracods that manifest their motility only after their transfer into the spermatheca (Lowndes, 1935). Probably, also the Oniscidea sperm, normally devoid of motility, may acquire a temporary capacity of movement during the interaction with the female gamete; the temporary acquisition of motility could be result from the release of molecules from egg surface or the occurrence of appropriate physiological conditions.

This hypothesis, which could be considered fanciful, is supported by:

(1) the observation reported by Reibisch (1926) of a worm-like movement of the sperm head of Caprellidae (whose organization is largely comparable to that of the Oniscidea sperm) and the report at of a slow worm-like movement of the sperm tail of *Talitrus* (Amphipoda) placed in contact with eggs taken from the brood pouch of the female immediately after their deposition in this structure (Williamson, 1951);
(2) the observation that the Oniscidea sperm, during their storage into the female genital tract, lose frequently the tail and it is therefore likely that egg fertilization is realized only by the sperm head (Longo and Trovato, 2008).

The results of our research, which made it possible, for the first time, to detect through the use of FITC-phalloidin a consistent presence of F-actin into the sperm head of *A. peraccae*, provide a strong support to the hypothesis previously reported; the ultrastructural investigation has confirmed the presence of F-actin in the amorphous part of the acrosome, in the cytoplasmic region between the acrosome and nucleus and also within the thin cytoplasmic ring surrounding the apical portion of the nucleus itself.

The specificity of the interaction between phalloidin and F-actin was confirmed by the control experiments.

Such a large amount of F-actin could reasonably be linked with the need of an acquisition of motility, even if only temporary, carried by the sperm at the time of its interaction with the female gamete.

Will be further investigations, especially experimental ones, to provide the answer to this fascinating enigma.

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