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Sperm morphology of the neotropical harvestman *Iporangaia pustulosa* (Arachnida: Opiliones): Comparative morphology and functional aspects

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Abstract

We describe herein the sperm morphology of the harvestman *Iporangaia pustulosa*. Adult males were dissected, the reproductive tract was schematized and the seminal vesicle was processed by light, transmission and scanning electron microscopy. The male reproductive tract is composed of a tubular testis, two deferent ducts, a seminal vesicle, a propulsive organ and a penis, similar to that observed in other Opiliones. The spermatozoa from the seminal vesicle are oval, aflagellate and immotile, presenting a nucleus surrounding an invagination of the cytoplasm, as well as a complex acrosome and projections on the cell surface. In the testis, spermatozoa are devoid of projections. In the seminal vesicle, they gradually acquire the projections with tufts adhering to it. Consequently, spermatozoa in various distinct stages of projection development can be found in the seminal vesicle. We believe that these projections (1) could help transport sperm along the male and perhaps female reproductive tracts; (2) are used to anchor the spermatozoa inside the female spermatheca in order to avoid mechanical displacement by the genitalia of other males and (3) may play a role in oocyte recognition. We propose that the evolution of aflagellarity in Opiliones is related to the unique morphology of the female reproductive tract. Since eggs are fertilized on the tip of the ovipositor just prior to being laid, there is no advantage favoring sperm mobility. Additionally, female sperm receptacles are small and males that produced small spermatozoa would have a higher chance of fertilizing more eggs.

Keywords: Aflagellate sperm; Harvestman; Sexual selection; Sperm competition; Surface projections

1. Introduction

The phylum Chelicerata comprises more than 100,000 described species and is the most diversified arthropod group considering sperm morphology (reviewed in Alberti, 1995, 2000). Especially within the class Arachnida, the spermatozoa present great morphological variation, including filiform flagellate forms as observed in the order Scorpiones, spiraled forms in the orders Pseudoscorpiones, Amblypygi, Araneae, and Ricinulei, and even spherical and aflagellate forms in the orders Opiliones, Palpigradi, Solifugae, and Acari. Arachnid spermatozoa may also vary in length, ranging from less than 2 μm in some harvestmen to nearly 1 mm in ticks (Alberti, 1995, 2000).

Most studies describing sperm morphology in Opiliones were published in the 1970s and 1980s (Reger, 1969; Juberthie and Manier, 1976, 1977a,b,c, 1978; Juberthie et al., 1976; Tripepi, 1983; Jones and Cokendolpher, 1985). These studies focused on species from the northern hemisphere belonging to the families Sironidae (suborder Cyphophthalmi), Phalangiidae (suborder Eupnoi), Nemastomatidae (suborder Dyspnoi), and Cosmetidae (suborder Laniatores). In all cases, the aflagellate spermatozoa are solely comprised of a nucleus and an acrosome, which has not always been properly identified. The spermatozoa are densely concentrated in the seminal vesicle, rendering recognition of their organelles very difficult (review in Juberthie and Manier, 1978).

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Although a general framework of sperm morphology exists for the Opiliones, there are still many uncertainties on ultrastructural characteristics, the motility process, and the fusion of gametes in these species in which the acrosome is absent. Since the early papers on the subject, the methodology has changed considerably with improvements in instrumentation. Ultrastructural analysis using the current techniques and equipment could provide new information regarding the composition and location of the organelles, including acrosome, nucleus, centrioles, and mitochondria, and also increase our understanding of their structure and function. Moreover, detailed information on sperm morphology in arachnids may also provide useful characters for both phylogenetic analysis (e.g., Alberti and Peretti, 2002; Alberti, 2005) and studies of sexual selection (e.g., Morrow, 2004).

In this study we describe the male reproductive tract and spermatozoa morphology of the harvestman *Iporangaia pustulosa* Mello-Leitão 1935 (Laniatores: Gonyleptidae). Females of *Iporangaia pustulosa* lay eggs on the undersurface of shrub leaves growing at the margin of streams and the eggs are covered by an abundant transparent mucus coat. The offspring is guarded by the males, which may be found resting at the leaf base or on the upper surface of the leaf containing the egg-batch. Males copulate with several females and the batches are generally composed of eggs in several stages of embryonic development. Females are iteroparous and copulate with several males throughout their lives (Machado et al., 2004). This is the first study to investigate a representative of the neotropical Gonyleptidae, which is one of the largest family in the order and has been the focus of many recent behavioral studies (see references in Machado and Raimundo, 2001; Machado, 2002; Hara et al., 2004). Since the reproductive biology of *I. pustulosa* has been recently reported (Machado et al., 2004), we also integrate our morphological data with behavioral information in this harvestman species.

2. Methods

Twenty adult males of *Iporangaia pustulosa* were collected at the Parque Estadual Intervales (24°14′S; 48°04′W; 800 m alt.), close to the municipality of Ribeirão Grande, southern São Paulo State, Brazil (for details on this site see Leonel, 1994). Voucher specimens were deposited in the Museu de Zoologia da Universidade de São Paulo (MZSP) and Museu de História Natural da Universidade Estadual de Campinas (ZUEC).

2.1. Male reproductive tract anatomy

Live males were ventrally dissected in 0.1 M sodium phosphate buffer using a stereoscopic microscope. The reproductive tract was removed and a schematic drawing was made.

2.2. Light microscopy

2.2.1. Spermatozoa suspension

Drops of sperm obtained from the seminal vesicle, suspended in 0.1 M sodium phosphate buffer, were spread on glass slides, fixed in 4% paraformaldehyde for 30 min at room temperature and quickly washed in the same buffer. To study general characteristics, the suspensions were stained with toluidine blue, washed, mounted and observed with a photomicroscope. To study the nuclear structure, sperm suspensions were stained with 0.2 μg/ml 4,6-diamino-2-phenylindole (DAPI) in PBS for 30 min, washed in running water and immersed in 0.1 M McIlvane buffer for 5 min in the dark, at room temperature. They were mounted and photographed with a microscope equipped with a BP 360-370 filter (Olympus BX60).

2.2.2. Histology

Seminal vesicles were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 12 h at 4 °C, rinsed in 0.1 M sodium phosphate buffer, dehydrated in acetone and embedded in epoxy resin. The tissues were sectioned at 1–2 μm, mounted on microscope slides, stained with toluidine blue, pH 4.0 and photographed with a microscope (Olympus BX60).

2.3. Transmission electron microscopy

Testis and seminal vesicles were fixed in 2.5–3% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate or sodium phosphate buffers for intervals of 12–72 h, at 4 °C. They were postfixed in 1% osmium tetroxide in the same buffer for 3–5 h, dehydrated in acetone and embedded in epoxy resin. For better protein and microtubule preservation, other testis and seminal vesicles were fixed in 2.5% glutaraldehyde and 1% tannic acid in 0.1 M sodium phosphate buffer, with 1.5% sucrose and 5 mM calcium chloride for 3 days at 4 °C. They were washed in phosphate buffer and stained with 1.5% uranyl acetate for 2–5 h at room temperature before dehydration in acetone and epon embedding (Dallai and Afzelius, 1990). Sections, stained with uranyl acetate and lead citrate, were observed in a LEO 906 Zeiss electron microscope.

2.4. Scanning electron microscopy

Sperm suspensions were spread on round cover slips and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer with 1.5% sucrose and 5 mM calcium chloride for 15 min, rinsed in the buffer for 15 min and post fixed in 1% osmium tetroxide. After rinsing in buffer and dehydrating in ethanol or acetone, they were critical point dried, gold sputtered and observed in a JEOL 5800LV scanning electron microscope.

3. Results

The reproductive tract of *Iporangaia pustulosa* is comprised of a tubular U-shaped testis connected to two deferent ducts that transfer spermatozoa into a large seminal vesicle. Following the seminal vesicle, there is a propulsive organ and an eversive penis (Fig. 1).

The spermatozoa of *I. pustulosa* obtained from the seminal vesicles are dispersed in the lumen (Figs. 2 and 3) and present no apparent motility. They are aflagellate, oval and average 12 μm in length by 2 μm in width, tapering to fine points (Figs. 3 and 4). Spermatozoa from the seminal vesicle consist of a nucleus surrounding an invagination of the cytoplasm, as well as an acrosome and many projections on the cell surface (Figs. 5–8).

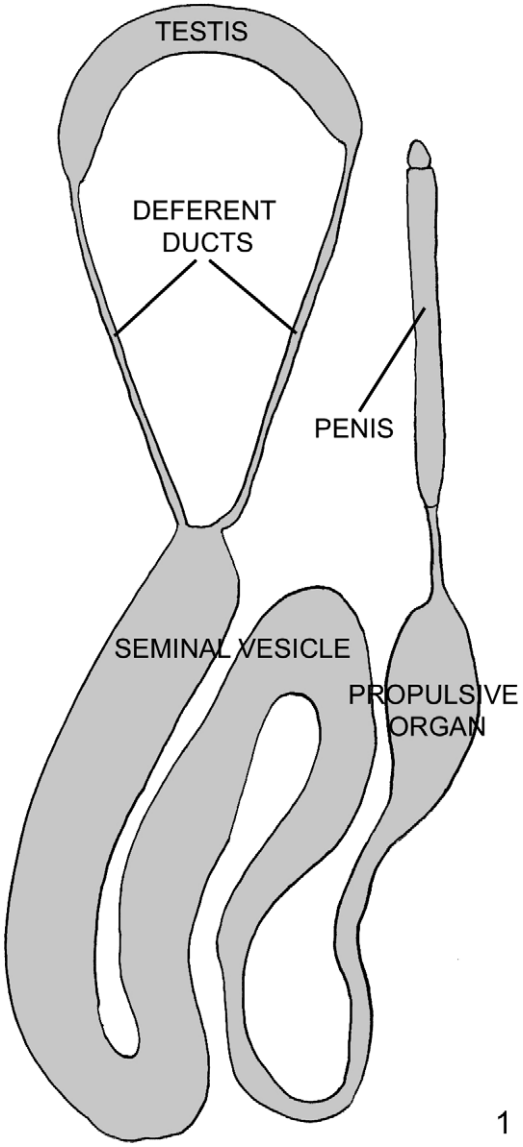


Fig. 1. Schematic drawing of the male reproductive tract of *Iporangaia pustulosa*.

The nucleus is uniformly condensed, occupying approximately 90% of the spermatozoon volume, resembling the shape of this cell (Figs. 4–6).

The invagination, a cytoplasmic portion that is engulfed by the nucleus during the spermiogenesis, conforms to the general oval pattern, appearing pear-shaped in cross sections, remaining open on the surface opposite to the acrosome (Figs. 7 and 8). This cytoplasm is less electron-dense than the nucleus and is devoid of organelles. No microtubular structures, such as an axoneme or centriole, were found. The acrosome is partially immersed in the nucleus, opposite to the nuclear opening, and is comprised of a globular acrosomal vesicle in a cup-shaped layer of remaining cytoplasm, which underlies the vesicle and contains associated folded membranes and mitochondria-like structures (Figs. 8–10). Externally, a thin, smooth extracellular layer covers the acrosome.

Cell suspensions from the seminal vesicle, viewed in scanning electron microscopy, showed different types of spermatozoa: a thinner cell with a smooth surface, measuring about 11.42 μm by 1.7 μm, and a wider one with a rough surface, measuring about 14.12 μm × 3.2 μm. In all the suspensions observed, the number of wider spermatozoa was considerably higher than the thinner ones (Fig. 11). Transmission electron microscopy revealed the presence of a great number of projections covering the cell surface in the seminal vesicle, corresponding to the rough surface observed with the scanning electron microscope (Fig. 12).

All of the observed sperm types presented the same intracellular structures, with the same arrangement. In the testis, the late spermatids and early spermatozoa presented a simple, irregular waving plasma membrane without external coats (Fig. 13).

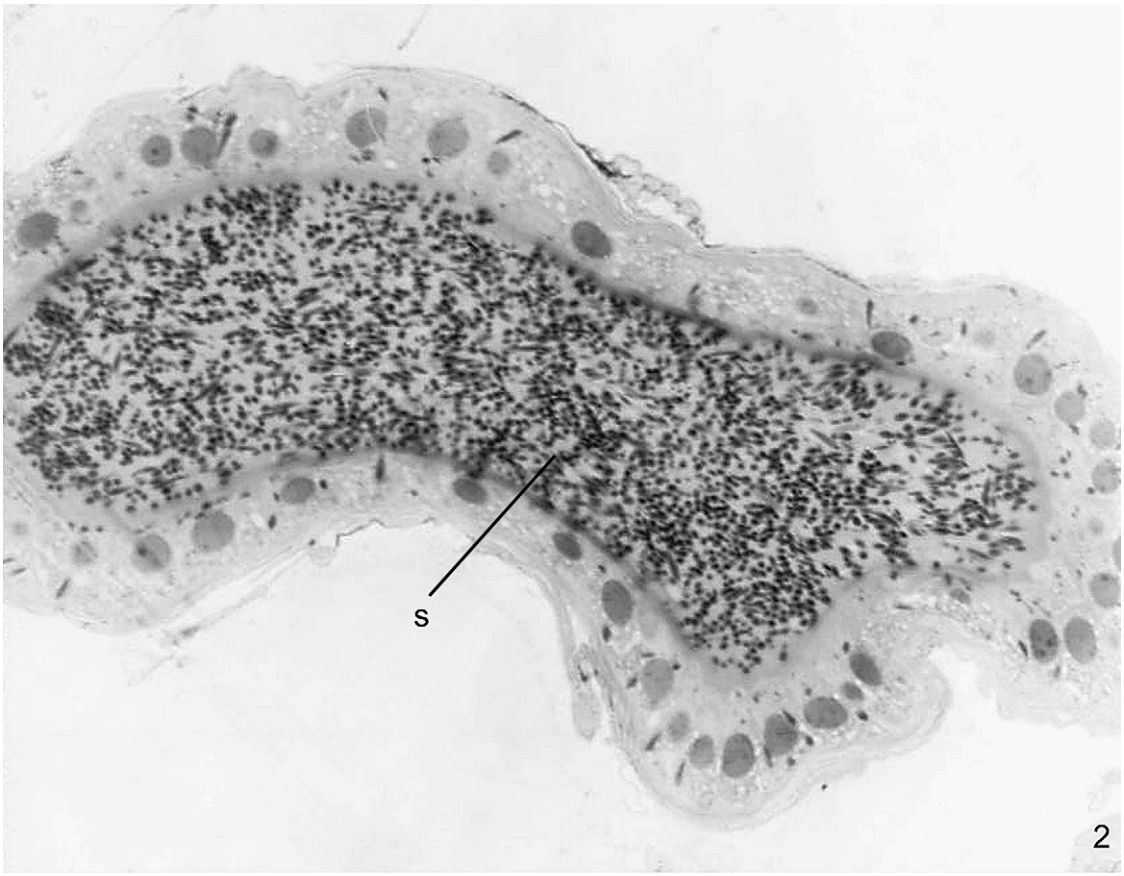
After leaving the testis, the sperm membrane forms regular scallops filled with a dense material and small irregular external tufts adhering to each scallop (Figs. 14 and 15). Then, the spermatozoa gradually acquire projections adhering to the scallops of plasma membrane (Figs. 16 and 17) except above the acrosome (Fig. 5). These projections are covered by an amorphous material on their tips, resembling a glycocalyx and a thin electron-lucent layer (Figs. 16 and 18). As a result, spermatozoa in various distinct stages of projection development can be found in the seminal vesicle.

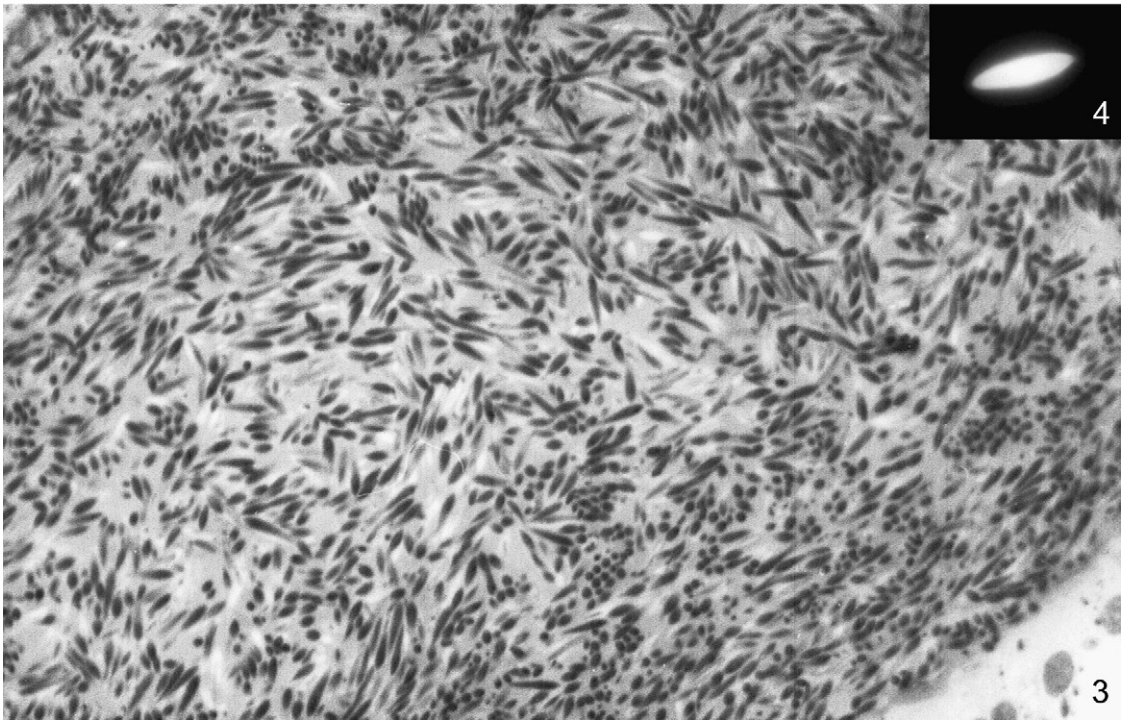
In the proximal region of the seminal vesicle, in relation to the testis, more spermatozoa are found with short projections with a delicate linear covering. In the medial region, they occur in approximately equal numbers, while in the terminal region the majority of the spermatozoa have long projections.

4. Discussion

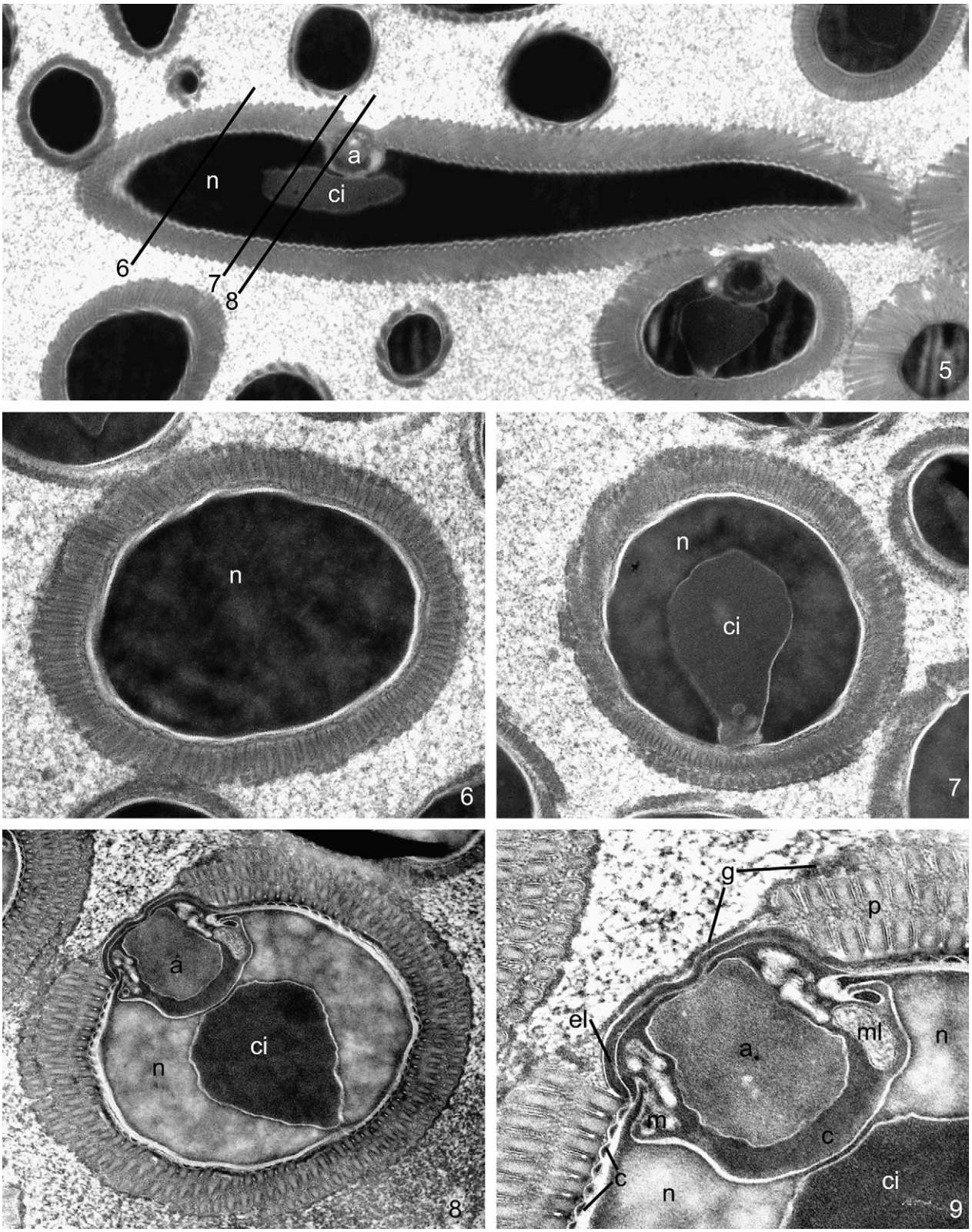
4.1. Comparative morphology and functional aspects

The reproductive tract of *Iporangaia pustulosa* has the same components as previously described in the literature for other harvestman species (Berland, 1949; Juberthie, 1965; Cokendolpher and Jones, 1991). There is a tubular testis





Figs. 2–4. Light microscopy. (2) Spermatozoa (s) dispersed in the lumen of the seminal vesicle (×67). (3) Large magnification of spermatozoa dispersed in the lumen of the seminal vesicle (×120). (4) Nucleus stained with DAPI technique (×300).



Figs. 5–9. Transmission electron microscopy (TEM). (5) Longitudinal section of the spermatozoon showing the nucleus (n); the acrosome (a) and the cytoplasmic invagination (ci). The lines represent the cross sections of Figs. 6–8 (×12,900). (6–8) Cross sections corresponding to different regions of the spermatozoon shown in Fig. 5 (×27,500), (×24,300), (×29,400). a, acrosome; ci, cytoplasmic invagination; n, nucleus. (9) Detail of the acrosomal region. (×79,800). a, acrosome; c, cytoplasm; ci, cytoplasmic invagination; el, extracellular layer; g, glycocalyx; m, folded membranes; ml, mitochondria like structure; n, nucleus; p, projections.

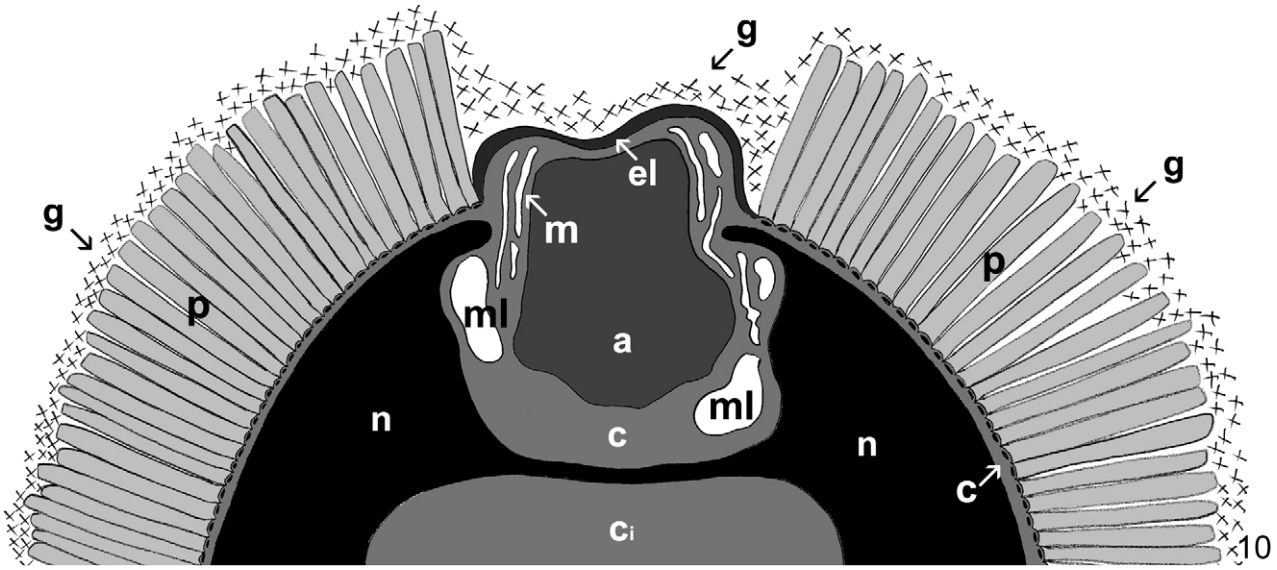


Fig. 10. Schematic drawing of the acrosomal region. a, acrosome; c, cytoplasm; ci, cytoplasmic invagination; el, extracellular layer; g, glycocalyx; m, folded membranes; ml, mitochondria like structure; n, nucleus; p, projections.

and a large seminal vesicle, suggesting that males are able to produce large amounts of sperm (Birkhead and Moeller, 1998). Two deferent tubules connect the testis to the seminal vesicle, where the sperm is stored. A propulsive organ is located immediately in front of the penis, which is probably related to the eversion of the male genitalia through hydraulic pressure of the hemolymph (J.W. Shultz, personal communication).

An extensive review of the literature revealed that aflagellate sperm independently evolved in at least 36 taxonomic groups, including representatives of many arthropod orders (Morrow, 2004). Aflagellate sperm are apparently produced by all species of the order Opiliones, except for members of the suborder Cyphophthalmi, which retain a non-motile axoneme in the sperm (Alberti, 1995, 2000, 2005).

As a representative of the suborder Laniatores, the spermatozoa of *I. pustulosa* lack a flagellum. However, contrary to prior studies of most harvestman species (e.g., Juberthie and Manier, 1976, 1977a,b,c; Tripepi, 1983) no evidence of centrioles was encountered in spermatozoa of *I. pustulosa*. Even after applying a specific fixation for microtubules using tannic acid (Dallai and Afzelius, 1990), we did not identify these structures.

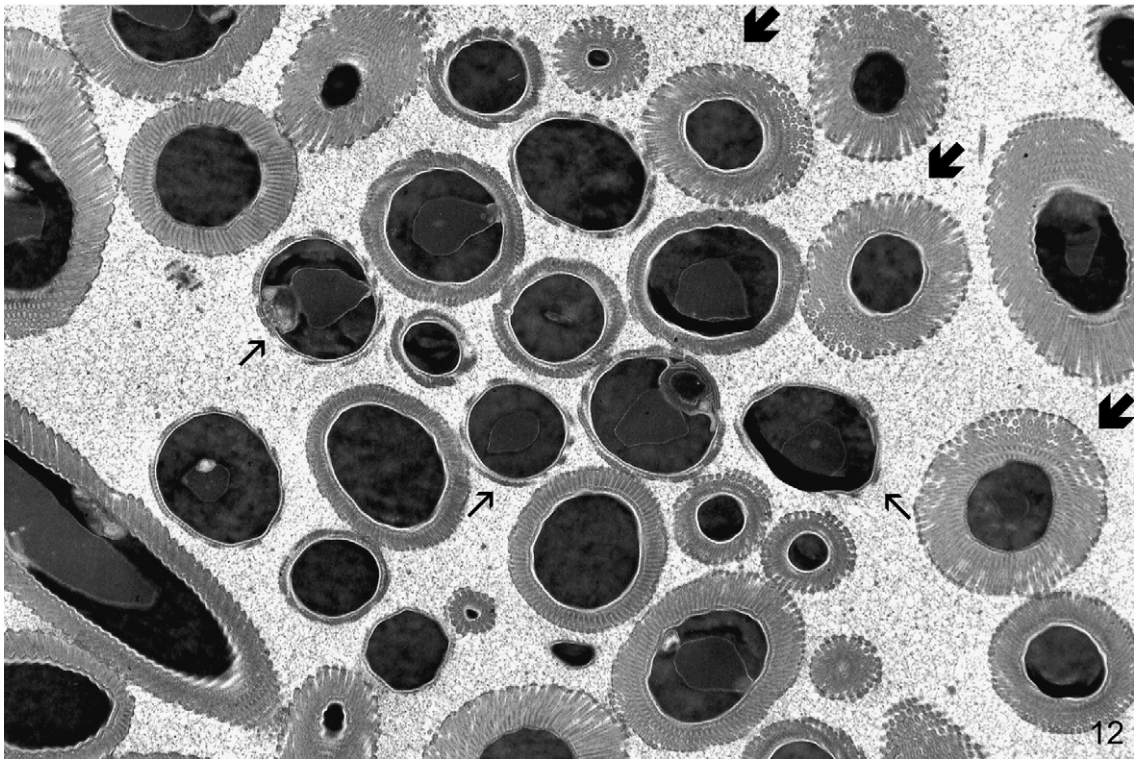
The ultrastructure of the acrosome of *I. pustulosa* has never been described in other harvestmen species. A globular acrosomal vesicle, as occurs in *I. pustulosa*, was observed only for the Laniatores *Epedanellus tuberculatus*, *Cynortoides cubanus* (Juberthie and Manier, 1977c) and *Vonones sayi* (Jones and Cokendolpher, 1985), but for these three species, the descriptions are not detailed. In *V. sayi*, the acrosome represented ''an electron translucent structure protruding from the side of the spermatozoa connected to a dense structure embedded in the spermatozoa wall'', as described by the authors. We consider that the ''dense structure'' described for *V. sayi* is the same as the acrosome of *I. pustulosa*, while their ''electron translucent structure'' is really a large secretion deposit similar to what we also found covering different regions of the spermatozoa (not shown in this study).

In general, the harvestman acrosome is a flat dense structure as observed in the Dyspnoi *Mitostoma pyrenaeum* and *Nemastoma bimaculatum* (Juberthie and Manier, 1977a) and in the Eupnoi *Phalangium opilio* (Tripepi, 1983). In the Cyphophthalmi *Siro rubens* (Juberthie et al., 1976) and in the Dyspnoi *Trogulus nepaeformis* (Juberthie and Manier, 1977b) and *Ischyropsalis luteipes* (Juberthie and Manier, 1976), the flat dense acrosome possesses a rod inserted into the nucleus.

The cytoplasmic invagination penetrating the nucleus is not a unique characteristic of *I. pustulosa* and has been previously found in representatives of the suborders Laniatores, such as the cosmetids *Vonones sayi* (Jones and Cokendolpher, 1985), *Cynortoides cubanus* and *Epedanellus tuberculatus* (Juberthie and Manier, 1977c), as well as in the Eupnoi, such as the sclerosomatid *Leiobunum* sp. (Reger, 1969); and Dyspnoi, such as the nemastomatids *Mitostoma pyrenaeum* and *Nemastoma bimaculatum* (Juberthie and Manier, 1977a).

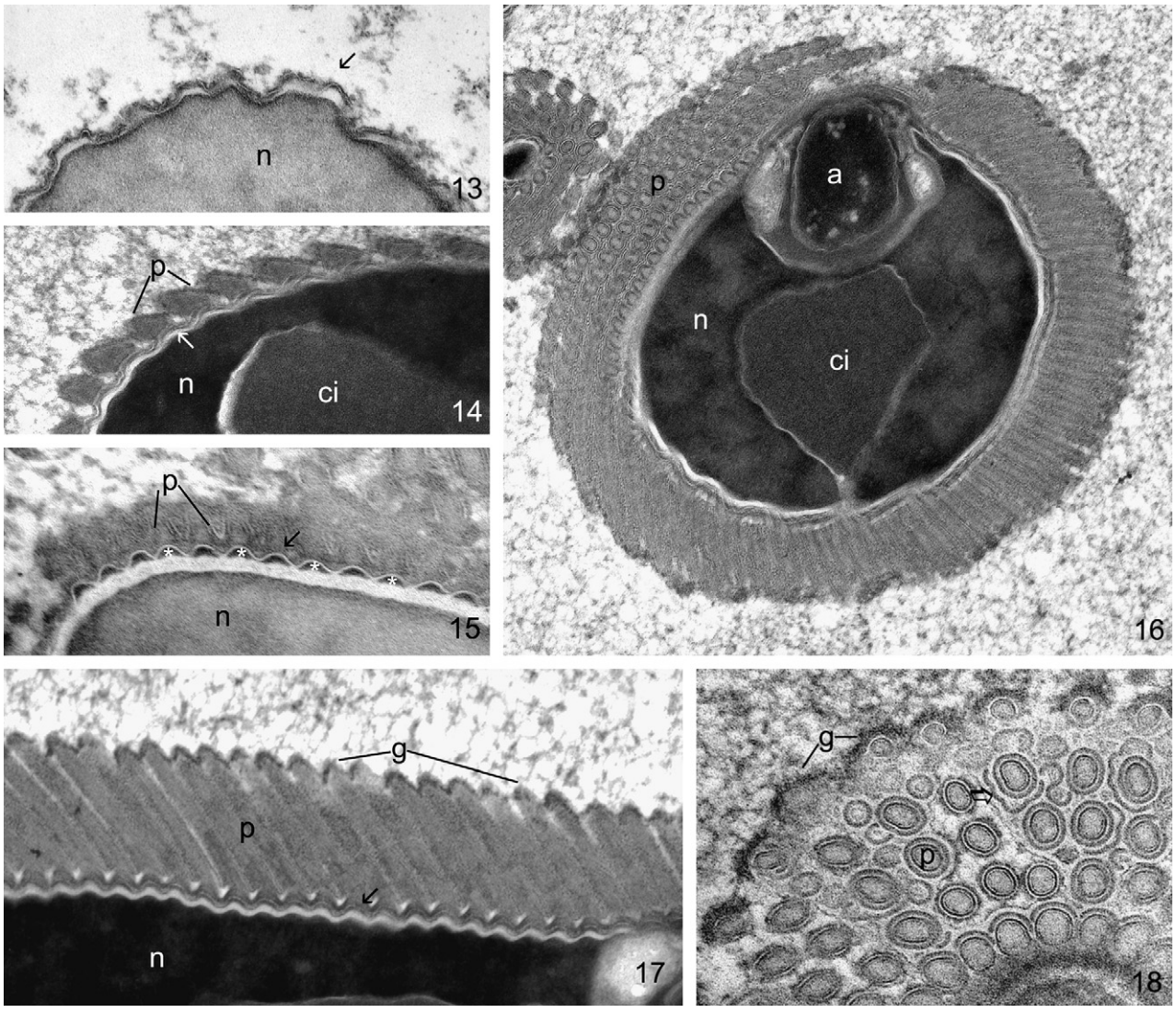
The ultrastructure of spermatozoa obtained from the seminal vesicle of *I. pustulosa* is particularly similar to that of the cosmetid *V. sayi*, in which the cytoplasmic invagination, the homogeneously condensed nucleus, the acrosome and surface projections were also reported (Jones and Cokendolpher, 1985).

Unfortunately, most prior studies do not report the morphology of sperm obtained from the seminal vesicle. The projections of *I. pustulosa* and *V. sayi* occur only in spermatozoa from the seminal vesicle. Although the contents of the seminal vesicle have already been investigated in the



Figs. 11 and 12. Two sperm morphologies: observed in Scanning (Fig. 12) and Transmission (Fig. 11) Electron Microscopes. The thinner one with smooth surface (thin arrow) and the wider one with rough surface (large arrows). In Fig. 12. notice the presence (large arrows) and absence (thin arrows) of projections on the surface of the spermatozoa representing both surfaces observed in Fig. 11 (×5,100) (×10,200).

phalangiid *Phalangium opilio* these structures were not found (Tripepi, 1983). It is possible that this morphological feature is restricted to the suborder Laniatores, or at least to the superfamily Gonyleptoidea. Projections of the surface are also found in the aflagellate sperm of the Solifugae *Oltacola gomezi* and *Procleobis patagonicus* (Alberti and Peretti, 2002) and in the aflagellate sperm of some Acari (Reger, 1961, 1963, 1971; Witalinski and Dallai, 1994; Alberti,



Figs. 13–18. TEM. (13) Spermatozoon in the testis with simple and irregularly waving plasma membrane (arrow). (×36,000) n, nucleus. (14) Plasma membrane (arrow) with irregular small tufts and early projection. (×55,700). ci, cytoplasmic invagination; n, nucleus; p, projections. (15) Plasma membrane (arrow) with regular scallops filled with dense material (*). (×98,900). n, nucleus; p, projections. (16) Spermatozoon showing developed projections around the cell (×26,900). a, acrosome; ci, cytoplasmic invagination; n, nucleus; p, projections. (17) Longitudinal section showing the detail of the projections with glycocalyx. (×41,900). g, glycocalyx; n, nucleus; p, projections; arrow, plasma membrane. (18) Cross section of the projections (p) with linear covering (large arrow) and glycocalyx (g) (×80,900).

2000). These projections found in different groups do not necessarily represent the same function.

We believe that the projections on the cell surface of *I. pustulosa* sperm are extracellular structures, and do not represent microvilli as observed in *Vonones sayi*. First, the mature germ cells in the testis do not present projections and, in this late spermiogenesis stage, these cells no longer possess the organelles necessary for developing the projections. Second, we believe that the scalloped membrane represents a modified plasma membrane; therefore, these projections are externally located. The thin electron-lucent layer and the amorphous material located on the projections, as well as the dense layer over the acrosome, probably present carbohydrate constituents, similar to a glycocalyx.

The morphologically different spermatozoa of *I. pustulosa* do not represent a true dimorphism. They can be considered different developmental stages that occur during their maturation process. The thin spermatozoa, with a smooth surface, are cells that recently exited the testis, while the wide ones with a rough surface are mature cells. We believe that the projections are developed during the final, extratesticular spermatic maturation; therefore, their presence identifies mature cells. The origin of these projections is unknown, but we believe that the epithelial cells from extratesticular ducts (deferent ducts and seminal vesicle) produce the material that will be organized into these complex coats since the complete projections were found only posterior to the testis.

Projections of the cell surface are also found in the aflagellate sperm of some Acari (Reger, 1961, 1963, 1971; Witalinski and Dallai, 1994; Alberti, 2000); however, in this group, the projections are not extracellular structures. According to Reger (1961, 1963), Feldman-Muhsam (1986) and Witalinski

and Dallai (1994), the sperm motility is due to these foldings. Since we observed no sperm motility *in vitro* and we consider these projections extracellular structures, we cannot affirm this activity. Although, the role of the projections in Opiliones sperm remains unclear, we propose here three non-mutually exclusive hypotheses to explain their function. First, the rough projections may facilitate sperm transport through the male and perhaps female reproductive tracts. A second possibility is that the projections are used to anchor the spermatozoa inside the female spermatheca to prevent mechanical displacement by the genitalia of other males. Third, the presence of carbohydrate tufts on the projection tips suggests that they may play a role in oocyte recognition, as occurs in other animals.

4.2. Sexual selection and sperm morphology

One of the most important selective forces in the evolution of sperm morphology is sperm competition (Birkhead and Moeller, 1998). Since aflagellate sperm is probably less costly to produce, both in terms of energy and time, selection could favor the loss of the sperm flagellum and any other motile mechanisms in monandrous species (Morrow, 2004). Conversely, species showing paternal care are expected to present numerous male strategies to insure paternity. In these species, male–male competition is likely to be intense either by means of sperm displacement of previous males or through sperm–sperm interaction (Birkhead and Moeller, 1998). Recently, Morrow (2004) found no evidence suggesting that the evolution of aflagellate sperm could be linked to the removal of selective pressures generated by sperm competition.

In this paper we provide information on sperm morphology of the neotropical harvestman *I. pustulosa*, whose males take care of eggs and early hatched nymphs (Machado et al., 2004). Since females are polyandrous, sperm competition is typically severe, since low confidence of paternity reduces the benefits and increases the costs of male caring behavior (Kokko and Jennions, 2003). In this scenario, sperm should be highly motile, but our results show that the sperm of *I. pustulosa* are aflagellate, and direct observation indicates that they are incapable of independent movement.

Observations of some taxonomic groups indicate that the loss of the flagellum is to some extent gradual (Morrow, 2004). In these cases, an intermediate stage occurs where the flagellum first becomes immotile in phylogenetically basal groups before the axoneme degenerates entirely in derived groups. Opiliones provide an example of this sequence of events since species of the basal suborder Cyphophthalmi retain a non-motile axoneme within the sperm (Alberti, 1995). The complete loss of the axoneme in the suborders Eupnoi, Dyspnoi, and Laniatores, however, is probably not correlated with relaxation of the selection pressure from sperm competition on sperm morphology.

Recent studies on species of Cyphophthalmi suggest that the first harvestmen transferred the sperm via spermatophores (Karaman, 2005). Females probably stored the spermatozoa in the sperm receptacles, placed near the tip of the ovipositor, as they do presently (Juberthie and Manier, 1978). Since eggs are fertilized at the tip of the ovipositor just prior to being laid (Blanc, 1880; de Graaf, 1882), sperm do not need to travel; thus, there is no pressure favoring sperm mobility. This hypothesis may also apply to the remaining suborders of Opiliones because the position of the sperm receptacles is the same.

Another hypothesis for the evolution of aflagellarity in Opiliones is related to the size of the sperm receptacles, which is very small when compared to other arachnid groups, such as spiders (Foelix, 1996) and scorpions (Sissom, 1990). Due to their small size, it is possible that these organs have a limited capacity to store sperm. In this context, the loss of the flagellum is expected because this structure would needlessly occupy space inside the sperm receptacles and because the spermatozoa are not engaged in a race to reach the eggs. It is noteworthy that the two hypotheses raised here are not mutually exclusive, and both rely on the unique morphology of the female ovipositor to explain the evolution of aflagellarity in the order Opiliones.

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