Ultrastructure of spermatozoa of different species of Neogoveidae, Sironidae and Stylocellidae (Cyphophthalmi: Opiliones)

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ABSTRACT Contrib. Nat. Hist. 12: 53–69.

The present investigation is based on four species belonging to three cyphophthalmid families (Neogoveidae: *Metagovea* sp.; Sironidae: *Suzukielus sauteri*; Stylocellidae: *Fangensis insulanus*, *Stylocellus* sp.) investigated for the first time with respect to spermatogenesis. Results of previous studies on three further species of Sironidae (*Siro rubens*, *Siro exilis*, *Cyphophthalmus duricorius*) are also included. Spermatogenesis in Cyphophthalmi has been characterised by sperm-dimorphism. That means two different types of spermatozoa develop, the fertile eusperm and the non-fertile parasperm. However, this peculiarity was until now only known from members of the family Sironidae. The results presented here on representatives of two further families indicate that this peculiar and apomorphic feature is a general trait of Cyphophthalmi. The sironid eusperm develop through a flagellate euspermatid. In later stages of spermiogenesis the flagellum will be retracted into the sperm cell body resulting in the formation of a crypt and the axoneme forming a ring of microtubules. Since a crypt is present in all observed cyphophthalmids studied so far, it is likely that this retraction of a flagellum occurs also in other cyphophthalmids. The eusperm are generally dish-like (in different variations). The parasperm are bigger than the eusperm and they differ in shape, the former being characterised by their many long microvilli. The observed species differ strikingly with respect to the structure of the eusperm. Furthermore, an acrosomal complex may be present or lacking. The shape of the nucleus, structure of the crypt, the arrangement of the microtubules, and the location of the mitochondria may be different. In addition specific peculiarities are observed, e.g., radiating microvilli in *Stylocellus* sp. and dense inclusions in *Metagovea* sp. First phylogenetic implications of the results are indicated.

Introduction

Cyphophthalmi or mite harvestmen represent a group which is in recent publications considered as representing the sister group of all the other Opiliones (e.g., Shultz 1998, Giribet & al. 1999, 2002). It comprises about 170 species in at least 6 families Troglosironidae, Sironidae, Pettalidae, Stylocellidae, Neogoveidae and Ogoveidae (Giribet 2000, Pinto-da-Rocha & al. 2007, Giribet: http://giribet.oeb.harvard.edu/Cyphophthalmi/). The group is rather poorly investigated, in particular with regard to internal anatomy and fine structure. The internal anatomy was studied by Janczyk (1956). Further aspects were investigated by, e.g., Juberthie (1961, 1964, 1967), Juberthie & al. (1976), Martens (1979), and Gutjahr & al. (2006). Only recently, it became evident that sperm transfer likely occurs via spermatophores (Karaman 2005, Novak 2005, Schwendinger & Giribet 2005). In 1964 Juberthie described the sperm balls in *Siro rubens* consisting of spermatozoa and so-called protective cells surrounding a center of secretion. It was later shown by electron microscopy that these spheres are produced in a peculiar type of double or dichotomous spermatogenesis (Juberthie & al. 1976). The species produces two types of sperm: fertile sperm (later called eusperm) and aberrant, non-fertile sperm (= the protective cells; later called parasperm; terms from Healy & Jamieson 1981). Alberti (1995, 2005, Alberti & Michalik 2004) observed the same phenomenon in two other species of Sironidae, *Cyphophthalmus duricorius* (at that time included in the genus *Siro*) and *Siro exilis*. This double spermatogenesis is a singular phenomenon in Arachnida and is certainly apomorphic (see Alberti 2005 considering also Amblypygi and Uropygi). A further remarkable observation reported by Juberthie & al. (1976) from *S. rubens* and also found by Alberti (2000, 2005) in *C. duricorius* is the development of a flagellum-like process in early stages of spermatogenesis which is later withdrawn into the cell body resulting in the formation of a peripheral ring of microtubules. This was an important observation since the spermatozoa of all other Opiliones are entirely devoid of a flagellum (Juberthie & Manier 1978, Jones & Cokendolpher 1985, Moya & al. 2007), i.e. the sperm cells are of the aflagellate type. The case of Sironidae could also support the conclusion of Weygoldt & Paulus (1979a,b) and Paulus (2004) that coiled spermatozoa are a synapomorphy of (at least part of the) epectinate Arachnida. Hence it is remarkable that Sironidae show plesiomorphic traits in sperm development (formation of a flagellum) on the one hand and highly singular characteristics on the other hand (double spermatogenesis, sperm balls). Furthermore, the investigations of Alberti (2000, 2005) revealed remarkable differences between the species. Whereas *S. rubens* has a typical acrosomal complex

composed of an acrosomal vacuole and an acrosomal filament, *C. duricorius* is entirely devoid of this fundamental component of the spermatozoa of most Metazoa. Since it has long been known that sperm characteristics may be useful for phylo-genetic/systematic considerations (e.g., Franzén 1956, 1970, Wingstrand 1972, Baccetti & Afzelius 1976, Baccetti 1979, Kohnert & Storch 1984, Jamieson & al. 1999, Alberti 1980a,b, 1990, 1991, 2000, 2006, Alberti & Weinmann 1985, Alberti & Peretti 2002, Klann & al. 2005; Michalik & al. 2006, Dunlop & Alberti 2008), the present study was initiated to show, whether the peculiar sperm dimorphism is a common feature of Cyphophthalmi or is restricted to Sironidae. Furthermore, this study should reveal the basic characteristics of cyphophthalmid sperm and their modifications to make these characteristics available for further interpretations in a phylogenetic or evolutionary context. Though Cyphophthalmi are considered to be monophyletic as was also shown for Stylocellidae and Pettalidae, this is not evident for the other families based solely on morphology (see Giribet & Boyer 2002). More recently molecular data also found support for the monophyly of Neogoveidae and Troglosironidae, but not of Sironidae (Boyer & al. 2007).

Material and Methods

The following species were investigated:

Sironidae: *Cyphophthalmus duricorius* Joseph, 1868 from Slovenia (leg. G. Alberti) and Styria (Austria; leg. R. Schuster);

Sironidae: *Suzukielus sauteri* (Roewer, 1916) from Mt. Takao (Japan; leg. S. Boyer, G. Giribet & N. Tsurusaki);

Stylocellidae: *Fangensis insulanus* Schwendinger & Giribet, 2005 from Ko Siray (Thailand; leg. P. Schwendinger),

Stylocellidae: *Stylocellus* sp. from Gunung Bonde, Sulawesi (Indonesia; leg. R. Clouse, G. Giribet & C. Rahmadi);

Neogoveidae: *Metagovea* sp. from the Reserva Natural del Río Ñambí (Colombia; leg. L. Benavides & G. Giribet).

Specimens were kept alive and dissected and fixed in ice-cold buffered glutaraldehyde (3,5%; cacodylate buffer: pH 7.4, 0.1M). After two hours in fixative, the buffered glutaraldehyde was diluted (4:1) and mailed to Greifswald where further processing occurred. Alternatively, specimens were sent alive to Greifswald and processed as just described. After rinsing the specimens several times with buffer solution, postfixation in 2% aqueous OsO_4 occurred for two hours. Then again the specimens were rinsed with buffered

solution and dehydration with graded ethanol solutions (60%, 70%, 80%, 96%, absolute ethanol) followed. The specimens were transferred into Spurr's epoxy resin (Spurr 1969) or Araldite. Polymerization occurred at 60°C. Specimens were sectioned (70 nm) using a Diatome diamond knife and were stained according to Reynolds (1963). The sections were studied in a Zeiss EM 10A transmission electron microscope. Semithin sections (400 nm) stained according to Richardson & al. (1960) were used for light microscopy (Olympus BX60) for general orientation.

Results

In the following, the mature spermatozoa of the species mentioned above belonging to three of the six families are shown. Details on spermatogenesis will be published elsewhere. In all species sperm balls including eu- and parasperm were found. Whereas the parasperm are rather similar, the eusperm reveal remarkable structural differences.

Sperm balls: All species investigated until now show the formation of sperm balls (Figs. 1–5). These consist of various distinctly structured secretions, large parasperm located in the periphery, and eusperm positioned slightly deeper in the sphere. Most of the center of the sperm ball is filled with secretion (presently not known for *Fangensis insulanus*). Whereas numbers of parasperm and eusperm are similar in the sperm balls of most of the investigated species, a remarkable bias towards parasperm was observed in *Stylocellus* sp. (Fig. 4A).

Parasperm: Parasperm are aflagellate and rather similar in all investigated species (Figs. 1–5). They are more or less boat-shaped, with many long microvilli extending towards the center of the sperm ball. Whereas in the sironid and neogoveid species the microvilli-bearing side of the cell is slightly concave (Figs. 1b, 2b, 5b), in both stylocellid species it frequently shows a slight convex shape (Figs. 3b, 4b). The cytoplasm does not show distinct structures except for *Stylocellus* sp. in which peculiar electron-lucent areas may be present (Fig. 4a, b). Mostly, the cytoplasm appears to be largely unstructured or even degenerated as does the nucleus, which is located opposite to the microvilli-bearing side.

Eusperm: The always aflagellate eusperm are species-specifically differentiated (Figs. 1–6). They are more or less dish-like with a distinct crypt opening on one side with a small pore, which in *Suzukielus sauteri* has a distinct border (Figs. 1c, 6a). The crypt always contains numerous microvilli, which are arranged in a conspicuous parallel order in *Metagovea* sp. (Figs. 5c, 6f). The nucleus is mostly very electron dense (*Metagovea* is an exception) and

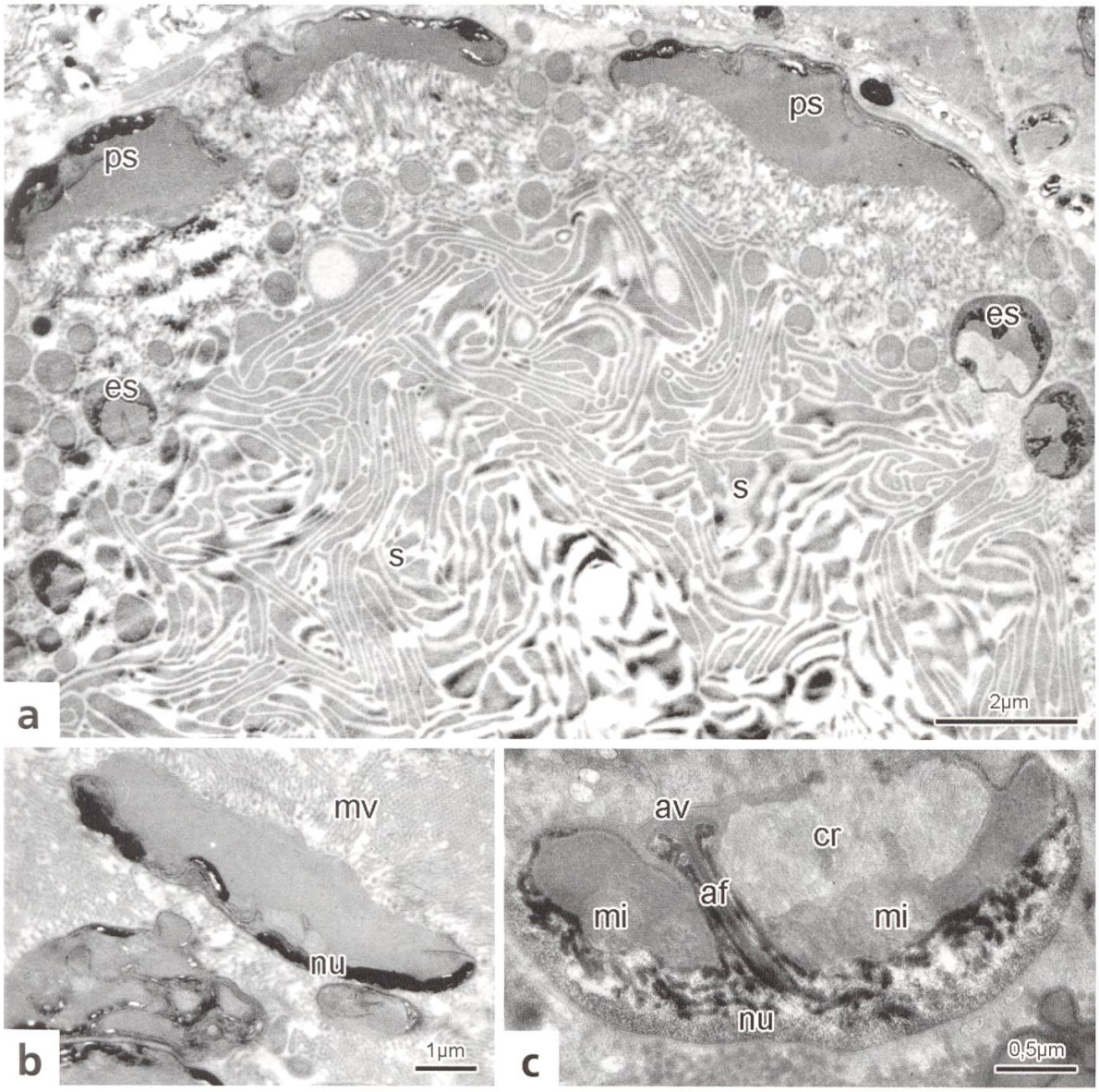

Fig. 1. *Suzukielus sauteri* (Sironidae). – a: Sperm ball: Parasperm (ps) peripherally, eusperm (es) more centrally, secretion (s): thin peripheral sheets, spherical droplets, irregular or flat structures centrally. – b: Parasperm: shape of a boat, numerous microvilli (mv) extending towards the center of the ball, cytoplasm and nucleus (nu) largely degenerated. – c: Eusperm: Oval to cup-like, opening of crypt with conspicuous border, umbrella-like nucleus opposite the crypt (cr) with a process extending to opposite cell membrane where it contacts a typical acrosomal complex consisting of a flat acrosomal vacuole (av) and an acrosomal filament (af) extending into the nucleus. Mitochondria (mi) are partly sunken into the nucleus. Ring of microtubules (mt, see *S. rubens* Fig. 6b) not recognizable. Except for the latter structure, eusperm of *S. sauteri* are rather similar to that of *Siro rubens* described by Juberthie & al. (1976).

is located close to the side opposite to the crypt. This part of the nucleus is umbrella-like in the four sironids and in the neogoveid species following the shape of the cell body (Figs. 1c, 2c, 5,c, 6a–c, f). In the sironids *Siro rubens* and *Suzukielus sauteri* the stalk of the 'umbrella' is directed towards the side of the cell where the crypt opens (Figs. 1c, 6a, b). In the sironid *Cyphophthalmus duricorius* (Figs. 2c, 6c) and in the neogoveid *Metagovea* sp. (Figs. 5c, 6f) such a nuclear/umbrella stalk is lacking. In contrast, the nucleus of the

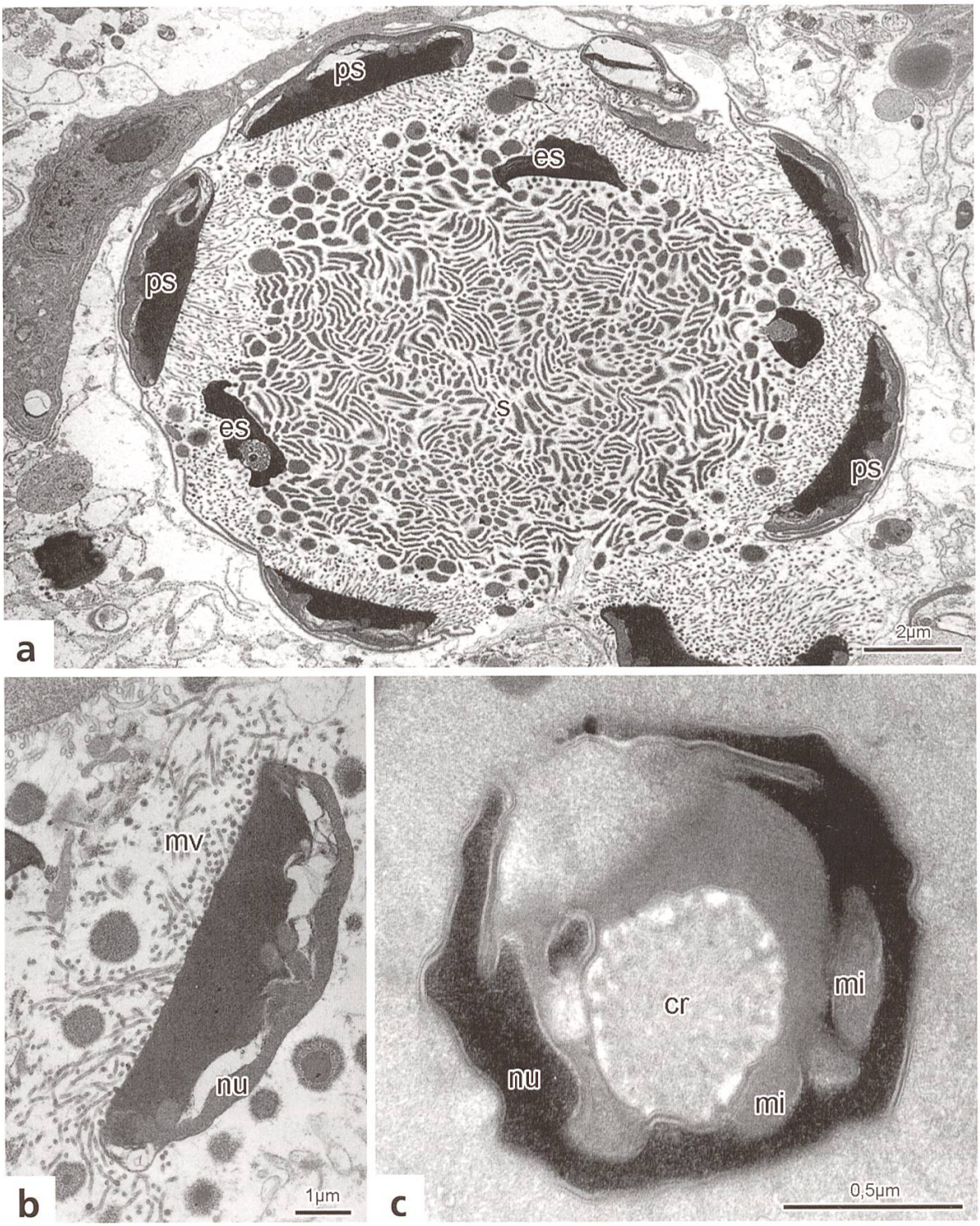

Fig. 2. ***Cyphophthalmus duricorius*** **(Sironidae) (partly based on Alberti 2005). – a: Sperm ball: Similar to** ***Suzukielus sauteri*****: parasperm (ps),eusperm (es), secretion (s). – b: Parasperm: Similar to** ***S. sauteri*****: microvilli (mv), nucleus (nu). – c: Eusperm: Oval to cup-like, central crypt (cr) with microvilli, nucleus (nu) opposite the crypt and umbrella-like but without nuclear process, mitochondria (mi) are partly sunken into the nucleus, acrosomal complex lacking, no ring of microtubules recognizable.**

stylocellids is bell-shaped with the borders of the 'bell' directed to the side of the cell opposite the crypt (Figs. 3c, 4c, 6d, e). The handle of the 'bell' is directed towards the side where the crypt opens. A remarkable feature is the acrosomal complex, which is a fundamental characteristic of metazoan sperm.

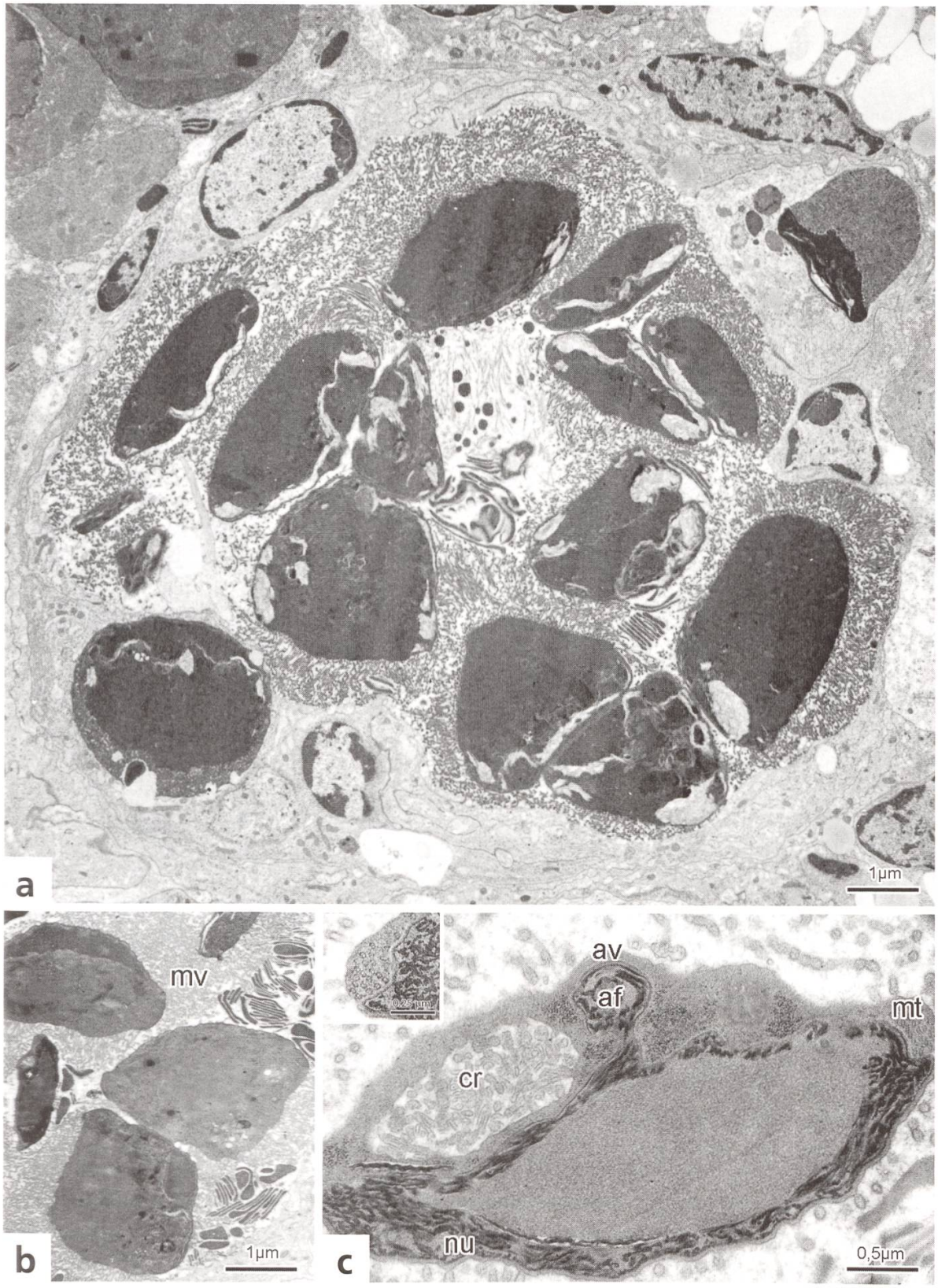

Fig. 3. ***Fangensis insulanus*** **(Stylocellidae). – a: Sperm ball: Incomplete informations available only, at the moment; eu- and parasperm are present and various secretions. – b: Parasperm: Rather stout cells with reduced internal components, many long microvilli (mv). – c: Eusperm: Slightly elongated cells with crypt (cr) filled with microvilli. Nucleus (nu) of peculiar bell-shape. The handle of the 'bell' points against the cell membrane aside of the crypt and touches a small, inconspicuous acrosomal vacuole (av), a rather long acrosomal filament (af) extends into the nucleus. The cytoplasm enclosed by the bell-shaped nucleus is homogeneous, mitochondria are found externally of the 'bell'. A ring of microtubules (doublets, mt) is evident. Inset: Doublets of microtubules in higher magnification.**

In Chelicerata it consists of an acrosomal vacuole and a subacrosomal substance which plesiomorphically is partly modified into an acrosomal filament (perforatorium) and penetrates into a nuclear canal (Alberti 2000). Such an acrosomal complex was found in the sironids *S. rubens* (Juberthie & al. 1976) and *S. sauteri* (Fig. 1c, 6a, b) but not in *C. duricorius* (Fig. 2c, d, 6c). The complex is also found in the stylocellids (Figs. 3c, 4c, 6d, e), but is lacking in the neogoveid *Metagovea* sp. (Figs. 5c, 6f). In all the species possessing a nuclear process (i.e. "umbrella-stalk, bell-handle"), this process is directed against a small acrosomal vacuole, positioned close to the opening of the crypt, and contains the acrosomal filament. In those species (*C. duricorius*, *Metagovea* sp.) lacking the acrosomal complex, a nuclear process is also absent. Two species show further structures not seen in the other species. *Metagovea* sp. has peculiar dense inclusions piled around the crypt opening (Figs. 5c, 6f) and *Stylocellus* sp. shows a number of radiating microvilli, which frequently appear as a row of pearls due to regular constrictions (Figs. 4c, 6e). As mentioned already, the eusperm (as the parasperm) are aflagellate. However, a more or less conspicuous ring of doublets of microtubules in the periphery of the cell is observed in a number of species (e.g., *S. rubens*, *F. insulanus*, *Stylocellus* sp.; Figs. 1c, 3c, 6b, d, e).

Discussion

It is very likely that double spermatogenesis is a characteristic of all Cyphophthalmi since it was seen in all the investigated taxa of species representing three families. However, since recent phylogenetic results suggest that Pettalidae may be the sister family to the remaining Cyphophthalmi (Boyer & al. 2007), the double spermatogenesis could be a synapomorphy of the non-pettalid clade. This question will obviously require study of the spermatogenesis in pettalids. All species observed so far also produce spherical sperm aggregations (also observed in preliminary observations on *Siro exilis*; Alberti 2005). Most remarkable is the diversity of eusperm. Since these are the fertilizing sperm this was to be expected. Basic components of the eusperm are: acrosomal complex, nucleus, and few mitochondria. These components are fundamental and occur in many sperm cells (Baccetti & Afzelius 1976). Also remarkable is the varying shape of the nucleus (umbrella-like, bell-like) and the lack of an acrosomal complex in a sironid (*Cyphophthalmus duricorius*) and a neogoveid species (*Metagovea* sp.), certainly an apomorphic (perhaps analogous) feature. A free flagellum is lacking in mature spermatozoa of all investigated Cyphophthalmi. The presence of a ring of microtubules seen in

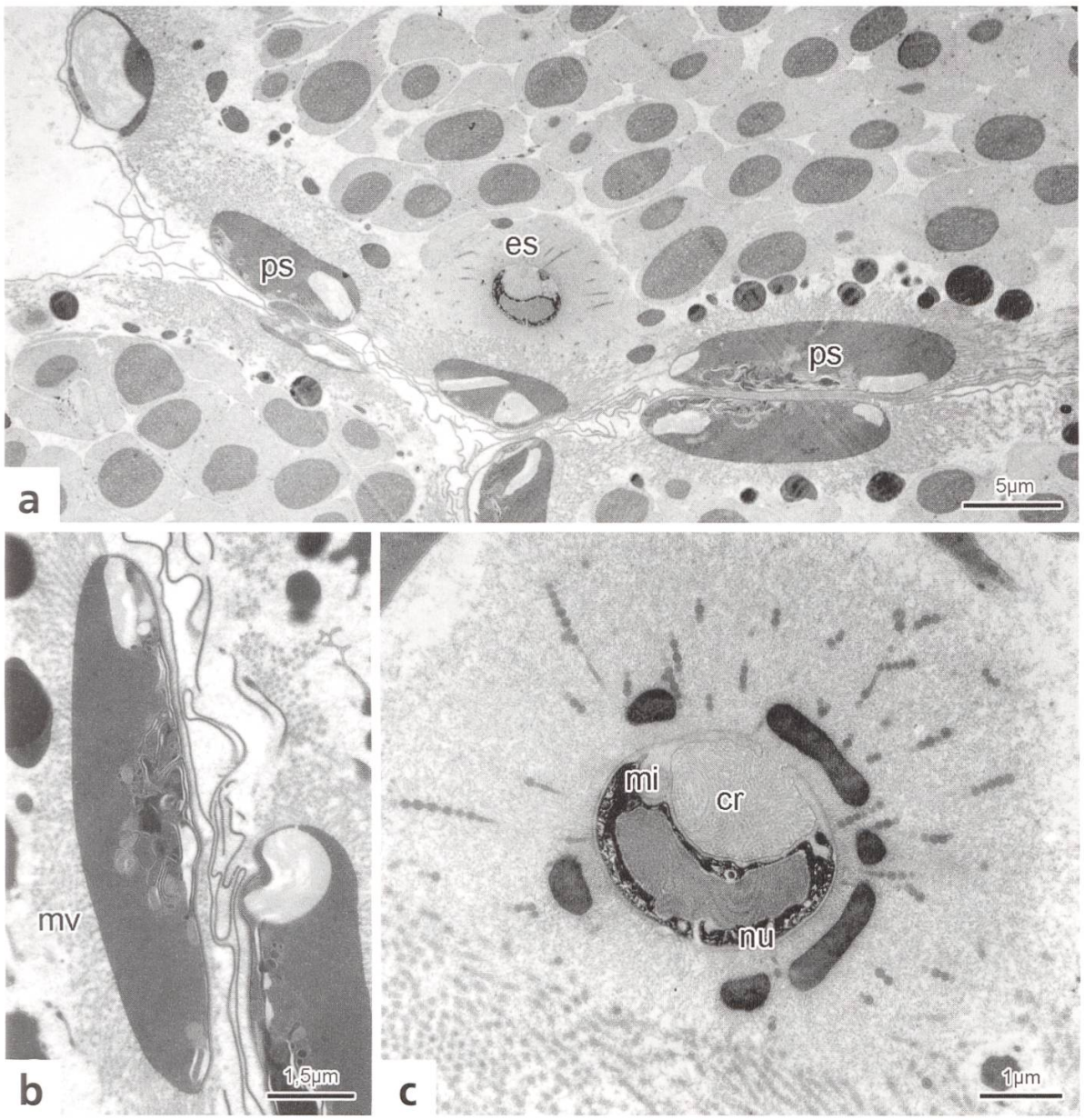

Fig. 4. *Stylocellus* sp. (Stylocellidae). – a: Sperm ball: Components as usual, but eusperm (es) are relatively rare, parasperm (ps) at the periphery. – b: Parasperm: Shaped like a boat with numerous microvilli (mv) extending to the center of the sphere, cytoplasm contains distinct lucent areas and dark structures of irregular appearance. – c: Eusperm: Spherically shaped, crypt (cr) as usual, nucleus (nu) bell-shaped surrounding homogeneous field of cytoplasm, the peripheral borders of the 'bell' come very close leaving only a small opening. The handle of the 'bell' extends to the cell membrane alongside of the crypt and contains an acrosomal filament. The acrosomal vacuole is very inconspicuous. Few mitochondria (mi) are positioned as in *F. insulanus*. A ring of microtubules seems to be present, but is inconspicuous. The most remarkable feature is the presence of long microvilli extending radially from the cell surface into a fine secretion surrounding the cell. The microvilli often appear as a row of pearls. Occasionally it was seen that the secretion between the microvilli condensed to dense bodies.

some species is therefore a remnant of the withdrawn flagellum. The apparent absence of the ring in some species is difficult to interpret, though, and it might be a consequence of suboptimal fixation conditions and/or extended condensation of the cytoplasm that may hide the tubules. A fundamental and

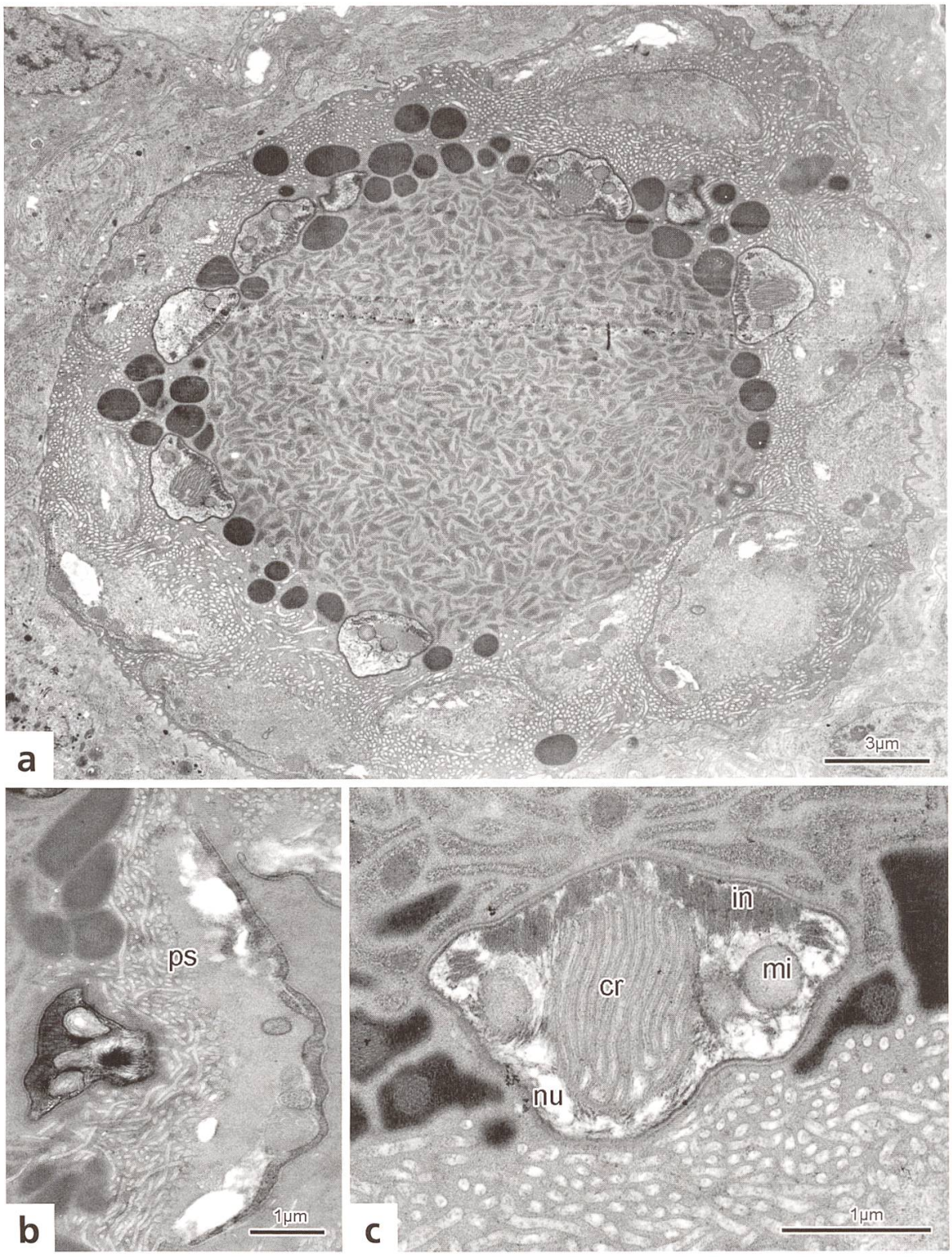

Fig. 5. *Metagovea* sp. (Neogoveidae). – a: Sperm ball: Components as in other species. – b: Parasperm (ps): Rather stout cells, similar to other species. Mitochondria are present. – c: Eusperm: Cup-shaped, crypt (cr) relatively large and centrally located. Its microvilli are quite stout and parallel. Nucleus (nu) is situated opposite the crypt and umbrella-like. No nuclear process, however. Mitochondria (mi) are seen in the small region between nucleus and crypt. No acrosomal complex, instead a number of dense inclusions (in) are piled around the opening of the crypt. A ring of microtubules was not detectable.

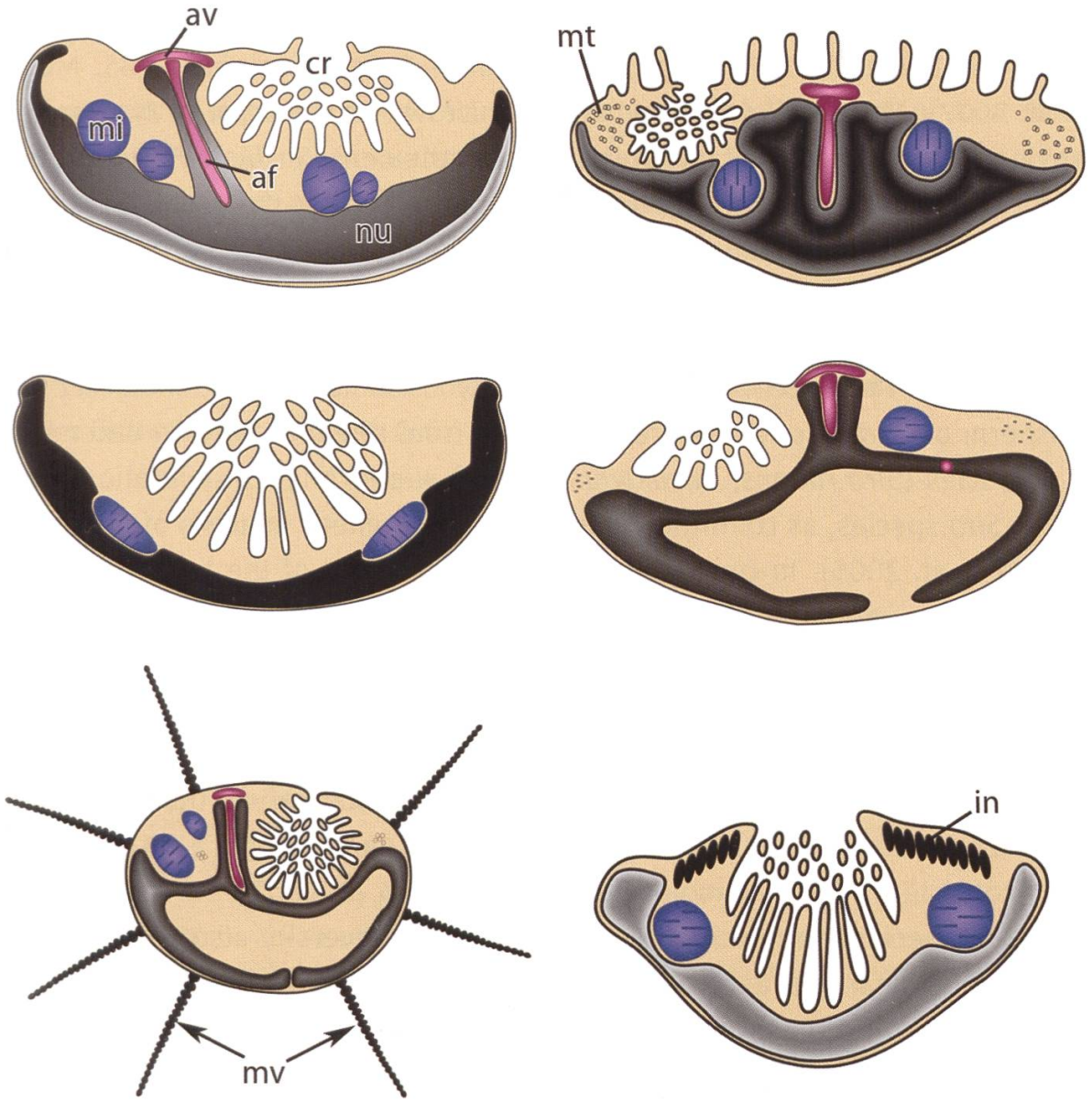

Fig. 6. Drawings of eusperm of: – a: *Suzukielus sauteri* (Sironidae). – b: *Siro rubens* (Sironidae) (acc. to Juberthie et al. 1976). – c: *Cyphophthalmus duricorius* (Sironidae) (acc. to Alberti 1995, 2005). – d: *Fangensis insulanus* (Stylocellidae). – e: *Stylocellus* sp. (Stylocellidae). – f: *Metagovea* sp. (Neogoveidae). Abbreviations: af, acrosomal filament; av, acrosomal vacuole; cr, crypt; in, dense inclusions; mi, mitochondrium; mt, doublets of microtubules; mv, radiating microvilli.

characteristic structure of cyphophthalmid sperm cells is the crypt. In sironids it is formed during spermatogenesis around the base of the flagellum and hence corresponds to a flagellar tunnel (or collar) seen frequently in spermatogenesis of Chelicerata with flagellate sperm (e.g., Fahrenbach 1973, 1999, Alberti & Weinmann 1985, Alberti & Janssen 1986, Alberti 2000, 2005, Michalik & al. 2004, Michalik & Alberti 2005). However, the formation of numerous microvilli within the crypt is a specific character of the investigated Cyphophthalmi. Since the crypt was found in all cyphophthalmid species investigated, it seems likely that all these species also show a transitional flagellum dur-

ing spermatogenesis. A crypt does not occur in other Opiliones (Reger 1969, Juberthie & Manier 1978, Tripepi 1983, Jones & Cokendolpher 1985, Moya & al. 2007). The parallel arrangement of microvilli in the crypt in *Metagovea* sp. is a peculiar feature of this species as are the dense inclusions around the crypt opening. Another peculiarity related to the crypt is the pronounced border of its opening in *Suzukielus sauteri*. *Stylocellus* sp. differed from all the other species in presenting peculiar microvilli-like processes radiating from the cell surface of the sperm cell. Also, the presence of relatively few eusperm in each sperm ball seems remarkable. Finally it should be mentioned that the eusperm of *C. duricorius* differed strikingly from those of the two other sironid species (*Siro rubens, Suzukielus sauteri*) supporting its separation from other *Siro* species, as confirmed in a recent phylogenetic analysis of Sironidae (Boyer & al. 2005). In contrast, the eusperm studied of the two stylocellid species – though differing in some details – are rather similar (e.g., shape of nucleus, acrosomal complex). With regard to the Stylocellidae our results thus support those of Giribet & Boyer (2002) who placed the genus *Fangensis* in this family (see also Schwendinger & Giribet 2005). Further, the profound differences between the three species of Sironidae probably reflect the conclusion of Giribet & Boyer (2002) that this family may be paraphyletic, a result that is also consistent with new molecular data (Boyer & al. 2007). Further studies on more species are required to understand this diversity also with respect to the functional relevance of the peculiar sperm balls and their exceptional components. Of relevant importance are members of the families Pettalidae, Troglosironidae and Ogoveidae. Molecular data analyses of Troglosironidae have suggested a sister group relationship to Neogoveidae (e.g., Giribet & Boyer 2002; Boyer & al. 2005; Schwendinger & Giribet 2005), a result worth investigating with respect to sperm ultrastructure. It would also be interesting to study the two genera *Metasiro* and *Parasiro*. The monospecific genus *Metasiro* from the southeastern USA was formerly classified in the family Sironidae, but it has recently been transferred to Neogoveidae (Giribet 2007). The Western Mediterranean genus *Parasiro* has a morphology that differs strikingly from that of other sironids (e.g., lack of anal glands, presence of teeth and pegs on the claws of the walking legs) and recent molecular analyses suggest that this genus may have had an independent origin from that of other sironids (Boyer & al. 2007). We see in the study of spermiogenesis a whole new suite of characters of great relevance to continue studying the evolution of this primitive group of Opiliones.

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Dedication

This study is dedicated to UD Dr. Konrad Thaler, unforgettable as an exemplary scientist and personality.

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