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Sperm ultrastructure and spermiogenesis of Coniopterygidae (Neuroptera, Insecta)

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ABSTRACT

The spermiogenesis and the sperm ultrastructure of several species of Coniopterygidae have been examined. The spermatozoa consist of a three-layered acrosome, an elongated elliptical nucleus, a long flagellum provided with a 9+9+3 axoneme and two mitochondrial derivatives. No accessory bodies were observed. The axoneme exhibits accessory microtubules provided with 13, rather than 16, protofilaments in their tubular wall; the intertubular material is reduced and distributed differently from that observed in other Neuropterida. Sperm axoneme organization supports the isolated position of the family previously proposed on the basis of morphological data.

1. Introduction

Neuropterida (Neuroptera *sensu lato*) comprise the orders Raphidioptera (snakeflies), Megaloptera (alderflies and dobsonflies) and the extremely heterogeneous Neuroptera (lacewings). The first modern approach towards systematization of the Neuroptera order identifies three suborders: Nevrorthiformia, Myrmeleontiformia and Hemerobiiformia (Aspöck, 1992, 1993, 1995). The morphology of the larval head capsule of Coniopterygidae relates them to Hemerobiiformia (Aspöck et al., 2001; Aspöck, 2002). Coniopterygidae are characterized by a very small size in comparison with other taxa of Neuroptera; body, wings and (often) legs covered with wax granules from specialized hypodermal glands (a feature which gives the insects their common name of "dusty wings"); a strong reduction of wing venation and an unusual structure of genitalia (McEwen et al., 2001).

Coniopterygidae are one of the most enigmatic families of the Hemerobiiformia. They form an isolated family that arose early from the main neuropteran phylogenetic lineage (Withycombe, 1925; Meinander, 1972). The internal reproductive organs of the adult males differ from those of other Neuroptera. Males of Coniopterygidae exhibit a penis which, instead, lacks in all the other families. In contrast with what occurs in the other Neuroptera, no spermatophores are formed in Coniopterygidae. Testes appear normal in the larval instars, but progressively degenerate in the pupa so that in the adult only a ventral oval receptacle filled with spermatozoa and secretion is evident; this single receptacle is considered to be a seminal vesicle. A wide *ductus ejaculatorius* leads from the *vesicula seminalis* to the penis (Withycombe, 1925; Meinander, 1972). Comparative analysis of the ovary structure further supports the placement of Coniopterygidae into a separate lineage of Neuroptera (Kubrakiewicz et al., 1998).

Spermatozoa from representatives of Raphidioptera and Megaloptera have been studied by Afzelius and Dallai (1988). Among Neuroptera, sperm ultrastructure has been examined in Myrmeleontidae and Ascalaphidae (Afzelius and Dallai, 1979), Chrysopidae and Hemerobiidae (Baccetti et al., 1969; Phillips, 1970) and Mantispidae (Dallai et al., 2005). From these studies a relationship of Neuropterida with Coleoptera has been suggested, mainly based on the general structure of the flagellar components. Moreover, in Mantispidae atypical (paraspermatozoa) and functional (euspermatozoa) spermatozoa have been described, the former characterized by a giant axoneme provided with large accessory microtubules (Dallai et al., 2005).

The aim of this paper is to verify whether also the sperm structure can support the isolated position of Coniopterygidae within Neuroptera. The results we present here are of interest for future studies dealing with the phylogenetic relationship of Neuroptera. Moreover, our ultrastructural analysis of spermiogenesis in this unconventional natural model is also relevant for the general knowledge of the cell biology of the axoneme and in particular about the central complex.

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2. Materials and methods

2.1. Animal species

Conwentzia psociformis (Curtis, 1834) (Coniopterygidae, Neuroptera), from Principina, near Grosseto, Italy
Conwentzia sp., from Ferrara and from different sites near Siena, Italy
Coniopteryx sp., from the neighbourhood of Siena, Italy
Semidalis vicina (Hagen, 1861), from Maruggio, near Taranto, Italy
Semidalis aleyrodiformis (Stephens, 1836), from the neighbourhood of Sassari, Italy
Semidalis sp., from Maruggio, near Taranto, Italy
Libelloides longicornis (Linnaeus, 1764) (Ascalaphidae, Neuroptera), from Farma Valley, near Siena, Italy
Odagmia sp. (Simuliidae, Diptera), from the neighbourhoods of L'Aquila, Italy

Moreover, larvae of *C. psociformis* and *S. aleyrodiformis*, collected from *Pistacia lentiscus* (Linnaeus, 1753) in Principina (GR), Italy, were reared at room temperature in test-tubes containing leaves of *P. lentiscus*. Larvae were fed daily with a small water drop containing 5% of sucrose, up to their pupation. Pupae 1, 2, 3, 6 and 8 days old of *C. psociformis* were also studied.

2.2. Light microscopy (LM)

Live larvae and adults of *C. psociformis* were photographed with an Olympus SZX12 stereo light microscope equipped with a Zeiss AxioCam MRc5 digital camera.

For measurements of sperm length, free spermatozoa were obtained from adult males by dissecting their seminal vesicles in 0.1 M phosphate-buffer (PB), pH 7.2, with 3% sucrose. Free spermatozoa were then mounted in 90% glycerol and photographed by interference-contrast microscopy with a Leica DMRB light microscope equipped with a Zeiss AxioCam MRc5 digital camera.

2.3. Transmission electron microscopy (TEM)

Testes and seminal vesicles were isolated from larvae, pupae and adult males by dissection in PB. Part of the material was fixed for 2 h at 4 °C in 2.5% glutaraldehyde in 0.1 M PB pH 7,2, to which 3% sucrose was added; after rinsing with PB, the material was post-fixed for 1 h in 1% osmium tetroxide, rinsed again in PB, dehydrated in ethanol and embedded in Epon–Araldite. The remaining material was processed according to Dallai and Afzelius (1990) using 1% tannic acid in the glutaraldehyde fixation (but omitting osmic post-fixation), then en block stained in 1% uranyl acetate and rinsed in PB.

Ultrathin sections obtained with a Reichert Ultracut II E ultramicrotome, were routinely stained and then observed with a Philips CM 10 electron microscope operating at 80 kV.

3. Results

The third-instar male larva of *C. psociformis* (Fig. 1A) has small gonads that do not show spermatogenetic activity since spermatogenesis begins only after pupation. For pupation, the larva spins a flat circular cocoon of white silk provided with an inner and outer envelope. Male gonads of *C. psociformis* isolated from 1-day-old pupae consist of two ovoidal testes connected to a small seminal vesicle. Spermiogenesis occurs during pupation; early spermatids can be found in 6-day-old pupae. Anteriorly, spermatids contain a flattened acrosome and an elongated nucleus with diffuse chromatin. In cross-section the nucleus has an almost circular appearance and shows three infoldings of the nuclear envelope (Fig. 2A,B) at the level of which a dense material is present and interrupts the continuity of the row of microtubules encircling the nucleus (Fig 2A,B). The two mitochondrial derivatives have an elongated shape in cross-section and are surrounded by microtubules. The axoneme for most of its length has a 9+9+0 pattern with accessory tubules appearing during their formation still connected with the B-subtubule of microtubular doublets; no central tubules are visible (Fig. 2C) except in a short distance just beneath the centriolar region in which three central microtubules are present.

In the 8-day-old pupa, the spermatids have progressed along their maturation; they are elongated cells with reduced cytoplasm. The nucleus surrounded by a row of microtubules contains a meshwork of dense chromatin filaments (Fig. 3A). In cross-section, on the nuclear tip the acrosome appears as a lenticular structure provided with a narrow axial subacrosomal cavity (Fig. 3A); a short posterior prolongation of the acrosome parallels the nucleus (Fig. 3A). In a posterior cavity of the nucleus a bundle of seven microtubules is visible surrounded by the centriole adjunct material (Fig. 3A). Beneath this region is present the centriolar region that shows doublets associated with the proximal end of the accessory tubule; five microtubules are present in the axial part (Fig. 3C,D). Further behind the 9+9+3 axonemal model is visible (Fig. 3A,E). The three central microtubules are disposed orderly in a triangular pattern. Each microtubule shows two opposite short projections reaching the adjacent microtubules (Fig. 3E). The axonemal tip consists of only the peripheral doublets. The mitochondrial derivatives have elliptical shape in cross-section and show a crystallized axis in their matrix close to the axoneme; they are still surrounded by a row of microtubules (Fig. 3E).

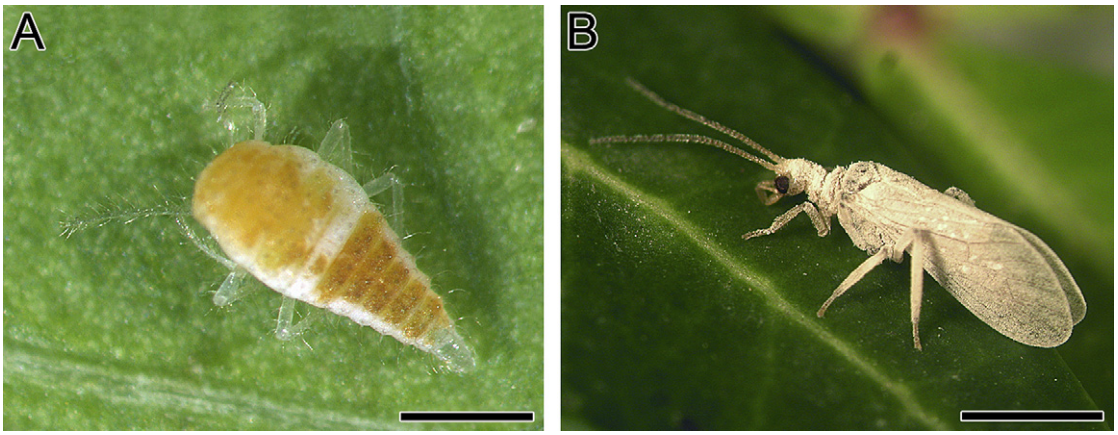

Fig. 1. *Conwentzia psociformis.* LM. (A) Third-instar larva. (B) Adult. Scale bars: 1 mm in A, 2 mm in B.

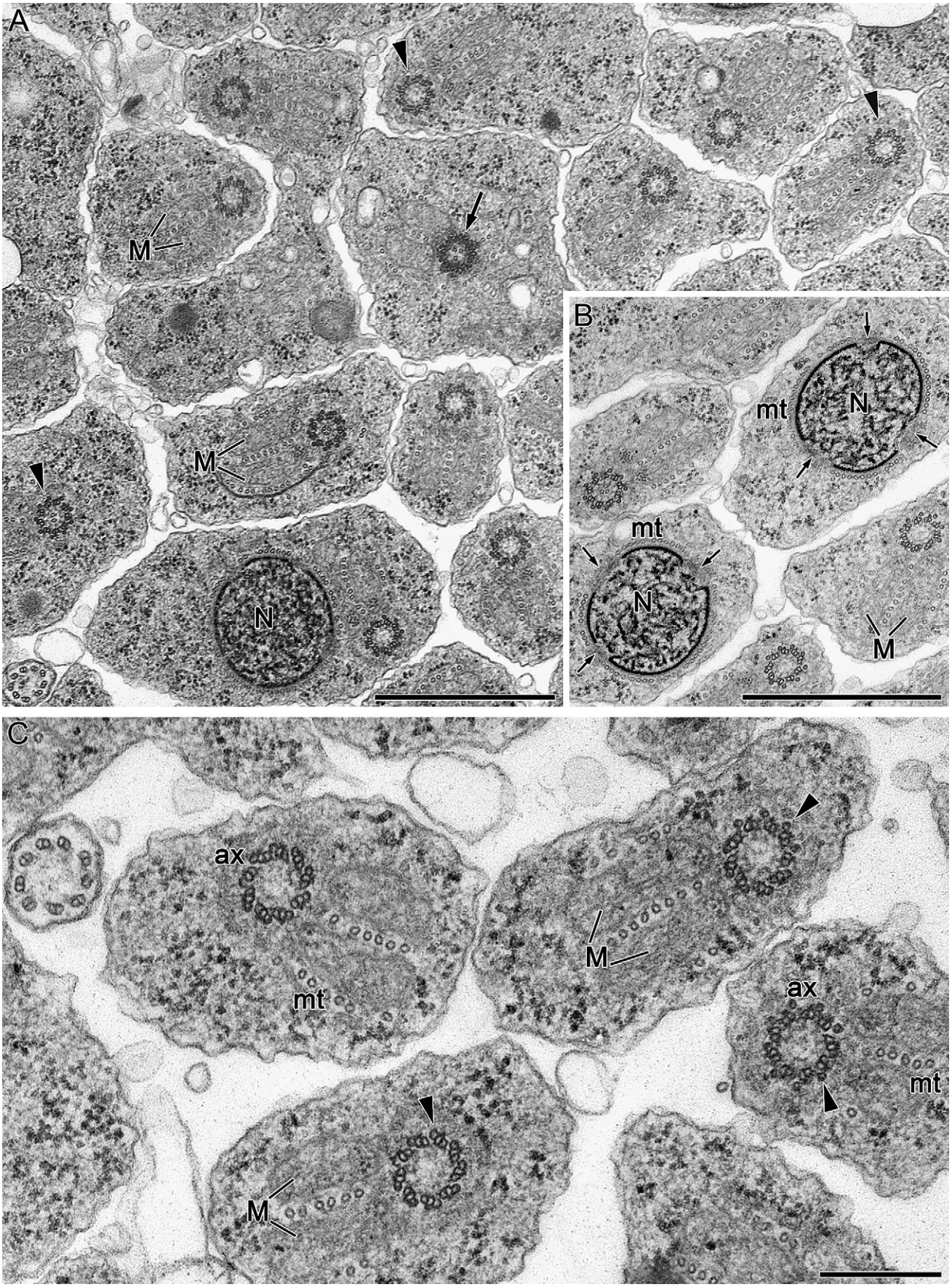

Fig. 2. *Conwentzia psociformis*. TEM. (A–C) Six-day-old pupa. Cross-sections through testes, showing early spermatids. (A) Note the presence of three microtubules in the axial part of the axoneme in centriolar region (arrow) while two or no microtubules (arrowheads) are present in the other axonemes. A large nucleus (N) and mitochondrial derivatives (M) are surrounded by a row of microtubules. (B) Spermatid nuclei (N) showing three infoldings (arrows) of the nuclear envelope. At these levels the layer of microtubules (mt) is interrupted and dense material is present at these levels. M, mitochondrial derivatives. (C) Cross-section through spermatids showing the beginning of the formation of the accessory tubules from the B-subtubule of each doublet (arrowheads). The axoneme (ax) is still formed as a 9+9+0 pattern. The mitochondrial derivatives (M) are surrounded by microtubules (mt). Scale bars: 1 μm in A and B, 0.3 μm in C.

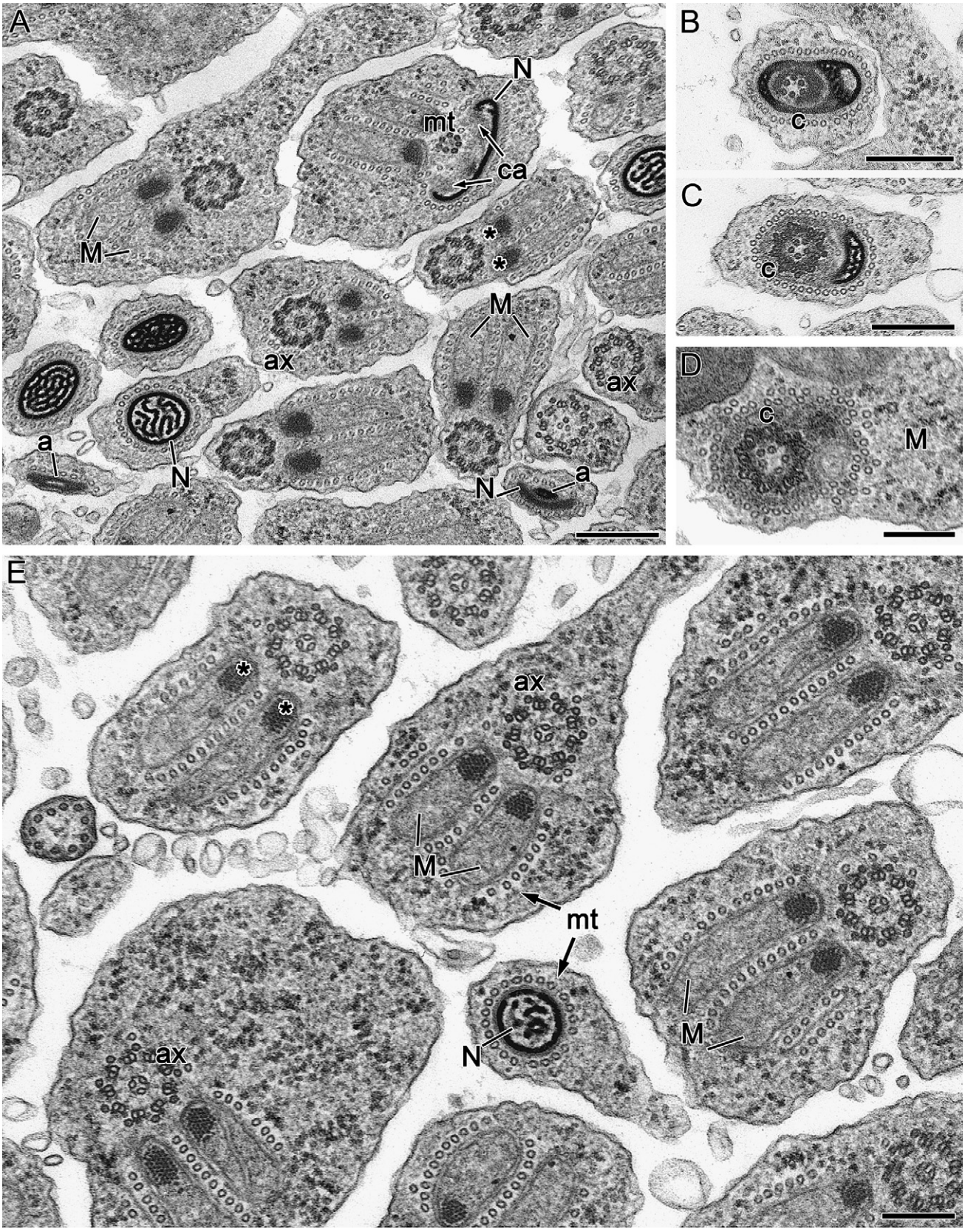

Fig. 3. *Conwentzia psociformis*. TEM. (A–E) Eight-day-old pupa. Cross-sections through testes showing aged spermatids. (A) At this level the spermatids show a 9+9+3 axoneme (ax) and two elongated mitochondrial derivatives (M) with a beginning of crystallization of the inner material (asterisks). The nucleus (N) shows dense chromatin filaments. Beneath the nucleus (N) a little amount of centriolar adjunct material (ca) is visible close to seven singlet microtubules (mt). Acrosomes (a) are also visible. (B–D) Details of the centriolar region (c) with axial microtubules. (E) Late spermatids with the 9+9+3 axoneme. N, nucleus; M, mitochondrial derivatives; (asterisks), crystallization of the inner material of the mitochondrial derivatives; ax, axoneme; mt, microtubules. Scale bars: 0.3 μm in A–C, 0.2 μm in D and E.

In the mature male (Fig. 1B) testes are either missing or very reduced in size, while a large seminal vesicle is visible. In the seminal vesicle of all examined species many living 80–90 μm long spermatozoa are present, which are motile when they are dispersed in buffer solution (Fig. 4A). A cross-section through the seminal vesicle shows a compact mass of secretion into which numerous orderly disposed spermatozoa are embedded (Fig. 4B–E). Spermatozoa are filiform, show a short flattened apical acrosome, a long elliptical nucleus which becomes very flat in the apical region, and a long flagellum with a sinusoidal appearance (Fig. 4A). The acrosome is 0.8 μm long and shows a posterior thin region 0.25 μm long, which runs parallel to the nucleus (Fig. 4B,D). In cross and longitudinal sections, a narrow subacrosomal cavity is visible (Fig. 4B,C). A space, only 13 nm thick, is evident between the acrosomal vesicle and the plasma membrane; in this narrow space a dense material, forming the extra-acrosomal material, seems to be present (Fig. 4C). The flagellum consists of an axoneme and of two mitochondrial derivatives. Accessory bodies are lacking between the two above-mentioned structures (Fig. 4E–H). The axoneme has a 9+9+3 pattern with accessory tubules provided with 13 protofilaments in their tubular wall. Intertubular material is present only on the outer side of doublets while that associated with the accessory microtubule is missing (Fig. 4F–H). The three central tubules still show short dense projections towards the neighbouring tubules (Fig. 4F–H). Microtubular doublets are provided with both dynein arms and radial spokes. The posterior flagellar region shows only a single 9+2 axoneme, the accessory tubules being missing; in the tail posterior tip also the two central microtubules are missing (not shown).

4. Discussion

Previous spermatological data (Afzelius and Dallai, 1994) and ovary ultrastructure (Büning, 2006) suggested a sister-group relationship of Neuropterida with Coleoptera. In particular, the distribution of the intertubular material in the axoneme other than the structure of accessory tubules supported this hypothesis. However, the sperm architecture does not allow to indicate a further relationship within any of the orders into which Neuropterida are commonly divided. As we reported above, Coniopterygidae are an isolated family of Hemerobiiformia and, according to Aspöck (2002), "they would be criticized in any place of the cladogram". On the basis of the larva head capsule, Aspöck proposed that Coniopterygidae might be associated with higher Hemerobiiformia, while their supposed relationship with Sisyridae appears to be only weakly founded (Aspöck, 2002). Moreover, molecular data indicated a close relationship of Coniopterygidae with Dilaridae (Haring and Aspöck, 2004). Though limited to a few species, the results presented here on sperm structure of Coniopterygidae indicate the expression of an unusual axonemal pattern, thus confirming the originality of the group and the difficulties to find a clear relationship with other taxa of Neuroptera.

We followed spermiogenesis along sequential steps of pupa instars and observed the progressive formation and differentiation of the sperm components. We also confirm the progressive degeneration of testes and the occurrence of only an evident seminal vesicle in adult males (Meinander, 1972).

Our results first indicate that the assemblies of the nine outer doublets and of the central microtubules are temporally distinct processes, the latter being assembled later. This finding is consistent with the information coming from the study of flagellar assembly in *Trypanosoma,* showing that the central pair and the outer doublet microtubules constitute different assembly units and pose different requirements for microtubule nucleation; the assembly of the nine doublets is templated by the basal body and does not require γ-tubulin, while the nucleation of the central pair singlets is a γ-tubulin-dependent process which takes place in a definite region internal to the basal body (McKean et al., 2003).

Other results have also indicated that the assembly of the central pair microtubules is under a distinct control and poses different molecular requirements (Raff et al., 2000; Nielsen et al., 2001).

The occurrence of an unusual number of singlet microtubules within the central apparatus, though not a common feature among insects, has been already described in other species belonging to different orders (reviewed by Dallai et al., 2006); however, the available information on axoneme assembly provide no clue to understand the molecular basis of this unusual phenotype; several factors have been implicated in the assembly of the central pair, but all the mutations that have been till now described to affect central microtubules result in their disappearance, rather than in their increase in number (reviewed by Mencarelli et al., 2008). In Coniopterygidae, the presence of three central microtubules is likely to be related to a second unusual feature of these spermatozoa, that is, a bundle of up to seven microtubules that run internally to the basal body partly continuing in the central singlets of the axoneme. This microtubular bundle appears to be formed in close association with the so-called centriole adjunct, a mass of electrondense material that is associated with the proximal region of the basal body and is located within an invagination of the nuclear envelope. In *Drosophila* spermatozoa, this material has been suggested to contain γ-tubulin (Wilson et al., 1997), and its possible role as a microtubule-assembly site has been suggested by other structural studies of insect spermatogenesis (Paccagnini et al., 2007). Thus, this bundle of microtubules is likely to originate from a redundant process of microtubule nucleation.

Results presented in this paper show that the sperm of Coniopterygidae are characterized by the presence of three central microtubules in the axoneme and by accessory microtubules with the same structure, that is, they are both formed by 13 protofilaments, rather than by 16, as it more commonly occurs among insects. This observation suggests the occurrence of some relationship between the two classes of singlet microtubules, possibly due to some shared structural constraints. This possibility has been also indicated by the analysis of *Drosophila* transgenic flies carrying modified tubulin isotypes, which has shown that the different subsets of axoneme microtubules—doublets and singlets—have separable requirements for α- and β-tubulin structure (Fackenthal et al., 1995; Hutchens et al., 1997).

The amount of centriolar adjunct material is very reduced in the spermatozoa and this could be related with the lack of accessory bodies in the Coniopterygidae spermatozoa; a correlation among the presence of the centriole adjunct material and that of accessory bodies has been demonstrated in spermatozoa of many other insect orders. The presence of evident accessory bodies and of an expanded centriole adjunct in the neuropteran species studied so far supports this claim (Jamieson et al., 1999). With regard to the mitochondrial derivatives, Coniopterygidae show a partial crystallization of the material in the matrix. A similar pattern was found in the alderfly *Sialis lutaria*, while in the Raphidioptera and Neuroptera studied, the mitochondrial matrix is filled with crystallized material (Jamieson et al., 1999).

Coniopterygidae seem to have a three-layered flattened acrosome and an elliptical nucleus. The acrosome has a short and narrow subacrosomal space, but it is difficult to establish whether the material constituting the *perforatorium* occurs. The small size of the acrosome and the narrow space between acrosomal and plasma membrane make it also difficult to establish the presence of an extra-acrosomal layer. Thus, the appearance of the acrosome is less clear than that described by Afzelius and Dallai (1979, 1988) in *Libelloides* sp. or in *S. lutaria*, which have a clear tri- and bi-layered acrosome, respectively. However the presence of a tri- and bi-layered acrosome is shared by many insect orders (Jamieson et al.,

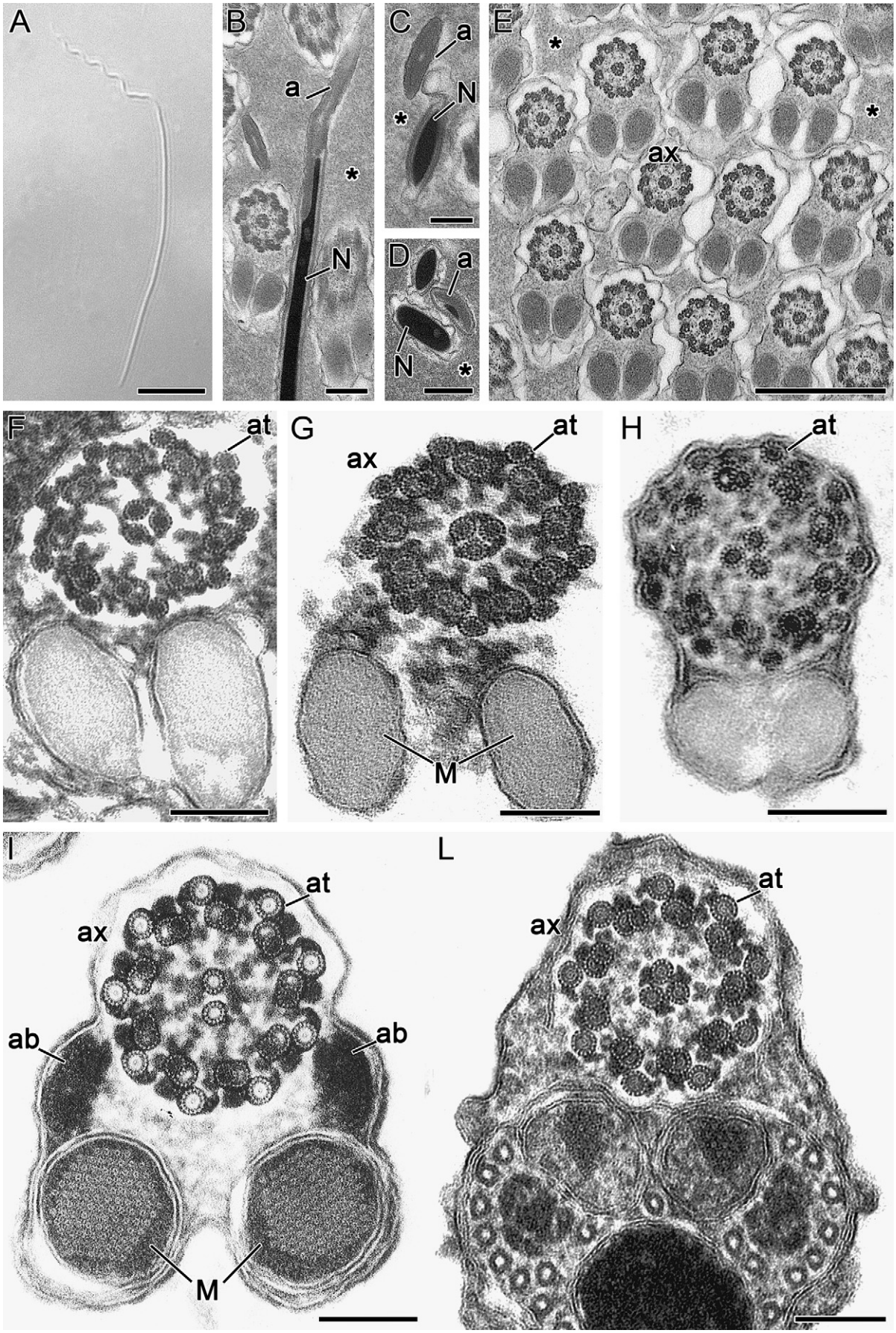

Fig. 4. (A) Interference-contrast micrograph of *Semidalis* sp. living sperm. Note the sinusoidal pattern of the flagellar region. (B-L) TEM. (B–D) Cross-sections and longitudinal sections through seminal vesicle showing the nucleus (N) and the three-layered elliptic acrosome (a); (asterisks), secretion in the lumen of the seminal vesicle. (E) Cross-sections through the spermatozoa from the seminal vesicle. The axoneme (ax) and the two mitochondrial derivatives (M) have acquired their final appearance. The sperm are embedded in a dense secretion (asterisks). (F–H) Cross-sections through sperm tails of three species of Coniopterygidae after tannic acid preparation, showing the 9+9+3 axoneme (ax) and the two mitochondrial derivatives (M). Note that the accessory microtubules (at) have 13 protofilaments in their tubular wall. (F) *Conwentzia psociformis*. (G) *Semidalis* sp. (H) *Coniopteryx* sp. (I) Cross-section through a sperm tail of *Libelloides longicornis* (Neuroptera, Ascalaphidae) showing the 9+9+2 axoneme (ax), the two accessory bodies (ab) and the two mitochondrial derivatives (M). Note that the accessory microtubules (at) have 16 protofilaments in their tubular wall. (L) Cross-section through a sperm tail of *Odagmia* sp. (Diptera, Simuliidae) showing the 9+9+3 axoneme (ax). Note that the number of protofilaments in the accessory tubules (at) is 15. Scale bars: 10 μm in A, 0.2 μm in B and D, 0.1 μm in C, 0.5 μm in E, 0.1 μm in F–L.

1999) and apparently is a plesiomorphic character. Thus, this feature cannot contribute to clarify the phylogenetic relationships of the family.

A sperm axoneme with a 9+3 pattern was found in Arachnida spermatozoa, such as spiders (Baccetti et al., 1970; Alberti, 1990; Dallai et al., 1995; Michalik, 2006) and some "Pedipalpi" (Alberti, 2000). The presence of three central microtubules in the sperm flagellar axoneme, instead, is not common among insects. A 9+9+3 model is shared by a few unrelated insect species; this is the case of dipteran Simuliidae (Fig. 4L), which are provided with an axoneme recalling at first glance that of Coniopterygidae. An accurate observation of material prepared by tannic acid impregnation (Dallai and Afzelius, 1990), however, allowed the demonstration that accessory tubules of Coniopterygidae consist of 13 rather than 15 protofilaments in their tubular wall, as it occurs in Simuliidae. Moreover, the projections starting from each of the three central tubules have a different organization in the two groups. In Coniopterygidae each single microtubule shows two projections towards the other two adjacent microtubules, while in simulid diptera each microtubule has a single projection oriented counterclockwise (compare Fig. 4F with L). Axonemes with patterns varying from 9+9+0 to 9+9+3 have also been detected in the psocid *Elipsocus westwoodi* (King and Ahmed, 1989) and a single 9+3 was described in several porricondylid dipterans (Dallai et al., 1996). Considering the accessory tubules and the intertubular material of Coniopterygidae it appears that these structures have an organization quite different from those occurring in the other Neuropterida. Within this taxon the sperm axoneme of the following species was studied: *Raphidia* sp. (Raphidioptera) and *S. lutaria* (Megaloptera) (Afzelius and Dallai, 1988); *Myrmeleon formicarius* and *Libelloides* sp. (Afzelius and Dallai, 1979); *Chrysopa carnea*, *C. prasina*, *C. formosa*, *Eumicromus paganus* (Baccetti et al., 1969); and *Chrysopa* sp. (Phillips, 1970) (all Neuroptera). The accessory tubules of all Neuropterida that have been examined so far consist of 16 protofilaments in their tubular wall. Thus, the axonemal pattern results are quite similar among the species. The only exception described in the group is the giant tubules present in the sperm axoneme of the atypical spermatozoa (paraspermatozoa) of *Perlamantispa perla* (Dallai et al., 2005). These accessory tubules have a large diameter and contain up to 40 protofilaments in their tubular wall. In Neuropterida, the intertubular material is distributed in a peculiar manner with a counter-clockwise beak-like expansion on one side of the accessory tubules and a more extended dense material adhering to the outer surface of the doublet microtubules (Fig. 4I). In Coniopterygidae, the intertubular material does not exhibit the beak-like expansion on one side of the accessory microtubules and the dense material in contact with the doublet microtubules is reduced. Altogether, the data set observed indicates that Coniopterygidae have realized several autapomorphies, thus making it difficult to establish their relationship with any other taxon of the group. The above-reported study, together with the already known unusual morphological features (Withycombe, 1923, 1925; Meinander, 1972), i.e., the number of Malpighian tubules, the progressive involution of testes, the presence of copulatory organs and finally, the small body size with the peculiar wax secretory granules, confirm the isolate position of Coniopterygidae.

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